

Arnold von Eckardstein
Christoph J. Binder *Editors*

Prevention and Treatment of Atherosclerosis

Improving State-of-the-Art Management
and Search for Novel Targets

EAS



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Preface

We are proud editors of this follow-up edition of the well-received *Handbook of Experimental Pharmacology* Volume 170 “Atherosclerosis: Diet and Drugs,” which was published more than 15 years ago. Since then, major progress has been made in the primary and secondary prevention of atherosclerotic cardiovascular diseases (ASCVDs), thanks to improved control of the classical risk factors with well-established causality in the pathogenesis of ASCVDs, namely, LDL-cholesterol, blood pressure, diabetes, and prothrombotic states. The five chapters in the first part of this book summarize the progress in this evidence-based state-of-the-art management of ASCVDs as well as the perspectives of ongoing drug developments towards better control of these risk factors. The five subsequent chapters of Part II address the state of research and development towards the control of risk factors which are well established by epidemiology but still equivocal due to negative, contradictory, or missing outcomes of randomized controlled trials [triglycerides, HDL-cholesterol, Lipoprotein(a), obesity, nutraceuticals]. Vascular and immune cells, adipose tissue as well as microbiota are important in the pathogenesis of atherosclerosis and intensely investigated. The validation of some anti-inflammatory drugs (e.g., colchicine or methotrexate) or targets (interleukin 1 beta) by clinical trials has been started and generated promising results in first trials. The six chapters of Part III describe the ongoing translational efforts directed to unravel and validate novel target molecules for drug development. Hypothesis-free systems biology approaches have become an important strategy also in atherosclerosis research, which are covered by the four chapters of Part IV. Genomics, transcriptomics, proteomics, and metabolomics have successfully helped to identify novel players in the pathogenesis of atherosclerosis or validate the causality of candidate molecules or processes in the development or clinical manifestation of ASCVDs.

This *Handbook of Experimental Pharmacology* on atherosclerosis is part of the educational activities of the European Atherosclerosis Society (<https://www.eas-society.org>). EAS finances the open access publication of this handbook, to foster the training and promotion of early career scientists and the dissemination of old and new knowledge on ASCVD and their risk factors to the scientific and medical community as well as the lay public. As the editors of this Handbook of Experimental Pharmacology, we gratefully acknowledge not only this financial but also the moral and intellectual support by the Executive Committee of EAS. We applaud the

authors of the 19 chapters for their engagement and reliable cooperation. Finally, we thank Prof. Martin Michel for his sustained interest and guidance as well as Susanne Dathe, Alamelu Damodharan, and Anand Ventakachalam from Springer Nature who supported us with patience and enthusiasm in the production of this book.

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For more than 50 years, the Society's expertise has been used to teach clinicians how to manage lipid disorders and how to prevent atherosclerosis. Through its publications, EAS creates a framework for discussion of new developments in the field. Activities cover the prevention and care of atherosclerosis and its clinical manifestations, and also basic science in the atherosclerosis field. With live and online educational activities including Advanced Courses, Rare Lipid Disorder Courses, and its Annual Congress, EAS supports exchange of knowledge between scientists and clinicians. Many lectures from these events are recorded and available on the Society's educational platform, EAS Academy. By reaching out to involve young scientists and researchers also from related disciplines, EAS strengthens and expands development of the field now and in the future. These activities will ultimately lead to improved healthcare for persons with cardiovascular disease and lipid disorders.

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Part I

**Improving the Treatment of Established
Targets**



Diet, Lifestyle, Smoking

Lale Tokgozoglu, Vedat Hekimsoy, Giuseppina Costabile,
Ilaria Calabrese, and Gabriele Riccardi

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Abstract

Cardiovascular disease is the leading cause of death globally. The past few decades have shown that especially low- and middle-income countries have undergone rapid industrialization, urbanization, economic development and market globalization. Although these developments led to many positive changes in health outcomes and increased life expectancies, they all also caused inappropriate dietary patterns, physical inactivity and obesity. Evidence shows that a large proportion of the cardiovascular disease burden can be explained by behavioural factors such as low physical activity, unhealthy diet and smoking. Controlling these risk factors from early ages is important for maintaining cardiovascular health. Even in patients with genetic susceptibility to cardiovascular disease, risk factor modification is beneficial.

Despite the tremendous advances in the medical treatment of cardiovascular risk factors to reduce overall cardiovascular risk, the modern lifestyle which has led to

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greater sedentary time, lower participation in active transport and time spent in leisure or purposeful physical activity, unhealthy diets and increased exposure to stress, noise and pollution have diminished the beneficial effects of contemporary medical cardiovascular prevention strategies. Therefore attenuating or eliminating these health risk behaviours and risk factors is imperative in the prevention of cardiovascular diseases.

Keywords

Cardiovascular diseases · Diet · Lifestyle · Prevention

The global burden of disease has dramatically shifted from communicable to non-communicable diseases, making cardiovascular disease (CVD) the leading cause of death in Europe as well as most parts of the world. According to 2017 European Cardiovascular Disease Statistics, there are more than 11 million new cases of cardiovascular disease in Europe, and cardiovascular disease accounts for 45% of all deaths. Europe is in the midst of an epidemiologic, economic, social and nutritional transition. The past few decades have shown that especially low- and middle-income countries have undergone rapid industrialization, urbanization, economic development and market globalization. Although these developments led to many positive changes in health outcomes and increased life expectancies, they all also caused inappropriate dietary patterns, physical inactivity and obesity. Fat and energy consumption is increasing mostly in Eastern Europe, and smoking remains a key public health issue despite decline in some countries.

The causal factors for CVD are well defined today. Genetic susceptibility, environmental factors and lifestyle are the most important determinants of cardiovascular health. Preventable or treatable risk factors such as high blood pressure, high cholesterol, diabetes, smoking and obesity play an important role in the development of cardiovascular disease as well as lifestyle, socioeconomic and environmental conditions. We also know that socioeconomic deprivation increases the dependence on alcohol and tobacco use as well as leading to consumption of unhealthy foods. When we look at the ranking of European countries according to GDP per capita, we see that cardiovascular risk goes hand in hand with low income.

Although genetic susceptibility is extremely important in the development of cardiovascular disease, even the subjects with high genetic risk derive benefit from healthy lifestyles. In a recent study, genetic risk was determined by a polygenic risk score of up to 50 single-nucleotide polymorphisms and adherence to a healthy lifestyle consisting of 4 factors (no current smoking, no obesity, healthy diet and regular physical activity). A favourable lifestyle was associated with a 50% lower relative risk of CAD in all three groups of low, intermediate and high genetic risk (Khera et al. 2016). A healthy lifestyle will modify and decrease traditional risk factors like hypertension, diabetes, dyslipidemia and obesity. In addition lifestyle modification has positive effects beyond attenuation of traditional risk factors. To maintain cardiovascular health, it is important to strive for optimal levels of four

health behaviours (non-smoking, body mass index $<25 \text{ kg/m}^2$, physical activity and ideal diet) and three health factors (untreated total cholesterol $<200 \text{ mg/dL}$, untreated blood pressure $<120/<80 \text{ mmHg}$ and fasting blood glucose $<100 \text{ mg/dL}$) (Lloyd-Jones et al. 2010).

Although there have been tremendous advances in the medical treatment of cardiovascular risk factors in the past decades, the same cannot be said for lifestyle. The modern lifestyles have led to greater sedentary time, lower participation in active transport and time spent in leisure or purposeful physical activity, unhealthy diets and increased exposure to stress, noise and pollution.

1 Diet

The evidence that dietary factors influence the development of CVD derives mainly from epidemiological observations and from clinical studies on the impact of dietary changes on traditional risk factors such as plasma lipids, blood pressure or glucose levels.

In fact, epidemiological studies clearly indicate that higher consumption of fruit, non-starchy vegetables, nuts, legumes, fish, vegetable oils, yogurt and whole grains, along with a lower intake of red and processed meats and foods high in refined carbohydrates and salt, is associated with a reduced incidence of cardiovascular events. Moreover, they indicate that the replacement of animal fats, including dairy fats, with PUFAs or other vegetable sources of fats as well as fibre-rich carbohydrate foods can decrease the risk of CVD (Forouhi et al. 2018; Sacks et al. 2017).

Conversely, the evidence from randomized controlled trials (RCTs) is based on few studies that provide, in some cases, conflicting results. In this respect, the difficulties of performing clinical trials that adequately test nutritional interventions for CVD prevention cannot be underestimated since these studies require thousands of participants followed for years or decades. As to the lack of concordance between studies, this is due not only to methodological problems, particularly inadequate sample size or the short duration of many trials, but also to the difficulty of evaluating the impact of a single dietary factor independently of any other change in the diet (Forouhi et al. 2018).

These limitations suggest caution in interpreting the results of RCTs or even meta-analyses of RCTs in relation to the effect of a single dietary change on CVD, particularly where they diverge from the existing global evidence (Catapano et al. 2016). Therefore, in evaluating the role of diet in the prevention of cardiovascular diseases, we will consider not only RCTs on the impact dietary modifications on CVD events but also observational studies and clinical trials on the effects on major CVD risk factors.

Table 1 Summary of the effects of dietary fatty acids, carbohydrates and salt on cardiovascular risk factors and cardiovascular events

	LDL-C	Triglycerides	HDL-C	Plasma glucose	Insulin sensitivity	Blood pressure	CVD
Trans fatty acids	↑↑	-/↑	↓↓	-	↓↓	-	↑↑
SAFA	↑↑	-	-/↑	-	↓↓	↑	↑
MUFA	-	-	-	↓	↑	↓/-	↓
PUFA n-6	-	↓	↓	↓/-	↑	↓/-	↓
PUFA n-3	-	↓↓ (at high dose)	-	-	-	↓ (at high dose)	↓ (at high dose)
Refined starch	-	↑	↓	↑	↓	-	-
Sugars	-	↑↑	↓	↑ (long term)	↓↓	↑/-	↑
Dietary fiber	↓↓	-/↓	-	↓↓	↑	↓	↓
Salt	-	-	-	-	-	↑	↑

↑ increase; ↓ decrease; - no effect

1.1 Dietary Fat (Table 1)

1.1.1 Effects on CVD Risk Factors

The available evidence supports current dietary guidelines recommending that saturated fatty acid (SAFA) intake be reduced and replaced with unsaturated fatty acids. This approach aims at improving blood lipid and lipoprotein profile in order to reduce the risk of coronary heart diseases, since high LDL cholesterol and elevated plasma triglyceride concentrations are established independent CVD risk factors (Catapano et al. 2016; Graham et al. 2012; Reiner 2013; Reiner et al. 2011). A reduction in dietary SAFA intake can be achieved through the isocaloric replacement with unsaturated fatty acids, mainly monounsaturated (MUFA, mostly cis-oleic acid) or polyunsaturated fatty acids (PUFAs) or with dietary carbohydrates. According to the results of meta-analyses of RCTs, replacing 5% of calories from SAFA with MUFA or PUFA (predominantly linoleic acid; C18:2n-6) is able to significantly reduce LDL cholesterol levels by 0.21 mmol/L (8 mg/dL) and 0.28 mmol/L (11 mg/dL), respectively. In addition, this dietary approach is able to significantly improve insulin sensitivity and reduce triglyceride levels, mostly in the postprandial period; notably, the triglyceride-lowering effect is more pronounced with n-6 PUFA than with MUFA. As for HDL cholesterol levels, there is no relevant effect when SAFA is replaced by MUFA, whereas a small decrease occurs when they are substituted by n-6 PUFA (Mensink et al. 2003). Replacing SAFA with refined carbohydrates also reduces LDL cholesterol levels (by 0.16 mmol/L, 6.2 mg/

dL), but has untoward effects on other lipoproteins, namely, HDL cholesterol and plasma triglyceride levels.

Dietary trans-fatty acids (TFAs) are partially hydrogenated fatty acids formed when oils are solidified to produce margarine. Overall, the average intake of trans-fatty acids in western countries is presently low and derives mainly from foods of industrial origin. Quantitatively, dietary trans-fatty acids increase LDL cholesterol levels to the same extent as saturated fat, although, at variance with SAFA, they induce a marked reduction of HDL cholesterol levels (Mensink et al. 2003).

Among the various types of dietary fat, the most relevant effect on plasma triglycerides is achieved with long-chain *n*-3 polyunsaturated fatty acids; however, in order to reach an intake sufficient to induce a clinically relevant triglyceride-lowering effect (usually 25–30%), it is necessary to rely on foods artificially enriched with *n*-3 polyunsaturated fat or on pharmacological supplements. Dietary fatty acids may also affect other non-lipid CVD risk factors, particularly, blood pressure (Rasmussen et al. 2006; Zock et al. 2016). In fact, the results from the DIVAS study – a 16-week parallel group RCT on individuals with moderate CVD risk – showed that the isocaloric replacement of 9.5–9.6% of calories from SAFA with MUFA or *n*-6 PUFA attenuated the increase in night systolic pressure (–4.9 mmHg, $p = 0.019$) and reduced E-selectin. In line with this finding, a multicentre study in which our group was involved demonstrated that in healthy individuals, the consumption of a diet rich in MUFA for 3 months, compared to a SAFA-rich diet, significantly decreased diastolic blood pressure, provided that total fat intake was not exceedingly high (below 35–40% energy intake) (Rasmussen et al. 2006). The beneficial effects of replacing SAFA with unsaturated fat have been summarized in a meta-analysis reporting significantly lower systolic and diastolic blood pressure with high-MUFA diets (Schwingshackl et al. 2011). As to *n*-3 PUFA, lower blood pressure levels were observed only in hypertensive subjects given >3 g/day as pharmacological supplement (Miller et al. 2014).

Dietary fatty acids are also able to influence insulin sensitivity and, more in general, glucose metabolism; this was suggested by a recent meta-analysis of RCTs indicating that increased PUFA intake is able to improve long-term glycaemic control and reduce insulin resistance (Imamura et al. 2016). In particular, replacing 5% of calories from SAFA partially replacing SAFA or carbohydrates with PUFA or MUFA improved both blood glucose control and insulin sensitivity; moreover, PUFA increased also insulin secretion (Imamura et al. 2016). Other clinical trials have shown that the isocaloric replacement of SAFA with MUFA or PUFA improved insulin sensitivity in healthy subjects (Vessby et al. 2001) and also reduced hepatic fat accumulation (Summers et al. 2002). In line with these findings, Bozzetto and colleagues showed that partially replacing SAFA and carbohydrates with MUFA for an 8-week period induced a clinically relevant reduction of hepatic fat content (29%) in type 2 diabetic patients (Bozzetto et al. 2012).

1.1.2 Effects on CVD Events

TFAs represent one of the few dietary components unanimously considered as deleterious in relation to the cardiovascular risk. Data in the literature are very

consistent in showing that TFA intake is associated with a higher risk of CVD and sudden death; this relationship is stronger than with any other nutrient (Mozaffarian et al. 2006). In fact, a meta-analysis of four prospective cohort studies has shown that a 2% increase in energy intake from TFAs is associated with a 23% higher incidence of coronary heart disease (CHD) (Mozaffarian et al. 2006).

In recent years, concern has been expressed regarding the impact of dietary saturated fatty acids (SFAs) on cardiovascular risk. The uncertainty stems from recent meta-analyses failing to find an association between the amount of SFA in the habitual diet and the incidence of CVD (Chowdhury et al. 2014; Siri-Tarino et al. 2010). However, other meta-analyses of prospective studies and RCTs as well as large longitudinal observations clearly indicate that high SFA is linked with a small but potentially important increase in cardiovascular risk (Jakobsen et al. 2009; Li et al. 2015); recently, a Cochrane systematic review has shown that a low-saturated-fat diet induces a 17% reduction in cardiovascular events compared to a control diet (Hooper et al. 2015).

Inconsistencies are due to multiple reasons and, in particular, to the way the data are evaluated; in fact, the meta-analysis by Siri-Tarino overadjusted the outcomes by correcting them for plasma lipid levels, thus eliminating one of the major pathophysiological links between SFA intake and CVD (Siri-Tarino et al. 2010). More in general, in RCTs evaluating the effects of SFAs on CVD, the choice of the foods/nutrients utilized in the control diet to replace SFAs (for instance, unsaturated fat versus refined grains) can influence the effect observed and significantly modify the outcomes of the comparison. The importance of SFA as a risk factor for CVD has been recently underlined by recommendations of scientific societies and public health authorities (Sacks et al. 2017; USDA 2016).

PUFA intake is associated with lower CVD risk, as shown in a meta-analysis of 13 prospective cohort studies (310, 602 subjects) reporting that dietary linoleic acid (LA) intake is inversely associated with the risk of CHD incidence (15%) and mortality (21%) (Farvid et al. 2014).

The available data on the relationship between dietary cholesterol and CVD are inconsistent, probably due to the difficulty to evaluate the impact of dietary cholesterol independently of SAFA that is present in many cholesterol-rich foods. However, a recent meta-analysis from six prospective US cohorts in which many of the possible confounders were properly accounted for showed that each additional 300 mg of dietary cholesterol consumed per day (roughly one egg) was significantly associated with higher risk of CVD incidence (17%) and mortality (18%) (Zhong et al. 2019).

1.2 Dietary Carbohydrates (Table 1)

1.2.1 Effects on CVD Risk Factors

Dietary carbohydrates are the main determinants of postprandial blood glucose levels, which represent an important and independent risk factor for cardiovascular diseases, not only in diabetic patients but also in individuals with normal fasting

glucose values (Rivellese et al. 2012). There is a wealth of data indicating that a high intake of refined carbohydrates affects also plasma insulin and triglyceride levels, both at fasting and in the postprandial period, as well as HDL-cholesterol levels (Katan et al. 1997; Riccardi and Rivellese 1991; Sacks and Katan 2002). However, it is worth highlighting that dietary carbohydrates are a heterogeneous class of nutrients that include not only sugars and refined starches but also dietary fibre (i.e. non-starch polysaccharides), with different chemical structures and physical forms and therefore different metabolic effects in relation to their digestion and intestinal activity (Giacco et al. 2016). In line with this concept is the recent evidence supporting the hypothesis that the quality of dietary carbohydrates rather than their amount plays a relevant role in the prevention and development of major cardiovascular risk factors (Reynolds et al. 2019; Riccardi and Costabile 2019). The main indicators of carbohydrate quality refer essentially to the amount of fibre and the glycaemic index of carbohydrate-rich foods as well as the sugar content.

There is consolidated evidence of the beneficial role of consuming low-glycaemic index carbohydrates on blood glucose control, HbA1c levels, fasting and postprandial triglyceride levels and HDL cholesterol levels (Augustin et al. 2015; Riccardi et al. 2008; Thomas and Elliott 2010). However, the best available evidence of the beneficial metabolic impact of low-glycaemic index foods comes from studies in people with type 2 diabetes.

In the last decades, several clinical trials have been conducted to investigate the possible effects of different types of fibre – i.e. fibre from whole grain, legumes, fruit and vegetables and fibre supplements – on body weight changes, blood glucose metabolism, plasma lipids and blood pressure control. The overall evidence shows that soluble fibre, mainly β -glucans from oat and barley but also inulin, guar gum, glucomannan, pectin and psyllium principally found in fruit, vegetables and legumes, are able to significantly reduce plasma LDL-cholesterol levels, improve blood pressure (mainly β -glucan and psyllium) and induce a small reduction in body weight (Bozzetto et al. 2018). Moreover, there is evidence of a triglyceride-lowering effect of dietary fibre and whole grain during the postprandial period (Bozzetto et al. 2014; De Natale et al. 2009; Giacco et al. 2014).

These results support the current nutritional recommendations to replace refined grains with whole grains and increase dietary fibre intake to at least 25–29 g per day to reduce the incidence of the main cardiometabolic risk factors.

Among refined carbohydrates, specific attention should be paid to simple sugars, particularly fructose, whose adverse effects (at amounts higher than 10% of total energy) on human health have been highlighted. According to evidence from observational and intervention studies, the consumption of high fructose-sweetened beverages increases fasting and postprandial triglycerides levels, especially in subjects with obesity and hypertriglyceridemia (Chiavaroli et al. 2015; Stanhope et al. 2009), and also has adverse effects on visceral fat deposition, blood pressure and insulin sensitivity (Stanhope et al. 2009).

1.2.2 Effects on CVD Events

Since dietary carbohydrates are a heterogeneous class of nutrients with different metabolic effects, the evaluation of the relationship between the total amount of carbohydrates in the diet and the incidence of cardiovascular diseases is misleading. In fact, a recent prospective study in 135,335 individuals from 613 communities in 18 countries has shown, in contrast with the results from meta-analyses of several large cohort studies in North America and Europe, that a high carbohydrate intake is associated with higher risk of total mortality (Dehghan et al. 2017). The reasons for the inconsistency have been highlighted by a recent meta-analysis performed on data from four US communities showing that both very high and very low carbohydrate diets were associated with increased mortality: the lowest mortality was observed in people in whom carbohydrates represented 50–55% of their energy intake (Seidemann et al. 2018). However, the quality of carbohydrate-rich foods rather than their quantity has the strongest effect on the development of major health outcomes; in this respect, what really matters is the presence of vegetable fibre and/or sugar in the diet.

In fact, a recent meta-analysis reports a 15–30% decrease in all-cause and cardiovascular-related mortality and in the incidence of coronary heart disease between the highest and the lowest dietary fibre intake. The relationship between fibre intake and CVD was linear although the greatest risk reductions were observed for individuals with a fibre intake of 25–29 g/day, mainly provided by cereal fibre; a habitual consumption of 40–50 g/day of whole grains was associated with a risk reduction of 20–30% (Reynolds et al. 2019).

On the other hand, a higher consumption of sugar is associated with an increased risk of coronary events (17% for every serving/day increase in sugar-sweetened beverages consumption) (Xi et al. 2015).

1.3 Salt (Table 1)

1.3.1 Effects on CVD Risk Factors

Extensive scientific evidence shows that reducing dietary salt (sodium chloride) intake significantly decreases systolic and diastolic blood pressure in adults with or without hypertension (He et al. 2013; Stamler et al. 2018). Recent data from the international multicentre population INTERMAP study reported a positive association between salt intake and blood pressure values also within the normal range (Zhou et al. 2019). In addition to sodium reduction, an increase in potassium intake is beneficial to prevent and control blood pressure in people with hypertension, with no adverse effects on plasma lipid concentrations (Aburto et al. 2013a). In line with these findings, current nutritional recommendations include a potassium intake of at least 3.5 g per day in adults. Therefore, a diet that combines low sodium and high potassium intakes is more effective in reducing blood pressure and hypertension risk. An example of this type of approach is the DASH diet, recommended for the non-pharmacological management of hypertension. This diet includes also changes in the quality of fat – promoting unsaturated fat and carbohydrates – favouring

unrefined ones. Recent results from a systematic review and meta-analyses of RCTs support the beneficial effect of the DASH diet not only on blood pressure but also on other cardiovascular risk factors, including total and LDL cholesterol concentrations (Siervo et al. 2015).

1.3.2 Effects on CVD Events

Two recent meta-analyses of cohort studies showed a direct relationship between sodium intake and stroke, CVD and CHD mortality; a higher sodium intake was significantly associated with an increased risk of CVD (12%) and CHD (32%) mortality, in addition to a much higher risk of stroke mortality (63%) (Aburto et al. 2013b; Poggio et al. 2015). Consistently, two recent meta-analyses of clinical trials showed that dietary salt reduction induces a significantly lower incidence (−20%) of CVD (Adler et al. 2014; He and MacGregor 2011).

Actually, in many parts of the world, the average sodium consumption (mainly derived from dietary salt intake and food additives, such as sodium glutamate) is above that recommended by guidelines: less than 5 g of salt/day (equivalent to about 2.3 g of sodium daily) (WHO Guidelines Approved by the Guidelines Review Committee 2012).

In the last decades, the science of human nutrition has shifted from a reductionistic approach focused on specific nutrients to a broader view emphasizing the role of food groups/dietary patterns (van Horn et al. 2016). This paradigm change is due to convincing scientific evidence showing that human health is indeed influenced by single nutrients but also by their complex interactions and by their interplay with other bioactive substances present in foods. These are likely to act synergistically, and, therefore, their impact on human health may not be appreciated unless evaluated within the context of the whole diet. Furthermore, characteristics other than nutrient combinations (i.e. physical features of the foods, technological processes, cooking procedures) may influence the absorption and bioavailability of nutrients and in turn modulate their metabolic effects. Therefore, there is growing attention to the identification of dietary patterns associated to the risk of disease or death. The Mediterranean diet is one of dietary patterns for which strong evidence from observational and intervention studies has accumulated on the benefits in the primary and secondary prevention of cardiovascular disease and other major chronic diseases, such as type 2 diabetes, cancer and probably cognitive impairment (de Lorgeril et al. 1999; Estruch et al. 2018; Sofi et al. 2014).

The Mediterranean diet is characterized by a food pattern resembling the healthy food choices outlined above and, in particular, by the regular consumption of plant foods – including vegetables, pulses, nuts, fruits and unrefined cereals – and fish as well as a low intake of red and processed meats and whole fat dairy products; the main source of fat is extra virgin olive oil (Table 1). The optimal nutrient distribution and the balanced food choices of this dietary pattern have supported it as a useful model for the implementation of a healthy diet at the population level. Its strengths are not only the evidence in support of its beneficial impact on cardiovascular disease prevention but also its very deep cultural roots that are the source of the large body of

Table 2 Summary of lifestyle measures and healthy food choices for managing total cardiovascular risk

Dietary recommendations should always take into account local food habits; however, interest in healthy food choices from other cultures should be promoted
A wide variety of foods should be eaten. Energy intake should be adjusted to prevent overweight and obesity
Consumption of fruits, vegetables, legumes, nuts, wholegrain cereal foods and fish (especially oily) should be encouraged
Foods rich in trans-fatty acids should be avoided totally; foods rich in SFAs (tropical oils, fatty or processed meat, sweets, cream, butter, regular cheese) should be replaced with the above foods and with monounsaturated fat (extra virgin olive oil) and polyunsaturated fat (non-tropical vegetable oils) in order to keep SFA intake of <10 En% (<7% in the presence of high plasma cholesterol values)
Salt intake should be reduced to <5 g/day by avoiding table salt and limiting salt in cooking and by choosing fresh or frozen unsalted foods; many processed and convenience foods, including bread, are high in salt
For those who drink alcoholic beverages, moderation should be advised (<10 g/day for women and <20 g/day for men), and patients with hypertriglyceridaemia should abstain
The intake of beverages and foods with added sugars, particularly soft drinks, should be limited, especially for persons who are overweight, have hypertriglyceridaemia, metabolic syndrome or diabetes
Physical activity should be encouraged, aiming at regular physical exercise for at least 30 min/day every day
Use of and exposure to tobacco products should be avoided

culinary recipes to make a healthy choice a gastronomical adventure (Catapano et al. 2016) (Table 2).

2 Physical Activity

Having a sedentary life is a cardiovascular risk factor in itself. The amount of time spent being sedentary is increasing all over Europe even in young people. Watching TV, being stationary in front of a computer and using vehicles for transportation are the disadvantages of modern living. Especially in middle-aged females, sedentary lifestyle is almost as risky as smoking (Brown et al. 2015). Several studies have shown that sedentary behaviour increases cardiovascular risk. One of the largest prospective registries was the PURE study where physical activity was recorded in 130, 843 participants without existing CVD for 6.9 years. Compared with low physical activity (<600 metabolic equivalents [MET] × minutes per week or <150 min per week of moderate-intensity physical activity), moderate (600–3,000 MET × minutes or 150–750 min per week) and high physical activity (>3,000 MET × minutes or >750 min per week) were associated with graded reduction in mortality and major cardiovascular events (Lear et al. 2017).

Experimental studies that mimicked sedentary behaviour have shown greater postprandial glucose and insulin levels during bouts of prolonged sitting compared

with individuals taking frequent standing or walking breaks (Pulsford et al. 2017). Compared with prolonged sitting, breaking up sitting time with intermittent, even light-intensity activity, can increase expression of anti-inflammatory and antioxidative pathway modulators (Latouche et al. 2013). A recent meta-analysis investigated the association between sedentary behaviour and incident CVD events using data from nine prospective cohort studies and showed that risk increased with increased sedentary time, with the highest risk being in more than 10 h a day of sedentary time (Pandey et al. 2016). A recent study has shown that sustained physical activity but not weight loss was associated with improved survival in coronary heart disease (Moholdt et al. 2018).

Physical activity has a positive effect on body weight, blood pressure, blood glucose, lipid levels, endothelial function, autonomous regulation and coagulation. The summary of all these effects leads to a decrease in cardiovascular disease incidence and mortality. Physical activity has both acute and chronic effects on blood pressure. With increased physical activity, blood pressure is lowered especially in subjects with prehypertension compared to those with normal blood pressure (Physical Activity Guidelines Advisory Committee 2018). Physical activity combined with calorie restriction can contribute to weight loss and supports the maintenance of weight loss. The risk of type 2 diabetes mellitus is greatly reduced with physical activity, and this benefit is observed irrespective of body weight. Engaging in 150–300 min a week of moderate-intensity physical activity can reduce the risk of developing type 2 diabetes mellitus by 25–35% (Physical Activity Guidelines Advisory Committee 2018). Regular physical activity or exercise can affect serum lipid levels favourably. Men and women who exercise regularly have significantly lower LDL-C and VLDL and higher HDL-C levels compared to age- and gender-matched sedentary controls (Vodak et al. 1980; Wood et al. 1976). Similarly, moderate physical exercise decreases LDL-C and triglycerides and increases HDL in male survivors of myocardial infarction (Ballantyne et al. 1982). The effect of exercise on lipids varies in exercise intervention studies, and there appears to be a minimum exercise volume to increase HDL-C (Kodama et al. 2007). There are additional antiatherogenic effects of physical activity besides those affecting traditional cardiovascular disease risk factors (Green et al. 2017). Exercise intensity improves vascular endothelial function in a dose-dependent manner for aerobic activities (Ashor et al. 2015). Endothelial function is improved with exercise even in the absence of changes in classical risk factors like lipids levels, blood pressure, glucose or BMI (Green et al. 2008). Acute- and moderate-intensity exercises increase shear stress, which stimulates endothelium-dependent vasodilatation through the increased synthesis of nitric oxide (Erkens et al. 2017). Regular exercise training also leads to increased coronary artery size and dilatation capacity (Haskell et al. 1993) and increased luminal diameter of conduit arteries (Green et al. 2008). Exercise training can reduce the wall thickness of conduit arteries and increases the development of coronary collateral blood vessels (Thijssen et al. 2012). Unhealthy gut microbiota has recently been shown to increase the risk of CVD (Fu et al. 2015; Lanter et al. 2014). Regular physical activity or endurance exercise training can positively alter the human gut microbiota by increasing

bacterial diversity, increasing faecal concentrations of short-chain fatty acids and increasing the proportion of healthy bacterial species (Allen et al. 2018; Bressa et al. 2017; Estaki et al. 2016). A recent study on endurance training has shown that exercise increases telomerase activity and length, whereas resistance training did not show these effects (Werner et al. 2019). Ideally, a combination of resistance and endurance training should be implemented (Reiner et al. 2019).

Physical activity is any activity that moves the skeletal muscles and consumes energy, whereas systematic physical activity, like swimming and running, is described as exercise. In healthy individuals, regular physical activity decreases all-cause and cardiovascular mortality by 20–30% (Lollgen et al. 2009; Moore et al. 2012; Sattelmair et al. 2011). Regular physical activity increases cardiorespiratory fitness (The American College of Sports Medicine 2014). Cardiorespiratory fitness is associated with reduced prevalence of CVD risk factors and improves prognosis (Harber et al. 2017). A meta-analysis of 33 studies in over 100,000 individuals observed that every 1 estimated MET increase in CRF was associated with 13 and 15% reductions in all-cause and CVD mortality (Nauman et al. 2017). Cardiorespiratory fitness is important even in the presence of other risk factors and shown to be protective even in men with metabolic syndrome (Katzmarzyk et al. 2004) and obesity (Moholdt et al. 2017).

Although all agree that any level of physical activity is better than none, there is controversy about the optimal dose of physical activity for CVD prevention. The intensity and frequency of exercise should be personalized and adapted to a person's needs as well as lifestyle. We also need to keep in mind that there is a high degree of interindividual variation in cardiorespiratory fitness responses to exercise.

The 2016 ESC prevention guideline recommends (Piepoli et al. 2016) for healthy adults of all ages to perform at least 150 min a week of moderate-intensity or 75 min a week of vigorous-intensity aerobic physical activity (PA) or an equivalent combination. For additional benefits in healthy adults, a gradual increase in aerobic PA to 300 min a week of moderate-intensity or 150 min a week of vigorous-intensity aerobic PA, or an equivalent combination thereof, is recommended. Multiple sessions of PA should be considered, each lasting ≥ 10 min and evenly spread throughout the week. Clinical evaluation, including exercise testing, should be considered for sedentary people with cardiovascular risk factors who intend to engage in vigorous PAs or sports.

Even low-level physical activity, such as low-dose running or commuting to work by bicycle, has been associated with a lower incidence of obesity, arterial hypertension, dyslipidaemia and diabetes mellitus (Grontved et al. 2016). The most recent Physical Activity Guidelines for Americans from the US Department of Health and Human Services has stated that benefits related to physical activity start even earlier and are easier to obtain than was previously thought according to recent evidence (Physical Activity Guidelines Advisory Committee 2018). These guidelines state that although one should try to get to guideline-recommended goals, the threshold at which health benefits begin to start is less than 150 min a week for most outcomes. There is no lower limit to the benefits of physical activity in reducing cardiovascular disease risk. However, getting to goal helps in obtaining benefits for the greatest

number of outcomes. Additional physical activity confers additional benefits, and health risk does not seem to increase with high amounts of physical activity, even beyond 3–5 times the 150 min a week recommendation in healthy individuals. Compared with inactive adults, meeting the goals is associated with a 14% reduced risk of developing coronary heart disease (Physical Activity Guidelines Advisory Committee 2018).

2019 ACC/AHA Guideline on the Primary Prevention of Cardiovascular Disease (Arnett et al. 2019) also recommends that adults should engage in at least 150 min per week of accumulated moderate-intensity or 75 min per week of vigorous-intensity aerobic physical activity to reduce atherosclerotic cardiovascular disease. For adults unable to meet the minimum physical activity recommendations, engaging in some moderate- or vigorous-intensity physical activity, even if less than this recommended amount, and decreasing sedentary behaviour can be beneficial to reduce CVD risk.

Although some exercise is beneficial for all, for those with known cardiovascular disease, the dose of exercise needs to be individualised to prevent adverse outcomes. The risk of having an adverse cardiovascular event during exercise is very low, especially during light exercise. However, it is best to do a risk assessment and tailor the exercise program according to the need of the individual. Light- and moderate-intensity exercise can be done with minimal risk, whereas intensive exercise should be preceded by a medical evaluation, especially for middle-aged people. The cardioprotective effects of regular PA, whether performed in low or high volumes, are clear and extend across all ages, gender and race (El Saadany et al. 2017; Nes et al. 2017; O'Donovan et al. 2018). Whether overexercising in healthy individuals can have unfavourable consequences has been questioned. In some studies, long-term strenuous exercise has been shown to increase the risk of atrial fibrillation (Gorenek Chair et al. 2017). It is never too late to start exercising. The Aerobics Center Longitudinal Study has shown that men who were in the lowest percentile of CRF at their first examination but fit at the time of their second examination years later had a 52% reduction in CVD mortality compared with men who remained unfit (Blair et al. 1995). For every one estimated MET increase, all-cause and CVD mortalities were reduced by 15% and 19%, respectively (Lee et al. 2011).

3 Smoking

Smoking is one of the most important modifiable risk factors for CVD. Furthermore, stopping smoking is the most cost-effective prevention intervention. It is well known that smoking and using all forms of tobacco products cause CVD and increase mortality (Mons et al. 2015; Teo et al. 2006). Because tobacco use even in small amounts increases cardiovascular risk, reducing the number of cigarettes or the amount of tobacco consumption is not sufficient. Even smoking one cigarette per day carries a risk (Hackshaw et al. 2018). Therefore there is no safe level of smoking and quitting is mandatory. Passive smoking also increases cardiovascular risk, and

studies have shown that second-hand smoke exposure increases CVD and triggers adverse events in non-smokers (Lv et al. 2015).

Cigarette smoke contains more than 4,000 chemicals and toxins like nicotine, carbon monoxide, cadmium and oxidants (Smith and Fischer 2001). Exposure to these chemicals affects all stages of atherosclerotic vascular disease unfavourably. Endothelial dysfunction due to reduced NO bioavailability and activation of NF- κ B will lead to functional and eventually physical damage to the endothelium (Collins 1993; Rahman and Laher 2007). Nicotine has been shown to promote MMP expression in smooth muscle cells and decreased collagen synthesis in the arterial wall impairing the stability of the plaque (Carty et al. 1996; Raveendran et al. 2004) Cigarette smoke increases systemic inflammation (Csordas et al. 2008). The catecholamines are increased in the circulation triggering vasospasm (Zhu and Parmley 1995). Smoking also alters the balance between pro- and anticoagulant factors towards procoagulation. Plaque thrombogenicity is increased with increased plasma tissue factor, VWF, thrombin and thrombomodulin and activated platelets (Markuljak et al. 1995; Miller et al. 1998). Upon smoking cessation, several of these prothrombotic changes revert to normal. Blood viscosity is also increased with smoking (Lowe et al. 1980). All of these effects contribute to plaque formation, vulnerability and thrombus development. Interestingly, there is a genetic susceptibility to the atherothrombotic effects of smoking. Different variants have been described that alter one's susceptibility to the negative effects of smoking (Wang et al. 1996).

Smoking dependence is a chronic disease that needs to be managed with patience, expertise and time. The ESC prevention guidelines recommend the following strategy for smoking cessation (Piepoli et al. 2016): systematically inquire about smoking status at every opportunity; unequivocally urge all smokers to quit; determine the person's degree of addiction and readiness to quit; agree on a smoking cessation strategy, including setting a quit date, behavioural counselling and pharmacological support; and arrange a schedule of follow-up ("five As" for a smoking cessation strategy for routine practice – ask, advise, assess, assist, and arrange). In case where advice and motivational interventions fail, drug-based interventions can be used to aid quitting smoking. Nicotine replacement therapies, bupropion and varenicline, have been shown to be helpful in increasing quitting (Cahill et al. 2013; Hughes et al. 2014; Stead et al. 2012). Electronic cigarettes emitting an aerosol containing nicotine have been developed to aid quitting; however, they may also emit toxic gases that may increase the risk of cardiovascular and pulmonary diseases, arrhythmias and hypertension (Benowitz and Fraiman 2017). Patients often are concerned about weight gain after smoking cessation. However a recent study from the cohorts of the Nurses' Health Study (NHS), (NHS II) and the Health Professionals Follow-Up Study (HPFS) has shown that even if the smoking cessation was accompanied by substantial weight gain, this did not decrease the benefits of quitting smoking on reducing cardiovascular and all-cause mortality and cardiovascular mortality decreased in all weight-change groups (Hu et al. 2018).

Modern lifestyles expose us to air pollution and occupational noise which also have a negative impact on cardiovascular diseases. It has been shown that the risk of

STEMI rises within hours of exposure to air pollutants (Sahlen et al. 2019). Furthermore, several investigations support that inhalation of ambient particulate matter triggers pulmonary and systemic inflammation resulting in metabolic syndrome and cardiopulmonary disease (Clementi et al. 2019). Exposure to high occupational noise is also associated with increase in hypertension, cardiovascular disease and cardiovascular mortality (Skogstad et al. 2016; Teixeira et al. 2019).

A healthy lifestyle is critically important to improve cardiovascular health and increase the control of risk factors. Healthy lifestyles should be adapted from birth to childhood and maintained throughout the lifespan. This can be possible only with creating health-promoting environments and improving healthcare policies, the interaction between the patient and the physician and education of the patient and the public about healthy lifestyles.

The WHO has put together a global action plan for the prevention and control of non-communicable diseases (WHO 2013). The goal is to reduce the risk of premature non-communicable disease deaths by 25% by 2025. According to this plan, reductions in tobacco use, salt intake, physical inactivity, harmful use of alcohol, raised blood pressure as well as stopping the diabetes and obesity epidemics are the goals. Early action and aggressive implementation of this plan may decrease the cardiovascular disease epidemic if the individual WHO targets are met and healthcare systems are strengthened. At the individual level, it is important for us to promote healthy lifestyles to everyone, including patients who already have CVD since it is never too late to benefit from a healthy lifestyle.

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Blood Pressure-Lowering Therapy

Isabella Sudano , Elena Osto , and Frank Ruschitzka 

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Abstract

Extensive evidence demonstrates that lowering blood pressure can substantially reduce the risk of atherosclerotic cardiovascular disease and death.

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In light of the latest 2018 European Society of Cardiology/European Society of Hypertension Joint Guidelines, we summarize the current recommendations about lifestyle intervention strategies, pharmacotherapy, and device-based treatments for the management of arterial hypertension. Special attention is given to direct effects exerted by some antihypertensive drugs targeting vascular wall cell components that are involved in the pathogenesis of atherosclerosis.

Keywords

Blood pressure medical treatment · Cardiovascular risk factors · Device therapy · Hypertension · Hypertension-driven atherosclerotic complications · Lifestyle interventions

Abbreviations

ACE	Angiotensin-converting enzyme
Ang II	Angiotensin II
ARBs	Angiotensin II receptor blockers
BP	Blood pressure
CCBs	Calcium channel blockers
CVD	Cardiovascular disease
ESC	European Society of Cardiology
ESH	European Society of Hypertension
MR	Mineralocorticoid receptor
NO	Nitric oxide
ROS	Reactive oxygen species
SHF	Swiss Heart Foundation
SNSF	Swiss National Science Foundation

1 Introduction

Continuous progress in understanding the epidemiology, pathophysiology, and pharmacology of arterial hypertension has consistently improved the possibility of an efficient and safe treatment of elevated blood pressure (BP).

Extensive evidence demonstrates that lowering BP can substantially reduce the risk of cardiovascular disease (CVD) and death with similar proportional reductions across various population subgroups. Every 10 mmHg systolic BP reduction significantly diminished the risk of major CVD events (RR 0.80, 95% CI 0.77–0.83), coronary heart disease (0.83, 0.78–0.88), stroke (0.73, 0.68–0.77), heart failure (0.72, 0.67–0.78), and all-cause mortality (0.87, 0.84–0.91) (Ettehad et al. 2016).

Arterial hypertension is characterized by structural and functional changes in blood vessels, which lead to increased arterial stiffness, vascular inflammation, endothelial dysfunction, fatty streaks, early atherosclerotic plaque, plaque progression, and plaque rupture. Vice versa, arterial stiffness, endothelial dysfunction, and vascular inflammation may also contribute to increased BP. Several lifestyle

Table 1 From ESC/ESH guidelines for hypertension, Eur Heart Journal 2018 (©ESC/ESH 2018)

Table 3. Classification of office blood pressure ^a and definitions of hypertension grade ^b			
Category	Systolic (mmHg)		Diastolic (mmHg)
Optimal	<120	and	<80
Normal	120–129	and/or	80–84
High normal	130–139	and/or	85–89
Grade 1 hypertension	140–159	and/or	90–99
Grade 2 hypertension	160–170	and/or	100–109
Grade 3 hypertension	≥180	and/or	≥110
Isolated systolic hypertension ^b	≥140	and	<90

BP blood pressure, SBP systolic blood pressure

The same classification is used for all ages from 16 years

^aBP category is defined according to seated clinic BP and by the highest level of BP, whether systolic or diastolic

^bIsolated systolic hypertension is graded 1, 2, or 3 according to SBP values in the ranges indicated

interventions and drugs lowering BP were demonstrated to improve endothelial function, decrease arterial stiffness and vascular inflammation, and ultimately prevent the development and/or progression of atherothrombosis.

After exclusion of the main causes of a secondary hypertension, lifestyle modifications should be suggested as BP and cardiovascular risk lowering strategy to every patient with arterial hypertension. However, sooner or later, most patients diagnosed with arterial hypertension will require a pharmacological therapy.

According to the 2018 ESC/ESH Guidelines (Williams et al. 2018), the necessity of a pharmacological therapy will be defined by the grade of arterial hypertension (see Table 1), the cardiovascular risk, and the presence of hypertension-mediated organ damage or concomitant diseases such as a history of cardiovascular events, diabetes mellitus, or chronic kidney disease. The 2018 Guidelines suggest as general rule to reduce office BP below 140/90 mmHg aiming to reach a BP around 130/80 mmHg; see Table 1.

The ultimate goal of antihypertensive therapy is the prevention of cardiovascular events. The higher the absolute cardiovascular risk, the more likely it is that a patient will benefit from a more aggressive BP goal. However, although cardiovascular events generally decrease with more intensive lowering of BP, the risk of adverse effects, cost, and patient inconvenience increase as more medication is added (Ettihad et al. 2016; Williams et al. 2018). See Fig. 1.

This recommendation together with good clinical judgment and shared decision-making between patients and care providers should guide our BP-lowering therapy.

2 Non-pharmacological Therapy

Treatment of hypertension should always include non-pharmacological therapy (Fig. 1) (Williams et al. 2018).

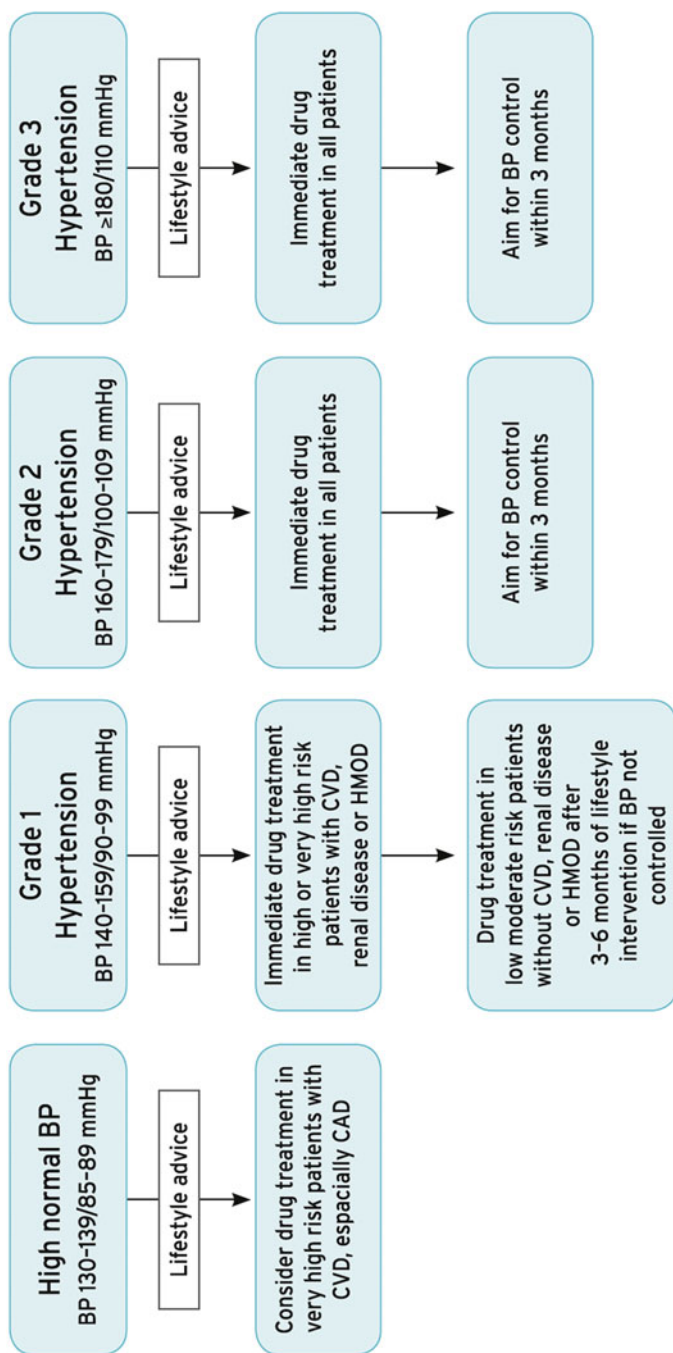


Fig. 1 From ESC/ESH guidelines for hypertension, Eur Heart Journal 2018 (©ESC/ESH 2018)

The ESC/ESH Guidelines (Williams et al. 2018) suggested the following lifestyle changes as contributors for reducing BP and cardiovascular risk to the majority of patients with arterial hypertension: healthy diet including dietary sodium restriction and moderation of alcohol consumption, overweight reduction, regular physical activity, and cessation of consumption of any product containing tobacco or nicotine.

2.1 Salt Restriction

In general, a healthy diet should avoid any excess: moderate sodium reduction is associated with a decrease in BP in hypertensive and normotensive individuals of a maximum of 4.8–2.5 mmHg systolic and 1.9–1.1 mmHg diastolic, respectively (He and MacGregor 2003).

Young patients with hypertension usually are salt-resistant, while older patients as well as obese individuals or patients with diabetes mellitus and chronic kidney disease are characterized by increased salt sensitivity (Weinberger 1996).

Evidence supporting very strong reduction of salt intake is weak. The effect of reduced dietary sodium on cardiovascular event rates remains unclear (Bibbins-Domingo et al. 2010; He et al. 2011; He and MacGregor 2011; Taylor et al. 2011).

Prospective cohort studies have reported an overall increased risk of mortality and cardiovascular events on high sodium intake, but to date, no prospective randomized controlled trial has provided definitive evidence about the optimal sodium intake to minimize cardiovascular events and mortality. However, it was reported that reducing sodium intake below 3 g of sodium per day further reduced BP, but paradoxically was associated with an increased risk of all-cause and cardiovascular mortalities in both the general population and in hypertensive patients, suggesting a J-curve phenomenon (Mente et al. 2016).

Increased potassium intake is associated with BP reduction and may have a protective effect, thereby modifying the association between sodium intake, BP, and CVD. Increasing potassium intake (e.g., including high intake of vegetables and fruits in the diet) could be a problem in patients with diabetes and chronic kidney disease and cannot be applied to all hypertensive patients (Bernabe-Ortiz et al. 2020; Binia et al. 2015; Mente et al. 2016; Miller et al. 2016).

Therefore, every patient with arterial hypertension independently of his/her sodium sensitivity should reduce the sodium consumption and avoid processed and frozen food, which frequently is rich in salt.

2.2 Reduce Alcohol Intake

For a long time, positive linear associations between alcohol consumption, BP, prevalence of hypertension, and cardiovascular risk have been established.

The Prevention and Treatment of Hypertension Study (PATHS) investigated the effects of reduced alcohol consumption on BP; the intervention group had a modest

1.2/0.7 mmHg lower BP than the control group at the end of the 6-month follow-up period. A meta-analysis of 56 epidemiological studies suggested that reduction of alcohol consumption, even for light-moderate drinkers, might be beneficial for cardiovascular health (Holmes et al. 2014). Binge drinking can cause strong increases of BP (Mancia et al. 2013).

Hypertensive men and women, who drink alcohol, should be advised to limit their consumption to 14 units and 8 units per week, respectively (one unit is equal to 125 mL of wine or 250 mL of beer). Alcohol-free days during the week and avoidance of binge drinking are also recommended (Williams et al. 2018).

2.3 Weight Loss and Avoidance of Overweight and Obesity

Weight loss in overweight or obese individuals leads to a significant reduction in BP, independently of exercise and dietary sodium restriction (Appel et al. 2006; Cohen and Gadde 2019), and has multiple beneficial effects against pathologic factors leading to high BP and to end-organ damage in this patient population (Cohen and Gadde 2019).

Weight loss is associated with decreased intra-abdominal pressure exerted on vessels by the excessive visceral fat deposition. Another beneficial effect of weight loss is the amelioration of insulin resistance, which is associated with renal sodium reabsorption and increased sympathetic tone in obesity. Moreover, chronic inflammation associated with overweight/obesity promotes vascular aging, favoring the onset of hypertension. Inflammation also contributes to arterial stiffness and impairs the physiologic anti-contractile effect of perivascular adipocytes on adjacent small arteries (Viridis et al. 2015; Aghamohammadzadeh et al. 2013). Decrease in body weight with lifestyle management, although effective in the short term, is difficult to sustain in the longer-term follow-up. Indeed, the overall efficacy of lifestyle interventions in reducing cardiovascular outcomes has been questioned by the results of the Action for Health in Diabetes (Look AHEAD) Study (Look, Ahead Research Group et al. 2013; Semlitsch et al. 2016). Currently, the most effective pharmacological treatments against obesity include glucagon like peptide-1 (GLP-1) receptor agonists and bariatric surgery. GLP-1 receptor agonists, in particular liraglutide, are cornerstones in antidiabetic therapy, which also have shown positive effects in reducing BP and CVD mortality (Helmstadter et al. 2020; Pi-Sunyer et al. 2015; Mingrone et al. 2015). Liraglutide is currently available also as weight-loss medication in obese patients without diabetes and has promising effects improving hypertension and cardiovascular risk profile over 1-year treatment (Pi-Sunyer et al. 2015; Fonseca et al. 2014; le Roux et al. 2017).

Bariatric surgery has a sustained effect on weight loss, which is superior to pharmacological and lifestyle modifications. Considerable weight loss after bariatric surgery corresponds to high rates of remission of hypertension (Pareek et al. 2019). The GATEWAY trial showed that a reduction of $\geq 30\%$ of the total number of antihypertensive medications while maintaining controlled blood pressure occurred in 83.7% of the patients randomized to receive the Roux-en-Y gastric bypass plus

antihypertensive medical therapy compared with only 12.8% patients from the control group with pharmacological therapy alone. Indeed, remission of hypertension was present in almost 50% of patients randomized to gastric bypass, whereas no patient randomized to control therapy was free of antihypertensive drugs at 12-month follow-up (Schiavon et al. 2018).

2.4 Regular Physical Activity

Epidemiological studies suggest that regular aerobic physical activity is beneficial for both reducing BP and decreasing CVD event rates and mortality (Franklin et al. 2020; Williams et al. 2018). As a result and along with the notion that “more exercise is better,” more and more normotensive and hypertensive adults have increased their participation in high-intensity interval training or competitive long distance endurance events. However, the quality and intensity of the physical activity is very important. Recent evidence suggests that beyond a safe upper limit, exercise may result in deleterious cardiovascular adaptations. For instance, exercise-induced hypertension and the race distances may contribute to the occurrence of myocardial fibrosis detectable by MRI in asymptomatic triathletes (Tahir et al. 2018).

Aerobic endurance training, dynamic resistance training, and isometric training reduce resting systolic BP and diastolic BP by mean 3.5/2.5, 1.8/3.2, and 10.9/6.2 mmHg, respectively, in general populations. Regular physical activity of lower intensity and duration lowers BP less than moderate- or high-intensity training, but is associated with at least a 15% decrease in mortality in cohort studies (Rossi et al. 2012). Thus, the current ESC/EHS Guidelines recommend at least 30 min of moderate dynamic exercise (walking, jogging, cycling, or swimming) on 5–7 days per week (Williams et al. 2018). The impact of isometric exercises on BP and CVD risk is less well-established, although it can be part of a comprehensive treatment regimen (Chrysant 2010).

3 Pharmacological Therapy for the Treatment of Arterial Hypertension

Current evidence suggests that treatment and gradual control of hypertension by the use of the major classes of antihypertensive drugs exert positive effects on atherosclerosis. Randomized controlled trials provided robust evidence that five drug classes lower BP and prevent CVD events. They are therefore recommended by the 2018 ESC/ESH Guidelines (Williams et al. 2018) for the treatment of hypertension: angiotensin-converting enzyme (ACE) inhibitors, angiotensin II receptor blockers (ARBs), beta-blockers, calcium channel blockers (CCBs), and diuretics (thiazides and thiazide-like diuretics such as chlorthalidone and indapamide). ACE-I or ARBs alone or in combination with a calcium antagonist or a diuretic (thiazide-like to be preferred to thiazide) are considered for the first-line treatment. The use of beta-blockers is limited to special indications.

3.1 Who Should Be Treated with Pharmacological Therapy?

The benefits of antihypertensive therapy are clear in the majority of patients with arterial hypertension, but still controversial in subgroups. They include patients with Grade 1 hypertension but without manifest CVD, patients with white coat or masked hypertension and low cardiovascular risk, patients with an estimated 10-year cardiovascular risk <10%, and patients older than 75 years of age who are non-ambulatory or living in nursing homes.

Randomized trials demonstrated that treating hypertension with any antihypertensive therapy reduces cardiovascular morbidity and mortality. According to the ESC/ESH Guidelines, antihypertensive drug therapy should be initiated without any delay in patients with hypertension Grades 2 or 3 or after some time under non-pharmacological therapy in individuals with very high cardiovascular risk and Grade 1 or high-normal BP (Fig. 1). The decision to initiate drug therapy should be individualized and involve shared decision-making between patient and provider.

3.2 Choice of Initial Antihypertensive Agents

Multiple studies and meta-analyses conclude that the degree of BP reduction rather than the kind of antihypertensive medication is the major determinant of reduction in cardiovascular risk in patients with hypertension (Ettehad et al. 2016).

However, not all antihypertensive drugs are equally effective in reducing cardiovascular events and only few reduced mortality (van Vark et al. 2012).

Recommendations for the use of specific classes of antihypertensive medications are based upon clinical trial evidence of decreased cardiovascular risk, BP-lowering efficacy, safety, and tolerability. Most patients with hypertension will require more than one BP medication to reach their BP goal. As the consequence, the new guideline for treatment of hypertension suggests to use combination therapy at early stage and, if possible, a fixed-dose single-pill combination medication to improve adherence (Williams et al. 2018). Having multiple available classes of BP medications, practitioners and clinicians can individualize therapy based upon individual patient characteristics and preferences.

The following drugs are suggested for the start of monotherapy or combination therapy of arterial hypertension:

- ACE inhibitors
- ARBs
- Long-acting CCBs (most often a dihydropyridine such as amlodipine)
- Thiazide-like or thiazide-type diuretics

The previous 2013 Guidelines (Mancia et al. 2013) favored monotherapy for the start of treating hypertension. However, only a minority of patients reach the target blood pressure level under monotherapy, and the combination of two drugs is much more efficient than increasing the dose of a single drug (Williams et al. 2018).

Therefore, the current 2018 Guidelines limit the start of monotherapy to low-risk patients with stage 1 hypertension whose SBP is <150 mmHg, very high-risk patients with high-normal BP, or frail older patients; see Table 1.

3.3 Combination Therapy

Single-agent therapy will not adequately control BP in most patients whose baseline systolic BP is 15 mmHg or more above their goal. Combination therapy with drugs from different classes has a substantially greater BP-lowering effect than doubling the dose of a single agent (Wald et al. 2009).

When more than one agent is needed to control BP, a therapy with a long-acting ACE inhibitor or ARB in combination (fix if this is possible) with a long-acting dihydropyridine CCB or a diuretic is the first choice. Combination of an ACE inhibitor or ARBs with a thiazide diuretic is considered less beneficial, when hydrochlorothiazide instead of thiazide-like diuretic (chlorthalidone or indapamide) is used (Burnier et al. 2019; Williams et al. 2018). ACE inhibitors and ARBs should **not** be used together.

Chlorthalidone and indapamide have been used in several RCTs showing cardiovascular benefits, and these agents are more potent per milligram in lowering BP and have a longer duration of action compared with hydrochlorothiazide without any evidence of more side effects (Williams et al. 2018).

As such, even if head-to-head RCTs are missing, this data suggests that thiazide-like diuretics such as chlorthalidone and indapamide should be preferred over classical thiazide diuretics (e.g., hydrochlorothiazide and bendrofluazide) (Williams et al. 2018; Burnier et al. 2019; Roush et al. 2015).

The Danish Cancer Registry and the Danish Prescription Registry examined the association between the use of hydrochlorothiazide (HCTZ) and the risk of basal cell carcinoma, squamous cell carcinoma, and nodular melanoma (Pedersen et al. 2018a, 2018b, 2019). These two case-control studies showed that high cumulative doses of HCTZ (>50 g) are associated with a dose-dependent increase in the risk of non-melanoma skin cancer, but not of melanoma. The increase of risk was only small for squamous cell carcinoma and negligible for basal cell carcinoma. These studies have several limitations including the investigation of a pale-skinned population and the lack of information on genetic predisposition, sun habits, and ultraviolet exposure. Moreover, the risk reduction of death due to lower BP by HCTZ was much stronger than the small risk increase for squamous cell carcinoma by HCTZ. In general, statistically significant associations from observational studies do not prove any causal relationship.

The next step is the combination of RAAS blocker, Ca antagonists, and thiazide/thiazide-like diuretics.

If BP is not sufficiently controlled by this triple combination therapy, a mineralocorticoid receptor (MR) antagonist (i.e., spironolactone or eplerenone) may be added (Williams et al. 2015).

In patients with difficult-to-treat/resistant hypertension, a beta-blocker, an alpha-blocker, or a direct arterial vasodilator could be added.

Generally, concomitant use of beta-blockers and non-dihydropyridine CCBs should be avoided, as both drug classes reduce heart rate.

3.4 Direct Effects of Antihypertensive Drugs on Atherosclerosis

Apart from lowering blood pressure and thereby removing an important risk factor, some antihypertensive drugs appear to exert direct effects on vascular cells that are involved in the pathogenesis of atherosclerosis.

3.4.1 ARBs and ACE Inhibitors

ARBs and ACE inhibitors directly affect the renin-angiotensin-aldosterone system (RAAS), by blocking the binding of angiotensin II (Ang II) to the AT1 receptor and decreasing the production of Ang II, respectively. Hypertension promotes and accelerates the atherothrombotic process via inflammatory mechanisms linked to activation of oxidative stress by Ang II, which subsequently leads to endothelial dysfunction and development of atherogenic lesions and plaques. Endothelial dysfunction is observed in the early stages of atherosclerosis. A healthy endothelium induces vasodilatation and has antioxidant and anti-thrombotic effects. The dysfunctional endothelium releases less of nitric oxide (NO) and other protective molecules, has a disrupted redox balance, and acquires pro-constrictive and pro-thrombotic phenotypes (Flammer et al. 2012; Sudano et al. 2011). A dysfunctional endothelium has been associated with cardiovascular risk factors including diabetes mellitus or impaired glucose metabolism, hypertension, cigarette smoking, dyslipidemia, obesity, and/or metabolic syndrome (Flammer et al. 2012; Sudano et al. 2011).

RAAS antagonists, as well as some dihydropyridine CCBs, possess ancillary and synergistic effects that increase NO bioavailability, reduce oxidative stress, and suppress inflammatory responses, thereby improving both endothelial activity and vascular function (Safar and Smulyan 2007; Sudano et al. 2011; Taddei et al. 2002).

3.4.2 Diuretics

Among the diuretics, thiazide-like diuretics such chlorthalidone and indapamide but not hydrochlorothiazide were found to improve endothelial function (Dell'Omo et al. 2005; Vinereanu et al. 2014). Indapamide also reduces arterial stiffness (Agnoletti et al. 2013).

3.4.3 Calcium Antagonists

Dihydropyridine CCBs lower BP mainly through vasodilation and reduction of peripheral resistance. Several clinical studies have demonstrated that they have clinical benefits in patients with CVD. Some studies have indicated that dihydropyridine CCBs have anti-atherogenic effects beyond their BP-lowering effects (Silva et al. 2019; Sudano et al. 2011). In fact, in several animal models, dihydropyridine CCBs were found to suppress the formation of atherosclerotic

lesions. It is well-known that the production of reactive oxygen species (ROS) is involved in the progression of atherosclerosis by stimulating the production of inflammatory factors such as chemokines, cytokines, and adhesion molecules (Mason 2002; Ishii et al. 2012). Dihydropyridine CCBs can suppress ROS generation and subsequent inflammatory actions in vascular cells and arterial walls. Furthermore, several reports have revealed that dihydropyridine CCBs suppress the expression of adhesion molecules, thereby inhibiting monocyte adhesion to endothelial cells, which is an early step in the pathogenesis of atherosclerosis. Dihydropyridine CCBs also suppress proliferation and migration of smooth muscle cells both *in vitro* and *in vivo* (Mason 2002; Ishii et al. 2012). In macrophages, dihydropyridine CCBs decrease cholesterol accumulation and intracellular cholesterol esterification and increase cholesteryl ester hydrolysis. Moreover, dihydropyridine CCBs suppress the expression of matrix metalloproteinases, which affects the stability of atheromatous plaques. Interestingly, recent studies have revealed that the anti-atherosclerotic effects of dihydropyridine CCBs are mediated, at least in part, via the activation of peroxisome proliferator-activated receptor- γ (Ishii et al. 2012).

3.4.4 Beta-Blockers

In general, beta-blockers usually do not have any effect on endothelial function and atherothrombosis. The only exception is nebivolol, thanks to its high selectivity as beta1-blocker. Nebivolol inhibits the proliferation of human coronary smooth muscle and endothelial cells (Brehm et al. 2001). The specific vasorelaxant properties of nebivolol are mediated by endothelium-dependent NO release and antioxidant activity (do Vale et al. 2018). Unfortunately, nebivolol treatment in patients with non-obstructive coronary artery disease was associated with greater plaque progression and constrictive remodeling as compared to atenolol (Hung et al. 2016). Carvedilol, a nonselective blocker with additional adrenergic receptor antagonist activity, has also been shown to exert beneficial actions against endothelial dysfunction through its antioxidant effects (Bank et al. 2007), although the molecular mechanisms have not yet been fully clarified (Viridis et al. 2011).

3.4.5 Mineralocorticoid Receptor Antagonists

The MR antagonists, spironolactone and eplerenone, have been shown to reduce morbidity and mortality, in part, by blunting the adverse effects of aldosterone on endothelial function and inflammation involved in the development and complications of atherosclerosis. Recent evidence highlight that pharmacological blockade or genetic deletion of endothelial MR blunt vascular inflammation including expression of adhesion molecules, leukocyte-endothelial interactions, and plaque inflammation. Of note, in preclinical studies endothelial MR inhibition is protective only in male, but not in female mice (Moss et al. 2019). Thus, gender- and sex-specific actions of the MR in vascular function and atherosclerosis, so far still poorly investigated, will deserve future attention also in the clinical setting (Shen et al. 2017). Sympathetic hyperactivity with rising catecholamine levels and adrenergic receptors stimulation is a common feature of many CVDs, including

hypertension. This is associated with endothelial NO synthase uncoupling and a pro-constrictive vascular phenotype on adjacent small arterial vessel wall components, such as smooth muscle and endothelial cells. Activation of MR signaling in the perivascular adipose tissue surrounding small arteries contributes to β -adrenoceptor overstimulation. Thus, MR antagonists targeting the endothelium and the perivascular adipocytes surrounding small arteries may achieve a dual benefit in hypertension with involvement of sympathetic over-activation, as, for instance, in overweight and obesity (Victorio et al. 2016).

4 Perspectives of Future Antihypertensive Therapy

4.1 Unresolved Medical Needs

Despite large evidence confirming the importance of lowering BP and the availability of many effective and well-tolerated antihypertensive drugs, BP control rate is unfortunately not as high as it should. This is, at least in part, related to poor adherence to lifelong antihypertensive therapy but also, in a minority of patients, due to “difficult to treat” or “resistant” hypertension.

According to the guidelines, resistant or difficult-to-treat hypertension is defined as: *blood pressure that is not controlled to goal despite adherence to an appropriate regimen of three antihypertensive drugs of different classes (including a diuretic) in which all drugs are prescribed at suitable antihypertensive doses* (Williams et al. 2018). Pseudo-resistance as well as secondary causes of hypertension should be excluded before this diagnosis is made.

Pseudo-resistance results from some or all of the following (Williams et al. 2018):

- Inaccurate blood pressure measurement (e.g., use of an inappropriately small blood pressure cuff, not allowing a patient to rest quietly before taking readings)
- Poor adherence to blood pressure medications
- Poor adherence to lifestyle and dietary approaches to lower blood pressure
- Suboptimal antihypertensive therapy, due either to inadequate doses, an inappropriate drug combination, or exclusion of a diuretic from the antihypertensive regimen
- White coat hypertension
- Extracellular volume expansion
- Increased sympathetic activation
- Ingestion of substances that can elevate the blood pressure, such as nonsteroidal anti-inflammatory drugs (NSAIDs) or stimulants
- Secondary or contributing causes of hypertension

4.2 New Drug Developments

Recent research has not yielded major advances in treatment of hypertension: no new targets were identified for development of antihypertensive drugs. In fact, trends show a dramatic slowing of research and development for novel blood pressure-lowering drugs. The reasons are manifold: the field is crowded with relatively effective drugs; there is a lack of major new discoveries and targets; and there are many challenges in developing blockbuster drugs. A short look in clinicaltrials.gov shows that while the development of new classes of antihypertensive drugs is apparently waning, the most current activities by big pharmaceutical companies focused on developing new combination pills, including fixed-dose combination drugs with the exception of a new ARB (fimasartan) and the compound AGSCT101, which is actually tested versus carvedilol. Fimasartan is an Ang II receptor antagonist with selectivity for the AT-1 receptor subtype, developed in 2012 by a Korean company (Boryung Pharmaceutical) as an oral antihypertensive drug (Fimasartan 2011; Chi et al. 2011). Fimasartan reduced BP with a good tolerability profile in a large-scale observational population study – Safe-KanArb (Park et al. 2013). The K-MetS study (Park et al. 2017) included 10,601 patients with metabolic syndrome and evaluated long-term effects of fimasartan on major adverse cardiovascular outcomes.

A recent study evaluated the effects of fimasartan and amlodipine therapy on carotid atherosclerotic plaque inflammation using 18F-fluorodeoxyglucose positron emission tomography imaging. Both drugs similarly decreased carotid atherosclerotic plaque inflammation in patients with acute coronary syndrome (Oh et al. 2019).

Concerning AGSCT101, a new antihypertensive drug developed by Ahn-Gook Pharmaceuticals Co., Ltd., the details are scarce. The Phase III Clinical Trial to Evaluate the Antihypertensive Effect of AGSCT101 Versus Carvedilol in Patient with Stage 1 to 2 Essential Hypertension is described in clinicaltrials.gov. Unfortunately, data about mechanism of action of this drug and status of recruiting of the described study are missing.

4.3 Device Therapy

Various device-based therapies such as renal denervation, carotid baroreceptor stimulation, creation of an arteriovenous fistula, or endovascular carotid body modification have emerged, principally targeted at the treatment of resistant hypertension.

Most data are available for renal denervation. The principle of renal denervation is to destroy some of the sympathetic nerves around the renal artery leading to lower sympathetic nervous activity and lower BP. The first results on renal denervation were obtained with devices using radiofrequency application in the open label SYMPPLICITY HTN-1 and SYMPPLICITY HTN-2 trials, along with several case series and observational studies. The SYMPPLICITY HTN-3 trial proved safety, but was unable to show efficacy of renal denervation using a radiofrequency catheter when

compared with sham treatment in patients with severe resistant hypertension on multiple medications (Williams et al. 2018). Post hoc analyses of the SYMPPLICITY HTN-3, however, revealed important information concerning patient selection, difference in adherence to antihypertensive medication in the treatment groups, a higher use of antihypertensive drugs in the sham group, as well as technical failure in performing renal denervation, which led to a revision of renal denervation technology and technique. Based on this background, several novel, sham-controlled studies have been conducted and are, in part, published. SPYRAL HTN-OFF MED (Townsend et al. 2017; Bohm et al. 2020), SPYRAL HTN-ON MED (Kandzari et al. 2018), as well as RADIANCE-HTN SOLO (Azizi et al. 2018) showed significant and consistent reductions in BP, both office and ambulatory, in patients with and without concomitant antihypertensive.

The SPYRAL HTN-ON MED was recently published and showed the superiority of catheter-based renal denervation compared with a sham procedure to safely lower BP in the absence of antihypertensive medications (Bohm et al. 2020). Catheter-based renal denervation is superior to a sham procedure to safely lower BP in the absence of antihypertensive medications.

Less data are available about the effect of carotid baroreceptor stimulation and endovascular carotid body modification both techniques aiming to reduce BP through reduction of sympathetic tone and obtained by creation of an arteriovenous fistula. Concerning carotid baroreceptor stimulation, the first-generation device reduced BP in controlled and uncontrolled clinical trials, while controlled clinical trials proving efficacy in BP reduction do not exist for the currently available second-generation carotid sinus stimulator (Jordan et al. 2019; Heusser et al. 2020).

Some, mostly uncontrolled, studies suggest that other techniques such as baroreflex amplification and carotid body modulation may lead to reduction of BP in patients with difficult-to-treat hypertension. However more evidence on safety and efficacy from ongoing large randomized sham-controlled trials is needed before baroreflex amplification and carotid body modulation can be implemented in routine clinical practice (Groenland and Spiering 2020).

Last, but not least, the possibility to safely reduce BP in patients with uncontrolled hypertension by creating a central iliac arteriovenous anastomosis was tested. The ROX CONTROL HTN study (Lobo et al. 2015) tested this hypothesis using the novel arteriovenous ROX Coupler (ROX Medical, San Clemente, CA, USA).

This small study (44 patients treated and 39 patients in the standard of care group) enrolled and showed that creation of an arteriovenous anastomosis was associated with significantly reduced BP.

The actual ESC/ESH Guidelines do not recommend the use of any device-based therapies for the routine treatment of hypertension, unless in the context of clinical studies and RCTs (Williams et al. 2018).

Nevertheless, device-based therapy for hypertension is a fast-moving field, and new data especially data from study evaluating renal denervation are expected in the near future and could change this recommendation.

5 Conclusion

Hypertension represents one of the most important modifiable risk factors for stroke, heart failure, myocardial infarction, and chronic kidney disease, contributing significantly to the global burden of CVD.

The initial assessment of a patient with hypertension is essential for choosing effective therapy. All cardiovascular risk factors as well as hypertension-mediated target organ damage and presence of comorbidities should be taken into account.

The treatment of hypertension should focus on the overall health of the patient, focusing on reducing the risk of future cardiovascular events.

For a long-lasting adherence, it is important that patients are actively involved in the process of choosing the best BP-lowering strategies.

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Glycaemic Control in Diabetes

D. Müller-Wieland, J. Brandts, M. Verket, N. Marx, and K. Schütt

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Abstract

Reduction of glucose is the hallmark of diabetes therapy proven to reduce micro- and macro-vascular risk in patients with type 1 diabetes. However glucose-lowering efficacy trials in type 2 diabetes didn't show major cardiovascular benefit. Then, a paradigm change in the treatment of patients with type 2 diabetes has emerged due to the introduction of new blood glucose-lowering agents. Cardiovascular endpoint studies have proven HbA1c-independent cardioprotective effects for GLP-1 receptor agonists and SGLT-2 inhibitors. Furthermore, SGLT-2 inhibitors reduce the risk for heart failure and chronic kidney disease. Mechanisms for these blood glucose independent drug target-related effects are still an enigma. Recent research has shown that GLP-1 receptor agonists might have anti-inflammatory and plaque stabilising effects whereas

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SGLT-2 inhibitors primarily reduce pre- and after-load of the heart and increase work load efficiency of the heart. In addition, reduction of intraglomerular pressure, improved energy supply chains and water regulation appear to be major mechanisms for renoprotection by SGLT-2 inhibitors. These studies and observations have led to recent changes in clinical recommendations and treatment guidelines for type 2 diabetes. In patients with high or very high cardio-renal risk, SGLT-2 inhibitors or GLP-1 receptor agonists have a preferred recommendation independent of baseline HbA1c levels due to cardioprotection. In patients with chronic heart failure, chronic kidney disease or at respective risks SGLT-2 inhibitors are the preferred choice. Therefore, the treatment paradigm of glucose control in diabetes has changed towards using diabetes drugs with evidence-based organ protection improving clinical prognosis.

Keywords

GLP-1 receptor agonists · Heart failure · SGLT-2 inhibitors · Type 2 diabetes

Risks for clinical complications of micro- and macro-vascular disease appear to be increased in patients with type 1 and type 2 diabetes. Whereas the relation between elevated glucose levels and microvascular complications seems to be directly correlated and generally specific for diabetes. The relation to macrovascular risk, however, is more complex, multifactorial and not exclusive for diabetes. The link between type 2 diabetes and macrovascular risk is extending beyond glucose levels and may be related to obesity, fat partitioning, and insulin resistance, a concert also called metabolic syndrome.

In the following, we provide epidemiological evidence for increased vascular risk in diabetes, discuss the role of glucose control and introduce the cardiovascular outcome trials (CVOTs) for GLP-1 receptor agonists and SGLT-2-inhibitors. These trials provide direct clinical evidence for cardio-renal protection, independent of baseline HbA1c levels, leading to major changes in treatment algorithms in current guidelines. In conclusion, we will allude to the emerging of efficient obesity treatments, which might ultimately alter pathophysiological features of the metabolic syndrome and potential targets for atherosclerosis.

1 Cardiovascular Risk in Diabetes

The Emerging Risk Factors Collaboration analysed individual data from a total of more than one million people with more than 135,000 deaths (Danesh 2015). The relative risk of mortality was increased approximately twofold in the presence of diabetes or myocardial infarction, almost fourfold for the combination and almost sevenfold for additional stroke.

In the Swedish National Diabetes Registry, 318,083 patients with type 2 diabetes were compared with nearly 1.6 million matched controls over 5–6 years for

cardiovascular endpoints and all-cause mortality (Sattar et al. 2019). Patients diagnosed with diabetes before age 40 years had on average a mean twofold increased risk of all-cause mortality, 2.72-fold increased risk of cardiovascular mortality, 4.77-fold increased risk of heart failure and 4.33-fold increased coronary risk. The risk was age-dependent and was no longer increased in a person older than 80 years old. Due to the increased risk of mortality in patients with diabetes, a database of care providers was used to analyse its relative risk in 31,987 newly diagnosed patients with diabetes compared to 162,656 controls over a 9.5-year period (Zucker et al. 2017). During this period, 14% of patients with diabetes died, but only 8% of matched controls without diabetes died. When adjusted for age, sex, socioeconomic status, obesity, smoking and comorbidities incident diabetes was associated with a 38% higher relative risk of all-cause mortality. A systematic review summarised published data over 10 years (2007–2017) on the prevalence of cardiovascular disease in nearly five million people with diabetes (Einarson et al. 2018). 32.2% of patients with type 2 diabetes had clinically manifest cardiovascular disease, 14.9% had clinically manifest heart failure and 10% had myocardial infarction. In 9.9% of cases, cardiovascular disease was the cause of death, which represented 50.3% of the total death rate.

Patients with type 1 diabetes are notably well analysed in the Swedish national registry. In this registry, the patient's mean age between 1998 and 2011 was 35.8 years. Patients were followed for a mean of about 8 years and compared to random control individuals by age, sex and region. Patients with type 1 diabetes had a significantly higher overall mortality of 8.0% compared to 2.9% in subjects without diabetes, corresponding to a relative risk of 3.52 (Lind et al. 2014). Recently, this registry analysed the prognostic significance of 17 risk factors for death, i.e. mortality from all causes, acute myocardial infarction or stroke. Of the 32,611 patients with type 1 diabetes in this Swedish registry cohort, 5.5% died over the course of 10.4 years. The strongest predictors of death and cardiovascular endpoints were HbA1c, albuminuria, diabetes duration, systolic blood pressure and blood LDL cholesterol concentration. An increase of approximately 1.0% in HbA1c was associated with a 22% higher risk. Furthermore, HbA1c levels <7.0% were associated with a significantly lower risk (Rawshani et al. 2019).

2 Microvascular End-Organ Damage and Cardiovascular Risk

Microvascular alterations with increased risk for end-organ damage, such as retino-, nephro- and neuropathy, are increased in diabetes. The duration and intensity of hyperglycaemia appear to play a major role. One clinical relevant question for cardiovascular risk stratification is whether microvascular “end-organ damage” in turn also increases the risk for macrovascular complications. To address this question, a population-based cohort study of 49,027 patients with type 2 diabetes analysed the association between the combined endpoint consisting of cardiovascular death, nonfatal myocardial infarction, stroke and the cumulative presence of retinal, nephro- and peripheral neuropathy over 5.5 years (Brownrigg et al. 2016).

Each microvascular end-organ damage was associated with an increased cardiovascular risk by approximately 35–40%. The accumulation of relative cardiovascular risk in the presence of one, two or three microvascular diseases increased by 32%, 62% and 99%, respectively. Similar trends were seen for cardiovascular death, all-cause mortality and hospitalisation for heart failure.

Therefore, microvascular end-organ damage appears to indicate or even mediate increased cardiovascular risk. In the case of a possible mediating role, common links discussed include damage to the microvascular pathway including endothelial dysfunction. This is clinically relevant because microvascular complications and endothelial dysfunction can be directly influenced by elevated blood glucose concentrations. However, the incidence as well as progression of end-organ damage is also modulated by other factors, such as increased blood pressure, smoking, etc. (Climie et al. 2019; Ziegler et al. 2020).

3 Role of Hyperglycaemia for Micro- and Macro-vascular Complications

Diabetes is not only associated with an increased micro- and macro-vascular risk, but also with a variety of non-vascular changes or syndromes. Since diabetes per se is currently (still) defined by hyperglycaemia, we will briefly discuss the possible roles of hyperglycaemia as an indicator, mediator or modulator of late complications.

Microvascular complications seem to be essentially caused by hyperglycaemia. However, it has to recurrently persist to display clinical consequences or complications. Though, macrovascular complications are more likely to be caused by other factors, such as insulin resistance, altered fat ectopic accumulation (e.g. in the liver, skeletal muscle or heart – see below), dyslipidaemia, hypertension, hypercoagulability, etc. Therefore, these complications are not diabetes-specific and can occur even before diagnosis, e.g. prediabetes or metabolic syndrome (Rask-Madsen and Kahn 2012; Kahn et al. 2019). Numerous non-vascular changes are not specific to diabetes, but are observed more frequently in patients with diabetes, e.g. fatty liver, COPD, certain forms of cancer, depression, cognitive disorders, osteoporosis, disorders of the gastrointestinal tract as well as the genitourinary system, skin changes, increased risk of infection, cheiroarthropathies, etc.

The role of hyperglycaemia in the many facets of vascular and other late complications may vary, i.e. hyperglycaemia may be a mediator, a modulator or just an indicator of increased risk. Moreover, the “damaging thresholds” of hyperglycaemia fluctuate for different micro-, macro- and non-vascular complications. One reason for this may also be that functional loss of the target organ, like retina or glomerular filtration, is not only a direct consequence of microvascular changes, but also involves other non-vascular cells, e.g. pericytes, podocytes, etc. Hitherto, it remains unclear how hyperglycaemia can exactly cause microvascular or organ damage. One common hypothesis is that hyperglycaemia leads to epigenetic and thus long-term changes that are unlikely to be quickly reversible by good blood glucose control. Furthermore, metabolically oriented

hypotheses assume that the function, synthesis and degradation of proteins through glycosylation or increased substrate supply for various metabolic pathways can indirectly or directly lead to cell and tissue disorders, e.g. endothelium, nerve cells, proteins in the blood, e.g. of coagulation factors as well as connective tissue proteins. The formation of advanced glycosylation end-products (AGE), increase in sorbitol or hexosamine metabolic pathways, intracellular activation of protein kinase C through an increase in diacylglycerol as well as other signalling cascades and gene regulatory mechanisms play a decisive role. In addition to glycosylation, the formation of reactive oxygen radicals and superoxides in the mitochondria could also have a key biological role in this complex process in a subcellular level. Clinically, it is important to note that glycosylation is usually irreversible, i.e. a near-normal blood glucose control does not acutely lead to a “dissolution” of already manifested alterations, but ensures that no further changes occur and that the cellular metabolism and the pattern of modified proteins reconstitute themselves over (long) time. Therefore, early and long-term control of blood glucose metabolism, especially in younger people who still have a long life expectancy, remains a clinical concept.

In this respect, it is recognised that early and sufficient glucose control may reduce the incidence and possible progression of vascular disease. However, dissolution and cure of already established glucose-related damage, as it occurs in older patients with type 2 diabetes, longer disease duration, or prevalent atherosclerotic cardiovascular disease, appears not feasible. Therefore, it is a milestone that new drug classes like GLP-1 receptor agonists and SGLT-2-inhibitors have HbA1c-independent complication-protective effects even in the short-term.

4 Role of Hyperglycaemia in Reducing Vascular Risks in Type 1 Diabetes

The hypothesis that therapeutic lowering of HbA1c and thus hyperglycaemia is associated with a reduction in late complications has been proven in patients with type 1 diabetes by the DCCT (Diabetes Control and Complications Trial) study. The DCCT study has shown as a proof-of-concept study that early and effective blood glucose lowering can significantly reduce the incidence and progression of microvascular complications in patients with type 1 diabetes; similar associations were found in further follow-up for macrovascular complications. It could be that macrovascular complications or atherosclerosis develops quite differently in type 2 diabetes than in type 1 diabetes, or that the DCCT study examined very young patients in whom the many other modulators of vascular risk (see above) do not yet have the decisive significance as in later adulthood or in most patients with type 2 diabetes. The DCCT study was initiated by the National Institute of Diabetes and Digestive and Kidney Disease (NIDDK) from 1983–1993 with 1,441 patients with type 1 diabetes mellitus and became a milestone for the treatment of patients with type 1 diabetes. This study showed that intensified insulin therapy lasting a mean of 6.5 years halved the incidence and progression of microvascular sequelae compared to conventional therapy, which was associated with a significant difference in

HbA1c (The Diabetes Control and Complication Trial 1993). After a mean follow-up of 17 years in >90% of the initially enrolled patients, the cardiovascular risk was significantly reduced by 42% in the intensified treatment group, and the reduction in HbA1c was significantly associated with this (The Diabetes Control and Complication Trial/Epidemiology of Diabetes Interventions and Complications (DCCT/EDIC) Study Research Group 2005). In the EDIC (Epidemiology of Diabetes Interventions and Complications) study, patients have now been followed up for over 30 years. In summary, after 30 years the following was concluded (Zinman et al. 2014):

- Hyperglycaemia is the primary modifiable mediator of late complications in type 1 diabetes.
- Near-normal glucose control reduces the incidence and progression of microvascular complications, such as retinopathy, nephropathy and neuropathy.
- Intensive diabetes therapy reduces cardiovascular complications in type 1 diabetes.

Recently, the concept and importance of good glycaemic control has been further explored and discussed in the DCCT/EDIC trial in relation to cardiovascular complications (Riddle and Gerstein 2019). A mediation analysis and multivariable models show (Bebu et al. 2019) that the quality of adjustment of traditional risk factors accounts for only about 50% of the cardioprotective effect of improved metabolic control. HbA1c can be an indicator for changes in other parameters, e.g. lipids, albuminuria, etc., but this explains <10% of the effect. Therefore, about 40% of the cardioprotective effect remains for HbA1c or elevated glucose concentrations per se. Thus, the therapeutic concept is further confirmed that HbA1c lowering in patients with type 1 diabetes is clinically relevant for reducing the incidence and progression of micro- and macro-vascular late complications. The future must show whether the predictability of the clinical prognosis for late complications through other parameters, such as the duration of glucose control in the desired range, so-called time in range (TiR), has a benefit alongside or in addition to HbA1c (Battelino et al. 2019).

5 Efficacy Trials of Glucose Lowering in Type 2 Diabetes

The UKPDS trial randomly assigned 5,102 patients with newly diagnosed type 2 diabetes to intensive glucose control with sulphonylurea or insulin or to management with diet alone. Those who were overweight at study entry also could be randomised in the intensive arm to receive metformin (UKPDS 33 1998; Stratton et al. 2000). In the insulin and sulphonylurea analyses, HbA1c levels of 7.0% vs. 7.9%, respectively, were significantly associated with a relatively decreased risk for a composite endpoint of all diabetes-related complications by 12%, and microvascular disease risk by 25% during an average follow-up of 10 years. Intensive control showed a trend towards decreased risk of myocardial

infarction and no effect on stroke. In overweight patients, metformin yielded better glucose control (HbA1c 7.4% vs. 8.0%) and significantly decreased relative risk for myocardial infarction by 39% and for all-cause mortality by 36%. The long-term follow-up of the UKPDS trial cohort suggests a “legacy” of cardiovascular benefit of early and tight glycaemic control. Similarly, this was a finding observed in the long-term follow-up of the Diabetes Control and Complications Trial (DCCT) in patients with type 1 diabetes. However, the results of the following efficacy trials in patients with longer diabetes duration were not so promising.

The ACCORD trial compared intensive vs. standard glucose control in 10,251 patients with type 2 diabetes who had high CVD risk, achieving an HbA1c of 6.4% vs. 7.5% (Gerstein et al. 2008). This trial was halted early due to an excess of all-cause mortality in the intensively treated group (257 vs. 203 events; $p = 0.04$), with no significant difference observed in the primary composite cardiovascular disease endpoint of cardiovascular death, myocardial infarction, and stroke. The ADVANCE trial enrolled 11,140 patients with type 2 diabetes who had cardiovascular disease, microvascular disease, or another vascular risk factor at study entry (Heller 2009). Patients randomly received intensive glucose control with gliclazide plus other drugs in the intensive arm, compared with standard control with other drugs. Similar to the ACCORD trial, the ADVANCE trial did not show statistically significant improvement in the composite outcome of cardiovascular death, myocardial infarction, and stroke with intensive control (achieved HbA1c of 6.4% vs. 7.0%), despite having 1,147 events. In the Veterans Affairs Diabetes Trial (VADT), 1791 US veterans with type 2 diabetes and inadequate glucose control randomly received either intensive or standard glucose control (Duckworth et al. 2009). Despite a wide separation in glucose control levels (HbA1c of 6.9% vs. 8.4%) and ascertainment of 499 primary major adverse cardiovascular events (MACEs), this trial also found no significant improvement in cardiovascular outcomes with intensive control. From post hoc analyses of data for each of these trials and supported by the long-term observations from UKPDS in patients with newly diagnosed diabetes at study entry, the concept has emerged that more intensive glycaemic control may be safer and may have more favourable cardiovascular effects when used earlier in the course of diabetes. In contrast, patients with a longer duration of type 2 diabetes and more intense glucose control has only resulted in modest impact on cardiovascular events over time. In this respect, cardiovascular safety studies are of interest, because they aim to assess the safety of a new drug and possibly a mainly glucose independent superiority effect on predefined endpoints, like cardiovascular or renal complications.

6 Cardiovascular Safety Studies in Type 2 Diabetes

Cardiovascular safety studies are designed in such a way that the substance under investigation is compared with placebo, but that the risk parameter to be influenced, in this case HbA1c or blood glucose, should also be comparably reduced in the placebo arm of the study according to study protocol to achieve glycaemic

equipoise. Therefore, this design tests not only the safety of a therapeutic molecule, but also its effect on cardiovascular risk, independent of blood glucose-lowering. Overall, insulin glargine (The ORIGIN Trial Investigators 2012), insulin degludec (Marso et al. 2017) as well as acarbose (Holman et al. 2017b), and sulphonylurea therapy (in directly tested, see Vaccaro et al. 2017, Rosenstock et al. 2019a) are cardiovascular neutral. In this respect it is worthwhile to mention that pioglitazone has been shown to reduce relative risk for myocardial infarction and stroke in a special patient population (subgroup of the PROACTIVE trial), i.e. patients after stroke or transient ischaemic attack (Kernan et al. 2016). Cardiovascular safety studies were conducted for the DPP4 inhibitors alogliptin, linagliptin, saxagliptin and sitagliptin; there is no corresponding prospective endpoint study for vildagliptin. These endpoint studies (Zannad et al. 2015 EXAMINE, Rosenstock et al. 2019b CARMELINA, Scirica et al. 2013 SAVOR-TIMI 53, Green et al. 2015 TECOS) showed safety, but not superiority, in patients with pre-existing cardiovascular disease or multiple risk factors for the primary endpoint, which is in most cases composed of cardiovascular death, myocardial infarction and stroke. In the SAVOR-TIMI (Saxagliptin Assessment of Vascular Outcomes Recorded in Patients with Diabetes Mellitus-Thrombolysis In Myocardial Infarction) 53 trial, 16,492 patients with type 2 diabetes and manifest cardiovascular disease or risk factors were randomised and followed up for a mean of 2.1 years. The primary endpoint, i.e. cardiovascular safety, was proven. However, compared to placebo, a higher hospitalisation due to heart failure was observed in the treatment group with 3.5% vs. 2.8%. For this reason, saxagliptin is not recommended in patients with pre-existing heart failure. Thus, GLP-1-RAs have shown to be cardiovascular protective and SGLT-2 inhibitors provide consistent evidence for cardio- and nephroprotection. Therefore, we will focus on the clinical evidence and potential mechanisms related to these observations.

7 GLP-1 Receptor Agonists (GLP-1-RA)

Therapeutically used GLP (glucagon like peptide)-1 receptor agonists act directly via the GLP-1 receptor, which leads not only to a glucose-dependent metabolically mediated release of insulin from the beta cells, but also to inhibition of glucagon release from the pancreatic alpha cells. Most of them are injected subcutaneously once daily or weekly, depending on the preparation. In addition to effective HbA1c lowering, this therapy also leads to clinically relevant weight loss.

The cardioprotective evidence for GLP1 receptor agonists is based on the results of 7 placebo controlled endpoint trials: LEADER for liraglutide (Marso et al. 2016b); SUSTAIN-6 for subcutaneous semaglutide (Marso et al. 2016a), HARMONY for albiglutide (Hernandez et al. 2018), REWIND for dulaglutide (Gerstein et al. 2019a), and AMPLITUDE-O for efglenatide (Gerstein et al. 2021). These trials demonstrated a significant reduction in the 3-point MACE endpoint (cardiovascular death, nonfatal myocardial infarction, or nonfatal stroke). In the PIONEER-6 study with oral semaglutide, the primary endpoint was negative, but cardiovascular

death and all-cause mortality were reduced (Husain et al. 2019). In the studies with lixisenatide (Pfeffer et al. 2015) and long acting formulation (LAR) of exenatide (Holman et al. 2017a), the primary endpoint was negative. The clinical results of these trials compared with placebo are summarised in Table 1. The results appear heterogeneous for the individual endpoints, which could be due to the study design and different patient populations. Effects on a reduction in the hospitalisation rate due to heart failure were not observed in any study (Lim et al. 2018). Since the curves between treatment and placebo generally only began to diverge after about 1 year, it is assumed that the cardioprotective effects are rather due to a modulation of atherosclerotic processes (Marx and Libby 2018), see Fig. 1.

In Fig. 1, we have distinguished between direct and indirect mechanisms, which we cannot discuss in detail here and therefore refer to recent reviews (Drucker 2016, Nauck et al. 2017; Müller et al. 2019, Giorgino et al. 2020, Nauck et al. 2020). In case of indirect mechanisms, the reduction of blood pressure and weight are certainly in the foreground, whereby interestingly, ectopic lipid accumulation in the liver (Armstrong et al. 2016; Newsome et al. 2021) and epicardial fat (Iacobellis and Villasante Fricke 2020) is also reduced, see below. In addition, there are first indications that certain intraepithelial T lymphocytes in the intestine modulate metabolism and the risk of atherosclerosis as well as that GLP-1 could play an important role as a mediator (He et al. 2019). In terms of direct mechanisms, it must be emphasised that endothelial function, plaque stabilisation and anti-inflammatory effects are of particular importance for the clinical risk posed by plaque (Libby and Hansson 2019). All these mechanisms can be modified by incretin hormones in experimental studies. Therefore, it is thought that GLP-1 receptor agonists may also directly influence the development and stability of atherosclerotic plaque (Rakipovski et al. 2018; Kahles et al. 2018). For example, GLP-1-receptors in various plaque cells like endothelial cells, vascular smooth muscle cells, monocytes, and macrophages are coupled to responses which might modulate plaque biology (Nauck et al. 2020). Similar to ROS formation, oxLDL activation of inflammatory cells and adhesion molecules are reduced. eNOS formation in endothelial cells is increased. After GLP-1- receptor stimulation, foam cell formation is reduced as well as their caspase-mediated apoptosis and the necrotic core in plaque. Interestingly, GLP-1 receptor agonist treatment of cells reduced MMP expression, potentially leading to less degradation of extracellular matrix and thereby increased plaque stability. It is also interesting that GLP-1 receptor agonists may favourably influence the consequences of an ischaemic event (Giblett et al. 2016; Brott 2015; McCormick et al. 2015).

In addition, reduction in progression of chronic kidney disease and clinical renal endpoints were positive as secondary endpoints in LEADER (Mann et al. 2017) and REWIND (Gerstein et al. 2019b). This effect might relate to GLP-1-RA treatment-associated reduction in the progression of albuminuria and mild natriuresis possibly by some inhibition of NHE-3 in the tubulus system.

Table 1 Overview of the cardiovascular outcome trials for GLP1-RA

Study drug	ELIXA Lixisenatide	EXSCEL Exenatide	LEADER Liraglutide	SUSTAIN 6 Semaglutide	Harmony outcomes Albiglutide	PIONEER 6 Semaglutide	REWIND Dulaglutide	AMPLITUDE- O Efglenatide
Year	2015	2017	2016	2016	2018	2019	2019	2021
Patients (n)	6,068	14,752	9,340	3,297	9,463	3,183	9,901	4,076
Median follow-up (years, IQR)	2.08 (NR)	3.2 (2.2–4.4)	3.8 (NR)	2.1 (NR)	1.5 (NR)	1.3 (0.03–1.6)	5.4 (5.1–5.9)	1.81 (1.69–1.98)
MACE HR (95% CI)	1.02 (0.89–1.17)	0.91 (0.83–1.00)	0.87 (0.78–0.97)	0.74 (0.58–0.95)	0.78 (0.68–0.90)	0.79 (0.57–1.11)	0.88 (0.79–0.99)	0.73 (0.58–0.92)
MACE definition	Death from CV causes, nonfatal stroke, nonfatal MI, or unstable angina	Death from CV causes, nonfatal MI, or nonfatal stroke	Death from CV causes, nonfatal (including silent) MI or nonfatal stroke	Death from CV causes, nonfatal MI, or nonfatal stroke	Death from CV causes, nonfatal MI, nonfatal stroke, or urgent coronary revascularisation for unstable angina	Death from CV causes, nonfatal MI, or nonfatal stroke	Nonfatal MI, nonfatal stroke, and death from CV causes or unknown causes	Nonfatal MI, nonfatal stroke, or death from CV or undetermined causes)
Event rate (rate/1,000 patient-year)								
Treatment	64	37	34	32.4	45.7	29	23.5	39
Placebo	63	40	39	44.4	58.7	37	26.6	53
Inclusion criteria								
Age (years)	≥30	≥18	≥50	≥50	≥40	≥50	≥50	≥18

HbA1c (%)	5.5–11.0	6.5–10.0	≥7.0	≥7.0	≥7.0	n/a	≤9.5	≥7.0
BMI (kg/m ²)	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
eGFR (mL/min/1.73 m ²)	≥30	≥30	n/a	≥30	≥30	≥30	≥15	≥25
ASCVD	Established	Any level of risk	Established or high risk	Established	Established or ≥1 risk factor	Established or ≥1 risk factor	Established or ≥2 risk factor	Established or CKD + ≥1 risk factor

ASCVD atherosclerotic cardiovascular disease, BMI body mass index, C_{cr} creatinine clearance, CI confidence interval, CKD chronic kidney disease, CV cardiovascular, eGFR estimated glomerular filtration rate, HF heart failure, HR hazard ratio, IQR interquartile range, i.v. intravenous, MACE major adverse cardiovascular event, MI myocardial infarction

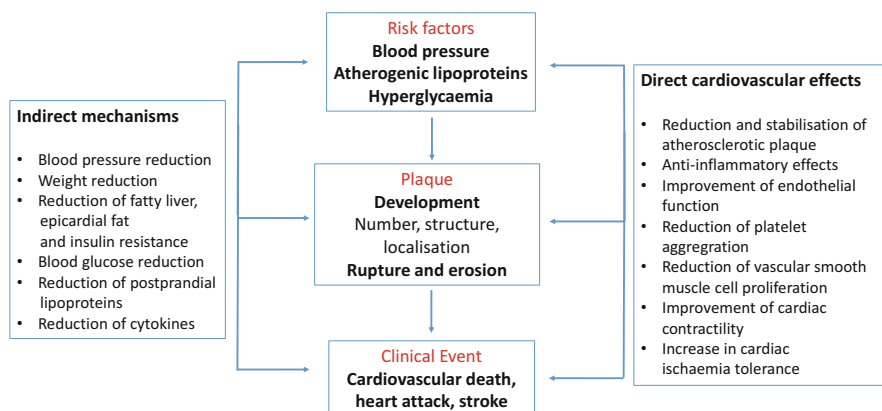


Fig. 1 Potential mechanisms and concepts for modulation of atherosclerotic plaque formation, rupture and erosion and their cardiovascular consequences by GLP-1 receptor agonists. For further explanation see text

8 SGLT (Sodium Glucose Transporter) 2 Inhibitors

SGLT-2 inhibitors are a group of blood glucose-lowering substances that lead to an increased excretion of glucose in the urine by reducing the activity of the transporter selectively in the proximal tubulus of the kidney by approximately 40–60%. CVOTs have been published for canagliflozin, dapagliflozin, empagliflozin, ertugliflozin, and sotagliflozin, mentioned here in alphabetical order and are summarised in Table 2.

The EMPA-REG OUTCOME study (Zinman et al. 2015) enrolled 7,020 patients with pre-existing cardiovascular disease over a mean observation period of 3.1 years and investigated the effect of 10 or 25 mg empagliflozin daily in addition to existing medication. The primary endpoint was a composite of cardiovascular death and nonfatal myocardial infarction and stroke and was significantly reduced with a relative risk reduction (RRR) of 14%; the event rate was 12.1% in the placebo group and 10.5% with empagliflozin treatment. There was no difference between the two doses of empagliflozin. Component analysis showed no effect on nonfatal myocardial infarction or stroke, but cardiovascular mortality was significantly reduced with an RRR of 38%, which also reduced all-cause mortality. In addition, hospitalisation due to heart failure was also significantly lower with an RRR of 35%, an effect that became significant after only a few weeks. The effect on renal function was further analysed. The prespecified secondary renal endpoint was worsening nephropathy (progression to macroalbuminuria, doubling of serum creatinine, initiation of renal replacement therapy or death due to renal causes) and incidence rate of albuminuria. The incidence or relative risk of worsening nephropathy was significantly reduced by 44% with empagliflozin (Wanner et al. 2016). In the primary CANVAS programme (Neal et al. 2017) and in the CREDENCE study (Perkovic

Table 2 Overview of the cardiovascular outcome trials for SGLT2i

	EMPA-REG OUTCOME	CANVAS Programme	DECLARE-TIMI 58	SOLOIST-WHF	VERTIS
Study drug	Empagliflozin	Canagliflozin	Dapagliflozin	Sotagliflozin	Vertugliflozin
Year	2015	2017	2018	2020	2020
Patients (n)	7,020	10,142	17,160	1,222	8,246
Median follow-up (years, IQR)	3.1 (2.2–3.5)	2.4 (NR)	4.2 (3.9–4.4)	0.76	3.5 (NR)
MACE HR (95% CI)	0.86 (0.74–0.99)	0.86 (0.75–0.97)	0.93 (0.84–1.03)	0.67 (0.52–0.85)	0.97 (0.85–1.11)
MACE definition	Death from CV causes, nonfatal MI or nonfatal stroke	Death from CV causes, nonfatal MI or nonfatal stroke	CV death, MI or ischaemic stroke	Deaths from CV causes and hospitalisations and urgent visits for HF	Death from CV causes, nonfatal MI or nonfatal stroke
Event rate (rate/1,000 patient-year)					
Treatment	37.4	26.9	12.2	510	39
Placebo	43.9	31.5	14.7	763	40
Inclusion criteria					
Age (years)	≥18	≥30	≥40	18–85	≥40
HbA1c (%)	7.0–9.0 (with OAD) 7.0–10.0 (without OAD)	7.0–10.5	6.5–12.0	≥ 6.5	7.0–10.5
BMI (kg/m ²)	≤45	n/a	n/a	n/a	≥18
eGFR (mL/min/1.73 m ²)	≥30	≥30	C cr ≥60 mL/min	≥30	≥30

(continued)

Table 2 (continued)

ASCV	EMPA-REG OUTCOME Established	CANVAS Programme Established or ≥ 2 risk factors	DECLARE- TIMI 58 Established or multiple risk factors	SOLOIST-WHF Hospitalised for HF and i.v. diuretic therapy	VERTIS Established
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ASCV atherosclerotic cardiovascular disease, BMI body mass index, CI confidence interval, C cr creatinine clearance, CV cardiovascular, eGFR estimated glomerular filtration rate, HF heart failure, HR hazard ratio, IQR interquartile range, i.v. intravenous, MACE major adverse cardiovascular event, MI myocardial infarction, OAD oral antidiabetic therapy

et al. 2019), the SGLT-2 inhibitor canagliflozin also significantly reduced the risk of the combined cardiovascular endpoint (cardiovascular death, nonfatal myocardial infarction or stroke) by 14% and renal endpoints. This confirmed the concept of cardio-renal risk reduction by a second substance, and the question now arose whether this principle of action could also be observed with a third substance and, above all, also in patients with lower cardio-renal risk. In the DECLARE-TIMI 58 trial, 17,160 patients with type 2 diabetes were studied over a mean of 4.2 years with and without dapagliflozin 10 mg; 10,186 of these patients had no previous atherosclerotic disease (Wiviott et al. 2019). The primary safety analysis confirmed non-inferiority of dapagliflozin for MACE (major cardiovascular events), and the primary efficacy analysis showed a significantly lower event rate for the combined endpoint of cardiovascular death and hospitalisation for heart failure (HHF), although this outcome was substantially driven by HHF. A sub-analysis of 3,586 patients with pre-existing myocardial infarction showed that compared with placebo, dapagliflozin significantly reduced the relative risk of MACE by 16% with an absolute risk reduction of 2.6% (Furtao et al. 2019). The effect of dapagliflozin on nephropathy has been investigated as a renal composite secondary endpoint ($\geq 40\%$ decrease in eGFR in mL/min/1.73 m², new manifestation of ESRD, or death due to renal or cardiovascular causes). The relative risk for this renal endpoint was significantly reduced by 24% with dapagliflozin. In the 671 patients with heart failure and impaired left ventricular pump function, dapagliflozin also significantly reduced not only hospitalisation for heart failure but also cardiovascular death with a relative risk reduction of 45% compared to placebo (Kato et al. 2019). Ertugliflozin was investigated in the VERTIS CV trial in 8246 patients with type 2 diabetes and cardiovascular disease over a mean of 3.5 years (Cannon et al. 2020). Ertugliflozin was noninferior to placebo for MACE and clinical renal endpoints. However, results were consistent with other SGLT2-inhibitors in respect of reducing hospitalisation for heart failure in a respective prespecified group and reduction in decline rate of eGFR. Sotagliflozin, an SGLT2 inhibitor with possibly some SGLT-1 blocking potential, was investigated in patients with diabetes and recent worsening of heart failure (SOLOIST-WF) and in patients with diabetes and chronic kidney disease (SCORED) resulting in a significant risk reduction for cardiovascular death and hospitalisation for heart failure in patients with heart failure (Bhatt et al. 2020b) or chronic kidney disease with or without albuminuria (Bhatt et al. 2020a).

In consideration of these very impressive trial results with major impact on risk for heart failure and renal complications to a large extent non-related to baseline HbA1c and glucose-lowering capacity in these trials, the question was emerging, whether SGLT2-inhibitors reduce also clinical risk primarily in patients suffering from chronic heart failure or kidney disease with and also without diabetes. Four studies have been published so far, the DAPA-HF and EMPEROR-Reduced trials for chronic heart failure and CREDENCE for patients with chronic kidney disease (CKD) in patients with diabetes and the DAPA-CKD trial in CKD patients with and also without diabetes.

In the DAPA-HF study (McMurray et al. 2019), 4,744 patients with manifest heart failure and impaired left ventricular function (LVEF $\leq 40\%$, NT-proBNP

≥ 600 pg/mL) were enrolled and treated with dapagliflozin 10 mg or placebo in addition to existing standard therapy. The study was event-driven and ran for approximately 3 years. The relative risk for the primary endpoint was significantly reduced by 26% and was composed of cardiovascular death, hospitalisation or urgent medical visit for heart failure. There was no difference between the 42% of patients with or without diabetes. In the EMPEROR-Reduced trial (Packer et al. 2020) 3730 patients with chronic heart failure and reduced left ventricular ejection fraction ($\leq 40\%$) were investigated over a mean period of 16 months. The relative risk for the primary combined endpoint being cardiovascular death and hospitalisation for heart failure was significantly reduced by 25% regardless of the presence or absence of diabetes. Therefore, both studies show the benefit of SGLT2-inhibition in patients with clinically relevant chronic heart failure independent of diabetes status. These data have impacted current guidelines for the treatment of patients with chronic heart failure, SGLT2-inhibition being an evidence-based treatment option.

In patients with chronic kidney disease, CREDENCE (Perkovic et al. 2019) was the first study to investigate the effect of an SGLT-2 inhibitor, in this case 100 mg canagliflozin OD, primarily on renal function. The primary renal endpoint was composed of end-stage renal failure (defined by need for dialysis, kidney transplantation or drop in eGFR to < 15 mL/min/1.73 m²), doubling of serum creatinine levels or death due to renal or cardiovascular causes. 4,401 patients with type 2 diabetes, an eGFR between 30 and 90 mL/min/1.72 m² and albuminuria of > 300 –5,000 mg/g creatinine were included and studied for a mean of 2.62 years. Canagliflozin significantly reduced the relative risk for the primary renal endpoint by 30%, the relative risk for end-stage renal failure by 32% and its combination with creatinine doubling and renal death by 34%. DAPA-CKD (Heerspink et al. 2020) randomly assigned 4,304 patients with an eGFR of 25–75 mL/min/1.73 m² and a urinary albumin-to-creatinine ratio of 200–5,000 receiving 10 mg dapagliflozin or placebo over a median time of 2.4 years. The relative risk for the combined endpoint of sustained decline in the eGFR of at least 50%, end-stage kidney disease, or death from renal causes was significantly lower by 44% in the dapagliflozin arm compared to the placebo group. This effect was irrespective of having type 2 diabetes or not. Therefore, SGLT-2 inhibitors are also finding their position in guidelines for the treatment of chronic kidney disease. However, one should be aware that currently there are still differences in specifications of their label in patients with reduced eGFR in different countries and regions of the world.

9 Mechanisms of Action for Cardio-Renal Protection Through SGLT-2 Inhibition

SGLT-2 transports glucose in exchange for sodium in the proximal tubule of the kidney and reabsorbs approximately 90% of the 180 g of glucose normally filtered daily in primary urine, for review see Heerspink et al. (2016); Lytvyn et al. (2017); Verma and McMurray (2018); Marton et al. (2021). SGLT-2 inhibitors selectively

SGLT-2 inhibitors: Potential mechanisms for cardiac protection

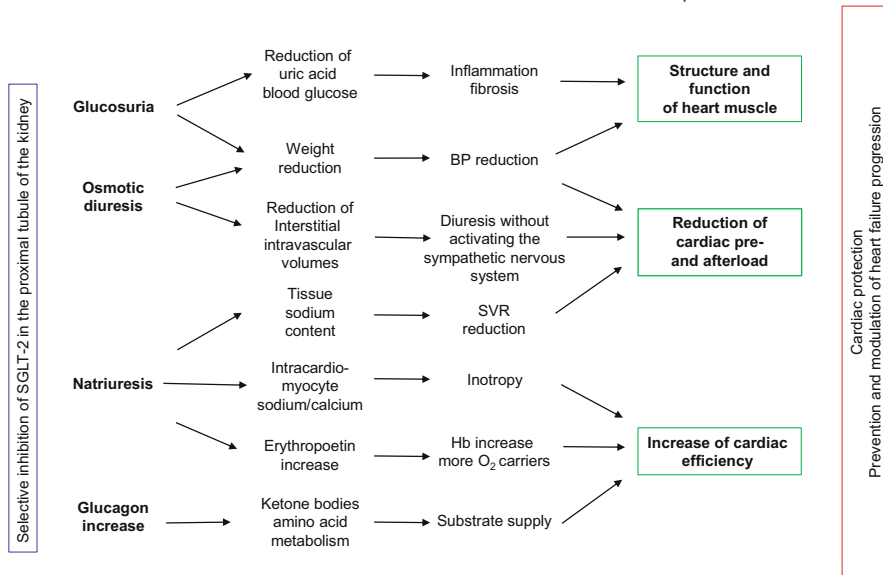


Fig. 2 Potential mechanisms and concepts of cardiac protection by SGLT-2 inhibitors. For further explanation see text

prevent the transporter with high affinity by an average of about 40–60%. This inhibition leads to glucosuria, associated osmotic diuresis, increased natriuresis and an increase in glucagon levels. Figure 2 summarises the principal mechanisms of action and outlines how the cardioprotective effects associated with SGLT-2-inhibitor therapy are currently imagined.

Glucosuria leads to a loss of calories, approx. 70–80 g over 24 h, corresponding to approx. 300 kcal, and weight reduction of approx. 2–3 kg, half of which is due to the reduction of fat mass. The other half is due to the osmotic diuresis of approx. 300 mL/24 h. In contrast to diuretics, this, in combination with natriuresis, leads to an interstitial fluid mobilisation without a significant reduction in intravascular volume, and thus not to an activation of the sympathetic nervous system or an increase in the pulse rate. These effects also cause a reduction in peripheral vascular resistance and, in combination, a reduction in blood pressure of 4–6 mmHg systolic (1–2 mmHg diastolic), which can be even more pronounced at elevated blood pressure levels or in combination with diuretics.

In addition, natriuresis and possibly direct interaction of SGLT-2 inhibitors in the myocardium with sodium proton exchanger-1 (NHE-1) may favourably alter the intracellular ratio of sodium and calcium. The increased natriuresis, also through additional inhibition of tubular NHE-3, probably leads indirectly in the medulla of the kidney to higher consumption of oxygen and thus renally to an increased formation of erythropoietin and an increase in the formation of red blood cells and the oxygen carrier haemoglobin. In a mediation analysis of the cardiovascular

endpoint study of empagliflozin, this appears to be clinically relevant for understanding the observed effect in cardioprotection (Inzucchi et al. 2018).

An increase in glucagon levels of about twofold by SGLT-2 inhibitors is associated with an approximately twofold increase in the concentration of ketone bodies during therapy. Ketone bodies in turn can be effective substrates (especially α -hydroxy-butyrate) for cardiac muscle and possibly also lead to altered metabolism of branched-chain amino acids in the cardiac muscle, which can be disturbed in diabetic cardiomyopathy (Wanner and Marx 2018). In summary, therapy with SGLT-2 inhibitors results in a rapid and effective pre- and afterload reduction of the heart as well as possibly a clinically relevant favourable change in metabolism and intracardiomyocyte electrolytes, stabilising the structure and function of the myocardium and increasing work efficiency.

For renoprotection, the key mechanism is probably increased natriuresis in the proximal tubule, leading to activation of the tubuloglomerular feedback (TGF) mechanism. The natriuresis caused by SGLT2 inhibition is sensed by the macula densa in the juxtaglomerular apparatus and leads to a counter-regulation, i.e. specifically a reduction of glomerular filtration by constriction of the vas afferens, probably mediated by adenosine. This lowers the intraglomerular pressure and thus also leads to a reduction in albuminuria and in the long term to a stabilisation and reduced progressive decrease in the glomerular filtration rate. This fascinating observation now also makes the hyperfiltration in the early phase of a renal change in patients with diabetes understandable, because an increased activity of the SGLT-2 transporter in diabetes leads to increased reabsorption of sodium, thus reduction of TGF and vasodilation of the vas afferens and thus hyperfiltration of the glomerulum.

In addition to the aforementioned hypotheses of reduction of cardiac pre- and after-load and intraglomerular pressure reduction by tubuloglomerular feedback mechanisms, a recent hypothesis involves metabolic adaptations. Under SGLT-2-inhibitor treatment, there is an increased caloric loss and an about twofold increase in glucagon levels with a consecutive increase in ketone bodies. These endocrine-metabolic changes provide both a better energy efficient substrate utilisation for the heart and the metabolism switch to a state of chronic hypometabolism and water deprivation. This state is compensated in the long-run by appropriate adaptation of energy homeostasis or metabolic rate and water conservation. This adaptation process, called “aestivation”, is evolutionary conserved enabling adaptation to energy and water shortage (Marton et al. 2021). This metabolic switch induced by SGLT-2-inhibition might play a role in improved function and reduced damage progression of different organs, most extensively clinically proven for heart and kidney.

10 Change in Guidelines and Clinical Recommendations

Patient-centred care is the focus of most guidelines and new recommendations (Davies et al. 2018; Consentino et al. 2019; Buse et al. 2020; Landgraf et al. 2020; American Diabetes Association 2021). Based on the clinical evidence provided

recently and discussed above, guidelines nowadays don't put HbA1c in focus for decision about treatment strategy in type 2 diabetes, but rather the clinical picture and risk of cardio-renal complications in the foreground. Evidence-based means that the results of large studies, especially cardiovascular endpoint studies, must be translated into practice.

The recent ESC recommendations on “Diabetes, prediabetes and cardiovascular disease” (Consentino et al. 2019), produced in collaboration with the EASD, have incorporated the extensive data from large cardiovascular endpoint studies in recent years with new antidiabetic agents and have led to a new and more specific positioning of metformin and cardioprotective blood glucose-lowering drugs in patients with high and very high cardio-renal risk and diabetes mellitus. The decisive factor for the choice of a glucose-lowering substance for cardiovascular risk reduction is primarily not an HbA1c target anymore, but the risk stratification of patients with diabetes according to the ESC recommendations 2019:

Very high risk patients are those with diabetes and established cardiovascular disease or end organ damage or three or more risk factors or early onset of type 1 diabetes of long duration (>20 years). Patients with diabetes duration ≥ 10 years without end-organ damage but with another risk factor are classified in the high risk category. The moderate-risk category includes young patients (type 1 diabetes under 35 or type 2 diabetes under 50 years) with diabetes duration under 10 years without other risk factors.

The new algorithm for cardiovascular risk reduction with antihyperglycaemic agents in untreated type 2 diabetic patients – regardless of HbA1c level – focuses on the high/very high risk categories. Patients with atherosclerotic cardiovascular disease or high/very high risk should receive an SGLT2 inhibitor or GLP1 receptor agonist monotherapy as a class Ia recommendation, according to the evidence from the studies above. In patients with existing or increased risk for heart failure, SGLT-2 inhibitors are recommended. Similar recommendation belongs to renal protection.

Although the effect of metformin on risk for myocardial infarction was better than expected from degree of glucose lowering in the UKPS trial, it has been questioned whether metformin should continue to be first-line therapy in all patients with type 2 diabetes. The CVOTs described above suggest that in these patient groups GLP-1 RAs and SGLT2-inhibitors should be first choice driven by evidence and not metformin. This is supported by further evaluations of these CVOTs indicating that metformin has no modulating effect on risk protection by GLP-1RA or SGLT2-inhibitors (Marx 2020; Sattar and McGuire 2021).

11 Perspectives: Fat Partitioning as a New Target of Diabetes Drugs

Like treatment of chronic heart failure or chronic kidney disease in patients with and without diabetes by SGLT2-inhibitors initially developed for diabetes, it is also interesting to note that new incretin-based drugs, like “twincretins”, reach mean body weight reductions between 8 and 12 kg and absolute reductions in HbA1c of

around 2.0% (Rosenstock et al. 2021; Frías et al. 2021). Results of these kind of studies might help decipher new networks between fat tissue, diabetes and atherosclerosis related risks. In this respect, increased and abnormally distributed fat might be a new target. Numerous clinical and pathogenetic studies have changed our understanding of adipose tissue in such a way that the amount of adipose tissue is not the only essential pathophysiological phenomenon, but in particular the abnormal deposition of fat in other cells or organs, called ectopic lipid accumulation or fat partitioning (Blüher 2016; Crewe et al. 2017; Scherer 2016; Stefan et al. 2008; Unger 2002). When the normal storage capacity and plasticity of subcutaneous adipose tissue and its ability to adapt to increased energy balance are exhausted, there is recruitment of inflammatory cells in adipose tissue and increased deposition of fat in other cells, such as visceral fat cells, and in cells of the liver and cardiovascular system. The deposition of fat alters the function of these cells, e.g. resistance to insulin in the liver, release of mediators from visceral fat, or possibly changes in contractility in the case of cardiac muscle cells. A clinically important and classic example of ectopic lipid accumulation is Non-Alcoholic Fatty Liver Disease (NAFLD), which is often associated with insulin resistance, obesity and diabetes (van Heerebeek and Paulus 2016) and diastolic dysfunction of the heart (van Wagner et al. 2015). A growing number of studies show that intracardiomyocyte fat is associated with altered cardiac cell function including heart failure and epicardial fat (EAT) cells may influence cardiac function and cardiovascular risk through release of humoral, inflammatory and metabolic mediators (Unger 2002; McGavock et al. 2007; Fontes-Carvalho et al. 2014). Recently it has been shown that in patients with diabetes EAT volume was independently associated with coronary calcium score (Cosson et al. 2021) and that extracellular vesicles from EAT facilitate atrial fibrillation and induce proinflammatory and fibrotic responses in cell culture (Shaihov-Teper et al. 2021). Furthermore, considering potential cardiovascular benefit of greater weight reductions, as we have learned from bariatric surgery (Sjöström et al. 2004), CVOTs of these new treatment options are awaited with great curiosity. In addition, these degrees of fat reduction and glucose-lowering open up possibilities to reach “remission” of type 2 diabetes (Lean et al. 2018). Overall perspectives in the treatment of type 2 diabetes are to identify different subgroups with different risk profiles for late complications and to better understand major pathomechanisms that can then be therapeutically targeted therapeutically (Ahlquist et al. 2019; Zaharia et al. 2019).

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LDL-Cholesterol-Lowering Therapy

Angela Pirillo, Giuseppe D. Norata, and Alberico L. Catapano

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Abstract

The causal relation between elevated levels of LDL-C and cardiovascular disease has been largely established by experimental and clinical studies. Thus, the reduction of LDL-C levels is a major target for the prevention of cardiovascular disease. In the last decades, statins have been used as the main therapeutic approach to lower plasma cholesterol levels; however, the presence of residual lipid-related cardiovascular risk despite maximal statin therapy raised the need to develop additional lipid-lowering drugs to be used in combination with or in alternative to statins in patients intolerant to the treatment. Several new drugs have been approved which have mechanisms of action different from statins or impact on different lipoprotein classes.

Keywords

Cardiovascular disease · Dyslipidemias · Ezetimibe · Familial hypercholesterolemia · Hypercholesterolemia · Hypertriglyceridemia · Lipid-lowering drugs · Low-density lipoprotein cholesterol · Low-density lipoprotein receptor · Proprotein convertase subtilisin/kexin type 9 · Statins

1 Introduction

Atherosclerosis is a chronic inflammatory disease affecting arterial wall and characterized by a progressive accumulation of lipids in the subendothelial space. Epidemiological and genetic evidence suggests low-density lipoprotein cholesterol (LDL-C) as a causal factor in cardiovascular disease (Ference et al. 2017), and the results of a large number of randomized clinical trials definitely proved that decreasing LDL-C levels translates in a proportional reduction of the risk of atherosclerotic cardiovascular (CV) events (Baigent et al. 2005, 2010, 2011; Cannon et al. 2015a; Sabatine et al. 2017a; Schwartz et al. 2018).

Populations of modern societies present with LDL-C levels largely exceeding those believed to be physiological. Optimal plasma concentration of LDL-C ranges around 25 mg/dL, since above this value the LDL receptor (LDLR) is saturated (Brown and Goldstein 1986); furthermore, individuals with genetically determined low levels of LDL-C are healthy and exhibit very low incidence of CV events (Glueck et al. 1997; Cohen et al. 2006). These observations, coupled to the results of clinical trials testing the most recent lipid-lowering drugs (i.e., PCSK9 inhibitors), which have shown that LDL-C levels may be lowered well below the values recommended by guidelines without safety concerns (Robinson et al. 2017; Sabatine et al. 2017b), provided the notion that reaching very low LDL-C levels may safely confer additional clinical CV benefits. This concept led to a substantial reduction of the LDL-C goals recommended by the most recent guidelines for the management of dyslipidemias (Mach et al. 2020).

Statins, which represent the cornerstone for the treatment of hypercholesterolemia, have shown approximately a 20% reduction in the risk of cardiovascular events per each mmol/L LDL-C reduction (Baigent et al. 2005); a more intensive intervention was associated with a greater reduction of the incidence of major vascular events (Baigent et al. 2010), suggesting that the higher the degree of reduction of LDL-C levels, the greater the benefit in terms of reduction of CV events.

Although statin monotherapy could reduce LDL-C levels to a large extent (up to –50% with the highest doses of most potent statins), this might not be enough to reach the desired goals based on the individual CV risk, and therefore additional approaches are needed.

This is even more important in patients with familial hypercholesterolemia (FH) which are considered among those with the highest cardiovascular risk, due to the exposure to high plasma LDL-C levels from birth (Nordestgaard et al. 2013). FH is caused by mutations in genes encoding proteins involved in the LDL catabolism, such as LDL receptor (LDLR), apolipoprotein B (apoB), proprotein convertase subtilisin/kexin type 9 (PCSK9), and LDLR adaptor protein (LDLRAP); mutations in LDLR gene account for the vast majority of FH cases (Nordestgaard et al. 2013). Heterozygous FH (HeFH) patients are characterized by two- to threefold elevation in plasma cholesterol levels and development of coronary atherosclerosis at an early age (usually after 30 years); homozygous FH (HoFH) patients, which include either patients with a significantly reduced LDLR activity (2–30% residual activity) or receptor-negative subjects, characterized by a residual LDLR activity <2%, exhibit a more severe cardiovascular condition, due to the exposure to very high LDL-C levels from birth, childhood coronary heart disease, and premature death from myocardial infarction (prior to 20 years of age) if untreated (Cuchel et al. 2014; Sniderman et al. 2014).

These aspects indicate that the choice of the most appropriate therapeutic approach should be done taking into account the LDL-C target and the distance of LDL-C from the target which are dictated by the cardiovascular risk of the patient, and thus the therapy needs to be tailored to each patient. While statins are the first approach, combination therapy is critical when certain LDL-C goals should be achieved (Norata et al. 2013a; Toth et al. 2016; Russell et al. 2018).

2 Statins in the Prevention of Cardiovascular Disease

Statins are competitive inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme of cholesterol synthesis pathway. Following the reduction of intracellular cholesterol synthesis, hepatocytes upregulate surface LDLR expression to increase LDL uptake, resulting in the reduction of circulating LDL-C levels. A large number of clinical trials have established the efficacy of statins in reducing cardiovascular morbidity and mortality in both primary and secondary prevention (Baigent et al. 2010; Chan et al. 2011; Mills et al. 2011a, b; Tonelli et al. 2011; Naci et al. 2013; Taylor et al. 2013). A meta-analysis of 14 randomized clinical trials including >90,000 participants

showed a significant reduction in coronary heart disease (CHD) mortality (-19% , $p < 0.0001$), myocardial infarction or coronary death (-23% , $p < 0.0001$), and fatal or nonfatal stroke (-17% , $p < 0.0001$) (Baigent et al. 2005). Overall, there was a significant 21% proportional reduction in the incidence of major vascular events per mmol/L LDL-C reduction (Baigent et al. 2005). A linear relationship between proportional reduction in incidence of major cardiovascular events and mean absolute LDL-C reduction was reported (Baigent et al. 2005). The analysis of more intensive versus less intensive statin regimens showed that treatment with more intensive regimens resulted in an additional 15% ($p < 0.0001$) reduction in major vascular events (Baigent et al. 2010), suggesting that further reducing LDL-C levels safely translates into a further clinical benefit.

Despite the proven efficacy of statin therapy, a relevant percentage of patients still experience cardiovascular events, even in the presence of well-controlled LDL-C levels due to a “residual risk” (Ahn and Choi 2015), likely related to abnormalities in other lipid parameters (such as high triglycerides and low HDL-c) which may account for this effect particularly in specific groups of patients (including obese patients or those with metabolic syndrome or type 2 diabetes) (Fruchart et al. 2014). Moreover, although statin therapy represents the first approach for the treatment of FH, its efficacy is strictly related to the presence of a functional LDLR and thus may be effective in HeFH or in receptor-defective HoFH (Nordestgaard et al. 2013; Cuchel et al. 2014). In HoFH patients carrying null mutations on LDLR gene, statins induce a modest reduction of LDL-C ($\sim 20\%$) likely through pathways LDLR-independent, which however is not sufficient to reduce massively LDL-C levels and reach the goals suggested for their risk category (Hovingh et al. 2013). In addition, although statins are overall well tolerated, statin-associated muscle symptoms may lead to therapy discontinuation and limit clinical benefit (Banach et al. 2015). Muscle-related adverse events occur mainly with high statin doses which are commonly used in high cardiovascular risk patients, but they have been reported also in patients presenting with comorbidities, due to pharmacokinetic interactions with other drugs (Chatzizisis et al. 2010; Taha et al. 2014). Several pharmacological approaches are available for the management of hypercholesterolemia in patients intolerant to statins, including the possibility to combine a low dose of a statin with another cholesterol-lowering drug which is acting by a complementary mechanism of action (Pirillo and Catapano 2015). Several non-statin drugs approved for the treatment of dyslipidemia, however, failed to further reduce the incidence of cardiovascular events when added to current statin therapy, with the exception of ezetimibe (Ridker 2014; Cannon et al. 2015a) and, more recently, PCSK9 inhibitors (Sabatine et al. 2017a; Schwartz et al. 2018).

3 Non-statin Cholesterol-Lowering Drugs

In recent years several therapeutic approaches have been investigated and developed with the aim of reducing plasma cholesterol levels with a mechanism of action complementary to that of statins. Among them, ezetimibe and, more recently, the

monoclonal antibodies targeting PCSK9 have been approved, while other molecules including bempedoic acid and PCSK9 gene silencing are in an advanced phase of clinical development.

3.1 Ezetimibe

Ezetimibe is a lipid-lowering drug acting as an inhibitor of intestinal cholesterol absorption through the inhibition of the sterol transporter Niemann-Pick C1L1 (NPC1L1) protein (Davis and Veltri 2007). This protein, highly expressed at the brushborder membrane of intestinal epithelial cells, plays a central role in the intestinal absorption of cholesterol and the regulation of cholesterol plasma levels (Wang 2007; Wang and Song 2012). The observation that individuals carrying loss-of-function variants in *NPC1L1* have lower LDL-C levels and a 53% relative risk reduction of CHD (Stitzel et al. 2014) has suggested that this protein could represent a potential pharmacological target for the treatment of hypercholesterolemia.

Ezetimibe has a complementary mechanism of action as compared to statins. Statins, by inhibiting cholesterol synthesis pathway, induce the upregulation of hepatic LDLR, thus resulting in increased uptake of LDL particle from the circulation. This effect prompts a feedback mechanism which increases cholesterol absorption in the intestine, thus partially affecting the efficacy of statin therapy. In this context, ezetimibe as monotherapy, by inhibiting intestinal cholesterol absorption, will reduce plasma cholesterol levels, but the effect would be mitigated by the induction of increased cholesterol synthesis in the intestine and the liver (Descamps et al. 2011). Therefore, the combination statin+ezetimibe will reduce both cholesterol synthesis and absorption, thus representing a valuable approach for further reducing plasma LDL-C levels beyond what was observed with statins in monotherapy.

Indeed several clinical trials have shown that adding ezetimibe to statin therapy results in a further 15–20% LDL-C level reduction (Catapano et al. 2005, 2006; Mikhailidis et al. 2007; Norata et al. 2013a). A pooled analysis of over 21,000 subjects from 27 clinical trials showed that co-administration of ezetimibe with a statin produces a greater LDL-C-lowering effect than statin alone in a wide range of patients (Morrone et al. 2012). Furthermore, the reduction in LDL-C levels was more impressive in patients with CHD or CHD risk-equivalents treated with ezetimibe added to current statin dose compared with patients who titrated the statin dose (Foody et al. 2013). This difference has been reported also in diabetic patients treated with ezetimibe+statin, who showed a 24.6% LDL-C reduction compared with a 10.9% reduction observed in those receiving a doubled statin dose; similarly the entire lipid profile was improved more in patients receiving the combination therapy (Sakamoto et al. 2015). A meta-analysis of available randomized clinical trials showed that the addition of ezetimibe to ongoing simvastatin, atorvastatin, or rosuvastatin therapy results in a greater reduction of LDL-C in high CV risk patients compared with doubling the statin dose (Lorenzi et al. 2019).

The combination ezetimibe+statin has been shown to be effective also in FH patients with residual LDLR activity, who achieve lower levels of total cholesterol and LDL-C than patients treated with statin alone (Gagne et al. 2002; Pisciotta et al. 2007; Kastelein et al. 2008).

Adding ezetimibe to statin therapy was also shown to provide a clinical benefit. The SHARP trial showed that ezetimibe+simvastatin administration for 4.9 years in patients with moderate-to-severe kidney disease reduced LDL-C levels by 0.85 mmol/L (33 mg/dL) and decreased the incidence of first major atherosclerotic events (including nonfatal MI, coronary death, non-hemorrhagic stroke, or arterial revascularization) by 17% compared with placebo (Baigent et al. 2011). The *IMPROVE-IT* trial compared the effect of a 6-year administration of ezetimibe +simvastatin or simvastatin alone in patients with a recent acute coronary syndrome (Cannon et al. 2015a). The combination therapy reduced LDL-C level more than simvastatin alone (24% further reduction), resulting in a significant 6.4% lower relative risk reduction of the primary composite endpoint (cardiovascular death, nonfatal myocardial infarction, unstable angina requiring hospitalization, coronary revascularization, nonfatal stroke) (Cannon et al. 2015a). Specific subgroups of patients benefit more from the combination therapy, including women (12% reduction of the relative CV vs 5% in men) (Toda Kato et al. 2015), older patients (<65 years HR 0.98; ≥75 years HR: 0.80), and diabetic patients (15% reduction versus 2% in non-diabetics) (Giugliano et al. 2018). Combined therapy was also superior to simvastatin alone in reducing the incidence of non-hemorrhagic stroke (hazard ratio 0.78, $P = 0.008$) (Wiviott et al. 2015). Adverse event incidence was similar in all subgroups (Wiviott et al. 2015).

3.2 PCSK9 Inhibitors

Proprotein convertase subtilisin kexin 9 (PCSK9) is a serine protease involved in the regulation of hepatic low-density lipoprotein receptor (LDLR) expression and, as a consequence, in the control of plasma LDL-C levels (Fig. 1). In fact, secreted PCSK9 binds to LDLR, and when the complex LDLR/PCSK9 is internalized, the conformational change of LDLR induced by PCSK9 impairs LDLR recycling on cell surface while making it more susceptible to degradation within lysosomes. This leads to a reduced LDLR surface expression, reduced LDL uptake, and therefore increased LDL-C plasma levels (Leren 2014; Norata et al. 2016; Seidah et al. 2019). Individuals carrying loss-of-function mutations in *PCSK9* gene have lower levels of LDL-C and reduced risk of cardiovascular disease (Cohen et al. 2005, 2006; Kathiresan 2008; Benn et al. 2010; Kent et al. 2017), while gain-of-function mutations are associated with an increased risk of premature cardiovascular disease (Naoumova et al. 2005; Hopkins et al. 2015; Qiu et al. 2017). Based on these observations, PCSK9 inhibition has been suggested as a possible approach for the control of hypercholesterolemia (Fig. 1).

Despite the liver is the major organ expressing PCSK9, other tissues express this protein, including the kidney, pancreas, and brain (Norata et al. 2016); this

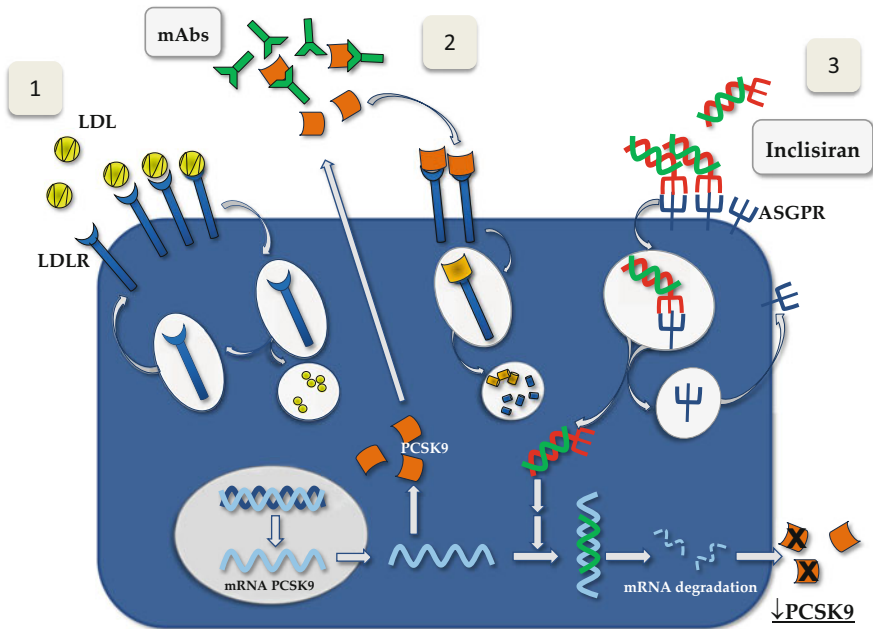


Fig. 1 Mechanism of action of PCSK9 inhibitors. In the absence of PCSK9 (1), LDL binds to LDLR. After internalization, the LDL-LDLR complex dissociates, LDL undergoes lysosomal degradation, and LDLR is recycled to the cell surface. In the presence of PCSK9 (2), LDLR cannot dissociate from LDL and is degraded within the lysosomes, leading to increased levels of circulating LDL-C. Monoclonal antibodies (mAbs) neutralize secreted PCSK9, thus inhibiting its binding to the LDLR and dampening PCSK9-induced LDLR degradation. Inclisiran (3) is a siRNA which is internalized by specific hepatic receptors (ASGPR1) and acts intracellularly by binding to PCSK9 mRNA, thus inducing its degradation and reducing the production of the protein. LDL, low-density lipoprotein; LDLR, LDL receptor; PCSK9, proprotein convertase subtilisin/kexin type 9

observation raises the question of whether the pharmacological inhibition of PCSK9 may also result in extrahepatic effects with a clinical relevance. PCSK9 deficiency in animal models and loss-of-function mutations in humans were associated with increased risk of new-onset diabetes (Ferenc et al. 2016; Da Dalt et al. 2019), pointing to a role for PCSK9 and LDLR on cholesterol metabolism in pancreatic beta cells (Perego et al. 2019). Available data from clinical trials with anti-PCSK9 monoclonal antibodies excluded this hypothesis (Sabatine et al. 2017a; Schwartz et al. 2018); data from long-term post-marketing surveillance are awaited to clarify this aspect.

Different approaches have been tested or are under evaluation for targeting PCSK9 (Seidah et al. 2019), including two fully human monoclonal antibodies against circulating PCSK9, evolocumab and alirocumab, which inhibit the activity of secreted PCSK9 (approved for the treatment of hypercholesterolemia), and, more recently, a small interfering RNA approach which inhibits PCSK9 synthesis in the liver now under clinical evaluation.

3.2.1 Evolocumab

Several phase 2 trials have shown that evolocumab efficiently reduces LDL-C levels in hypercholesterolemic subjects either as monotherapy or as add-on to background lipid-lowering therapy (LLT) (Giugliano et al. 2012; Koren et al. 2012) and was effective also in FH patients (Raal et al. 2012; Stein et al. 2013) and in statin-intolerant patients (Sullivan et al. 2012).

The PROFICIO (Program to Reduce LDL-C and Cardiovascular Outcomes Following Inhibition of PCSK9 in Different Populations) program of evolocumab included phase 3 clinical trials aimed at assessing the effectiveness of evolocumab in comparison with placebo or ezetimibe across a broad population of patients with hypercholesterolemia. In monotherapy, evolocumab efficiently reduced LDL-C levels compared with either placebo or ezetimibe (Koren et al. 2014); when added to a moderate- or high-intensity statin therapy, evolocumab reduced LDL-C levels more efficiently than adding placebo or ezetimibe, and most patients achieved LDL-C levels <70 mg/dL, compared with the group receiving ezetimibe (Robinson et al. 2014). Similar results were obtained when hypercholesterolemic patients were treated for 52 weeks with evolocumab added to different lipid-lowering therapy with a significant 57% LDL-C level reduction that was maintained throughout the study period, independent of background therapy (Blom et al. 2014). Evolocumab also induced a decrease in percent atheroma volume (PAV) in statin-treated patients (Nicholls et al. 2016).

The efficacy of PCSK9 has been tested also in patients with statin intolerance. In the phase 3 GAUSS-2 study, statin-intolerant patients were treated with evolocumab or ezetimibe for 12 weeks: evolocumab reduced LDL-C levels more efficiently than ezetimibe, and the incidence of myalgia among treated patients was low (Stroes et al. 2014). These findings have been confirmed by the GAUSS-3 trial (Nissen et al. 2016).

Specific studies have evaluated the effect of evolocumab also in FH patients (Raal et al. 2012, 2015b). The RUTHERFORD-2 study showed a significant reduction of LDL-C levels (~60%) in HeFH patients treated with evolocumab (Raal et al. 2015b). The pilot study TESLA part A, performed in six receptor-defective and two receptor-negative HoFH patients, showed that patients with defective LDLR activity had a significant reduction in their LDL-C levels following the treatment with evolocumab (~23%), while, as expected on the basis of the mechanism of action, receptor-negative patients did not respond to the therapy (Stein et al. 2013) further confirming that the mechanism by which evolocumab reduces LDL-C levels is primarily through the upregulation of residual LDLR activity. In the phase 3 TESLA part B trial, a ~31% LDL-C reduction was observed; the analysis of LDL-C reduction according to LDLR mutation status showed that the type of mutation causing FH is the major determinant of evolocumab-induced response (Raal et al. 2015a). An interim analysis of the open-label TAUSSIG trial confirmed that evolocumab reduces LDL-C levels by approximately 20% in HoFH patients (with the exception of receptor-negative patients), a reduction that persisted up to 48 weeks, with a high variability in the percent change of LDL-C from baseline (Raal et al. 2017).

Patients who completed one of the phase 2 or 3 studies have been recruited for the open-label trials OSLER-1 (for phase 2) and OSLER-2 (for phase 3); the analysis of combined data showed that evolocumab reduced LDL-C levels by 61% compared with patients treated with standard therapy; this reduction was sustained through 48 weeks and translated into a lower rate of all CV events (Sabatine et al. 2015). The final report of the OSLER-1 study showed a persistent LDL-C reduction over 5 years with a good safety profile (Koren et al. 2019). The benefit of this massive LDL-C reduction translated also in a clinical benefit. Indeed the FOURIER trial showed that, after a median follow-up of 2.2 years, in statin-treated patients with CVD and LDL-C ≥ 70 mg/dL, evolocumab significantly reduced by 15% the risk of the primary endpoint (including cardiovascular death, myocardial infarction, stroke, hospitalization for unstable angina, or coronary revascularization) and by 20% the secondary endpoint (cardiovascular death, myocardial infarction, or stroke) (Sabatine et al. 2017a). The clinical benefit was present in all patient categories, with patients at the highest CV risk showing the greatest absolute risk reduction (Sabatine et al. 2017b, 2018; Bonaca et al. 2018). A prespecified analysis of the FOURIER trial showed a linear relationship between LDL-C levels and CV outcomes down to LDL-C < 0.5 mmol/L ($< \sim 20$ mg/dL), without safety concerns (Giugliano et al. 2017b). The therapy with evolocumab did not increase the risk of new-onset diabetes and did not worsen glycemia (Sabatine et al. 2017b) nor did affect negatively the cognitive function (Giugliano et al. 2017a). Altogether these results indicate that evolocumab can be used safely used to reduce LDL-C levels and the risk of CV events in patients at high CV risk despite current optimized lipid-lowering therapy.

3.2.2 Alirocumab

Alirocumab is a fully human monoclonal antibody against PCSK9. As for evolocumab, several phase 2 trials have been performed in different patient groups; these trials have reported a significant alirocumab-induced reduction of LDL-C levels in patients taking statins as well as in patients with FH (40% up to 73%) (McKenney et al. 2012; Roth et al. 2012; Stein et al. 2012b; Dufour et al. 2017).

The *ODYSSEY* program included 14 phase 3 trials on alirocumab, aimed at evaluating the efficacy and safety of alirocumab alone or in combination with other lipid-lowering therapies in different groups of hypercholesterolemic patients. As monotherapy, alirocumab reduced LDL-C levels more efficiently than ezetimibe (47.2% vs 15.6%) (Roth and McKenney 2015). Then alirocumab was tested in high CV risk populations as add-on to maximum tolerated statin \pm other LLT: the COMBO I and II studies showed that alirocumab induced a greater reduction of LDL-C levels compared with either placebo (48.2% vs 2.3%) (Kereiakes et al. 2015) or ezetimibe (50.6% vs 20.7%) (Cannon et al. 2015b). In the *ODYSSEY LONG TERM* trial, the addition of alirocumab to the maximal tolerated dose of statin produced a 61% reduction of LDL-C levels at week 24, and this reduction persisted up to week 78 (52.4%) (Robinson et al. 2015). Adjudicated major CV events in a post hoc analysis were lower in alirocumab group than in placebo group (1.7% vs 3.3%), with cumulative probability of event curves tending to diverge over time

(Robinson et al. 2015). The ODYSSEY OPTIONS I and II trials compared the effects of adding alirocumab to atorvastatin or rosuvastatin, adding ezetimibe, or doubling the statin dose; these trials reported the greatest LDL-C level reductions in patients treated with alirocumab as add-on (Bays et al. 2015; Farnier et al. 2016).

A specific trial was designed to address the efficacy and safety of alirocumab in statin-intolerant patients, with a statin rechallenge arm. In the ODYSSEY ALTERNATIVE trial, alirocumab produced greater LDL-C reduction compared with ezetimibe in patients intolerant to statins (45% and 14.6% at week 24, respectively), and a higher percentage of patients reached the recommended LDL-C goal (41.9% vs 4.4%, respectively) (Moriarty et al. 2015). Skeletal muscle-related adverse events were the most common in all treatment groups, but significantly lower with alirocumab vs atorvastatin (HR 0.61, 95% CI, 0.38–0.99, $P = 0.042$) (Moriarty et al. 2015).

The ODYSSEY program included also clinical trials specifically designed to evaluate the effect of alirocumab in HeFH patients. In the ODYSSEY FH I (North America, Europe, South Africa) and FH II (Europe) studies, HeFH patients with an inadequate control of LDL-C levels despite maximally tolerated LLT received alirocumab and showed 57.9% (FH I) and 51.4% (FH II) LDL-C level reductions with alirocumab versus placebo; these reductions were maintained up to week 78 (Kastelein et al. 2015). Similar reductions were observed in HeFH patients presenting with higher LDL-C levels (≥ 4.1 mmol/L; ≥ 160 mg/dL) despite maximally tolerated LLT, in agreement with previous observations (Kastelein et al. 2014; Robinson et al. 2015). The treatment with alirocumab allowed the discontinuation or, at least, prolonged the time to next apheresis treatment in HeFH patients undergoing weekly or biweekly lipoprotein apheresis at baseline (ODYSSEY ESCAPE trial) (Moriarty et al. 2016). The ODYSSEY OLE study, which included HeFH patients who had completed one of the four phase 3 parent studies (FH I, FH II, LONG TERM, HIGH FH), showed a durability of alirocumab-induced LDL-C lowering over time (Farnier et al. 2018).

The ODYSSEY OUTCOMES trial tested the hypothesis that the treatment with alirocumab may reduce the risk of CV events in patients with a recent acute coronary syndrome having LDL-C levels exceeding the recommended goals for this risk category despite high-intensity statin therapy (Schwartz et al. 2018). After a median follow-up of 2.8 years, the risk of composite primary endpoint was significantly reduced by 15% in alirocumab-treated patients, and the greatest absolute reduction was observed in patients with the highest baseline LDL-C levels (≥ 100 mg/dL) (Schwartz et al. 2018). During the study, LDL-C levels were reduced by 62.7% at 4 months and 54.7% at 48 months (Schwartz et al. 2018).

Altogether, the results obtained in these studies have shown a significant efficacy of this pharmacological approach, which, by inducing a remarkable and sustained reduction of LDL-C levels beyond that obtained with statins with/without other LLT, translates into a greater CV benefit.

3.3 Lomitapide

Microsomal triglyceride transfer protein (MTP) localizes in the endoplasmic reticulum (ER) of hepatocytes and enterocytes and acts by transferring triglycerides and cholesteryl esters from the ER membrane to the nascent apoB (Hooper et al. 2015) (Fig. 2). MTP plays an essential role in the assembly and secretion of apolipoprotein B-containing lipoproteins, including very-low-density lipoproteins (VLDL) and chylomicrons (Hooper et al. 2015). Loss-of-function mutations in the gene encoding for MTP (*MTP*) result in a reduced synthesis of VLDL and chylomicrons, increased apoB degradation, and lower levels of circulating LDL-C; based on these observations, MTP has been proposed as a possible target for the pharmacological control of hypercholesterolemia, particularly for the treatment of HoFH (Sirtori et al. 2014). Lomitapide is an MTP inhibitor which reduces LDL-C levels independent of LDLR (Fig. 2), and preclinical studies in animal models have shown that MTP inhibition resulted in the reduction of atherogenic lipoprotein levels (Wetterau et al. 1998; Shiomi and Ito 2001).

Lomitapide has been approved for the treatment of HoFH (Berberich and Hegele 2017), based on the results of several randomized clinical trials. A phase 2 study showed that the administration of lomitapide for 16 weeks in monotherapy efficiently reduced LDL-C levels in six HoFH patients (5 LDLR-negative and 1 LDLR-defective) (Cuchel et al. 2007); overall the drug was well tolerated, and the most serious adverse events were elevations in liver aminotransferase levels and hepatic fat accumulation (Cuchel et al. 2007). This study highlighted that, although lomitapide was highly effective in reducing LDL-C levels, it also induced liver steatosis in treated patients, casting doubts about long-term safety.

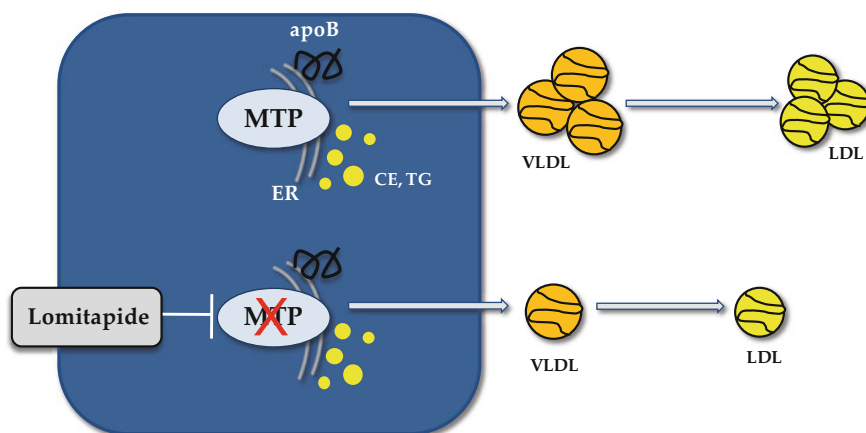


Fig. 2 Mode of action of lomitapide. MTP mediates the transfer of cholesteryl esters (CE) and triglycerides (TG) to apolipoprotein B (apoB) resulting in the synthesis of VLDL and chylomicrons. Lomitapide inhibits the activity of MTP, thus reducing the production of apoB-containing lipoproteins

To address this aspect, lomitapide was administered to 29 HoFH patients in a single-arm, open-label, phase 3 study in addition to conventional lipid-lowering therapy (Cuchel et al. 2013). Mean LDL-C levels were significantly reduced by 50% at the end of efficacy phase (week 26) with some patients able to discontinue or increase the interval between apheresis treatments (Cuchel et al. 2013). At the end of the study (week 78), LDL-C reduction was partially attenuated (−38%); changes in the concomitant lipid-lowering therapies or adjustment of the lomitapide dose in patients experiencing adverse effects can likely explain this result (Cuchel et al. 2013). Many patients reported gastrointestinal adverse reactions, which were controlled by moving patients on a very-low-fat diet. Increased levels of ALT, AST, or both, observed in some patients, were transient, successfully managed by dose reduction or temporary interruption, and were not related to changes of liver function. A modest increase (8.6%) in hepatic fat content was observed (Cuchel et al. 2013); the clinical consequence of this rise in liver fat is still unclear and requires further investigation with longer-term trials in a higher number of patients to assess whether it could have metabolic consequences.

Data from the real-world setting seem to suggest that safety profile of lomitapide is similar to that reported by clinical trials and have confirmed the need of a careful monitoring of transaminase levels and the adherence to a low-fat diet which may minimize gastrointestinal side effects (Roeters van Lennep et al. 2015). A longer follow-up is required to further support safety and establish whether lomitapide therapy may translate into a clinical cardiovascular benefit. To this aim, the LOWER registry for patients treated with lomitapide was created (Blom et al. 2016); it also includes the CAPTURE substudy which is aimed at investigating the effect of lomitapide on the atheroma regression and/or stabilization in the carotid artery and aorta (Blom et al. 2016).

Lomitapide is a weak inhibitor of CYP3A4, thus it might decrease statin metabolism (Tuteja et al. 2014), and therefore careful monitoring for adverse events when these two drugs are administered in combination is required.

3.4 Mipomersen

Elevated levels of apoB, the main apoprotein of all atherogenic lipoproteins, represent an established risk factor in atherosclerosis; mutations in apoB gene causing familial hypobetalipoproteinemia, characterized by very low levels of apoB and LDL-C, have been associated with a reduced risk of coronary disease (Peloso et al. 2019). Furthermore, a Mendelian randomization study showed that genetic variants resulting in reduced apoB levels are associated with comparable lower CHD risk per unit difference in apoB (Ference et al. 2019a), strengthening the concept that the clinical benefit of lipid-lowering therapies may be proportional to the absolute change in apoB levels. Thus, apoB may be a pharmacological target to reduce hypercholesterolemia.

In parallel with the possibility to inhibit MTP activity, it is also possible to target the key structural protein of VLDL and LDL to inhibit lipoprotein biosynthesis

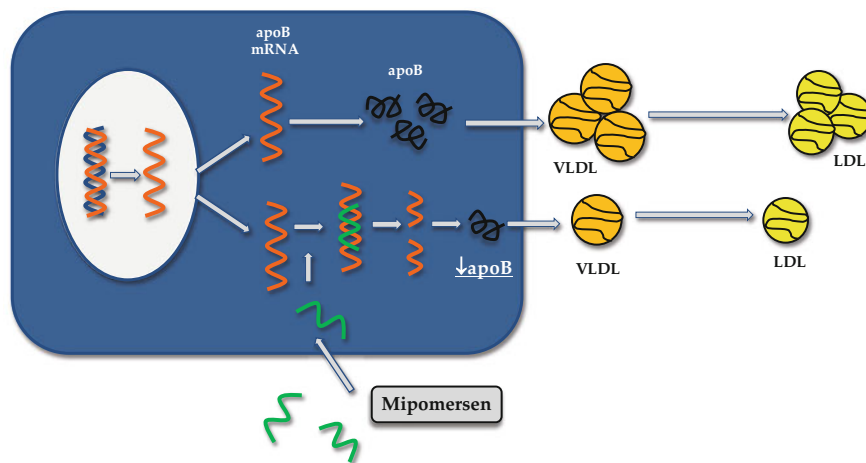


Fig. 3 Mechanism of action of mipomersen. Mipomersen is an antisense oligonucleotide (ASO) that inhibits the synthesis of apolipoprotein B. It binds to the cognate mRNA forming a substrate for a nuclease, thus resulting in mRNA degradation and reduced production of apoB and apoB-containing lipoproteins

(Norata et al. 2013a). Mipomersen is a second-generation antisense oligonucleotide against the coding region of human apolipoprotein B mRNA which, by silencing apoB, results in the reduction of all apoB-containing lipoproteins. The effect of mipomersen is independent of LDLR and thus represents a suitable approach for the treatment of FH with severe LDLR mutations (Crooke and Geary 2013) (Fig. 3). Several trials have shown the lipid-lowering efficacy of mipomersen either as monotherapy or as add-on to current lipid-lowering therapies (Kastelein et al. 2006; Akdim et al. 2010a, b, 2011; Raal et al. 2010; McGowan et al. 2012; Stein et al. 2012a; Visser et al. 2012). A recent meta-analysis on data from 13 randomized clinical trials confirmed that mipomersen significantly reduced LDL-C levels by ~26%, with an overall improvement of all lipid parameters (Fogacci et al. 2019). Nevertheless, mipomersen therapy was associated with several side effects such as injection site reactions, flu-like symptoms, hepatic steatosis, and hepatic enzyme elevations which lead to an elevated number of patients discontinuing the treatment (Fogacci et al. 2019).

The most important side effect expected from the mechanism of action is an increased content of TG in the liver which raised some concerns regarding mipomersen therapy. While short-term studies have in fact reported increased steatosis in mipomersen-treated patients, longer-term trials (up to 104 weeks) showed a return to normal of hepatic triglyceride levels (Sahebkar and Watts 2013; Toth 2013). Further, the analysis of liver biopsies from mipomersen-treated patients revealed a histopathological feature of simple steatosis without signs of inflammation or fibrosis (Hashemi et al. 2014).

In HeFH patients treated with mipomersen, apoB and LDL-C were reduced by ~20% with 200 mg/week dose and by more than 30% with 300 mg/week dose (Akdim et al. 2010b), with the most common adverse effect being injection site erythema (97%). In the same study, four patients treated with mipomersen (three of which in the highest dose group) experienced transaminase elevations $>3 \times$ ULN with signs of hepatic steatosis (Akdim et al. 2010b). Due to the long half-life of mipomersen, LDL-C levels remain lower than baseline for as long as 2–3 months after the last injection (Akdim et al. 2010b). Similar results have been reported in HeFH patients with CAD (Stein et al. 2012a). Mipomersen is also effective in HoFH patients, with a 24.7% mean LDL-C reduction from baseline observed on top of their maximal tolerated lipid-lowering therapy, associated with an overall improvement of plasma lipid profile (Raal et al. 2010). Also in these patients, the most common adverse event was injection site reactions, followed by ALT elevations ≥ 3 times ULN which occurred in four mipomersen patients and one patient showing also a significant increase in hepatic fat content (Raal et al. 2010).

A 2-year interim report of a long-term trial showed that mipomersen provides sustained reduction in apoB and LDL-C levels. Of note the median liver fat increased during initial 6–12 months, but returned to baseline with longer treatment, suggesting that a liver metabolic adaptation might occur (Santos et al. 2015). Long-term treatment with mipomersen not only reduces LDL-C and other atherogenic lipoprotein levels but may also reduce the incidence of CV events in patients with FH (Duell et al. 2016).

4 Cholesterol-Lowering Drugs Under Clinical Development

4.1 Inclisiran

Small interfering RNA (siRNA) induces the degradation of a specific mRNA which halts protein synthesis (Norata et al. 2013b). Inclisiran is a specific siRNA which targets PCSK9 mRNA and blocks intracellular PCSK9 synthesis in the liver, as opposed to monoclonal antibodies against PCSK9 which block circulating protein (Fig. 1).

Preclinical studies have shown that a siRNA targeting PCSK9 (ALN-PCS) was effective in reducing plasma PCSK9 and LDL-C levels (Frank-Kamenetsky et al. 2008), and in healthy volunteers, a single intravenous dose of ALN-PCS showed a mean 70% reduction in circulating PCSK9 plasma levels and a 40% reduction in LDL-C levels (Fitzgerald et al. 2014).

Inclisiran (ALN-PCSsc) is a siRNA against PCSK9 conjugated to triantennary N-acetylgalactosamine carbohydrate structure allowing the compound to be recognized by the hepatic asialoglycoprotein receptors, which is selectively expressed in hepatocytes, thus resulting in a specific liver uptake. In healthy volunteers, inclisiran induced a dose-dependent reduction of plasma PCSK9 levels (up to 83.8%) and LDL-C levels (up to 59.7%) (Fitzgerald et al. 2017). The ORION clinical program was started to test the efficacy and safety of inclisiran in people with

atherosclerotic CVD and elevated LDL-C levels despite the maximum tolerated dose of LLT, as well as in patients with FH. In the phase 2 trial ORION-1, conducted in patients at high CV risk with elevated LDL-C levels treated with the maximum possible dose of a statin with or without additional LLT, individuals received a single dose or two doses (at days 1 and 90) of inclisiran or placebo (Ray et al. 2017). Inclisiran dose-dependently reduced PCSK9 and LDL-C levels (Ray et al. 2017). At day 240, both PCSK9 and LDL-C were significantly lower than at baseline in all inclisiran regimen groups (Ray et al. 2017). The rate of adverse events was similar in inclisiran and placebo groups, and also injection site reactions were rare and similar to those reported with monoclonal antibodies (Ray et al. 2017; Leiter et al. 2019). The treatment with inclisiran every 6 months resulted in durable LDL-C level reductions over 1 year (Ray et al. 2019b). The *ORION-3 study* is an ongoing open-label extension study of ORION-1 comparing the effect of long-term dosing of inclisiran or evolocumab administration; inclisiran will be administered on day 1 and every 180 days thereafter for up to 4 years (NCT03060577). The interim results showed a favorable safety and tolerability profile for inclisiran, with infrequent, mild, and transient injection site reactions, and no evidence of myalgias or liver or renal adverse events that were related to inclisiran. A robust LDL-C reduction was observed (51% in all patients), and the time-averaged response showed no loss of effect over 3 years (Kastelein 2019).

The phase 3 trials ORION-9, ORION-10, and ORION-11 were designed to assess the efficacy and safety of inclisiran in HeFH, ASCVD, and/or risk equivalent patients with stable LLT. The ORION-9 showed that LDL-C were reduced by 50% at day 210, time-averaged percent change of LDL-C days 90–540 was 45%, and a durable and potent LDL-C-lowering effect was observed over 18 months, independent of the FH genotype; the rate of adverse events was comparable among groups (Raal et al. 2020). Similar effects were reported in the ORION-10 and ORION-11 trials, with prespecified exploratory analysis on CV events showing a lower rate in patients treated with inclisiran compared to placebo (Ray et al. 2020). Inclisiran has been also tested in four HoFH patients in the ORION-2 pilot study, showing a persistent PCSK9-lowering effect independent of the specific mutation and an LDL-C-lowering effect related to the severity of mutation (maximum –43%) (Raal 2019). The ORION-5 trial (NCT03851705) is evaluating the effect of placebo or inclisiran in 45 HoFH patients in a 6-month double-blind period, and then all patients will receive inclisiran during an 18-month open-label follow-up period; the study is expected to end June 2021.

The ongoing ORION-4 aims to assess whether PCSK9 inhibition by inclisiran 300 mg safely lowers the risk of major atherosclerotic cardiovascular events in $\geq 15,000$ patients with pre-existing ASCVD during a median treatment duration of 5 years (NCT03705234). Estimated primary completion date is December 2024.

4.2 Bempedoic Acid

Bempedoic acid is a new drug that inhibits the synthesis of cholesterol by inhibiting the activity of ATP citrate lyase (ACLY), the enzyme catalyzing the transformation of citric acid into oxaloacetate and acetyl-CoA (Fig. 4). The inhibition of ACLY, by blocking cholesterol biosynthesis, results in an increased hepatic expression of the LDL receptor (Pinkosky et al. 2016) and a consequent reduction in the circulating levels of LDL-C. Based on these observations, targeting ACLY activity appears to be a promising approach to lower LDL-C levels. A recent Mendelian randomization study, comparing variants in ACLY gene (mimicking the effect of bempedoic acid) with variants in HMGCR gene (mimicking the effect of statins), seems to suggest that inhibiting ACLY translates into cardiovascular protection (Ference et al. 2019b); in fact, the reduction of the risk of major cardiovascular events for each decrease of 10 mg/dL in LDL-C levels was similar for genetic variants that mimic bempedoic acid or statin activity (Ference et al. 2019b).

Bempedoic acid is a pro-drug, which is converted into its active form by a hepatic enzyme not expressed in skeletal muscle (very-long-chain acyl-CoA synthetase-1, ACSVL1), which should reduce the potential risk of muscle-related adverse events and may thus represent an important approach in patients intolerant to statins.

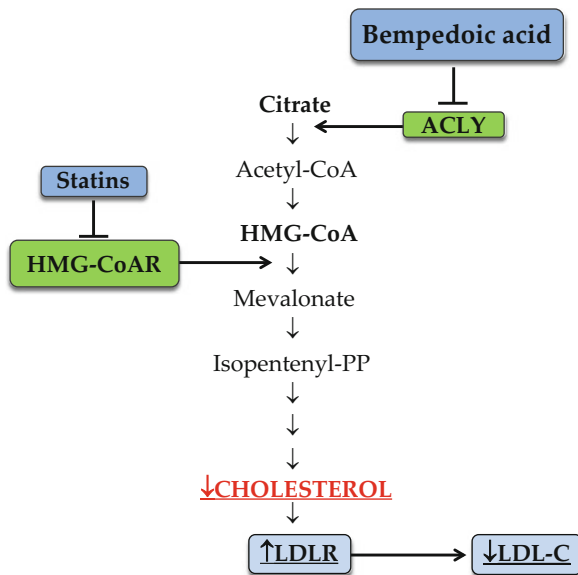


Fig. 4 Mechanism of action of bempedoic acid. Bempedoic acid inhibits cellular cholesterol biosynthesis by blocking the activity of ATP citrate lyase (ACLY), an enzyme upstream of hydroxymethylglutaryl-CoA reductase (the target of statins). Therefore cells upregulate the expression of the LDLR to compensate for reduced cholesterol biosynthesis. A specific enzyme, ACSVL1 (very-long-chain acyl-CoA synthetase-1), expressed in the liver but not in other peripheral tissues, converts bempedoic acid into the active form thus conferring hepatoselectivity to the drug

Bempedoic acid was also reported to activate the AMP-activated protein kinase (AMPK) subunit beta1 (Pinkosky et al. 2013), however this subunit appears to be less relevant in humans, and therefore whether bempedoic acid positively impact carbohydrate metabolism in humans remains to be demonstrated.

Bempedoic acid dose-dependently lowered LDL-C levels up to 27% in hypercholesterolemic patients, independent on their TG levels (Ballantyne et al. 2013). Non-HDL-C and apoB levels and LDL particle number were also significantly reduced (Ballantyne et al. 2013). Hypercholesterolemic patients with type 2 diabetes showed an even greater LDL-C reduction following the treatment with bempedoic acid (−43%) (Gutierrez et al. 2014). The treatment with bempedoic acid did not result in a worsening of glycemic control, which is another relevant concern associated with statin therapy (Gutierrez et al. 2014). Two studies have specifically evaluated the effect of bempedoic acid in hypercholesterolemic patients intolerant to statins. One study reported a 32% LDL-C level reduction after 8 weeks of treatment (vs a −3.3% with placebo), with an incidence of muscle-related adverse events similar in the two groups (Thompson et al. 2015). In another study, bempedoic acid alone reduced LDL-C levels up to 30%, compared with a 21% reduction observed with ezetimibe; the greatest reduction was observed in patients treated with the combination bempedoic acid+ezetimibe that also improved the levels of other lipids (43–48%) (Thompson et al. 2016). These reductions were similar in statin-tolerant and statin-intolerant patients, and frequencies of muscle-related adverse events were low in all treatment groups (Thompson et al. 2016).

In patients with persistently elevated LDL-C despite a background statin therapy, bempedoic acid induced a significant LDL-C reduction compared with placebo (17%–24% vs 4%), but muscle-related adverse events had similar frequencies in all groups (Ballantyne et al. 2016). Similar results were obtained in patients treated with bempedoic acid added to high-dose atorvastatin background therapy, who showed a 22% LDL-C level reduction compared with placebo and an overall improved lipid profile (Lalwani et al. 2019). In this study, the addition of bempedoic acid to atorvastatin background therapy resulted in an increased exposure (<30%) to atorvastatin, which is suggestive of a weak interaction between the two drugs likely without clinical relevance (Lalwani et al. 2019). The results of these studies suggest that bempedoic acid, alone or in combination with ezetimibe, may represent a valuable approach for the treatment of hypercholesterolemic patients, including those intolerant to statins.

The phase 3 CLEAR program (Cholesterol Lowering via Bempedoic Acid, an ACL-Inhibiting Regimen) includes five studies, four of which have been completed. The CLEAR Harmony and the CLEAR Wisdom studies showed 12.6 and 17.4% LDL-C level reductions in patients with high LDL-C despite receiving maximum tolerated LLT; both studies showed similar incidence of adverse events (Goldberg et al. 2019; Ray et al. 2019a). The CLEAR Serenity study evaluated the effect of bempedoic acid or placebo in patients with a history of intolerance to at least two statins, showing a significant reduction in LDL-C levels (−23.6% vs −1.3% with placebo) and other lipid parameters, with similar incidence of adverse events in the treatment groups (Laufs et al. 2019). Similar results were obtained in the CLEAR

Tranquility trial, which showed that bempedoic acid in combination with ezetimibe further reduced LDL-C levels compared with ezetimibe alone (28.5%) in statin-intolerant patients, with safety profiles comparable between treatment groups (Ballantyne et al. 2018). An ongoing study (CLEAR Cardiovascular Outcomes) is evaluating whether treatment with bempedoic acid treatment reduces the risk of cardiovascular events in patients at high cardiovascular risk with statin intolerance. The results of this study are expected for 2022.

5 The Future of Cholesterol Lowering

5.1 ANGPTL3-L_{Rx}

Angiopoietin-like 3 (ANGPTL3) is an endogenous inhibitor of LPL and endothelial lipase (EL), two enzymes playing an important role in lipoprotein metabolism (Tikka and Jauhiainen 2016). Individuals carrying LOF mutations in the *ANGPTL3* gene have lower levels of TG and LDL-C and a significantly reduced CV risk (Dewey et al. 2017; Stitzel et al. 2017; Tarugi et al. 2019). In agreement with this observation, the analysis of plasma levels of ANGPTL3 showed that subjects in the lowest ANGPTL3 level tertile have a significantly reduced risk of myocardial infarction compared to subjects with levels in the highest tertile (Dewey et al. 2017; Stitzel et al. 2017). Based on these observations, ANGPTL3 has been suggested as a possible pharmacological target for the treatment of mixed hyperlipidemias, and for FH patients, due to its cholesterol-lowering effect which is independent of LDLR (Wang et al. 2015). Two different strategies are under investigation to inhibit ANGPTL3: a monoclonal antibody (evinacumab) and an antisense oligonucleotide (ASO, ANGPTL3-L_{Rx}) (Fig. 5), both reducing plasma LDL-C and TG levels.

Evinacumab is a fully human monoclonal antibody targeting ANGPTL3 able to reduce LDL-C (up to 23%) and TG (up to 76%) levels in healthy volunteers (Dewey et al. 2017). When tested in HoFH patients, including two null homozygotes and one compound heterozygote with two null alleles, evinacumab added to ongoing LLT further reduced LDL-C levels by 49% (Gaudet et al. 2017). Recently, a phase 3 trial confirmed that evinacumab halves LDL-C levels in HoFH patients independent of the causative mutation, with 47% of patients achieving LDL-C levels <100 mg/dL (<http://www.prnewswire.com/news-releases/regeneron-announces-positive-topline-results-from-phase-3-trial-of-evinacumab-in-patients-with-severe-inherited-form-of-high-cholesterol-300901035.html>). Ongoing studies are assessing, alongside the lipid-lowering effect, the safety and long-term tolerability (up to 192 weeks) of evinacumab therapy in HoFH patients and the potential development of anti-drug antibodies. Evinacumab is also tested in patients with HeFH or non-FH but with a history of atherosclerotic cardiovascular disease with LDL-C levels persistently elevated despite receiving maximally tolerated LLT.

ANGPTL3-L_{Rx} is an antisense oligonucleotide containing three GalNac residues to promote the specific recognition by hepatic ASGPR1 receptors (Graham et al. 2017). In preclinical studies, the treatment with ANGPTL3-L_{Rx} significantly

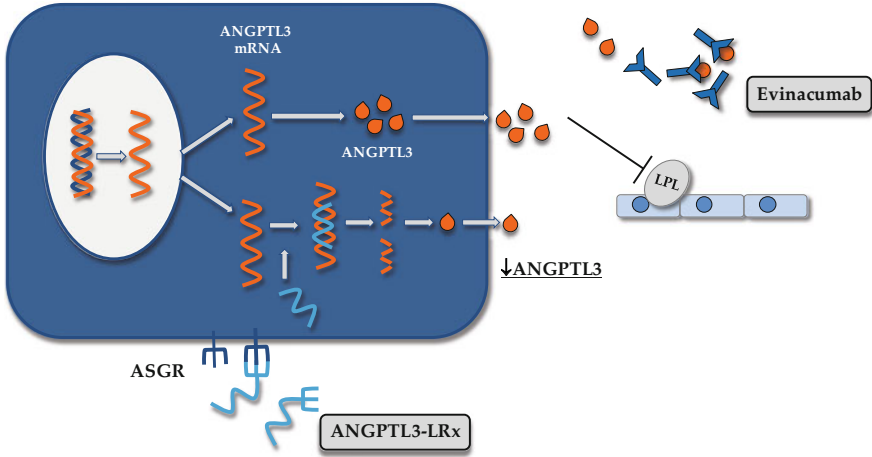


Fig. 5 Mechanism of action of antisense oligonucleotides. ANGPTL3-Lrx and APOA(a)-Lrx are antisense oligonucleotides containing three GalNAc residues which promote the specific recognition by hepatic ASGPR receptors. Once internalized, they inhibit protein synthesis of ANGPTL3 or apo(a), respectively, by binding the corresponding mRNAs and inducing their degradation

reduced ANGPTL3 protein levels, which translated into a reduction of plasma TG and LDL-C, hepatic TG content, progression of atherosclerosis, and improvement of insulin sensitivity (Graham et al. 2017). In healthy subjects with elevated TG levels, ANGPTL3-L_{Rx} produced a dose-dependent reduction in circulating levels of ANGPTL3 (47–84%), TG (33–63%), LDL-C (1.3–33%), VLDL-C (28–60%), and apoCIII (19–59%) (Graham et al. 2017). No serious adverse events were reported during treatment, and no evidence of pro-thrombotic effects, bleeding episodes, and reduction of platelet count was reported compared to ASOs of previous generations (Graham et al. 2017).

6 Conclusions

In the last decade, several novel therapeutic approaches have been tested and some approved for reducing plasma lipids and lipoproteins. Some drugs are already available in clinical practice; others are at late stages of development. From a pharmacological perspective, in addition to statins, bempedoic acid was shown to produce an effective inhibition of cholesterol biosynthesis in the liver, while other molecules inhibit cholesterol absorption in the intestine (ezetimibe), inhibit lipoproteins synthesis in the liver and the intestine (lomitapide), or promote lipoprotein catabolism (PCSK9 inhibitors or ANGPTL3 inhibitors). Interest is also emerging on the possibility to target Lp(a).

The new technology of antisense oligonucleotides and small interfering RNAs has clearly opened a fast track for drugs targeting pathways likely to be causal to CVD. The availability of these approaches will definitely improve the management of dyslipidemias, particularly hypercholesterolemias.

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Antithrombotic Therapy: Prevention and Treatment of Atherosclerosis and Atherothrombosis

R. H. Olie, P. E. J. van der Meijden, H. M. H. Spronk, and H. ten Cate

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Abstract

Atherosclerosis is a multifactorial vascular disease that develops in the course of a lifetime. Numerous risk factors for atherosclerosis have been identified, mostly inflicting pro-inflammatory effects. Vessel injury, such as occurring during erosion or rupture of atherosclerotic lesions triggers blood coagulation, in attempt to maintain hemostasis (protect against bleeding). However, thrombo-inflammatory mechanisms may drive blood coagulation such that thrombosis develops, the key process underlying myocardial infarction and ischemic stroke (not due to embolization from the heart). In the blood coagulation system, platelets and coagulation proteins are both essential elements. Hyperreactivity of blood coagulation aggravates atherosclerosis in preclinical models. Pharmacologic inhibition of blood coagulation, either with platelet inhibitors, or better documented with anticoagulants, or both, limits the risk of thrombosis and may potentially reverse atherosclerosis burden, although the latter evidence is still based on animal experimentation.

Patients at risk of atherothrombotic complications should receive a single antiplatelet agent (acetylsalicylic acid, ASA, or clopidogrel); those who survived an atherothrombotic event will be prescribed temporary dual antiplatelet therapy (ASA plus a P2Y₁₂ inhibitor) in case of myocardial infarction (6-12 months), or stroke (<6 weeks), followed by a single antiplatelet agent indefinitely. High risk for thrombosis patients (such as those with peripheral artery disease) benefit from a combination of an anticoagulant and ASA. The price of gained efficacy is always increased risk of (major) bleeding; while tailoring therapy to individual needs may limit the risks to some extent, new generations of agents that target less critical elements of hemostasis and coagulation mechanisms are needed to maintain efficacy while reducing bleeding risks.

Keywords

Anticoagulants · Antiplatelet therapy · Aspirin · Atherosclerosis · Atherothrombosis · Clopidogrel · Coagulation · Platelets · Thrombosis

1 Atherogenesis and the Role of Blood Coagulation Components

Atherosclerosis is a multifactorial vascular disease that develops in the course of a lifetime. Numerous risk factors for atherosclerosis have been identified, mostly inflicting pro-inflammatory effects, hence, the term “chronic inflammatory disease” (Ross 1999). The blood coagulation system has a primary role in maintaining hemostasis, preventing fatal bleeding (Spronk et al. 2003a). A second function relates to wound healing in a broader sense. Blood coagulation becomes activated in response to any vascular injury causing contact between the vascular matrix and/or cells with blood. This clotting process likely is meant to seal the wound

surface and help to repair the underlying wound bed. Platelets, coagulation, and fibrinolysis factors, as well as fibrin are key elements in this wound healing process, and the administration of some of these components (platelets, fibrinogen) is also studied in clinical trials on wound healing of the skin (Hoffman 2018; Opneja et al. 2019). In the vasculature, pro-atherogenic changes of the vascular endothelium, involving endothelial cell (EC) activation, dysfunction, or damage (exposing subendothelial matrix), trigger the coagulation system in an attempt to vascular wound healing.

1.1 Blood Coagulation: Impact on Vascular Endothelial Cells

The blood coagulation system consists of different pathways that together provide the hemostatic plug or, in pathological situations, the thrombus. Platelets and coagulation proteins act in concert to build the fibrin-platelet clot on top of the damaged vessel wall, while the fibrinolytic system helps to limit clot formation and acts to restore blood flow upon (partial) clot lysis.

In atherogenesis, early inflammatory endothelial perturbation (Gimbrone Jr. and Garcia-Cardena 2016) may trigger activation of platelets that together with leukocyte populations provide a first thrombo-inflammatory response to injury (Fig. 1) (Messner and Bernhard 2014). Activation of platelets (but also other cells) yields extracellular vesicles (EVs) that promote thrombo-inflammatory reactions, amplifying fibrin formation (Badimon et al. 2017). Extracellular vesicles are particles that are naturally released from nearly all kinds of cells; carry a cargo of proteins, RNA, and lipids from the parent cell; and are thus considered to be key components in cell-cell communication. Repeated and/or ongoing inflammatory pressure (endogenous factors like oxidized lipoproteins, glycated end products, homocysteine, etc. and exogenous factors like smoking and other sources of particulate matter) challenges the vascular endothelium to become activated and permeable for inflammatory cells, lipids, and other toxic components (Mozaffarian et al. 2008).

Disruption of the endothelial cell barrier is induced or aggravated by pro-inflammatory cytokines, metalloproteases, and cellular enzymes like elastase and trypsin as well as by the influence of coagulation serine proteases like factor Xa and thrombin, acting on protease-activated receptors (PARs) (Coughlin 2005; Posma et al. 2016; Ruf 2018). Physiologically, PARs are expressed at the endothelial cell surface to mediate endothelial *protective* effects of activated protein C (APC), through activation of PAR1 (Mosnier et al. 2012). Besides activation of protein C, the thrombin-thrombomodulin (TM) complex also converts plasma pro-carboxypeptidase B2 (proCPB2 or thrombin-activatable fibrinolysis inhibitor, TAFI). Activated TAFI (TAFIa) inhibits plasmin formation and hence stabilizes fibrin clots by inhibiting plasmin generation and fibrinolysis (Fujiwara et al. 2012; Morser et al. 2010; Myles et al. 2003; Shao et al. 2015). TAFIa also has anti-inflammatory properties including the inactivation of pro-inflammatory mediators like bradykinin, anaphylatoxins C3a and C5a, and thrombin-cleaved OPN (Myles et al. 2003; Naito et al. 2013; Nishimura et al. 2007; Relja et al.

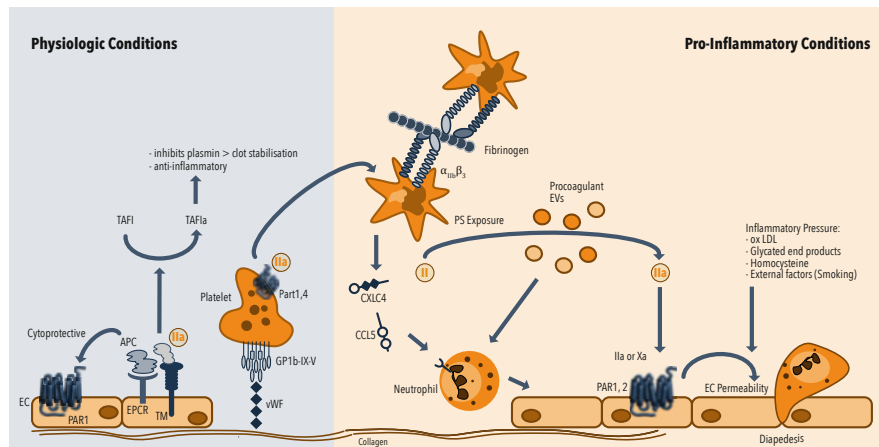


Fig. 1 Interactions of platelets, leucocytes, coagulation factors, and vascular endothelial cells in atherogenesis and atherothrombosis. Under *physiologic conditions*, thrombin (IIa) supports endothelial cell integrity through endothelial cell-mediated APC and TAFIa generation. PARs are expressed at the endothelial cell (EC) surface to mediate cytoprotective effects of APC through activation of PAR1. APC is activated by the thrombin-thrombomodulin (TM) complex, which also converts TAFI into activated TAFI (TAFIa), which has anti-inflammatory properties. Under *pro-inflammatory conditions*, several pro-inflammatory cytokines alter PAR expression patterns and downregulate the protective cellular receptors like TM and EPCR. The result is a shift to prothrombotic and offensive functions of PAR-EC interactions. Thrombin-induced PAR1 activation in combination with endogenous factors like smoking and oxidized lipoproteins challenges the vascular endothelium to become permeable for inflammatory cells. Damage of the endothelial layer results in exposure of highly reactive subendothelial proteins, such as collagen. Under arterial shear stress, vWF bound to collagen enables platelet adhesion via the glycoprotein (GP)Ib-IX-V complex. Activation of platelets yields extracellular vesicles that promote thrombo-inflammatory reactions, including attraction of leukocytes. Activated platelets deposit chemokines, such as CXCL4 and CCL5, on the inflamed endothelium, thereby recruiting leukocytes, which bind to platelets adhering to the (sub)endothelium. Platelets and leukocytes secrete pro-inflammatory and pro-angiogenic factors that further support atherogenesis. Traces of thrombin activate platelets through PAR1 and PAR4. These activated platelets provide a procoagulant phosphatidylserine (PS)-rich surface on which coagulation factors can gather, ultimately leading to the conversion of prothrombin (II) to thrombin (IIa). Thrombin-induced PAR activation induces inflammatory damage to the vascular endothelium. EC endothelial cells, APC activated protein C, PAR protease-activated receptor, EPCR endothelial cell protein C receptor, TM thrombomodulin, TAFI thrombin-activatable fibrinolysis inhibitor, GPIb-IX-V glycoprotein Ib-IX-V, vWF von Willebrand factor, CXCL4 chemokine ligand 4, CCL5 chemokine ligand 5, PS phosphatidylserine, EV extracellular vesicle

2013). This way, under physiologic conditions thrombin supports endothelial cell integrity through endothelial cell-mediated APC and TAFIa generation.

Under inflammatory pressure, pro-inflammatory cytokines alter PAR expression patterns and downregulate protective cellular receptors like TM and endothelial cell protein C receptor (EPCR) (Esmon 1995, 2014). The result is a shift from anticoagulant (and protective) to prothrombotic (and offensive) functions of

serine protease-PAR-EC interactions (Fig. 1). In the case of PAR-1, the shift from protective (APC induced) to inflammatory (thrombin induced) is referred to as “biased” signaling (Griffin et al. 2015).

1.2 Platelets and Extracellular Vesicles

Mouse studies provide mechanistic insight in the pro-atherogenic functions of blood components including platelets, EVs, and coagulation proteins. Platelets and platelet-derived EVs are important messengers of various mediators including microRNAs that modify inflammation and atherogenesis. A range of studies provided evidence for involvement of EVs on various key pathways in atherogenesis, including inflammation, calcification, and cell trafficking (Badimon et al. 2017; Paone et al. 2019; Alique et al. 2018; Bouchareychas and Raffai 2018; Miller et al. 2016). Ongoing research is addressing ways to utilize some of these functions of EVs including transportation of microRNA for potential therapeutic purposes in atherosclerosis (Yin et al. 2015). Majority of EVs are platelet-/megakaryocyte-derived and contain cytokines, RNA species, coagulation factors, etc. EVs can interact with, e.g., leukocytes and regulate activity (Vasina et al. 2013).

Platelets roll and adhere to activated endothelium (Schulz and Massberg 2012). Platelets promote the differentiation and activation of leukocytes (Lievens and von Hundelshausen 2011). Activated platelets deposit chemokines, mainly chemokine ligand 4 (CXCL4) and chemokine ligand 5 (CCL5), on the inflamed endothelium, thereby recruiting leukocytes and exacerbating atherosclerosis (von Hundelshausen et al. 2005; Huo et al. 2003). Also, secreted chemokines attract leukocytes, which bind to platelets adhering to the (sub) endothelium. Platelets and leukocytes secrete pro-inflammatory and pro-angiogenic factors that further support atherogenesis (Semple et al. 2011). Multiple (receptor) interactions between platelets-leukocytes and platelets-vessel wall have been observed in inflammatory conditions such as atherosclerosis (Koupenova et al. 2018; Koenen 2016). Platelet-neutrophil interactions and the release of neutrophil activation products including neutrophil extracellular traps (NETs) may also trigger coagulation.

Cross talk between platelets and coagulation is another important mechanism that operates at different levels. Traces of thrombin activate platelets through PAR1, with PAR4 activation as a delayed, secondary mechanism. Activated platelets provide a phosphatidylserine (PS)-rich surface on which coagulation reactions take place (tenase and prothrombinase complex formation). Upon induction with strong agonists such as a combination of thrombin and collagen, PS exposure allows binding of Gla domain-containing coagulation factors which enhances the activity of coagulation factor complexes. This procoagulant platelet response is supported by the secretion of coagulation factors (prothrombin, Factor V, Factor 8 transcript, Factor XIII, and fibrinogen) and modified by anticoagulation factors (e.g., antithrombin, tissue factor pathway inhibitor (TFPI), protein S).

Platelet-derived factor V may be an important determinant of local prothrombinase formation, driving atherogenesis (Ren et al. 2017). Additional factors that are secreted by platelets are tissue factor (TF) (the origin still somewhat controversial) and protein disulfide-isomerase (PDI) that acts to de-encrypt TF in an active conformation. Polyphosphate (PolyP) is secreted that may activate factor XII. Platelet-bound factor XI drives coagulation in an angiotensin-dependent manner (Kossmann et al. 2017). Another mechanism that bridges platelets and coagulation is by binding coagulation factors via the glycoprotein (Gp) complexes GPIb-V-IX, GPIIb/IIIa, and GPVI (Swieringa et al. 2018). Finally, platelets alter fibrin structure (via regulating local thrombin concentration) and mediate clot contraction. This process involves different platelet populations, including procoagulant platelets that primarily stimulate fibrin formation in the periphery of the clot, associated with clot contraction (Nechipurenko et al. 2019). In the course of this process, thrombin-activated platelets become fragmented, a process that limits further dissemination of activated platelets (Kim et al. 2019).

1.3 Antiplatelet Agents and Atherosclerosis

From the described thrombo-inflammatory mechanisms, one may infer that platelet inhibition with antiplatelet medication could potentially attenuate atherogenesis and atherosclerosis. In addition to the inhibition of thromboxane A2 (TXA2) production, aspirin (acetylsalicylic acid (ASA) being the active component) increases platelet nitric oxide (NO) synthesis, protects NO from its inactivation, and improves endothelial dysfunction (Russo et al. 2017). Aspirin also has anti-inflammatory effects, but whether the doses used to prevent platelet aggregation are sufficient to produce meaningful anti-inflammatory effects in humans remains uncertain. In mice, low-dose aspirin improves vascular inflammation and stabilizes plaques (Cyrus et al. 2002) or limits plaque severity, maybe related to reduced fractalkine levels (Liu et al. 2010). In humans, aspirin has been shown to reduce the levels of pro-inflammatory cytokines including interleukin (IL)-6 and monocyte colony-stimulating factor (Ikonomidis et al. 1999) and to protect the endothelium against inflammatory challenge (Kharbanda et al. 2002). In spite of these and other possible protective effects, the net effect on human atherosclerosis remains uncertain (Tousoulis et al. 2016). With regard to thrombosis, literature points to effects of aspirin on fibrin clot formation and stability that may contribute to its antithrombotic action (Gurbel et al. 2019).

Evidence for a role of the P2Y₁₂ receptor in atherogenesis comes from studies in mice with a double apoE and P2Y₁₂ deletion genotype that show reduced lesion area, increased fibrous content at the plaque site, and decreased monocyte/macrophage infiltration of the lesions in the double knockout animals as compared to control apoE^{-/-} mice (Li et al. 2012; West et al. 2014). The P2Y₁₂ inhibitor clopidogrel reduced levels of p-selectin, e-selectin, monocyte chemoattractant protein-1, and platelet-derived growth factor β , reduced macrophage and T-cell

infiltration in atherosclerotic lesions, and delayed the development and progression of de novo atherosclerosis (Heim et al. 2016).

In preclinical models clopidogrel attenuated atherosclerosis (Heim et al. 2016; Afek et al. 2009; Takeda et al. 2012), although these protective effects were not evident in other studies (Schulz and Massberg 2012; West et al. 2014). Similarly, ticagrelor reduced the initiation of atherosclerosis in apoE^{-/-} mice (Schirmer et al. 2012) although this effect was not observed by West et al. (West et al. 2014). Discrepancies between the positive and negative studies may be related to drug dose, timing, and duration of treatment (Nylander and Schulz 2016). Furthermore, stabilization and reduced necrotic core were seen in mice with established plaques at 20 weeks of age, for ticagrelor (Buchheiser et al. 2011).

1.4 Coagulation Proteases

Coagulation proteins are for the large part synthesized in the liver and secreted in blood after posttranslational modification. Many of the proteins are zymogens (or proenzymes) that require limited proteolysis to become fully active. It requires one or more triggers like tissue factor (TF) and amplifiers like thrombin, to activate a cascade response yielding fibrin formation at the sites of injury. The liver also makes a series of anticoagulant proteins including antithrombin and protein C that act to dampen the coagulation cascade physiologically. A fairly large number of coagulation proteins are also expressed outside the liver; this can be *constitutive* (like protein S that is for 50% synthesized in vascular EC; tissue factor pathway inhibitor (TFPI) that is partially liver, partially megakaryocyte derived; factor VIII from liver sinusoidal endothelial cells; TF expressed in fibroblasts of the arterial adventitia) or *inducible* under conditions of inflammation: an example is production of TF in blood leukocytes (monocytes/macrophages, neutrophils) and possibly platelets, vascular smooth muscle cells (VSMC), and other cell types (Grover and Mackman 2018). Other proteins like factor VII (Wilcox et al. 2003) and factor X (Sanada et al. 2017) can be expressed by VSMC and fibroblasts. Proteins like factor XI, XII, and prothrombin have been demonstrated in extravascular localizations including atherosclerotic vessels, but whether these are deposited or locally synthesized remains unknown (Borissoff et al. 2010; Wilcox 1994; Soardi et al. 1961). Of interest, coagulation enzymes including thrombin and aPC participate in the control of hematopoiesis in the bone marrow (Nguyen et al. 2018), which may be relevant for the response to stress situations including inflammation.

Within atherosclerotic lesions, coagulation proteins like TF and factors VII and X contribute to form catalytic complexes driving thrombin and fibrin formation (Borissoff et al. 2010). The local formation of fibrin and its split products are modifiers of angiogenesis and cell trafficking within the plaques (Fay 2004; Binder et al. 2017; Badimon and Vilahur 2014; Spronk et al. 2018). The important role of plasminogen activator (receptor)-plasmin and plasminogen activator

1 (PAI-1) system in regulating controlled proteolysis in atherosclerosis, with impact on inflammation (Foley 2017), falls beyond the scope of this chapter.

In general, hypercoagulability and/or thrombophilia has a modest but detectable effect on atherosclerosis, at least coronary artery disease (CAD), peripheral artery disease (PAD), and other manifestations of atherosclerosis (Borissoff et al. 2012; Kleinegris et al. 2013; Lowe and Rumley 2014). This association is not unequivocal, and the lack of protective effect of specific coagulation deficiencies such as in hemophilia, which is not associated with reduced burden of atherosclerosis, argues against very strong influence of coagulation activity in human atherogenesis (Biere-Rafi et al. 2012; Kamphuisen and ten Cate 2014). Nevertheless, evidence from preclinical models is quite striking (Borissoff et al. 2011). Coagulation proteases tend to aggravate atherogenesis toward atherosclerosis in mouse models of atherosclerosis, mostly apoE^{-/-} mice, under pressure of a Western-type diet. In these mice, any hypercoagulable effect introduced by backcrossing apoE^{-/-} mice on a specific mouse with increased procoagulant tendency such as factor V Leiden, or TM^{Pro/pro} mutation, results in worsening atherogenesis. In contrast, mice with a less procoagulant phenotype like FXI^{-/-}, FVII^{+/-}, or some of the FVIII^{-/-} traits tend to be protected against atherosclerosis progression (Borissoff et al. 2011; Shnerb Ganor et al. 2016; Mackman 2016).

Application of direct oral anticoagulants that inhibit thrombin (dabigatran) or factor Xa (rivaroxaban) slows down atherosclerosis in apoE^{-/-} mice. At least five of such studies document protection against progression of atherosclerosis, including improved plaque stability, while on dabigatran (Lee et al. 2012; Kadoglou et al. 2012; Borissoff et al. 2013; Pingel et al. 2014; Preusch et al. 2015). One protective mechanism may involve the dabigatran-mediated attenuation of pro-inflammatory M1 macrophages in the vessel wall, observed in Lldr^{-/-} mice (Feldmann et al. 2019).

Detrimental effects of dabigatran exposure have also been published; in a diabetic rat model, dabigatran exposure caused increased platelet reactivity, increased coronary lipid deposition, as well as increased PAR4 expression in vessels (Scridon et al. 2019).

Rivaroxaban, the direct factor Xa inhibitor, also attenuates atherosclerosis in apoE^{-/-} mice (Zhou et al. 2011; Hara et al. 2015) or even reverses existing atherosclerosis in apoE^{-/-} mice (Posthuma et al. 2019). Overall, most studies suggest that applying direct oral anticoagulants (DOAC) in atherogenic mice slows down but also stabilizes atherosclerotic lesions and alter the plaque phenotype toward more (Borissoff et al. 2013) or sometimes less stability features (Seehaus et al. 2009), the latter depending on age and sex and probably additional factors like diet. The observed regression on rivaroxaban similarly showed diminished instability markers, but the actual mechanisms explaining diminished plaque volume on anticoagulation remain to be determined (Posthuma et al. 2019).

These proof-of-concept studies support important roles for coagulation proteases FXa and thrombin in driving atherogenesis and altering the phenotype in

different directions. Inhibiting factor Xa to actually reverse atherosclerosis in mice raises many new questions regarding the underlying mechanisms of factor Xa-mediated cell signaling and its impact on plaque progression/regression. The protection is thought to involve reducing the impact of FXa and thrombin on PAR1 and 2 activation, respectively. The importance of these PARs in regulating atherosclerosis is emerging; recently, protection against atherosclerosis in PAR2^{-/-} × apoE^{-/-} mice was shown, associated with reduced activity of nuclear factor-κB (NFκB)-regulated inflammation (Hara et al. 2018). However, the importance of coagulation proteases as compared to other possible ligands remains to be demonstrated.

2 From Atherosclerosis to Atherothrombosis

During atherogenesis there are different possible stages of *atherothrombosis* development that could be characterized by combinations of acute or acute on chronic thrombus formation. The occurrence of atherothrombosis is not a single event in time; in series of autopsy studies, it was convincingly demonstrated that coronary thrombi of all stages of development can be found in patients that suddenly died (Kramer et al. 2010). These studies demonstrate that atherothrombosis is part of an ongoing process with temporary, sometimes partial occlusions, subsequent remodeling of thrombus and vessel, gradually changing the vessel wall morphology, and affecting the lumen (Mastenbroek et al. 2015).

Atherogenesis and atherosclerosis may go through several stages of disease. A first and early stage relates to “perturbation” of the vascular endothelium (Nawroth et al. 1984; de Groot et al. 1987), referring to a state of endothelial cell activation associated with a disturbance of the anticoagulant/procoagulant balance, in a pro-inflammatory and thrombotic direction. It could be imagined that this perturbation may in fact be a first trigger in specific types of arterial thrombosis that may form in absence of visible atherosclerosis with sensitive imaging of coronary arteries. A second, more frequent scenario is the formation of a thrombus based on a damaged atherosclerotic plaque. Since long, two fundamentally different scenarios, plaque erosion versus plaque rupture, are recognized (Fuster et al. 1992a, b; Arbab-Zadeh et al. 2012).

While plaque rupture was previously recognized as the dominant pathophysiologic mechanism, recent studies suggest a gradual shift toward less plaque rupture and persistently frequent plaque erosion. One hypothesis for this change in time is declining impact of smoking and increased attention for and management of cardiovascular risk factors (e.g., statins, smoking cessation) that might translate into vascular protective effects and a more stable plaque phenotype (Quillard et al. 2017; Pasterkamp et al. 2017). Currently, about 30% of ST-elevation myocardial infarction (STEMI) is thought to result from eroded plaque lesions (Libby 2013).

Although known for a long time and recognized also as a predilection site for atherothrombosis in the aforementioned autopsy studies, erosion probably triggers fundamentally different mechanisms than rupture of a vulnerable cap (Quillard

et al. 2017). Plasma from patients with STEMI showed higher levels of epidermal growth factor and thrombospondin-1 in patients with intact fibrotic cap versus those with ruptured cap lesions, while interferon-inducible T-cell alpha chemoattractant (I-TAC) was lower in coronary blood from intact cap lesion subjects (Chandran et al. 2017). In thrombectomy specimens from these patients, I-TAC mRNA expression levels were markedly increased in patients with eroded lesions. Eroded lesions are characterized by fewer inflammatory cells, abundant extracellular matrix, and the presence of neutrophil extracellular traps (NETs). Eroded plaques contain more myeloperoxidase (MPO)-positive inflammatory cells; MPO is a marker of NETs. Patients with plaque erosion have more MPO in peripheral blood than those with plaque fissure (Ferrante et al. 2010). In addition, components from extracellular matrix including hyaluronan that interact with prohemostatic proteins like fibrinogen, fibrin, and fibronectin are important erosion determinants (Pedicino et al. 2018; Wight 2018). Inflammation and NETs may be complimentary mechanisms to trigger blood coagulation toward thrombus formation. Ruptured lesions typically express inflammatory cells, matrix containing oxidized lipids, and subendothelial proteins from the matrix and on inflammatory cells, including procoagulant tissue factor, factors VII and X, as well as platelets localizing and activating proteins including von Willebrand factor (vWF), collagen, and thrombospondin (Quillard et al. 2017; Pasterkamp et al. 2017). If, indeed, there are fundamental differences in the phenotypes of eroded versus ruptured lesions, it may be anticipated that thrombus formation also follows different pathways. On the other hand, it has been suggested that the main difference between eroded and ruptured plaque is the absence of direct contact between blood components and the necrotic core (Badimon and Vilahur 2014). Better insight into the mechanisms involved in atherothrombosis related to erosion or rupture of plaques is important in order to tailor antithrombotic protection in a more mechanistically founded direction. As will be discussed below, current antithrombotic management does not yet consider such differences.

3 Antithrombotic Therapy: Clinical Principles and Applications

3.1 Single Antiplatelet Agents: Mode of Action and Side Effects

Aspirin is the common name for acetylsalicylic acid, a compound that acts by inhibiting prostaglandin synthesis in different cells; the relevant antithrombotic effect is thought to be mostly based on inhibition of the cyclooxygenase-1 production of thromboxane A₂ in platelets (Gresele 2002). It is prescribed at doses between ± 75 and 325 mg od, mostly related to regional differences (higher doses more common in the USA than Europe). Its intake results in irreversible inhibition of platelet activation and aggregation. To achieve continued effect, daily intake of aspirin is needed to suppress newly formed platelets, about 10% per day.

Clopidogrel is a prodrug that needs metabolization in the liver (Coukell and Markham 1997). It is ingested as a single dose of 75 mg/day, and the active

metabolite shows a large interindividual variation in blood. This variability is partly explained by genetic polymorphisms encoding cytochrome P450 (CYP) 2C19, the hepatic enzyme involved in biotransformation of the prodrug clopidogrel to its active metabolite (Shuldiner et al. 2009). The active metabolite interferes with the binding of ADP to the P2Y12 receptor. This pharmacodynamic variation translates into variation in clopidogrel effectiveness after percutaneous coronary intervention (PCI) (Mega et al. 2010; Sibbing et al. 2009). The interindividual variation in response to clopidogrel, commonly known as clopidogrel “resistance,” was first identified in patients with coronary disease, occurring up to 25% of cases in platelet function testing (depending on the test used). Given the association between clopidogrel high on-treatment platelet reactivity (HTPR) and increased incidences of major adverse cardiovascular events (MACE), several studies addressed the question whether dose adjustment based on platelet function testing would correct this problem. Unfortunately, none of the studies demonstrated that test-based adjusted clopidogrel dosing could improve clinical efficacy of such interventions (Price et al. 2011; Collet et al. 2012). In part, the variation in clopidogrel activity is “corrected” for by the development of more potent P2Y12 inhibitors with a more predictable pharmacodynamic profile including prasugrel, ticagrelor, and cangrelor.

The principal side effect of aspirin is bleeding, and the regular use of aspirin increases the risk of particularly gastrointestinal bleeding twofold (Garcia Rodriguez et al. 2016). Risk factors for bleeding should therefore be taken into account, including (recent) GI ulcer (*H. pylori* infection may be additional factor), old age, and use of interacting medication including other antiplatelet therapy (APT), NSAIDs, COX-2 selective inhibitors, oral anticoagulants, and corticosteroids. In individual decision-making, balancing the pros (risk of atherothrombotic complications) and the cons (mostly bleeding) needs to be done, and certain decision support tools like the app “Aspirin Guide” can be helpful in this regard. In general, the risk of GI bleeding with aspirin can be effectively reduced with proton pump inhibitors (PPI), better than with histamine-2 receptor antagonists (Mo et al. 2015; Szabo et al. 2017). Recently, a fixed-dose combination of aspirin and omeprazole (Yosprala) was approved by the FDA, hoping that the simultaneous intake of these agents would improve adherence by reducing gastric side effects (Veltri 2018). Although clopidogrel does not interfere with prostaglandins in gastric mucosal tissue, its use is also associated with an increased risk of bleeding, of which GI bleeds are the most common type. The standard use of PPI in patients on clopidogrel has been disputed; in fact there is evidence that its concomitant use is associated with an increased risk of MACE (Bundhun et al. 2017). Compared to clopidogrel, the newer and more potent P2Y12 inhibitors prasugrel and ticagrelor are associated with an increase in the risk of major bleeding. These agents are generally not prescribed as single APT, although ticagrelor may be used as single agent in selected PAD patients (Hiatt et al. 2017).

3.2 Primary Prevention in the Population; Selecting the Right Subject?

In patients with atherosclerotic vascular disease, antithrombotic medication has been applied since the late 1950s of the past century. Traditionally, platelets were regarded key players in atherothrombosis; hence much focus has been put on antiplatelet agents, initially mostly aspirin. Given the efficacy/safety profile, *primary prevention* with aspirin has been extensively studied. Recent studies and meta-analyses of decades of large trials refute a major role for aspirin in primary prevention. Exceptions may be subjects with diabetes in whom aspirin showed to reduce the incidence of major vascular events including myocardial infarction, at a price of increased major bleeding risk (Group ASC et al. 2018). Otherwise, primary prevention with aspirin in apparently healthy subjects may require additional risk factors like coronary calcification to yield sufficient net clinical benefit. A recent discussion paper on the pros and cons of aspirin for primary prevention in elderly subjects shows the jury is still out (McNeil et al. 2018a, b, c; Fernandes et al. 2019).

3.3 Primary Prevention in Subjects with Atherosclerosis

Primary prevention (of MACE) with aspirin or other APT is warranted in all subjects with *symptomatic* atherosclerotic disease, including angina, or symptomatic PAD. Here, the risk/benefit ratio is clearly in favor of indefinite APT. In patients with any evidence of coronary artery disease, indefinite single APT with aspirin is recommended. This policy is adjusted in case of emerging interventions like PCI; see further.

Remarkably, aspirin was not better than placebo in patients with *asymptomatic* lower extremity artery disease (LEAD), in spite of the fact that their mortality is comparable to patients with symptomatic disease (Fowkes et al. 2010). In symptomatic PAD, there is a certain preference for the P2Y12 inhibitor clopidogrel over aspirin, as the PAD subgroup in the CAPRIE trial that compared aspirin with clopidogrel in subjects at high risk for cardiovascular complications showed better antithrombotic efficacy for clopidogrel at comparable bleeding risk (Table 1) (Committee 1996). However, both aspirin and clopidogrel are still used for this indication. Stronger acting APT like ticagrelor may be used as alternative in patients with PAD in case of failure, allergy, or “resistance” to aspirin or clopidogrel, based on the EUCLID trial showing non-inferiority of ticagrelor versus clopidogrel (Hiatt et al. 2017). Table 1 presents an overview of different therapeutic strategies in symptomatic patients with stable CAD or PAD, referring to the large trials supporting these strategies.

The principle of combining APT was established in the CURE trial, comparing the efficacy and safety of aspirin plus clopidogrel with aspirin alone in the secondary prevention of MACE in patients with coronary disease (Mehta et al. 2001). Since then, dual antiplatelet therapy (DAPT) has become a cornerstone treatment in secondary prevention following PCI (see further). However, the

Table 1 Studies evaluating different antithrombotic strategies in patients with coronary artery disease (CAD) and peripheral artery disease (PAD) (adapted from Olie et al. (2018))

Therapeutic strategy	Stable coronary artery disease (CAD); outcome		Stable peripheral artery disease (PAD); outcome			Bleeding complications in PAD and CAD patients		
	MACE	Ref.	MACE	Ref.	MALE	Ref.	Bleeding	Ref.
Aspirin	↓	[1]	↓/–	[1, 2]	↓	[3, 4]	↑	[1-4]
MONOTHERAPY compared to aspirin								
Clopidogrel	↓	CAPRIE [5]	↓	CAPRIE [5]			↑	CAPRIE [5]
Ticagrelor			↓ ^a	EUCLID [6]	– ^a	EUCLID [6]	↑	EUCLID [6]
Rivaroxaban 5 mg B.I.D.	–	COMPASS [7]	–	COMPASS [7]	↓	COMPASS [7]	↑↑	COMPASS [7]
COMBINATION THERAPY compared to aspirin								
Aspirin + clopidogrel	↓	CHARISMA [8, 9]	–	CHARISMA [10]	–	CHARISMA [10]	↑↑	CHARISMA [8-10]
Aspirin + ticagrelor	↓↓	PEGASUS-TIMI 54 [11]	↓↓ ^b	PEGASUS-TIMI 54 [12]	↓↓ ^b	PEGASUS-TIMI 54 [12]	↑↑	PEGASUS-TIMI 54 [11, 12]
Aspirin / DAPT + vorapaxar	↓↓↓	TRA2°P-TIMI 50 [13]	–	TRA2°P-TIMI 50 [13, 14]	↓↓	TRA2°P-TIMI 50 [13, 14]	↑↑↑	TRA2°P-TIMI 50 [13, 14]
Aspirin + VKA	↓↓↓	WARIS, ASPECT [15, 16]	–	WAVE [17]	↓	WAVE [17]	↑↑↑	WARIS, ASPECT, WAVE [15-17]
DAPT + rivaroxaban 2.5 mg B.I.D. (post-ACS)	↓↓↓	ATLAS ACS2-TIMI 51 [18]					↑↑↑	ATLAS ACS2-TIMI 51 [18]
Aspirin + rivaroxaban 2.5 mg B.I.D.	↓↓↓	COMPASS [7]	↓↓↓	COMPASS [7]	↓↓↓	COMPASS [7]	↑↑	COMPASS [7]

Study population, control groups, and definition of primary efficacy and safety outcomes are highly variable between different studies. This table is based on the authors' interpretation of clinical trials and meta-analyses and is clearly not based on head-to-head comparisons of the different therapeutic strategies

- (↓) to (↓↓↓) indicates modest to strong decrease in MACE/MALE
 (↑) to (↑↑↑) indicates modest to strong increase in bleeding complications
 (–) indicates no beneficial effect compared to aspirin monotherapy
 (↓/–) indicates contradictory results

Gray boxes indicate that these results are not available and/or not applicable
 MACE major adverse cardiovascular events, MALE major adverse limb events, DAPT dual antiplatelet therapy, VKA vitamin K antagonist, B.I.D. bis in die, twice a day, ACS acute coronary syndrome, ref. reference

- Table adapted from Olie et al. (2018)
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Table 1 (continued)

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^aNo direct comparison with aspirin monotherapy but compared to clopidogrel monotherapy

^bIn patients with concomitant PAD and CAD

concept also triggered studies as to the potential benefits DAPT would offer in patients with high-risk cardiovascular disease. Large trials like CHARISMA (Bhatt et al. 2006) tested this concept, the outcome of which was however negative in failing to show improved efficacy while increasing the bleeding risk. Consequently, DAPT is not recommended in any patient for *primary* prevention of MACE, unless there are subject-specific reasons for this more potent combination.

In PAD, in general, *combined* antiplatelet therapy does not add benefit to the patient and increases bleeding risk; for this reason, it is only applied for short-term use, e.g., after endovascular interventions (Hess et al. 2017). The use of oral anticoagulants (mostly vitamin K antagonists) in therapeutic intensity is not indicated in patients with PAD, except for those that underwent venous bypass grafting (Dutch Bypass Oral Anticoagulants or Aspirin (BOA) Study Group 2000). The most recent regimen studied in patients with high-risk vascular disease, including PAD, is the combination of an anticoagulant rivaroxaban 2.5 mg bd plus low-dose aspirin, which reduced cardiovascular mortality as well as major acute limb events in patients with PAD (patients with LEAD or carotid artery disease) (Anand et al. 2018). From a mechanistic perspective, targeting platelets and the plasmatic coagulation system seems rational, given the postulated pathophysiologic mechanisms discussed above. Moreover, targeting FXa, herewith also inhibiting the formation of thrombin, reduces the potential interactions between these proteases and cellular PARs. Thus, vascular protective effects may be an additional consequence of a strategy that includes an anticoagulant (although the clinical evidence is still weak).

3.4 Secondary Prevention of Atherothrombosis in Patients with Arterial Vascular Disease

Secondary prevention with APT, following myocardial infarction or ischemic stroke (not due to cardiac embolism, thus including a spectrum of non-embolic strokes), is straightforward and based on class 1A evidence.

Combined APT (dual (DAPT) antiplatelet therapy) is indicated in all settings of acute coronary syndrome, with or without PCI with stent placement (DAPT for 6–12 months, longer in selected cases) (Roffi et al. 2016; Authors/Task Force et al. 2014; Amsterdam et al. 2014). In acute ischemic stroke, DAPT is only indicated for a very limited duration, up to 6 weeks, due to the observed increased bleeding risk (Wang et al. 2013; Johnston et al. 2018). Following these time windows, single APT will be continued indefinitely, comprising of aspirin in most patients with CAD and aspirin or, more commonly, clopidogrel in patients after atherothrombotic stroke (Hackam and Spence 2019).

In most combined APT regimens, aspirin remains an element; in patients with CAD, DAPT comprises aspirin plus a P2Y₁₂ inhibitor, prasugrel or ticagrelor (unless contraindicated clopidogrel, a weaker P2Y₁₂ inhibitor, is used in combination with aspirin). Although the thienopyridine prasugrel is a prodrug like clopidogrel, it only requires a single oxidation step to form its active metabolite,

and it seems to be not affected by genetic variations in CYP enzymes. Compared to clopidogrel its use is associated with an increase in the risk of major bleeding, especially among those with age ≥ 75 years or body weight < 60 kg, and is contraindicated in patients with prior stroke (Wiviott et al. 2007). Ticagrelor, an oral, direct acting, reversible P2Y₁₂ receptor antagonist, provides faster and greater platelet inhibition with less patient-to-patient variation (Wallentin et al. 2009).

Despite intensification of APT in recent years, an approximately 10% risk for recurrent ischemic events at 1 year after coronary events still remains (Wiviott et al. 2007; Wallentin et al. 2009). With blockade of the TXA₂ pathway and the P2Y₁₂ receptor, platelets can still be activated by thrombin, via the PAR1 and PAR4 receptor on their surface (Olie et al. 2019). Vorapaxar is an orally administered, competitive PAR1 antagonist that blocks thrombin-mediated platelet activation via PAR1, without inhibiting other modes of thrombin activity, such as fibrin formation, protein C activation, and PAR4 activation. On top of standard antiplatelet therapy (consisting of aspirin in almost all cases and a thienopyridine or dipyridamole in a significant proportion), addition of vorapaxar led to significant reduction in rates of ischemic cardiovascular events in patients with stable CAD or PAD, but at the price of increased major bleeding, limiting its use in clinical practice (Morrow et al. 2012). Non-platelet-mediated effects of PAR inhibition on the vascular endothelium have been speculated on, since PAR1 is also present on endothelial cells and VSMCs, where it mediates mitogenic effects (Posma et al. 2016). Thus, PAR1 inhibition might be effective in reducing vascular remodeling and consecutive progression of atherosclerosis.

In patients with atherosclerotic disease, oral anticoagulants were not routinely applied until recently. One reason for not using anticoagulants was the assumption that atherothrombosis primarily is a platelet-dependent phenomenon, given the efficacy data of all APT trials. A second reason to refrain from anticoagulants was the concern of cholesterol embolization that could follow anticoagulant-associated instability and plaque rupture; however, the evidence for a causal effect of anticoagulants is poor.

Early trials with vitamin K antagonists (van Bergen et al. 1994; Smith et al. 1990) in CAD patients had provided proof of principle that inhibition of coagulation may be of additional benefit in atherothrombotic disease, although in daily practice, their use was practically abandoned due to markedly increased bleeding and more effective protection by DAPT. Nonetheless, after the introduction of direct oral anticoagulants (DOACs), the role of these anticoagulants in secondary prevention of ischemic events was re-evaluated in several trials (Olie et al. 2018). Addition of low-dose rivaroxaban (2.5 mg twice daily) on top of APT reduced major adverse cardiac events both in patients with acute coronary syndrome (in the ATLAS-ACS-2 TIMI 51 trial (Mega et al. 2012)) and in patients with stable CAD or PAD (in the COMPASS trial (Eikelboom et al. 2017)). In COMPASS also cardiovascular mortality was reduced in patients receiving the combination of vascular doses of rivaroxaban (2.5 mg bd) and aspirin as compared to either agent alone, which means a breakthrough in the efficacy of antithrombotic management (Coppens et al. 2019).

4 The Effects of Antithrombotic Therapy on the Vessel Wall and Atherogenesis: Clinical Relevance?

Platelets are pivotal in atherogenesis, but the impact of antiplatelet agents beyond inhibition of platelet activation and aggregation (including the procoagulant role platelets play in catalyzing phospholipid-dependent coagulation reactions) remains controversial. As discussed, low-dose aspirin may have local anti-inflammatory effects in the vessel wall, but evidence for diminished atherosclerosis related to aspirin intake is not present.

Human studies revealed several anti-inflammatory effects and reduction in platelet CD40 ligand and CD62, respectively, associated with clopidogrel (less evident in those with clopidogrel “resistance”) and prasugrel, both thienopyridines (summarized in (Nylander and Schulz 2016)). Ticagrelor had anti-inflammatory effects including reduced formation of platelet-neutrophil aggregates in inflammation models and more profound reduction in Il-6 in a human sepsis model as compared to clopidogrel, suggesting that stronger inhibition of the P2Y12 receptor (ticagrelor) may provide greater anti-inflammatory effect (Nylander and Schulz 2016). Off-target effects of ticagrelor on endothelial function and vascular biomarkers have been speculated on, but a recent study comparing ticagrelor to prasugrel and clopidogrel found no evidence for any non-platelet-mediated effects in post-acute coronary syndrome patients (Ariotti et al. 2018).

The previous generation of oral anticoagulants, the vitamin K antagonists (VKA), has defined effects on the vasculature due to inhibition of carboxylation of specific vitamin K-dependent proteins like matrix Gla protein, a protein involved in the inhibition of both medial and intimal calcification. Ample mouse studies support these effects, where VKA rapidly induces calcification that can be partially prevented by additional vitamin K administration (Spronk et al. 2003b). Whether the VKA achieved inhibition of FXa and thrombin generation also in part affects atherosclerosis remains difficult to dissect, because of the overwhelming effect of calcification. Whether VKA affects plaque *stability* in humans is still uncertain. Observational studies suggest more plaque instability in patients on VKA due to intraplaque hemorrhage particularly upon prolonged exposure and higher intensity of anticoagulation (Mujaj et al. 2018; Li et al. 2014). However, in the Rotterdam study, the effects were comparable for anticoagulants (VKA) and aspirin, so the specificity and causal contribution of anticoagulation versus antiplatelet effects remain unproven. More direct evidence for effects of inhibiting coagulation protease needs to come from studies with DOACs. Two studies that either randomized patients to rivaroxaban or warfarin (Lee et al. 2018) or performed propensity score matching yielding three populations that used DOAC, warfarin, or no anticoagulants (Plank et al. 2018) were recently published. Both studies show diminished calcification and less instability features in patients on DOAC versus VKA. These somewhat preliminary data seem to point to a possibly favorable impact of DOAC (hence, FXa/thrombin inhibition) on atherosclerosis and the vessel wall.

5 Novel Antiplatelet and Anticoagulant Targets

In spite of the available array of antithrombotic agents, there is still a need for further improvement. One of the critical downsides of all antithrombotic agents is a risk of bleeding, linked to the potency of the drug. Thus, more effective antiplatelet agents like prasugrel or ticagrelor will generally inflict more bleeding risk than the less potent P2Y₁₂ inhibitor clopidogrel. Combinations of APT like in DAPT or even triple therapy will also show increased bleeding risk as compared to single agents.

Although the current class of DOAC has a better safety profile with regard to intracranial bleeding as compared to VKA, there is still a substantial risk of other clinically relevant bleeding complications, including gastrointestinal bleeds. Combined anticoagulant and APT therapy also has increased bleeding potential, and even the relatively low doses of rivaroxaban in the COMPASS regimen, combined with aspirin, increase the risk of major bleeding (Eikelboom et al. 2017).

With existing antiplatelet drugs, a therapeutic ceiling seems to be reached, whereby increased potency is offset by elevated bleeding risk. Ongoing research on developing new antiplatelet drugs therefore focuses on identifying targets that inhibit thrombosis while maintaining hemostasis. Developing thrombi at sites of endothelial injury are now known to be composed of two distinct regions: the hemostatic plug (composed of highly activated platelets and rich in fibrin) and the propagating platelet thrombus (composed of platelets in a low activation state) (Stalker et al. 2013). The latter region of the propagating thrombus is regulated by phosphatidylinositol 3-kinase- β (PI3K β), glycoprotein (GP)IIb/IIIa outside-in signaling, and activation of protein disulfide-isomerase (PDI). Inhibition of these factors that regulate thrombus propagation seems to protect against thrombotic occlusion while preserving hemostasis. Therefore, novel agents include PI3K β inhibitors, PDI inhibitors, conformation-specific targeting of activated GPIIb/IIIa, and selective inhibition of GPIIb/IIIa outside-in signaling (McFadyen et al. 2018).

Other candidate drugs include inhibitors of the GPIb-vWF axis, novel PAR1 and PAR4 inhibitors, and blockade of platelet GPVI-mediated adhesion pathways. The GPIb-IX-V receptor binds to vWF during injury and under conditions of high shear stress, allowing early platelet adhesion to the subendothelium. Therefore, various inhibitors of this axis have been developed. Although two antibodies against vWF (ARC1779 and caplacizumab) have demonstrated to have antithrombotic effects, their development has been halted owing to an increased incidence of bleeding (Markus et al. 2011; Muller et al. 2013). However, additional agents, directly targeting GPIb or the vWF binding domain, are under development. As discussed, the use of the currently available PAR-1 antagonist vorapaxar is limited by substantially increased rates of bleeding. Besides orthosteric antagonists like vorapaxar, another class of PAR1 inhibitors, called paramodulins, has been developed. These target the cytoplasmic face of PAR1, contrary to blocking the ligand-binding site like vorapaxar, which inhibits all signaling downstream of the PAR1 receptor. This may allow paramodulins to selectively block platelet and endothelial cell activation mediated by PAR1 while maintaining the cytoprotective signaling pathways in endothelial cells (Aisiku et al. 2015). As

thrombin activates platelets via both PAR1 and PAR4, also the PAR4 receptor is currently under investigation as a target for platelet antagonism (McFadyen et al. 2018). Another promising target is the interaction between GPVI and collagen. The observation that GPVI is platelet-specific, in combination with the fact that patients with GPVI deficiency usually suffer from only a mild bleeding phenotype, has led to strategies targeting GPVI. Phase II trials, studying the anti-GPVI agent Revcept in patients with stable CAD and symptomatic carotid stenosis, are currently underway (Majithia and Bhatt 2019).

In recent years, the contribution of the proteins of the contact system (factors VIII, IX, XI, and XII, prekallikrein, and high-molecular-weight kininogen) to the process of atherothrombosis has gained more attention. Factor XII-deficient humans have a normal hemostatic capacity, while animal models have revealed an important role of factor XIIa-driven coagulation in arterial thrombosis (Kuijpers et al. 2014). Furthermore, factor XIIa contributes to inflammation through the activation of the inflammatory bradykinin-producing kallikrein-kinin system (Nickel et al. 2017; Long et al. 2016). Thus, pharmacological inhibition of factor XII(a) may not only be a safer therapeutic strategy (by inhibition of thrombosis while preserving hemostasis) but also has additional beneficial anti-inflammatory and anti-atherogenic effects. Currently, factor XII(a) and its activator polyphosphate are being studied as potential targets for prevention of thrombosis. However, factor XIIa also stimulated the fibrinolytic pathway (Long et al. 2016), and inhibition may thus have potential prothrombotic side effects. Moreover, when thrombosis is initiated by TF exposure, small amounts of thrombin generated by extrinsic tenase have the potential to activate FXI, thereby bypassing FXII inhibition. Therefore, FXI inhibition may be a better target than FXII inhibition. Furthermore, besides attenuation of coagulation, factor XI deprivation has also been shown to slow down atherogenesis in apoE/factor XI double knockout mice (Shnerb Ganor et al. 2016). Several potential strategies to target FXI are currently under investigation, including antisense oligonucleotides (ASOs) that reduce hepatic synthesis of FXI, monoclonal antibodies that suppress FXIa generation and inhibit FXIa activity, and aptamers that block the binding site and small molecules that bind reversibly to the active site of FXIa and inhibits its activity (Weitz and Chan 2019). Clinical phase 2 studies with FXI-directed ASOs, monoclonal antibodies against FXIa, and an oral FXIa inhibitor have been performed (Buller et al. 2015) or are currently underway (Weitz and Chan 2019).

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Part II

Novel Drug Developments Addressing Predefined Targets



Metabolism of Triglyceride-Rich Lipoproteins

Jan Borén and Marja-Riitta Taskinen

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Abstract

Triglycerides are critical lipids as they provide an energy source that is both compact and efficient. Due to its hydrophobic nature triglyceride molecules can pack together densely and so be stored in adipose tissue. To be transported in the aqueous medium of plasma, triglycerides have to be incorporated into lipoprotein particles along with other components such as cholesterol, phospholipid and

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associated structural and regulatory apolipoproteins. Here we discuss the physiology of normal triglyceride metabolism, and how impaired metabolism induces hypertriglyceridemia and its pathogenic consequences including atherosclerosis. We also discuss established and novel therapies to reduce triglyceride-rich lipoproteins.

Keywords

Apolipoprotein B · Chylomicrons · Hypertriglyceridemia · Metabolism · Therapies · Triglyceride-rich lipoproteins (TRL) · Very low density lipoproteins (VLDL)

1 Introduction

Interest in triglyceride-rich lipoproteins (TRLs) has for long been rather low, but recent results demonstrating that TRLs are causally associated with atherosclerotic cardiovascular disease (ASCVD) have generated major interest in these lipoproteins. Hypertriglyceridemia is quite common today and approximately 25% of US adults are estimated to have hypertriglyceridemia (triglyceride [TG] level ≥ 1.7 mmol/L). TRLs are synthesized in the liver as very low-density lipoproteins (VLDL) and in the intestine as chylomicrons. During lipolysis TRLs are converted to atherogenic cholesterol-ester enriched lipoprotein remnant particles. Dysregulation of the normal metabolism of TRLs leads to excess formation of these atherogenic lipoprotein remnant particles (Chapman et al. 2011; Nordestgaard and Varbo 2014; Boren et al. 2014; Dallinga-Thie et al. 2016). Since humans are postprandial most of the day, we continuously generate atherogenic remnant particles. Consequently, the continuous generation of remnants after each meal may be an important causal risk factor for the development of atherosclerosis. Genetic studies have also identified key regulators of the metabolism of TRLs and major emphasis is now directed at evaluating their potential as novel candidate targets for dyslipidemia and premature ASCVD risk (Dallinga-Thie et al. 2016). Here we discuss how TRLs are synthesized and metabolized.

2 Hepatic Formation and Secretion of VLDL

The assembly of VLDL is a complex process and involves a stepwise lipidation of apoB100, the principal apolipoprotein on VLDL, in the liver (Olofsson et al. 2000; Olofsson and Boren 2005). ApoB100 is a large protein consisting of one globular N-terminal structure, two domains of amphipathic β -sheets and two domains of amphipathic α -helices (Segrest et al. 2001). ApoB differs from other apolipoproteins in that it is highly hydrophobic. Therefore, it cannot equilibrate between different lipoproteins but remains bound to the particle on which it was secreted into plasma. Thus, every VLDL particle contains one molecule of apoB100. This is generally

thought to be explained by the presence of antiparallel β -sheets with a width of approximately 30 Å, which form very strong lipid-binding structures (Segrest et al. 2001).

The lipidation cascade starts with a cotranslational transfer of triglycerides to nascent apoB polypeptides during the assembly of nascent VLDL mediated by the microsomal triglyceride-transfer protein (MTP) in the rough endoplasmic reticulum (ER) (Fig. 1) (Boren et al. 1992; Rustaeus et al. 1998). The critical role of MTP in VLDL assembly is demonstrated by the rare, autosomal-recessive disorder abetalipoproteinemia. The disorder results from mutations in the gene encoding the large subunit of MTP and is characterized by nearly a complete absence of apoB-containing lipoproteins including VLDL. To date, over different 30 mutations

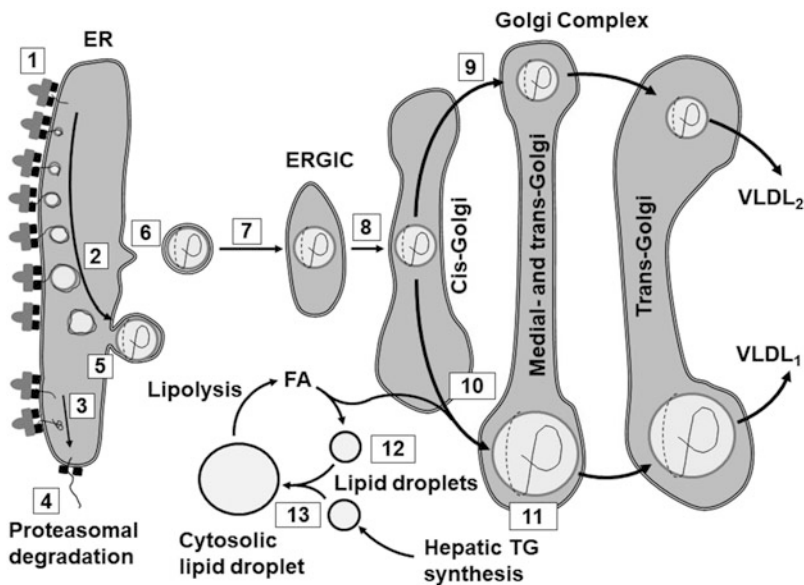


Fig. 1 Assembly and secretion of apoB100-containing lipoproteins. ApoB is synthesized and translocated into the lumen of the endoplasmic reticulum (ER) (1). The growing nascent apoB polypeptide is cotranslationally lipidated by the lipid transfer protein MTP to form a partially lipidated pre-VLDL particle (2). If apoB fails to be lipidated and acquire a correct protein folding (3), it is sorted to posttranslational degradation (4). The triglyceride-poor pre-VLDL particle exits the ER by Sar1/CopII vesicles that bud off (6) from specific sites on the ER membrane (Gusarova et al. 2003). The vesicles fuse to form the ER Golgi intermediate compartment (ERGIC) (7), which then fuses with the cis-Golgi (8). The triglyceride-poor particles are either secreted as smaller VLDL₂ particles (9) or further lipidated (10) to form mature triglyceride-rich VLDL₁ particles, which are then secreted (11). The formation of triglyceride-rich VLDL₁ particles is highly dependent on the presence of triglyceride-containing cytosolic lipid droplets. These lipid droplets are formed as small primordial droplets from microsomal membranes (12) and increase in size by fusion (13). The triglycerides within the droplets undergo lipolysis and are re-esterified (14) before they lipidate the triglyceride-poor VLDL to form triglyceride-rich VLDL. Hepatic triglycerides originate from influx of free fatty acids, hepatic de novo lipogenesis (DNL), or hepatic uptake of lipoprotein particles

in the MTP gene have been described for ABL (Lee and Hegele 2014). Absence of MTP leads to premature proteosomal degradation of nascent apoB and therefore absence of VLDL and chylomicron production. The patients are characterized by hypocholesterolemia and the absence of apoB-containing lipoproteins (Paquette et al. 2016).

The cotranslational lipidation stabilizes the nascent apoB polypeptide and results in the formation of a nascent pre-VLDL lipoprotein particle (Bostrom et al. 1988). The immature pre-VLDL undergoes further lipidation in the secretory pathway, forming a triglyceride-poor VLDL particle (Stillemark-Billton et al. 2005). This particle can either be secreted from the liver as a smaller VLDL particle (i.e., VLDL₂) or undergo further lipidation to form a larger triglyceride-rich (i.e., VLDL₁) (Stillemark-Billton et al. 2005; Stillemark et al. 2000). The lipidation cascade is still not fully understood, but has been shown to involve several proteins including the GTP-binding protein ADP-ribosylation factor 1 (ARF-1) (Asp et al. 2005).

The conversion of smaller triglyceride-poor VLDL particles to large triglyceride-rich VLDL likely involves the fusion of cytoplasmic lipid droplets to the smaller VLDL particle. Thus, this bulk addition of triglycerides differs from the initial stepwise addition of triglycerides. The formation of the large mature VLDL particles is therefore dependent on the presence of cytosolic lipid droplets (Wiggins and Gibbons 1992; Salter et al. 1998; Gibbons et al. 2000). Therefore, it's not surprising that hepatic accumulation of triglycerides, non-alcoholic fatty liver disease (NAFLD), is linked to oversecretion of large VLDL₁ particles (Adiels et al. 2006a, b). However, not all forms of fatty liver disease are linked to increased hepatic secretion of VLDL₁, indicating that the hepatic stores of triglycerides in some genetic forms of NAFLD, like PNPLA3, are not accessible for VLDL formation. However, the molecular mechanisms are still unclear. Interestingly, the amounts of triglycerides that are added to triglyceride-rich poor VLDL seem to be constant. Thus, subjects with type 2 diabetes secrete more – not larger – VLDL₁ particles than non-diabetic controls (Adiels et al. 2005, 2006a, b). Thus, bulk addition of triglycerides from the cytoplasmic lipid droplets seems to be a highly regulated process.

3 Regulators of Hepatic VLDL Secretion

Hepatic triglyceride accumulation stimulates hepatic VLDL₁ secretion, and the sources for liver fat are: (1) plasma fatty acids generated by lipolysis of the peripheral adipose tissue; (2) fatty acids synthesized in the liver from carbohydrates through hepatic de novo lipogenesis (DNL); (3) fatty acids that come from the diet; and (4) hepatic uptake of triglyceride-rich lipoproteins (TRLs) (Parks and Hellerstein 2006; Barrows and Parks 2006). Most of the hepatic triglycerides originate from circulating fatty acids, since the hepatic uptake of fatty acids is not regulated. Thus, increased levels of circulating fatty acids are directly connected to increased hepatic uptake of fatty acids (Tamura and Shimomura 2005). Lipolysis of adipose tissue

(in particular the visceral adipose tissue) is the principal contributor (approx. 80%) of the plasma NEFA pool (Tamura and Shimomura 2005). It is therefore not surprising that visceral adiposity is strongly associated with NAFLD and oversecretion of VLDL₁ particles (Parks and Hellerstein 2006; Barrows and Parks 2006; Farquhar et al. 1965; Parks et al. 1999; Havel 1961; Donnelly et al. 2005).

Normally the hepatic DNL plays a minor role (<5%) (Barrows and Parks 2006), but in conditions of increased plasma glucose and hyperinsulinemia it has been shown to generate ≈25% of liver triglycerides (Donnelly et al. 2005). The explanation is that glucose is the substrate for hepatic DNL and that hyperinsulinemia is linked to increased expression of factors needed for hepatic DNL such as SREBP1-c (Browning and Horton 2004; Shimomura et al. 1999), the carbohydrate response element-binding protein (ChREBP) (Koo et al. 2001), and PPAR γ (Edvardsson et al. 1999; Chao et al. 2000; Westerbacka et al. 2007).

In addition, there is evidence that VLDL₁ and VLDL₂ are regulated independently. Ethanol overconsumption seems to stimulate VLDL₁ production in humans (Fielding et al. 2000), whereas endogenous cholesterol synthesis correlates with VLDL₂-apoB but not VLDL₁-apoB production (Prinsen et al. 2003). This finding may explain why VLDL₂, but not VLDL₁, is increased in patients with increased plasma cholesterol such as moderate hypercholesterolemia (Gaw et al. 1995) and familial hypercholesterolemia (James et al. 1989).

4 Synthesis and Secretion of Chylomicrons from the Intestine

Chylomicrons are synthesized in the enterocytes of the small intestine and each chylomicron contains one molecule of apoB48. The apoB48 protein corresponds exactly to the N-terminal 48% of apoB100. The explanation for this is that both proteins are encoded by the same gene. The mRNA for apoB48 is generated from the apoB100 mRNA by a posttranscriptional editing process during which a deamination of a cytidine (at nucleotide 6,666) to a uridine converts a glutamine codon to a stop codon. The mechanism has been extensively reviewed (Davidson and Shelness 2000; Anant and Davidson 2001; Wang et al. 2003). The assembly of chylomicrons is a highly complex multistep process, and less is still known about chylomicron assembly than VLDL assembly (Xiao et al. 2019; Hussain et al. 2005). However, it is known that in addition to MTP, intestinal assembly of chylomicron requires Sar1 GTPase, which is critical for the intracellular transport of apoB48-containing particles from ER to the Golgi (Julve et al. 2016).

The newly synthesized chylomicrons carrying dietary lipids and fat-soluble vitamins are secreted through lacteal endothelial gaps that are present in the postprandial phase into the venous system blood system through the lymphatic system. Thus, unlike other nutrients dietary lipids bypass the hepatic portal system.

Over the last years several surprising findings have been made (Lambert and Parks 2012). First, studies have demonstrated that the intestine stores triglycerides and that lipids secreted after a meal may have been consumed in an earlier meal

(Mattes 2002; Robertson et al. 2002; Chavez-Jauregui et al. 2010). This may explain the early rise in postprandial plasma triglycerides since the intestine does not have to absorb dietary lipids and then form chylomicrons, but instead start secreting stored triglycerides in chylomicrons. We have also realized that the release of chylomicrons is linked to a taste–gut–brain axis (Khan and Besnard 2009). Interestingly, chylomicrons can be secreted when fat (Mattes 2009) or glucose (Robertson et al. 2003) is merely tasted but not consumed. Lastly, contrary to what was believed, recent studies have shown that apoB48-containing particles are secreted not only as chylomicrons but also as less triglyceride-rich lipoprotein particles (isolated in the VLDL density range) both in the fasting state and postprandially (Bjornson et al. 2019a, b).

5 Disorders of the Synthesis of TRLs

Abetalipoproteinemia (ABL) (also known as the Bassen–Kornzweig syndrome) is a rare autosomal-recessive disease that is characterized by very low plasma concentrations of TG and cholesterol (under 30 mg/dL) and undetectable levels of LDL and apoB. The rare recessive genetic disease is caused by loss-of-function mutations in the MTTP gene encoding for the microsomal triglyceride-transfer protein (MTP).

Clinic: Mutations in MTP leads to impairment of the formation of triglyceride-rich VLDL and chylomicrons. Patients with ABL (and compound heterozygous and homozygous FHBL) have therefore very low plasma total cholesterol and generally low plasma triglycerides. LDL-C when measured by direct methods, and apoB, will be absent or their concentrations will be very low. Patients may display neurological, hematological (acanthocytosis on peripheral blood smear and anemia), and gastrointestinal symptoms due to deficiency in lipophilic vitamins and fat malabsorption (Paquette et al. 2016). The deficiency of vitamin E could lead to severe neurological disorders including spinocerebellar degeneration with ataxia and retinitis pigmentosa (Welty 2014). In addition, the impaired secretion of hepatic triglycerides may lead to hepatic steatosis (Welty 2014). The clinical phenotype and severity differs as the type and combination of MTTP mutations influence the clinical phenotype and treatment response (Paquette et al. 2016). Subjects who carry a single MTTP mutation may have normal plasma lipid levels or may have LDL-cholesterol and apoB concentrations similar to those seen in heterozygous familial hypobetalipoproteinemia (Lee and Hegele 2014; Paquette et al. 2016).

Treatment: Early diagnosis and treatment is important to prevent neurologic complications of this disease. Reversal of existing neurologic disease can also be achieved. Treatment involves a low-fat diet, supplementation with essential fatty acids and high oral doses of fat-soluble vitamins, vitamins A and E (Paquette et al. 2016; Welty 2014; Linton et al. 1993). High dose of oral fat-soluble vitamins bypasses the chylomicron pathway, and vitamins are carried via the portal circulation (Lee and Hegele 2014; Paquette et al. 2016).

Chylomicron retention disease (CRD). In addition to MTP, chylomicron formation requires Sar1 GTPase, one of the subunits of the coat protein (COPII) complex, which is critical for the vesicular transport of apoB-48-containing particles from endoplasmic reticulum to the Golgi (Julve et al. 2016). Loss-of-function mutations in SAR1B, the gene encoding Sar1 homolog B GTPase causes CRD (also known as Anderson disease) (Julve et al. 2016), a rare autosomal-recessive disorder characterized by an intestinal defect in lipid transport due to a failure of chylomicron formation in enterocytes (Julve et al. 2016).

Clinic: The failure to synthesize chylomicrons results in severe malabsorption with steatorrhea, fat-soluble vitamin deficiency, low blood cholesterol levels, and failure to thrive in infancy (Julve et al. 2016).

Treatment: Same as ABL.

Familial hypobetalipoproteinemia (FHBL) is an autosomal codominant disorder characterized by apoB <5th percentile and LDL-cholesterol usually between 20 and 50 mg/dL (Welty 2014; Linton et al. 1993). Over 60 different mutations in apoB producing truncated forms of apoB, ranging from apoB to apoB89, have been reported (Welty 2014; Linton et al. 1993). These truncated forms of apoB are named according to the percent length of the native apoB100 molecule. Truncated forms of apoB shorter than apoB30 are seldom detectable in human plasma as lipoproteins since these truncated proteins undergo intracellular degradation. Although one allele is affected only in heterozygous FHBL, the plasma levels are normally closer to one quarter to one third of normal, due to low hepatic secretion of the truncated forms of apoB combined with decreased production and increased clearance of VLDL and LDL produced by the normal allele (Welty et al. 1997; Elias et al. 1999; Aguilar-Salinas et al. 1995; Parhofer et al. 1996).

Clinic: Heterozygous FHBL is often asymptomatic and not diagnosed unless a lipid profile is obtained. In contrast, the clinical presentation of homozygous FHBL is similar to ABL. Early diagnosis of homozygous FHBL is therefore important. As the hepatic secretion of triglyceride-rich lipoproteins is impaired, FHBL has been shown to associate with hepatic steatosis and mild elevation of liver enzymes (Welty 2014). In 32 FHBL subjects, the hepatic fat content was increased to $14.0 \pm 12.0\%$ compared to $5.2 \pm 5.9\%$, respectively, for 33 controls matched for age, sex, and indices of adiposity (Tanoli et al. 2004).

Treatment: For homozygous FHBL treatment involves a low-fat diet, supplementation with essential fatty acids and high oral doses of fat-soluble vitamins, vitamins A and E (Welty 2014; Linton et al. 1993).

6 Metabolism of Triglyceride-Rich Lipoproteins

After secretion of chylomicrons and VLDL, the lipoproteins are exposed to lipoprotein lipase (LPL) on the capillary endothelial cells within adipose tissue, skeletal muscle, and the heart, leading to hydrolyzation of the triglycerides, allowing the delivery of non-esterified free fatty acids (NEFA) to adipose tissue, skeletal muscle and the heart.

As the triglycerides are removed from the particles, they shrink and their density increases (Goldberg 1996); chylomicrons become chylomicron remnants, and large triglyceride-rich VLDL₁ particles become smaller VLDL₂ and subsequently intermediate density lipoproteins (IDL). The IDL particles can be further hydrolyzed to LDL particles by action of the hepatic lipase (HL). Since all human TRLs contain a substantial amount of cholesterol esters, hydrolysis of triglycerides leads to enrichment of cholesterol esters. Consequently, TRL remnants are enriched in cholesteryl esters (Dallinga-Thie et al. 2010).

Although roughly 80% of the increase in postprandial plasma triglycerides consists of chylomicrons and their remnants (Cohn et al. 1993), the majority of particles (around 80%) comprise of liver-derived VLDL and their remnants (Karpe et al. 1995; Schneeman et al. 1993). Also, the area under the curve for apoB100 is 10-fold higher than that of apoB48 (Vakkilainen et al. 2002), and the production rate of apoB100 is 15–20 times higher than that of apoB48 (Lichtenstein et al. 1992; Welty et al. 1999). We have earlier shown that chylomicrons and VLDL particles are not cleared equally by the lipoprotein lipase pathway, and that chylomicrons seem to be the preferred substrate (Adiels et al. 2012). Therefore, the major contribution to an atherogenic lipoprotein profile from chylomicrons is likely its interference with apoB100 catabolism.

ApoB-containing particles with a diameter of about 70 nm or smaller can penetrate the arterial endothelial layer, and subsequently become retained in the artery wall. Thus, cholesterol-rich remnants (i.e., both chylomicron remnants and VLDL remnants) can lead to cholesterol deposition in growing lesions, accelerated atherosclerosis, and enhanced CVD risk in a similar manner as LDL.

Genetic deficiency of LPL leads to the rare autosomal-recessive disorder *familial LPL deficiency*. These patients usually display milky plasma (accumulation of chylomicrons) and very severe hypertriglyceridemia with episodes of abdominal pain (pancreatitis), eruptive cutaneous xanthomata, and hepatosplenomegaly.

7 Deciphering the Pathogenesis of Hypertriglyceridemia

Insulin resistance and hypertriglyceridemia are associated with an atherogenic dyslipidemia characterized by prolonged postprandial hyperlipidemia, accumulation of small dense LDL (sdLDL) and low HDL cholesterol. The mechanism that leads to the formation of sdLDL is well clarified; the cholesteryl ester transfer protein (CETP) transfers triglycerides from VLDL₁ to LDL. This results in formation of triglyceride-rich LDL. These lipoprotein particles are the preferred substrate for hepatic lipase (HL) that depletes triglycerides from the triglyceride-rich LDL. As large triglyceride-rich VLDL₁ particles are the substrate for CETP, accumulation of TRLs is a prerequisite for sdLDL formation (Adiels et al. 2006b; Packard 2003; Georgieva et al. 2004). The enzymes also act on HDL, resulting in the formation of sdHDL that are efficiently removed from circulation. The combined action of CETP and HL thus results in the formation of sdLDL and low HDL cholesterol (Verges 2005; Taskinen 2003). Several studies indicate that increased sdLDL is associated

with increased CVD risk (Austin et al. 1990; Lamarche et al. 1997; Gardner et al. 1996; Vakkilainen et al. 2003). However, it is still unclear if sdLDL is a marker of an atherogenic dyslipidemia or causatively linked to the increased CVD risk (Sacks and Campos 2003).

To elucidate the pathophysiology of the hypertriglyceridemia in obese subjects we have performed a series of kinetic studies with stable isotopes. These studies have shown that the impaired lipid metabolism is caused by dual mechanisms: increased secretion of triglyceride-rich VLDL₁ from the liver and delayed clearance of TRLs from the circulation (Borén et al. 2015; Taskinen et al. 2011). This is illustrated in Fig. 2 by two pathways: a *synthesis pathway* and a *clearance pathway*. Interestingly, the *synthesis pathway* explained only 20% of the variation of plasma triglycerides (Borén et al. 2015). In contrast, the *clearance pathway* explained 50% of the variation in the total plasma triglycerides. Thus, the impaired catabolism of VLDL₁-triglycerides is the most important determinant of the plasma triglyceride concentration in subjects with abdominal obesity and dyslipidemia.

The *synthesis pathway* includes liver fat and total fat mass as these remained independent predictors of VLDL₁-triglyceride secretion rate in a stepwise multivariable regression analysis (Borén et al. 2015). Increased liver fat is linked to impaired regulation of VLDL production and a continuous oversecretion of VLDL₁ (Adiels

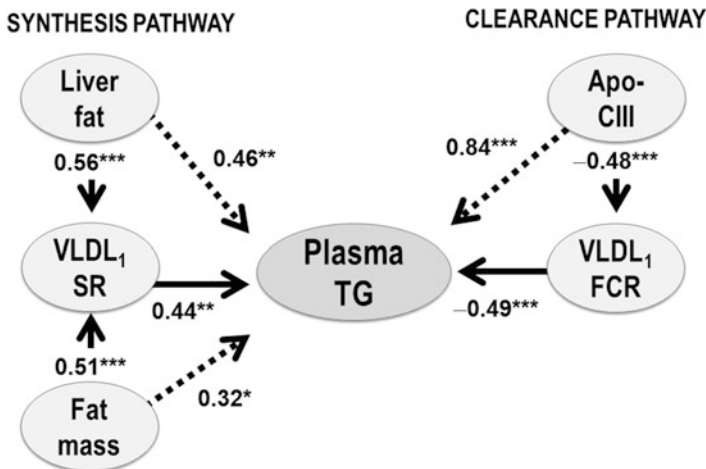


Fig. 2 Predictors of VLDL₁-triglyceride secretion and catabolism. Liver fat content ($P < 0.01$) and total fat mass ($P < 0.05$) are important independent predictors of VLDL₁-TG secretion rate (SR). The plasma concentration of apoC-III correlated strongly with plasma TG and the fractional catabolism of VLDL₁-TG. VLDL₁-TG kinetics explained 76% of the variation in the total plasma triglycerides. Of these, $\approx 20\%$ was explained by the secretion pathway, whereas $\approx 50\%$ was explained by the clearance pathway. Thus, indices of catabolism were stronger predictors of plasma triglycerides than parameters of secretion. The associations between liver fat and fat mass vs plasma TG (dotted lines) are likely secondary and mediated via VLDL₁ SR. Likewise, the direct effect of apoC-III on plasma TG (dotted line) is likely explained by effect(s) of apoC-III beyond lipoprotein lipase-independent pathways of triglyceride metabolism (Borén et al. 2015)

et al. 2006b; Poulsen et al. 2016) (Fig. 2), and adipose tissue is the major source of fatty acids in the NEFA pool that determines the hepatic uptake of fatty acids. In the *clearance pathway*, the plasma concentration of apoC-III is shown to correlate strongly with plasma triglycerides and clearance of VLDL₁-triglycerides.

8 Regulation of Hydrolysis of TRLs and the LPL Pathway

The clearance of triglycerides is directly linked to the lipolysis of TRLs by LPL (Ginsberg et al. 1986). Full activity of LPL requires the interaction with the transport protein glycosylphosphatidylinositol anchored high density lipoprotein binding protein 1 (GPI-HBP1) and the lipase maturation factor 1 (LMF 1) present at the surface of capillaries (Sandesara et al. 2019). The LPL activity is modulated by several regulators including apoC-I, apoC-II, apoC-III, angiopoietin-like 3 (ANGPTL3), ANGPTL4 and ANGPTL8 (Kersten 2014). Insulin and apoC-III are the key regulators of LPL activity. Insulin stimulates the expression of LPL in endothelial cells whereas apoC-III inhibits LPL activity and thereby reduces the clearance of TRLs (Ginsberg and Brown 2011; Zheng et al. 2010; Yao 2012). The seminal role of LPL for the catabolic rate of TRLs and the conversion of large VLDL particles into smaller particles is demonstrated in studies in subjects with LPL gene mutations (Ooi et al. 2012). In addition, strong evidence supports the critical roles of apoC-III and apoE for suppressing or stimulating, respectively, clearance of apoB-containing lipoproteins from the circulation (Mendivil et al. 2010; Sacks 2015; Zheng et al. 2007).

ApoC-III is displacing apoC-II, an activator of LPL, from lipoprotein surfaces and thus impairing the actual lipolytic process (Sacks 2015; Gordts et al. 2016; Larsson et al. 2013). In addition, apoC-III has a wide range of actions on triglyceride metabolism beyond its LPL-dependent effects (Taskinen and Boren 2015; Norata et al. 2015). For example, apoC-III interferes with the binding of apoB and apoE to hepatic lipoprotein receptors including heparin sulfate proteoglycan receptor (HSPG), low-density lipoprotein receptors (LDLR), and LDLR related protein 1 receptor (LRP1). This raises the option that high apoC-III would also inhibit the receptor mediated hepatic uptake of TRL remnants (Huff and Hegele 2013). The fact that an apoC-III ASO (antisense oligonucleotides) that inhibits apoC-III synthesis greatly reduced serum triglycerides in subjects with *familial LPL deficiency* demonstrates that apoC-III inhibits also hepatic clearance of remnants by LPL-independent pathways (Gaudet et al. 2014). However, recent results indicate that apoC-III inhibits turnover of TRLs primarily through a hepatic clearance mechanism mediated by the LDLR/LRP1 axis, since apoC-III ASO treatment in LDLR/LRP1 deficient mice did not lower plasma TG levels (Gordts et al. 2016). Interestingly, we have recently reported that apoC-III metabolism is significantly perturbed in subjects with type 2 diabetes and that the apoC-III secretion rate was markedly higher in subjects with diabetes compared with BMI-matched non-diabetic subjects (Adiels et al. 2019). Improved glycemic control with liraglutide therapy reduced significantly apoC-III secretion rate and, thereby, apoC-III levels in type

2 diabetic subjects (Matikainen et al. 2019). These findings suggest that glucose homeostasis is a regulator of apoC-III metabolism and that the secretion rate of apoC-III seems to be an important driver for the elevation of TRLs in type 2 diabetes.

Angiopoietin-like protein (ANGPTL) family includes three members (ANGPTL3, ANGPTL4, and ANGPTL8) that are important modulators of lipoprotein metabolism (Kersten 2017; Christopoulou et al. 2019; Romeo et al. 2009; Li et al. 2020). Both ANGPTL3 and ANGPTL4 are endogenous inhibitors of LPL, and loss-of-function (LOF) mutations in ANGPTL3 and 4 associate with low triglyceride levels (Romeo et al. 2009; Minicocci et al. 2013; Zhang 2012) and reduced CVD risk (Dewey et al. 2017; Myocardial Infarction Genetics Investigators CAEC et al. 2016; Dewey et al. 2016). ANGPTL3 deficiency has been reported to reduce hepatic VLDL secretion and lower LDL-cholesterol (Wang et al. 2015a). Interestingly, ANGPTL3 gene silencing has been shown to associate not only with reduced hepatic secretion of apoB-containing lipoproteins, but also with enhanced uptake of particles via the LDL receptor. This likely explains the reduction of IDL cholesterol levels in subjects with familial hypobetalipoproteinemia (Xu et al. 2018). Consequently, targeting ANGPTL3 and ANGPTL4 genes has emerged as a promising goal for triglyceride lowering therapies (Tsimikas 2018; Gaudet et al. 2017a; Keech and Jenkins 2017; Bauer et al. 2016).

The inhibitory action of ANGPTL8 on LPL function requests the presence of ANGPTL3 (Kersten 2017; Haller et al. 2017; Luo and Peng 2018), as ANGPTL8 seems to enhance the inhibitory action of ANGPTL3 on LPL (Chi et al. 2017). Interestingly, these two proteins seem to work together to orchestrate responses of both glucose and lipid metabolism in fasting and in feeding (Wang et al. 2015b). Interestingly, ANGPTL3 is exclusively expressed in the liver being as a true hepatokine while ANGPTL8 is expressed both in adipose tissue and in the liver. The co-operative action of these two proteins seems to regulate the uptake of triglyceride-derived fatty acids either in the adipose tissue for storage or in cardiomyocytes and skeletal muscle for oxidation (Li et al. 2020; Vatner et al. 2018; Davies 2018).

ApoE plays a pivotal role in both triglyceride and cholesterol metabolism (Marais 2019). It predominantly associates with triglyceride-rich lipoproteins to mediate the clearance of their remnants after enzymatic lipolysis in the circulation (Marais 2019; Mahley and Huang 2007; Nakajima et al. 2019). Plasma levels of apoE and other lipids and lipoproteins are under strong genetic influence by *APOE* polymorphism – a combination of two genetic variants (rs429358 and rs7412) giving rise to six common *APOE* genotypes, ϵ 22, ϵ 32, ϵ 33, ϵ 42, ϵ 43, and ϵ 44 (Marais 2019; Mahley and Huang 2007). Both ϵ 2 and ϵ 4 alleles are associated with unfavorable lipid profiles, and the ϵ 4 allele is a strong genetic risk factor for Alzheimer disease and by far the strongest hit in genome-wide association studies of longevity. The apoE variants relate to different amino acids at positions 112 and 158: cysteine in both for apoE2, arginine at both sites for apoE4, and respectively cysteine and arginine for apoE3 that is viewed as the wild type. High levels of plasma apoE have been shown to associate with increased risk of ischemic heart disease (Rasmussen et al. 2019). Hence both a quantitative importance of plasma apoE levels and a qualitative

genetically determined effect appear to be important for cardiovascular disease (Rasmussen et al. 2019).

9 Role of Triglyceride-Rich Lipoproteins in Atherogenesis

It is well established that hypercholesterolemia is causatively linked to atherosclerotic cardiovascular disease and that lowering of cholesterol-rich LDL levels reduces cardiovascular events (Boren et al. 2020). However, cholesterol-lowering medication only prevents up to half of these events. Recent advances in human genetics indicate that the remaining “residual risk” of ASCVD is linked to elevated plasma triglyceride levels. Since triglyceride itself is not thought to contribute to atherogenesis, a consensus view has emerged that the remaining risk is linked to increased formation of “remnant” particles. These are derived from TRLs in the blood when the triglycerides are removed by the enzyme LPL (Boren et al. 2020). The remnant particles are not efficiently lowered by the available cholesterol-lowering medications.

To enter the artery wall, lipoproteins must cross the endothelium by transcytosis, a vesicular transport process. While chylomicrons and large VLDLs cannot undergo transcytosis because of their size, smaller chylomicron and VLDL remnants can and do penetrate the arterial wall. Thus, TRL remnants, in addition to LDL, may be retained in the arterial wall (Chapman et al. 2011). Even though remnant particles remain richer in triglycerides than cholesterol, their large size means that they contain up to twofold more cholesterol content per particle than LDL. However, the relative atherogenicity of remnants relative to LDL remains unclear.

10 Therapies to Reduce Triglyceride-Rich Lipoproteins

Lifestyle changes to lower plasma triglycerides – The first approach to lower moderately increased plasma triglycerides is to alter lifestyle (Laufs et al. 2020). Focus on a healthier diet and physical activity are cornerstones of lifestyle recommendations. Patients should reduce net caloric intake and lessen intake of sucrose, fructose, and alcohol. Diets rich in saturated fatty acids should be replaced with food enriched in monounsaturated and polyunsaturated fat (Laufs et al. 2020). It should be remembered though that scientific evidence for dietary recommendations is sparse. In addition, it is genuinely hard to persuade patients to change lifestyle and to make lifestyle changes that last. Thus, pharmaceutical approaches are often required.

Pharmacological therapies to lower plasma triglycerides – All commonly used cholesterol-lowering drugs as statins, ezetimibe, PCSK9 inhibitors only discreetly reduce triglyceride levels (around 5–15%), even though statins are somewhat more efficiently in reducing triglycerides and TRL remnants than PCSK9 inhibitors. Fibrates, omega-3-fatty acids, and niacin are somewhat more efficiently in reducing triglyceride levels (25–45%).

Fibrates are agonists of peroxisome proliferator-activated receptor- α (PPAR- α), acting via transcription factors regulating on lipid and lipoprotein metabolism. The drug has good efficacy in lowering fasting TG levels, as well as postprandial triglycerides and TRL remnants, albeit with marked interindividual variation. In addition, a small LDL-C increase may be observed in subjects with high triglyceride levels. The cardiovascular benefits have been shown to be heterogeneous and less robust than that of statins; when used as monotherapy, fibrates have been demonstrated to reduce CVD risk. However, when used in combination with statins no further reduction in CVD risk was demonstrated, although subgroup analysis indicates that hypertriglyceridemic patients with low HDL-C may benefit from such combination therapy. Results from ongoing trials using pemafibrate, a selective peroxisome proliferator-activated receptor alpha (PPAR α) receptor agonist, will show if this approach will be successful (Pradhan et al. 2018).

For decades, omega-3-fatty acids have been used to lower plasma triglycerides and to prevent CVD (Bays et al. 2008). The results from two recent clinical trials using different omega-3 fatty acids have been mixed and somewhat confusing. The REDUCE-IT trial used icosapent ethyl omega-3 fatty acid (4 g daily). The results were positive and resulted in a 25% reduction in CVD and a 20% reduction in plasma triglyceride levels. Interestingly, the treatment also reduced plasma C-reactive protein by 40% (Bhatt et al. 2019). These results strengthen the link between plasma triglycerides and CVD (Myocardial Infarction Genetics Investigators CAEC et al. 2016; Do et al. 2013, 2015). However, the reduction of cardiovascular event was independent of plasma triglyceride levels both at baseline and on treatment, indicating that the reduction of cardiovascular events was only modest due to changes in TRL levels. One potential explanation for the clinical benefits could be the marked attenuation of the postprandial response by eicosapentaenoic acid, the hydrolytic product of icosapent ethyl, as 58% of the study participants had type 2 diabetes that commonly have prolonged postprandial hypertriglyceridemia (Taskinen and Boren 2015). In contrast to the positive outcome from the REDUCE-IT trial, the STRENGTH trial using another omega-3 fatty acid formulation (a combination of eicosapentaenoic acid and docosahexaenoic acid) failed to demonstrate any clinical benefit. The explanation(s) for the different outcomes is still unclear. Possible reasons include that the two trials studied different type of omega-3 fatty acids, and that the REDUCE-IT trial used mineral oil as placebo which may have adverse effects, whereas STRENGTH used a corn oil placebo. A recent Cochrane review of 86 randomized controlled trials with 162,796 participants concluded “evidence suggests that increasing long-chain omega-3 slightly reduces risk of coronary heart disease mortality and events, and reduces serum triglycerides” (Abdelhamid et al. 2020).

The 2019 ESC/EAS guidelines for the management of dyslipidemias recommend that statin treatment remains the first choice for managing high triglycerides (triglycerides >200 mg/dL or 2.3 mmol/L) (Mach et al. 2020). However, the guidelines have taken account of evidence from REDUCE-IT and recommend n-3 PUFAs (particularly icosapent ethyl 2 \times 2 g daily) in high-risk patients with persistently elevated plasma triglycerides (between 135 and 499 mg/dL or 1.5 and

5.6 mmol/L) despite statin treatment. In high-risk patients at LDL-C goal with TG >200 mg/dL or >2.3 mmol/L, fenofibrate or bezafibrate may be considered in combination with statins (Mach et al. 2020).

11 Development of Novel Interventions

Efficient interventions to reduce plasma levels of TRLs and TRL remnants are still missing, and development of the development of strategies to treat the large numbers of hypertriglyceridemic individuals who currently remain at high risk of ASCVD despite optimal treatment according to current guidelines is urgently needed. Genetic studies have demonstrated that apoC-III and angiopoietin-like protein 3 (Angptl3) are critical regulators of triglyceride metabolism, and both have been developed as drug targets.

Statins and omega-3 fatty acids modestly reduce plasma apoC-III levels by less than 20% (Ooi et al. 2008; Maki et al. 2011; Morton et al. 2016; Dunbar et al. 2015). However, development of antisense oligos and siRNA has made it possible to develop high efficiency therapies. For example, antisense therapeutic oligonucleotides conjugated with N-acetyl galactosamine-conjugated (GalNAc) adducts (i.e., the ligand of the hepatic asialoglycoprotein receptor) have been developed. These are very efficient in reducing *APOC3* expression (Graham et al. 2013). For example, results from the APPROACH trial, a 52-week randomized, double-blind, phase 3 in 66 patients with familial chylomicronemia syndrome, demonstrated that the drug resulted in an impressive 77% decrease in plasma triglyceride levels (Witztum et al. 2019). The antisense therapeutic oligonucleotides have also been shown to markedly lower plasma apoC-III and triglycerides levels in subjects with severe or uncontrolled hypertriglyceridemia (Gaudet et al. 2015; Gouni-Berthold 2017) and in subjects with diabetic dyslipidemia (Digenio et al. 2016). Intriguingly, the intervention not only improved the diabetic dyslipidemia, but also improved whole-body insulin sensitivity (by 57%).

To target Angptl3, both a monoclonal antibody and therapeutic oligonucleotides have been developed. Recent results have demonstrated that anti-ANGPTL3 therapies reduce both marked hypertriglyceridemia (around 75% reduction) and severely elevated LDL-cholesterol in subjects with familial hypercholesterolemia (around 23% reduction) (Gaudet et al. 2017b). The finding that anti-Angptl3 lowers LDL-C in subjects lacking functional LDL receptors indicates that the underlying mechanism is independent of the LDL receptor pathway. In line, a GalNAc-modified antisense-oligonucleotide has recently been shown to reduce both plasma triglycerides and LDL-cholesterol (by 63.1% and 32.9%, respectively) (Graham et al. 2017). Interestingly, results from murine models indicate that the antisense-oligonucleotide seems to reduce hepatic steatosis. These results have prompted ongoing human studies.

Other ongoing projects involve lipoprotein lipase gene therapy, oral inhibitors of intestinal DGAT1 to reduce dietary fat absorption and triglyceride synthesis, and treatments targeting apoC-III and Angptl4 (Laufs et al. 2020).

12 Conclusion

Whether plasma triglycerides constitute an independent risk factor for CVD has been debated for decades, but there is now strong support for a causative role of TRLs in CVD. These studies equally indicate that cholesterol-enriched TRL remnant play a key role in the pathophysiology of atherosclerotic vascular disease. We are now beginning to understand the complex regulation of triglyceride metabolism. Hopefully, this molecular understanding will be translated into targeted treatment for the atherogenic dyslipidemia associated with hypertriglyceridemia.

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High Density Lipoproteins: Is There a Comeback as a Therapeutic Target?

Arnold von Eckardstein

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Abstract

Low plasma levels of High Density Lipoprotein (HDL) cholesterol (HDL-C) are associated with increased risks of atherosclerotic cardiovascular disease (ASCVD). In cell culture and animal models, HDL particles exert multiple potentially anti-atherogenic effects. However, drugs increasing HDL-C have failed to prevent cardiovascular endpoints. Mendelian Randomization studies neither found any genetic causality for the associations of HDL-C levels with differences in cardiovascular risk. Therefore, the causal role and, hence, utility as a therapeutic target of HDL has been questioned. However, the biomarker “HDL-C” as well as the interpretation of previous data has several important limitations: First, the inverse relationship of HDL-C with risk of ASCVD is neither linear nor continuous. Hence, neither the higher-the-better strategies of previous drug developments nor previous linear cause-effect relationships assuming Mendelian randomization approaches appear appropriate. Second, most of the drugs previously tested do not target HDL metabolism specifically so that the futile trials question the clinical utility of the investigated drugs rather than the causal role of HDL in ASCVD. Third, the cholesterol of HDL measured as HDL-C neither exerts nor reports any HDL function. Comprehensive knowledge of structure-function-disease relationships of HDL particles and associated molecules will be a pre-requisite, to test them for their physiological and pathogenic relevance and exploit them for the diagnostic and therapeutic management of individuals at HDL-associated risk of ASCVD but also other diseases, for example diabetes, chronic kidney disease, infections, autoimmune and neurodegenerative diseases.

Keywords

Apolipoprotein A-I · CETP · Cholesterol efflux · Fibrate · HDL mimetic · High density lipoproteins · Nicotinic acid

1 Introduction

Low plasma levels of high density lipoprotein (HDL) cholesterol (HDL-C) are associated with increased risks of atherosclerotic cardiovascular diseases (ASCVD), notably coronary heart disease (CHD) (Emerging Risk Factors Collaboration 2009; Madsen et al. 2021). HDL particles exert a broad spectrum of biological activities many of which are considered as anti-atherogenic, for example mediation of cholesterol efflux from macrophage foam cells and reverse transport of cholesterol to the liver, promotion of endothelial integrity and function, inhibition of inflammation by suppression of myelopoiesis and transmigration of leukocytes through the endothelium as well as macrophage activation, inhibition of lipid oxidation as well as inactivation of oxidized lipids (Fig. 1) (Von Eckardstein and Kardassis 2015; Robert et al. 2021; Rohatgi et al. 2021). Furthermore, atherosclerosis could be

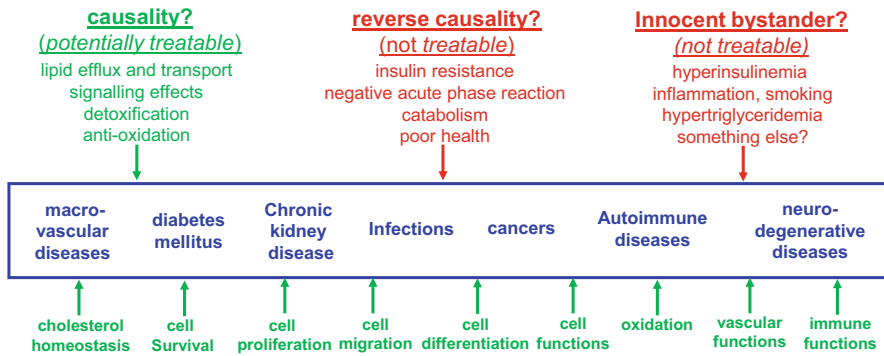


Fig. 1 Possible pathophysiological relationships of low HDL cholesterol with its associated diseases (modified from Von Eckardstein and Kardassis 2015)

decreased or even reverted in several animal models by transgenic over-expression or exogenous application of apolipoprotein (apoA-I), i.e. the most abundant protein of HDL (Hoekstra and Van Eck 2015; Lee-Rueckert et al. 2016). However, in humans, drugs increasing HDL-C such as fibrates, nicotinic acid (niacin), or inhibitors of cholesteryl ester transfer protein (CETP) have failed to prevent fatal or non-fatal cardiovascular endpoints (Keene et al. 2014; Riaz et al. 2019). Infusions of reconstituted HDL (rHDL) did not lead to regression of atherosclerosis in coronary or carotid arteries (He et al. 2021). Moreover, in several inborn errors of human HDL metabolism and genetic mouse models with altered HDL metabolism, low or high HDL-C levels were not always associated with the differences in cardiovascular risk and atherosclerotic plaque load, respectively, that were expected from epidemiology (Hoekstra and Van Eck 2015; Lee-Rueckert et al. 2016; Zanoni and von Eckardstein 2020). For example, the loss of scavenger receptor B1 (SR-BI) function aggravates the risk of ASCVD events in human carriers of SCARB1 mutations and promotes atherosclerosis in Scarb1 knock-out mice despite increasing HDL-C levels (Hoekstra and Van Eck 2015; Lee-Rueckert et al. 2016; Zanoni et al. 2016). Because of these ambiguous data, the causal role of HDL in the pathogenesis of atherosclerosis as well as the suitability of HDL-C as a therapeutic target is nowadays scrutinized if not doubted (Madsen et al. 2021; März et al. 2017). Both the previous euphoria and the current skepticism in the discussion of HDL's role in health and disease, specifically in ASCVD but also beyond, have been suffering from several misconceptions, which are described in the first part of this review. As the conclusion, several perspectives for the clinical exploitation of HDL are presented in the second part.

2 Possible Reasons for HDL-C's Clinical Futility

2.1 Lack of Causality

Mendelian randomization studies have been a successful tool to support the causality of LDL cholesterol (LDL-C) in atherosclerosis: Single nucleotide polymorphisms (SNPs) and rare genetic variants that are associated with lower or higher LDL-C levels are associated with lower and higher risk, respectively, of ASCVD events. The associations of genetically determined LDL-C with ASCVD risk are even stronger than the associations of measured LDL-C. This is because the genetic information includes both time and dosage of exposure to the harmful LDL-C whereas the measured LDL-C only records the dosage of the harm (Borén et al. 2020). Mendelian randomization studies also support causality of hypertriglyceridemia and elevated apoB levels as well as hypertension in the pathogenesis of ASCVD (Benn and Nordestgaard 2018). Conversely, this genetic strategy rather excluded genetic causality of HDL-C and apoA-I levels in the manifestation of ASCVD, at least after maximal adjustment for confounding lipid traits such as apoB and triglyceride levels (Richardson et al. 2020; Voight et al. 2012). However, it is important to note the limitations of Mendelian Randomization studies. With respect to HDL-C the most important limitation is the assumption of a continuous relationship between the risk factor and the clinical endpoint. This is true for the association of LDL-C or nonHDL-C with major cardiovascular events but not for HDL-C, where no difference in risk is observed among individuals with HDL-C levels above the 60th percentile (Emerging Risk Factors Collaboration 2009; Johannesen et al. 2020; Madsen et al. 2017; see Sect. 2.2).

HDL-C levels below the widely accepted risk thresholds of 1.0 mmol/L or 40 mg/dL are frequently confounded by other risk factors of ASCVD, notably hypertriglyceridemia, manifest diabetes mellitus type 2 (T2DM) or impaired fasting glucose, smoking, chronic inflammatory diseases (chronic obstructive lung disease, rheumatic diseases) or biomarkers of inflammation (e.g., elevated C-reactive protein), overweight or obesity (Fig. 1; Assmann et al. 1996; Damen et al. 2017). Due to the links of HDL metabolism with the metabolism of triglyceride-rich lipoproteins, it has been suggested that low HDL-C is an indirect long-term indicator of postprandial hypertriglyceridemia and hence exposure of atherogenic remnants like elevated glycated hemoglobin A1c is a long-term marker of disturbed glucose metabolism but a non-causal risk factor of glycation-induced organ damage (Langsted et al. 2020).

2.2 Epidemiology and Human Genetics Disprove “the Higher the Better” Concept

The association of HDL-C with risk of ASCVD events has been described for decades to be inverse. The resulting widespread reception of HDL-C as the “good cholesterol” led to the application of “the higher the better” strategies to both patient counselling and drug development. However, the meta-analysis of 68 population

studies with more than 300,000 participants and 2,785 incident myocardial infarctions by the Emerging Risk Factors Collaboration found the unadjusted risk of myocardial infarction gradually decreasing from the first decile to the eighth decile (i.e., until about 1.5 mmol/L or 58 mg/dL) but no significant changes at higher levels of HDL-C. After adjustment for possible confounders, statistically significant dose-dependent risk decreases happen within the lower six deciles until about 50 mg/dL (1.3 mmol/L) but not above this threshold (Emerging Risk Factors Collaboration 2009). Similar observations were made in more than 110,000 and 630,000 participants of the Copenhagen General Population (CGPS) and Copenhagen City Heart Studies (CCHS) and CANHEART studies (Madsen et al. 2017; Wijeysondera et al. 2017). In Denmark, the decreases in risk of cardiovascular events reached plateaus at 1.5 mmol/L in men and 2.0 mmol/L in women (Madsen et al. 2017). In Canada, below the reference interval ranging from 50 to 60 mg/dL, the incidence of ASCVD events increased with every decreasing 10 mg/dL interval of HDL-C. Above the threshold of 60 mg/dL, the ASCVD risks were overall significantly lower compared to the reference interval, but did not differ between the increasing 10 mg/dL strata, neither in men nor in women (Wijeysondera et al. 2017). Of note, the associations of HDL-C with total as well as disease-specific mortalities including cardiovascular mortality are even parabolic (U-shaped): Both in the Danish and Canadian studies, the inverse associations of HDL-C with total mortality reached their nadirs at 1.8–1.9 mmol/L (70–75 mg/dL) and 2.3–2.4 mmol/L (90–95 mg/dL) in men and women, respectively. Beyond these thresholds, the risk of dying became gradually higher with further increasing HDL-C levels (Ko et al. 2016; Madsen et al. 2017).

The discontinuous and even parabolic associations of HDL-C with cardiovascular morbidity and mortality, respectively, have been largely ignored both in the execution of Mendelian randomization studies and in the design of randomized controlled studies that aimed at the lowering of cardiovascular risk by increasing of HDL-C: both have been based on the assumption of continuous the-higher-the-better associations. The majority of the trials on fibrates, niacin, or CETP inhibitors did not define any upper limit of HDL-C for inclusion into the trial (Table 1). No Mendelian Randomization study restricted the analysis to the ranges where changes in HDL-C are associated with changes in risk, e.g. to the lower five or six deciles (Richardson et al. 2020; Voight et al. 2012). Of note, a large register study of a lipid clinics in Boston among individuals with HDL-C below 25 mg/dL (0.8 mmol/L) found an increased prevalence of ASCVD events in carriers of mutations in the genes of APOA1, ABCA1, LCAT, and LPL (Geller et al. 2018). Also studies in Dutch and Canadian families affected by loss of function mutations in APOA1, ABCA1, or LCAT found the prevalence of ASCVD events increased among mutation carriers, but only if HDL-C was below the fifth percentile (Abdel-Razek et al. 2018; Tietjen et al. 2012). Conversely, mutations in the genes of CETP, SCARB1, and LIPG, which cause increases in HDL-C show heterogeneous associations with ASCVD. Loss of function mutations in LIPG encoding endothelial lipase do not alter the risk of ASCVD (Voight et al. 2012). The associations of loss of function mutations in CETP and SCARB1 with ASCVD are controversial: Rare

Table 1 The majority of “HDL” trials did not define any cut-off level of HDL cholesterol for inclusion or exclusion of participants

Trial	Drug	Type of prevention	HDL-C inclusion criterion	HDL-C (mmol/L) at baseline
HHS	Gemfibrozil	Primary	Not defined	1.22 ± 0.28
BIP	Bezafibrate	Secondary	<1.16 mmol/L	0.89 ± 0.14
VA-HIT	Gemfibrozil	Secondary	<1.05 mmol/L	0.89 ± 0.18
FIELD	Fenofibrate ^a	Diabetes mellitus	Not defined	1.10 ± 0.26
ACCORD ^a	Fenofibrate ^a	Diabetes mellitus	<1.42 mmol/L	0.98 ± 0.21
AIM-HIGH ^a	Niacin ^a	Secondary	<1.05 mmol/L	0.91 ± 0.16
HPS2-THRIVE ^a	Niacin ^a	Secondary	Not defined	1.14 ± 0.30
ILLUMINATE ^a	Torcetrapib ^a	Secondary	Not defined	1.26 ± 0.31
DALOUTCOME ^a	Dalcetrapib ^a	Secondary	Not defined	1.10 ± 0.30
ACCELERATE ^a	Evacetrapib	Secondary	<2.07 mmol/L	1.18 ± 0.30
REVEAL ^a	Anacetrapib	Secondary	Not defined	1.04 ± 0.26

HHS = Helsinki Heart Study (Frick et al. 1987), BIP = Bezafibrate Infarction Prevention (BIP) 2000, VA-HIT (Rubins et al. 1999), FIELD = Keech et al. (2005), ACCORD Study Group (2010); AIM-HIGH Investigators (2011), HPS2-THRIVE Collaborative Group (2014); ILLUMINATE (Barter et al. 2007b), Dal-OUTCOME (Schwartz et al. 2012), ACCELERATE (Lincoff et al. 2017; HPS3/TIMI55–REVEAL Collaborative Group 2017)

^aCombined with statins vs. statins alone. *HDL-C* HDL cholesterol

SCARB1 mutations were associated with increased CVD risk in one study but not in another (Helgadottir et al. 2018; Zanoni et al. 2016). CETP deficiency was originally associated with reduced risk of ASCVD and increased life expectancy but later studies found diverse associations of loss of function mutations in CETP with ASCVD, namely increased risk in the Honolulu Heart Study but decreased risk in a Japanese population study (Moriyama et al. 1998; Yamashita and Matsuzawa 2016; Zhong et al. 1996). Likewise, the common polymorphisms in CETP which are associated with lower CETP mass and activity, LDL-C, and triglycerides but higher HDL-C were showed diverse associations with ASCVD in different studies: meta-analyses found lower risks of ASCVD associated with loss of function alleles of CETP (Kathiresan 2012; Niu and Qi 2015), but there are several individual studies which found the opposite (Agerholm-Larsen et al. 2000; Borggreve et al. 2006). Loss of function mutations in APOC3 cause higher HDL-C levels and reduce cardiovascular risk (Crosby et al. 2014; Pollin et al. 2008), but this may reflect proatherogenic features of apoC-III beyond its influence on HDL-C and triglyceride levels (Riwanto et al. 2013; Zewinger et al. 2020; Zvintzou et al. 2017) (see also Sect. 3.2).

2.3 Limitations of HDL Modifying Drugs

The futility of the most recent randomized controlled trials (RCTs) on fenofibrate (ACCORD Study Group 2010; Keech et al. 2005), nicotinic acid (AIM-HIGH Investigators 2011; HPS2-THRIVE Collaborative Group 2014), and cholesteryl ester transfer protein (CETP)-inhibitors (ILLUMINATE, Dal-OUTCOME, ACCELERATE, REVEAL) (Barter et al. 2007b; HPS3/TIMI55-REVEAL Collaborative Group 2017; Lincoff et al. 2017; Schwartz et al. 2012) is frequently used as the argument to question the causality of HDL in the pathogenesis of atherosclerosis. However, this conclusion overlooks that – except the CETP inhibitor dalcetrapib (Schwartz et al. 2012) – none of these drugs is specifically altering HDL-C. Especially fibrates and nicotinic acid exert stronger effects on other lipoprotein traits than on HDL-C. Thus, their failure to reduce ASCVD events should primarily prompt to scrutinize the suitability of these pharmacological strategies rather than the causality of HDL in ASCVD. Moreover, one should be oblivious to meta-analyses that demonstrated futility of fibrates or nicotinic acid if combined with statins but efficacy if used as monotherapies (Keene et al. 2014; Riaz et al. 2019). Likewise, genetic studies indicate that potential efficacy of CETP inhibitors in ASCVD prevention may be hampered by the combination with statins (Ference et al. 2017).

2.3.1 Neither Fibrates nor Nicotinic Acid Specifically Target HDL Metabolism

Fibrates are agonists of the peroxisome proliferator agonist receptor alpha (PPAR α). As such they regulate the transcription of several genes which are relevant in the metabolism of HDL metabolism (e.g., APOA1, PLTP, SCARB1) but also triglyceride-rich lipoproteins (Montaigne et al. 2021; Zandbergen and Plutzky 2007). As the result, fibrates cause increases in HDL-C of maximally 15% and decreases in triglycerides of 25–50%. The rather moderate effect on HDL-C is partially explained by the induction of APOA1 and SCARB1 genes, which enhances production and catabolism of HDL, respectively. As the result, the flux of HDL and probably reverse cholesterol transport are affected by fibrates more profoundly than reflected by changes in HDL-C. Triglycerides rather than HDL-C were the most profoundly altered lipoprotein traits. The two gemfibrozil utilizing trials – the primary prevention Helsinki Heart Study (Frick et al. 1987) and the secondary prevention study VA-HIT (Rubins et al. 1999) – were the only ones which found significant reductions of ASCVD events by the fibrate intervention vs. placebo. Only three trials (VA-HIT, BIP, and ACCORD) pre-defined plasma levels of HDL-C as inclusion criterion (ACCORD Study Group 2010; Bezafibrate Infarction Prevention Study 2000; Rubins et al. 1999). For ACCORD, the threshold was rather high with 55 mg/dL (1.42 mmol/L) (ACCORD study group 2010). Post-hoc analyses of the fibrate trials demonstrated relative risk reductions for subgroups of patients with HDL-C and triglycerides levels <35 mg/dL (0.9 mmol/L) and >200 mg/dL (2.3 mmol/L) ranging from 27% (FIELD, Keech et al. 2005) to –65% (Helsinki

Table 2 Effects of lipid modifying drug classes on lipoprotein traits and prevention of major cardiovascular events (MACE)

Drug class	LDL-C (max $\Delta\%$)	Triglycerides (max $\Delta\%$)	HDL-C (max $\Delta\%$)	MACE reduction
Statins	-50	-40	+10	Yes
Ezetimibe	-20	-10	0	Yes
Resins	-10	+20	0	Yes
PCSK9-inhibitors	-60	-20	+10	Yes
Fibrates	-10	-40	+15	Controversial: Yes, if without statins or post hoc, if HDL-C low and triglycerides elevated
Omega-3 fatty acids	+10	-35	0	Controversial
Nicotinic acid	-15	-30	+20	Controversial: Yes, if without statins
CETP inhibitors	-40	-15	+130	Anacetrapib: Yes Dalcetrapib & evacetrapib: No Torcetrapib: Adverse

CETP cholesteryl ester transfer protein, *HDL-C* High density lipoprotein cholesterol, *LDL-C* low density lipoprotein cholesterol, *MACE* major cardiovascular events, *PCSK9* proprotein subtilisin kexin type 9 convertase

Heart Study) (Sacks et al. 2010). Currently the *Pemafibrate to Reduce Cardiovascular Outcomes by Reducing Triglycerides IN patiENTs With diabeTes* (PROMINENT) trial tests prospectively the efficacy of the novel combined PPAR α /PPAR δ agonist pemafibrate (NCT03071692) (Pradhan et al. 2018).

Nicotinic acid (niacin) is an agonist of the G-protein coupled receptor GPR109A (HM74A or PUMA-G) (Offermanns 2014). As such, it primarily inhibits the lipolysis in adipocytes and secondarily, by reducing the free fatty acid flux, the lipogenesis and VLDL production in the liver. Reduced free fatty acid exposure may also promote ABCA1 activity in the liver and hence the production of nascent HDL (Chapman et al. 2010; KAmanna et al. 2013). In addition CETP activity was found decreased upon treatment with nicotinic acid due to direct and indirect inhibitory effects via production as well as activity of the protein and diminished pool of VLDL and hence acceptor particles, respectively (Chapman et al. 2010). In addition, nicotinic acid lowers plasma levels of LDL-C and lipoprotein(a) (Lp(a)). Despite these multiple beneficial effects on lipoproteins, in both the AIM-HIGH and HPS-THRIVE trials, the combination of statins with nicotinic acid was not superior to statin monotherapy in preventing ASCVD events (Table 2) (AIM-HIGH Investigators 2011; HPS2-THRIVE Collaborative Group 2014). Only AIM-HIGH defined inclusion criteria based on HDL-C (<1.05 mmol/L or <40 mg/dL). However, post-hoc analyses did not find any evidence that low HDL-C defines a subgroup of patients who benefit from nicotinic acid (Guyton et al. 2013; HPS2

THRIVE Collaborative Group 2014) However, in a meta-analysis monotherapy of nicotinic acid was found effective in reducing cardiovascular morbidity and mortality (Keene et al. 2014). Because of futility and the occurrence of flushes as very unpleasant and frequent side effects, nicotinic acid is no longer available for treatment in many countries.

2.3.2 CETP Inhibitors Block Rather than Promote Reverse Cholesterol Transport

CETP links the metabolism of HDL and apoB containing lipoproteins by exchanging cholesteryl esters of HDL against triglycerides of VLDL and LDL (Chapman et al. 2010). As the result of inhibiting this exchange, the most effective CETP inhibitors – torcetrapib, evacetrapib, and anacetrapib – cause increases of HDL-C by 75 (Torcetrapib) to 130% (Evacetrapib) and decreases of LDL-C by 25% (torcetrapib) to 40% (Anacetrapib) (Barter et al. 2007b; HPS3/TIMI55–REVEAL Collaborative Group 2017; Lincoff et al. 2017). The weaker CETP inhibitor dalcetrapib increases HDL-C by 30% without causing any drop in LDL-C (Schwartz et al. 2012). CETP inhibitors also decrease Lp(a) by up to 35% through an as yet unknown mechanism (Gencer and Mach 2020). Despite their at first sight beneficial effects on lipoprotein traits, three trials were prematurely stopped; the ILLUMINATE trial because of excess morbidity and mortality in the torcetrapib arm possibly due to off target effects of torcetrapib (Barter et al. 2007b). ACCELERATE and dal-OUTCOME were stopped prematurely because of futility of evacetrapib and dalcetrapib, respectively (Lincott et al. 2017; Schwartz et al. 2012). Only the combination of statin with anacetrapib in the REVEAL trial showed some superiority towards statin only therapy (HPS3/TIMI55–REVEAL Collaborative Group 2017). However, with a 9% relative risk reduction or the primary endpoint, the added value of anacetrapib was small and attributed to the decrease in LDL-C rather than to the increase in HDL-C. Because of the parallel successful development of PCSK9 inhibitors, which are much more effective in lowering LDL-C and event rates, the development of anacetrapib was stopped. Dalcetrapib, however, is further developed towards a personalized indication: post-hoc analyses of the Dal-OUTCOME study revealed that the carrier status for a mutation in the adenylate cyclase subtype 9 encoding ADCY9 gene discriminated individuals who did or did not benefit from dalcetrapib treatment by lower ASCVD event rates (Tardif et al. 2015). However, the same mutation discriminated responders and non-responders neither to anacetrapib nor to evacetrapib in the REVEAL and ACCELERATE trials, respectively (Hopewell et al. 2019; Nissen et al. 2018). Conversely, the interaction of CETP with ADCY9 was recapitulated in genetic mouse models (Rautureau et al. 2018). The Dal-GenE trial currently investigates prospectively, whether patients selected for the ADCY9 genotype benefit from treatment with dalcetrapib (NCT02525939) (Tardif et al. 2020).

At first sight the negative outcomes of the CETP inhibitor trials were surprising, not only because of the beneficial effects on the lipoprotein profile but also because several large genetic studies demonstrated lower prevalences or incidences of cardiovascular events among carriers of low activity CETP alleles (Kathiresan

2012). However, later population genetic studies showed an interaction between CETP and HMGCR alleles. In the presence of HMGCR alleles that reduce HMG-CoA reductase activity and thereby mimic treatment effects of statins, CETP alleles that cause low CETP activity and mimic the effects of CETP inhibitors did not confer any additional cardiovascular risk reduction (FERENCE et al. 2017). Likewise, torcetrapib treatment reduced atherosclerosis in apoE3 Leiden* CETP transgenic mice, if provided as monotherapy but not if provided in combination with statins (de Haan et al. 2008) whereas anacetrapib treatment enhanced the anti-atherogenic effect of atorvastatin (Kühnast et al. 2015). Nevertheless, the question is raised if the anti-atherogenicity of CETP inhibition depends on the capacity of the LDL receptor pathway: if this is fully functional, for example, as the result of statin treatment, CETP will promote reverse cholesterol transport and should not be blocked (von Eckardstein 2020). Only in situations, where LDL removal by the LDL receptor pathway is compromised, it may be useful to withhold cholesterol from LDL by CETP inhibition for hepatic removal through LDL receptor independent pathways involving direct HDL/receptor interactions, for example with SR-BI. As an alternative explanation, it was proposed that CETP inhibition renders HDL dysfunctional by prolonging the half-life of HDL particles and thereby making them susceptible to adverse alterations in the lipid and protein composition or oxidative and enzymatic modifications of protein or lipid components. However, the classical function of HDL, mediation of cholesterol efflux from macrophages was rather increased upon treatment of humans or animals with Evacetrapib, Anacetrapib, or Dalcetrapib (Brodeur et al. 2017; Metzinger et al. 2020; Nicholls et al. 2015; Simic et al. 2017; Tardif et al. 2015). Interestingly endothelial functions were not improved or even impaired in apoE3 Leiden* CETP transgenic mice upon treatment with evacetrapib and anacetrapib, respectively, despite increasing CEC and paraoxonase activity (Simic et al. 2017).

2.3.3 Combination with High-Intensity Statins: The Winner Takes it All

The combination of statins with fenofibrate, nicotinic acid, or CETP inhibitors was motivated by post-hoc meta-analyses of statin trials, which found the residual risk of patients treated with statins to be significantly associated with low HDL-C levels (Boekholdt et al. 2013). A closer look to post-hoc analyses of individual trials, however, reveals that these associations became weaker the lower LDL-C levels were reached. For example, in the WOSCOP study, where mean levels of LDL-C were lowered from 5.0 mmol/L in the placebo arm to 3.6 mmol/L in the pravastatin arm, low baseline levels of HDL-C were associated with increased risk of ASCVD events in both treatment groups (West of Scotland Coronary Prevention Study Group 1998). However, more than 10 years later in the JUPITER study (LDL-C at baseline <3.37 mmol/L), both baseline and on-treatment levels of HDL-C were significantly associated with residual risk only in the placebo group with a mean on treatment LDL-C of 2.8 mmol/L, but not in the rosuvastatin group with an on treatment mean LDL-C level of 1.42 mmol/L (Ridker et al. 2010). Similar discrepant observations were made in the secondary prevention trials CARE and LIPID vs. TNT: HDL-C levels explained part of the residual risk in both placebo and pravastatin groups of

CARE and LIPID (mean baseline LDL-C 3.80 mmol/L, on treatment LDL-C 2.85 mmol/L) (Sacks et al. 2000). However, in the TNT trial (baseline LDL-C 2.55 mmol/L), low HDL-C increased ASCVD risk in the low-dose atorvastatin group (on treatment LDL-C 2.60 mmol/L) but not in the high dose atorvastatin group (on treatment LDL-C 2.0 mmol/L) (Barter et al. 2007a). It thus appears that the optimized control of LDL-C by high intensity statin therapy alleviates the residual risk associated with low HDL-C levels. In this regard it is also noteworthy that contemporary observational studies in general populations as well as in patients with clinically manifest ASCVD find weaker associations of HDL-C with first and recurrent cardiovascular events, respectively, than historical studies which recruited their participants in the pre-statin era (Bolibar et al. 2000; Colantonio et al. 2016; Schwartz et al. 2012). These secular trends are usually explained by the generally improved risk factor control. However, one must also be aware of the change in the methodology of HDL-C measurements that occurred in parallel with the triumphal procession of statins. Since about 1990, non-traceable and biased homogenous assays have replaced the previous cholesterol quantification after manual precipitation of apoB containing lipoproteins. One can hence not exclude that changes in the analytics affected the prognostic value of HDL-C (Miller et al. 2010).

2.4 Wrong Biomarker “the Good Cholesterol”

By contrast to the disease causing cholesterol in LDL (Borén et al. 2020), the cholesterol in HDL (that is HDL-C) neither exerts nor reflects any of the potentially anti-atherogenic activities of HDL. HDL-C is only a non-functional surrogate marker for estimating the HDL pool size without deciphering the heterogeneous composition and, hence, functionality of HDL (Rohatgi et al. 2021; Annema and von Eckardstein 2013, 2016). Differences in the molar content of apoA-I, phosphatidylcholines, cholesterol, and cholesteryl ester cause differences of HDL subclasses in shape, size, and charge. HDL particles carry hundreds of different quantitatively minor proteins and lipid species many of which are not just passive cargo (like cholesterol) but biologically active and susceptible to quantitative and qualitative modifications by diseases or interventions (Rohatgi et al. 2021, Annema and von Eckardstein 2013, 2016). These functionally active components hence have a much bigger chance than HDL-C to serve as a causal biomarker that can be exploited towards the development, targeting, and monitoring of therapies.

The most obvious candidate for a functional HDL biomarker is the plasma concentration of apoA-I which is not only a mandatory structural component of the bulk of HDL but also exerts several biological activities of HDL, for example activation of ABCA1 and LCAT to efflux and esterify cholesterol, respectively, or binding to SR-BI and other HDL receptors. In both epidemiological and clinical studies, apoA-I levels show inverse associations with ASCVD events, which however are not stronger than those of HDL-C (Emerging Risk Factors Collaboration 2009). Neither did Mendelian Randomization studies unravel any causal genetic

relationship between apoA-I levels and ASCVD (Karjalainen et al. 2020; Richardson et al. 2020).

Other widely investigated HDL biomarker candidates include numbers and sizes of HDL particles. However, the outcomes of their evaluation in epidemiological and clinical studies are controversial. Some studies found HDL particle number (HDL-P) superior to HDL-C (Chandra et al. 2015; Kuller et al. 2007; Mackey et al. 2012; Otvos et al. 2006; Singh et al. 2020), others vice versa (El Harchaoui et al. 2009; Mora et al. 2009; Parish et al. 2012; Qi et al. 2015). Interestingly, within the JUPITER trial HDL-P was superior to HDL-C in the prediction of events among statin treated probands but inferior among placebo treated probands (Mora et al. 2013). Some studies found small HDL particles more strongly related with outcomes than large HDL particles (Ditah et al. 2016; Kim et al. 2016; McGarrah et al. 2016; Silbernagel et al. 2017), other studies found the opposite (Li et al. 2016; Arsenault et al. 2009). A recent meta-analysis of four studies concluded similar strong associations of small, medium, and large HDL particles with the incidence of ASCVD events (hazard ratio and 95% confidence interval 0.91 and 0.87 to 0.96) (Wu et al. 2018). With a hazard ratio and 95% confidence interval of 0.82 and 0.78 to 0.87, the total number of HDL particles showed stronger associations. Interestingly a recent Mendelian Randomization study found protective associations between the concentration of medium and – less so but also significantly – small HDL particles with coronary artery disease (Zhao et al. 2021). Drug interventions in lipoprotein metabolism result in diverse changes of HDL particle size and numbers. For example, treatment with nicotinic acid and CETP inhibitors increases HDL-C levels more profoundly than HDL-P, reflecting the shift to larger particles. Vice versa, upon treatment with fibrates, HDL-P increases more strongly than HDL-C (Rosenson et al. 2015).

3 Consequences and Perspectives

3.1 The Search for Novel HDL-Biomarkers

The further development of HDL as a therapeutic target is mainly limited by the availability of biomarkers that reflect the functional and causal role of HDL in the pathogenesis of atherosclerosis. To this end, bioassays of HDL function were recently developed and validated in population and clinical studies. Among them, cholesterol efflux capacity (CEC) has been investigated most extensively. In these studies, different macrophage cell lines treated with different drugs to enhance the cellular cholesterol efflux machinery were utilized as donors of radioactively or fluorescently labeled cholesterol. ApoB depleted plasma or serum was used as acceptors and as surrogate of HDL to avoid laborious ultracentrifugation. The heterogeneity of assays together with the heterogeneity of populations investigated has contributed to some discrepant findings (Anastasiou et al. 2018). Nevertheless, a recent meta-analysis of eight prospective studies and more than 10,000 participants with more than 3,000 events found a significant inverse association of CEC with

ASCVD events (HR 0.86, 95% CI: 0.76–0.98). In a subgroup of five studies also mortality was related to CEC (HR 0.77, 95% CI: 0.80–1.0). Although CEC correlates with HDL-C, the associations of CEC with cardiovascular outcomes were independent of HDL-C (Soria-Florido et al. 2020). However, the concept of CEC as a proxy of HDL functionality has several limitations. First, as a laborious and difficult if not impossible to standardize bioassay it is a research rather than diagnostic tool, primarily for proof of concept studies and secondarily for the identification of functional molecular markers (Anastasius et al. 2018). Second, CEC should not be considered as an overall proxy of HDL functionality because other functions of HDL neither correlate nor share molecular determinants with CEC (Cardner et al. 2020). Third, although most intensively investigated, it is not clear that mediation of cholesterol efflux is the most relevant atheroprotective function of HDL. In fact, changes in CEC upon treatment with CETP inhibitors did not predict correctly the clinical outcomes of these drug interventions. They led to increases in CEC of apoB-free sera or plasmas but not to any reduction in cardiovascular event rates and coronary atherosclerosis, respectively (Brodeur et al. 2017; Metzinger et al. 2020; Nicholls et al. 2015; Simic et al. 2017; Tardif et al. 2015).

Despite these limitations, CEC has been used as the reference to develop molecular biomarkers that can be measured in clinical laboratories. One example is the derivation of an algorithm which integrates the information of differently sized HDL particles as measured by NMR. The estimated NMR-based CEC correlated very well with the in vitro measured CEC ($R^2 > 0.8$) and predicted incident CHD events with a hazards ratio of 0.86; 95% CI, 0.79–0.93, adjusted for traditional risk factors and HDL-C (Kuusisto et al. 2019). Another example is a proteomic score integrating the information of apolipoproteins A-I, C-I, C-II, C-III, and C-IV showed good correlation with CEC as well as significant association with the presence of coronary artery disease and cardiovascular mortality independently of clinical risk factors including conventionally measured concentrations of apoA-I and apoB (Jin et al. 2019; Natarajan et al. 2019). Replication studies are needed to validate these surrogate scores of CEC.

Several laboratories have used tandem mass spectrometry to search for protein or lipid components of HDL as functional biomarkers. The most recent update of the HDL Proteome Watch data bank (<http://homepages.uc.edu/~davidswm/HDLproteome.html>; accessed July 15, 2021) documents more than 200 proteins which were identified in HDL by at least three of 40 independent studies and are therefore considered as highly confident components of HDL. Even higher numbers of lipid species were identified by mass spectrometry of HDL (Cardner et al. 2020; Kontush et al. 2013). The concentrations of these molecules vary from less than 1 $\mu\text{mol/L}$ to more than 1 mmol/L (Annema and von Eckardstein 2013; Rohatgi et al. 2021). Already in view of the average HDL particle concentration of about 20 $\mu\text{mol/L}$ it is clear that only some lipids (e.g., unesterified cholesterol, cholesteryl esters, phosphatidylcholines) or proteins (e.g., apoA-I) are present on each particle with several copies. Other low abundant lipids (e.g., sphingosine-1-phosphate, oxysterols) and proteins (apoM or LCAT) are dispersed throughout different particles. Interestingly, these molecules are non-randomly distributed among HDL

particles. For example, the presence of sphingosine-1-phosphate is linked to the presence of its chaperone apoM (Christoffersen et al. 2011). By combining two immunoaffinity chromatography procedures, one with anti-apoA-I antibodies and one with an antibody against one of 16 other HDL-associated proteins, 16 HDL subclasses with distinct proteomes and little intraindividual variation over 3–24 months were identified (Furtado et al. 2018). Many proteins of each HDL subspecies exert related functions, for example in lipid transport, hemostasis, oxidation, or inflammation suggesting that specific functions beyond cholesterol efflux are exerted by distinct subspecies of HDL rather than the bulk of HDL. In agreement with this concept, a recent systems biology approach found distinct functions of HDL determined by clusters of distinct proteins and lipids carried by HDL with little overlap between the functions (Cardner et al. 2020). Moreover, in four prospective nested case–control studies, the presence or absence of distinct proteins was found to determine the association of apoA-I levels with incident cardiovascular events (Sacks et al. 2020). For example, apoA-I levels in particles that contain apoE or apoC-I but not their apoE or apoC-I- free counterparts showed the expected inverse association with incident ASCVD events. Vice versa, apoA-I levels in apoC-III-free particles but not particles containing apoC-III showed the expected inverse association with incident ASCVD events (Sacks et al. 2020). Cholesterol levels in apoC-III containing HDL even showed a positive association with incident ASCVD (Jensen et al. 2018). Moreover, apoC-III containing HDL was found to interfere with the capacity of HDL to inhibit the apoptosis of endothelial cells and to promote efflux from macrophages (Riwanto et al. 2013; Zvintzou et al. 2017). This makes apoC-III an interesting target for therapy beyond lowering of triglycerides (Zewinger et al. 2020). Other studies found the enrichment of HDL with either pulmonary surfactant protein B or serum amyloid protein A associated with increased risk of mortality in patients with diabetic end-stage nephropathy, heart failure, or CHD (Emmens et al. 2018; Kopecky et al. 2015; Zewinger et al. 2015).

Several mass spectrometric studies demonstrated gross alterations in the lipidome of HDL in patients with acute or chronic CHD as well as changes in response to statin therapy or body weight reduction (Cardner et al. 2020; Khan et al. 2018; Meikle et al. 2019; Orsoni et al. 2016; Sutter et al. 2015). However, to date, only signatures of lipid species in total plasma but not in HDL have been explored for their prognostic performance in prospective studies (Hilvo et al. 2019; Mundra et al. 2016). NMR-based studies identified some more general lipid traits of HDL to be associated with incident disease. However, they represent classes or subclasses rather than species of lipids and they are strongly intercorrelated with each other as well as measures of particle size or numbers so that they are not pursued as biomarkers beyond the latter indices (Cardner et al. 2020; Hafiane and Genest 2015; Rosenson et al. 2011).

3.2 Ongoing and Novel Drug Developments

After the failure of CETP inhibitors, only few drug developments targeting HDL have been continued or newly started. Some of the latter targets are pleiotropic and HDL is a bystander rather than the focus of these drug developments.

3.2.1 Reconstituted HDL, apoA-I Mimetic Peptides, and Recombinant LCAT

After infusions of artificially reconstituted HDL (rHDL) were found to reduce atherosclerosis in hypercholesterolemic rabbits, several formulations of rHDL were developed for investigation of their atheroprotective effects (He et al. 2021). Because rather large amounts of protein are needed, only short-term applications in acute clinical settings are feasible, for example in patients with acute coronary syndrome (ACS). rHDL containing phosphatidylcholines together with the recombinant apoA-I Milano variant (ETC-216, MDCO-216) or recombinant wild type apoA-I plus sphingomyelin (Cer001) or apoA-I isolated from plasma (CSL111, CSL112) were initially tested in phase II trials for their short-term effects on coronary atherosclerosis which was assessed by intravascular ultrasound of ACS patients. Whereas initial studies showed some regression of coronary atherosclerosis upon treatment with ETC-216 or CSL111 (Tardif et al. 2007; Nissen et al. 2003), later larger studies with MDCO-216 or Cer001 did not (Nicholls et al. 2018a, b). Neither did Cer001 cause regression or prevent progression of carotid atherosclerosis in patients with genetic HDL deficiency (Zheng et al. 2020). Currently only one formulation – CSL112 – is further pursued by a large randomized and controlled phase III trial (ApoA-I Event Reducing in Ischemic Syndrome II = AEGIS II). Seventeen thousand four hundred patients with myocardial infarction are randomized to 4 weekly infusions of either 6 g CSL112 or placebo within 5 days of the event (Gibson et al. 2021). The primary outcome is the time to first occurrence of the composite of CV death, MI, or stroke through 90 days. Secondary outcomes include the total number of hospitalizations for coronary, cerebral, or peripheral ischemia through 90 days and time to first occurrence of the composite primary outcome through 180 and 365 days. Results are expected to become available in 2023.

In addition to rHDL containing full length apoA-I, also apoA-I mimetic peptides are developed for treatment of atherosclerosis. They showed promising results in vitro and in preclinical animal models. Three of them have been tested for safety and effects on HDL-C and HDL function. For two of them – L4F and D4F – results have been reported. They did not cause any changes in HDL-C or anti-inflammatory HDL functions. No results have been reported for ETC642. The clinical development of FX-5A is planned (Wolska et al. 2021).

Application of recombinant LCAT is an alternative strategy to increase HDL-C or promote HDL metabolism by substitution of components (Freeman et al. 2020). In a randomized controlled study 32 patients were treated with three weekly injections of different dosages of recombinant LCAT (MEDI6012) or placebo (Bonaca et al. 2021). Compared to placebo, MEDI6012 caused dose-dependent increases of

HDL-C by 66% to 144% at day 19. Interestingly the initial bolus injection led to a more than 40% increase of HDL-C within 30 min. MEDI6012 caused neither any severe side effects nor the generation of neutralizing antibodies. In an ongoing phase IIb trial (REAL-TIMI 63B), the application of 2 dosages of MEDI6012 to ACS patients is currently investigated for its effect on infarct size in more than 400 ACS patients (<https://clinicaltrials.gov/ct2/show/NCT03578809>). Half of the patients receive additional 4-weekly injections of MEDI6012 or placebo for 12 weeks for investigation of LCAT's effects on coronary calcification. Of note, recombinant LCAT is also attractive for the use as enzyme replacement in familial LCAT deficiency, however with the goal to prevent the development and progression of nephropathy in these patients (Freeman et al. 2020; Vaisman et al. 2019).

3.2.2 Apabetalone

Apabetalone (RVX208) is an inhibitor of bromodomain and extraterminal (BET) proteins that regulate the expression of multiple genes by interference with histone acetylation. RVX208 was initially developed because it strongly induced APOA1 gene expression in cultivated hepatocytes and caused a profound increase of HDL-C and apoA-I levels in non-human primates by 90 and 60%, respectively (Ghosh et al. 2017). Also mice responded with substantial increases of HDL cholesterol. Atherosclerosis was suppressed in apoE deficient mice. However, in humans treatment with apabetalone caused very moderate increases in HDL-C and apoA-I levels but also reduced CRP levels. The development of the drug was stopped after a recent phase III trial (BETonMACE) in patients with acute coronary syndrome and type 2 diabetes did not reveal any reduction of clinical events compared with placebo. (Ray et al. 2020).

3.2.3 PPAR Modulators

The PPAR α modulator Pemaafibrate is developed primarily for the treatment of hypertriglyceridemia and the related cardiovascular risk. In a phase II dose finding study, pemaafibrate dose-dependently decreased triglycerides and increased HDL-C by up to 42% and 21%, respectively, compared to 30% and 14% by fenofibrate (Arai et al. 2017). In hypertriglyceridemic patients, treatment with pemaafibrate caused increases of HDL-C by about 16% as well as CEC (Yamashita et al. 2018). In apoE2 knock-in mice, pemaafibrate increased HDL-C, CEC as well as macrophage-to-feces reverse cholesterol transport, and reduced the extent of atherosclerotic lesions (Hennuyer et al. 2016). The PROMINENT trial investigates the effect of pemaafibrate vs. placebo on cardiovascular outcomes of 10,000 participants with diabetes mellitus type 2, triglycerides 200–499 mg/dL (2.26–5.64 mmol/L), HDL-C level \leq 40 mg/dL (1.03 mmol/L) during a maximal follow-up of 5 years (Pradhan et al. 2018).

3.2.4 ANGPTL3 and Endothelial Lipase

Angiopoietin-like protein 3 (ANGPTL3) is mainly produced by the liver and an endogenous inhibitor of both lipoprotein lipase and endothelial lipase (EL). After the discovery of loss of function mutations in the ANGPTL3 gene as a cause of

panhypolipoproteinemia and reduced cardiovascular risk (Arca et al. 2020), antisense oligonucleotides as well as monoclonal antibodies were developed for the treatment of hypertriglyceridemia and hypercholesterolemia. In fact, treatment of hypertriglyceridemia with the antisense oligonucleotide Vupanorsen and refractory hypercholesterolemia with the monoclonal antibody Evinacumab caused pronounced decreases of triglyceride levels and LDL-C but also HDL-C (Gaudet et al. 2020; Rosenson et al. 2020). The clinical implication of the 20–30% decrease in HDL-C is not known but very likely reflects the increased activity of EL upon ANGPTL3 inhibition (Wu et al. 2020).

Conversely, also EL inhibitors are developed with the aim to increase HDL-C. Treatment of non-human primates with the monoclonal anti-EL antibody MEDI5884 dose-dependently increased HDL-C and apoA-I levels by up to 100% and 30%, respectively (Le Lay et al. 2021). In a phase I study, human volunteers also experienced increases in HDL-C and apoA-I as well as particle number and size. CEC and anti-inflammatory activities of HDL were also improved. However, endothelial lipase inhibition also caused increases in LDL-C, albeit more profoundly in non-human primates than in humans. In non-human primates this unwanted effect could be blocked by PCSK9 inhibition. Nevertheless, one must wonder whether the risk of increasing LDL-C is well taken, especially since loss-of-function alleles of LIPG encoding EL do not confer any cardiovascular risk reduction despite increasing HDL-C thus questioning the clinical utility of EL inhibition (Voight et al. 2012).

3.2.5 ApoC-III Inhibition

Antisense oligonucleotides against apoC-III (Volanesorsen) exert pronounced triglyceride lowering effects by reducing the production of VLDL as well as by promoting lipolysis and remnant removal by disinhibiting lipoprotein lipase and remnant receptors, respectively. Probably secondarily to the lowering of triglycerides, interference with apoC-III in patients with chylomicronemia also leads to increases of HDL-C levels by 40% (Witztum et al. 2019). Although not tested, one must assume that Volanesorsen also decreases the content of apoC-III in HDL. In view of the positive rather than inverse association of apoC-III containing HDL with cardiovascular outcomes (Jensen et al. 2018; Sacks et al. 2020); as well as the noxious effects of apoC-III on HDL functionality towards cholesterol efflux and endothelial survival and inflammation (Riwanto et al. 2013; Zewinger et al. 2020; Zvintzou et al. 2017), one may hypothesize that apoC-III inhibition also exerts anti-atherogenic effects by improving HDL function. However, this hypothesis needs to be tested.

3.2.6 HDL-C Lowering Therapies: Probucol and Androgens

For a long time, lowering of HDL-C has been considered as a safety issue in drug development. This has changed as the causal role of HDL in ASCVD has been questioned. Even more so, the association of high HDL-C with increased mortality and morbidity for certain diseases (Bowe et al. 2016a; Ko et al. 2016; Madsen et al. 2017) raises the question whether under certain conditions HDL-C lowering may be useful. In view of the genetic association of loss of function mutations in SR-BI with

increased cardiovascular risk (Zanoni et al. 2016) and the finding of increased atherosclerosis in *Scarb1* knock-out mice (Hoekstra and van Eck 2015), especially therapies that lower HDL-C by upregulation of SR-BI in the liver may be interesting.

Probucol is an old drug which was originally developed to exploit its anti-oxidative effects on LDL. Although it was shown to induce regression of atherosclerosis and xanthomas and has been rather widely used in Japan, the development and clinical application of probucol has not been consequently pursued (Yamashita et al. 2015). The main reason was the about 30% lowering of HDL-C. Activation of CETP and SR-BI has been elucidated as the underlying mechanism. In the most recent PROSPECTIVE trial, 876 Japanese patients with CHD and LDL-C \geq 140 mg/dL without medication or those treated with lipid-lowering drugs received optimal lipid-lowering treatment together with placebo or probucol 500 mg/day. After 3 years, LDL-C and HDL-C were 8.5 mg/dL and 16.3 mg/dL, respectively, lower in the probucol than in the placebo group. The event rates did not differ significantly between the groups, although by trend, CHD events happened less frequently in the probucol group (Arai et al. 2021). Interestingly, the combined analysis of the PROMINENT and IMPACT trials showed reductions of cerebrovascular events, however in the absence of any effect on carotid atherosclerosis (Yamashita et al. 2021). Although futile, the data raise the question of whether probucol is a treatment option for patients with high HDL-C.

The stimulatory effects of testosterone on hepatic SR-BI expression are probably the main reasons for the substantial differences in HDL-C levels between males and females (Chiba-Falek et al. 2010; Langer et al. 2002). Even more so, the HDL-lowering effects of testosterone have contributed to the caution on the use of testosterone for the treatment of the aging male syndrome, transgender patients, or female sexual dysfunction as well as for male contraception (Thirumalai et al. 2015; Wu and von Eckardstein 2003). The effects of testosterone replacement on hard cardiovascular endpoints have not been investigated. However, a recent randomized controlled trial in 1007 men with overweight or obesity as well as disturbed or manifest diabetes at baseline showed benefits of 1,000 mg intramuscular testosterone vs. placebo injection on glycemic control and incidence of diabetes during 2 years of follow-up (Wittert et al. 2021).

3.3 Other Disease Targets

As late onset diseases, ASCVDs have not been rate limiting in the evolution of species. Therefore, one must envisage that the broad spectrum of HDL's protective functions has rather evolved to prevent other diseases or secure survival and healing of their victims. Such diseases or their clinical complications may serve as more appropriate targets than ASCVD for the therapeutic exploitation of HDL (Von Eckardstein and Rohrer 2016). Indeed, recent epidemiological and genetic studies unraveled several associations of HDL-C and genetic loci intimately related to HDL metabolism with non-cardiovascular diseases as well as mortality (Table 3) (Kjeldsen et al. 2021a; Madsen et al. 2021). Please note the diverse directions of

Table 3 Role of HDL in different diseases according to epidemiology, therapeutic interventions, human genetics, and animal experiments

Diseases	Association in observational studies	Clinical benefit in intervention studies	Genetic association	Animal experiments
Atherosclerotic cardiovascular diseases	Discontinuously inverse (until 60th percentile)	No (CETP inhibitors) or subgroups (fibrates)	No	Gene-dependent
Diabetes	Inverse	(yes) (post-hoc: CETP inhibitors, rHDL)	Yes (Mendelian gene scores) No (candidate genes)	Yes (ABCA1, APOA1)
Chronic kidney disease	Parabolic	Not investigated	Yes	Yes (LCAT)
Infections	Parabolic	Not investigated	Yes	Yes (APOA1, CETP, rHDL)
Autoimmune disease	Inverse	Not investigated	Not investigated	Yes (APOA1, SCARB1, S1P)
Age-related macular degeneration	Positive	Not investigated	Yes	(Yes) (ABCA1)
Alzheimer's disease	Discontinuously positive (>95th percentile)	Not investigated	No (Mendelian gene scores) Yes (GWAS, e.g., ABCA1)	(Yes) (ABCA1)

these associations which reach from inverse (diabetes, autoimmune diseases) over parabolic (infections, chronic kidney disease, mortality) to positive (Alzheimer's disease, age related macular degeneration), re-emphasizing that the kinetics of HDL metabolism and HDL function rather than the concentration of HDL particles are relevant.

3.3.1 Diabetes

Low levels of HDL-C are frequent in patients with diabetes mellitus type 2. This finding even precedes the manifestation of hyperglycemia and is hence an indicator of increased risk for incident diabetes (Haase et al. 2015; Schmidt et al. 2005; von Eckardstein et al. 2000; White et al. 2016; Wilson et al. 2007). Because of multiple effects of insulin on HDL metabolism, most of which are indirect via free fatty acids

or triglyceride-rich lipoproteins, these associations have been explained for a long time by reverse causality: diabetes and pre-diabetes cause low HDL-C rather than vice versa (Parhofer 2015; Vollenweider et al. 2015; von Eckardstein and Widmann 2014). However, increasing evidence from *in vitro* as well as *in vivo* studies indicates that HDL exerts protective functions on the function and survival of pancreatic beta cells as well as on the sensitivity of target cells to insulin (Cochran et al. 2021; Manandhar et al. 2020; Vollenweider et al. 2015; von Eckardstein and Widmann 2014; Yalcinkaya et al. 2020). Also mitochondrial function and thereby cellular energy metabolism is modulated by HDL (Lehti et al. 2013). In humans, the potentially anti-diabetic effects of HDL are best illustrated by the acute glucose lowering effect of rHDL infusion (Drew et al. 2009) as well as by the findings of post-hoc analyses of the CETP inhibitor trials: Participants who received the CETP inhibitors showed better glycemic control and experienced less often new-onset diabetes as compared to the placebo treated controls (Barter et al. 2011; Masson et al. 2018; Menon et al. 2020; Schwartz et al. 2020). Mendelian randomization studies yielded controversial results on the genetic causality of HDL-C in diabetes (Fall et al. 2015; Haase et al. 2015; White et al. 2016).

3.3.2 Chronic Kidney Disease

The Veterans Administration study showed a parabolic association of HDL-C with >30% declining estimated glomerular filtration rate (eGFR) or the incidence of eGFR <60 ml/min and also provided evidence for genetic causality (Bowe et al. 2016b). Mutations in APOA1, APOE, APOL1, and LCAT are causes of genetic nephropathies (Strazzella et al. 2021). However, it is not clear whether their pathogenesis involves HDL: Certain missense mutations in APOA1 cause familial amyloidosis, which also affect other organs (Zanoni and von Eckardstein 2020). Specific mutations in APOE cause lipid glomerulopathy which however has been suggested to develop in response to disturbed interactions of apoB containing lipoproteins with the LDL receptor or due to accumulation of the structural defective apoE (Saito et al. 2020). ApoL1 is the trypanolytic factor which is transported by a minor subfraction of HDL (Friedman and Pollak 2020). Certain apoL1 variants that protect the host from infections with *Trypanosoma brucei rhodesiense* and *gambiense* dramatically increase the risk of chronic kidney disease, notably focal segmental glomerulosclerosis, in their African and Afroamerican carriers (Friedman and Pollak 2020). LCAT deficiency is a classical HDL deficiency syndrome causing a nephropathy that eventually progresses to end-stage renal disease (Pavanello and Calabresi 2020). However, the pathogenic mechanism depends on the accumulation of lipoprotein X rather than the absence of HDL (Vaisman et al. 2019). This is best illustrated by the absence of nephropathy in patients with fish-eye disease where partial loss of LCAT causes the same decrease in HDL as classical LCAT deficiency, however no nephropathy (Pavanello and Calabresi 2020).

Treatment with fibrates improves albuminuria but worsens glomerular filtration rate (Speer et al. 2021). Niacin has no effect on renal function. Small studies also indicate protective effects of probucol towards acute kidney injury, for example of patients exposed to contrast agents (Xin et al. 2019). The pleiotropic effects of these

drugs on lipoprotein metabolism and the lack of association between changes in renal function and HDL-C under their treatment do not allow any conclusion on the role of HDL modifying therapies for prevention or treatment of CKD. Furthermore the exploitation of HDL towards kidney disease is also hampered by the currently unknown mechanisms how HDL exerts renal protection by HDL. Since small HDL particles are undergoing glomerular filtration and tubular re-uptake by the megalin/cubilin co-receptors, it may be that HDL delivers protective molecules to the kidney, for example sphingosine-1-phosphate (Bisgaard and Christoffersen 2019; Strazzella et al. 2021).

3.3.3 Infections

The Copenhagen General Population study found U-shaped associations between HDL-C and the incidence of infections (Madsen et al. 2018). The associations with bacterial infections were stronger than with viral infections. Upon limited adjustment, gastroenteritis, bacterial pneumonia, skin and urinary tract infections, as well as sepsis were more prevalent among individuals with HDL-C <1.0 mmol/L as compared to individuals with higher HDL-C. Upon full adjustment the associations with gastroenteritis and pneumonia remained significant (Madsen et al. 2018). A preliminary Mendelian randomization analysis with two loci (CETP and LIPC) provided initial evidence of genetic causality (Madsen et al. 2018). Genetic causality also exists for the association of low HDL-C with the incidence of sepsis as well as with the chance of survival in patients with sepsis (Trinder et al. 2020). Evidence from population studies as well as experiments in genetic animal models points to the importance of CETP in this process (Blauw et al. 2020; Trinder et al. 2019, 2021). However also HDL particles per se as well as specific structural components of HDL exert several antibacterial activities such as binding and removal of lipopolysaccharides, protection of epithelial and endothelial barriers, or modulatory effects on leukocyte functions (Catapano et al. 2014; Meilhac et al. 2020; Pirillo et al. 2015; Robert et al. 2021; Rohatgi et al. 2021; Trakaki and Marsche 2021). The special association of HDL-C with gastroenteritis may also mirror an important role of HDL that is locally produced in the intestine (Ko et al. 2020). Similarly, the protection from pneumonia may mirror the high exposure of the lung to newly synthesized HDL due to first pass effects (Gordon et al. 2016). In sepsis models, genetically modified mice overexpressing human apoA-I showed improved survival (Meilhac et al. 2020; Morin et al. 2015). Interestingly, the clinical development of CSL111 was originally aiming at the treatment of sepsis since their infusion into volunteers exerted several beneficial effects on inflammation, coagulation, and fibrinolysis (Pajkrt et al. 1996, 1997). Most recent experiments in preclinical sepsis models demonstrated better survival of mice treated with CSL111 (Tanaka et al. 2020). The better chances of survival from sepsis by patients carrying low CETP activity alleles suggest CETP inhibitors as interesting drugs for the treatment of patients with sepsis (Trinder et al. 2019, 2021). However, an important caveat comes from the ILLUMINATE study where torcetrapib treatment was associated with excess mortality due to infections (Barter et al. 2007b).

Although the association of HDL-C with the incidence of viral infections was not statistically significant in the Copenhagen General Population Study (Madsen et al. 2018), it is noteworthy that at least in vitro HDL or apoA-I interferes with the entry or fusion of viruses with target cells (Meilhac et al. 2020; Pirillo et al. 2015). HDLs also induce viral inactivation by immune cells and protect cells from virus-induced damage (Pirillo et al. 2015). The COVID19 pandemic also raised the question of whether HDL interferes with SARS-CoV2 infections. In the UK Biobank study, a linear inverse and independent association was found between pre-infection HDL-C levels and the risk of hospitalization for severe COVID19 (Hilser et al. 2021; Lassale et al. 2021). Mendelian Randomization rather excluded any causal role of HDL in preventing SARS-CoV2 infection. (Hilser et al. 2021). Nevertheless, since SR-BI is an entry route of several viruses including SARS-CoV2 into cells (Pirillo et al. 2015; Wei et al. 2020), competition of this interaction by HDL is an intriguing hypothesis. In addition, the protective effects of HDL on the survival and function of cells may also help infected cells, for example of the lung epithelium or the endothelium (Robert et al. 2021), to combat and survive the entered viruses. Interestingly, dalcetrapib is currently tested towards its effect on the course of COVID19 infections. (Talasaz et al. 2021; <https://clinicaltrials.gov/ct2/show/NCT04676867>).

Finally, HDL also exerts protective activities towards infections with protozoa. The best example is the protection of humans from *Trypanosoma brucei* by apoL1 transported by a subfraction of HDL containing also haptoglobin related protein. This complex kills *Trypanosoma brucei* by causing lysosomal swelling (Friedman and Pollak 2020). A similar HDL-related mechanism appears operative towards *Leishmania* (Samanovic et al. 2009).

3.3.4 Autoimmune Diseases

In an analysis of more than 110,000 participants of The Copenhagen General Population and the Copenhagen City Heart Study, low HDL-C concentrations were associated with elevated risk of developing the composite end point of 42 different autoimmune diseases (Madsen et al. 2019). Among them, the associations of celiac disease, idiopathic thrombocytopenic purpura, Sjögren's disease, diabetes type 1, inflammatory bowel diseases, and Graves' disease showed the strongest and individually significant associations with HDL-C. Currently, no data are available to prove or disprove causality of these associations (Madsen et al. 2021). Neither are the mechanisms understood. They may involve immunomodulatory effects of HDL which are relevant in the development of auto-immunity (Catapano et al. 2014; Pirillo et al. 2015; Rohatgi et al. 2021; Trakaki and Marsche 2021) or protective effects of HDL towards organs attacked by the immune system so that the onset of organ damage or failure and thereby the clinical diagnosis of manifest disease is delayed. For example, the anti-apoptotic effects of HDL on pancreatic beta cells may delay the loss of insulin production in the course of progressing type 1 diabetes (von Eckardstein and Widmann 2014; Yalcinkaya et al. 2020). For inflammatory bowel diseases, preclinical models generated evidence for the therapeutic potential of HDL: Intestinal inflammation was increased in ApoA1 knock-out mice but decreased in mice which overexpressed human APOA1 or were fed with

apoA-I mimetic peptides (Gerster et al. 2014; Meriwether et al. 2019; Nowacki et al. 2016).

3.3.5 Cancer

Several epidemiological studies found inverse associations between HDL-C and cancer in general as well as specific cancers such as breast cancer or colorectal cancer (Ganjali et al. 2021; Madsen et al. 2021; Pirro et al. 2018). Currently, there is no evidence of causality. However, several confounders of low HDL-C are associated with increased risk of several cancers, for example smoking, overweight and obesity, type 2 diabetes, or chronic inflammatory diseases. There is hence some likelihood that low HDL-C is a confounder of other causal risk factors rather than reflecting loss of anti-cancer functions. Even if HDL is not causally related to cancer, it will offer opportunities for therapeutic or diagnostic exploitation: probably to satisfy their high need of cholesterol for growth, many cancers show a high expression of lipoprotein receptors including SR-BI (Hoekstra and Sorci-Thomas 2017; Kinslechner et al. 2018; Velagapudi et al. 2018). This can be exploited by using rHDL for the delivery of anti-cancer drugs or tracers for imaging. In fact, according to experiments in preclinical models showed promising results (Morin et al. 2018; Rajora and Zheng 2016).

3.3.6 Behind the Blood Brain Barrier: Alzheimer's Disease and Age Related Macular Degeneration

Until recently, only cross-sectional studies and smaller prospective cohort studies described associations of HDL-C with neurodegenerative diseases including Alzheimer's disease (AD) and age related macular degeneration (AMD). They reported discrepant associations ranging from inverse over none to positive (Kjeldsen et al. 2021a). The situation became clearer but also surprising by recent reports of analyses in the Copenhagen General Population and Copenhagen City Heart Studies. High HDL-C levels >95th percentiles increase the risk of dementia and AD (Kjeldsen et al. 2021b). This association became even more prominent after adjustment for APOE genotypes. Also the risk of AMD was found to increase with HDL-C and even more so with apoA-I levels (Nordestgaard et al. 2021) confirming data on more than 30,000 individuals from the EYE-RISK and European Eye Epidemiology Consortia (Colijn et al. 2019). Of note, in that study higher HDL-C was most strongly associated with increased risk of early AMD. Mendelian Randomization studies found evidence for genetic causality of higher HDL-C for the higher risk of AMD but not AD (Burgess and Smith 2017; Chen et al. 2010; Fan et al. 2017; Neale et al. 2010; Ostergaard et al. 2015; Proitsi et al. 2014), the latter perhaps because of the non-linear relationship. CETP, APOE, and LIPC were important drivers of the genetic association between HDL-C and AMD (Colijn et al. 2019; Chen et al. 2010; Neale et al. 2010). However, candidate gene approaches as well as genome-wide association studies found ABCA1 as a genetic determinant of both AD and AMD risks (Bellenguez et al. 2020; Nordestgaard et al. 2015; Fritsche et al. 2016). Likewise, tissue specific knock-out experiments in mice indicate that loss of ABCA1 function in neurons and retinal pigment epithelial cells

compromise neurocognitive and retinal functions, respectively (Behl et al. 2021; Storti et al. 2019). These discrepant associations of AD and AMD with high HDL-C levels in peripheral blood but locally reduced cholesterol efflux in the brain and retina probably reflect the tight separation of these compartments by the blood brain barrier. Some HDL functions in the brain are executed by HDL-like particles that contain apoE instead of apoA-I and that are produced by astrocytes within the central nervous system (Button et al. 2019). Especially the association of APOE genotypes with risk of AD, although mechanistically not resolved, has been traditionally ascribed to apoE endogenously produced by the CNS rather than supplied by the systemic circulation. Of note however, also the concentration of apoE in plasma of peripheral blood has been associated with risk of AD (Rasmussen et al. 2018). Moreover, anti-apoA-I immunoreactivity is found in the brain. These findings indicate a limiting role of the blood brain barrier for any protective role of HDL in CNS diseases such as AD and AMD. Any therapeutic exploitation of HDL for CNS diseases will have to address the interaction of HDL with the blood brain barrier, either as a target of HDL's protective actions, for example in amyloid beta clearance, or as a barrier that must be surmounted by HDL to exert protective functions within the CNS (Button et al. 2019; Robert et al. 2021). The latter is also important for the use of HDL-like nanoparticles that are currently investigated as vehicles for drug delivery into the brain (D'Arrigo 2020; Kadiyala et al. 2019; Kim et al. 2020).

3.4 Implications for Nowadays' Clinical Practice

As the result of the futile intervention trials, HDL-C unlike LDL-C has not become any treatment goal (Grundy et al. 2019; Mach et al. 2020). However, HDL-C continues to be part of ASCVD risk assessment, both directly and indirectly by using HDL-C for the calculation of nonHDL cholesterol or even LDL cholesterol (Grundy et al. 2019; Mach et al. 2020; Martin et al. 2013; Sampson et al. 2020). Especially in asymptomatic patients without any lipid modifying treatment, a low HDL-C level is considered as a risk factor of developing ASCVD. As such, HDL-C is a component of most clinical risk prediction rules that are promoted by guidelines for the prevention of ASCVD (Grundy et al. 2019; Mach et al. 2020). Unfortunately, these algorithms do not realize the discontinuous relationship of HDL-C with risk, but still de-escalate risk estimates in individuals with very high HDL-C levels. This may be a reason why, for example, in the Copenhagen City Heart study the inclusion of HDL-C impaired rather than improved the prognostic performance of SCORE promoted by ESC and EAS (Mortensen et al. 2015). With the same reasoning, clinical laboratories as well as clinicians and practitioners should stop the still widely spread clinical practice to calculate total cholesterol/HDL-C- or LDL-C/HDL-C ratios because they may underestimate the risk of individuals with high HDL-C (de Wolf et al. 2020; Nordestgaard et al. 2020). The same concern may relate to the atherogenic index – the logarithmically transformed ratio of the molar concentrations of plasma triglycerides to HDL-C – especially because some studies found the joint presence of high HDL-C and hypertriglyceridemia associated with

increased ASCVD risk (Jeppesen et al. 1998; von Eckardstein et al. 1999). Finally, low HDL-C continues to be a component of definitions for the metabolic syndrome, which indicates increased risks not only for ASCVD but also for diabetes and other obesity related diseases (Alberti et al. 2009).

Whether reflecting compromised anti-atherogenic functions or indicating indirectly a proatherogenic situation, the finding of low HDL-C levels should prompt physicians and patients to optimize the control of other risk factors (März et al. 2017). The lost association of low HDL-C with increased risk upon intensive statin therapy indicates the importance of consequent LDL-C lowering in these patients. Additional important measures include cessation of smoking, correction of obesity and overweight, and treatment of hypertension. In view of the inconsistent outcomes of according randomized controlled trials it is a matter of uncertainty and controversy whether or not hypertriglyceridemia which frequently confounds low HDL-C should be targeted by drug treatment (Ginsberg et al. 2021).

The discussion on therapeutic consequences of high HDL-C levels is in its infancy. It is not clear whether the associations of high HDL-C with increased mortality and risks of CKD, infectious diseases, AD, or AMD are causal. An important potential confounder is excess alcohol consumption (Madsen et al. 2021). Potential candidates for HDL-C lowering drugs are probucol, ANGPTL3 inhibitors, or androgens. In the absence of HDL-C lowering treatments with proven efficacy, it is advisable to focus on risk factor control also in patients with high HDL-C as described for patients with low HDL-C.

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Lipoprotein(a)

Florian Kronenberg 

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Abstract

Lipoprotein(a) [Lp(a)] is an atherogenic lipoprotein with a strong genetic regulation. Up to 90% of the concentrations are explained by a single gene, the *LPA* gene. The concentrations show a several-hundred-fold interindividual variability ranging from less than 0.1 mg/dL to more than 300 mg/dL. Lp(a) plasma concentrations above 30 mg/dL and even more above 50 mg/dL are associated with an increased risk for cardiovascular disease including myocardial infarction, stroke, aortic valve stenosis, heart failure, peripheral arterial disease, and all-cause mortality. Since concentrations above 50 mg/dL are observed in roughly 20% of the Caucasian population and in an even higher frequency in African-American and Asian-Indian ethnicities, it can be assumed that Lp(a) is one of the most important genetically determined risk factors for cardiovascular disease.

Carriers of genetic variants that are associated with high Lp(a) concentrations have a markedly increased risk for cardiovascular events. Studies that used these genetic variants as a genetic instrument to support a causal role for Lp(a) as a cardiovascular risk factor are called Mendelian randomization studies. The principle of this type of studies has been introduced and tested for the first time ever with Lp(a) and its genetic determinants.

There are currently no approved pharmacologic therapies that specifically target Lp(a) concentrations. However, some therapies that target primarily LDL cholesterol have also an influence on Lp(a) concentrations. These are mainly PCSK9 inhibitors that lower LDL cholesterol by 60% and Lp(a) by 25–30%. Furthermore, lipoprotein apheresis lowers both, Lp(a) and LDL cholesterol, by about 60–70%. Some sophisticated study designs and statistical analyses provided support that lowering Lp(a) by these therapies also lowers cardiovascular events on top of the effect caused by lowering LDL cholesterol, although this was not the main target of the therapy. Currently, new therapies targeting RNA such as antisense oligonucleotides (ASO) or small interfering RNA (siRNA) against apolipoprotein(a), the main protein of the Lp(a) particle, are under examination and lower Lp(a) concentrations up to 90%. Since these therapies specifically lower Lp(a) concentrations without influencing other lipoproteins, they will serve the last piece of the puzzle whether a decrease of Lp(a) results also in a decrease of cardiovascular events.

Keywords

Apolipoprotein(a) · Association study · Cardiovascular disease · Copy number variation · Lipoprotein(a) · Lp(a) · Mendelian randomization · Therapy

1 Introduction

Lipoprotein(a) [Lp(a)] is one of the strongest genetically determined risk factors for cardiovascular disease (CVD) (Kronenberg and Utermann 2013). It contains besides an LDL particle an additional apolipoprotein that is called apolipoprotein

(a) [apo(a)]. This apolipoprotein shows a high homology with plasminogen. After more than 50 years of research, the physiological function of Lp(a) is still unexplained. An astonishing characteristic of Lp(a) is the more than 1,000-fold range of concentrations between individuals from almost zero to more than 300 mg/dL (Kronenberg and Utermann 2013). The distribution of Lp(a) is very skewed in most populations: for example, roughly 50% of the Europeans have concentrations below 10 mg/dL and about 25% have concentrations above 30 mg/dL.

2 Sites of Production and Catabolism of Lp(a)

Apo(a) is synthesized in the liver (Tomlinson et al. 1989; Kraft et al. 1989). Variation in Lp(a) concentrations among individuals is determined by the rate of production rather than by differences in the catalytic rate (Rader et al. 1994). After secretion of apo(a), it binds to LDL-apoB lysine-binding residues by its lysine-binding sites with subsequent forming of a disulfide bond. Whether the assembly occurs in the circulation at the hepatic surface or intracellularly is a matter of debate (Koschinsky and Marcovina 2004).

The site and mechanism of catabolism is discussed controversially. Studies in mice revealed that the liver is the main route of Lp(a) catabolism (Cain et al. 2005). The finding of an arteriovenous difference of Lp(a) concentrations in the renal circulation (Kronenberg et al. 1997), of apo(a) fragments in urine (Mooser et al. 1996), and of a disturbed Lp(a) metabolism in kidney disease (Frischmann et al. 2007) has suggested also a role of the kidney in Lp(a) catabolism. The receptors involved in the catabolic process are a matter of debate as recently comprehensively reviewed (McCormick and Schneider 2019) and include lipoprotein receptors such as the LDL receptor, VLDL receptor, LDL receptor related proteins (LRP1 and LRP2), toll-like and scavenger receptors (e.g., CD36 and SR-BI), carbohydrate receptors or lectins, and plasminogen receptors.

3 Physiology and Pathophysiology of Lp(a)

The physiological function of Lp(a) is still in the dark. It is believed that Lp(a) has proatherogenic and prothrombotic properties (Fig. 1). The apo(a) glycoprotein has a high degree of homology to plasminogen (McLean et al. 1987) suggesting that Lp(a) might not only be a link between the cholesterol transport system in plasma and the fibrinolytic system but may also act as a modulator of the balance between blood clotting and fibrinolysis. At least in vitro, Lp(a) indeed interferes with the blood clotting/fibrinolytic cascades at several steps (Boffa and Koschinsky 2016). As reviewed extensively (Koschinsky and Marcovina 2004; Boffa and Koschinsky 2016), this includes the inhibition of streptokinase and urokinase-mediated activation of plasminogen by the tissue-type plasminogen activator (t-PA), inhibition of t-PA in solution, fibrin and fibrinogen binding, competition with plasminogen and t-PA binding for soluble fibrinogen, competition with plasminogen for binding to

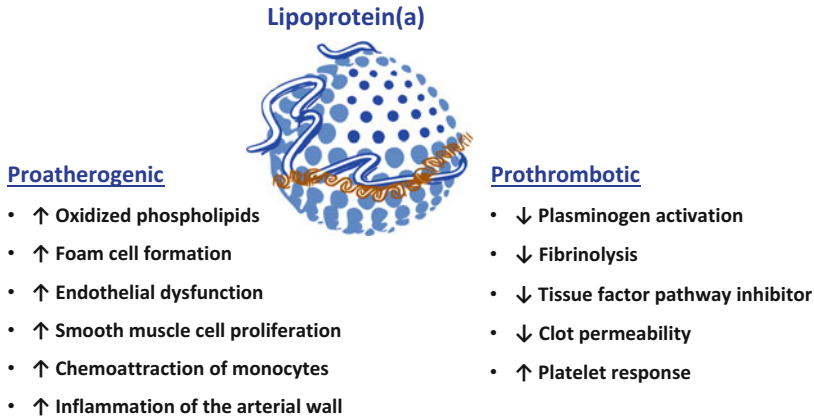


Fig. 1 Proatherogenic and prothrombotic properties of Lp(a)

cellular receptors, and enhancement of the plasminogen-activator-inhibitor PAI-1 activity. Considering that many individuals have low Lp(a) concentrations and some of them even almost no Lp(a) and others have concentrations far above 100 mg/dL, one might expect major effects on the balance between clotting and fibrinolysis which, however, have not convincingly been described by *in vivo* data. Epidemiologic and genetic studies did not provide support for a thrombogenic role and if there is any, it can only be found at very high Lp(a) values above the 95th percentile (Nordestgaard and Langsted 2016). Interestingly, there are reports that the situation might be different in childhood where high Lp(a) concentrations were found to be accompanied with venous thromboembolism (Nowak-Gottl et al. 1999).

A further property of Lp(a)/apo(a) is the interaction with components of the extracellular matrix including fibrin, fibronectin, tetranectin, proteoglycans, and β 2-glycoprotein (Köchler et al. 1997). The domains in apo(a), which mediate binding to fibrin/fibrinogen, are lysine-binding sites in KIV-8 and KIV-10 (Koschinsky and Marcovina 2004). Binding of Lp(a) to fibrin has been proposed as a mechanism to deliver cholesterol to sites of injury and wound healing of the vascular wall with the negative side effect that Lp(a) also deposits cholesterol in growing atherosclerotic plaques and inhibits fibrinolysis at the plaque surface. Furthermore, high Lp(a) levels impair activation of transforming growth factor- β by downregulation of plasmin generation, thereby contributing to smooth muscle cell proliferation (Grainger et al. 1993). The published data provide clear evidence that Lp(a) can interfere with many key reactions of clotting/fibrinolysis *in vitro* and is deposited in atherosclerotic plaques. Moreover, effects on monocytes/macrophages result in foam cell formation (Poon et al. 1997). Lp(a) induces chemoattractant activity of monocytes and induces macrophage expression of interleucin-8 (IL-8) (Klezovitch et al. 2001).

The connection between innate immune system and Lp(a) has been further strengthened by the identification of Lp(a) as the major plasma carrier of oxidized phospholipids (OxPLs) (Bergmark et al. 2008) which can stimulate many

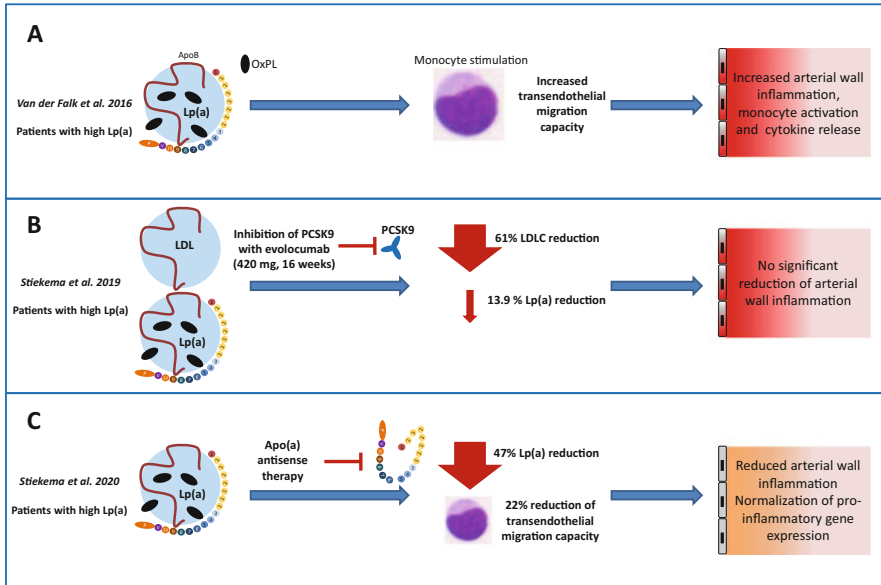


Fig. 2 Impact of Lp(a) on arterial wall inflammation. Panel (a) Individuals with high Lp(a) ($n = 30$, median 108 mg/dL) present significantly increased arterial wall inflammation as assessed by magnetic resonance imaging, 18-F fluorodeoxyglucose (18F-FDG) uptake PET/CT and SPECT/CT, and activated monocytes assessed by transendothelial migration and monocyte priming and challenge assays (van der Valk et al. 2016). Panel (b) 420 mg of the PCSK9 inhibitor evolocumab vs. placebo over 16 weeks reduced LDL-C consistently but did not improve arterial wall inflammation in individuals with high Lp(a) ($n = 129$, median ≈ 80 mg/dL), despite modest concomitant Lp(a) reduction (-13.9%) (Stiekema et al. 2019). Panel (c) High Lp(a) was associated with a pro-inflammatory transcriptome in monocytes. Treatment with different regimes of AKCEA-APO(a)-L_{RX} resulted in a 47% reduction of Lp(a) and in reductions of the pro-inflammatory signatures in the transcriptome, the transendothelial migration capacity, and the expression of chemokines and toll-like receptors on the monocytes surface (Stiekema et al. 2020; Coassin and Kronenberg 2020)

pro-inflammatory pathways in the arterial wall. The binding-site for oxidized phospholipids has been identified in the protein moiety of Lp(a), specifically in the KIV type 10 domain of apo(a) (Leibundgut et al. 2013). Levels of Lp(a) and OxPL in human plasma are highly correlated and it is therefore not unexpected that this also results in an association of OxPL levels with cardiovascular disease (Tsimikas et al. 2005, 2010; Kiechl et al. 2007). These oxidized lipid species are recognized by pattern recognition receptors of innate immune cells and trigger the whole cascade of inflammatory processes that can finally lead to plaque destabilization (Boffa and Koschinsky 2019). During recent years a series of publications (Fig. 2) from the group around Eric Stroes investigated the impact of Lp(a) and LDL-C on arterial wall inflammation and found that individuals with elevated Lp(a) concentrations have increased arterial inflammation and enhanced peripheral blood mononuclear cell trafficking to the arterial wall compared with subjects with normal Lp(a).

Monocytes isolated from subjects with high Lp(a) concentrations showed an increased capacity to transmigrate the endothelium and produce pro-inflammatory cytokines. This was mediated by OxPLs and subsequent blocking of the OxPLs by specific antibodies also reduced the pro-inflammatory responsiveness of the monocytes (Fig. 2a) (van der Valk et al. 2016). The subsequent publications revealed that a pronounced reduction of LDLC by PCSK9 inhibitors is not sufficient to substantially reduce the arterial wall inflammation (Fig. 2b) (Stiekema et al. 2019). In a further investigation they examined whether patients with cardiovascular disease and elevated Lp(a) concentrations experience anti-inflammatory effects following large reductions of Lp(a) by apo(a) antisense therapy. They observed in a first step that circulating monocytes of healthy individuals and patients, both having high Lp(a) concentrations, are characterized by a markedly pro-inflammatory gene expression profile with several pathways of the innate immune system being upregulated. In an intervention with apo(a) antisense therapy the authors showed that the resulting 47% lowering of Lp(a) concentrations was indeed capable of reversing the pro-inflammatory gene expression signature to levels close to that of controls with normal Lp(a) concentrations. This was accompanied with a 22% functional reduction in transendothelial migration capacity of monocytes *ex vivo* (Fig. 2c) (Stiekema et al. 2020; Coassin and Kronenberg 2020). These findings proposed a further mechanism how Lp(a) might mediate cardiovascular disease and added a new layer to our still shallow understanding of the exact pathophysiological mechanisms by which high Lp(a) causes cardiovascular disease.

4 Genetic Control of Lp(a) Concentrations

Lp(a) concentrations are not much influenced by age, sex, fasting state, inflammation (Kronenberg 2014a; Langsted et al. 2014) and lifestyle factors such as diet or physical activity. However, the concentrations are under strict genetic control. Family studies revealed a heritability estimate of Lp(a) concentrations of about 90% (Austin et al. 1992; Lamon-Fava et al. 1991; Utermann 1989). Lp(a) is therefore the lipoprotein with the strongest genetic control. The discovery of the size polymorphism of apo(a) in serum (Utermann et al. 1987) which is based on a variable number of the so-called kringle-IV (K-IV) repeats in the *LPA* gene (Utermann 1989; Kraft et al. 1992; Lackner et al. 1991, 1993) resulted in the identification of the *LPA* gene as the major gene for Lp(a) levels. Each of these up to more than 40 repeats has a size of 5.6 kB which results in a highly polymorphic and informative copy number variation (CNV).

There exists a pronounced inverse correlation between the number of K-IV repeats and Lp(a) concentrations. Individuals expressing a low number of K-IV repeats resulting in the so-called small apo(a) isoforms (up to 22K-IV repeats) have on average markedly higher Lp(a) concentrations than individuals carrying only large apo(a) isoforms (more than 22K-IV repeats) (Kronenberg and Utermann 2013). This K-IV size polymorphism of apo(a) explains about 20–80% of the

variability of Lp(a) concentrations depending on the ethnicity (Utermann et al. 1987).

Interestingly, Lp(a) levels of unrelated individuals carrying the same isoform size combination can still vary by up to 200-fold (Cohen et al. 1993; Perombelon et al. 1994). On the other hand, same-sized alleles within families which are identical-by-descent show typically a less than 2.5-fold variation in Lp(a) concentrations (Perombelon et al. 1994). This is a clear indication that further genetic variants exist that regulate the Lp(a) concentrations in addition to the isoform size (Cohen et al. 1993; Coassin et al. 2017). The search for single nucleotide polymorphisms in the *LPA* gene region had a first peak in 2009 with the description of the two SNPs rs10455872 and rs3798220 which since then are regularly used in hundreds of association studies (Clarke et al. 2009). However, there are many more SNPs in the wider *LPA* gene region which are associated with Lp(a) concentrations and genome-wide association studies (GWAS) have explored this in a systematic way (Li et al. 2015; Mack et al. 2017; Ober et al. 2009). In a recent GWAS meta-analysis in 13,781 individuals from 5 populations we identified 2001 SNPs in a 1.76 MB large region around the *LPA* gene. 48 of these SNPs were associated independently of each other with Lp(a) concentrations (p -values below 5×10^{-8}) (Mack et al. 2017). However, that does not mean that each of these SNPs is causally (functionally) related to Lp(a) concentrations. Many of these SNPs are probably in linkage disequilibrium with another SNP that is causally influencing Lp(a) concentrations. A typical example is rs75692336 which showed the strongest association with Lp(a) concentrations in a statistical model in our GWAS which adjusted besides age and sex also for the apo(a) isoform size. This SNP turned out to be a proxy SNP for the real culprit, a splice-site variant (G4925A) in the kringle-IV type 2 which is not easily accessible for genotyping (Coassin et al. 2017) and therefore not found in GWAS analyses (Mack et al. 2017).

Besides the *LPA* gene region, the *APOE* gene was found to be independently associated with Lp(a) concentrations. Especially the SNP which is responsible for the APO E2 allele (rs7412) decreased Lp(a) concentrations by 3.34 mg/dL or $\approx 15\%$ of the mean values of the population (Mack et al. 2017). The recent GWAS in 293,274 White British individuals revealed two further loci, the *CETP* locus as well as the *APOH* locus, the latter encodes for beta2-glycoprotein I (Hoekstra et al. 2021).

The major part of the *LPA* gene is a so-called camouflaged gene since up to 70% of the coding sequence harboring the K-IV type 2 with up to more than 40 repeats is not easily accessible to modern sequencing technologies. Each copy of the K-IV type 2 has a size of 5.6 kb. Until recently almost no sequence information was available for that region. Our group recently developed an ultra-deep sequencing strategy for this region and a variant analysis pipeline and reported the first map of genetic variation in the KIV-2 region. By sequencing 123 Central-European individuals and reanalyzing public data of 2,504 individuals from 26 populations, we found 14 different loss-of-function and splice-site mutations, as well as >100 partially even common missense variants (Coassin et al. 2019). One of these variants is the above-mentioned splice-site variant (G4925A) which has a frequency of about 22% and tremendously decreases Lp(a) concentrations by more than 30 mg/dL

(Coassin et al. 2019). Another variant is the R21X in the KIV-2 region which was not easy to analyze until recently. It is a likely causal SNP resulting in a nonsense mutation, which leads to a truncated protein that is rapidly degraded (Parson et al. 2004). We recently developed a highly sensitive allele-specific qPCR assay and genotyped R21X in 10,910 individuals from three populations and showed that R21X carriers have significantly lower (-11.7 mg/dL) mean Lp(a) concentrations (Di Maio et al. 2020). A study by Morgan and colleagues investigated two very rare variants, R990Q (rs41259144) located in the KIV type 4 and R1771C (rs139145675) located in the kringle V which were present in four null Lp(a) individuals. These two variants are hypothesized to impair the ability of the protein folding and thereby circumventing its processing to maturity for secretion (Morgan et al. 2020). Other detected mutations are currently under investigation to explain the functional consequences of these variants. It might very well be that some of these variants explain the large ethnic and interindividual differences in Lp(a) concentrations.

5 Lp(a) Concentrations and Risk for CVD

5.1 Searching for Lp(a) Thresholds Associated with an Increased Coronary Artery Disease Risk

The evidence is quite strong that high Lp(a) concentrations are associated with an increasing risk for cardiovascular disease (Kronenberg and Utermann 2013; Cegla et al. 2019). The Copenhagen City Heart Study observed for individuals from a general population with concentrations between 30 and 76 mg/dL (corresponding to the 67th–90th percentile) a 1.60-fold increased risk for incident myocardial infarction compared to individuals with Lp(a) concentrations below 5 mg/dL (corresponding to the lower 22% of the population). This risk increased to 1.90 for persons with Lp(a) concentrations between 77 and 117 mg/dL (90th to 95th percentile) and to 2.60 for individuals with Lp(a) concentrations above 117 mg/dL (>95th percentile) (Kamstrup et al. 2009) (Fig. 3, panel (a)). The concentration threshold for an increased risk has been discussed controversially and an European Atherosclerosis Society (EAS) consensus statement proposed 50 mg/dL (Nordestgaard et al. 2010). Most importantly, such a threshold corresponds to the 80th percentile of the concentration distribution in a Caucasian population and means that 20% of the population have probably an increased risk for CVD due to elevated Lp(a) concentrations. From a standpoint of public health relevance, this makes Lp(a) a very important risk factor for CVD.

The most recent data from the UK Biobank with more than 460,000 study participants followed for more than 11 years are in line with a rather linear increase in risk for CVD with increasing Lp(a) concentrations. In that sufficiently powered study the risk started already to increase above the median of Lp(a) which was 19.6 nmol/L (corresponds to roughly 8 mg/dL) (Patel et al. 2021). Of course, this raises the question what is a clinically meaningful increase in risk? The same study revealed also that Lp(a) is not only a risk factor in non-White populations but also in

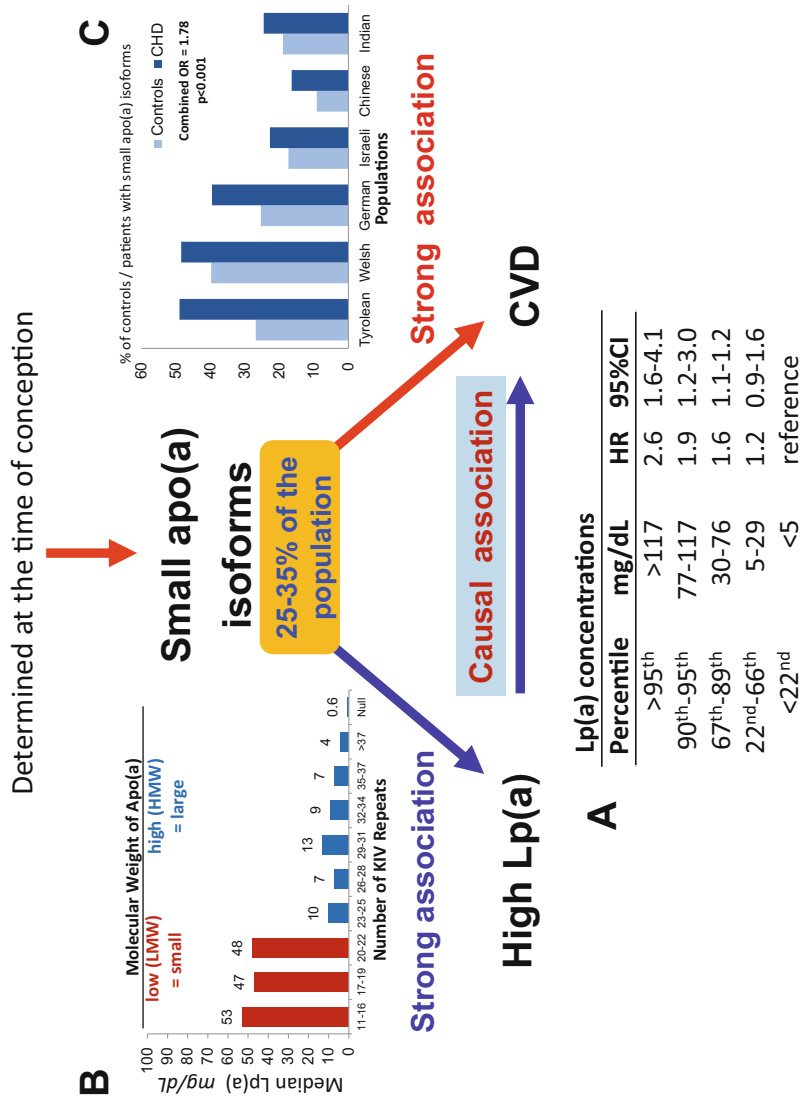


Fig. 3 Mendelian randomization approach to demonstrate a causal association between Lp(a) concentrations and cardiovascular disease. Panel (a) shows the association between elevated Lp(a) concentrations and cardiovascular disease (CVD) as shown in the Copenhagen City Heart Study (Kamstrup et al. 2009).

Black or Asian people (Patel et al. 2021), a matter of major discussions over the past. This is in line with earlier results from the ARIC study (Virani et al. 2012), the MESA Study (Guan et al. 2015), or the Dallas Heart Study (Lee et al. 2017), but in contrast to NHANES III or the INTERHEART Study which did not found Lp(a) to be associated with CVD in non-Hispanic Blacks (Brandt et al. 2020) or Africans (Pare et al. 2019), respectively.

The recent statement by the new ESC/EAS guidelines for the management of dyslipidemia might have introduced more confusion than clarification by stating that “Lp(a) measurement should be considered at least once in each adult person’s lifetime to identify those with very high inherited Lp(a) levels >180 mg/dL (>430 nmol/L) who may have a lifetime risk of ASCVD equivalent to the risk associated with heterozygous familial hypercholesterolaemia.” (Mach et al. 2019) The first part of this sentence to measure Lp(a) at least once in each individual is indeed a forward-looking advice important for risk stratification and in the near future maybe also for therapeutic interventions. However, some interpret the second part of this statement as the introduction of a new threshold for risk stratification. However, this is not the case since they mention that these values are associated with “very high inherited risk.” The risk is already markedly increased at lower levels as discussed above. These very high levels are only observed in far less than 1% of White people and should not be used as an excuse either not to screen or to decline any therapeutic options to lower Lp(a) concentrations with currently available therapies such as lipid apheresis or with therapies that will become available in the future. This part of the guidelines and the cited literature did not show the data and the calculations which resulted in this threshold in a convincing and reproducible way (Mach et al. 2019). It has therefore to be considered with caution and needs further clarification.

5.2 Lp(a) and Other Vascular Diseases

Besides numerous studies on coronary artery disease, during recent years several studies found even a strong association between high Lp(a) concentrations and stroke (Erqou et al. 2009; Langsted et al. 2019a; Zhang et al. 2019), aortic valve calcification stenosis (Arsenault et al. 2014; Cairns et al. 2017; Capoulade et al. 2018; Chen et al. 2018; Kamstrup et al. 2014; Thanassoulis et al. 2013; Vongpromek et al. 2015; Vuorio et al. 2019; Perrot et al. 2019), heart failure (Kamstrup and Nordestgaard 2016) as well as peripheral arterial disease (Dieplinger et al. 2007;

Fig. 3 (continued) Panel (b) shows the association between the number of K-IV repeats in the *LPA* gene and Lp(a) concentrations: individuals with small apo(a) isoform have markedly higher median Lp(a) concentrations than individuals with large apo(a) isoforms (Laschkolnig et al. 2014). Panel (c) shows the preponderance of small apo(a) isoforms in patients with CVD when compared to controls (Sandholzer et al. 1992). Since a low number of K-IV copies (11–22 copies) is associated with high Lp(a) levels and high Lp(a) levels are associated with CVD, it follows that a low number of K-IV copies has to be associated with CVD if the association of Lp(a) with CVD is causal. Figure is taken and adapted with permission from reference (Kronenberg 2016b)

Gurdasani et al. 2012; Laschkolnig et al. 2014). A recent GWAS for peripheral arterial disease identified for the *LPA* gene region the strongest signal which was even more pronounced than for the *CDKN2B* region on chromosome 9p21 or for the LDL receptor (Klarin et al. 2019). This makes Lp(a) a risk factor for many CVD endpoints although the strength of the association might differ between the various entities of endpoints. Studies that investigated the association of the *LPA* gene region with all-cause mortality (Langsted et al. 2019b) demonstrated also a significant association in case they were sufficiently powered. A sufficient study size and number of endpoints is in this context a prerequisite since the finding of an association with all-cause mortality is mainly driven by death in the wider context of CVD.

5.3 Differences Between Primary and Secondary Prevention Studies

There is strong evidence for an association between high Lp(a) concentrations and future CVD events in studies in whom participants were free of CVD at baseline (primary prevention studies). However, findings are inconsistent for study populations with preexisting CVD at baseline (secondary prevention studies). A meta-analysis of 11 secondary prevention studies found that elevated Lp(a) predicted major adverse cardiovascular events with an odds ratio of 1.40 (95% CI 1.15–1.71, $p < 0.001$) with a considerable heterogeneity between studies. When the authors stratified the studies into those with high (≥ 130 mg/dL) versus low (< 130 mg/dL) average on-treatment LDL-C, the association for Lp(a) was still significant for the studies with high average LDL-C (OR = 1.46, 95% CI 1.23–1.73) but not for those with low average LDL-C (OR = 1.20, 95% CI 0.90–1.60 (O'Donoghue et al. 2014). This has raised many discussions since especially first intervention studies to specifically lower Lp(a) would target patients with established CVD. A seminal review on secondary prevention studies elucidating the reasons why some of these studies did not find an association has recently been published by Boffa and colleagues (2018). The authors distinguished between major potential general “confounders” and confounders related to Lp(a) which might have influenced the findings of some of the secondary prevention studies. As general confounders they discussed the restrictive inclusion and exclusion criteria of randomized controlled trials, the lack of statistical power, differences in clinical management between primary and secondary prevention trials and finally an index event bias. Index event bias may underlie paradoxical findings whereby risk factors that are well established to contribute to pathological events do not appear to predict recurrence of these events (Dahabreh and Kent 2011). A famous example is hypertension which increases the risk for a first stroke about fourfold but is not associated with stroke recurrence (Smits et al. 2013). As confounders related to Lp(a) the authors mentioned the use of log-transformed Lp(a) levels, secondary changes in Lp(a) concentrations due to the events (consider that Lp(a) is thought to be an acute-phase protein), and issues related to the measurement of Lp(a) (e.g., standardization of the assays, isoform-dependency of the assays, or effects of sample handling and storage on Lp(a) measurement) (Boffa et al. 2018).

5.4 Is Lp(a) an Independent Risk Factor for CVD?

There is ample evidence that Lp(a) is an independent risk factor for CVD (Kronenberg and Utermann 2013). That means that high Lp(a) can increase the risk for CVD even if other classical risk factors are not present. And if combined with other risk factors the entire risk of a particular person increases further. Whether this risk increase is linear or exponential is much easier to predict on the population level than for a given person. Risk prediction is associated with uncertainties as long as we do not know or measure all factors that contribute to the risk of a disease. This has recently been demonstrated quite convincingly in the Malmö Diet and Cancer Study with almost 30,000 study participants and more than 4,122 incident coronary artery disease cases during a long median observation period of 21.3 years. The authors calculated polygenic risk scores for each individual using 6.24 million SNPs and observed that the lifetime risk for coronary artery disease increased from about 16% in those 10% of the participants with the lowest polygenic risk scores to more than 45% in the 10% of the population with the highest score. Most interestingly, this was independent from traditional risk factors meaning that in each traditional risk factor category (low, medium, borderline, and high) the risk increased two- to threefold for those with a high genetic risk score category compared to a low genetic risk score category (Hindy et al. 2020). This clearly demonstrates that a single risk factor including Lp(a) should never be seen “isolated” without considering the other risk factors as good as possible. This can be seen best when looking at the distribution of CVD risk contributors such as systolic blood pressure or LDL cholesterol in individuals who remain free and those who develop CVD over the upcoming years: there is always a substantial overlap in the distribution of these factors as already shown in the ancient studies from the Framingham cohort (Kannel et al. 1964) and lately by cohorts from Finland and Sweden (Ripatti et al. 2010). This explains also why not everybody with high Lp(a) will develop CVD.

With Lp(a) and LDL-C a very special situation is present since almost each method of LDL-C measurement (or calculation by formulas) includes also the cholesterol content present in the LDL particle of Lp(a). It is estimated that the proportion of cholesterol in the Lp(a) particle is about 30% of the Lp(a) mass (Kinpara et al. 2011), although some discuss an even higher value of 45% (Kronenberg et al. 2004) which also depends on the apo(a) isoform size. In some individuals with high Lp(a) concentrations the amount of cholesterol derived from Lp(a) can be quite substantial as in the roughly 5% of the general population with an Lp(a) concentration above 100 mg/dL. In these persons the cholesterol content included in the LDL-C measurement but originating from Lp(a) would be 30 to 45 mg/dL. In persons with 200 mg/dL this would contribute 60 to 90 mg/dL to the LDL-C measurement. This additional amount of LDL-C can even result in a diagnosis (or misclassification) of hypercholesterolemia which is actually caused by high Lp(a) concentrations (Langsted et al. 2016). A very recent study, however, reported that the percentage of Lp(a) cholesterol relative to Lp(a) mass varied from 5.8% to 57.3% (Yeang et al. 2021). Especially this low percentage of cholesterol might require further investigation and validation.

A further consequence from this measurement issue might become of clinical relevance, especially when a patient is given a statin and shows no response or a low response to this LDL-C-targeting treatment. A reason for this might be a high Lp(a) concentration: statins do not lower Lp(a) concentrations. If Lp(a) is very high, LDL-C might be misclassified as high which could result in a “wrong” indication for a statin under particular circumstances. When the targeted “true” LDL-C (the LDL-C without the Lp(a) cholesterol) is already in the target range, a treatment with a statin might result in a lower response than expected from the uncorrected LDL-C value (Scanu and Hinman 2002; Miltiadous et al. 2006).

6 What Evidence Do We Have for a Causal Association of High Lp(a) with CVD?

When a biomarker is changed in diseased patients, the important discussion starts whether this biomarker is a risk factor or a risk marker as illustrated recently (Kronenberg 2019a). In case of a risk factor, this parameter is causally related to disease and it might become an interesting drug target. If it is a biomarker, this parameter might be interesting for diagnostic purposes because it is changed secondarily to the disease and points the physician to the disease. It would not make sense to develop drugs which influence that parameter (see recent discussion on this issue in reference (Kronenberg 2016a). Mendelian randomization studies illustrated in Fig. 3 provide a strong support for causality. Genetic variants that are strongly associated with high Lp(a) concentrations (Fig. 3, panel (b)) show also an increased risk with CVD (Fig. 3, panel (c)) which underscores the causal link between high Lp(a) concentrations and CVD. Actually Lp(a) was the first practical example applying this approach almost a decade before the term “Mendelian randomization” has been coined: at the beginning of the 1990s data on the small apo(a) isoforms were used as genetic instrument: small isoforms with up to 22 K-IV repeats were associated with a significantly increased risk for coronary heart disease in six different populations (Sandholzer et al. 1992). A later meta-analysis including 7,382 coronary heart disease cases and 8,514 controls identified a 2.08-fold increased risk for carriers of small apo(a) isoforms (Erqou et al. 2010). Later approaches that investigated *LPA* variants on the DNA level either by the number of KIV repeats identified by pulsed-field gel electrophoresis or by the sum of KIV repeats of the two alleles by quantitative PCR or by the investigation of SNPs that are associated with high Lp(a) concentrations revealed the same finding (Clarke et al. 2009; Kamstrup et al. 2009; Kraft et al. 1996). On the other hand, genetic variants that are associated with low Lp(a) concentrations are obviously protective from CVD (Coassin et al. 2017; Di Maio et al. 2020; Lim et al. 2014). These strong associations make Lp(a) probably the most important genetic risk factor for CVD if we keep in mind the high frequency of small apo(a) isoforms or variants which go along with high Lp(a) concentrations in the population (Kronenberg 2016b).

Therapy	Effect on Lipoproteins	Effect on Clinical Events
RNA-targeting therapies: Antisense oligonucleotides (ASO) and short interfering RNA (siRNA) against apo(a)	Specifically reduce Lp(a) up to 90%	Trials in progress
Lipoprotein apheresis	Reduces LDL and Lp(a) by 60-70% (but also other parameters)	Decreases CVD events up to more than 80% (even in those where LDL-C was already very low → Lp(a) effect)
PCSK9 inhibitors	Reduce LDL by 60% and Lp(a) by 25-30%	Reduce CVD outcomes which might partially be attributable to the Lp(a)-lowering
Statins	Reduce LDL-C by 30-60% and there are observations of an Lp(a) increase	Clear decrease in CVD events with long-term outcome data. Lp(a) effect needs further investigations

Fig. 4 Effect of therapeutic interventions on lipoproteins and clinical outcomes

7 RNA-Targeting Therapies to Specifically Lower Lp(a)

Based on the epidemiological and genetic studies the next and most consequent logical step is the therapeutic lowering of Lp(a) and the investigation whether such a therapy also decreases the number of CVD events (Fig. 4). To specifically accomplish an Lp(a)-lowering without influencing other lipoproteins, two main approaches are currently available and both selectively reduce the synthesis of apo(a) in the liver (Landmesser et al. 2020) and are therefore the optimal approach not only to lower Lp(a) but also to serve the last piece of the puzzle to demonstrate that a specific lowering of Lp(a) will also reduce CVD events.

7.1 Antisense Oligonucleotides (ASO) Against Apolipoprotein(a)

In principle, antisense oligonucleotides are 13–20 nucleic acid long and bind to the target RNA. As soon as the single-stranded ASOs are taken up by the cell, they bind directly to the mRNA creating a duplex that forms a complex with the intracellularly available RNase H1. This finally mediates the target mRNA cleavage preventing the production of the targeted protein (Landmesser et al. 2020).

Using this principle to target apo(a), already the first phase I trial with IONIS-APO(a)_{Rx} given subcutaneously in various amounts and numbers of dosages

revealed a potent Lp(a)-lowering effect up to almost 80% (Tsimikas et al. 2015). In further trials it has been shown that this therapy results also in a reduction of oxidized phospholipids and a reduced monocyte inflammatory activation that returned close to baseline levels after stopping the medication. Furthermore, a new chemistry was used in this next trial in which a modified IONIS-APO(a)_{Rx} antisense oligonucleotide is conjugated with a GalNAc3 complex (IONIS-APO(a)-L_{Rx}). This formulation targets the drug to the hepatocyte via the asialoglycoprotein receptor, making it 30 times more potent than the parent antisense oligonucleotide. This enabled the administered dose to be reduced 10-fold, thereby improving its tolerability. The highest dose administered resulted in a 92% mean reduction of Lp(a) with no serious side effects (Viney et al. 2016). The most recent trial included 286 patients with established CVD and Lp(a) concentrations of at least 60 mg/dL (Tsimikas et al. 2020a). Administration of APO(a)-L_{Rx} resulted in dose-dependent decreases in Lp(a) concentrations with up to 72% at 60 mg every 4 weeks, and 80% at 20 mg every week, as compared with 6% with placebo. There were no significant differences between any APO(a)-L_{Rx} dose and placebo with respect to platelet counts, liver and renal measures, or influenza-like symptoms. The most common adverse events were injection-site reactions (Tsimikas et al. 2020a).

Based on those data the Lp(a)HORIZON trial (NCT04023552: <https://clinicaltrials.gov/ct2/show/study/NCT04023552>) started recently and will recruit 7,680 patients with the key inclusion criteria of Lp(a) ≥ 70 mg/dL at the screening visit, an optimal LDL cholesterol lowering treatment, an optimal treatment of other CV risk factors, and a myocardial infarction or an ischemic stroke ≥ 3 months to ≤ 10 years prior to the screening visit or a clinically significant symptomatic peripheral artery disease. Patients will be injected monthly 80 mg of the drug (now called TQJ230 or Pelacarsen) or placebo subcutaneously. The estimated completion date is April 2024.

7.2 Short Interfering RNA (siRNA) to Target Apo(a)

The siRNAs are chemically synthesized and a GalNAc-siRNA conjugate selectively enters the hepatocyte via receptor-mediated endocytosis using the asialoglycoprotein receptor (similar as for ASO). After this, the double-stranded siRNA is released from the endosome and the two RNA stands dissociate into the sense and antisense strand. The antisense strand forms a highly stable complex with the RNA-induced silencing complex (RISC) which induces the cleavage of the target mRNA, degradation by exonucleases, and reduced synthesis of the protein of interest. The complex of siRNA with RISC is highly stable which results in a long-term cleavage of the targeted transcripts with a suppression of the protein production lasting more than 6 months (Landmesser et al. 2020).

Currently, two drugs under Phase I and II trials are using the siRNA principle: the first one is AMG890 (also called oipasiran) with a phase 1 (NCT03626662) and a phase 2 trial (NCT04270760). Obviously the phase 1 trial showed an Lp(a) reduction of more than 90% that persisted for 3–6 months (Abstract by Koren MJ et al.;

Circulation 2020;142:A13951). The second one is SLN360: a phase 1 trial with 88 patients is currently under way and is expected to be completed in November 2022.

8 Other Lipid-Lowering Drugs and Therapies with Possible Influence on Lp(a) Concentrations and Clinical Outcomes

Before reviewing the various other approaches of lowering Lp(a) (Fig. 4), there are two issues which should be stressed when considering the results from the trials which investigated the Lp(a) lowering effects of various interventions:

- Most previous therapeutic options were not developed and were not aiming for a specific Lp(a)-lowering but reached out for other lipoproteins and the Lp(a)-lowering is a concomitant observation. Therefore, it is hard to disentangle whether an effect observed in an intervention group is an effect of Lp(a)-lowering or an effect of the influence on other lipoproteins or risk factors which were the primary target of that intervention.
- In many cases, studies were not designed to reach out for patients with high or solely high Lp(a) concentrations but targeted other lipoproteins such as high LDL-C or low HDL-C. Therefore in many cases the median Lp(a) levels were often as low as the median in general populations and these studies do not necessarily reflect the study population one would reach out to study the therapeutic effect in a high Lp(a) group.

8.1 Lipoprotein Apheresis

The data available on lipoprotein apheresis are coming mostly from Germany and are very impressive in terms of a 60–70% lowering of LDL-C and Lp(a) concentrations as well as the massive reduction of CHD outcomes. Of course, it is a sawtooth picture of the Lp(a) changes that can be observed with a decrease of Lp(a) from before to immediately after apheresis of up to more than 70%. After the apheresis session the Lp(a) concentrations increase again and the interval mean values were calculated to be roughly 35% lower compared to the values before the session (Julius et al. 2019).

In one of the first studies patients were included whose Lp(a) concentrations were above the 90th percentile. Lipoprotein apheresis not only reduced Lp(a) by 73%, but also coronary events by 86% (Jaeger et al. 2009). Interesting was the observation that in the subgroup of patients who had a very low Lp(a)-corrected LDL-C already before the onset of apheresis, the reduction in coronary heart disease events was about the same as in the other group in which LDL-C could additionally be lowered by apheresis. This means that in the first group, the reduction of coronary heart disease events is more likely due to the decrease in Lp(a) concentrations (Jaeger et al. 2009). Similar results for reducing Lp(a) concentrations and coronary heart disease

events were also shown in other investigations (Leebmann et al. 2013; Roeseler et al. 2016). A significant decline of the mean annual cardiovascular event rate was observed from 0.58 ± 0.53 in the period 2 years before regular lipoprotein apheresis to 0.11 ± 0.15 thereafter (Roeseler et al. 2016). Similar observations have been made in an US-American study (Moriarty et al. 2019).

Lipoprotein apheresis studies have been criticized since they were hard to control due to the lack of randomization and blinding. A recent small study has tried to overcome this problem in 20 patients with refractory angina pectoris and mean Lp(a) concentrations at 110 mg/dL. These patients underwent 3 months of blinded weekly lipoprotein apheresis or sham, followed by crossover. Despite the relatively short-term therapy, this resulted in a significant decrease in angina pectoris frequency, an increase in myocardial perfusion reserve, an increase in exercise capacity, and a decrease in total carotid wall volume (Khan et al. 2017).

A specific approach to study the effect of Lp(a)-lowering came from a small study applying a specific Lp(a) apheresis with sheep polyclonal monospecific antibodies against human apo(a). This intervention decreased Lp(a) on average by 73% without significant changes in true LDL-C and other risk factors. The mean percent diameter stenosis of the coronary arteries after 18 months decreased by 5% in the Lp(a) intervention group and increased by 5% in the control group that received only statins (Safarova et al. 2013).

8.2 PCSK9 Inhibitors

PCSK9 inhibitors primarily target LDL-C and lower this atherogenic lipoprotein by roughly 60%. However, Lp(a) concentrations are also lowered by 25–30% (Table 1). There are two therapeutic principles: (1) a monoclonal antibody against PCSK9 given every 2 weeks or monthly as used in evolocumab and alirocumab or (2) a small interfering RNA (siRNA) that targets the mRNA of PCSK9 as used in inclisiran which will be given twice yearly.

In a post-hoc analysis of the FOURIER Trial including 25,096 patients with established atherosclerotic CVD, the PCSK9 inhibitor evolocumab reduced the risk of CVD outcomes by 23% in patients with a baseline Lp(a) > median of 37 nmol/L (≈ 15 mg/dL), and by 7% in those \leq median (p -value interaction = 0.07). The conclusion was the same when 120 nmol/L (≈ 50 mg/dL) instead of the median was used as grouping threshold. The study observed a significant relationship with a 15% lower risk (95% CI, 2–26%; $p = 0.0199$) per 25 nmol/L (≈ 10 mg/dL) reduction in Lp(a) after adjusting for the change in LDL-C (Table 1) (O'Donoghue et al. 2019). This would mean that even a relatively small lowering of Lp(a) should result in a clinical benefit.

Recently published data from pre-specified analysis of the ODYSSEY Outcomes trial showed that Lp(a)-lowering by alirocumab contributes to a reduction of major adverse cardiovascular events (MACE) independently of LDL-C-lowering in 18,924 patients with recent acute coronary syndrome and LDL-C ≥ 70 mg/dL despite intensive or maximum tolerated statin treatment (Bittner et al. 2020). Of course,

Table 1 Calculations from post-hoc analyses from PCSK9 trials as well as Mendelian randomizations studies to estimate the effect of Lp(a)-lowering on clinical outcomes

Publication	Study description	Main findings
<i>Post-hoc analyses of PCSK9 inhibitor trials</i>		
FOURIER trial (O'Donoghue et al. 2019)	Intervention study of 25,096 patients treated by evolocumab vs. placebo in patients with established CVD	Lp(a) > median of 37 nmol/L: HR = 0.77 (0.76–0.88) vs. ≤median: HR = 0.93 (0.80–1.08) Lp(a) >120 nmol/L: HR = 0.75 (0.64–0.88) vs. ≤120 nmol/L: HR = 0.89 (0.79–1.01)
ODYSSEY OUTCOMES trial (Bittner et al. 2020)	18,924 patients with recent acute coronary syndrome and LDL-C ≥ 70 mg/dL despite intensive or maximum tolerated statin treatment	Proportion of MACE reduction attributable to changes in Lp(a) increased from 4% to 11% to 25% for Lp(a) levels at the 25th, 50th, and 75th percentiles, respectively. Lowering of Lp(a) by 42 mg/dL would be required to lower the MACE rate by 22%
Pooled data from 10 controlled phase 3 ODYSSEY trials (Ray et al. 2019)	4,983 patients with established CVD or presence of CVD risk factors without established CVD or heterozygous familial hypercholesterolemia	Relative risk reduction per 25% reduction of Lp(a) adjusted for LDL-C changes: for group with Lp(a) ≥50 mg/dL: HR = 0.60 (95% CI 0.39–0.92), $p = 0.0201$ for group with Lp(a) <50 mg/dL: HR = 0.94 (95% CI 0.81–1.09), $p = 0.3837$
<i>Mendelian randomization studies</i>		
Burgess et al. (2018)	5 population-based prospective cohort and case-control studies including 20,793 individuals with CHD and 27,540 controls. Lp(a) concentrations were measured in 34,276 individuals using various assays	A 101.5-mg/dL change (95% CI, 71.0–137.0) in Lp(a) concentration had the same association with CHD risk as a 38.67-mg/dL change in LDL-C level
Lamina and Kronenberg (2019)	13,781 individuals from the Lp(a)-GWAS-consortium from 5 primarily population-based studies in whom Lp(a) was measured with the same assay	A 65.7 mg/dL change (95% CI 46.3–88.3) in Lp(a) concentration had the same association with CHD risk as a 38.67-mg/dL change in LDL-C level
Madsen et al. (2020)	2,527 individuals from the Copenhagen general population study with a history of CVD	Using data from a secondary prevention setting revealed that a ≈ 55 mg/dL change in Lp(a) concentration is required to have the same association with CHD risk as a 38.67-mg/dL change in LDL-C level. Using genetic data revealed that an absolute lowering of Lp(a) of 66 mg/dL would be required

Table is taken and updated from (Kronenberg 2019b)

the major contribution to the risk reductions comes from the reduction of Lp(a)-corrected LDL-C which is the primary target of the PCSK9 inhibitors. However, alirocumab additionally reduced Lp(a) by a median of 23%. The LDL-independent contribution to the proportion of MACE reduction attributable to changes in Lp(a) caused by alirocumab treatment increased from 4% to 11% to 25% for Lp(a) levels at the 25th, 50th, and 75th percentiles, respectively (Table 1) (Bittner et al. 2020).

Furthermore, pooled data from 10 controlled phase 3 ODYSSEY trials have been analyzed. The authors observed a 12% relative risk reduction in MACE per 25% reduction in Lp(a) which was no longer significant after adjustment for LDL-C changes. In subgroup analysis, the association between Lp(a) reduction and MACE remained significant in a fully adjusted model among participants with baseline Lp(a) ≥ 50 mg/dL (Table 1) (Ray et al. 2019).

The siRNA therapy inclisiran has been studied in 501 patients using different dosages and revealed median Lp(a) reductions from baseline to day 180 from -14% to -18% in the single-dose groups and -15% to -26% in the 2-dose groups. The Lp(a) reduction did not reach statistical significance in any of the dosing groups which was probably caused by the very wide interindividual variability in the Lp(a) reduction (Ray et al. 2018). It needs to be seen how pronounced the Lp(a) reductions will be in the phase III trials.

8.3 Statins

There is currently a major discussion whether statins increase Lp(a) concentrations or not. Due to the structure of Lp(a) containing an LDL particle, the LDL receptor was always an interesting candidate for Lp(a) removal. This idea was supported by the observations that Lp(a) levels are markedly elevated in patients with familial hypercholesterolemia (Utermann 1989; Utermann et al. 1989). It was therefore very surprising when first studies by Kostner et al. (1989) and later by O'Donoghue et al. (2014) observed an increase in Lp(a) concentrations after successful lowering of LDL cholesterol by statins. This is in line with the results of the JUPITER Study: patients under rosuvastatin treatment showed even a statistically significant positive shift in the overall Lp(a) distribution that was not observed in the placebo group (Khera et al. 2014; Kronenberg 2014b). Recently, Willeit and colleagues collated patient-level data from seven randomized, placebo-controlled, statin outcome trials including 29,069 patients with repeated Lp(a) measurements. 14,536 patients were randomly allocated statin treatment. The effect of statin therapy on Lp(a) concentrations was heterogeneous across studies: the pooled percentage change was -0.4% with three trials showing a mean increase (between 2 and 15%) and four trials showing a mean decrease (between -1 and -13%) in Lp(a) concentrations (Willeit et al. 2018). A further subject-level meta-analysis included 5,256 patients (1,371 on placebo and 3,885 on statin) from six randomized trials. All six trials used the same Lp(a) assay. The mean percent change from baseline ranged from 8.5% to 19.6% in the statin groups and -0.4% to -2.3% in the placebo groups. When various statins were compared, the mean percent change from baseline ranged

from 11.6% to 20.4% in the pravastatin group and 18.7% to 24.2% in the atorvastatin group (Tsimikas et al. 2020b). A study in patients with dyslipidemia included 39 patients who first initiated statin treatment and a control group of 42 patients who were already on stable statin treatment for at least 4 months. Overall, Lp(a) concentrations did not increase significantly in both groups. However, when the analysis was stratified for the apo(a) isoforms, it was found that Lp(a) levels increased significantly from 66.4 to 97.4 mg/dL in patients with small apo (a) isoforms in the initiation group, but not in the control group and not in patients having only large apo(a) isoforms (Yahya et al. 2019). This interesting observation needs confirmation in larger studies.

The findings that statins might increase Lp(a) concentrations triggered discussions whether the benefit of statins by lowering LDL-C might be diminished or outweighed by the increase in Lp(a) concentrations especially in patients who have already increased Lp(a) concentrations before statin therapy is started. The JUPITER trial observed for the extended endpoint that patients with Lp(a) concentrations above the median reduced their risk for events by 28% (95% CI 3–48%) whereas those with baseline Lp(a) concentration below the median reduced the risk by 54% (95% CI 31–68%) (*p*-value for interaction = 0.10) (Khera et al. 2014). The meta-analysis by Willeit et al. found for those patients on statins a 47% higher risk when the Lp(a) levels were above compared to below 50 mg/dL (HR = 1.48, 95% CI 1.23–1.78). The risk was only 23% higher in the controls when Lp(a) was above compared to below 50 mg/dL (HR = 1.23, 95% CI 1.04–1.45%). This has been interpreted that the Lp(a)-associated risk becomes an even stronger predictor of residual risk when LDL-attributable risk is reduced with statin treatment (Willeit et al. 2018). Since this analysis did not use one of the groups as reference group for all comparisons (e.g., the controls with Lp(a) levels below 50 mg/dL), the risk estimates cannot simply be compared and might therefore be misinterpreted resulting in the idea that statins should be stopped in case of high Lp(a) concentrations. However, the probable risk benefit from lowering LDL-C by a statin outweighs the moderate increase in Lp(a) levels in most patients. This can be extrapolated from the study by Willeit et al. (see Fig. 2 of that publication): the cumulative risk was 21.7% in the placebo group (3,148 cases within 14,533 patients) which is higher compared to 17.9% in the statin-treated group (2,603 cases within 14,536 patients). This corresponds to a risk reduction to 0.83 by statins. Ethical considerations would not allow to test in a randomized controlled trial whether the avoidance of statins in patients with high Lp(a) concentrations would result in a benefit. In any case, the recent observations need careful attention and Lp(a) concentrations might better be monitored especially in patients who start statin therapy and having elevated Lp(a) concentrations since the observed increase under statin therapy in single patients can be sometimes very significant (Tsimikas et al. 2020c). In such patients a change to PCSK9 inhibitors might be an option to consider.

8.4 Drugs That Are Probably No Longer Followed for Lp(a)-Lowering Potential

There are some drugs that were not specifically designed to lower Lp(a) but showed an interesting Lp(a)-lowering effect. However, various reasons often related to the primary target and the observed side effects or efficacy revealed that they were stopped after clinical trials or they are no longer followed in extended clinical trials.

The CETP inhibitors anacetrapib (HPS3/TIMI55–REVEAL Collaborative Group et al. 2017; Thomas et al. 2017), evacetrapib (Nicholls et al. 2016), and TA-8995 (Ford et al. 2014) have in common that they not only increase HDL-C tremendously, but they also decrease Lp(a) between 25 and 40%. However, outcome studies using these drugs did not show a benefit or were even harmful in terms of CVD events. Both the thyroid analogue eprotirome (Ladenson et al. 2010) and the MTP inhibitor lomitapide (Samaha et al. 2008) lower Lp(a). Mipomersen, an antisense oligonucleotide targeting apoB mRNA lowers Lp(a) by roughly 30% but is restricted to very specific patient groups and countries due to its side effects (Raal et al. 2010).

In earlier times, niacin was thought to be a good candidate in treating Lp(a) elevation. However, in patients with optimally low levels of LDL-C, and despite favorable effects on HDL-C, triglycerides, and Lp(a), two clinical outcome trials failed to show any incremental clinical benefit on cardiovascular events with niacin when added to simvastatin (Boden et al. 2011; Landray et al. 2014). Following these trials, niacin is no longer available in most countries. However, besides other limitations these trials were not designed to examine the clinical effect in the patients with elevated Lp(a).

9 Therapeutic Lowering of Lipoprotein(a): How Much Is Enough?

On the eve of the introduction of specific Lp(a)-lowering therapies, the question has been raised, how much Lp(a) should be lowered to result in a clinical benefit. As recently discussed (Kronenberg 2019b), there are two approaches to get a rough estimate, which are a genetic estimation by using a Mendelian randomization approach or post-hoc analyses from clinical trials which target primarily LDL-C and as a “collateral yield” also lower Lp(a).

The Mendelian randomization approach uses genetic data from association studies on Lp(a) and LDL-C concentrations as well as association studies on CHD to estimate the required Lp(a)-lowering effect size to show the same association with CHD risk-lowering as a 38.67-mg/dL (1 mmol/L) therapeutic reduction in LDL-C. The latter is only used for benchmarking reasons. This intriguing idea used by Burgess and colleagues revealed that Lp(a) would have to be lowered by 101.5 mg/dL to show the same effect as lowering LDL-C by 38.67 mg/dL (Burgess et al. 2018). This number was probably markedly overestimated since the main study on which these results are based on had a median Lp(a) concentration twofold to threefold higher compared to other studies of the same ethnicity. We therefore

repeated these calculations using the same approach and the same data basis except for the estimates of various SNPs on Lp(a) concentrations. For these we used our own data from almost 14,000 individuals in whom Lp(a) was measured within one laboratory and with median Lp(a) levels in the range of expectations for typical Caucasian populations (Mack et al. 2017). We calculated that an Lp(a)-lowering of roughly 65 mg/dL would be required instead of more than 100 mg/dL (Lamina and Kronenberg 2019). These data were mainly based on population-based data and it was not clear whether these hold also true for secondary prevention patients. Exactly this situation has very recently been tested in a Danish study (Madsen et al. 2020). They used population-based data from individuals with a history of CVD who were followed after their initial event. Since this equals a secondary prevention setting, they calculated that plasma Lp(a) should be lowered by 50 and 99 mg/dL for 5 years to achieve 20 and 40% MACE risk reduction in secondary prevention. Accordingly, for a 22% MACE reduction, a reduction of Lp(a) by 55 mg/dL would be required. Interestingly, when the authors used genetic data for short-term risk reduction, they found that an absolute Lp(a) reduction of 66 mg/dL would be needed to obtain a MACE reduction of 22%. This is exactly the same number as calculated by our group but using primary prevention studies (Lamina and Kronenberg 2019).

For all these studies it has to be added that the comparison with an LDL-lowering of 38.67 mg/dL was only used as a benchmark which results for LDL-C in a 22% lowering of MACE (Baigent et al. 2010). Of course, even smaller lowering might be beneficial as has been demonstrated for LDL-C.

The data from Mendelian randomization studies gave the impression that Lp(a) would have to be lowered by a higher extent than LDL-C to have the same clinical benefit. The ratio would be 2.6 to 1.0 according to Burgess and colleagues (2018) or 1.7 to 1.0 according to two other studies (Lamina and Kronenberg 2019; Madsen et al. 2020). This would mean that LDL-C would have markedly higher atherogenic properties than Lp(a) which is not necessarily supported by data. The post-hoc analyses of interventional studies with PCSK9 inhibitors provide some major hope that even a smaller Lp(a)-lowering might already show a clinical benefit (Table 1). As discussed above, the FOURIER study described a 15% lower risk per 25 nmol/L (≈ 10 mg/dL) reduction in Lp(a) after adjusting for the change in LDL-C (O'Donoghue et al. 2019). From the recently published ODYSSEY Outcomes trial it can be calculated that a lowering of Lp(a) by 42 mg/dL would be required to lower the MACE rate by 22% (Bittner et al. 2020) which brings the above-mentioned ratio of required Lp(a)/LDL-C-lowering closer to 1.0. Furthermore, the pooled data from 10 controlled phase 3 ODYSSEY trials also showed that the relative risk reduction per 25% reduction of Lp(a) adjusted for LDL-C changes was higher for the group of patients with Lp(a) ≥ 50 mg/dL with an HR = 0.60 (95% CI 0.39–0.92, $p = 0.0201$) (Ray et al. 2019).

Limitations for this Mendelian randomization approach come from various assumptions which have to be considered with cautions: first, it is unclear whether Lp(a) and LDL-C particles have a similar cumulative effect on CVD over time, or with other words, whether they have the same atherogenic potential? This is not necessarily the case since there is some evidence that even Lp(a) particles of

different apo(a) isoforms have different atherogenic potential (Kronenberg et al. 1999; Saleheen et al. 2017). Second, besides an atherogenic nature of Lp(a) some data point also to a thrombogenic nature (Boffa and Koschinsky 2016; Romagnuolo et al. 2018). If this is also the case in vivo, the pathogenic mechanism of Lp(a) might even be stronger than for LDL-C and the lowering of Lp(a) would have an additional benefit on the thrombogenic axis.

All the available observations from post-hoc analysis of intervention studies and from Mendelian randomization studies are helpful for the planning of future trials. These trials will have to consider the uncertainties connected with these data such as more or less wide confidence intervals of the estimates. One of these uncertainties are introduced by the insufficiently standardized Lp(a) assays used in the various studies. This became obvious when looking at the data presented by Burgess et al. with the above-mentioned very high Lp(a) concentrations in their main cohort (Burgess et al. 2018). An overestimation of the Lp(a) concentration by an insufficiently standardized assay might result in a misclassification of patients to be at high risk and an enrollment of patients which would otherwise not be appropriate for the study. Therefore, major efforts should be invested in the screening phase for suitable study patients to avoid misclassification of patients by inappropriate Lp(a) assays (Kronenberg and Tsimikas 2019; Scharnagl et al. 2019).

10 Conclusions

The causal association between high Lp(a) concentrations and CVD is strongly supported by genetic studies. Post-hoc analyses following intervention studies with PCSK9-inhibitors that targeted primarily LDL-C but additionally lower also Lp(a) provide some evidence that an additional lowering of Lp(a) besides LDL-C is beneficial. However, these studies were not designed to target patients with high Lp(a) levels and are therefore limited. Mendelian randomization studies provide further strong support that lowering Lp(a) might be beneficial.

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Nonalcoholic Fatty Liver Disease

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Abstract

Nonalcoholic fatty liver disease (NAFLD) is considered the hepatic manifestation of the metabolic syndrome (MetS) and comprises one of the largest health threats of the twenty-first century. In this chapter, we review the current state of knowledge of NAFLD and underline the striking similarities with atherosclerosis. We first describe current epidemiological data showing the staggering increase of NAFLD numbers and its related clinical and economic costs. We then provide an overview of pathophysiological hepatic processes in NAFLD and highlight

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the systemic aspects of NAFLD that point toward metabolic crosstalk between organs as an important cause of metabolic disease. Finally, we end by highlighting the currently investigated therapeutic approaches for NAFLD, which also show strong similarities with a range of treatment options for atherosclerosis.

Keywords

Atherosclerosis · Epidemiology · NAFLD · Pathogenesis · Treatment

Abbreviations

ALT	Alanine transaminase
ASK1	Apoptosis signal-regulating kinase 1
CCR2/CCR5	C-C chemokine receptor type 2/C-C chemokine receptor 5
CKD	Chronic kidney disease
CPT-1	Carnitine palmitoyltransferase 1
DPP4	Dipeptidyl peptidase-4
ESLD	End-stage of liver disease
FXR	Farnesoid X receptor
GCKR	Glucokinase regulator
GLP1R	Glucagon-like peptide-1 receptor
HCC	Hepatocellular carcinoma
HDL	High-density lipoproteins
HmG-CoA	3-Hydroxy-3-methylglutarylcoenzyme A
HR-QOL	Health-related quality of life
KCs	Kupffer cells
LDL	Low-density lipoproteins
LPS	Lipopolysaccharide
LSECs	Liver sinusoidal endothelial cells
LXR	Liver X receptor
MetS	Metabolic syndrome
NAFLD	Nonalcoholic fatty liver disease
NASH	Nonalcoholic steatohepatitis
OCA	Obeticholic acid
oxLDL	Oxidized low-density lipoproteins
PBPLA3	Patatin-like phospholipase domain-containing 3
PCOS	Polycystic ovary syndrome
PPAR α	Peroxisome proliferator-activated receptor alpha
ROS	Reactive oxygen species
SGLT2	Sodium-glucose transport protein 2
SREBP1c	Sterol regulatory element-binding protein 1
T2DM	Type 2 diabetes mellitus

THR	Thyroid hormone receptors
TM6SF2	Transmembrane 6 superfamily 2
UDCA	Ursodeoxycholic acid
VLDL	Very low-density lipoprotein

1 Epidemiology

1.1 Definition, Prevalence, and Incidence of NAFLD

Being the most prevalent chronic liver disease worldwide (Li et al. 2019), nonalcoholic fatty liver disease (NAFLD) covers a diseases spectrum, initiating with hepatic steatosis which is defined by the presence of $\geq 5\%$ hepatic fat (referred to steatosis) in the absence of any secondary cause of hepatic steatosis such as chronic viral hepatitis and alcohol consumption (21 drinks/week in men and 14 drinks/week in women) (Chalasanani et al. 2012, 2018). In a second, more advanced stage, hepatic steatosis may advance into nonalcoholic steatohepatitis (NASH), which is characterized by a combination of hepatic steatosis and inflammation in the presence or absence of fibrosis. Finally, NASH can progress into advanced-stage liver diseases such as cirrhosis and hepatocellular carcinoma (HCC) (Angulo 2002; Anstee and Day 2013; Calzadilla Bertot and Adams 2016; Chalasanani et al. 2018; Fazel et al. 2016; Jou et al. 2008; Sayiner et al. 2016; Younossi and Henry 2016; Younossi et al. 2018a). Being the hepatic component of the metabolic syndrome (MetS) (Chalasanani et al. 2012), NAFLD is commonly associated with other metabolic disorders such as obesity, which is also linked to the development of cardiovascular diseases such as atherosclerosis (Chalasanani et al. 2018; Paoletti et al. 2006). Considering these links between NAFLD, obesity, and atherosclerosis, it is no surprise that NAFLD has become the most prevalent liver disease worldwide (Li et al. 2019).

Indeed, in the last three decades, the prevalence of NAFLD has increased at a constant rate. Numbers from a study from Younossi et al. showed the evolution of NAFLD prevalence in the United States from 1988 to 2008 ranging from 5.51% (1988–1994) to 9.84% (1999–2004) and 11.01% (2005–2008), indicating a twofold increase over two decades (Younossi et al. 2011a). At a global level, it is currently estimated that NAFLD affects about 25% of the general population. At the other hand, NASH has been calculated at 2–5% of the general population and so far represents the minority of NAFLD patients (10–20%) (Younossi et al. 2016a). However, while hepatic steatosis seems less harmful for the liver, patients suffering from steatosis are at increased risk for cardiac-related death (Targher et al. 2016). Therefore, steatotic patients should be monitored even at an early stage of the disease. For the future, Estes et al. predicted the NAFLD population to increase with 21% in 2030, expecting a staggering 100.9 million NAFLD patients, of which 27.00 million patients would also suffer from NASH, the latter indicating a 63% increase in prevalence compared to current numbers (Estes et al. 2018).

From a regional perspective, the pooled incidence of NAFLD in the West (being Europe and Northern America) was estimated to be 28 per 1,000 persons per year (Chalasanani et al. 2018; Younossi et al. 2016b). As mentioned previously, NAFLD has been reported as the most common liver disease in the United States (Bellentani and Marino 2009). However, within the Asian population, recent metadata also indicated the global incidence rate of NAFLD at 50.9 cases per 1,000 individuals per year (Li et al. 2019). Indeed, while it was initially perceived as a “Western disease,” NAFLD is now highly prevalent in all continents with the highest rates reported in South America (31%) and the Middle East (32%), followed by Asia (27%), the United States (24%), and Europe (23%), while being less common in Africa (14%) (Younossi et al. 2016b).

Taken these numbers into account, and especially those indicating the exponential increase of NASH patients, it is clear that NAFLD poses one of the largest burdens on current healthcare systems, emphasizing the urge for early and fast treatment to prevent further escalation of this disease.

1.2 Association with Other Diseases

NAFLD has been reported to be strongly linked to obesity, with a prevalence as high as 80% in obese patients and only 16% in individuals with a normal BMI and without metabolic risk factors (Bellentani et al. 2000; Williams et al. 2011). Relevantly, obesity seems to play a role in both the initial process leading to simple steatosis but also to its progression toward NASH (Polyzos et al. 2017). Indeed, it has been demonstrated that the risk of NASH development is lower in lean than overweight/obese individuals (Sookoian and Pirola 2018). Next to NASH, also patients suffering from hepatic fibrosis tend to be rather obese than non-obese (86% vs. 27%, respectively) (Fassio et al. 2004).

Besides obesity, it has been suggested that NAFLD is also tightly linked to cardiovascular diseases (Patil and Sood 2017). Recently, clinical observations indicated that NASH increases atherosclerosis and cardiovascular risks by local overexpression of inflammatory mediators, endothelial damage, and regulators of blood pressure (Targher et al. 2009, 2010). Others confirmed that NAFLD is independently associated with atherosclerosis progression (Targher et al. 2010). Additionally, other studies demonstrated that NAFLD patients have impaired flow-mediated vasodilatation (Villanova et al. 2005), increased carotid artery intimal-medial thickness and an increased prevalence of carotid atherosclerotic plaques compared to healthy subjects (Sookoian and Pirola 2008), independently of obesity and other established risk factors. These observations therefore emphasize the link between NAFLD and atherosclerosis.

Additionally, NAFLD has also been suggested as a risk factor for gastrointestinal tract malignancies, such as colorectal cancer (Lindenmeyer and McCullough 2018; Muhidin et al. 2012; Wong et al. 2011). Also, NAFLD is also associated with chronic kidney disease (CKD) (Musso et al. 2014), which is defined by the presence of kidney damage or a reduced glomerular filtration rate for 3 months or more

(Levey et al. 2005). Specifically, it has been suggested that the prevalence of CKD in NAFLD patients is between 4 and 40% (Marcuccilli and Chonchol 2016). This association has been partly explained by the NAFLD-associated microvascular alterations that also affect the kidney (Musso et al. 2016). Moreover these microvascular alterations have been linked to cerebrovascular disease, potentially contributing to cognitive impairment (Lombardi et al. 2019). Finally, polycystic ovary syndrome (PCOS), a condition that leads to the production of higher-than-normal amounts of male hormones in women, is also associated with NAFLD (Vassilatou 2014; Wu et al. 2018a). However, the clinical significance and its pathophysiological basis remain to be further investigated.

1.3 Clinical, Economic, and Social Burden of NAFLD

In spite of its increased prevalence over the last decades, to our knowledge, the clinical burden of NAFLD has not been characterized in detail (Boursier et al. 2018; Lam et al. 2016; Mullerova et al. 2019; Welte et al. 2012). Notwithstanding that all stages of NAFLD contribute to its clinical burden, the more progressive stages of NAFLD are expected to have the largest impact (Sayiner et al. 2016). As a progressive form of NAFLD, NASH is currently the second leading cause for liver transplantation in the United States and even the leading cause for liver transplantation in females (Noureddin et al. 2018). Furthermore, it is known that NAFLD has become one of the leading causes for cirrhosis (Kadayifci et al. 2008), the end-stage liver disease which is associated with high risks for development of bacterial infections leading to hospitalization (Albillos et al. 2014; Li et al. 2018; Singal et al. 2014). Additionally, the presence of advanced fibrosis (stage ≥ 2) in NAFLD has been directly associated with liver-related mortality (Angulo et al. 2015; Younossi et al. 2011b). There is also accumulating evidence that NAFLD is an important risk factor for hepatocellular carcinoma (HCC), which is the fifth most common type of cancer and third most common cause of cancer mortality (El-Serag and Rudolph 2007; Piscaglia et al. 2016; Younossi et al. 2015a). Translating the latter described observations into exact numbers, a recent study showed that in 2015, 28,000 deaths (2.2% of all deaths in the NAFLD population) were related to cirrhosis, HCC, or liver transplantation, while 162,560 deaths (accounting for 12.8% of all NAFLD deaths) were due to cardiovascular diseases (Angulo et al. 2015; Ekstedt et al. 2015). Indeed, these data point toward an important role for cardiovascular diseases in NAFLD-related mortality. In line with the previously estimated increased prevalence of NAFLD in the future, reports have indicated that the total number of deaths resulting from NAFLD will increase 44%, reaching 1.83 million deaths at an annual basis by 2030 (Estes et al. 2018). Regarding to the healthcare expenditure, Lam et al. demonstrated that more frequent clinical visits are associated with improved outcomes in pediatric NAFLD patients, substantiating the importance of frequent monitoring and follow-up to manage NAFLD progression (Lam et al. 2016). Moreover, Boursier et al. evaluated the hospitalization of NAFLD-/NASH-related end-stage liver disease (ESLD) patients by a 7-year follow-up study in France. This report described that ESLD patients experience

more hospitalization per year (over 400%), which are longer (400% increase in length) and are associated with a 300% increase in hospitalization costs (Boursier et al. 2018). These numbers therefore indicate the gigantic investments that are required to manage NAFLD.

In line with the increasing prevalence of NAFLD, the economic costs related to NAFLD have also been predicted to rise in the future. In the United States alone, approximately 103 billion dollars are annually spent on NAFLD-related costs, while in European countries (i.e., France, the United Kingdom, Germany, and Italy), these costs account for approximately €35 billion (Abdelmalek 2016). Of particular concern is the rising prevalence of obesity as well as the increase in general healthcare costs, which contributes to a tenfold increase in the current economic burden of NAFLD by 2025 (Abdelmalek 2016; Younossi et al. 2016a). Several studies also investigated the relationship between NAFLD patients and healthcare utilization and associated costs. These studies demonstrated that the number of outpatient visits for patients with NAFLD significantly doubled over time and underlined the fact that, between 2005 and 2010, the healthcare costs of inpatients and outpatients were increased 5% and 10%, respectively (Baumeister et al. 2008; Younossi et al. 2014, 2015b). Additionally, a study of the hepatology clinics in the West Suffolk area of the United Kingdom showed that the total annual hepatology budget for these specialized clinics was £130,000, including £58,000 for resources and £72,000 for clinic attendances. Moreover, the latest research estimating the economic burden of NASH patients by using a Markov decision analytic model demonstrated that lifetime costs of all NASH patients was approximately \$222.6 billion in the United States in 2017, with \$95.4 billion reflecting the advanced NASH population (Younossi et al. 2019).

From an individual perspective, NAFLD patients have to contend with a range of symptoms such as fatigue, decreased physical activity, and emotional health impairment which affect their quality of life (health-related quality of life (HR-QOL)) (Golabi et al. 2016; Loria et al. 2013; Younossi and Henry 2015, 2016). Several studies have demonstrated that NAFLD patients had poorer HR-QOL compared to other chronic liver diseases and also showed that NAFLD-related fatigue associated with impairments in physical functioning (Afendy et al. 2009; Dan et al. 2007; Newton et al. 2008). Potential explanations for the decrease of HR-QOL in NAFLD patients that have been raised are related to obesity and psychological processes as well as psychiatric issues such as depression and anxiety (Stewart and Levenson 2012; Surdea-Blaga and Dumitrascu 2011; Weinstein et al. 2011). Indeed, several studies have demonstrated that depressive disorders, as well as anxiety disorders, are more frequent in patients with NAFLD/NASH and are associated with more advanced liver histological abnormalities, such as severe hepatocyte ballooning (Elwing et al. 2006; Macavei et al. 2016; Stewart et al. 2015; Weinstein et al. 2011).

Altogether, these numbers substantiate the impact of NAFLD on healthcare, economy, and also the daily life of individual patients. To prevent further escalation of the disease, it is therefore of utmost importance to increase the understanding of the disease to find approaches to diagnose and treat (and if possible prevent) the progression of NAFLD.

2 Pathophysiology of NAFLD

As NAFLD comprises a spectrum of diseases, multiple pathophysiological processes are involved including dysregulation of lipid metabolism, increased hepatic inflammation, and the presence of hepatic fibrosis. However, how patients with steatosis develop inflammation is still unclear, leaving a blind spot in the understanding of how NASH exactly arises. Nevertheless, scientists have succeeded in unraveling several disease processes in NAFLD, which appear to show striking similarities with disease processes described in atherosclerosis. Furthermore, increasing evidence links different metabolic organs to NAFLD development, emphasizing the presence of metabolic crosstalk during NAFLD.

2.1 Intrahepatic Disturbances During NAFLD

The liver constitutes a key role in regulating whole body metabolism, which involves a complex interplay between different hepatic cell types ranging from hepatocytes as parenchymal cells to Kupffer cells (KCs), stellate cells, and liver sinusoidal endothelial cells (LSECs) among other cell types. As such, each of these cell types is influenced by pathophysiological processes during NAFLD, eventually leading to hepatic disturbances at whole organ level.

2.1.1 Lipo- and Glucotoxicity

Due to low physical activity and increased consumption of fats, lipotoxicity has arisen as one of the main players to contribute to NAFLD pathogenesis (Ibrahim et al. 2011). Lipotoxicity is defined by the excess generation of cytosolic lipids (mainly triglycerides and subtypes of free fatty acids) that have direct adverse effects on metabolic pathways of the cell (Schaffer 2016). Under normal conditions, triglycerides and free fatty acids are stored in adipose tissue, where they can be employed as energy source during periods of energy deprivation or during extreme exercise (Muro et al. 2014). However, in obesity, when the storage capacity of adipose tissue is exceeded, free fatty acids accumulate in ectopic organs, including the liver (but also in the arteries). As such, hepatic steatosis (Wojcik-Cichy et al. 2018) develops, resulting in the formation of adverse metabolites that hamper normal cellular physiology. For example, an excess of free fatty acids such as palmitic or stearic acids induces the generation of toxic metabolites, leading to caspase-dependent apoptosis of hepatocytes (Kakisaka et al. 2012; Pfaffenbach et al. 2010) but also of cardiomyocytes (Drosatos and Schulze 2013; Zou et al. 2017) and endothelial cells (Artwohl et al. 2008; Chinen et al. 2007). Indeed, while free fatty acid-induced apoptosis of hepatocytes is a key feature of lipotoxicity in the context of NAFLD (Trauner et al. 2010), other reports have shown that excess palmitate induces endoplasmic reticulum stress and apoptosis in the context of atherosclerosis as well (Erbay et al. 2009). Therefore, free fatty acid-induced apoptosis of parenchymal cells appears to be a shared mechanism between NAFLD and atherosclerosis development.

Besides inducing apoptosis, free fatty acid influx into hepatocytes also influences the function of key enzymes and nuclear receptors involved in hepatic de novo lipogenesis, fatty acid oxidation, and cholesterol metabolism, thereby further disturbing hepatic lipid metabolism. Indeed, expression levels of acetyl-coenzyme-A carboxylase 1, a key enzyme in fatty acid metabolism (Barber et al. 2005), were shown to decrease in advanced stages of NASH compared to individuals with steatosis (Nagaya et al. 2010). Moreover, in the transcriptional levels, liver X receptor (LXR), a nuclear receptor involved with regulation of cholesterol, fatty acid, and glucose metabolism (Kalaany and Mangelsdorf 2006), correlated with intrahepatic inflammation and fibrosis in NAFLD patients (Ahn et al. 2014; Ni et al. 2017). In line, macrophage-targeted delivery of LXR agonist inside the atherosclerotic plaque reduced atherosclerosis progression (Guo et al. 2018), pointing toward a key function of LXR in both NAFLD and atherosclerosis development.

Lipotoxic responses also affect LSECs, a type of non-parenchymal cell that is specifically involved in maintaining hepatic vascular tone and quiescence of hepatic stellate cells that are responsible for the fibrotic response. Upon treatment with oxidized lipids (Zhang et al. 2014) or palmitic acid (Matsumoto et al. 2018), LSECs directly or indirectly triggered the release of reactive oxygen species (ROS) production (Peters et al. 2018), which influences mechanisms related to inflammation and fibrosis (Ni et al. 2017).

Increased hepatic fat accumulation has also been associated with reduced levels of high-density lipoproteins (HDL) and increased levels of total plasma cholesterol, low-density lipoproteins (LDL), and very low-density lipoprotein particles (Koruk et al. 2003), the latter being involved with hepatic lipid export. Moreover, besides triglycerides and fatty acids, it has become evident that cholesterol is a key player in inducing hepatic inflammatory responses (Caballero et al. 2009; Plat et al. 2014; van Rooyen et al. 2011). Indeed, in the context of obesity-associated diseases, it was shown that cholesterol levels are associated with hepatic inflammation (Musso et al. 2003; Puri et al. 2007) and atherosclerosis (FERENCE et al. 2017) in humans. In agreement with these data, it has previously been shown that omitting cholesterol from the diet was able to prevent liver inflammation in hyperlipidemic and atherosclerosis-prone mice (Wouters et al. 2008), pointing toward cholesterol as a significant risk factor for early onset of NASH and progression of atherosclerosis.

In addition to lipotoxicity, glucotoxicity is a metabolic condition linked to increased intake of dietary sugars, resulting in hyperglycemia which may cause hepatotoxic effects by increasing steatosis (Mota et al. 2016). For instance, it was shown that high carbohydrate intake plays a role in de novo lipogenesis and hepatic steatosis (Ackerman et al. 2005; Neuschwander-Tetri et al. 2012), presumably via activation of lipogenic enzymes such as fatty acid synthase and stearoyl-CoA desaturase-1 (Maslak et al. 2015). In addition, high-fructose intake was shown to correlate with the severity of fibrosis in NAFLD patients (Basaranoglu et al. 2015; Mota et al. 2016) and carbohydrate intake associated with the progression of coronary atherosclerosis (Mozaffarian et al. 2004).

Glucotoxic and lipotoxic products, including free fatty acids, cholesterol, and ceramides, among others (Han et al. 2008), are also involved in the activation of cellular stress responses. For instance, it has been shown that saturated long-chain fatty acids can disturb metabolic fluxes, thereby increasing the production of harmful lipid intermediates (Kakisaka et al. 2012). These intermediates can promote the release of ROS, leading to oxidative stress and hence the progression from steatosis to NASH (Matsuzawa et al. 2007; van Herpen and Schrauwen-Hinderling 2008) and the development of atherosclerosis (Nowak et al. 2017).

Overall, these evidences show that triglycerides, fatty acids, and cholesterol overload disturb essential processes in the liver that result in NAFLD features, placing lipids at the center of NAFLD development. Moreover, as these processes show striking similarities with disturbances present in atherosclerosis, lipotoxicity is a denominator linking NAFLD to atherosclerosis.

2.1.2 Oxidative Stress and Mitochondrial Dysfunction

As mentioned in the previous paragraph, part of the lipotoxic response involves the generation of ROS, resulting in oxidative stress. Oxidative stress comprises a state during which there is an imbalance between generation of ROS at one hand and an inability to detoxify (i.e., via antioxidant mechanisms) these oxygenated intermediates (Masarone et al. 2018). As a consequence, free radicals, peroxides, and related products are generated and react with biological components such as proteins, DNA, but also lipids (Finkel and Holbrook 2000). Indeed, considering the increased amount of lipids present during NAFLD conditions, larger quantities of lipid peroxidation products are present in the liver of NAFLD patients (Sumida et al. 2013) and contribute to the transition toward more serious stages of NAFLD (Busch et al. 2017; Feldstein et al. 2010). In addition, ROS is known to mediate endoplasmic reticulum stress, thereby causing the formation of misfolded proteins, which is a critical factor in NAFLD (Ashraf and Sheikh 2015) as well as the progression of atherosclerosis (Hotamisligil 2010; Tabas 2010). Moreover, cholesterol oxidation products that are part of oxidized low-density lipoproteins (oxLDL) are majorly involved in inflammatory and fibrotic responses in the liver (also further discussed in next section) (Bieghs et al. 2013).

Considering the key role of mitochondria in cellular oxygen consumption and production of ROS, lipotoxic influences on mitochondria have the potential to further aggravate oxidative stress (Dominguez-Perez et al. 2019). Under physiological conditions, fatty acid transport into the mitochondria is mediated via carnitine palmitoyltransferase 1 (CPT-1) in order to stimulate beta-oxidation. Nevertheless, the expression of *Cpt-1* was shown to be reduced in NAFLD (Kohjima et al. 2007), findings that were further supported by Francque et al., showing that peroxisome proliferator-activated receptor alpha (*PPARα*), an important nuclear receptor regulating CPT-1, inversely correlated with disease severity in patients with NASH (Francque et al. 2015). By using isolated mitochondria, it was also shown that short chain ceramides increase mitochondrial permeability due to the generation of ceramide channels and increased cytochrome C

release (Colombini 2010), thereby mediating toxic effects. Moreover, mitochondrial cholesterol accumulation caused mitochondrial dysfunction (Balboa et al. 2017), and based on studies in the context of neurotoxicity (Barbero-Camps et al. 2014), mitochondrial cholesterol may play a role in endoplasmic reticulum stress and subsequent apoptosis.

2.1.3 Hepatic Inflammation and Fibrosis

An essential pathophysiological process during NAFLD that also unites the lipotoxic response with the generation of oxidative stress is the presence of hepatic inflammation which can progress into hepatic fibrosis. In contrast to the uptake of non-modified LDL, it has been established that the uptake of oxLDL contributes to cholesterol-induced foam cell formation and metabolic inflammation (Lara-Guzman et al. 2018) in NASH (Houben et al. 2017) but also in the context of atherosclerosis (Binder et al. 2003). Moreover, the accumulation of oxidized lipids into the lysosomal compartment of macrophages activates inflammatory cascades including inflammasome complexes and apoptosis (Bieghs et al. 2013; Grebe et al. 2018; Hendriks et al. 2013; Jerome 2010). Indeed, recent studies show that specific inhibition of the NLRP3 inflammasome not only reverses hepatic inflammation and fibrosis (Mridha et al. 2017) but also reduces atherosclerotic lesion development (van der Heijden et al. 2017), pointing toward an important role for the inflammasome in chronic inflammatory diseases (Cai et al. 2017; Duewell et al. 2010; Pan et al. 2018). Furthermore, cholesterol-mediated activation of inflammasomes decreases cholesterol efflux, thereby disturbing the regulation of bile acid metabolism. Previously, it was indeed shown that mice lacking the bile acid receptor farnesoid X receptor (FXR) had pro-atherogenic lipoproteins (Mencarelli and Fiorucci 2010) and increased hepatic bile acid levels (Sinal et al. 2000), pointing toward a potential role for FXR in cholesterol-induced liver inflammation. Indeed, improving cholesterol efflux in hepatic macrophages by overexpressing *Cyp27a1*, an enzyme responsible for the conversion of cholesterol into bile acids, reduced hepatic inflammation and fibrosis in an experimental model (Hendriks et al. 2015). Via accumulation of oxidized lipids into lysosomes, also disturbances in autophagy contribute to increased levels of inflammation both during NAFLD (Wu et al. 2018b) and atherosclerosis (Martinet and De Meyer 2009). Besides cholesterol, also other lipids such as phospholipids (Lee et al. 2012) and fatty acids (Reinaud et al. 1989), can (non)enzymatically interact with free radicals, triggering inflammation by a wide variety of underlying processes (Houben et al. 2017).

While macrophages play a key role in the inflammatory response, hepatic stellate cells are the main drivers of the fibrotic response (Friedman 1993). After a damaging insult, stellate cells are activated, thereby secreting collagens and related matrix proteins that lead to generation of scar tissue or fibrosis (Peters et al. 2018; Schneiderhan et al. 2001), a pathological process also described in atherosclerosis (Ostovaneh et al. 2018). Relevantly, Chu et al. recently demonstrated that exposing hepatic stellate cells to fatty acids resulted in an increased secretion of CCL20, resulting in a switch from a quiescent to an activated

hepatic stellate cell. These findings were further confirmed in humans, showing increased circulating CCL20 protein levels in patients with NAFLD-related fibrosis (Chu et al. 2018). Further data based on an elegant co-culturing system using primary liver cells pointed toward CCL5 as an important hepatic stellate cell-derived chemokine capable of mediating steatosis and pro-inflammatory responses in initially healthy hepatocytes (Kim et al. 2018). Moreover, *in vivo* induction of CCL5 in response to high-fat diet was also shown to serve as an important regulator of vascular remodeling, revealing a role for CCL5 and its receptor in atherogenesis (Lin et al. 2018). Therefore, multiple reports indicate that lipids enable fibrotic responses by influencing hepatic stellate cells.

2.2 Metabolic Crosstalk in NAFLD

As previously mentioned, the capacity of adipose tissue to store lipids determines the quantity of free fatty acids to be released into the circulation under high lipid conditions. However, besides its storage capacity, adipose tissue is known as a “secretory” organ, releasing adipokines and adipocytokines that influence other organs (Ouchi et al. 2011). For this reason, lipid-induced adipose tissue function increases the release of adipocytokines such as TNF α , IL6, IL18, and ANGPTL, leading to inflammatory responses in other metabolic organs such as the liver (Ouchi et al. 2011; Reilly et al. 2015). Moreover, the release of these adipokines also influences circulating immune cells, contributing to a state of chronic inflammation (Bijnen et al. 2018; Mancuso 2016; Nakamura et al. 2014). Due to this systemic impact, it is not surprising that adipokines also influence atherosclerosis development. Indeed, adipose tissue-released TNF α directly influenced atherosclerosis development (Tanaka and Sata 2018). Besides modulating inflammation, the increased release of free fatty acids also hinders the anti-lipolytic role of insulin, aggravating insulin resistance (Engin 2017; Sears and Perry 2015).

Another extrahepatic organ that has been linked to NAFLD development is the thyroid. Being an endocrine organ, the thyroid secretes hormones that have a role in the regulation of energy homeostasis including the metabolism of cholesterol and fatty acids (Sinha et al. 2018). Specifically, hypothyroidism is characterized by increased serum LDL and HDL levels and decreased triglyceride levels (Duntas 2002). Besides indirectly influencing hepatic lipid metabolism by modulating circulating lipid levels, thyroid hormones also directly affect hepatic lipid metabolism mainly via the presence of hepatic thyroid hormone receptors (THR) (Sinha et al. 2018). THRs are nuclear hormone receptors that function as ligand-dependent transcription factors influencing downstream metabolic genes (Davis et al. 2016) but also disturb other metabolic transcription factors such as PPAR γ , LXR, and sterol regulatory element-binding protein 1 (SREBP1c) (Araki et al. 2009; Wang et al. 2015). For this regulatory role on hepatic lipid metabolism, THR agonists were also considered for the management of hepatic steatosis (Cable et al. 2009) but later observed adverse effects resulted

in discontinuation of these clinical trials (Lammel Lindemann and Webb 2016). Nevertheless, thyroid hormones analogues (rather than THR_s) are still considered as potential future NAFLD treatment (Perra et al. 2008).

Another organ that has gained attention in the context of NAFLD is the brain. At one hand, NAFLD-related inflammation has been demonstrated to influence microglia in the brain, leading to alterations in microvasculature of the brain (Ghareeb et al. 2011; Kim et al. 2016). Furthermore, NAFLD-associated endothelial dysfunction and the procoagulant state were linked to the same microvascular alterations, which may contribute to disturbances in brain circulation, damage, and cognitive impairment (Lombardi et al. 2019). Besides the link to the aforementioned cerebrovascular diseases (Airaghi et al. 2018), other brain-related associations have been established to NAFLD. Firstly, a recent report from Horwath et al. demonstrated that endoplasmic reticulum stress in the subfornical organ of the brain, a brain region previously linked to appetite (Matsuda et al. 2017), directly mediated hepatic steatosis, thereby directly linking the brain to the liver in the context of NAFLD. Moreover, Weinstein et al. recently linked NAFLD to lower cerebral brain volume hinting at a more profound role for the brain in NAFLD (Weinstein et al. 2018). Finally, as a regulation center for energy metabolism, brain regions such as the arcuate nucleus in the hypothalamus sense the metabolic status and govern food intake (Schwartz et al. 2000), making an obvious link to obesity-related NAFLD. An essential hormone involved with the homeostatic regulation of energy and acting via the hypothalamus is leptin (Kwon et al. 2016). Notably, variants of leptin receptors associated with increased NAFLD susceptibility, pointing toward a potential role for hypothalamic leptin sensitivity in NAFLD (Zain et al. 2013). Additional evidence linking hypothalamic inflammation to hepatic steatosis further substantiated the potential involvement of the hypothalamus in NAFLD (Valdearcos et al. 2015).

To end, multiple reports have indicated the involvement of the gastrointestinal tract to play a role in NAFLD development. Under physiological circumstances, the intestinal lining serves as a physical barrier that separates the host from contents in the gut. Disruption of this barrier leads to intestinal permeability (Winer et al. 2016), allowing for leakage of intestinal bacteria and other products into the circulation. Indeed, leakage of lipopolysaccharide (LPS) derived from intestinal bacteria into the circulation (Kitabatake et al. 2017) can activate KCs in the liver (Ye et al. 2012), thereby directly resulting in NASH development (Kitabatake et al. 2017; Wigg et al. 2001). Relevantly, gut-derived serum LPS was similarly associated with atherosclerosis development, reaffirming the tight link between NASH and atherosclerosis (Pastori et al. 2017). Though there are limited studies providing a causal role of the gut microbiome in NAFLD pathogenesis, the current amount of evidence suggests that the gut microbiota are at least involved with the development of NAFLD (Gregory et al. 2015; Kaden-Volynets et al. 2018; Martinez-Guryn et al. 2018; Turnbaugh et al. 2006) (Wang et al. 2018). Other well-known factors linking the gut to NAFLD are bile acid metabolism (Dumas et al. 2006; Tremaroli and Backhed 2012), bacterial-derived short-chain fatty acids (Canfora et al. 2019), and the toxic compounds dimethylamine and

trimethylamine that were converted by bacteria from choline (Spencer et al. 2011; Wang et al. 2011). In line with our other descriptions, each of these compounds has also been associated with atherosclerosis development (Chambers et al. 2018; Charach et al. 2018; Tang et al. 2013).

Based on these evidences, it is clear that the development of NAFLD is linked to pathophysiological processes that arise in other (metabolic) organs. This information fuels a view of NAFLD being a complex, systemic disease influenced by a range of other organs. It is therefore likely that future management of NAFLD will require a systemic rather than a liver-specific approach.

2.3 Genetic Predisposition to NAFLD

NAFLD is considered a polygenic disease, implying the involvement of a variety of genetic factors in predisposing individuals to disease onset. While mutations in the patatin-like phospholipase domain-containing 3 (PNPLA3) gene were initially associated with hepatic steatosis (Romeo et al. 2008), other reports have also correlated the PNPLA3 variation to NASH progression (BasuRay et al. 2019; Valenti et al. 2010). Similarly, PNPLA3 genetic variants are also associated with carotid atherosclerosis in younger patients NAFLD (Petta et al. 2013). Though PNPLA3 variants were recently linked to the ubiquitylation processes (BasuRay et al. 2017), the exact underlying mechanism explaining the onset of NAFLD is still unclear.

Additionally, based on several population studies, it was recently described that mutations in the transmembrane 6 superfamily 2 (TM6SF2), a key regulator of very low-density lipoprotein (VLDL) export, correlated with NASH progression (Manne et al. 2018) and cardiovascular disease (Li et al. 2018), most likely via changes in plasma lipids. Indeed, plasma lipids appear to be one of the common denominators predicting severity of both NAFLD and coronary artery disease (Brouwers et al. 2019).

Further genetic screenings for NAFLD revealed that glucokinase regulator (*GCKR*) (Santoro et al. 2012) and lysophospholipid acyltransferase 7 (known as *MBOAT7*) (Mancina et al. 2016), key enzymes for glucose metabolism and reacylation of phospholipids, respectively, as well as neurocan were associated with NAFLD development (Speliotes et al. 2011). Yet, a more recent study focusing on the aforementioned NAFLD-risk alleles (*PNPLA3*, *TM6SF2*, *GCKR*, and *LYPLALI*) substantiated the heterogeneity of the NAFLD phenotype between patients, emphasizing the complexity of the disease (Sliz et al. 2018). As such, though genetic predisposition may influence disease onset, other pathophysiological factors that are independent of genetic predisposition are likely a stronger contributor to explain NAFLD development.

A line of research that has received increased attention is the influence of epigenetic changes on NAFLD development (Eslam et al. 2018). Epigenetic changes are induced by modifications in the regulators of DNA such as DNA methylation reactions, histone proteins, chromatin structure, and RNA-based

mechanisms resulting in changes in genes expression (Eslam et al. 2018). These epigenetic modifications influence aging-related processes which contribute to NALFD (Horvath et al. 2014) but can also be transmitted to the progeny, thereby combining genetic and environmental factors involved in the development of disease. Mice that were rechallenged with a high-fat diet after being exposed to this diet during fetal life showed more severe hepatic steatosis, inflammation, and fibrosis (Bruce et al. 2009). This influence of a detrimental fetal environment on NAFLD has been further substantiated by studies linking intrauterine growth retardation to increased risk of developing NAFLD (Nobili et al. 2007; Suomela et al. 2016; Valenti and Romeo 2016). Furthermore, methylation patterns of genes involved in insulin signaling associated with the presence of NASH, which disappeared after bariatric surgery (Ahrens et al. 2013). As such, though being in its infancy, epigenetic modifications are expected to have an important role on NAFLD progression (Eslam et al. 2018).

3 Therapeutics

The involvement of different mechanisms in the pathogenesis of NAFLD also adds a level of complexity in finding appropriate therapeutic options to improve the different aspects of NAFLD. While therapies to reduce hepatic steatosis are known, a major problem is reversing the inflammatory component in the liver. Indeed, at present, no effective therapeutic approaches exist for reducing hepatic inflammation (Houben et al. 2017). From market size perspective, NASH-related therapeutics generated \$1,179 million in 2017 and is estimated to reach \$21,478 million by 2025 (Shinde 2018), pointing toward the huge demand for NASH treatments. Due to the magnitude of this health concern and its potential impact on healthcare, multiple treatments are currently being investigated with the aim to decrease inflammation and fibrosis (Oh et al. 2016). In this section, we provide a selection of currently investigated therapeutic approaches for NAFLD and demonstrate that these approaches are also investigated in the context of atherosclerosis (see Fig. 1). From this perspective, we further highlight the link between NAFLD and atherosclerosis.

3.1 Dietary/Lifestyle Intervention and Bariatric Surgery

Dietary changes and lifestyle interventions resulting in weight reduction are currently the first-line therapy for NAFLD patients (Sumida and Yoneda 2018). Indeed, dietary restriction is the most effective way to reduce liver fat (Marchesini et al. 2016; Patel et al. 2015). Furthermore, it has been suggested that hepatic triglyceride content normalizes after a few weeks under a strictly hypocaloric diet (Patel et al. 2015), i.e., low fat and low carbohydrate, which has been proposed as the optimal composition of a diet for NAFLD patients (Asrih and Jornayvaz 2014). Apart from dietary changes, lifestyle modification

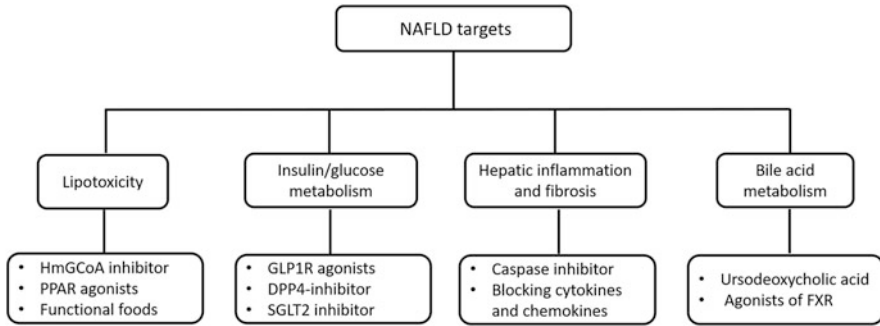


Fig. 1 Targets for NAFLD therapy. Besides exercise, changing the dietary pattern or surgical intervention and pharmacological intervention to improve NAFLD targets the pathological mechanisms of lipototoxicity, insulin/glucose metabolism, hepatic inflammation, fibrosis, as well as bile acid metabolism. *HmG-CoA* 3-hydroxy-3-methylglutarylcoenzyme A, *PPAR* peroxisome proliferator-activated receptor, *GLP1R* glucagon-like peptide-1 receptor, *DPP4* dipeptidyl peptidase-4, *SGLT2* sodium-glucose transport protein 2, *FXR* farnesoid X receptor

is another way to lose weight, for instance, via physical activity instead of sedentariness (Fabricatore 2007). However, compared to dietary restriction, physical activity is less effective in losing weight due to reduced caloric consumption as compared with dietary restriction (Marchesini et al. 2016; Zou et al. 2018). While dietary change and lifestyle intervention are able to reduce body weight, many patients cannot adhere to these interventions. Therefore, bariatric surgery, and more recently termed metabolic surgery (Sasaki et al. 2014), typically results in massive weight loss and in concordant improvements in liver histology (Dixon et al. 2004). Indeed, Mummadi et al. reported that the resolution rates of steatosis, steatohepatitis, and fibrosis were 91.6%, 81.3%, and 65.5% in 15 studies using paired liver biopsies after bariatric surgery (Mummadi et al. 2008). Recently, a 1-year follow-up study by Nickel et al. also supported bariatric surgery as an effective treatment for NAFLD (Nickel et al. 2018). However, as not all NAFLD patients qualify for bariatric surgery, other interventions are necessary to combat NAFLD and related symptoms.

3.2 Targeting Lipotoxicity

As accumulation of lipids inside the liver comprises an essential component in the development of NAFLD, multiple therapeutic approaches have aimed to reduce hepatic lipids with the objective to concordantly reduce hepatic inflammation and fibrosis. The best known example of cholesterol-reducing agents are statins, which are drugs aimed at inhibiting 3-hydroxy-3-methylglutarylcoenzyme A (HmG-CoA) reductase, the rate-limiting enzyme in the cholesterol biosynthesis pathway (Stancu and Sima 2001). Showing beneficial results in the context of atherosclerosis (Bittencourt and Cerci 2015), statins were also investigated in

NAFLD progression. Though some improvements were observed in hepatic damage and inflammation (Kargiotis et al. 2014), other reports declare only minor improvements or even increasing levels of inflammation and fibrosis when statins are administered over a longer period of time (Hyogo et al. 2008). Moreover, recent observations pointing toward the detrimental effects of statins on aging and associated processes (Cholesterol Treatment Trialists 2019; Izadpanah et al. 2015) raise drawbacks for using these drugs under certain conditions.

Finally, and potentially, the most promising pharmacological compound currently under investigation to regress NASH are agonists of PPAR. PPARs are nuclear receptor proteins exerting key regulatory functions as transcription factors on metabolism, among other physiological processes (Dubois et al. 2017). Currently, three types of PPARs (being PPAR α , PPAR β/δ , and PPAR γ) are known and used as targets to improve MetS-related symptoms. In the context of NAFLD, and specifically NASH, the PPAR γ agonist class thiazolidinediones has been shown to improve hepatic inflammation and advanced fibrosis (Bril et al. 2018; Musso et al. 2017). Furthermore, a new agonist, named elafibranor (or GFT505) that targets PPAR α and PPAR δ , was recently shown to improve hepatic inflammation and fibrosis, along with improvements in systemic inflammation, lipid, and glucose metabolism (Ratziu et al. 2016; Staels et al. 2013).

Due to these positive results, both thiazolidinediones and elafibranor are currently under clinical investigation for the treatment of NASH (Connolly et al. 2018). With regard to their application in atherosclerosis, thiazolidinediones have also been proven to slow progression of atherosclerosis in patients (Saremi et al. 2013), while elafibranor was so far not tested in this context. However, preliminary results in an atherosclerotic mouse model suggest that this latter PPAR α/δ dual agonist might also be beneficial in the context of atherosclerosis (Graham et al. 2005).

Besides pharmacological intervention, a more convenient manner of reducing lipids is by means of dietary intervention. Apart from following dietary regimens in which the composition of lipids, protein, and carbohydrates is modulated and caloric intake is minimized (Ratziu et al. 2015) in order to achieve improvements in energy metabolism (Kargulewicz et al. 2014; Perumpail et al. 2017), another approach is to increase the intake of food components named functional foods. Plant sterol and stanol esters are examples of such functional foods that have been proven to reduce serum total and LDL cholesterol (Lichtenstein and Deckelbaum 2001; Plat et al. 2019), leading to improvements in atherosclerosis (Kohler et al. 2017) and NAFLD (Plat et al. 2014). However, more studies are necessary to prove the potential benefit of plant sterol and stanol esters in NAFLD patients.

3.3 Targeting Insulin/Glucose Metabolism

As diabetes has been associated with several stages of NAFLD (Hazlehurst et al. 2016), researchers have investigated the impact of improving insulin and glucose metabolism in order to improve aspects of NAFLD. Firstly, glucagon-like peptide-1 receptor (GLP1R) agonists, which mimic the function of incretins, are currently

investigated in NAFLD (Gastaldelli and Marchesini 2016). GLP is a peptide derived from the L cells of the lower gastrointestinal tract (the small intestine and proximal colon) and known to enhance insulin secretion from pancreatic β cells and inhibit glucagon release from pancreatic α cells (Campbell and Drucker 2013; Ratziu et al. 2015). Whereas the GLP1R agonist exenatide enhanced hepatic steatosis (Tanaka et al. 2014), hepatic oxidative stress, and hepatic inflammation (Shao et al. 2018) and improved adipose tissue lipolysis in different in vivo models, the application of dulaglutide, lixisenatide, liraglutide, and, recently, semaglutide also shows promising results in terms of improvements in hepatic fat, damage, inflammation, and fibrosis (Armstrong et al. 2016; Cusi et al. 2018; Ipsen et al. 2018; Koutsovasilis et al. 2018; Petit et al. 2017; Rakipovski et al. 2018). As such, liraglutide (Armstrong et al. 2016) and semaglutide were under extensive clinical investigation. While liraglutide will not be further evaluated in phase 3 development, Novo Nordisk has initiated a phase 2b trial (NCT02970942) evaluating semaglutide versus placebo in 372 participants with stage F2-F3 fibrosis and NAS ≥ 4 with a score of at least 1 for each of the components (steatosis, ballooning, and lobular inflammation) (Connolly et al. 2018). Relevantly, as diabetes has also been linked to formation and progression of the atherosclerotic plaque (Beckman et al. 2002; Chait and Bornfeldt 2009; Katakami 2018), several GLP1R agonists have also been shown to improve atherosclerosis including exenatide, liraglutide, and semaglutide (Li et al. 2017; Marso et al. 2013; Rakipovski et al. 2018; Yang et al. 2017).

Besides the GLP1R agonists, another approach to improve the GLP1-related effects on insulin and glucose metabolism is by administration of dipeptidyl peptidase-4 (DPP4) inhibitors. DPP4 (also referred to as CD26) is an enzyme known to degrade GLP1. Hence, inhibition of DPP4 enhances the activity of GLP1. While administration of the DPP4 inhibitor sitagliptin has been successfully applied in diabetic patients (Derosa et al. 2015; Drucker and Nauck 2006), several reports demonstrated only minor to no beneficial effects on hepatic fat content or hepatic fibrosis (Cui et al. 2016; Joy et al. 2017). Relevantly, these negative results in the context of NAFLD were also confirmed in atherosclerosis, showing only minor effects on coronary artery plaque improvement (Katakami et al. 2018; Nozue et al. 2016).

Another class of pharmacological compounds that specifically improve glucose metabolism is inhibitors for sodium-glucose transport protein 2 (SGLT2), a transporter protein in the kidney responsible for the reabsorption of glucose (Hsia et al. 2017; Marshall 2018; van Baar et al. 2018). In contrast to the minor effects of the DPP4 inhibitors on NAFLD, the SGLT2 inhibitors canagliflozin, ipragliflozin, and luseogliflozin all show substantial improvements in hepatic steatosis, apoptosis, and fibrosis in in vivo models and NAFLD patients (Ito et al. 2017; Kabil and Mahmoud 2018; Shiba et al. 2018; Shibuya et al. 2018; Sumida et al. 2019). In line with the previously described similarities between NAFLD and atherosclerosis, SGLT2 inhibitors were shown to also positively impact atherosclerosis progression and development (Nakatsu et al. 2017; Nasiri-Ansari et al. 2018; Tanaka et al. 2016; Zelniker et al. 2019).

Together, several treatments aimed at improving insulin or glucose metabolism have positive effects on several aspects of NAFLD, substantiating the role of insulin and glucose metabolism in the progression of NAFLD. Moreover, therapeutic products that improve features of NAFLD also positively impact atherosclerosis, providing further evidence for the similarities between NAFLD and atherosclerosis.

3.4 Targeting Hepatic Inflammation and Fibrosis

Another therapeutic approach to ameliorate NAFLD is to directly target components of the inflammatory and/or fibrotic pathway, as these features are the main cause for hepatic symptoms in NAFLD patients and are also responsible for the development toward advanced liver diseases (Schuster et al. 2018). The caspase inhibitor, emricasan, is one of those investigated compounds targeting the inflammatory aspect of NAFLD. Specifically, caspases are enzymes involved with several physiological processes including inflammation, making them an attractive inflammatory drug target. Administration of emricasan to NAFLD patients showed improvements in hepatic damage (as evidenced by reductions in alanine transaminase (ALT) levels) (Shiffman et al. 2019). However, recent negative results with this compound have questioned its continuation for further clinical investigation (Garcia-Tsao et al. 2019). Another potential caspase-related target for inflammatory drugs is blocking the activation of inflammasomes (Schuster et al. 2018). Indeed, inhibition of the P2X7 receptor, which is known to activate the NLRP3 inflammasome (Amores-Iniesta et al. 2017), via SGM-1019 resulted in improvements in hepatic inflammation and fibrosis in mouse models and NASH patients (Dabbagh et al. 2018), substantiating its further clinical investigation in NAFLD.

Another way to reduce inflammation and fibrosis is by blocking the effect of cytokines and chemokines that propagate the inflammatory reaction. With this regard, the C-C chemokine receptor type 2/C-C chemokine receptor 5 (CCR2/CCR5) inhibitor cenicriviroc has been successfully created. Specifically, cenicriviroc reduced hepatic fibrosis, inflammation, as well as systemic inflammatory parameters in NAFLD patients and animal models (Friedman et al. 2018; Lefebvre et al. 2016; Tacke 2018). Currently, cenicriviroc is being evaluated in phase 3 trials, targeting patients with F2-F3 fibrosis and having an anticipated enrollment of 2,000 participants (Connolly et al. 2018). Additionally, inhibition of galectin-3, a protein belonging to the lectin family and previously linked to NASH severity, has provided promising first results (Harrison et al. 2016), which need to be further validated. Finally, inhibition of apoptosis signal-regulating kinase 1 (ASK1) has also been investigated as drug target to reduce hepatic inflammation and fibrosis. ASK1 is part of the mitogen-activated protein kinase family and has been shown an essential role in NASH development in patients and mouse models (Wang et al. 2017; Xiang et al. 2016; Zhang et al. 2018). In line with this observation, inhibition of ASK1 using selonsertib has shown impressive improvements in hepatic inflammation and fibrosis

(Loomba et al. 2017; Younossi et al. 2018b). Selonsertib is currently under evaluation in two phase 3 clinical trials (STELLAR-3 [NCT03053050] and STELLAR-4 [NCT03053063]) for the treatment of NASH (Connolly et al. 2018).

Similar to the dual therapeutic effects of approaches targeting insulin and glucose metabolism, therapeutic approaches targeting inflammation and fibrosis also show dual positive influences in atherosclerosis and NASH. Galectin-3 has, for example, been linked atherosclerotic plaque progression (Papaspayridonos et al. 2008), and its inhibition results in reductions of atherosclerotic lesion size in vivo (MacKinnon et al. 2013). However, compared to drugs targeting insulin and glucose metabolism, targeting inflammation and fibrosis pathways is less investigated in the context of atherosclerosis, as exemplified by no described clinical studies for selonsertib, emricasan, ASK1 inhibitors, or cenicriviroc.

3.5 Targeting Bile Acid Metabolism

Hepatic components that have been extensively linked to different aspects of NAFLD include bile acids. Bile acids have regulatory functions on lipid and glucose metabolism, impact gut microbiota composition, and influence hepatic inflammation and damage (Schuster et al. 2018), explaining why modulation of bile acid metabolism has been an attractive therapeutic target for NAFLD. Firstly, the hepatoprotective natural bile acid ursodeoxycholic acid (UDCA) has been shown to exert beneficial effects on immune function, has anti-apoptotic and insulin-sensitizing effects, and reduces harmful effects of reactive oxygen species (Kars et al. 2010; Ljubuncic et al. 1996; Rodrigues et al. 1998), all aspects present during NAFLD. Indeed, besides improvements in hepatic steatosis, inflammation, and damage in NASH animal models, two randomized controlled trials showed improvements in lobular inflammation and hepatic fibrosis along with reductions in ALT levels upon UDCA administration (Leuschner et al. 2010; Ratziu et al. 2011). However, other studies showed no effect of UDCA administration in NASH patients (Liechti and Dufour 2012), emphasizing the need for further investigation. In addition, agonists of FXR, a nuclear receptor that has been linked to NAFLD (Zhang et al. 2009), have also been tested in NAFLD. Obeticholic acid (OCA), a semisynthetic variant of chenodeoxycholic acid, showed reductions in steatosis and fibrosis (Fiorucci et al. 2005; Goto et al. 2018), and recently, the first promising results were published from the FLINT study, investigated OCA in NAFLD patients (Neuschwander-Tetri et al. 2015). Currently, OCA is being evaluated in the phase 3 study REGENERATE (NCT02548351) for the treatment of NASH (Connolly et al. 2018).

Strikingly, though bile acids are produced by the liver, multiple evidences have pointed toward their systemic effects on inflammation, cell death, and apoptosis (Chiang 2013). As such, recent evidences have also shown beneficial effects of UDCA (Bode et al. 2016) and OCA (Hageman et al. 2010; Moris et al. 2017) in atherosclerotic models.

4 Conclusion

As the hepatic component of the MetS, NAFLD comprises one of the largest global health threats of the twenty-first century. Though the exact etiology of why NAFLD patients progress from hepatic steatosis to hepatic inflammation and fibrosis is unclear, several studies have established key pathophysiological processes contributing to hepatic inflammation. Considering this large amount of processes involved with NAFLD (which have intra- and extrahepatic origins), it is clear that NAFLD is a complex, systemic disease with high interindividual variation, pointing toward combination therapies or personalized medicine as potential future directions for NAFLD. Moreover, due to this systemic nature, it is clear that NAFLD and atherosclerosis are very closely linked (Bieghs et al. 2012), implying the liver as a potential target to manage atherosclerosis.

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Prevention and Treatment of Atherosclerosis: The Use of Nutraceuticals and Functional Foods

Francesco Visioli and Andrea Poli

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Abstract

Nutritional interventions are effective and – in theory – easy to implement primary and secondary prevention strategies that reduce several risk factors of atherosclerosis and cardiovascular disease (CVD). Yet, because of (a) the severe impact of CVD in terms of mortality, morbidity, quality of life, and economy, (b) the proved role of LDL plasma concentrations as the most critical risk factor,

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and (c) the obstacles found both in terms of biological effects and compliance of the patient by an exclusively dietary intervention, food supplements or *nutraceuticals* are now valuable resources for physicians. As regards cholesterol control, several preparations are available in the market, and we will critically review them in this chapter.

Keywords

Atherosclerosis · Cardiovascular disease · Cholesterol · Diet · Functional foods · Nutraceuticals

1 Introduction

Atherosclerosis is multifactorial in nature and leads to various clinical forms of cardiovascular disease (CVD). Risk factors are manifold, but inflammation and elevated low-density lipoprotein (LDL) cholesterol are thought to be among the most important contributors (Alenghat and Davis 2019). In particular, Mendelian randomization studies and many intervention trials demonstrated that modifications of plasma LDL cholesterol concentrations are *casually* associated with cardiovascular risk (Ference 2015). As discussed in other parts of this book, variations of LDL predict cardiovascular risk in terms of amplitude and direction. Indeed, clinical trials have not yet allowed finding a lower limit of LDL concentrations to be achieved for optimal cardiovascular protection (Cholesterol Treatment Trialists et al. 2015).

Nutritional interventions are effective and – in theory – easy to implement primary and secondary prevention strategies that reduce several risk factors of atherosclerosis and CVD (Visioli and Poli 2019; Visioli et al. 2008). Focus on healthful food items, namely, fruit and vegetables and, hence, fiber, vitamins, (poly)phenols, and healthy fats (from extra virgin olive oil, vegetable oils, nuts, fatty fish) and low sodium improves diets and lessens CVD risk (Visioli and Hagen 2007; Visioli and Poli 2019). One notable example is the Mediterranean diet, which is associated with a significant improvement in health status and a significant reduction in overall mortality, as well as in morbidity and mortality from CVD and other major chronic diseases (Rees et al. 2019). Several systematic reviews of observational prospective studies have confirmed that greater adherence to such diet is associated with better health and greater longevity. Finally, the PREDIMED study provided important experimental proof of the cardioprotective properties of the Mediterranean diet (Martinez-Gonzalez et al. 2019). Consequently, physicians should pay particular attention to their patients' diets – even though curricular training is often suboptimal – and insist they preferentially adopt a plant-based regimen, enriched with fish and seafoods and with dairy products. Two limitations of dietary interventions are worth discussing. The first one is that the most commonly prescribed dietary and physical interventions have a limited (–1.5 to 5%) direct impact on LDL cholesterol levels, i.e., the foremost risk factor (Visioli and Poli 2019). The second and, perhaps, most important one is that extensive modifications of dietary habits are difficult to implement in real-life

settings. It is worth reminding that humans are hardwired to maximize energy intake and limit expenditure (i.e., the “thrifty” genotype). New neurocognitive training methods are being investigated to lose weight and reduce sweets and fats consumption more effectively. However, translation of such results to clinical practice will require time.

Because of (a) the severe impact of CVD in terms of mortality, morbidity, quality of life, and economy, (b) the proved role of LDL plasma levels as the most critical risk factor among a large collection of others, and (c) the obstacles found both in terms of biological effects and compliance of the patient by an exclusively dietary intervention, food supplements or *nutraceuticals* deserve an in-depth analysis because they are valuable resources for physicians (Poli et al. 2018; Poli and Visioli 2019). As regards cholesterol control, several preparations are available in the market, and we will critically review them in this chapter.

2 Red Yeast Rice

The most popular supplement for cholesterol control is red yeast rice (RYR), which originates from the fermentation of rice performed by the fungus *Monascus purpureus*. Its denomination comes from color of the fermenting rice, *Oryza sativa*. With fermentation, a group of molecules identified as monacolin K is synthesized in both lactone (K) and open ring acid forms (Ka). Both molecules undergo interconversion in the body (Poli et al. 2013). Fermentations can take place in liquid and solid mediums. The latter is less efficient than the former, where the overall production of monacolin K is lower (5–130 mg/L) but is also relatively simpler (Vendruscolo et al. 2016). Some studies have shown that environmental factors affect the production of secondary metabolites by *Monascus*. Examples include nitrogen sources and glutamic acid, which improve (from 48.4 to 215.4 mg/L) the production of monacolin K (Vendruscolo et al. 2016). Glutamic acid also increases the production of acetyl coenzyme A, which is a substrate for monacolin K. This line of research stems from the similarities with lovastatin synthetic genes (LNKS) in *Aspergillus*. Indeed, often unbeknownst to the lay public, monacolin K is chemically identical to lovastatin and, thus, strongly inhibits HMG-CoA reductase. RYR also contains monacolins J, L, X, and M that contribute to the inhibitory process to a lesser extent. A possible difference between RYR and lovastatin is their relative bioavailability: the former, according to a single study (Chen et al. 2013), is more bioavailable than the latter and, therefore, more effective on an mg-per-mg basis. Administered at doses of 3–10 mg/day, monacolin K reduces LDL cholesterol concentrations by 20–25% (Fogacci et al. 2019; Li et al. 2014). Its effects on HDL cholesterol are negligible; triglyceridemia is mildly reduced unless plasma triglyceride concentrations are elevated, in which case the effects of RYR are stronger (Li et al. 2014; Sahebkar et al. 2016).

As with statins, probably due to their effects on LDL plasma levels, monacolin K improves endothelial function, with a number of favorable consequences: from systolic blood pressure reduction to plaque stabilization (Strazzullo et al. 2007).

2.1 Untoward Effects

Even though RYR is perceived as “natural” and, hence, intrinsically safe, adverse effects of monacolin K are similar to those of statins, namely, lovastatin. These include elevated hepatic enzyme levels, gastrointestinal distress, and statin-associated myalgias (SAMS), i.e., muscle pain and weakness. Myositis (elevated creatinine phosphokinase [CPK] level) and rhabdomyolysis are more serious albeit highly infrequent complications of statin therapy (Stroes et al. 2015). Indeed, a recent review concluded that RYR is overall relatively tolerable and safe (Fogacci et al. 2019). Quite illogically, patients who experience statin-induced untoward effects often shift to RYR without telling their physicians. Some patients do experience a reduction of side effects, which could be explained by a “nocebo” effect. An alternative explanation for the higher tolerability of RYR products as compared with statins and observed in some studies (Becker et al. 2009) could be the lower doses of the active ingredient (2.5–3 mg) that are usually found in supplements sold in Europe. Indeed, patients who are truly intolerant to statins are not many, and the safety profile of this class of drugs is excellent.

One often overlooked issue is that monacolin K can interact with other drugs, as in the case of statin therapy. RYR should not be co-administered with drugs containing itraconazole, ketoconazole, erythromycin, clarithromycin, telithromycin, HIV protease inhibitors, cyclosporine, nefazodone, green tea, and grapefruit juice (≥ 0.2 L/day) because monacolin K and statins are metabolized by cytochrome P450 and – in particular – by isoenzyme 3A4. Finally, the co-administration of statins and RYR should be avoided for pharmacodynamic reasons (both have the same mechanism of action) and comparable side effects (Cicero et al. 2017; Fogacci et al. 2019).

As a final cautionary note, the quality of commercial RYR-based products is rather heterogeneous (Gordon et al. 2010). The amount of active principle was found to vary by up to 100 times; recently, moreover, in an EFSA document, highly variable amounts of monacolin K in the lactone and in the open ring form were found in RYR-based products found on the market (EFSA 2018). The open ring form usually accounts for about one third of the total monacolins in RYR, whereas synthetic lovastatin is almost 100% lactone. Considering that open ring monacolin K levels as low as 0.1% were found in some samples, the possibility that synthetic monacolin is fraudulently sold as RYR cannot be excluded (Gordon et al. 2010).

Because nutraceuticals are not regulated as drugs, red rice supplements are not always subjected to strict adverse event monitoring programs. Physicians should make patients aware of potential risk factors related to the use of RYR nutraceuticals to ensure safety, lack of allergenic compounds, and potential untoward effects (Dujovne 2017).

3 Phytosterols and Phytostanols

Phytosterols (PS) are triterpenes that are usually classified as *sterols* or *stanols*, according to the presence or absence of a double bond in position 5. PS are found in free or esterified forms: free sterols are integral part of the cellular wall of plants,

where they play important structural functions, whereas sterol esters represent storage products within the cell (Mamode Cassim et al. 2019). The only structural difference between sitosterol and cholesterol consists of an additional ethyl group present at position C-24 in sitosterol, which is probably at least in part responsible for its relatively poor intestinal absorption (Marangoni and Poli 2010).

There are more than 250 different PS molecules identified to date, β -sitosterol being the most abundant of them. Other plant sterols are present in vegetables and vegetable oils and include campesterol, stigmasterol, and dihydrobrassicasterol, at concentrations much lower than those of β -sitosterol; the saturated derivatives campestanol and sitostanol are found in almost negligible amounts in the vegetal kingdom (Marangoni and Poli 2010).

As mentioned, PS are mostly found in vegetables and derivatives such as vegetable oils; nuts, grains, and grain-derived products; and also sprouts, cabbages, cauliflowers, and green and black olives. Non-vegetable sources of PS include egg yolks, mammalian liver, and crustaceans. Commercially, PS are obtained from tall oil – which contains up to 80% of β -sitosterol – and the by-products of soybean oil production. Phytosterols are usually used as esterified forms to increase their solubility and allow for their incorporation into lipid-based foods (Marangoni and Poli 2010).

Considering that PS cannot be synthesized by humans, their circulating concentrations depend upon diet and absorption efficiency, as well as by its secretion in the gut by ABCG5-G5 transmembrane transporters. Depending on the type and amount of plant foods consumed, PS consumption and, in turn, PS blood concentrations vary within and between populations yet remain usually very low. Intervention studies report that higher phytosterols' intake as food supplements increases their circulating levels, while plant stanols supplementation decreases them. The absorption process is that of cholesterol and includes solubilized in mixed micelles, after the hydrolyzation of esters by a pancreatic ester hydroxylase. The specific carrier of cholesterol and PS is the Niemann-Pick C1-Like 1 (NPC1L1), i.e., the one that is specifically blocked by ezetimibe as discussed in other chapters of this book (Davis and Altmann 2009). Plasma levels of PS in humans are normally about 0.5% of those of cholesterol, because (1) the latter is synthesized in the liver, (2) PS is less efficiently absorbed by the small intestine, and (3) PS are rapidly excreted. Plant stanols concentrations in plasma are only 0.05% that of cholesterol (de Jong et al. 2003). Of note, PS play no known biological or functional role.

The main mechanisms responsible for the PS-induced reduction in cholesterol are their competition with cholesterol for incorporation into mixed micelles in the intestinal tract (Ras et al. 2014). As compared with cholesterol, PS are more readily hydrolyzed, and this process leads to a lower solubilization of cholesterol into micelles, which decreases their absorption and, in turn, increases fecal excretion of cholesterol and its metabolites. Co-crystallization of cholesterol and PS, leading to increased fecal excretion of cholesterol, may play a minor role.

Since PS absorbed from the gut are rapidly re-excreted in the gut lumen through the ATP-binding cassette transporters ABCG-5 and ABCG-8, located at the apical surface of the enterocyte, PS can have a rather long duration of action. This may

explain why similar effects can be obtained by administering PS in a single dose or divided into three canonical meals (Plat et al. 2000). If this is true, the daily intake pattern would not affect PS efficacy, and the reduced incorporation of cholesterol into mixed micelles may only partially be responsible for the cholesterol-lowering effect of PS taken with meals. On the other hand, administration of PS without a meal is less effective (−30%) than the same dose administered after a main meal (Doornbos et al. 2006). Patients need hence to be instructed to take PS after lunch or dinner.

In general, the cholesterol-lowering effect of PS is noticeable within few weeks and remains stable if supplementation is continuous (Ras et al. 2014). However, interruption of regular intakes reverses the effects of PS and brings cholesterol concentrations back to basal conditions. This is obvious, but patients are often satisfied with the results and stop taking PS without telling their physician. Finally, PS can be used as ad hoc preparations (beverages) or as functional ingredients of spreads, margarines, etc. (Ras et al. 2014).

This effect is additive to that of cholesterol synthesis inhibitors (statins), which compensatively tend to increase cholesterol absorption from the gut (Miettinen and Gylling 2003).

3.1 Untoward Effects

PS are safe, except for patients with homozygous sitosterolemia, who absorb between 15 and 60% of the ingested sitosterol compared to <5% of normal subjects. The only proven untoward effect of PS is the reduction of circulating fat-soluble vitamins. Physicians should, therefore, reinforce the advice to consume five or more servings of fruit and vegetables per day, which effectively maintains carotenoid levels in the normal range.

4 Berberine

Berberine is the active component of barberry (*Berberis vulgaris* L. family Berberidaceae), which grows in Asia and Europe. Barberry is an integral part of Iranian traditional medicine (Dong et al. 2013). Among its many purported actions, hypotensive activity, gastric secretory stimulation, choleric activity, increased tone of the digestive tract, anti-inflammatory activity, complement alternative pathway inhibition, delayed-type cutaneous hypersensitivity inhibition, antibacterial effects, antifungal effects, narcotic antagonist activity, and sedation have been suggested yet poorly investigated (Koppen et al. 2017). Of note, berberine is poorly bioavailable, i.e., ~1%, and pharmaceutical research is studying absorption enhancers and the use of nanoparticles or microemulsions to overcome this issue (Li et al. 2009; Liu et al. 2016).

Berberine is being mostly studied in Asian subjects and is being marketed in the West for its hypocholesterolemic activities. Indeed, some reviews indicate that

berberine can lower plasma cholesterol to an extent similar to that of statins, i.e., 20–30% (Johnston et al. 2017). This effect is greater in subjects with higher basal cholesterolemia and also depends on individual genetics.

In terms of mechanisms of action, berberine upregulates hepatic LDL receptor expression, likely by stabilizing LDL receptor mRNA through activation of extracellular signal-regulated kinase (ERK) pathways. Other studies suggest that berberine promotes LDL receptor expression through the inhibition of the proprotein PCSK9, which is also – likely – ERK-dependent (Dong et al. 2015). Another mechanism proposed for berberine is that it might interfere with intraluminal cholesterol micellarization, decreasing enterocyte cholesterol uptake, as shown in vitro in Caco-2 cells (Wang et al. 2014).

The effect of berberine on PCSK9 levels (which are compensatively increased during statin treatment) may explain the additive effect of berberine when administered with statins (Cicero et al. 2007). Nutraceuticals containing RYR and berberine are, in fact, quite popular.

The possibility that berberine also exerts a prebiotic effect, leading to a microbiota-mediated anti-atherosclerotic action, has recently been put forward based on animal studies (Zhu et al. 2018). Such effect would lead to reconsider the role of the amount of berberine unabsorbed from the gut.

4.1 Untoward Effects

Barberry per se is thought to be mildly toxic, but berberine is safe at therapeutic dosages. Gastrointestinal problems such as [chiefly] constipation, diarrhea, nausea, and abdominal distension are the most commonly reported side effects of berberine (Pirro et al. 2017). However, these effects diminish after reducing the dose of berberine. No other serious adverse events were reported in the trials that reported safety data, mainly obtained in Eastern countries. As with any other herbal treatment, herb-drug interactions should be carefully assessed in light of mutual cytochrome metabolism. Of note, repeated oral administration of berberine (0.3 g three times daily) decreases CYP2D6, CYP2C9, and CYP3A4 activities in healthy subjects (Guo et al. 2012).

5 Fiber

An adequate intake of fiber has multiple beneficial effects on human health that transcend their mere actions on plasma cholesterol (Rubin 2019).

In terms of cholesterol control, some meta-analyses did quantify the actions of beta-glucan (a class of non-starch polysaccharides: (1 → 3)(1 → 4)-β-D-glucan, which are highly viscous nondigestible fiber). A daily beta-glucan dose of 3 g reduces LDL cholesterol by 5–6%; no effects on other blood lipids have been reported (Cloetens et al. 2012). In addition to grains and cereals, namely, barley and oats, beta-glucan is available as ingredient of functional foods or as plain

supplement. Glucomannan, psyllium (a predominantly gelling polysaccharide mixture), and chitosan are other cholesterol-lowering kinds of fiber.

The mechanisms of action of soluble fibers such as beta-glucan are still elusive (Ho et al. 2016). The most plausible hypothesis is that fiber increases fecal excretion of cholesterol, bile acids, and other dietary fats via formation of gels in the intestine. It is therefore advisable to tell patients to increase their intake of water during supplementation with fiber to facilitate the formation of such gel-like substances. The high molecular weight fraction of beta-glucan appears to be the most effective one.

A prebiotic effect cannot on the other hand be ruled out, leading to an increased synthesis of SCFA (especially propionate), which, after absorption, may act as an inhibitor of cholesterol synthesis in the liver (Reis et al. 2017). At high dosage, beta-glucan also improves postprandial glycemic response (Ho et al. 2016).

5.1 Untoward Effects

The most frequently reported side effects of high fiber intake are gastrointestinal in nature and there is no tolerable upper limit for fiber. Precisely because fiber is nondigestible, it reaches the colon quite intact and undergoes fermentation by the microbiota. This can cause bloating and intestinal distress ranging from diarrhea to constipation (if water intake is inadequate). Co-administration of drugs also calls for caution because fiber can sequester active molecules and alter (usually decrease) bioavailability (Poli et al. 2018; Poli and Visioli 2019). Patients should be told to avoid such practice and to get used to fiber gradually.

6 Supplements in the Pipeline

After the policosanol fiasco, some molecules are being actively investigated for their potential role in hypercholesterolemia and dyslipidemia. We will briefly and critically review them here.

6.1 Astaxanthin

In light of its multiple biological actions, the xanthophyll carotenoid astaxanthin has been proposed as a suitable preventive and therapeutic agent in cardiovascular disease (Kishimoto et al. 2016). Astaxanthin exhibited lipid-lowering activity in laboratory mice supplemented diet with >0.03% of astaxanthin from *H. pluvialis* for 12 weeks, namely, lowering plasma TG concentrations. No significant differences in plasma TC and HDL-C concentrations between the control and treatment groups were reported (Yang et al. 2014). In a murine model of metabolic syndrome, a significant increase in HDL-C and a significant decrease of plasma TG levels and nonesterified fatty acids were induced by astaxanthin given at 50 mg/kg/day for

22 weeks. Moreover, astaxanthin decreased the size of the fat cells of white adipose tissue. In addition, lower adiponectin concentrations were recorded in the serum of obese, insulin-resistant type 2 diabetic rodents and humans, indicating a possible correlation between adiponectin concentrations and insulin resistance and fat accumulation (Stern et al. 2016).

The purported hypolipidemic effects of astaxanthin require further investigation, and its mechanisms of action are still unclear (Visioli and Artaria 2017). A meta-analysis by Ursoniu et al. (2015) indeed concluded that astaxanthin does not exert any significant effect in terms of plasma lipid profile. Of note, astaxanthin sourced from the microalgae *H. pluvialis* is the only one currently authorized for direct human consumption (Visioli and Artaria 2017), and consumers should be aware of possible contaminations with other sources of this carotenoid (as in the case of monacolin K). Further ad hoc investigations will eventually allow (or not) the addition of astaxanthin to the array of nutraceuticals for cholesterol control.

6.2 Hydroxytyrosol

Hydroxytyrosol (HT)'s activities on cholesterol and TGs are still equivocal and, likely, quantitatively irrelevant (Crespo et al. 2018). However, it is worth noting that HT has manifold biological actions that might prove useful to hypercholesterolemic patients (Tome-Carneiro and Visioli 2016). Such activities include thrombogenic potential reduction, anti-inflammatory actions, and inhibition of LDL oxidation as per an EFSA-authorized health claim (Crespo et al. 2018). Despite the hype, this last activity is of dubious relevance to human physiology, also in light of the antioxidant therapy debacle and the lack of appropriate and validated tests (Visioli et al. 2018). In any case, HT is being actively studied for its molecular actions, and accumulated data are strongly suggestive of its cardioprotective actions, regardless of the effects on cholesterol (Visioli et al. 2018).

6.3 Probiotics

Despite the lack of strong scientific evidence, the use of probiotics is increasing, and this includes the cardiovascular arena (Cavalcanti Neto et al. 2018).

Several possible mechanisms for cholesterol's intestinal removal by probiotics have been proposed (Shimizu et al. 2015). Examples include binding of cholesterol to cell surface (in a fashion similar to that of PS of fiber), uptake of cholesterol by growing cells, deconjugation of bile via bile salt hydrolase and subsequent coprecipitation of cholesterol, and some unclear physiological actions of short-chain fatty acid fermentation (Reis et al. 2017).

Prebiotics such as inulin and fructooligosaccharides are soluble, indigestible, viscous, and fermentable compounds that might play hypocholesterolemic actions either by decreasing cholesterol absorption and increasing its fecal excretion (similar to fiber as discussed above) or via production of short-chain fatty acids after their

fermentation by the microbiota. The use of synbiotics is, thus, often advocated for cholesterol control (Mahboobi et al. 2018).

We would like to make the readers aware of the fact that no health claim has been awarded for any probiotic strain as related to lowering cholesterol. The use of probiotics rests on theoretical grounds and some in vitro experiments. Future, well-controlled and large-scale human experiments will eventually support the use of probiotics in hypercholesterolemic patients.

6.4 Bergamot

Bergamot is a citrus fruit belonging to the genus *Citrus*, endemic to the Southern coastal area of Calabria (Italy). The derivatives of bergamot traditionally come from the essential oil contained in the peel and were obtained manually, by pressing. This process is now industrialized and allows the marketing of large quantities of preparations. The main use of bergamot essential oil (after de-furocoumarinization) is still in the food industry (for the flavoring of Earl Grey tea) and in the perfumery field, for its particular aroma. From a biomedical viewpoint, the most interesting phytochemical components of bergamot are the (poly)phenolic molecules present in its juice (commercially called bergamot polyphenolic fraction (BPF)) and the volatile terpenes that characterize the essential oil (Nauman and Johnson 2019).

The BPF exercises various actions on glycolipid metabolism, including the ability to reduce the hepatic accumulation of triglycerides, the partial inhibition of their synthesis, and the ability to inhibit the action of acyl-CoA cholesterol acyltransferase (ACAT) (Janda et al. 2016). The result is a lower production of apoB-containing lipoproteins. More recently, it has also been observed that the BPF is able to efficiently inhibit the pancreatic cholesterol ester hydrolase (pCEH), which leads to a reduction in the absorption of dietary cholesterol (Giglio et al. 2016).

Furthermore, the (poly)phenolic components of bergamot activate AMPK. AMPK is an important regulator of the metabolic pathways involved in the production of ATP in mammalian cells and acts as a sensor of AMP/ATP levels. The intracellular AMP increases when the energy state is low and binds to the AMPK to allow activation.

Finally, it is important to emphasize that the BPF can act directly as an HMG-CoA reductase inhibitor, thus exerting effects potentially similar to those of statins (Leopoldini et al. 2010).

In randomized, double-blind studies carried out in hyperlipemic and type 2 diabetes subjects, a remarkable effect of BPF was found in terms of reducing fasting blood glucose, LDL cholesterol, and triglycerides and increasing HDL cholesterol (Cai et al. 2017; Toth et al. 2015). In another study, a lecithin formulation (to increase bioavailability) of BPF, already successfully tested at the preclinical level, was used at a daily dose of 1,000 mg (corresponding to 400 mg of BPF) (Mollace et al. 2019). Lipid-lowering and hypoglycemic effects similar to those of BPF at a dose of 1,300 mg were recorded (Mollace et al. 2019). All these data must be, obviously,

confirmed in other randomized clinical trials before the BPF can become part of the therapeutic armamentarium (Nauman and Johnson 2019).

7 Conclusions

The decision to recommend a functional food or supplement/nutraceutical is integral part of the therapy and should be taken by physicians, not patients or other health professionals. This process includes the decision of which supplement to prescribe and at which dose, as well as timely checkups in order to monitor the safety and efficacy of treatment.

Furthermore, physicians should instruct patients on the importance and role of supplements, removing the “natural” halo with which they are often marketed. Because most supplements are not reimbursed by national healthcare systems, physicians should make sure their patients can sustain costs over time, considering that such treatment is often lengthy and in theory lifelong.

In this framework, some supplements and functional foods can effectively reduce plasma LDL cholesterol levels by 5–25%, either alone or in combination. As with pharmacological treatments, physicians must monitor the use of nutraceuticals and verify their regular use, their effects on lipid profile, as well as the eventual occurrence of untoward effects, many of which are typical of classic drugs. Should cardiovascular risk worsen remarkably, inclusion of ethical drugs is mandatory.

In conclusion, many effective and relatively safe nutraceuticals and functional foods are available and are backed by solid scientific evidence. Their use in cholesterol control and dyslipidemia is warranted in the frame of a comprehensive therapeutic strategy.

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Conflict of Interest None.

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Part III

Hypothesis Based Approaches to Unravel Novel Targets



Novel Adipose Tissue Targets to Prevent and Treat Atherosclerosis

Ludger Scheja and Joerg Heeren

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Abstract

Adipose tissue as a major organ of lipid and lipoprotein metabolism has a major impact on metabolic homeostasis and thus influences the development of atherosclerosis and related cardiometabolic diseases. Unhealthy adipose tissue, which is often associated with obesity and systemic insulin resistance, promotes the development of diabetic dyslipidemia and can negatively affect vascular tissue

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homeostasis by secreting pro-inflammatory peptides and lipids. Conversely, paracrine and endocrine factors that are released from healthy adipose tissue can preserve metabolic balance and a functional vasculature. In this chapter, we describe adipose tissue types relevant for atherosclerosis and address the question how lipid metabolism as well as regulatory molecules produced in these fat depots can be targeted to counteract atherogenic processes in the vessel wall and improve plasma lipids. We discuss the role of adipose tissues in the action of approved drugs with anti-atherogenic activity. In addition, we present potential novel targets and therapeutic approaches aimed at increasing lipoprotein disposal in adipose tissue, boosting the activity of heat-producing (thermogenic) adipocytes, reducing adipose tissue inflammation, and improving or replacing beneficial hormones released from adipose tissues. Furthermore, we describe the future potential of innovative drug delivery technologies.

Keywords

Adipokines · Atherosclerosis · Brown adipose tissue · Diabetic dyslipidemia · Hyperlipidemia · Inflammation · Insulin resistance · Lipid-lowering therapy · Lipoproteins · Obesity · Perivascular adipose tissue · Thermogenesis · Vascular remodeling · White adipose tissue

1 Introduction

Adipocytes are triglyceride-storing cells present in anatomically distinct adipose tissue depots throughout the mammalian body (Cinti 2001). Originally, two major subtypes, white and brown adipocytes, were identified, both playing an important role in energy metabolism. White adipocytes in white adipose tissue (WAT) store large amounts of triglycerides, typically in a single large lipid droplet, which can be hydrolyzed to provide other organs with free fatty acids (FFA) (Young and Zechner 2013). Brown adipocytes in brown adipose tissue (BAT) also store triglycerides, albeit in lower quantities, and, in many, smaller lipid droplets. When BAT is activated, for example, by exposure to a cold environment, brown adipocytes oxidize fatty acids, glucose, and other fuels in their numerous mitochondria to generate heat (Cannon and Nedergaard 2004). Under chronic cold exposure, adipocytes that are morphologically and functionally very similar to brown adipocytes, referred to as beige adipocytes, develop in WAT depots in a process called WAT browning (Bartelt and Heeren 2014). The number of thermogenic adipocytes thus increases when demand is high. As summarized in Fig. 1, white, brown, and beige adipocytes influence whole-body energy metabolism, lipoprotein levels (Scheja and Heeren 2016), and, through secretion of regulatory molecules, tissue homeostasis in other organs (Scheja and Heeren 2019). How these systemic effects of adipose tissues modulate the development of cardiovascular disease (CVD), and how they can be targeted therapeutically, is the topic of this chapter. Furthermore, local effects exerted by adipocytes found next to blood vessels (perivascular adipocytes) will be described and discussed.

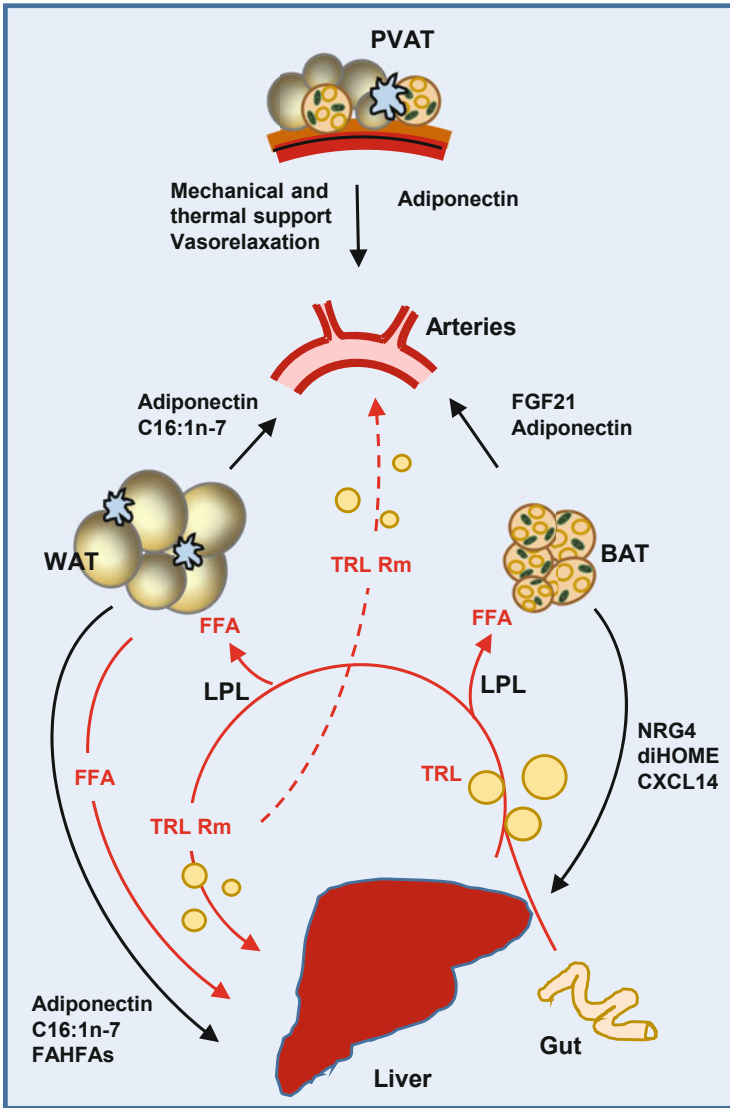


Fig. 1 Role of adipose tissues for cardiometabolic homeostasis under healthy conditions. In metabolic health, white and brown adipose can control systemic lipid homeostasis by regulating both the efficient clearance of triglyceride-rich lipoproteins and the release of fatty acids under catabolic conditions. Healthy adipose tissues harbor mostly anti-inflammatory immune cells (indicated by blue-colored immune cells) that are important to maintain tissue homeostasis. Under this condition, adipocytes mostly release beneficial hormones such as anti-inflammatory lipokines, adiponectin, and FGF21 that support vascular health by direct effects on arteries or indirectly by regulating hepatic lipid metabolism. Overall, healthy adipose tissues are associated with a state of metabolic flexibility that prevents atherosclerosis. *BAT* brown adipose tissue, *FFA* free fatty acids, *FGF21* fibroblast growth factor 21, *LPL* lipoprotein lipase, *NRG4* neuregulin 4, *PVAT* perivascular adipose tissue, *Rm* remnants, *TRL* triglyceride-rich lipoproteins, *WAT* white adipose tissue

2 Types of Adipose Tissue and Their Impact on Cardiovascular Disease

2.1 White Adipose Tissue

The bulk of triglycerides in the body is stored in subcutaneous and intra-abdominal WAT depots. The stored fatty acids can be released from white adipocytes by intracellular triglyceride lipolysis (Young and Zechner 2013). Norepinephrine, which is secreted by sympathetic nerves and signals through β -adrenergic receptors, is the major physiological trigger of lipolysis. β -Adrenergic stimulation elevates cyclic AMP, thereby activating adipose tissue triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL). This regulation ensures that in catabolic states such as fasting or endurance exercise, WAT releases FFA into the circulation to provide other organs with energy. In contrast, after feeding, intracellular lipolysis is suppressed by insulin, and WAT takes up dietary fatty acids from circulating triglyceride-rich lipoproteins (TRL). In this anabolic process, TRL triglycerides are hydrolyzed by lipoprotein lipase (LPL) bound to the luminal side of the capillary endothelium, and the FFA then pass through the endothelium to be taken up and esterified by the adipocytes (Kersten 2014). Apart from their role in fatty acid metabolism, white adipocytes are also an important source of adipokines (adipose peptide hormones) and other secreted regulatory molecules. Notably, only a few of them such as leptin and adiponectin are exclusively expressed in adipose tissue and act at the same time as a true endocrine hormone. In other words, most adipokines are also expressed by cells other than adipocytes and may act predominantly in a paracrine fashion (Scheja and Heeren 2019).

In prolonged periods of caloric surplus, i.e., when energy uptake exceeds energy expenditure, WAT depots expand by increasing adipocyte number (hyperplasia) and adipocyte size (hypertrophy). The resulting overweight or obesity is detrimental when adipocyte hypertrophy prevails (Klötting et al. 2010). In this state of unhealthy obesity, chronic low-grade inflammation occurs in WAT (Crewe et al. 2017), adipocytes become insulin resistant, and fatty acids cannot be stored efficiently any longer. Hence, they are ectopically deposited as part of various lipid species in other organs, a process believed to contribute to the systemic insulin resistance typically associated with obesity (Petersen and Shulman 2018). Another important consequence of limited WAT lipid storage in obesity is liver steatosis. Among other effects, this causes increased secretion of very low-density lipoproteins (VLDL) and thus promotes diabetic dyslipidemia (Scheja and Heeren 2016). Together with pro-inflammatory changes in adipokine patterns, dyslipidemia links expanded WAT depots, especially abdominal visceral fat (Lim and Meigs 2014), to the increased atherosclerosis risk observed in obese individuals (Fig. 2).

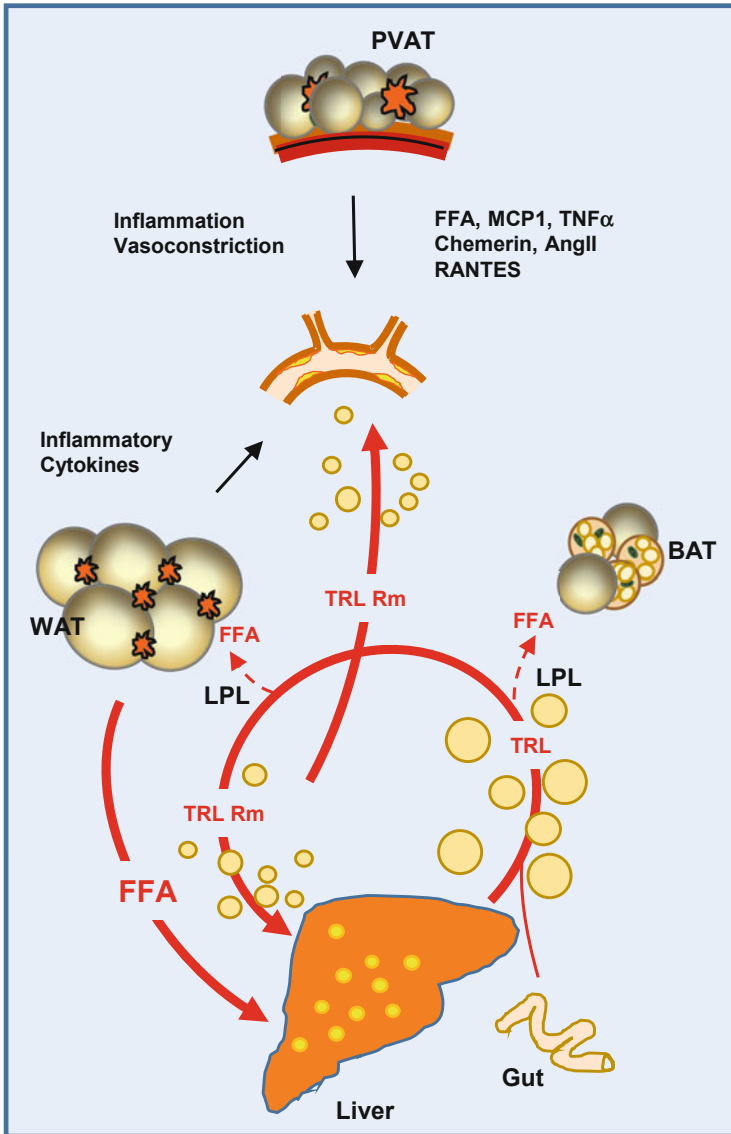


Fig. 2 Hypertrophy and inflammation in adipose tissues cause dyslipidemia and promote atherosclerosis. Chronic caloric surplus and low energy expenditure result in obesity, which is characterized by inflamed (indicated by red-colored immune cells) and hypertrophic adipose tissues as well as dysfunctional brown adipose tissues. Eventually, this causes atherogenic dyslipidemia due to impaired TRL clearance and enhanced fatty acid flux to the liver leading to increased lipoprotein secretion. The lower release of adipocyte-derived anti-inflammatory molecules (see Fig. 1) and pro-inflammatory cytokines in particular those released by PVAT promote inflammation and vasoconstriction. Overall, this creates an immunometabolic state that promotes atherosclerotic plaque formation

2.2 Thermogenic Adipose Tissue

Brown adipocytes present in distinct BAT depots (Zhang et al. 2018) generate heat to maintain body temperature by non-shivering thermogenesis (NST), a process that depends on the mitochondrial proton transporter uncoupling protein-1 (UCP1) that disconnects the respiratory chain from ATP synthesis (Cannon and Nedergaard 2004). NST is triggered by activation of lipolysis through increased β -adrenergic signaling and other catabolic stimuli (Bordicchia et al. 2012). Fatty acids released from the lipid droplets allosterically activate UCP1 and serve as mitochondrial fuel. During prolonged thermogenic stimulation by cold exposure, or other sustained catabolic stimuli such as burn trauma (Sidossis et al. 2015), mitochondria-rich beige adipocytes appear in WAT, a phenomenon called WAT browning (Bartelt and Heeren 2014). Although these adipocytes appear to be developmentally distinct, they are very similar to brown adipocytes with regard to gene expression, morphology, and function. Both brown and beige adipocytes exhibit a very high metabolic capacity and can combust large quantities of triglycerides and other sources of energy when activated. In cold-treated mice, BAT internalizes high amounts of triglycerides from circulating TRL (Bartelt et al. 2011). Under this condition, cholesterol-enriched TRL remnants are efficiently cleared by the liver where bile acid synthesis from cholesterol is increased (Worthmann et al. 2017). Furthermore, the flux of high-density lipoprotein (HDL) cholesterol from the periphery to the liver is enhanced (Bartelt et al. 2017). Consistent with the observed improved lipoprotein profiles, the size of atherosclerotic plaques was reduced in BAT-activated mice fed a cholesterol-rich diet compared to controls (Chang et al. 2012, Berbée et al. 2015). Of note, this beneficial outcome on atherosclerosis depends on lowering of plasma remnant cholesterol, as it was not observed in experiments with mouse models of severely compromised hepatic remnant clearance (LDL receptor (LDLR)- or apolipoprotein (apo) E-deficient mice) where BAT activation and thus TRL processing resulted in elevation of plasma remnant cholesterol (Berbée et al. 2015; Sui et al. 2019). Whether activated BAT or beige WAT has enough metabolic capacity to reduce atherosclerosis in humans needs to be shown. However, a recent, large epidemiological study indicates that high BAT mass inversely correlates with the risk of type 2 diabetes and major cardiometabolic diseases (Becher et al. 2020). Relative to body weight, BAT mass is smaller in humans than in rodents, and it apparently declines in obesity (van Marken Lichtenbelt et al. 2009). However, humans usually live under thermoneutrality, defined as an environmental temperature where the basal metabolic rate generates sufficient heat to maintain body core temperature. When mice are kept under thermoneutral conditions (ca. 30 °C; in dressed humans approximately 24 °C), BAT accumulates lipid and partially loses mitochondria, UCP1, and other determinants of thermogenic capacity (Kotzbeck et al. 2018). Thus, it is likely that in humans, reactivation of BAT and induction of beige WAT, for example, by repeated cold treatments, would profoundly increase metabolic rate and hence the capacity to metabolize atherogenic lipoproteins. Another issue is that metabolic imaging studies to quantify BAT activity in humans are usually done with radioactive glucose analogues. Suitable tracer analogues of

TRL, the major physiological fuel of BAT, still need to be developed. Most adipokines are expressed by both WAT and BAT, and due to its smaller size, BAT is probably less important. However, several adipokines are enriched in thermogenic adipose tissue, and some are induced and released from BAT under thermogenic stimulation or stress conditions (Villarroya et al. 2017). Overall, dysfunctional BAT is likely to influence atherosclerotic development especially by promoting dyslipidemia and inflammation (Fig. 2).

2.3 Perivascular Adipose Tissue

The adventitial layer of arteries is in close contact with adipocytes that form the perivascular adipose tissue (PVAT). PVAT is an important regulator of arterial function. It supports the blood vessel mechanically, by clearing FFA and by releasing paracrine factors that regulate vascular tone, inflammation, redox state, and smooth muscle cell proliferation (Costa et al. 2018). Given these multiple roles, it is not surprising that experimental stripping of arteries in rodents by mechanical or genetic means accelerates the development of atherosclerosis (Chang et al. 2012; Tian et al. 2013; Manka et al. 2014). Perivascular adipocytes may resemble white or brown adipocytes, depending on the anatomical location and the physiological state. For example, PVAT surrounding mesenteric arteries in mice was found to be similar to WAT (Gálvez-Prieto et al. 2008), whereas PVAT associated with the thoracic aorta contains brown-like adipocytes (Fitzgibbons et al. 2011). Of note, the latter adipocytes are functionally thermogenic, as they influence intravascular temperature in mice exposed to cold (Chang et al. 2012).

The quantity of PVAT in humans increases in obesity and is associated with cardiovascular disease (Britton et al. 2012). Mechanistic studies indicate that not only the volume but also altered properties of PVAT in obesity influence the development of atherosclerosis (Costa et al. 2018; Nosalski and Guzik 2017). One important alteration accelerating atheroma formation appears to be increased inflammation and infiltration of macrophages into PVAT (Henrichot et al. 2005; Skiba et al. 2017). Supporting this notion, expression of the pro-inflammatory molecule monocyte chemo-attractant protein-1 in transplanted PVAT is a determinant of neointima formation in a wire injury atherosclerosis model (Manka et al. 2014). Furthermore, PVAT-derived tumor necrosis factor- α is a causal factor for mitochondrial ROS production leading to aortic vasoconstriction in obese mice (Menezes da Costa et al. 2017). Inflammation promotes changes in the secretion of paracrine hormones from PVAT. For example, the release of adiponectin is reduced in obese, diabetic mice, leading to impaired insulin-dependent vasorelaxation (Meijer et al. 2013), whereas expression of the adipokine chemerin in PVAT confers vasoconstriction, and this is linked to obesity-induced hypertension (Ferland et al. 2018; Weng et al. 2017). These and several other hormones secreted by PVAT act as paracrine regulators of vascular tone, hypertension, and atherosclerosis (Nosalski and Guzik 2017) and thereby determine the health of arteries both under homeostatic and inflammatory circumstances (Figs. 1 and 2).

3 Therapies Targeting Adipose Tissue with Proven Clinical Efficacy in the Treatment of Atherosclerosis

3.1 Peroxisome Proliferator-Activated Receptor- γ (PPAR γ) Agonists

PPAR γ agonists of the thiazolidinedione (TZD) class are insulin-sensitizing drugs used for the treatment of type 2 diabetes. PPAR γ is a transcription factor critical for adipocyte differentiation, and WAT and BAT are the tissues with the highest expression. The main target for TZD-based diabetes therapy is dysfunctional hypertrophic adipose tissue where TZDs improve insulin signaling and lipid storage while inducing an anti-inflammatory adipokine profile. Together, these actions lead to less ectopic lipid deposition in liver and muscle and improved systemic insulin sensitivity (Yau et al. 2013). TZDs also have anti-atherogenic activity Saremi et al. 2013; Thorp et al. 2007. Whether these protective cardiovascular effects are mediated by PPAR γ expressed in adipocytes, for example, through improved thermogenesis and anti-inflammation (Chang et al. 2018), is unclear as the transcription factor is also expressed and mediates anti-atherosclerotic effects in immune, smooth muscle and endothelial cells (Murakami-Nishida et al. 2019; Subramanian et al. 2010; Qu et al. 2012, Ozasa et al. 2011). Nevertheless, several prospective clinical studies showed an improved plasma lipoprotein profile, reduced carotid artery intima media thickness, fewer cardiovascular disease events, and lower mortality in patients treated with the TZD pioglitazone compared to controls (Hanefeld 2009; Yau et al. 2013, Sartemi et al. 2013). Unfortunately, adverse effects, especially fluid retention, congestive heart failure, and bladder cancer, at least some of them target-related (Yau et al. 2013, Devchand et al. 2018), can offset the desired actions of the drug.

3.2 Niacin

Niacin (nicotinic acid) is a vitamin that, when applied orally at high doses, reduces atherosclerosis and cardiovascular mortality, especially in patients with metabolic syndrome (Superko et al. 2017). One anti-atherosclerotic mechanism of niacin is the improvement of diabetic dyslipidemia, including reduction in triglycerides and small dense LDL as well as the raising of HDL cholesterol (Kühnast et al. 2013). Signaling through the most important niacin receptor HCA2 (GPR109A, HM74) in adipocytes lowers cyclic AMP and reduces lipolysis and, hence, release of FFA. A long-standing hypothesis is that this improves diabetic dyslipidemia, as the decreased FFA flux to the liver entails reduced hepatic VLDL triglyceride secretion (Zeman et al. 2016). This notion has, however, been challenged by a paper describing that in both mice and humans, niacin and synthetic HCA2 ligands acutely lower plasma FFA whereas only niacin reduces plasma triglycerides and LDL cholesterol while elevating HDL (Lauring et al. 2012). Thus, the beneficial effects of niacin on diabetic dyslipidemia are at least in part independent of HCA2-mediated lipolysis. Of note, HCA2 is expressed and mediates anti-inflammatory effects in other artery

wall cell types, in particular macrophages, endothelial cells, and vascular smooth muscle cells (Graff et al. 2016). An important role of macrophages was suggested by a bone marrow transplantation study with LDLR-deficient ($Ldlr^{-/-}$) mice. In this study, niacin was not able to suppress intimal macrophage recruitment and atheroma formation in wild-type acceptor mice that received HCA2-deficient cells, whereas transplantation of HCA2 wild-type hematopoietic cells restored niacin efficacy. This effect was independent of plasma lipid levels (Lukasova et al. 2011). Taken together, niacin has anti-atherogenic properties by improving plasma lipoprotein concentrations especially under diabetic conditions and probably by direct anti-inflammatory effects on immune cells. In addition, niacin may exert anti-atherosclerotic effects through HCA2 by increasing the secretion of adiponectin (Plaisance et al. 2009) and by suppressing pro-inflammatory mediators in adipocytes (Digby et al. 2010).

3.3 Renin-Angiotensin System Blockade

Inhibitors of the renin-angiotensin system (RAS) are widely prescribed drugs for hypertension, known to reduce atherosclerosis in humans and preclinical models, to some degree through their anti-inflammatory activity (Ranjbar et al. 2019). Of note, all components of the RAS are expressed in PVAT (Gálvez-Prieto et al. 2008), and pre-clinical models indicate a role of PVAT RAS in atherosclerosis. For example, angiotensin II was increased in periaortic adipose tissue but not in the circulation or other adipose tissues of $Apoe^{-/-}$ mice with unilateral nephrectomy, a model of accelerated atherosclerosis. Of note, no increase in PVAT inflammatory markers was observed in the nephrectomized mice, and angiotensin receptor blockers (ARBs) reduced atheroma (Kawahito et al. 2013). In another study, aortic transplantation of PVAT from $Apoe^{-/-}$ mice fed a high cholesterol diet increased atherosclerosis in acceptor mice, whereas transplantation of PVAT from angiotensin II type 1 receptor knockout mice or PVAT from ARB-treated mice reduced atheroma (Irie et al. 2015). Taken together, it is plausible that PVAT in part mediates the anti-atherosclerotic efficacy of RAS inhibitors.

4 Novel Therapeutic Targets in Adipose Tissue for Treatment of Atherosclerosis

4.1 Promoting Lipoprotein Disposal and Lipid Storage in Adipose Tissues

WAT is a major site of TRL fatty acid disposal after a meal, and human studies showed that this process is frequently impaired in obese and diabetic subjects (Jacome-Sosa and Parks 2014; Kersten 2014). Improving TRL processing and lipid storage in white adipocytes is, therefore, a suitable approach to reduce plasma triglycerides and hence diabetic dyslipidemia. LPL is the gatekeeper of TRL disposal

in adipose tissue. The activity of the dimeric enzyme is controlled in a complex manner at the level of gene expression, assembly, translocation from adipocytes to the capillary lumen, and interaction of LPL with TRL particles (Kersten 2014). Insulin is the most important positive regulator of LPL activity, whereas angiopoietin-like-4 (ANGPTL4) and APOC3 are prominent negative regulators in adipose tissues (Kersten 2014). APOC3 has attracted a lot of attention in recent years, because plasma concentrations of this abundant apolipoprotein are a major determinant of plasma triglycerides and cardiovascular risk in the human population (Jørgensen et al. 2014; Crosby et al. 2014), explained by APOC3 inhibiting LPL as well as hepatic endocytosis of TRL remnants (Ramms and Gordts 2018; Taskinen et al. 2019). Recently, it became clear that the effect of APOC3 on lipoprotein receptor inactivation seems to be more relevant than its anti-lipolytic action (Gordts et al. 2016), especially as antisense-based reduction of APOC3 substantially lowered triglyceride levels in LPL-deficient patients (Gaudet et al. 2014). In addition to its role in dyslipidemia, APOC3 appears to directly act on arteries and to facilitate subendothelial accumulation of atherogenic particles (Taskinen et al. 2019). Overall, downregulation of APOC3 in the liver efficiently lowers triglycerides, and in part this effect is mediated via TRL disposal in adipose tissue.

ANGPTL4 negatively regulates LPL activity by preventing assembly of LPL and destabilizing the enzyme already during secretion from adipocytes (Dijk et al. 2018). Population-based genetic studies have consistently found reduced plasma triglycerides and reduced coronary artery disease risk in humans with a loss-of-function ANGPTL4 mutation (Bailetti et al. 2018; Stitzel et al. 2016; Dewey et al. 2016). A monoclonal antibody against ANGPTL4 suppressed plasma triglycerides in mice and monkeys (Dewey et al. 2016). Selective knockout of *Angptl4* in brown adipocytes of mice reduced TRL disposal only in BAT (Singh et al. 2018), supporting the notion that targeting ANGPTL4 in adipose tissue can be a means to specifically control organ-specific LPL activity. Importantly, LPL itself is also a drug target. Small molecules that bind to and activate LPL were identified in pharmaceutical screening efforts (Tsutsumi et al. 1993; Geldenhuys et al. 2014).

Another method to increase TRL disposal in adipose tissue is fibroblast growth factor (FGF) 21. FGF21 is an endocrine FGF isoform that fine-tunes systemic glucose and lipid metabolism (Bondurant and Potthoff 2018). At pharmacological doses, it increases insulin sensitivity and lowers blood glucose as well as lipids in mice (Kharitonov et al. 2005). Of note, lowering of triglycerides, but not glucose, was the most prominent effect of FGF21 in phase 1b clinical studies (Gaich et al. 2013; Talukdar et al. 2016). Mechanistic studies in mice showed that pharmacologically administered FGF21 acutely lowers plasma triglycerides and FFA. Tracer studies with labeled lipids demonstrated that this was due to higher uptake into WAT and BAT but not into other organs (Schlein et al. 2016). This effect was observed for both TRL-associated and albumin-bound fatty acids, indicating that FGF21 acts at least in part through stimulating fatty acid transport into adipocytes (Schlein et al. 2016).

An alternative target to boost lipoprotein disposal in adipose tissue is C-X-C chemokine motif receptor-7 (CXCR7). CXCR7 is also known as atypical chemokine

receptor-3, because it is not expressed in leukocytes (Berahovich et al. 2010). *Cxcr7^{-/-} Apoe^{-/-}* mice on Western-type diet had exacerbated hypercholesterolemia and atherosclerosis, whereas the selective CXCR7 agonist CCX771 attenuated plaque formation in *Apoe^{-/-}* mice (Li et al. 2014). CCX771 treatment lowered plasma triglycerides and VLDL cholesterol, which could be explained by increased VLDL clearance in visceral WAT but not BAT or other organs. This depot specifically exhibited increased LPL activity and reduced ANGPTL4 (Li et al. 2014).

Taken together, several targets and therapeutic approaches have been identified for the stimulation of lipoprotein clearance in adipose tissue with the aim to lower the plasma levels of atherogenic lipoproteins.

4.2 Boosting Thermogenic Activation

Activated thermogenic adipose tissue takes up energy at a high rate, and, at least in mice, a majority of calories is provided by TRL (Heine et al. 2018). BAT activation was shown to improve diabetic dyslipidemia, to increase reverse cholesterol transport and reduce atherosclerosis in APOE3-Leiden-CETP mice, an atherosclerosis-prone mouse model with humanized lipoprotein metabolism (Berbée et al. 2015; Bartelt et al. 2017). Reduction in atherosclerosis could be achieved by chronic β 3-adrenergic receptor (β 3-AR) stimulation using a synthetic agonist (Berbée et al. 2015), demonstrating feasibility of pharmacological intervention. Of note, the β 3-AR agonist mirabegron, an approved drug for overactive bladder, activates BAT in humans (Cypess et al. 2015), and chronic dosing with mirabegron induces WAT browning (Finlin et al. 2018). β 3-AR is highly expressed in adipocytes, and cardiovascular side effects of currently available β 3 agonists such as hypertension occur only at high doses and are in part due to cross-reactivity on β 1-ARs (Hainer 2016; Loh et al. 2019). Whether chronic BAT activation or induction of beige adipocytes by β 3-AR agonists is sufficient to improve diabetic dyslipidemia in humans needs to be shown and is currently under investigation. Importantly, boosting thermogenic adipocytes by β 3-AR agonists may counteract atherosclerosis independently of systemic lipoprotein metabolism via local activation of thermogenic adipocytes in PVAT. For example, a recent study demonstrated increased vascular temperature and reduced local inflammation in transgenic mice with higher thermogenic activity in PVAT (Xiong et al. 2017). Given that the common presence of BAT in adult humans has only been recognized recently (Celi 2009), it is likely that drugs directed at thermogenic adipose tissue targets other than β 3-AR, such as adenosine A_{2A} agonists (Gnad et al. 2014), will be developed in the future.

4.3 Targeting Inflammation in Adipose Tissue

Chronic, subclinical inflammation is a hallmark of insulin-resistant WAT in unhealthy obesity. Anti-inflammatory interventions in adipose tissue may slow or prevent the development of atherosclerosis by improving lipoprotein disposal,

normalizing systemic glucose homeostasis, and changing the secretome of adipose tissues in a favorable way. General anti-inflammatory therapies can have beneficial cardiovascular effects, as demonstrated by neutralizing antibodies directed against TNF α that reduce cardiovascular events in rheumatoid arthritis patients (Jacobsson et al. 2005) and raise plasma adiponectin levels (Nishida et al. 2008). Although immune cells are not organ-specific and found throughout the body, positive effects of many anti-inflammation therapies are likely to be at least in part mediated via inflammation in adipose tissue. This is especially true for PVAT that is in close proximity with atherosclerosis-prone arteries and exhibits a profound infiltration of macrophages, T-lymphocytes, and other immune cells during initiation and progression of atherosclerosis (Akoumianakis et al. 2017). Many anti-inflammatory proteins can be targeted in PVAT and have the potential to prevent and reduce atherosclerosis, as suggested by rodent studies. For example, the angiotensin 1–7 analogue AVE0991 that signals through the receptor Mas was demonstrated to selectively suppress inflammation in PVAT and reduce atherosclerosis in *ApoE*^{-/-} mice (Skiba et al. 2017). Receptors of the chemokine RANTES (CCL5) are associated with PVAT inflammation, and *Ccl5*^{-/-} mice are protected from perivascular inflammation (Mikolajczyk et al. 2016), while the RANTES receptor antagonist met-RANTES suppresses atherosclerosis in *Ldlr*^{-/-} mice (Veillard et al. 2004). Similarly, CXCL10 signaling regulates T cell infiltration in atherosclerosis, and genetic deficiency of the CXCL10 receptor CXCR3 (Veillard et al. 2005) or treatment with the CXCR3 antagonist NBI-74330 (van Wanrooij et al. 2008) attenuates atherosclerosis in hypercholesterolemic mice. Several other chemokines appear to play an important role in PVAT inflammation and atherosclerosis (Nosalski and Guzik 2017). Taken together, several promising targets to tackle atherosclerosis by reducing inflammation in adipose tissue depots have been identified in mouse experiments. Whether anti-atherosclerotic approaches targeting adipose tissue inflammation with proof of concept in rodents can be translated into human therapies needs, however, to be shown.

4.4 Hormones Derived from Thermogenic Adipose Tissue

Compared to WAT, BAT and other thermogenic fat depots have a small mass and thus are probably a minor source of most circulating adipokines. However, there are exceptions to the rule, and some hormones are enriched in BAT (Villarroya et al. 2017). Recent studies suggest that some of these hormones are potential targets to prevent or treat atherosclerosis. For example, transplantation of BAT into the visceral cavity of *ApoE*^{-/-} mice led to reduction in atherosclerotic plaque size. This was accompanied by increased plasma FGF21 and adiponectin, and the beneficial effect of BAT transplantation in this study could be blocked by β 3-AR antagonists (Kikai et al. 2018). Many anti-atherogenic activities of FGF21 have been identified in rodents (Jin et al. 2016; Domouzoglou et al. 2015). Therefore, it is plausible that elevated FGF21 mediates at least some of the effects in this model. Of note, adenosine A_{2A} receptor agonists, molecules that activate thermogenic

adipocytes in mice and humans (Gnad et al. 2014), were found to trigger FGF21 expression and secretion from BAT, and this was important to prevent hypertension-induced cardiac hypertrophy (Ruan et al. 2018). Thus, at least in mice, activated BAT is a meaningful source of cardio-protective and anti-atherosclerotic FGF21.

Moreover, the peptide neuregulin-4 and the linoleic acid derivative 12,13-dihydroxy-9Z-octadecenoic acid (diHOME) have recently been described as hormones enriched in and released by activated thermogenic adipocytes. They act in a paracrine fashion to potentiate thermogenic function and appear to exhibit beneficial metabolic functions in the liver and muscle (Nugroho et al. 2018; Stanford et al. 2018; Pellegrinelli et al. 2018; Guo et al. 2017; Lynes et al. 2017; Wang et al. 2014). It is tempting to speculate that these, and other pro-thermogenic hormones secreted by brown and beige adipocytes such as C-X-C motif chemokine ligand-14 (Cereijo et al. 2018), have a protective effect in atherosclerosis, for example, by increasing lipoprotein disposal or by modulating inflammation.

4.5 De Novo Lipogenesis-Derived Lipokines

Adipose tissue is a quantitatively relevant site of de novo lipogenesis (DNL), the endogenous synthesis of fatty acids from non-lipid precursors. One important feature of unhealthy obesity is increased DNL in the liver accompanied by decreased DNL in WAT (Eissing et al. 2013). Elevated hepatic DNL promotes insulin resistance and is a mechanism that worsens diabetic dyslipidemia by increasing triglyceride availability for VLDL production (Scheja and Heeren 2016). How decreased WAT DNL contributes to the development of metabolic disease has long been elusive. Research of the recent years has provided strong evidence that DNL-associated lipids secreted from adipocytes, coined lipokines, are part of the mechanism. The first lipokine to be identified was the FFA variant of palmitoleate (C16:1n-7), a major fatty acid produced by DNL that was found to be reduced in WAT of obese mice. Palmitoleate FFA turned out to be an anti-inflammatory, insulin-sensitizing molecule that improves systemic glucose homeostasis in mice (Cao et al. 2008). Palmitoleate was identified to reduce atherosclerosis via suppressing pro-inflammatory differentiation of macrophages, and *ApoE*^{-/-} mice on a Western-type diet supplemented with palmitoleate had significantly reduced plaque size compared to control mice (Çimen et al. 2016; Yang et al. 2019). Thus, WAT-derived palmitoleate can reduce atherosclerosis risk by modulating inflammation. It is important to note that WAT-derived palmitoleate is not or only marginally lower in obese, insulin-resistant compared to healthy humans (Eissing et al. 2013; Stefan et al. 2010). Nevertheless, dietary palmitoleate supplementation decreased inflammation and lowered LDL in human subjects (Bueno-Hernández et al. 2017; Bernstein et al. 2014), highlighting the anti-atherosclerosis potential of palmitoleate.

Other lipids proposed as adipose tissue-derived DNL-associated lipokines are fatty acid esters of hydroxy fatty acids (FAHFAs), a novel class of lipids (Yore et al. 2014). WAT levels of certain FAHFA species were shown to depend on DNL. Moreover, circulating levels of DNL-linked FAHFA were shown to be tightly

associated with insulin sensitivity in mice and in humans (Yore et al. 2014; Hammarstedt et al. 2018). In addition, oral FAHFA supplementation was demonstrated to improve glucose tolerance in mice (Yore et al. 2014), an FAHFA effect that has, however, been questioned by another group (Pflimlin et al. 2018). More research is warranted, especially with regard to FAHFA synthesis and degradation as well as FAHFA signaling mechanisms to better understand the role of this novel lipid class in metabolic regulation. Furthermore, studies addressing the association of DNL-derived FAHFAs with cardiovascular risk markers are needed to find out whether FAHFAs modulate the development of cardiovascular disease.

5 Future Directions

In the past decades, many adipokines, chemokines, and other regulatory molecules acting on or derived from adipose tissues that affect the development and progression of atherosclerosis have been identified. One major task will be to successfully translate these findings into the clinics. In some cases such as pioglitazone, cardiovascular clinical benefit is evident; however, adverse effects in other organs have precluded more widespread use or even led to the retraction of the drug. Here, adipose tissue targeted delivery using advanced drug delivery technologies could be a solution. Peptides that selectively bind to endothelium in WAT (Kolonin et al. 2004) and BAT (Azhdarinia et al. 2013), respectively, have been identified, and the WAT-selective peptide was successfully used for WAT-specific drug delivery (Xue et al. 2016). Although this WAT-directed drug delivery system is not suitable for oral application, proof of concept that adipose tissue depots can be targeted specifically was delivered.

Methods to achieve adipose-directed delivery of nucleic acids with the goal to overexpress a beneficial protein, or to modulate endogenous RNA levels, have been considerably advanced in the past years. For example, adipose tissue-directed expression of genes, or suppression using small hairpin RNAs, can be achieved by the means of adeno-associated virus vectors (O'Neill et al. 2014). Another promising approach is the delivery of silencing RNAs encapsulated by glucan shells, a method that specifically delivers the regulatory RNA species to WAT macrophages (Aouadi et al. 2013).

An alternative approach to molecular interventions in unhealthy adipose tissues would be the development of regenerative medicines based on bioelectric stimulation devices to increase the sympathetic tone in specific adipose depots or gene therapy and cell transplantation to restore functional adipocytes. Such innovative concepts and highly advanced technologies would provide a novel therapeutic strategy to target obesity-associated cardiovascular disease.

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Microbiome and Cardiovascular Disease

Hilde Herrema, Max Nieuwdorp, and Albert K. Groen

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Abstract

Atherosclerotic cardiovascular disease (ASCVD) is a prime example of a systems disease. In the initial phase, apolipoprotein B-containing cholesterol-rich lipoproteins deposit excess cholesterol in macrophage-like cells that subsequently develop into foam cells. A multitude of systemic as well as

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environmental factors are involved in further progression of atherosclerotic plaque formation. In recent years, both oral and gut microbiota have been proposed to play an important role in the process at different stages. Particularly bacteria from the oral cavity may easily reach the circulation and cause low-grade inflammation, a recognized risk factor for ASCVD. Gut-derived microbiota on the other hand can influence host metabolism on various levels. Next to translocation across the intestinal wall, these prokaryotes produce a great number of specific metabolites such as trimethylamine and short-chain fatty acids but can also metabolize endogenously formed bile acids and convert these into metabolites that may influence signal transduction pathways. In this overview, we critically discuss the novel developments in this rapidly emerging research field.

Keywords

ASCVD · Atherosclerosis · Bacteriophage · Bile acids · SCFA · TMAO

1 Introduction

Atherosclerotic cardiovascular diseases (ASCVD), including coronary heart disease and stroke, are still the main causes of death in the Western world. In the last decades, extensive research programs have attempted to unravel molecular mechanisms underlying these debilitating diseases. Substantial progress has been made particularly in identification of the causal role of low-density lipoprotein (Ference et al. 2017) and triglyceride-rich remnants (Nordestgaard 2016). Although the focal point in research is often directed to cholesterol-containing lipoproteins, the major predictive risk factor for ASCVD is age (Pencina et al. 2019). The vascular aging process that underlies ASCVD risk is a complex process in which lipoproteins and blood pressure interact with the vessel wall and a number of environmental factors exert a secondary influence. One of these factors, which has gained substantial interest in recent years, is the microbiome defined as all microorganisms that colonize the body. Although the vast majority of microbiome studies concentrate on the gut, the oral microbiome may play a significant role in the context of ASCVD development. In this overview, we will critically review recent literature that focuses on the role of the microbiome in ASCVD.

ASCVD is a prime example of a systems disease. The obligate substrate is deposition of cholesterol in the vessel wall. Once depot formation is initiated in the form of foam cells, a host of additional factors such as inflammatory processes further determine disease progression through a plethora of actions. Note that inflammatory processes may also control lipid levels in the blood, thereby exerting control on multiple steps in development of atherosclerosis. Until recently, most of the information about the importance of inflammation in ASCVD came from studies in animal models. The results of the CANTOS trial, which showed a beneficial effect on ASCVD events through antibody-mediated inhibition of interleukin-1 β , have added critical insight in the role of inflammation in ASCVD development in

humans (Ridker et al. 2017). Inflammation is an extremely complex process by itself, and it is therefore not surprising that novel inflammation-modulating factors pop up continuously as potential ASCVD influencers. Yet, the onset of the disease requires lipid disposition mediated primarily by apolipoprotein B (apoB)-containing lipoproteins. The dominant role of these lipoproteins was nicely exemplified by Mendelian randomization studies that make use of natural mutations in the genes encoding lipoproteins (reviewed in Holmes et al. 2017). This enabled studies into the effect of variations in circulating cholesterol carriers on ASCVD risk (Ference et al. 2017). In addition, LDL-C lowering trials using statins with or without ezetimibe or PCSK9 inhibitors have provided critical insight in the role of cholesterol carriers and ASCVD risk (Wright and Murphy 2016). Interestingly, there is linear relation between LDL-C levels and cardiovascular events in which zero disease risk was associated with circulating LDL-C levels of around 1 mM (Wright and Murphy 2016). This suggests that at this level, the risk of cardiovascular events is close to zero and no disposition of lipid in the vessel wall will occur. In the general population, however, the concentration of apoB-containing lipoproteins is much higher than 1 mM, and some degree of atherosclerotic plaques development is almost inevitable. Indeed in the 1960s of the last century, studies in young soldiers that died in the Korean and Vietnam wars demonstrated early signs of atherosclerotic plaque formation as early as 25 years of age in about 5% of the soldiers (Virmani et al. 1987). Fortunately, certainly at ages lower than 60 years, most atherosclerotic plaques are not symptomatic. A majority of current research initiatives therefore focuses on processes that induce vulnerability of the plaque to rupture, one of the main causes for acute coronary syndromes.

In short, lipid deposition in the vessel wall and complex inflammatory processes are critical for development of ASCVD. In addition, aging and environmental factors such as smoking and diet play an important role herein. More recently, both oral and gut microbiome have entered the spotlights. Before considering the role of the microbiome in ASCVD development in detail, we will first give an update on recent oral and gut microbiome literature and focus on host-microbiome interactions relevant for progression of ASCVD.

2 Gut and Oral Microbiome Communities: Potential Drivers of ASCVD?

The gut and oral microbiome are the first and second, respectively, largest and most complex communities of microorganisms in the human body and comprise bacteria, fungi, viruses, archaea, and protozoa. It is critical to point out that the vast majority of publications on the role of the gut and oral microbiome in human health and disease are heavily biased toward bacterial members of this community. The upcoming awareness of the critical role of other members of the microbiome to composition and function of the community – and thereby contribution to human metabolism – is likely to change this bias in the coming decade. We will first elaborate on the bacterial component of the (gut and oral) microbiome and their

postulated role in ASCVD development. Mechanistically, both the gut and oral microbiome are currently considered to affect human metabolism and ASCVD by interaction with the host immune system (gut and oral) and by conversion of dietary components into hormone-like signals or biologically active metabolites (gut) (Fig. 1).

The gut microbiome (estimated number of species >1,000, 1.5–2 kg per person) is a critical component of digestion, maintenance of gut barrier function, and immunomodulation. The predominant bacterial phyla are *Bacteroidetes* and *Firmicutes*, with *Actinobacteria*, *Proteobacteria*, and *Verrucomicrobia* being less abundant (Shetty et al. 2017). Methanogens are dominant among the archaea (Paterson et al. 2017). The vast majority of gut bacteria are shared among individuals at higher taxonomic levels (phylum). However, interindividual variation at lower taxonomic levels (species, strain) is very high. Alterations at this level, in particular reduced number and diversity of bacterial genes, have been associated with metabolic diseases including ASCVD (reviewed in Aron-Wisniewsky and Clément 2016; Liu et al. 2019; Tilg et al. 2019).

ASCVD risk factors have been reported to associate with the gut microbiome; for example, *Clostridiales* and *Clostridium* spp. correlate negatively with C-reactive protein, an inflammatory marker (Karlsson et al. 2012). The gut microbiome of ASCVD patients has been associated with decreased abundance of gut commensals such as *Bacteroidetes* (incl. *Bacteroides* and *Prevotella*) compared to healthy controls (Emoto et al. 2016). A metagenome-wide association study (Jie et al. 2017) in fecal samples of ASCVD patients and healthy controls reproduced findings on the reduced abundance of *Bacteroidetes* in ASCVD patients and further reported that these patients had reduced abundance of presumably beneficial short-chain fatty acid-producing bacteria such as *Roseburia intestinalis* and *Faecalibacterium prausnitzii*. Conversely, the microbiome of ASCVD patients was enriched in species belonging to the *Enterobacteriaceae* family, which are oftentimes associated with gut microbiome dysbiosis and (metabolic) disease development. Interestingly, the relative abundance of bacteria typical for the oral cavity, in particular *Streptococcus* spp., was also higher in the gut microbiome of patients with ACVD compared to healthy controls.

With over 700 bacterial species, the oral cavity comprises the second largest and most diverse microbiome community after the gut in the human body. These species are located in a plethora of very complex niches including the hard surfaces of the teeth as well as the soft mucus linings of the mouth. The mouth is the major entrance point to the human body. Bacteria from the oral cavity, in particular those associated with oral infectious diseases and adapted to thrive in an inflammatory environment (e.g., caries (tooth decay) and periodontitis (gum disease)), have been associated with “off-site” effects on systemic diseases such as ASCVD (Hajishengallis 2015).

Although the number of studies that have associated differences in gut and oral microbiome composition with ASCVD is plentiful, it is important to point out that data on a causal role for the gut microbiome in ASCVD development in humans is more difficult to find. The strongest evidence for a causal role of the gut or oral microbiome in progression of ASCVD has been derived from animal studies,

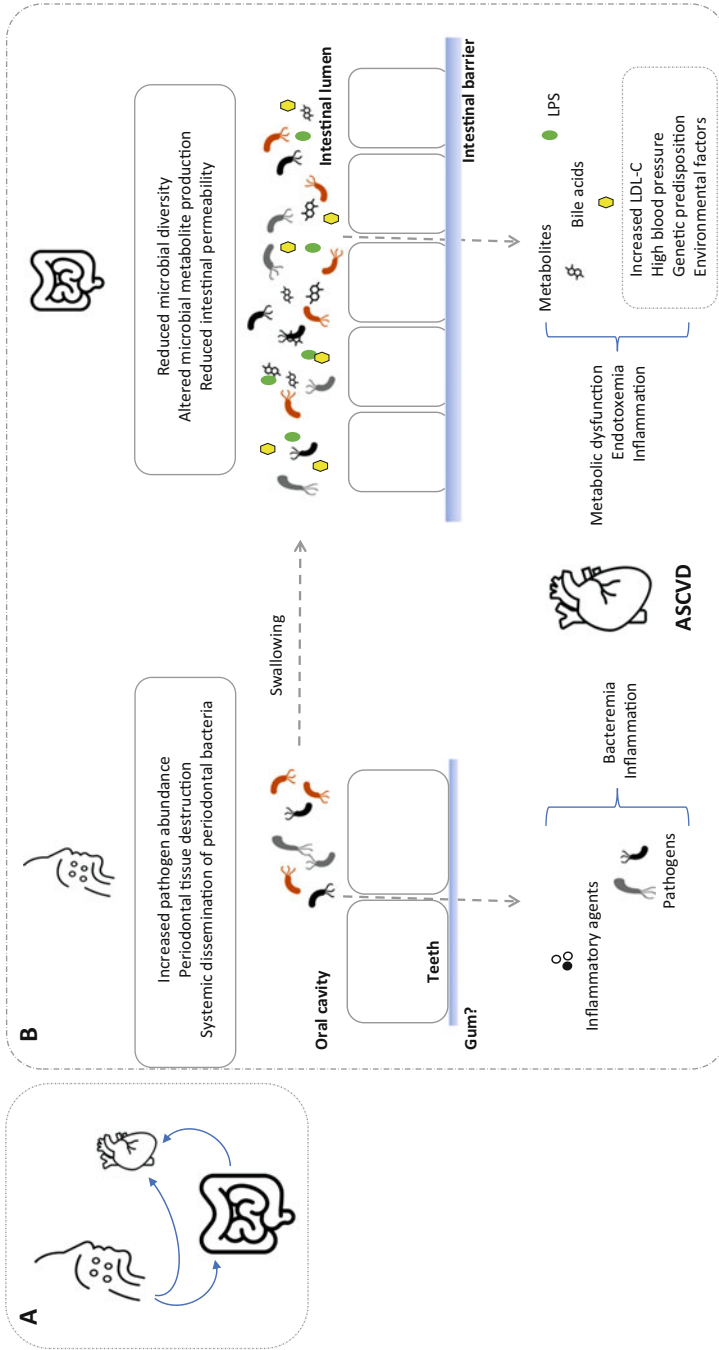


Fig. 1 Oral and gut microbiome have been implicated in the development of ASCVD (a). Increased pathogen abundance in the oral cavity, such as during periodontal disease, might reach the circulation and contribute to low-grade inflammation, a recognized risk factor for ASCVD. In addition, continuous swallowing of bacteria from the oral cavity has been suggested to alter gut microbiome composition, thereby contributing to ASCVD development (b). Please see text for details

Table 1 Overview of bacterial challenge studies carried out in germ-free mouse models

Mouse model	Diet + intervention(s)	Findings	Reference
ApoE ^{-/-} (gnotobiotic)	Chow, <i>Roseburia intestinalis</i> administration	Lesions and inflammation ↓ in <i>R. intestinalis</i> vs control	Kasahara et al. (2018)
ApoE ^{-/-} (GF and CONV-R)	Chow, HFD, choline supplementation (both diets)	Chow: lesions and CH ↓ in CONV-R vs GF HFD: lesions and CH = in CONV-R vs GF Choline suppl: TMAO ↑, lesions = in CONV-R vs GF	Jonsson et al. (2018)
ApoE ^{-/-} (GF and CONV-R)	Chow	Lesions, LPS, and inflammation ↑ in CONV-R vs GF LDL-C ↓ in CONV-R vs GF	Kasahara et al. (2017)
ApoE ^{-/-} (GF by abx)	Chow without/with choline	Lesions, CH, and foam cells ↓ in choline-abx vs choline-no abx	Wang et al. (2011)
ApoE ^{-/-} (GF and CONV-R)	Chow without/with cholesterol	Chow without CH: lesions ↓ in CONV-R vs GF Chow with CH: lesions = in CONV-R vs GF	Stepankova et al. (2010)
ApoE ^{-/-} (GF and CONV-R)	HFD	Lesions = in CONV-R vs GF	Wright et al. (2000)

in particular mouse models (Table 1). Germ-free (sterile) apolipoprotein E-null (ApoE ^{-/-}) mice were shown to have increased atherosclerotic plaques compared to conventionally raised counterparts when fed a chow diet (Stepankova et al. 2010). These data were confirmed by Lindskog et al. but only with respect to the chow diet (Jonsson et al. 2018). Conversely, when germ-free ApoE ^{-/-} mice were fed a high-fat/high-cholesterol diet, the absence of microbiota increased atherogenesis, but the extent was still reduced compared to conventionally raised mice (Jonsson et al. 2018). However, there is some controversy around these data because Kasahara et al. reported decreased atherosclerosis in germ-free ApoE ^{-/-} mice on a chow diet (Kasahara et al. 2017). Since chow diets are not well characterized and composition differs from batch to batch, the apparent discrepancy may well be caused by subtle changes in the diet used in the different studies.

Convincing data for a causal role of the microbiota in ASCVD development came from fecal microbiota transplantation (FMT) studies showing that atherosclerosis was induced when FMT was carried out with feces derived from mice with proven atherosclerosis (Gregory et al. 2015). The question arises which factor is responsible for the atherosclerosis aggravating effect induced by the microbiome. Activation of inflammatory signaling pathways by the gut microbiome, or components thereof, has received a lot of attention in the past decade. This is exemplified by observations that transplantation of a pro-inflammatory microbiome into atherosclerosis-prone LDLR^{-/-} mice accelerated phenotype development compared to LDLR^{-/-} mice receiving a control microbiome (Brandsma et al. 2019). Translocation of lipopolysaccharide (LPS)

across the intestinal wall into the blood seems a good mechanistic candidate. LPS is the major molecular component of the outer membrane of gram-negative bacteria, the most abundant bacteria in the gut (Raetz and Whitfield 2008). The lipid A component of LPS is a pathogen-associated molecular pattern, which activates Toll-like receptor 4 (TLR4) (Aderem and Underhill 1999). High-fat diet has been shown to increase gut permeability in mice. This may enhance the translocation of LPS into the circulation thereby inducing metabolic endotoxemia (Cani et al. 2007). In line, germ-free mice were demonstrated to be resistant to high-fat diet-induced insulin resistance and obesity (Rabot et al. 2010). Whether these results in mice can be translated to humans remains to be established.

Identification of atherosclerotic plaque-associated bacteria is suggestive of a direct role in plaque progression (Koren et al. 2011; Jonsson et al. 2017). Interestingly, many of these bacteria were also localized in the oral microbiome of patients with atherosclerosis. Indeed, a close association between periodontitis and ASCVD risk has been reported in a number of studies (Hajishengallis 2015). Gingival bleeding caused by periodontitis offers oral bacteria an easy entry into the circulation where they can attach to the atherosclerotic plaque. Whether they stay alive when bound to the plaque is not clear. As far as we are aware, no live bacteria have been cultured from plaques obtained during surgery. Yet, upon entry into the blood, the orally derived bacteria may be capable of activating endothelial cells, possibly leading to expression and secretion of metalloproteinases that in turn may decrease plaque stability. The activity of periodontitis has been shown to be reflected in systemic inflammation. This is important given the role of inflammation in the pathogenesis of ASCVD. Plasma levels of the inflammatory marker C-reactive protein (CRP) have been correlated with periodontitis status (Noack et al. 2005; Yoshii et al. 2009) as well as the pro-inflammatory cytokine IL-6 (Loos et al. 2005). These immune modulators may be either produced locally in the oral environment and subsequently secreted into the circulation or arise as a result of low-grade, short-lived bacteremia (Torres De Heens et al. 2010). Given the high prevalence of periodontitis in the adult population, treatment of this disease may be an important modality to reduce the incidence of ASCVD (Lobo et al. 2019).

Summarizing these studies, it seems fair to conclude that activation of an inflammatory pathway is a reasonable way via which bacteria may promote progression of ASCVD. Whether the prokaryotes that enter the circulation via the oral cavity or gut play a role in affecting plaque stability has not yet been shown. In a study in mice, Jonsson et al. (2017) did not find differences in bacterial content between stable and labile plaques. However, activation of the inflammatory component of ASCVD may not be the only way by means of which the microbiota exert influence.

2.1 Other Microbiome Community Members

Although *bacteria* and *archaea* indeed account for >99% of microbiome mass (Shkoporov and Hill 2019), it should be realized, however, that both the

oral and gut microbiome contain vast numbers of viruses, fungi, and – in most humans – protozoans. Although many of these less abundant community members have been linked to human disease (Hoffmann et al. 2013; Huseyin et al. 2017; Paterson et al. 2017; Laforest-Lapointe and Arrieta 2018), surprisingly little is known about trans-kingdom community-level interactions and the consequences thereof for human health. In order to deepen our understanding about the complexity of host-microbial interactions, it will be critical to address such interactions in the relevant ecosystem (e.g., the gut or oral cavity).

2.1.1 Viruses and Bacteriophages

Bacteriophages (phages), viruses of bacteria, are of particular interest because of their proven role in shaping microbial communities in many ecosystems (Fernández et al. 2018; Warwick-Dugdale et al. 2019). Furthermore, phages are abundantly present in the gut (estimated 1:1 ratio with bacteria), either as free phage or integrated into the bacterial genome as prophage (Reyes et al. 2012; Walk et al. 2016; Carding et al. 2017).

The many studies that have described a strong association of specific bacterial strains with ASCVD generally characterize the phylogenetic core, dynamics, and stability of the bacterial ecosystem by high-throughput 16S ribosomal RNA gene sequencing-based approaches. This precludes identification of integrated bacteriophage DNA in the bacterial DNA (see for recent reviews (Shetty et al. 2017; Hornung et al. 2018; Falony et al. 2019)). Increasingly accessible and affordable shotgun and long-read nanopore sequencing approaches together with rapidly emerging computational tools to unravel novel phage genomes are now rapidly solving part of the challenges phage researchers have faced in the past. These include the fact that the majority of gut bacteria (phage hosts) are strict anaerobes and thereby extremely difficult to culture. This has, until recent years, limited researchers to microscopic characterization of phages. The current collection of known gut phages therefore is a vast underrepresentation of the gut phageome. Interestingly, a healthy gut status in humans has been shown to mainly comprise integrated phages (Reyes et al. 2010; Minot et al. 2011), whereas cases of intestinal bowel disease have been associated with higher levels of free phages (Norman et al. 2015; Duerkop et al. 2018). Prophage integration has been shown to affect bacterial fitness and metabolic function in the gut (Duerkop et al. 2012; Hsu et al. 2019; Oh et al. 2019). Moreover, evidence for a direct role of phages in activation of the mammalian immune system, a critical element of ASCVD development, has recently been put forward (Gogokhia et al. 2019; Sweere et al. 2019). In line with long-standing observations that bacteriophages are able to pass the intestinal wall to enter the bloodstream, at least in experimental settings (Van Belleghem et al. 2019), these results support the urgency to carefully look into these viral members of the microbiome community in the gut and beyond. Implications for phages as modulators of (immune)metabolism are yet to be confirmed, but we predict this will be highly relevant for studies addressing the role of the microbiome in human ASCVD development.

3 Microbiome-Derived Metabolites

Gut bacteria are also considered to modulate human metabolism by production of small molecules including conversion of dietary components into hormone-like signals or biologically active metabolites (Fig. 2). It has been estimated that about 10% of the small molecules in the circulation are derived from the gut microbiome (Holmes et al. 2012). This estimate can very well be an underestimation because

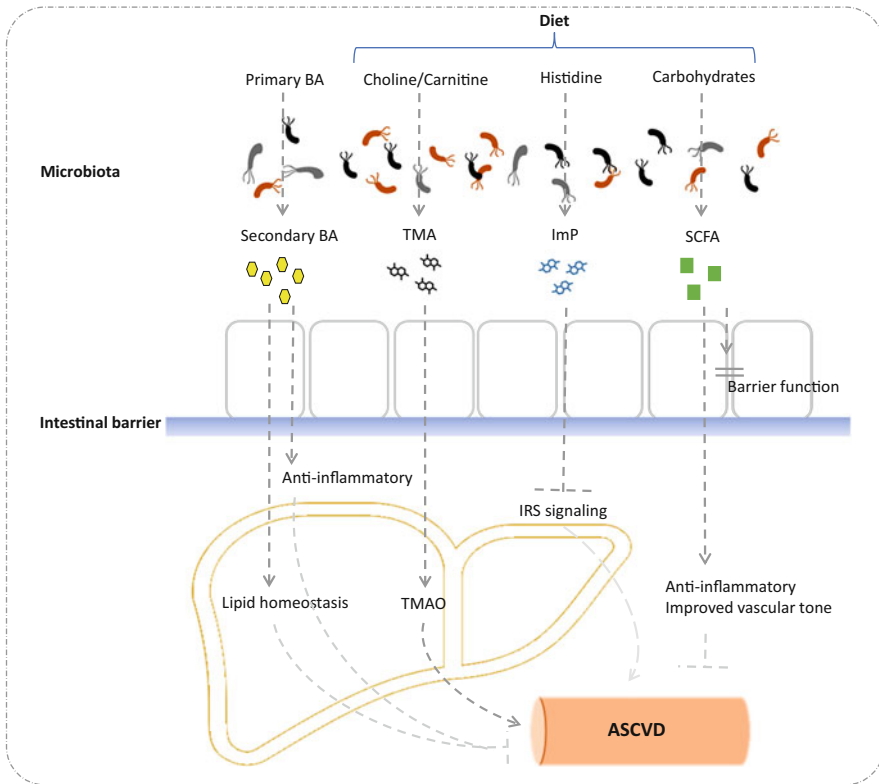


Fig. 2 Gut microbial metabolites have been implicated in ASCVD development. Primary bile acids are produced by the liver after which a small percentage is converted to secondary bile acids by colonic bacteria. Although evidence from human trials addressing if secondary read outs associated with ASCVD (e.g., inflammation and LDL-C levels) have been reported to be reduced by bacteria capable of converting primary to secondary bile acids. TMA is produced by the microbiota from choline or carnitine precursors. In the liver, TMA is converted to TMAO which has been extensively linked to ASCVD development. ImP is a microbial metabolite of histidine. ImP directly inhibits insulin receptor-mediated signaling thereby leading to insulin resistance, a significant risk factor for the development of ASCVD. Whether ImP indeed affects ASCVD development is yet to be established. SCFA is a microbial fermentation product of complex carbohydrates. Many health benefits have been addressed to SCFA and include improved gut barrier function and reduced inflammation. Please see text for details

despite the major advances in development of metabolomics in the last decade, most circulating metabolites whether endogenous or microbial have not yet been identified.

3.1 TMAO

A wonderful example of how a bacterial metabolite can be identified to exert effect on ASCVD comes from the studies of the Hazen group in Cleveland Clinic (Zhu et al. 2017; Koeth et al. 2019; Wu et al. 2014). They demonstrated that nutrients such as choline and carnitine can be converted to trimethylamine (TMA) by gut bacteria that express TMA lyases. The gas TMA subsequently diffuses from the intestine into the circulation and is converted to trimethylamine N-oxide (TMAO) in the liver by the hepatic enzyme flavine monooxygenase 3 (FMO3) (Brown and Hazen 2015). TMAO activates atherosclerosis in mouse models, and TMAO plasma levels have been shown to correlate with incidence of cardiovascular disease in humans in a number of studies (Zhu et al. 2016, 2017). Several other studies, however, failed to show this correlation (Heianza et al. 2017; Kaysen et al. 2015; Mueller et al. 2015; Aldana-Hernández et al. 2019). Zhu et al. showed that TMAO may exert its action via influencing blood platelet hyperresponsiveness and thrombosis, which provides a mechanistic link between TMAO and cardiovascular risk (Zhu et al. 2016, 2017). Recently, Chen et al. showed that TMAO may induce ER stress (Chen et al. 2019). Unravelling the metabolic pathways involved in TMAO metabolism provides a beautiful example how the interaction between microbial activity and host metabolism can be elucidated. By developing specific inhibitors of TMA lyases, the Hazen group (Koh et al. 2018) may have produced the tools to treat patients at risk for ACSVD due to increased TMAO (Wang et al. 2015). In the original paper in which the Hazen group introduced the TMAO pathway, additional ASCVD-associated peaks in the MS spectra were observed (Wang et al. 2011). Characterization of these putative metabolites has not yet been published, but it seems justified to suggest that there is more to come.

3.2 Imidazole-Propionate

Metabolites produced by microbial metabolism of aromatic amino acids are good candidates. Recently the histidine derivative imidazole-propionate (ImP) has been linked to insulin resistance in humans. By detailed analysis of portal blood obtained from obese diabetic patients compared to “healthy” (nondiabetic) obese controls, the group was able to single out ImP as one of the compounds strongly increased in portal blood from the diabetics (Koh et al. 2018). The group of Backhed subsequently characterized the molecular mechanism of action in great detail (Koh et al. 2018). Extensive mechanistic studies in mice revealed that ImP impairs insulin signaling via p38 protein kinase. Identification of ImP is very recent, and its

putative role in ASCVD has not been investigated yet. Since ASCVD is an almost inevitable comorbidity of diabetes, an aggravating effect of IMP on ASCVD may be expected to be published in the near future.

3.3 Short-Chain Fatty Acids

The short-chain fatty acids (SCFAs) butyrate, propionate, and acetate are produced from colonic fermentation of complex fibers by the gut microbiota. SCFAs are the main product of the digestive actions of the gut microbiota making them interesting candidates in the quest for microbial-derived metabolites influencing ASCVD. Lower levels of SCFA or SCFA-producing bacteria have been correlated with arterial stiffness, high blood pressure, and related end-organ damage (Pluznick 2013; Kim et al. 2018; Menni et al. 2018). Despite the abundant literature on diverse aspects of SCFA, metabolism insight in their impact on ASCVD in humans is limited to association studies. However, interesting effects have been observed in studies in animal models. A case in point is the recent study of Kasahara et al. in *Nature Microbiology* (Kasahara et al. 2018) that focused on the ameliorating action of butyrate on atherosclerotic plaque progression in ApoE $-/-$ mice. Germ-free ApoE $-/-$ mice were first colonized with a mixture of eight low butyrate-producing bacterial strains. Subsequently, mice were inoculated with the high butyrate-producing strain *Roseburia intestinalis* as well. This led to a significant reduction in atherosclerotic plaque size (compared to controls). Interestingly, butyrate, whether added to diet or produced by the bacteria, had no effect on plasma cholesterol or TMAO levels. The observed beneficial effect on plaque progression seemed mainly due to a tightening of gut barrier function which potentially reduces translocation of LPS in this animal model. This local effect of butyrate makes sense because this particular SCFA is almost completely metabolized by the colonic enterocytes. Besides this putative effect on gut barrier function, which clearly requires confirmation, SCFA have been implicated in modulation of inflammatory processes (Ohira et al. 2017). The G protein-coupled receptors GPR41 and GPR43 serve as SCFA receptors and have been shown to elicit intracellular signal transduction cascades mediated by mitogen-activated protein kinases (MAPKs) and protein kinase C (PKC) (den Besten et al. 2013). In addition, butyrate has been shown to inhibit histone deacetylases, thereby altering the acetylation state of histones and other proteins which may induce epigenetic changes in gene transcription (Vinolo et al. 2011). A direct effect of SCFAs on expression of COX1 and 2 and hence possibly on eicosanoid production, important regulators of inflammatory processes, has also been proposed (Nurmi et al. 2005; Al-Lahham et al. 2010).

Most studies aiming at increasing insight into the molecular mechanism via which SCFA exert influence on inflammatory processes derive from in vitro experiments with cultured cells or tissues (Vinolo et al. 2011). Butyrate and propionate have been shown to affect neutrophil function by increasing apoptosis via a caspase-dependent pathway (Aoyama et al. 2010). A problem

with most *in vitro* studies is that supraphysiological concentrations are used to show the effects making translation to the human situation difficult. Furthermore, acetate, butyrate, and propionate sometimes exhibit contrasting effects (Cavaglieri et al. 2003) leading to controversy on their modes of action. It can, however, not be excluded that the SCFA concentration required to elicit an anti-inflammatory response varies with the type of inflammation (Al-Lahham et al. 2010).

Confirmation of the anti-inflammatory properties that are often associated with SCFA comes from animal experiments. In ApoE knockout mice, feeding with butyrate reduced atherosclerotic lesions and lowered macrophage migration accompanied by a decrease in pro-inflammatory cytokines (Aguilar et al. 2014). In mice treated intraperitoneally with acetate, inflammatory processes after kidney injuries were decreased leading to attenuation of the detrimental effects of inflammation on renal function (Andrade-Oliveira et al. 2015). Conversely, after systemic administration of supraphysiological doses of SCFA, renal tissue inflammation was increased due to dysregulation of T-cell response (Park et al. 2016). In another study in mice, SCFA receptors GPR41 and GPR43 were found to be required for an inflammatory response to bacterial infection and thus a protective pro-inflammatory response (Kim et al. 2013). In rodent models of colitis, oral acetate administration was shown to be protective (Masui et al. 2013).

As far as we are aware, no outcome trials have been carried out focusing on the effect of SCFA on ASCVD. However, a few human studies looked at the effect of SCFA on inflammatory aspects. A recent study investigated the effect of colonic infusions of SCFA, in concentrations found in the gut, on fasting levels of cytokines in overweight and obese subjects. The pro-inflammatory cytokine IL-1 β decreased with a high acetate (60%) containing SCFA mixture compared to placebo and was significantly lower compared to a SCFA mixture containing high propionate (35%). Postprandial IL-1 β levels as well as other pro-inflammatory cytokines including TNF- α , IL-6, and IL-8 did not change in the obese subjects neither in the fasting nor in the postprandial period (Canfora et al. 2017). In the study by van der Beek et al. (2016), a tendency for lower fasting plasma TNF- α concentrations was found after distal colonic acetate infusion with a 100 mmol/L yet not with a 180 mmol/L, as well as after proximal colonic acetate infusion.

3.4 Other Microbiome-Produced Metabolites Associated with ASCVD

A number of studies have appeared recently that aimed to identify bacteria as well as metabolites associated with different stages of cardiovascular disease (Wang et al. 2019; Würtz et al. 2015; Kurilshikov et al. 2019; Liu et al. 2019). Using a metagenomics approach, Kurilshikov et al. could link metabolic pathways encoded in the various bacteria to ASCVD risk but assessed risk directly in only one of the studied cohorts by measuring carotid IMT. In the other cohorts, a metabolic risk score was calculated from 33 established ASCVD biomarkers. An advantage of the metagenomics approach is that functional relations between

ASCVD risk and microbial pathway can be identified. This is important because many bacterial strains share metabolic pathways. ASCVD risk is associated strongly with pathways involved in amino acid metabolism (Newgard 2017). Metabolomics was investigated in this study using the NMR-based Nightingale platform which focuses mainly on lipoproteins, and the expected relations between ApoB-containing lipoproteins and ASCVD risk were observed. Using a multi-omics approach, in which state-of-the-art metabolomics was combined with 16S rRNA sequencing, a number of metabolic pathways and co-abundant bacterial groups were identified to associate with ASCVD severity (Liu et al. 2019). Although ASCVD severity was determined using coronary angiography, which enables accurate diagnosis of the extent of plaque formation, this limits the number patients that can be studied. By grouping bacteria by co-abundance, functional properties of these groups could be predicted linking ASCVD risk to taurine, sphingolipid, ceramide, and benzene metabolism. Identification of xenobiotics links environmental variables directly to microbiota and host metabolism which could lead in future studies to identification of molecular mechanisms.

4 Bile Acids

The most important endogenous molecules that undergo microbial modifications are the family of bile acids (BA).

4.1 Bile Acid Metabolism

These molecules are produced exclusively by the liver via two pathways that start separately but fuse after four steps to share most of the subsequent steps in the parts that produce the primary bile acid chenodeoxycholic acid (Kuipers et al. 2014). The so-called classic bile acid synthesis pathway starts with the conversion of cholesterol into 7- α -cholesterol catalyzed by the enzyme 7- α -hydroxylase and produce either cholic acid or chenodeoxycholic acid. In humans, this has been postulated to be the major pathway, but this hypothesis requires experimental validation. After synthesis is completed via a complex pathway consisting of enzymatic steps in the cellular cytosol as well as mitochondria, the molecules are conjugated in peroxisomes with either glycine or taurine (Russell 2003; Chiang and Ferrell 2019). In humans the ratio glycine/taurine is mostly around 3; rodents predominantly conjugate with taurine (Kuipers et al. 2014). Another substantial difference between rodents and humans is the fact that rodents convert the hydrophobic chenodeoxycholic acid into the very hydrophilic muricholic acids. This completely alters BA function and precludes direct translation of rodent data to humans (Kuipers et al. 2014).

In mice and man, BA are stored in the gallbladder and are expelled into the duodenum primarily after initiating intake of food (Behar 2013). The consensus is that sensors in the small intestine register arrival of fat and protein and activate

gallbladder contraction through release of cholecystokinin, although also in the absence of food regular small contraction of the gallbladder must occur to maintain BA concentrations observed in the circulation (Sips et al. 2018). BA arriving in the terminal ileum are extremely efficiently absorbed via the sodium-dependent bile acid transporter (ASBT or SLC10A2) (Hagenbuch and Dawson 2004). Bile acids are highly toxic for bacteria which is probably an important reason why the small intestine is sparsely colonized relative to the colon. Depending on small intestinal motility and the bile salt hydrolase activity of the microbiota colonizing the small intestine, a small amount of bile acids enters the colon and is metabolized into a myriad of so-called secondary or more recently tertiary bile acids. The degree of metabolism strongly depends on the colonic microbiota composition of a given subject (Ridlon et al. 2006).

Secondary bile acids are hydrophobic and consequently highly toxic for bacteria; apparently bacteria that are able to dehydroxylate bile acids have evolved to create a toxic environment for their neighbors. Because of the fact that the secondary bile acids are hydrophobic, they can passively diffuse across the colonocyte cell membranes and enter the bloodstream. Whether this process is purely diffusion or whether transporters are also involved is not known. In humans, it is estimated that about 5% of the bile acid pool is not reabsorbed and is excreted via the feces. The variability may in part be caused by changes in absorptive capacity which might directly influence the risk on ASCVD. Note that although only 5% of the bile acids escapes the enterohepatic circulation, bile acid excretion is the major pathway for cholesterol export from the body, apart from neutral sterol excretion. Aging correlates negatively with bile acid synthesis (Einarsson et al. 1985); thus also cholesterol excretion via this route decreases with age pointing to a possible causal relation between BA excretion and ASCVD. The plasma concentration of taurocholate has been found to negatively correlate with longevity (Cheng et al. 2015). This could be due to increased absorptive capacity possibly increasing with age and also accounting for the decrease in synthesis, but this still has to be addressed experimentally. One report has described a negative correlation between bile acid synthesis rates and ASCVD events (Charach et al. 2018). Though highly interesting this study requires confirmation.

4.2 Regulation by Bile Acids

Besides the direct role of BA in cholesterol metabolism, they control diverse metabolic pathways via membrane and nuclear hormone receptors signaling. Particularly G-protein linked receptor TGR5 and the farnesoid X receptor (FXR) are important in this respect. Both receptors show a great preference for the more hydrophobic bile acids; hence microbial metabolism plays a major role in regulating BA control of metabolism.

The role of FXR in controlling progress of ASCVD is ambiguous. Hanniman et al. reported increased atherosclerosis development in FXR/ApoE double KO mice (Hanniman et al. 2005), whereas two other studies reported that loss of FXR

in low-density lipoprotein receptor $-/-$ (LDLR) mice and ApoE $-/-$ mice reduced atherosclerotic lesion size (Guo et al. 2006; Zhang et al. 2006). Although differences in gut microbiota composition and sex of the mouse models used may play a role, the exact nature of these discrepancies is unclear. In contrast, FXR stimulation with the FXR agonists PX20606 and WAY-362450 did prevent atherosclerotic plaque formation in ApoE KO, LDLR $-/-$, or CETP transgene LDLR $-/-$ models (Hartman et al. 2009; Hambruch et al. 2012). Additionally, FXR stimulation modulates inflammatory responses, thereby reducing pro-inflammatory cytokine production. The first FDA-approved FXR agonist obeticholic acid (OCA) is currently tested in human trials (Neuschwander-Tetri et al. 2015). Unexpectedly, OCA induced an increase in LDL cholesterol and a concomitant decrease in HDL-C (Nevens et al. 2016). The underlying mechanism is not clear, and the effect on ASCVD has not yet been assessed; but the induced phenotype makes OCA a less attractive option to treat atherosclerosis in humans.

The other important BA receptor, TGR5, has a high affinity for secondary BA in particular lithocholate (Klindt et al. 2015). TGR5 stimulation activates thyroid hormone deiodinase 2 which converts inactive thyroxine (T4) into active 3,5,3'-triiodothyronine 12 (T3) and stimulates energy expenditure (Watanabe et al. 2006). Interestingly, TGR5 activation has immunosuppressive effects. TGR5 has been shown to reduce cytokine expression via inhibition of nuclear translocation of NF- κ B (Pols et al. 2011; Yoneno et al. 2013). Furthermore, TGR5 activation with INT-777 inhibits the inflammasome, a major driver of the inflammatory component of ASCVD progression (Hao et al. 2017). In addition to its effects on immune cells, TGR5 also effects metabolism in endothelial cells (Keitel et al. 2007) where it may control nitric oxide (NO) production, through phosphorylation of endothelial nitric oxide synthase (eNOS) (Kida et al. 2013). The immunomodulatory functions of TGR5 make this receptor an interesting target to treat atherosclerosis, and because of its high affinity for lithocholic acid and deoxycholic acid, it may explain beneficial effects of the gut microbiota in ASCVD. So far, the effects of specific TGR5 agonists have only been studied in animal models; hence it is not clear whether the results can be translated to humans.

5 Summary and Future Perspectives

The etiology of ASCVD starts simple with disposition of lipids in the vessel wall but develops into an extremely complex myriad of aggravating and inhibiting factors when it progresses. Because so many factors are involved, it seems justified to assume that ASCVD develops in a unique way in any single patient. Up to now treatment of ASCVD mainly focuses on inhibiting the initiating factor, disposition of lipid in the vessel wall. Although perhaps successful in inhibiting progress of the disease, it does not induce regression of the plaques to a significant extent. Attempts to induce plaque regression have been very unsuccessful so far. Enhancing cholesterol efflux through increasing plasma HDL concentration has not worked

probably by a lack of understanding of the molecular mechanism of cholesterol efflux *in vivo*.

The question arises whether influencing the composition of the microbiome can help. As discussed in this review, the bacterial component of the microbiome can influence the process of ASCVD development at many different stages. Particularly, the oral microbiome can easily invade a patient suffering from a very common periodontitis. This causes a systemic inflammatory response potentially aggravating atherosclerotic plaque progression.

Bacteria can initiate production of harmful molecules such as TMA or ImP that are likely to contribute to ASCVD development. Since many bacterial species are capable of TMA or ImP production, it is challenging to develop strategies to, e.g., eradicate these bacteria. Antibiotics treatment has been shown to reduce TMAO production in humans (Craciun and Balskus 2012). However, it is critical to point out that there are many objections to using antibiotics as means to intervene in microbiome-mediated cues to ASCVD development. These include significant consequences of antibiotic use for the gut microbial community, risk to develop antibiotic resistance, and the fact that antibiotics use has been associated with increased progression of ASCVD (Heianza et al. 2019).

Early initiatives aiming to specifically reduce production of TMA (instead of the bacterium) have shown promising results in lowering TMAO levels and ASCVD risk, at least in mice. Inhibition of the activity of TMA lyase, which hydrolyses choline to TMA, using the choline analog DMB reduced atherosclerosis burden in ApoE^{-/-} mice fed a choline-rich diet (Wang et al. 2015). More recently, it was shown that strategies aiming to inhibit phospholipase D, a bacterial enzyme that frees choline from phosphatidylcholine lipids, might be an interesting novel target to reduce choline-derived production of TMA (Chittim et al. 2019). More upstream in the cascade of microbial-metabolite production, it might be beneficial to develop strategies that aim to reduce intake of precursors of the presumably harmful metabolites. Although it is too early to tell if reduction of choline intake or alternative dietary strategies to reduce TMAO production will prevent ASCVD development (Washburn et al. 2019), these initiatives might provide feasible and economic solutions for reduction of these and other (e.g., ImP from histidine) microbiome-derived atherogenic metabolites. Important in this context is that humans usually have very low coherence to dietary interventions. In addition, high interindividual differences in response to dietary interventions (Walker et al. 2011; Cotillard et al. 2013; Kovatcheva-Datchary et al. 2015) make diet a challenging intervention to alter the microbiome and (markers of) ASCVD development.

The microbial modification of primary into secondary bile acids is in part facilitated by the bacterial enzyme bile salt hydrolase (BSH). BSH activity has been postulated to alter cholesterol accumulation, inflammation, and atherosclerosis development (Tremaroli and Bäckhed 2012), and BSH activity is present in a very wide range of bacteria (Joyce et al. 2014). A modified *E. coli* strain carrying the BSH gene was shown to enhance expression of genes involved in cholesterol efflux, immune homeostasis, and energy metabolism in mice (Joyce et al. 2014).

In line, a dedicated intervention study using BSH-active *Lactobacillus reuteri* in hypercholesterolemic humans showed that this probiotic effectively lowered LDL-C compared to placebo-treated subjects (Jones et al. 2012). Of interest in this context is that many probiotic strains are characterized by BSH activity (Begley et al. 2006). Whether or not the BSH activity underlies the beneficial effects of probiotic strain administration on parameters of ASCVD risk/health remains to be determined. Nevertheless, many probiotic strains have been associated with ASCVD health. *Bifidobacteria* and *Lactobacillus plantarum* have been associated with lowering of cholesterol (Tahri et al. 1996). Interestingly, *Lactobacillus plantarum* (Nguyen et al. 2007) and *Lactobacillus rhamnosus* (Qiu et al. 2018) were also reported to decrease TMAO and atherosclerosis development in mice prone to develop the disease. Likewise, in hypercholesterolemic humans, *Lactobacillus rhamnosus* was reported to reduce cholesterol levels (Costabile et al. 2017).

One can speculate that beneficial bacteria may produce molecules that halt or even induce regression of the process. As far as we know, studies to find these compounds have not been carried out. A good strategy may be to use modern machine learning methods to analyze the plasma of subjects with atherogenic plasma profile that do not show signs of ASCVD.

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Smooth Muscle Cell-Proteoglycan-Lipoprotein Interactions as Drivers of Atherosclerosis

Sima Allahverdian, Carleena Ortega, and Gordon A. Francis

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Abstract

In humans, smooth muscle cells (SMCs) are the main cell type in the artery medial layer, in pre-atherosclerotic diffuse thickening of the intima, and in all stages of atherosclerotic lesion development. SMCs secrete the proteoglycans responsible for the initial binding and retention of atherogenic lipoproteins in the

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artery intima, with this retention driving foam cell formation and subsequent stages of atherosclerosis. In this chapter we review current knowledge of the extracellular matrix generated by SMCs in medial and intimal arterial layers, their relationship to atherosclerotic lesion development and stabilization, how these findings correlate with mouse models of atherosclerosis, and potential therapies aimed at targeting the SMC matrix-lipoprotein interaction for atherosclerosis prevention.

Keywords

Atherosclerosis · Biglycan · Diffuse intimal thickening · Extracellular matrix · Lipoprotein retention · Proteoglycans · Response to retention · Smooth muscle · Smooth muscle cells

1 Introduction

Smooth muscle is the involuntary nonskeletal form of muscle cells, present in hollow organs such as the stomach, intestine, bladder and uterus, the respiratory tract, and arteries and veins of the circulatory system. Smooth muscle cells (SMCs) in the artery muscular or “medial” layer contract or relax to redistribute blood throughout the body and to regulate local blood pressure and blood vessel volume. In addition, in humans SMCs are the predominant cell type in the pre-atherosclerotic intima and all stages of atherosclerotic lesion development (Allahverdian et al. 2018). In the intimal layer, SMCs take on alternate phenotypes and roles including increased proliferation, increased production of matrix proteoglycans, uptake of lipoproteins and foam cell formation, loss of SMC markers and expression of macrophage markers, and stabilization of arteries against plaque rupture. In this chapter we review extracellular matrix (ECM) production by SMCs and its critical role in mediating the lipoprotein retention that drives atherosclerotic lesion development. We correlate these findings in humans with mouse models of atherosclerosis and how modification of the SMC-derived proteoglycan-lipoprotein interaction might be targeted as a means of preventing or treating atherosclerosis.

2 Smooth Muscle Cell Phenotype Switch and Extracellular Matrix Production

Vascular smooth muscle cells (SMCs), unlike skeletal and heart muscle cells, are highly plastic and show a spectrum of phenotypes in the medial and intimal artery layers, with contractile and synthetic being the extremes (Owens et al. 2004). SMCs switch their phenotypes depending on genetic preprogramming and local environmental signals including cytokines and mechanical forces. The majority of SMCs constituting the medial layer of healthy arteries exhibit a contractile phenotype with high levels of cytoplasmic contractile myofilaments, a low proliferation rate, and low protein synthesis. On the other end of the spectrum, intimal SMCs can exhibit either

a contractile phenotype or a dedifferentiated, synthetic phenotype with reduced expression of contractile proteins and other differentiated SMC markers and high rates of proliferation and protein synthesis. The majority of SMCs in the intima are of synthetic phenotype. SMCs forming the fibrous cap over atheromas, however, retain SMC markers including α -SMA and show a more differentiated, contractile phenotype (Feil et al. 2014; Perisic Matic et al. 2016; Albarran-Juarez et al. 2016; Chappell et al. 2016). Although transdifferentiation of SMCs with partial or total loss of SMC markers and gain of some macrophage markers has been reported in both human and mouse atherosclerotic lesions, detailed studies have confirmed SMCs maintain their characteristics and do not function as macrophages (Feil et al. 2014; Allahverdian et al. 2014; Shankman et al. 2015; Vengrenyuk et al. 2015).

As the major cell type of the pre-atherosclerotic and atherosclerotic intima, the phenotypic characteristics and products generated by synthetic phenotype SMCs are key determinants of atherosclerotic lesion development (Nakashima et al. 2008). Some of these SMC products are components of the ECM (Wight 2018). ECM is a component of all tissues and organs including blood vessels that provides physical strength to the tissue and acts as a framework in which cells and other molecules are embedded and bound. In arteries, the composition of ECM varies in the intima, media, and adventitia, between healthy and atherosclerotic arteries, and with different stages of atherosclerosis. In particular, the marked increase in proteoglycan content of ECM in the pre-atherosclerotic diffuse intimal thickening (DIT) stage of intima development creates the conditions necessary for the initial retention of serum lipoproteins and initiation of foam cell and atherosclerotic lesion formation (Nakashima et al. 2008; Wight 2018).

3 Extracellular Matrix (ECM)

Extracellular matrix (ECM) is a non-cellular, well-organized, and highly dynamic three-dimensional network in which cells reside and function. ECM is comprised of both fibers and a non-fibrillar amorphous gelatinous material called ground substance. Fibrous protein components of ECM include collagens, elastin, fibronectin (FN), and laminins. The non-fibrillar substance consists of proteoglycans (including versican, perlecan, biglycan, and decorin), along with glycoproteins and hyaluronan. Proteoglycans consist of long chains of repeating disaccharides attached to a core protein, while glycoproteins are composed of short, highly branched chains of various monosaccharides with no repeating unit attached to a core protein (Fig. 1). Hyaluronan is a glycosaminoglycan molecule that has no protein core but forms a non-covalent complex with proteoglycans in the ECM.

ECM components interact with resident cells through their cell surface receptors and regulate their morphology as well as functions such as growth, migration, differentiation, and survival (Theocharis et al. 2016). The composition and structure of ECM varies from tissue to tissue, and alteration of this composition changes the overall structure of the tissue and the function and properties of the embedded cells. Moreover, growth factors, cytokines, and chemokines that bind to ECM molecules

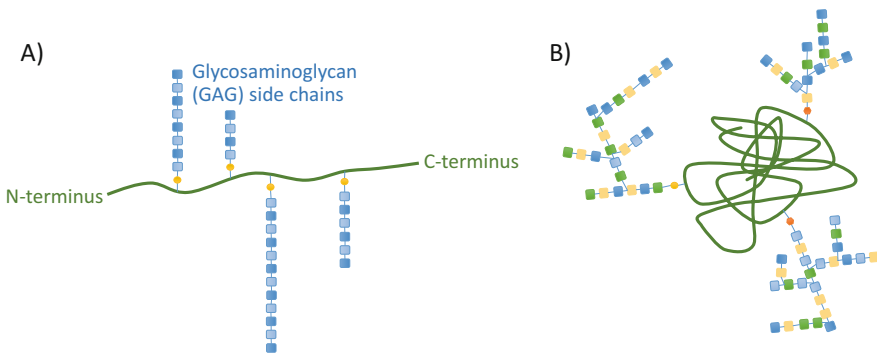


Fig. 1 Model structures of proteoglycans and glycoproteins. **(a)** Proteoglycans consist of a core protein (green line) with glycosaminoglycans (GAGs, blue beaded string), consisting of repeating disaccharide units of various lengths that are attached via O-glycosidic linkages (yellow circles) to the protein. Proteoglycans are distinguished by the size of their protein cores and by the number and type of GAGs attached to their protein cores. **(b)** Glycoproteins consist of a globular protein with a variety of monosaccharides (colored squares) covalently attached to the protein via O-glycosidic linkages (yellow circles) or N-glycosidic linkages (orange hexagon)

are released in response to different stimuli and modify local cellular function (Theocharis et al. 2016). All cell types synthesize and secrete ECM, with alteration of cellular properties due to physiological and pathologic conditions modulating the composition of ECM produced. In the following sections, we focus primarily on ECM produced by, and affecting the function of, arterial SMCs.

3.1 Fibrillar Matrix

Collagens, the most abundant protein in the human body and the major component of the ECM, have widespread distribution among tissues from bone and cartilage to vessel walls. Collagens are categorized into fibril-forming varieties including types I, II and III, present mainly in dermis, tendon, bone, and cartilage, and non-fibrillar collagens such as types IV and VIII, present in the basement membrane – the layer between the endothelium/epithelium and underlying cells – and fibril-associated forms including collagen types IX and XII, which do not form fibers themselves but are associated with collagen fibers (Theocharis et al. 2019). Collagens I and III are the main collagen forms in the medial and intimal layers of medium and large arteries and are the predominant collagens providing tensile strength to the vessel wall (Shekhonin et al. 1985; Frantz et al. 2010; Yue 2014). Collagens make up approximately 40% of all ECM in normal arteries, 10% of early atherosclerotic lesions, and 50% in advanced lesions (Wight 2018). Among the different subtypes, type I collagen is more highly expressed in SMC-rich fibrous caps compared to SMC-poor plaque shoulders. Collagen fibers are thus believed to play an important role in plaque stability (Rekhter 1999).

Elastin fibers are another major fibrillar component of ECM that provide recoil and elasticity to tissues undergoing repeated stretching, such as arteries. Elastic fibers constitute about 50% of the ECM in normal artery walls but only 20% of early and 10% of late atherosclerotic lesions (Wight 2018). Elastin is produced by assembly and crosslinking of its soluble precursor tropoelastin (Wise and Weiss 2009). Normal arteries contain mature crosslinked elastin fibers and negligible amounts of tropoelastin. Using atherosclerotic models, Phinikaridou et al. have shown that accumulation of tropoelastin is associated with plaque progression and instability. They also found that human endarterectomy specimens with ruptured plaques have higher tropoelastin content than stable plaques, suggesting this marker can be used as an indicator of plaque instability (Phinikaridou et al. 2018).

Fibronectin (FN) is another fibril-forming glycoprotein of the ECM. Fibronectin monomers are produced and secreted by cells to form FN fibers (Theocharis et al. 2016). The fibrillar form of FN is a homodimer with monomers linked at the cell surface by disulfide bonds. A soluble monomeric form of fibronectin is also present in plasma, which cells can utilize to assemble into fibers. Fibronectin fibers have binding sites for each other to form multimers. They can also bind to cell surface receptors such as integrins, as well as to collagens, heparin, and fibrin, and act as a bridge to make a network (Theocharis et al. 2016, 2019; Yue 2014). Fibronectin along with fibrinogen and vitronectin is the major component of the provisional matrix present in the intimal space during early stages of atherosclerosis. Fibronectin therefore plays an important role in initiation of the plaque but also later in formation of the fibrous cap to stabilize the plaque (Rohwedder et al. 2012; Finney et al. 2017).

The laminin family comprises 16 heterotrimeric glycoproteins (Theocharis et al. 2016). Each laminin fiber contains one α -, one β -, and one γ -chain in five, four, and three genetic variants, respectively. Laminins are a major component of the basement membrane underlying endothelial cells and surrounding SMCs in the intima. Laminin fibers of the basement membrane bind to each other to make a network to which cells adhere and interact with collagen IV to stabilize the overall structure. Interaction of laminin with collagen is mediated through bridging molecules like the proteoglycan perlecan. Laminin fibers interact with resident cells through multiple cell surface receptors including integrins to regulate cellular differentiation, adhesion, and migration (Theocharis et al. 2016, 2019).

3.2 Proteoglycans: Non-fibrillar Components of the ECM

Proteoglycans are complex macromolecules consisting of a core protein (e.g., versican; the entire proteoglycan molecule is named according to its core protein) to which one or more glycosaminoglycan molecules (GAGs) are covalently attached (Wight 2018) (Fig. 1). GAGs are linear negatively charged polysaccharides of varying length made up of repeating disaccharide units. There are six types of GAGs: chondroitin sulfate (CS), dermatan sulfate (DS), heparan sulfate (HS), heparin, keratan sulfate (KS), and hyaluronic acid (HA; also called hyaluronan). CS, DS, HS, and KS are linked to the different core proteins through O-glycosidic

Table 1 Summary of the various proteoglycans found in the arterial wall, their respective GAG chains, location, and their key role in atherogenesis and vascular homeostasis

Proteoglycan	Glycosaminoglycan (GAG)	Location	Role in atherogenesis and vascular homeostasis
Versican	CS	<ul style="list-style-type: none"> • Throughout the arterial wall • Enriched in areas with many SMCs (e.g., DIT, fibrous cap, and the edge of lipid cores) 	<ul style="list-style-type: none"> • Provides a matrix that prepares SMC for proliferation and migration
Biglycan	CS, DS	<ul style="list-style-type: none"> • Abundant in DIT, plaque core, and fibrous cap 	<ul style="list-style-type: none"> • Mediation of lipoprotein retention
Decorin	CS, DS	<ul style="list-style-type: none"> • Adventitia of normal and atherosclerotic arteries • Similar location as biglycan [e.g., deep (outer) intima in early atherosclerosis] but to a lesser extent • Enriched in the intima of atherosclerosis-resistant arteries 	<ul style="list-style-type: none"> • May contribute to plaque stability
Perlecan	HS	<ul style="list-style-type: none"> • Basement membrane between endothelial cells and intimal SMCs (abundant in mice but not humans) 	<ul style="list-style-type: none"> • In mice: permeability barrier and lipoprotein retention • In humans: unclear

linkage to make distinct PG families: CSPGs, DSPGs, HSPGs, and KSPGs. Binding of more than one type of GAG to a single core protein creates various PG subtypes. Hyaluronan is the only GAG that is synthesized at the cell membrane and not in the Golgi apparatus, and is only present in a protein-free form. Proteoglycans are abundant in the ECM and are also present on the cell surface and intracellularly. They interact with growth factors, cytokines, cell surface receptors, and other ECM molecules, through which they participate in multiple cellular functions including signaling, proliferation, migration, adhesion, differentiation, and apoptosis (Wight 2018; Theocharis et al. 2016; Yue 2014). Almost all cell types in the vessel wall can synthesize PGs, with SMCs being the major contributor. Proteoglycan content of the normal vessel wall is low, only 4% of total ECM, but it increases dramatically up to 50% in early atherosclerosis and 20% in late lesions (Wight 2018). There are more than 20 different PGs in blood vessels. As discussed below, the negative charge of PGs plays a critical role in the binding and retention of positively charged lipoproteins in the artery intima, a pivotal step in atherosclerotic lesion initiation. Key features of PGs involved in atherosclerotic lesion development are discussed here and summarized in Table 1.

3.2.1 Versican

Versican is a large aggregating CSPG (Wight 2018) that exists in at least four different isoforms, created by alternative splicing of mRNA from a single gene (V0–V3). These variants differ by the length of their core proteins and the number of GAGs attached. Versican forms complexes with hyaluronan, a long-chain GAG, to make high molecular weight aggregates. These aggregates provide a swelling pressure that resists tissue shrinkage and also a viscoelastic pericellular matrix that allows SMCs to change their shape to prepare them for proliferation and migration (Wight and Merrilees 2004). Immunostaining of the ECM of human coronary arteries showed the presence of versican in all layers of the artery wall but increased in areas of atherosclerotic arteries with high SMC content such as diffuse intimal thickening (DIT), fibrous cap, and the edge of the lipid core in advanced lesions (Gutierrez et al. 1997; Kolodgie et al. 2002). SMCs are the main source of versican, and its expression is upregulated by factors including platelet-derived growth factor (PDGF) and transforming growth factor- β 1 (TGF- β 1). Both PDGF and TGF- β 1 increase the number of versican transcripts and elongation of CS chains in cultured SMCs (Schonherr et al. 1991).

3.2.2 Biglycan

The small leucine-rich proteoglycan (SLRP) family includes small core proteins (biglycan, decorin) containing a number of leucine-rich repeats (LRRs) that can have CS, DS, or KS side chains (Wight 2018). Biglycan, together with decorin, belongs to the same class of SLRPs due to their homologous genes and proteins and the presence of ten LRRs sandwiched between cysteine-rich regions (Gutierrez et al. 1997). The N-terminal domain of biglycan typically contains two CS/DS side chains (Iozzo 1999) or two DSPG chains (O'Brien et al. 1998; Gutierrez et al. 1997). Biglycan is the primary proteoglycan found in the intima of normal and atherosclerotic human arteries (Little et al. 2008) and is enriched in areas such as DIT (discussed further in Sect. 4), the plaque core, and fibrous cap (Gutierrez et al. 1997). The presence of growth factors such as PDGF and TGF- β 1 transforms biglycan into “hyperelongated biglycan,” which has more sulfate incorporated, conveying higher negative charge and longer GAG chains (Schonherr et al. 1993), and therefore increased binding capacity to low density lipoprotein (LDL) and other apolipoprotein B (apoB)-containing lipoproteins (Little et al. 2002). Biglycan concentrated in the deepest part of DIT colocalizes strongly with apoB in early and advancing fatty streaks and plays a critical role in mediating lipoprotein retention in the artery wall (outlined further in Sect. 4 below).

3.2.3 Decorin

Decorin is another member of the SLRP family (Iozzo 1999). Decorin's N-terminus has a single CS/DS chain (Wight 2018). Decorin is expressed in the adventitia of normal and atherosclerotic arteries (Gutierrez et al. 1997). Decorin is also found, although to a somewhat lesser extent than biglycan, in the deep (outer) intima of coronary arteries in early stages of atherosclerosis, suggesting it also has a role in lipoprotein retention. It is also often found in association with collagen fibers in

atherosclerotic plaque and may have a role in plaque stability by increasing collagen fibrillogenesis (Wight 2018).

3.2.4 Perlecan

Perlecan, the most abundant HSPG in the mouse artery wall (Nakashima et al. 2008), is located in the basement membrane between the endothelial cells and intimal SMCs (Tran-Lundmark et al. 2008). Perlecan functions as a permeability barrier but also facilitates lipoprotein retention (Tran-Lundmark et al. 2008). Mice that were deficient in both HS-perlecan and apoE were shown to have higher flux but lower arterial retention of lipoproteins compared to apoE-single null mice (Tran-Lundmark et al. 2008). Human atherosclerotic lesions have low expression of perlecan, and the role of perlecan in development of human atherosclerosis is not clear.

4 Interactions Between SMCs and the ECM

In addition to SMCs being the primary source of proteoglycans and other ECM components in arteries, the composition and organization of ECM itself plays an important role in the regulation of SMC phenotype, i.e., the interaction between SMCs and ECM is *bidirectional*. Integrins, transmembrane receptors linking the intracellular SMC cytoskeleton to the ECM, play a critical role in this bidirectional signaling across the plasma membrane and regulation of SMC phenotype by ECM components (Finney et al. 2017; Adiguzel et al. 2009).

Fibrillar collagen I, a major component of ECM in the medial layer, induces a contractile and quiescent phenotype in human SMCs grown in culture, whereas monomeric (non-fibrillar) collagen type I, present in atherosclerotic plaque intima, enhances SMC proliferation, a characteristic feature of synthetic phenotype (Ichii et al. 2001; Koohestani et al. 2013; Yeh et al. 2012). This phenotypic shift was accompanied by alteration of gene expression in cultured human SMCs induced by polymerized compared to monomeric collagens (Ichii et al. 2001). Fibrillar collagen type I has also been shown to inhibit PDGF-induced proliferation and migration of SMCs (Raines et al. 2000). Collagen type IV and laminin, components of the basement membrane that surrounds arterial SMCs in the subendothelial space, induce a differentiated contractile phenotype in vascular SMCs grown in culture and diminish the synthetic phenotype induced by PDGF (Thyberg and Hultgardh-Nilsson 1994). In the same manner, integrins $\alpha 1\beta 1$ and $\alpha 7\beta 1$, which bind to collagen IV and laminin, respectively, are highly expressed in quiescent contractile SMCs and serve to maintain the spindle morphology and contractile phenotype of SMCs (Finney et al. 2017). Fibronectin, a major component of provisional matrix in the intima, induces both contractile and synthetic phenotype in vascular SMCs depending on the nature of integrins expressed by the local SMCs (Finney et al. 2017). Altogether various combinations of integrins expressed by SMCs and a variety of ECM components result in a complex SMC-ECM interaction that affects both SMC and ECM properties.

Smooth muscle cells also interact with non-fibrillar PG components of the ECM. Bingley et al. found that HSPGs extracted from rabbit aortae inhibit phenotypic change of the cultured SMCs and maintain them in a quiescent state (Bingley et al. 1998). Interestingly, they found that when applied in a periaortic gel following injury of rabbit carotid artery, HSPGs inhibit neointima formation (Bingley et al. 1998). Different subtypes of versican have been shown to alter SMC phenotype differently (Wight et al. 2014). Hyaluronan, present in both early and advanced lesions (Krolikoski et al. 2019), enhances proliferation and migration of SMCs (Evanko et al. 1999) and when overexpressed in SMCs of apoE-deficient mice accelerates the progression of atherosclerosis (Chai et al. 2005).

5 SMC, ECM, and Lipoprotein Interactions in Diffuse Intimal Thickening (DIT)

Diffuse intimal thickening (DIT) is a layer of thickened intima composed of SMCs, elastin, and PGs. DIT is initiated in utero and is present in all humans in atherosclerosis-prone arteries such as the coronary arteries and abdominal aorta by the age of 2 years (Nakashima et al. 2008). DIT represents a pre-atherosclerotic stage that provides the “soil” for the initial deposition and retention of lipoproteins and subsequent stages of atherosclerosis (Nakashima et al. 2008; Dubland and Francis 2016). Versican and biglycan are the most abundant proteoglycans in this DIT layer of atherosclerosis-prone arteries, while the thin intimal layer of atherosclerosis-resistant arteries, such as the internal thoracic artery, is enriched in decorin (Merrilees et al. 2001).

Using immunohistochemical techniques, Nakashima et al. showed that biglycan is localized in the deeper region of DIT in human coronary arteries (Nakashima et al. 2007). Lipoproteins that diffuse in from the plasma are trapped and deposited in the deep intima in the early stages of atherosclerosis due to a charge-charge interaction between this proteoglycan and apolipoprotein B of the lipoproteins. In an *in vitro* model, overexpression of biglycan in rat vascular SMCs increased lipoprotein binding to matrix formed by the cultured cells, while expression of a GAG-deficient mutated biglycan blocked this binding and accumulation (O’Brien et al. 2004). This observation and colocalization of biglycan with apoB- and apoE-containing lipoproteins in both early and advanced human (Nakashima et al. 2007; O’Brien et al. 2004) and murine lesions (Kunjathoor et al. 2002) indicate the presence of biglycan is crucial for the initial and ongoing deposition of lipids in the artery wall.

Unlike biglycan, immunohistochemical studies show versican is predominantly localized in the superficial part of the thickened intima of coronary arteries (Merrilees et al. 2001). Although *in vitro* studies have shown versican is capable of binding to LDL and has a larger number of binding sites for LDL compared to biglycan (Camejo et al. 1988), it has not been detected in lipoprotein-rich areas of atherosclerotic lesions nor colocalized with apoE or apoB epitopes in either human or mouse atherosclerotic lesions (Wight and Merrilees 2004; Kunjathoor et al. 2002).

Decorin, like biglycan, is distributed in the deep intima although at lower levels and colocalizes with lipids with less intensity compared to biglycan (Nakashima et al. 2007; Otsuka et al. 2015). In vitro studies have shown decorin links LDL with collagen type I (Pentikainen et al. 1997), and in vivo studies have demonstrated colocalization of decorin with collagens and apoB in atherosclerotic lesions (Nakashima et al. 2007; Riessen et al. 1994). Moreover, overexpression of decorin in rat SMCs after balloon injury of the carotid arteries resulted in compact collagen-rich lesions with reduced intimal thickness (Fischer et al. 2000). These findings suggest that decorin has a primary role in collagen organization in atherogenesis and a secondary role in lipid retention.

Perlecan is a heparan sulfate proteoglycan and the most abundant PG in the mouse artery wall, present in basement membranes in the intima and media (Nakashima et al. 2008). Human atherosclerotic lesions express only low amounts of perlecan (Tran et al. 2007). Like biglycan in humans, perlecan is involved in lipid retention in mouse atherosclerosis (Kunjathoor et al. 2002). Vikramadithyan et al. have shown that heterozygous deficiency of perlecan in chow-fed apoE-deficient mice results in more than 70% reduction in early-stage atherosclerosis lesion size. However, these authors did not find any difference in lesion size in advanced atherosclerosis (Vikramadithyan et al. 2004). Tran-Lundmark et al. also showed more than 50% reduction in lesion size in HS-deficient perlecan apoE^{-/-} mice compared to control apoE^{-/-} mice (Tran-Lundmark et al. 2008).

The expression of key artery wall ECM components and their roles including lipoprotein binding and fibrous cap formation are indicated in Fig. 2.

6 SMC-ECM Interaction in Lipid Retention

The ability of SMC-generated PGs in the human arterial intima to bind atherogenic apoB-containing lipoproteins mediates what is considered to be the critical and rate-limiting step in atherogenesis, from which subsequent steps of atheroma development ensue. This is the so-called “response-to-retention” hypothesis of atherosclerosis (Williams and Tabas 1995). The interaction between atherogenic lipoproteins and PGs involves an ionic or electrostatic interaction between positively charged amino acids of apoB on lipoproteins and negatively charged sulfate groups on the PGs generated by SMCs (Camejo et al. 1998; Boren et al. 1998). The role of deposition of lipoproteins in the intimal space and specific interactions of SMC-ECM molecules and apolipoproteins that mediate retention of lipoproteins are outlined below.

6.1 Deposition of Lipoproteins in the Artery Wall

Movement of lipoproteins from the plasma to the intimal space involves transport across the vascular endothelium via transcytosis, a process that is independent of LDL receptor function (Simionescu and Simionescu 1991). In contrast to

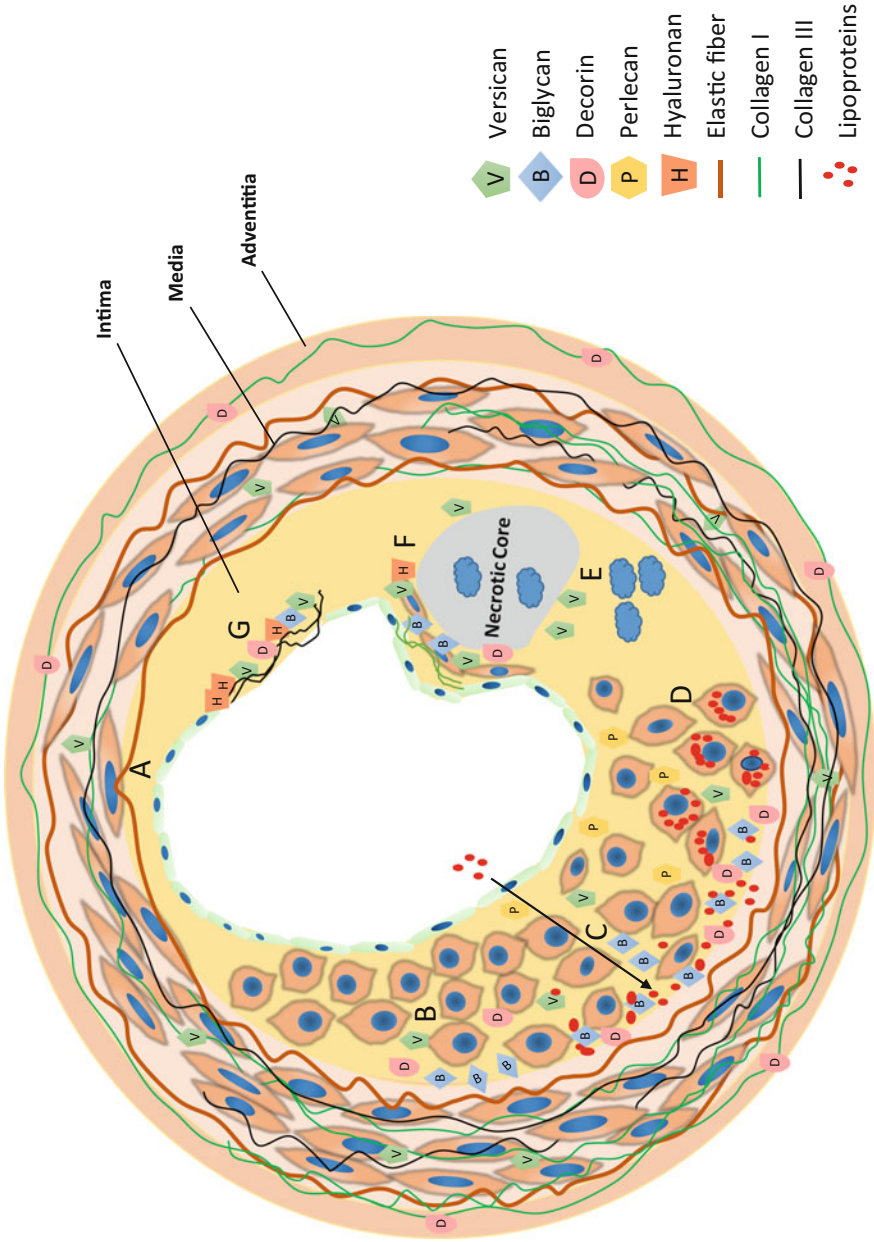


Fig. 2 Distribution of major ECM components in the normal artery wall and different stages of atherosclerosis. Composition of ECM varies in different layers of the artery wall and at different stages of atherosclerosis. (a) Collagen I and decorin in the adventitia, along with collagens I and III, elastic fibers, and versican

endocytosis and lysosomal degradation of lipoproteins mediated by the LDL receptor, endothelial transcytosis delivers lipoproteins including LDL and other apoB-containing lipoproteins including very-low-density lipoproteins (VLDL), VLDL remnants (intermediate-density lipoproteins), chylomicron remnants (Yang et al. 2018), and presumably lipoprotein (a) via endocytic uptake, intracellular transport, and exocytosis into the ECM of the artery wall (Simionescu and Simionescu 1991; Fung et al. 2018). Although transcytosis is the rate-limiting step in the movement of LDL and other lipoproteins into the subendothelial layer, it does not equate to the amount of LDL retained in the intimal space (Vasile et al. 1983). This was supported by the *in vivo* observation that the rate of LDL entry into normal rabbit aorta exceeded the rate of LDL accumulation (Carew et al. 1984). The retention of LDL occurs more extensively in lesion-susceptible areas such as the aortic arch compared with lesion-resistant arteries such as the descending thoracic aorta (Schwenke and Carew 1988). The difference in LDL accumulation between atherosclerosis-susceptible and atherosclerosis-resistant sites is also apparently independent of LDL permeability, as an *in vivo* comparison of healthy and atherosclerosis-susceptible arterial segments in rabbits showed they have similar LDL permeability (Schwenke and Carew 1989). These findings indicate that retention of apoB-containing lipoproteins, rather than the rate of transcytosis or endothelial permeability, is the rate-limiting factor in the amount of deposition of these lipoproteins in the artery wall. These studies formed the basis for Williams and Tabas' "response-to-retention" hypothesis, proposed in 1995, that highlighted the retention of apoB-containing lipoproteins as the major driver of atherogenesis (Williams and Tabas 1995).

Fig. 2 (continued) in the medial layer make up the majority of ECM in normal arterial walls. **(b)** Diffuse intimal thickening (DIT) is a thick layer of smooth muscle cells (SMCs) and proteoglycans (PGs) present in atherosclerosis-prone arteries beginning from birth. Biglycan, decorin, and versican secreted mainly by SMCs are the primary PGs in DIT, where biglycan and decorin are located in the deep intima and versican mainly in the superficial intima. **(c)** ApoB-containing lipoproteins that enter from the plasma via transcytosis through the endothelium are retained in the deep intima in early atherosclerosis due to a charge-charge interaction between apoB and glycosaminoglycan side chains of biglycan and decorin in the deep intima. **(d)** Trapped lipoproteins are modified, aggregated, and taken up by surrounding SMCs and macrophages to make foam cells (macrophages are omitted to emphasize the role of the ECM). Perlecan in mouse atheromas also binds lipoproteins but does not participate in this retention in humans. **(e)** Apoptosis/necrosis of foam cells leads to formation of a necrotic core in advanced atherosclerotic plaques, with versican being prominent at the edge. **(f)** Stable plaques are defined by the presence of a thick fibrous cap lying between the necrotic core and the luminal surface of the plaque. The fibrous caps consist of more differentiated SMCs and are enriched in versican, biglycan, and collagen type I, with lesser amounts of decorin and hyaluronan. **(g)** Unstable plaques with little or no fibrous cap show an intense staining for hyaluronan, versican, and collagen type III at the site of erosion and weak staining for biglycan and decorin

6.2 PG-Binding Sites in Lipoproteins

Lipoproteins delivered to the intima interact with the ECM via binding sites in their apolipoproteins (Usman et al. 2015). ApoB has been widely studied as the binding ligand of GAGs (Camejo et al. 1990; Srinivasan et al. 1988; Anber et al. 1997). Inhibition of the LDL-PG interaction by the presence of an anti-apoB antibody supports the observation that apoB is the binding site in lipoproteins (Sambandam et al. 1991). Modification of positively charged arginine and lysine residues in LDL abolished complex formation with CSPG, a GAG found in versican, biglycan, and decorin, indicating the importance of these amino acids in the apoB-PG interaction (Anber et al. 1997). The extent of apoB interaction with PGs is dependent on the degree of exposure of these positively charged residues in apoB (Camejo et al. 1990). The presence of hydrophobic, hydroxyl-containing serine and threonine residues around positively charged lysine and arginine residues increases the hydrophilicity of these segments and enhances their ability to interact with PGs (Camejo et al. 1990). In addition, the hydrolysis of surface phospholipids on LDL by secretory phospholipase A2 results in smaller and denser LDL particles that have greater exposure of PG-binding apoB segments, accounting for the increased affinity of small dense LDL for biglycan and decorin when compared to native LDL (Sartipy et al. 1999).

Site B of apoB100, residues 3,359–3,369, has been proposed to be the principal site of binding with PGs (Boren et al. 1998). A single-point mutation in this region of apoB100 severely affects proteoglycan interactions, without affecting LDL receptor binding (Boren et al. 1998). Furthermore, Cardin and Weintraub identified the GAG-binding sequence of apoB100 to be either $-X-B-B-X-B-X$ or $-X-B-B-B-X-X-B-X$ where X is a hydrophobic amino acid and B is either lysine or arginine (Cardin and Weintraub 1989). Chylomicron remnants, which are also atherogenic and deposit in the artery wall, contain the N-terminal 48% of apoB100 (apoB48) and therefore lack the GAG-binding Site B. GAG binding of apoB48 has been shown to be mediated by a lysine-rich cluster of amino acids in N-terminal residues 84–94, termed Site B-1b, exposed in this carboxyl-truncated form of apoB.

In addition to apoB, apoE and apoAI also colocalize with biglycan in human atherosclerotic plaques (O'Brien et al. 1998), but only apoE was found to have proteoglycan-binding domains (O'Brien et al. 1998; Olin et al. 2001). High-density lipoproteins (HDL) binding to GAGs appear to be limited to apoE-containing but not apoE-free HDL (Olin et al. 2001).

6.3 The Variability of Interactions Between Lipoproteins and Proteoglycans

Iverius initially demonstrated the interaction between GAGs and human plasma lipoproteins by equilibrating lipoproteins with a GAG-linked agarose gel, resulting in the interaction of LDL and VLDL, but not HDL, acetylated VLDL, or acetylated LDL, with heparin, DS, HS, and chondroitin-4-sulfate (Iverius 1972). Heparin and

CSPGs have the same binding site in apoB (Sambandam et al. 1991). The position of sulfation of GAGs was not found to be important; however increased sulfation, which confers higher negative charge, correlates with increased lipoprotein binding (Sambandam et al. 1991), confirming the electrostatic nature of the interaction between negatively charged GAGs and positively charged apoB-binding sites. The strength of the interaction between VLDL and LDL with different GAGs was heparin > DS > HS ~ C4-S (Iverius 1972). Contrary to this observation, Vijayagopal et al. observed greater affinity between LDL and CS than HSPG (Vijayagopal et al. 1983). This was supported by another publication that found CS/DSPG from bovine aorta was more potent than HS-PG in inducing complex formation with LDL and that the strongest complex formed is between LDL and CS/DSPG (Radhakrishnamurthy et al. 1990). Parallel with Iverius' observation, DSPG was determined to be more efficient in binding LDL than CSPG (Sambandam et al. 1991). The localization of lipoproteins in atherogenic plaques with biglycan and decorin (which may have DS side chains) as opposed to versican (a CSPG) aligns with this observation.

6.4 Effects of Binding and Retention of Lipoproteins

The retention of lipoproteins in the intima and their subsequent modification including oxidation and aggregation initiates a vicious cycle of further SMC proliferation and synthesis of elongated and more lipoprotein-attracting PGs that increase lipoprotein entrapment (Camejo et al. 1993; Chang et al. 2000). Macrophages infiltrating into the developing plaque secrete lipoprotein lipase, which can also act as a bridge between lipoproteins and PGs and thereby enhance lipoprotein retention. Macrophages also secrete inflammatory mediators that lead to further cell recruitment and plaque development (reviewed in Usman et al. 2015). The trapping of lipoproteins through interactions with PGs in the intima makes them a substrate for modifications including oxidation and aggregation. This converts the lipoproteins into ligands for scavenger receptors on macrophages and SMCs, leading to foam cell formation in both these cell types (Allahverdian et al. 2014; Wang et al. 2019). In this way, consistent with the response-to-retention theory of atherogenesis, SMC-dependent PG synthesis is the critical driver of subsequent stages of atherosclerotic lesion development.

7 Role of ECM Proteoglycans in Early Stages of Atherosclerosis Development in Animal Models

A key question, raised by the apparent pivotal importance of SMCs in producing the lipoprotein-binding PGs that drive lipoprotein retention and atherogenesis in humans, is how important this is in mice, the primary models used to study the determinants of atherosclerosis in man. Although small animals do not develop a pre-atherosclerotic SMC DIT layer like humans (Nakashima et al. 2008), several

studies provide evidence that the initial binding of apoB-containing lipoproteins to subendothelial PGs in the artery wall is also a key step in atherogenesis in animal models of atherosclerosis. Perlecan and biglycan, but not versican, a major PG detected in human plaques, appear early in developing lesions in apoE^{-/-} and LDLR^{-/-} mice underneath the endothelium and in the absence of intimal thickening (Kunjathoor et al. 2002). The presence of these PGs around areas of extracellular lipid deposition suggests that they also contribute to lipoprotein retention in murine models of atherosclerosis. This supports the theory of PG-dependent lipoprotein retention, although these PGs are likely secreted by endothelial cells or activated macrophages rather than SMCs in the first stage of mouse atherosclerosis (Kinsella et al. 1997; Nugent et al. 2000; Chang et al. 2012). In intermediate and advanced lesions, PGs and lipoproteins are localized in SMC-rich areas of the mouse intima (Kunjathoor et al. 2002). In another study, Thompson et al. found that overexpression of biglycan by SMCs in LDLR^{-/-} mice increases lipid retention and atherosclerosis development (Thompson et al. 2014). Moreover, apoE^{-/-} mice heterozygous for deletion of perlecan have less atherosclerotic lesion area at early stages compared to apoE^{-/-} mice with no deletion. This might be attributed partly to decreased lipoprotein retention in the perlecan^{+/-} mice (Vikramadithyan et al. 2004). In another study, Skalen et al. used transgenic mice expressing recombinant LDL with proteoglycan binding site-defective apoB and showed those mice develop significantly less atherosclerosis compared to mice expressing wild-type control LDL (Skalen et al. 2002). These studies demonstrate a key role for PGs for initial subendothelial lipid retention at early stages of mouse atherosclerosis and a likely role for SMC-derived PGs in later stages of murine models of this disease.

8 The Role of the ECM-SMC Interaction in Plaque Stability and Rupture

Stable plaque is defined by the presence of a thick fibrous cap lying between the necrotic core and luminal surface of the plaque. It consists of SMCs embedded in a collagen-proteoglycan matrix with varying degrees of macrophage and lymphocyte infiltration and has a critical role in maintaining integrity of the plaque. Unstable plaques are prone to erosion and rupture that leads to platelet adhesion and luminal thrombus formation, the most common cause of acute coronary syndromes and sudden cardiac death (Allahverdian et al. 2018). Plaque rupture involves breakage of a large lipid/necrotic core with large luminal thrombus, thin fibrous cap, and a dense infiltration of inflammatory cells (Kolodgie et al. 2004).

Extracellular matrix components of atherosclerotic lesions have a key role in maintaining stability of plaques and accumulate in topographically distinct patterns in different plaque types. Kolodgie et al. found that the fibrous caps of stable lesions show increased amounts of versican, biglycan, and collagen type I staining and significantly less decorin and hyaluronan (Kolodgie et al. 2002). With SMCs being the main producer of collagen and PGs in the fibrous matrix (Pietila and Nikkari 1983), it is not surprising that the number of SMCs in fibrous caps is directly

correlated with plaque stability (Allahverdian et al. 2018). One study found collagen VIII deficiency in apoE^{-/-} mice resulted in thinning of the fibrous cap, which suggests that this collagen plays an important role in protecting the plaque from rupture (Lopes et al. 2013).

Eroded plaques show an intense staining for hyaluronan, versican, and collagen type III at the plaque/thrombus interface and weak staining for biglycan and decorin. Cell surface receptor for hyaluronan, CD44, has been shown to be expressed by a subset of SMCs at the plaque/thrombus interface in erosions (Kolodgie et al. 2002) and mediates adhesion of platelets to hyaluronan (Day 1999). Exposure of versican-hyaluronan to flowing blood at the de-endothelialized surface of the eroded plaque promotes platelet attachment and may play an important role in the development of thrombosis via a CD44-dependent mechanism (Koshiishi et al. 1994).

Fibrous caps owe their strength and resistance to rupture to their collagen fiber content and therefore the content and synthetic capacity of collagen-producing SMCs (Doran et al. 2008; Libby 2008). Thinned fibrous cap areas contain very few SMCs with little to absent staining for PGs or hyaluronan and scattered strands of collagen type I (Kolodgie et al. 2002). Factors that alter the abundance or function of SMCs thereby alter ECM production by SMCs and plaque stability.

8.1 Factors Involved in Plaque Instability

Pro-inflammatory cytokines have been shown to reduce the number of intimal SMCs, thereby compromising ECM production (Geng et al. 1997; Kockx and Knaapen 2000). Various cytokines and growth factors, normally present in atherosclerotic plaques, also either stimulate or suppress collagen synthesis by vascular SMCs (Amento et al. 1991). Moreover, reduction of SMC density in plaque and the fibrous cap as a result of apoptosis results in diminished ECM production and may lead to plaque instability. von der Thusen et al. found that induction of vascular SMC apoptosis by overexpression of p53 in the fibrous cap of carotid plaques of apoE^{-/-} mice results in reduction of cell density, cap thinning, and plaque destabilization (von der Thusen et al. 2002). Low endothelial shear stress (ESS) has been shown to induce SMC apoptosis (Qi et al. 2008) and therefore indirectly affect collagen content of atherosclerotic plaques. Frontini et al. found that lipid loading of human vascular SMCs impairs their ability to assemble fibrillar collagen and fibronectin (Frontini et al. 2009), which may contribute to reducing plaque stability. Others found that enhancement of cholesterol efflux preserves the assembly of fibrillar collagen and fibronectin by SMCs (Beyea et al. 2012).

An imbalance between synthesis and degradation of ECM leads to thinning of the fibrous cap and plaque rupture. Members of the matrix metalloproteinases (MMP) and tissue inhibitor of metalloproteinase (TIMP) families can alter the quantity and composition of ECM and affect plaque stability (Newby et al. 2009). Although macrophages are the main source of MMPs in atherosclerosis (Shah et al. 1995), secretion of different MMPs by SMCs also plays an important role in cap thinning and plaque destabilization. Members of the MMP family with collagenase activity,

including MMP-1, MMP-8, MMP-13, and the activator of MMP collagenases, MMP-14, weaken the plaque by degrading collagen fibers (Libby 2008; Deguchi et al. 2005). The concentration, production, and expression of MMP-9 (Loftus et al. 2000), MMP-1, and MMP-13 (Sukhova et al. 1999) are significantly higher in unstable compare to stable carotid plaques.

Low endothelial shear stress (ESS) has also been shown to play an important role in plaque instability. In an *ex vivo* model of porcine carotid atherosclerosis, low ESS resulted in an increase in MMP expression (Gambillara et al. 2005). Low ESS is also associated with a diminished SMC and collagen content, a high MMP expression and activity, and a marked thinning of the fibrous cap in mouse carotid and porcine coronary plaques (Cheng et al. 2006; Koskinas et al. 2013).

9 Therapeutic Targets and Interventions

The critical role of SMC-dependent PG synthesis and lipoprotein retention in subsequent atherosclerosis development makes the PG-lipoprotein interaction a potentially profound and novel point of therapeutic intervention to prevent ischemic vascular disease. Vazquez and colleagues introduced the chP3R99 monoclonal antibody (mAb) against ECM GAGs as a potential therapeutic for atherosclerosis in 2012 (Soto et al. 2012). The mAb binds to sulfated GAGs such as heparin, HS, and DS and had the strongest affinity for CS in aorta (Soto et al. 2012). It prevented 70% of LDL-CS complex formation and abolished 80% of LDL oxidation *in vitro* (Soto et al. 2012). The mAb also demonstrated the capacity to (1) prevent foam cell formation by the inhibition of LDL retention and oxidation in the arteries of rats; (2) decrease the intima-media ratio, a measure of DIT formation; and (3) decrease macrophage infiltration in the aorta of New Zealand white rabbits (Soto et al. 2012). Thereafter, they delivered the mAb chP3R99 via subcutaneous injections to apoE^{-/-} mice fed a high-fat and high-cholesterol diet and determined that the vaccination stimulated an immune response against various sulfated GAGs via the induction of a secondary anti-idiotypic response (Ab2) that mirrors GAG antigenic determinants (Brito et al. 2012). The Ab2 gave rise to synthesis of anti-idiotypic antibodies (Ab3) that have a specificity homologous to that of Ab1 (Brito et al. 2012). Further research using chP3R99 in the same mouse model established the chP3R99-LALA variant restricted the progression of atherosclerosis in apoE^{-/-} mice by preventing initial CS-LDL complex formation (Delgado-Roche et al. 2015). Both the efficiency of the chP3R99-LALA mAb in initiating the anti-idiotypic cascade and anti-atherogenic benefits were dose-dependent in apoE^{-/-} mice but were independent of the sex and age of the mice (Sarduy et al. 2017). The mAb was also shown to decrease inflammation and halt lesion development of advanced stages of atherosclerosis in male apoE^{-/-} mice (Brito et al. 2017). These findings strongly confirm the relevance of the “response-to-retention” hypothesis of atherogenesis and provide the exciting possibility of developing an immunization that could block the PG-lipoprotein interaction lifelong as a novel means of preventing atherosclerosis.

10 Conclusion

Smooth muscle cells (SMCs) are the most abundant cell type in the healthy human artery wall as well as the pre-atherosclerotic and atherosclerotic intimal layer. The ECM components secreted by SMCs are critical factors in maintaining normal arterial architecture and stability but also in predicting propensity to atherosclerosis. In particular, the secretion of PGs by intimal SMCs promotes the initial and ongoing binding and retention of atherogenic lipoproteins in the artery wall. This retention of lipoproteins is necessary for further development of atherosclerosis, apparently in mice as well as in humans, making the proteoglycan-lipoprotein interaction a novel and exciting potential point of intervention for the future treatment and prevention of atherosclerosis.

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Anti-inflammatory and Immunomodulatory Therapies in Atherosclerosis

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Abstract

Hypercholesterolemia is a major risk factor in atherosclerosis development and lipid-lowering drugs (i.e., statins) remain the treatment of choice. Despite effective reduction of LDL cholesterol in patients, a residual cardiovascular risk persists in some individuals, highlighting the need for further therapeutic intervention. Recently, the CANTOS trial paved the way toward the development of specific therapies targeting inflammation, a key feature in atherosclerosis progression. The pre-existence of multiple drugs modulating both innate and adaptive immune responses has significantly accelerated the number of translational studies applying these drugs to atherosclerosis. Additional preclinical research has led to the discovery of new therapeutic targets, offering promising perspectives for the treatment and prevention of atherosclerosis. Currently, both drugs with selective targeting and broad unspecific anti-inflammatory effects have been tested. In this chapter, we aim to give an overview of current advances in immunomodulatory treatment approaches for atherosclerotic cardiovascular diseases.

Keywords

Adaptive immunity · Anti-inflammatory therapy · Atherosclerosis · Cardiovascular diseases · Immunotherapy · Inflammation · Innate immunity

1 Introduction

Atherosclerosis is a lipid-driven disease that is characterized by the formation of plaques in the subendothelial space of arteries. Therefore, lipid-lowering drugs such as statins are considered the treatment of choice as their reduction of circulating low-density lipoprotein (LDL) can prevent plaque formation and progression in patients (FERENCE et al. 2017; Borén et al. 2020). A crucial step in disease initiation involves the retention of LDL and other ApoB-carrying lipoproteins within the vascular wall due to their ability to bind intimal proteoglycans. Subsequent enzymatic and non-enzymatic modifications of these lipoprotein particles result in the formation of damage-associated molecular patterns (DAMPs) that trigger an inflammatory response. This includes the activation of endothelial cells by oxidized phospholipids and other lipid species. In response, the endothelium secretes chemoattractant cytokines like monocyte chemoattractant protein-1 (MCP-1), growth factors (macrophage colony-stimulating factor (M-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF)) and upregulates the expression of genes coding for adhesion molecules (VCAM-1 and ICAM-1), allowing the recruitment and differentiation of inflammatory monocytes. Monocytes recruited into the intima become lesional macrophages, which take up oxidized LDL and

aggregated LDL via scavenger receptors (CD36, SR-A), ultimately leading to foam cell formation – a rate-limiting step that greatly contributes to the development and progression of atherosclerotic plaques (Borén et al. 2020). Excess accumulation of modified lipoproteins, formation of foam cells, subsequent macrophage cell death, and impaired clearance of these apoptotic cells, all contribute to chronic inflammation in the vascular wall (Borén et al. 2020; Libby 2021). Studies over the past 20 years have focused on the chronic inflammatory nature of atherosclerosis and have demonstrated the detrimental role of inflammation in its pathophysiology. This work led to the consideration of markers of inflammation such as IL-6 and high-sensitivity C-reactive protein (hsCRP) as biomarker for risk stratification of cardiovascular patients (Ridker et al. 2000a, b, 2009). According to this concept, patients with signs of inflammation (i.e., elevated levels of hsCRP or IL-6) are considered at higher risk of experiencing a cardiovascular event during their lifetime compared to individuals without residual inflammation. Additionally, both innate and adaptive immunity have been found to play a crucial role in the initiation, progression, and rupture of atherosclerotic plaques (Gisterå and Hansson 2017; Wolf and Ley 2019; Libby 2021). Since the importance of the immune system in atherosclerotic plaque development is becoming increasingly clear, novel treatments that target both innate and adaptive immunity have been developed with the aim to complement already established lipid-lowering therapies (Bäck et al. 2019). This chapter aims to introduce the reader to these novel anti-inflammatory and immunomodulatory treatments, which range from small molecule drugs to antibodies and vaccination strategies.

To optimally target the inflammatory component of atherosclerosis, one needs to understand the chronic inflammatory nature of this specific process. Chronic inflammation is typically the consequence of a non-resolving inflammation due to an impaired clearance and persistent presence of the inciting trigger combined with a hampered removal of freshly recruited inflammatory cells (Tabas and Glass 2013). In atherosclerosis the main triggers of inflammation are sterile DAMPs that result from the pathological accumulation of lipids in the vascular wall. Therefore, optimal strategies targeting chronic inflammation are either directed toward limiting the generation and activity of disease-specific DAMPs or the inflammatory response they incite. Other strategies aim at dampening inflammation or enhancing the resolution processes. Clearly, the more targeted anti-inflammatory treatments are to a specific pathophysiology, the lower the risk of adverse effects such as a reduction of the host's capacity to fight infections. Thus, careful assessment of the risks and benefits of each anti-inflammatory strategy is needed during the development of these treatments. Modulating both the innate and adaptive responses offers promising possibilities to limit chronic inflammation in the vascular wall.

2 Targeting Innate Immune Responses

2.1 Targeting the NLRP3 Inflammasome

Accumulating evidence shows that the nucleotide-binding oligomerization domain, leucine-rich repeat-containing receptor (NLR) family pyrin domain-containing 3 (NLRP3) inflammasome plays a central role in plaque formation, growth, and stability. Its activation is typically necessary for the secretion of proinflammatory cytokines, such as IL-1 β and IL-18, which in turn activate the innate immune system. Ligation of both pattern recognition receptors (PRRs) and tumor necrosis factor receptor (TNFR) can activate the NF- κ B pathway, which promotes the transcription of proinflammatory cytokines like tumor necrosis factor- α (TNF- α) and interleukin IL-6 while also initiating the priming of the NLRP3 inflammasome. The latter involves the production of cytosolic NLRP3 protein as well as the inactive form of IL-1 family cytokines: pro-IL-1 β and pro-IL-18 (Kelley et al. 2019). Although several PRRs can trigger the formation of inflammasomes, such as NACHT, LRR, and PYD domains-containing protein 1 (NLRP1), NLR family CARD domain-containing protein 4 (NLRC4), and absent in melanoma 2 (AIM2), the NLRP3 inflammasome displays unique activation properties. A large variety of pathogen-associated molecular patterns (PAMPs) (microbial, viral, fungal molecules) and DAMPs (urate crystals) can activate NLRP3. In the context of atherosclerosis, cholesterol crystals (Duewell et al. 2010) have been suggested as key DAMPs driving NLRP3 activation. Importantly, NLRP3 also senses perturbations of cellular homeostasis such as lysosome rupture, ion channel disturbances (K⁺ efflux), and mitochondrial stress resulting from various stimuli (Kelley et al. 2019). In its activated form, NLRP3 can recruit the apoptosis-associated speck-like protein which contains a caspase recruitment domain (ASC) and the inactivated form of caspase-1 (pro-caspase-1). The proximity of pro-caspase 1 proteins within the complex leads to caspase-1 activation, which proceeds to the proteolytic activation of pro-IL-1 β , pro-IL-18, and the proapoptotic factor gasdermin D (GSDMD) involved in pyroptosis, a lytic cell death necessary for cytokine release.

With respect to atherogenesis, important insights came from studies by Duewell and colleagues, who showed that lipid accumulation in the vessel wall leads to the formation of cholesterol crystals which are taken up by macrophages where they activate the NLRP3 inflammasome (Rajamaki et al. 2010; Duewell et al. 2010). In parallel, many studies have investigated the role of NLRP3-derived cytokines (i.e., the IL-1 cytokine family: mainly IL-1 β , IL-1 α , IL-18, and IL-1R antagonist (IL-1Ra)), as well as proteins involved in NLRP3 activation (ASC, caspase-1) in murine atherosclerosis using transgenic mice. Targeting IL-1 signaling using recombinant IL-1Ra, which competes with IL-1 α and IL-1 β for binding of IL-1 receptors, in Apoe^{-/-} mice prone to atherosclerosis, resulted in reduced fatty streak formation. This indicates an important role of IL-1Ra in the suppression of lesion development during the early phase of the disease (Elhage et al. 1998; Isoda et al. 2004; Merhi-Soussi et al. 2005). Similarly, overexpression of IL-1Ra in Ldlr^{-/-} mice, fed a high-cholesterol/high-fat diet (containing cholate) for 10 weeks led to a reduction of

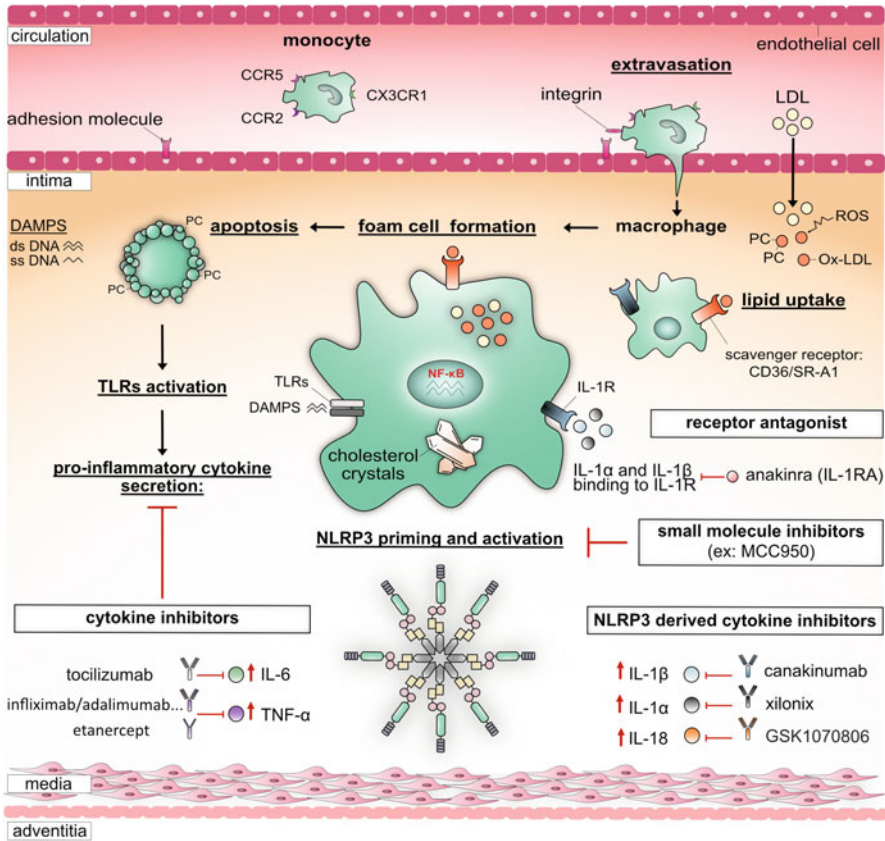


Fig. 1 Therapies targeting innate immune responses in atherosclerosis. Proinflammatory cytokines such as IL-6, TNF, IL-1, and IL-18 actively modulate atherosclerosis development. Therefore, several strategies aim at reducing their levels in circulation. The NLRP3 inflammasome greatly contributes to the secretion of such cytokines and several therapeutic approaches have been developed to prevent its deleterious effects. Drugs targeting NLRP3 are of three types: monoclonal antibodies targeting NLRP3 products (i.e., IL1- α -Xilonix, IL1- β -Canakinumab, IL-18-GSK1070806), recombinant proteins (i.e., IL-1-receptor antagonist-Anakinra), and NLRP3 direct inhibitors (i.e., MCC950). While some of these therapies were assessed in large clinical trials (i.e., Canakinumab), others remained inconclusive due to the low number of patients involved in the study (i.e., Xilonix), or were not investigated in humans yet. The potential benefit of such therapies still requires to be evaluated in robust clinical trials

atherosclerotic lesion area (Devlin et al. 2002). Taken together, these studies suggest that targeting the NLRP3 inflammasome using small molecule inhibitors or targeting NLRP3 products represents promising therapeutic targets for cardiovascular diseases (Fig. 1).

Regarding NLRP3 products, the two IL-1 isoforms (IL-1 α and IL-1 β) share molecular similarities but are produced by different cellular sources and seem to display different release mechanisms. The release of IL-1 α can be inflammasome-

dependent or independent, according to the nature of the activating stimulus (Groß et al. 2012). In contrast to IL-1 β , the precursor form of IL-1 α is active and acts as an alarmin. IL-1 α is constitutively expressed as a membrane-bound protein on the surface of various cell types including non-immune cells like epithelial cells. When cells expressing IL-1 α undergo cell death, IL-1 α is released and acts as an alarmin, inducing IL-1 β production and contributing to local inflammation. Additionally, IL-1 α can be produced by macrophages, in an inflammasome-independent manner (Freigang et al. 2013). IL-1 β on the other hand is mainly produced by hematopoietic cells like dendritic cells, monocytes, and macrophages with its activation requiring (in the majority of cases) the NLRP3 inflammasome. Importantly, IL-1 β has been detected in human atherosclerotic plaques and its circulating levels correlate with the severity of coronary atherosclerosis (Galea et al. 1996; Dewberry et al. 2000). Despite these discrepancies, the two IL-1 isoforms share similar downstream effector functions as they both signal via the IL-1 receptor family (IL-1R). Mechanistically, IL-1 signaling impacts various cell types within the atherosclerotic lesion, affecting plaque size and stability. It also contributes to the activation status of endothelial cells, which express adhesion molecules (VCAM-1 and ICAM-1) for the recruitment of leukocytes (Wang et al. 1995). Furthermore, IL-1 β induces MCP-1 secretion, which promotes monocyte recruitment and is strongly associated with cardiovascular risk (Jun et al. 2009).

The importance of IL-1 α has only recently been appreciated. A small study carried out in coronary artery disease (CAD) patients and healthy control subjects showed higher expression of IL-1 α in peripheral blood mononuclear cells (PBMC) of CAD patients (Wæhre et al. 2004). Additionally, IL-1 α has been described as the key driver of the senescence-associated secretory phenotype (SASP) of vascular SMCs that can promote vascular inflammation (Clarke et al. 2010; Gardner et al. 2015). Another study carried out by S. Freigang and colleagues also suggests that IL-1 α greatly contributes to vascular inflammation in atherosclerosis. Reconstitution of cholesterol fed Ldlr^{-/-} mice with bone marrow from IL-1b^{-/-}, IL-1a^{-/-} and wild-type mice resulted in dramatically reduced atherosclerosis in IL-1 α -deficient chimeras but not IL-1 β -deficient or wild-type chimeras (Freigang et al. 2013). Similarly, D. Gomez et al showed that antibody-mediated inhibition of IL-1 β in Apoe^{-/-} mice with established atherosclerosis failed to decrease lesion size but resulted in adverse plaque remodeling (Gomez et al. 2018). In contrast, other studies suggest beneficial effects of IL-1 β targeting in murine atherosclerosis (Kirii et al. 2003; Bhaskar et al. 2011).

Thus, IL-1 α and IL-1 β may affect plaque formation at different time points in lesion development (Kirii et al. 2003; Duewell et al. 2010; Bhaskar et al. 2011; Gomez et al. 2018; Vromman et al. 2019). While IL-1 α appears to be important for the early phase of atherosclerosis, IL-1 β seems to be more involved in the progression of established plaques (Vromman et al. 2019). Additionally, Vromman et al. showed that the anatomical location of plaques matters in the response to therapies targeting IL-1 signaling. For example, neutralization of IL-1 α or both isoforms using either an anti-IL1 α and/or an anti-IL-1 β monoclonal antibody reduces the lesion area in aortic roots but not in the brachiocephalic arteries in Apoe^{-/-} mice. Moreover,

compensatory outward remodeling was more impaired in the brachiocephalic artery compared to the aortic roots during early atherosclerosis.

In summary, the NLRP3 inflammasome and inflammasome-derived cytokines represent attractive points for therapeutic intervention in cardiovascular diseases. Careful design of preclinical studies will allow better dissection of the mechanistic and physiological aspects of IL-1 inhibition and improvement of the development of new therapies.

2.1.1 Targeting the IL-1 Signaling Pathway

Anti-IL-1 β (Canakinumab)

The recent CANTOS (Canakinumab. Anti-Inflammatory Thrombosis Outcome Study) trial has made a profound impact on the field of atherosclerotic cardiovascular disease. It showed for the first time in a large phase 3 clinical trial that therapeutic targeting of inflammation provides a clinical benefit to patients with stable atherosclerosis. The goal of the study was to evaluate the effect of canakinumab, a monoclonal antibody targeting IL-1 β , on adverse cardiac events in patients with a history of MI and elevated hsCRP (hsCRP ≥ 2 mg/L). 10,061 patients, most of whom were on statin therapy, received either placebo or canakinumab at three different doses: 50 mg, 150 mg, or 300 mg, every 3 months. The best results were obtained with the intermediate dose (150 mg) which led to a 15% reduction in the primary endpoint of nonfatal MI, nonfatal stroke, or cardiovascular death. However, as IL-1 β is also a crucial cytokine in the host immune response against bacterial infection, its inhibition also significantly increased the number of fatal infections (Ridker et al. 2017). Despite these non-negligible side effects, this study clearly demonstrates a beneficial effect of inhibiting IL-1 β signaling.

To better predict positive outcomes of canakinumab therapy in patients, an additional analysis of the CANTOS trial was conducted (Ridker et al. 2018b). Within the canakinumab treated group, patients with hsCRP levels < 2 mg/L following treatment had a 25% reduction in major adverse cardiovascular events while no significant benefits were observed in patients with levels ≥ 2 mg/L. Importantly, cardiovascular mortality and all-cause mortality were both reduced by 31% in patients with hsCRP levels < 2 mg/L. This was not the case in patients with levels ≥ 2 mg/L where no benefits were shown. This analysis clearly documented the need to assess the successful impact on inflammation to optimally appreciate the protective effect of this intervention.

Another analysis published by the CANTOS group indicates that targeting IL-1 β alone may not sufficiently alleviate CVD risk, as both IL-6 and IL-18 levels remain predictors of future cardiovascular events in patients receiving anti-IL-1 β treatment. Briefly, in patients treated with canakinumab, the risk of major adverse cardiac events (MACE) increased by 15% and 42% for each tertile increase in IL-18 and IL-6 levels, respectively. This suggests a residual inflammatory component that needs to be considered for further cardiovascular risk reduction. Drugs targeting the IL-6 cytokine are currently tested in clinical trials and might benefit patients with

sustained elevated IL-6 levels by preventing recurrent CVD events (Biasucci et al. 2020; Ridker et al. 2020).

IL-1 Receptor Antagonist (Anakinra)

Anakinra is an antagonist of the IL-1 receptor, blocking the action of both IL-1 α and IL-1 β isoforms. Since 2001, it has been used as a treatment in patients with rheumatoid arthritis (RA), who are also known to be at high risk of cardiovascular disease (Nurmohamed 2009; Avina-Zubieta et al. 2012; Primdahl et al. 2013). Anakinra was assessed in small clinical trials in patients with myocardial infarction: The “Virginia Commonwealth University-Anakinra Remodeling Trial 1-2-3” (VCU ART1-2-3) and the “Markers of inflammation in non-ST elevation acute coronary syndromes” (MRC ILA-HEART) study (Abbate et al. 2010, 2013, 2020; Morton et al. 2015). In VCU ART, this type of interleukin-1 blockade significantly reduced the systemic inflammatory response with a strong reduction of hsCRP levels in patients with ST-Elevation Myocardial Infarction (STEMI) as well as decreasing the incidence of heart failure. In the MRC ILA-Heart trial, a reduction of IL-6 and hsCRP levels was also observed in non-STEMI (NSTEMI) patients who received Anakinra daily for 2 weeks. However, by day 30, the hsCRP levels in patients treated with Anakinra were significantly higher than in the placebo group, and an unexpected increase of late recurrent ischemic events was observed. Interestingly, a recent study carried out by the Interleukin-1 Genetics Consortium supports the hypothesis that long-term IL-1 inhibition may be associated with an increase in cardiovascular events. In this study, individuals who carried four IL-1Ra raising alleles were found to have an increased odds ratio for coronary heart disease (1.15) compared to those carrying no IL-1Ra raising alleles. Additionally, the concentration of proatherogenic lipids, including LDL cholesterol, increased with each extra allele present (Freitag et al. 2015). However, the VCU-ART study showed no effect on recurrent ischemic events, while there was an increase of MACE upon Anakinra treatment after 1 year in the MRC ILA-Heart trial.

In summary, the relationship between IL-1 targeting and clinical outcomes seems more complex than originally thought. Currently attempted therapeutic strategies include targeting IL-1 β (Canakinumab) or both IL-1 α and IL-1 β with the human recombinant IL-1RA (Anakinra). Further understanding of the distinct roles of the different IL-1 isoforms would enhance our understanding of clinical trial data (CANTOS, VCU ART-1/2/3, MRC ILA-HEART) and improve future trial design.

2.1.2 NLRP3 Inhibitors

Despite the importance of the NLRP3 inflammasome in many inflammatory diseases, there is currently no approved inflammasome inhibitor for the treatment or prevention of atherosclerosis. Nevertheless, many small molecule compounds targeting the NLRP3 inflammasome have been developed, with promising results. Importantly, both canonical and non-canonical NLRP3 activation pathways can be targeted.

MCC950

MCC950 is a small molecule inhibitor of the NLRP3 pathway, with a mechanism which is yet to be fully understood (Coll et al. 2015). Nevertheless, it was found to reduce atherosclerotic lesion formation in *Apoe*^{-/-} mice. This was associated with decreased expression of the adhesion molecules ICAM-1 and VCAM-1, as well as reduced macrophage infiltration within plaques and no change in necrotic core size (Van Der Heijden et al. 2017). This early data suggests that MCC950 represents an interesting candidate to target NLRP3 induced inflammation in atherosclerosis.

Tranilast

Tranilast, which was originally used to treat allergy, was shown to inhibit NLRP3 by facilitating its ubiquitination and impairing ASC oligomerization (Huang et al. 2018; Chen et al. 2020). The effect of Tranilast on atherosclerosis was assessed in Watanabe heritable hyperlipidemic rabbits and resulted in decreased atherosclerosis upon treatment (Matsumura et al. 1999). Recently, another study carried out in mice showed that Tranilast decreased the initiation and progression of atherosclerosis in both *Ldlr*^{-/-} and *Apoe*^{-/-} prone atherosclerotic mice (Chen et al. 2020). Thus, pharmacologic manipulation of NLRP3 ubiquitination offers a new strategy for protection against atherosclerosis.

Despite encouraging results from animal models, many questions remain to be answered concerning the safety of NLRP3 inhibitors. It will be critical to further examine how these compounds affect the activity of other inflammasomes and how the host immune response is affected in the long run. The initiation of new clinical trials evaluating the benefit of NLRP3 inhibitors in the context of cardiovascular diseases will also depend on these factors.

2.2 Targeting TNF and Interleukins

2.2.1 Tumor Necrosis Factor (TNF)

Tumor necrosis factor (TNF) is a proinflammatory cytokine with a broad spectrum of functions and targets. It controls inflammatory responses by regulating leukocyte activation, maturation, and cytokine/chemokine release. TNF secretion can be triggered by the recognition of PAMPs and DAMPs, but also by cytokines and interferon such as IL-1, GM-CSF, TGF- β , TNF- α itself (autocrine loop), and IFN- γ (Zelová and Hošek 2013). It is mainly produced by macrophages and T cells, but many other cell types, including non-immune cells such as endothelial cells and smooth muscle cells (SMCs), can also secrete TNF. Importantly, TNF seems to be highly expressed in human atherosclerotic plaques (Rus et al. 1991; Canault et al. 2006). Ohta et al showed that TNF enhances the expression of ICAM-1, VCAM-1, and MCP-1 in endothelial cells, favoring monocyte recruitment into the vascular wall. Additionally, an increase of scavenger receptor A dependent uptake of oxidized LDL by macrophages has been described, as well as decreased IL-1 β and INF- γ mRNA expression, which together strongly support a deleterious role of TNF in murine atherosclerosis (Ohta et al. 2005; Xiao et al. 2009). Interestingly, recent

studies suggest that TNF might also be involved in the regulation of the NLRP3 inflammasome (Sode et al. 2014; Bauernfeind et al. 2016; McGeough et al. 2017).

TNF is a central mediator of the inflammatory response and its signaling mechanism is well described. Many biological agents interfering with TNF signaling have been developed and tested in clinical trials in the context of inflammatory diseases associated with high cardiovascular risk, such as rheumatoid arthritis (RA), psoriatic arthritis, and Crohn's disease. For example, treatment with TNF blockers (infliximab or etanercept) in RA is associated with a lower incidence of cardiovascular events (Jacobsson et al. 2005). Two meta-analyses also confirmed that RA patients treated with anti-TNF therapy have a lower risk of cardiovascular events in comparison with patients treated with disease-modifying antirheumatic drugs (DMARDs) (Barnabe et al. 2011; Roubille et al. 2015). Currently, five different anti-TNF drugs are approved for clinical use, which include soluble TNF-receptor (etanercept) and TNF monoclonal antibodies (adalimumab, infliximab, golimumab, and certolizumab pegol). The evidence for their use in atherosclerotic cardiovascular disease will be discussed below.

Adalimumab

While adalimumab has been used as a safe therapy in a myriad of inflammatory diseases (Burmester et al. 2020), no large-scale trial regarding its effect on atherosclerotic cardiovascular disease exists. A small study in RA patients showed that treatment with adalimumab improves endothelial-dependent vasodilatation (Gonzalez-Juanatey et al. 2006), findings which were confirmed in a second study by the same authors. In this latter study, a comparison of baseline and post-treatment (12 months) carotid artery wall thickness (intima-media) measurement in RA patients showed no statistical difference (Gonzalez-Juanatey et al. 2012). Nevertheless, these observations suggest that adalimumab may improve the state of subclinical atherosclerosis in RA patients. Improvement of endothelial cell function was also observed in a study involving 14 psoriatic patients (Avgerinou et al. 2011). After 12 weeks of adalimumab treatment, no significant change on hsCRP level compared to baseline was observed, but ICAM-1 expression was decreased and the flow-mediated dilation (FMD), used as a marker of endothelial dysfunction, was improved. A larger study (NCT01722214) included 107 psoriatic patients randomized to receive either adalimumab for 52 weeks or placebo for 16 weeks followed by adalimumab for 36 weeks (per-protocol setting). At 16 weeks, the adalimumab treatment group had no difference in vascular inflammation compared to the placebo control group. At the 52-week time point, there was no difference observed in the ascending aorta with adalimumab, but a modest increase in vascular inflammation in carotids was described (Bissonnette et al. 2017). Thus, in lieu of a large clinical trial, the effect of adalimumab in ASCVD remains unclear.

Golimumab

Golimumab is a fully-humanized monoclonal anti-TNF antibody approved for the treatment of RA, ankylosing spondylitis (AS), psoriatic arthritis, and ulcerative colitis. Golimumab has a greater affinity for the soluble (sTNF) vs. the

transmembrane form (mTNF). While no data exist regarding the therapeutic benefit of golimumab in atherosclerosis, a pilot study in patients with ASCVD provided encouraging results (Tam et al. 2014). This randomized, double-blind, placebo-controlled trial aimed to assess the efficacy of Golimumab in preventing atherosclerosis progression and arterial stiffness in AS patients. A total of 20 patients received 50 mg of Golimumab monthly, with 21 receiving placebo treatment, for 1 year. After 6 months, no significant change in vascular parameters (i.e. aortic stiffness, carotid intima/media thickness) was observed between the two groups. However, a significant progression of the mean intima-media thickness (IMT) was only seen in the placebo group and not in the golimumab group. Maximum IMT, pulse wave velocity (PWV), and augmentation index (Aix) remained unchanged (paired t-test analysis). Nevertheless, no significant difference concerning vascular parameters was demonstrated between the two groups after 1 year of treatment. Further large-scale studies are needed to fully validate the potential effects seen in this study.

In addition to these findings in AS patients, TNF inhibitors were found to improve the overall pathologic profile of RA and psoriasis patients at high cardiovascular risk. Therapeutic targeting of TNF has shown benefit in the prevention of atherosclerosis in RA (Del Porto et al. 2007), which might also be the case in psoriasis (Sattar et al. 2007). Interestingly, a recent meta-analysis that includes 7,697 coronary artery disease (CAD) patients and 9,655 control patients has investigated the association of *TNF* gene polymorphisms and CAD susceptibility (Huang et al. 2020). The authors concluded that there was no association of *TNF* 308G/A, 857C/T, 863C/A, and 1031 T polymorphisms with CAD susceptibility. However, the *TNF* 238G/A genotype showed a significant association with higher CAD susceptibility in the subgroup of Europeans and North Asians, which suggests a direct role of TNF in CAD. Additionally, several studies described an adverse effect of TNF inhibitors on lipid profiles and described an increase of total cholesterol and triglycerides levels (Curtis et al. 2012; Hassan et al. 2016). In summary, further large-scale studies are required to evaluate the potential benefits of anti-TNF agents.

2.2.2 IL-6

Interleukin-6 (IL-6) is a pleiotropic cytokine involved in different arms of the immune system. It can result in both pro- and anti-inflammatory outcomes which can be explained by the different signaling pathways engaged: the classical pathway, the trans-signaling pathway, and the blockade pathway. The classical pathway occurs in a few cell types such as T cells, hepatocytes, and monocytes. It involves the IL-6 receptor (IL-6R) and glycoprotein 130 (gp130), a signal transducer sub-unit of the IL-6 receptor. In the trans-signaling pathway, the mechanism involves the signal transducer gp130 as well as soluble IL-6 receptor (sIL-6R). In contrast to the classical pathway, trans-signaling can occur in any cell type expressing a membrane-bound gp130 protein. In this scenario, sIL-6 binds to sIL-6R and forms a complex that can activate signaling through gp130 resulting in a greater magnitude of proinflammatory state. IL-6 signaling can also be blocked via the soluble form of gp130 which acts as a natural inhibitor of trans-signaling by binding sIL-6/IL-6R complex (Jostock et al. 2001).

Despite inconclusive data from experimental models (Elhage et al. 2001; Song and Schindler 2004; Schieffer et al. 2004; Schuett et al. 2012), shreds of evidence from translational studies identify IL-6 as a key regulator of atherogenesis. Expression of IL-6 was detected in human atherosclerotic plaques (Seino et al. 1994; Rus et al. 1996; Schieffer et al. 2000). Several studies found that high levels of IL-6 are associated with increased cardiovascular risk in human (Mendall et al. 1997; Ridker et al. 2000b; Fisman et al. 2006; Zakai et al. 2007; Danesh et al. 2008; Lefkou et al. 2010). Among them, a study from J. Danesh and colleagues, who compared serial measurements of serum IL-6 between patients with a history of coronary heart disease and controls. Results showed that long-term exposure to circulating IL-6 was associated with an increased risk of coronary artery disease (Danesh et al. 2008). However, follow-up studies of the CANTOS trial showed that patients with elevated IL-6 levels after canakinumab treatment have no significant benefit as opposed to patients with lower IL-6 levels (Ridker et al. 2018a, 2020).

Collectively, these studies and the studies based on the CANTOS trial suggest an important role of IL-6 in cardiovascular diseases. Targeting the IL-6 axis may be crucial to reduce the residual inflammation responsible for the increased risk of cardiovascular events.

Several clinical trials tested the therapeutic benefit of targeting the IL-6 axis using Tocilizumab (Table 1). However, higher-powered studies are required to better evaluate the safety and efficiency of current IL-6 targeting agents, and appreciate their benefit in preventing atherosclerosis.

2.3 Targeting Chronic Inflammation Using Broad Anti-inflammatory Drugs

Given the importance of inflammation in atherosclerosis, the usage of broad anti-inflammatory drugs could potentially reduce the risk of atherosclerotic events. Well known for their anti-inflammatory properties, methotrexate and colchicine are widely used as main treatments in inflammatory diseases with a high risk of CVD, notably RA, psoriasis, and other rheumatologic disorders (Saag et al. 2008; Singh et al. 2012). The repurposing of these two drugs as a therapeutic approach against atherosclerosis was evaluated in several clinical trials discussed below.

2.3.1 Methotrexate

Treatment with low-dose methotrexate (LD-MTX) reduces the levels of circulating CRP, IL-6, TNF-alpha and cardiovascular events in RA patients (Choi et al. 2002; Wessels et al. 2008; Westlake et al. 2010; Roubille et al. 2015). An important feature of LD-MTX is its ability to increase adenosine production and stimulate the adenosine A_{2A} receptor, which has been shown to promote the expression of several proteins involved in reverse cholesterol transport, thus potentially reducing foam cell formation (Reiss et al. 2008). The potential benefit of LD-MTX in the treatment of atherosclerosis was recently assessed in the cardiovascular inflammation reduction trial (CIRT) which is discussed below.

Table 1 Tocilizumab therapy and consequences on CVD risk factors

Drug	Target	Approval	Cohort	Trial design	Effect	References
Tocilizumab	IL-6R	RA patients	RA patients	Non-randomized pilot study	<ul style="list-style-type: none"> Improved endothelial function and aortic stiffness ↑ CRP level Modification of total cholesterol levels 	Proterou et al. (2011)
			RA patients	Non-randomized pilot study	<ul style="list-style-type: none"> Side effect on lipid profile: consequences on cardiovascular risk were not assessed 	Strang et al. (2013)
			RA patients	Randomized, double-blind placebo-controlled	<ul style="list-style-type: none"> Side effect on lipid profile: consequences on cardiovascular risk were not assessed 	McInnes et al. (2015)
			Non-ST-elevation myocardial infarction (NSTEMI) patients	Randomized, double-blind placebo-controlled	<ul style="list-style-type: none"> Inflammation and primary percutaneous coronary intervention related to high-sensitivity troponin T release ↑ CRP level 	Kleveland et al. (2016)

Cardiovascular Inflammation Reduction Trial (CIRT)

The CIRT trial was performed in parallel with the CANTOS trial and aimed to determine if LD-MTX could reduce cardiovascular events in patients with stable atherosclerosis and high cardiovascular risk (Everett et al. 2013). The double-blind trial included 4,786 patients with a history of myocardial infarction or multivessel coronary disease, as well as either type 2 diabetes or metabolic syndrome. Patients were randomized and treated with either LD-MTX (2,391) or placebo (2,395). The final primary endpoint was a composite of nonfatal myocardial infarction, nonfatal stroke, cardiovascular death, and hospitalization for unstable angina that led to urgent revascularization. After a median follow-up of 2.3 years, the primary endpoint occurred in 201 patients in the methotrexate group and 207 patients in the placebo group (HR 0.96, 95% CI 0.79–1.16). Also, treatment with LD-MTX did not reduce plasma levels of CRP, IL-1 β , IL-6, TNF- α . No significant difference between the two groups for any component of the primary endpoint was found.

Considering the success of the CANTOS trial and the beneficial effect seen with LD-MTX in RA patients, the CIRT trial results are disappointing. While CANTOS targeted a specific and well-defined pathway, the CIRT trial used a drug with a large spectrum of anti-inflammatory effects with molecular consequences which are not yet understood. Besides the different mechanisms of action, an additional explanation for the discrepant results between CIRT and CANTOS may be differences in the study design of the two studies. Patients recruited to the CIRT trial had a lower residual inflammatory profile (median baseline CRP: 1.6 mg/L) compared to patients from the CANTOS trial, where patient recruitment was based on hsCRP levels ≥ 2 mg/L as a marker of high residual inflammation (median baseline CRP: 4.2 mg/L). In support of this explanation, treatment of RA patients with LD-MTX led to a reduction of hsCRP (Westlake et al. 2010) and decreased cardiovascular risk.

These data demonstrate that the design of future clinical trials needs to consider inclusion criteria that target individuals with a high inflammatory profile.

2.3.2 Low-Dose Colchicine

Colchicine is an inexpensive anti-inflammatory drug used in patients with gout, familial Mediterranean fever, and pericarditis. By preventing microtubule assembly, colchicine can disrupt inflammasome activation, microtubule-based inflammatory cell chemotaxis, phagocytosis, and other host immune mechanisms. The first benefit of colchicine in ischemic heart disease (IHD) has been observed in familial Mediterranean fever patients (Langevitz et al. 2001). Patients diagnosed with FMF show a sustained inflammatory response which puts them at a higher risk of suffering a heart attack in comparison with the general population. Langevitz and colleagues showed that treatment with colchicine lowered the risk of IHD in FMF patients compared to untreated patients with other inflammatory conditions and similar rates of risk factors. Also, patients taking colchicine achieved a frequency of IHD comparable to the general population. To assess the potential benefit of colchicine in atherosclerosis, several clinical trials such as the low-dose colchicine trial (LoDoCO), or more recently the colchicine cardiovascular outcomes trial (COLCOT), have been carried out and will be discussed below.

LoDoCo-MI and LoDoCo-1/2 Trials

In 2007, a small pilot study had demonstrated the potential of low-dose colchicine to reduce hsCRP levels, independently of lowering cholesterol (Atorvastatin), in patients with stable coronary artery disease (Nidorf and Thompson 2007). However, in the randomized and placebo-controlled LoDoCo (Low-Dose Colchicine)-MI study (237 patients), colchicine at 0.5 mg/day for 30 days failed to reduce hsCRP levels (≤ 2 mg/L) in patients with recent MI (Hennessy et al. 2019).

Subsequently, the LoDoCo (Low-Dose Colchicine) trial was designed as a prospective study to assess the efficiency and safety of long-term low-dose colchicine usage in patients with stable coronary disease. 532 patients on lipid-lowering medication and antithrombotic therapy were randomized and treated with a daily dose of colchicine (0.5 mg/mL) or no colchicine. After a median follow-up of 3 years, the primary outcome (acute coronary syndrome, out-of-hospital cardiac arrest, or non-cardioembolic ischemic stroke) had occurred in 5.3% of patients in the low-dose colchicine group, as opposed to 16% in the non-treated group (Nidorf et al. 2013). To validate the observations from the LoDoCo study, a large-scale study (LoDoCo 2) involving 5,522 patients was conducted. LoDoCo 2 confirmed the significant treatment benefit of low-dose colchicine in reducing CVD risk in patients with stable coronary disease. The primary endpoint was a composite of cardiovascular death, spontaneous (non-procedural) myocardial infarction, ischemic stroke, or ischemia-driven coronary revascularization. The secondary endpoint was a composite of cardiovascular death, spontaneous MI, or ischemic stroke. Primary endpoints occurred in 6.8% of patients in the low-dose colchicine group as opposed to 9.6% in the placebo-treated group, and secondary endpoints occurred in 4.2% in the colchicine group and 5.7% in the placebo group. However, an increase in death from non-cardiovascular diseases was observed in the colchicine treated group, which further justifies the need for more targeted therapies that limit undesirable side effects (Nidorf et al. 2019, 2020; Pradhan 2021).

COLCOT Trial

Another recent double-blind randomized placebo-controlled colchicine intervention study, named COLCOT (colchicine cardiovascular outcomes trial), aimed to assess the benefit of colchicine in patients with a recent acute MI (Tardif et al. 2019). In total, 4,745 patients were divided into two groups that were given oral colchicine at 0.5 mg/day or a placebo. The primary efficacy endpoint was a composite of death from cardiovascular causes, myocardial infarction, stroke, urgent hospitalization for angina leading to coronary revascularization, or resuscitated cardiac arrest. At a median of 23 months, the primary composite endpoint had occurred in 5.5% of the treatment group and 7.1% in the placebo group (hazard ratio, 0.77). Remarkably, the hazard ratio for stroke (HR = 0.26) and hospitalization for angina (HR = 0.50) indicate an important benefit in the colchicine treated group for these two particular primary endpoints. Overall, colchicine showed a good safety and tolerability profile despite a slight but significant increase in pneumonia occurrence (0.9%) in the colchicine group compared to the placebo group (0.4%). There were no differences in peripheral blood counts and CRP levels among the treated group compared to the

placebo. Recent follow-up analysis suggests that the main benefit of colchicine therapy occurs when treatment is initiated in the first 3 days post-MI (Bouabdallaoui et al. 2020).

Additionally, the repurposed usage of low-dose colchicine has shown multiple benefits for the treatment of stable coronary artery diseases. A recent pilot study carried out in 80 patients with recent acute coronary syndrome suggests that low-dose colchicine uptake on a regular basis can efficiently stabilize plaques on top of optimal medical therapy (Vaidya et al. 2018). Further upcoming data from large-scale trials such as CLEAR-Synergy (4,000 patients with ST-elevation myocardial infarction undergoing percutaneous coronary intervention (NCT03048825)) will help to validate its efficiency and tolerability in coronary diseases.

2.4 Targeting Chemokine Signaling

Considerable work has been carried out on the identification of inflammatory mediators that actively participate in the progression of atherosclerosis which has increased the number of potential new therapeutic targets. Among them, chemokines were identified as key players in leukocyte recruitment/adhesion and activation as well as promotion of local inflammation in arteries. The contribution of chemokines to plaque formation/destabilization and thrombus formation has been extensively reviewed elsewhere (Zernecke and Weber 2014).

Despite the proven relevance of specific chemokines in atherogenesis, the clinical evaluation of drugs targeting specific chemokines or chemokine pairings has not progressed much. This is mainly due to the complexity of the “working network” within which chemokines operate and their critical role in fundamental host defense.

Several chemokines and their receptors such as CCL5/CCR5 (including heterodimeric interactions), CCL2/CCR2, CX3CL1/CX3CR1 are an active part of disease progression (Combadière et al. 2008). Drugs targeting those axes were developed in the context of other diseases like human immunodeficiency virus infections and facilitate the establishment of new clinical studies involving patients with cardiovascular diseases. Currently, drugs used to disrupt chemokine–receptor interactions rely on four main strategies: the modification of the chemokine N-terminal domain, the synthesis of small molecules used as a receptor antagonist, the usage of specific antibodies targeting chemokines, and other drugs interfering with chemokines’ heterodimeric interactions. Here, we will focus on promising chemokine targeting therapies with available clinical data in the context of atherosclerosis.

2.4.1 Targeting the CCR5 Axis

Expression of CCL5 and CCR5 has been identified in atherosclerotic plaques more than 15 years ago (Wilcox et al. 1994; Pattison et al. 1996; Veillard et al. 2004). Atheroprotective effects resulting from the perturbation of CCR5/RANTES (Regulated on Activation, Normal T cell Expressed and Secreted) interactions

were described in several mouse studies. The genetic inactivation of CCR5 in chow or high-fat diet-fed Apoe^{-/-} mice was shown to be protective in advanced atherosclerosis, but not in the early phase of the disease. The atheroprotective effect was associated with a decrease of macrophage numbers in plaques and reduced levels of circulating IL-6. The plaque quality was also affected by an increased content of SMCs, promoting plaque stabilization (Kuziel et al. 2003; Braunersreuther et al. 2007; Quinones et al. 2007). Moreover, a natural variant of CCR5 (CCR5Δ32 polymorphism) which reduces the cell surface expression of CCR5, is associated with a reduced risk of coronary artery diseases and MI (Szalai et al. 2001; González et al. 2001). Thus, the CCR5 pathway appears to be an interesting potential target for antiatherosclerotic therapy.

Maraviroc

The CCR5 inhibitor maraviroc is one of the few small molecules targeting a chemokine/receptor interaction approved by the US Food and Drug Administration and the European Medicines Agency as a preventive treatment for HIV (2007). HIV-associated inflammation increases HIV patients' risk of cardiovascular disease (Shah et al. 2018), which suggests a potential role of maraviroc in modulating the atherogenic risk of these patients. This was confirmed preclinically as the treatment of atherosclerosis-prone Apoe^{-/-} mice with maraviroc has been found to reduce atherosclerotic lesion development (Cipriani et al. 2013).

A recent pilot study in HIV patients showed that maraviroc treatment decreases carotid intima-media thickness, reflecting potential anti-atherogenic effects (Francisci et al. 2019). Additionally, an anti-human CCR5 monoclonal antibody (HGS004-Human Genome Sciences), tested in a clinical trial in HIV patients, showed a good safety profile and could be an interesting candidate for atherosclerosis therapy (Lalezari et al. 2008). Finally, a phase 4 clinical trial evaluating the efficacy of maraviroc in modulating atherosclerosis in HIV patients has been completed (NCT03402815) and results are forthcoming. This data will provide crucial information regarding the future of CCR5 axis targeting therapy in atherosclerosis.

2.4.2 Targeting the CCR2 Axis

Many pieces of evidence from experimental models demonstrate the proatherogenic role of CCR2 and its ligand CCL2/MCP-1 (Monocyte chemoattractant protein 1) (França et al. 2017). Notably, CCR2 signaling can promote monocyte recruitment to atherosclerotic lesions and foam cell formation (Boring et al. 1998; Bobryshev 2006). A recent discovery by Winter et al showed that the recruitment of myeloid cells within atherosclerotic lesions is subject to circadian regulation (Winter et al. 2018). This establishes a new concept of chrono-pharmacology-based therapy for the treatment of atherosclerosis, which is particularly relevant for the usage of CCR2 antagonists given their role in the promotion of monocyte recruitment (Winter et al. 2018).

MNL1202

In an effort to develop a specific antagonist of CCR2, a humanized antibody MNL1202 has been generated and tested in a phase 2 clinical trial in patients at risk for CVD (with at least 2 risk factors for atherosclerotic CVD, hsCRP level >3 mg/L, and receiving or not receiving lipid-lowering drugs). The direct blocking of CCR2 with MLN1202 was well tolerated and significantly reduced hsCRP levels in the antibody-treated group compared to the placebo-treated group (Gilbert et al. 2011). Thus MLN1202 appears to be a promising candidate to target atherosclerosis-associated inflammation.

Despite encouraging preclinical results, few molecules targeting chemokines and their receptors have been approved for clinical use yet. The fundamental role of chemokine signaling during both innate and adaptive immune response suggests that its targeting likely critically impairs the host immune response. For example, treatment with the CCL5 variant Met-RANTES or deficiency in CCL5 delays viral clearance by macrophages and impairs T-cell function (Makino et al. 2002). In this regard, the specific targeting of chemokine heteromerization represents a good alternative as it poses fewer risks of immunological side effects than chemokine antagonists. Up to now, 11 heteromeric chemokine pairs have been reported (Koenen and Weber 2010), expanding the therapeutic possibilities. Importantly, preclinical studies are carried out in a pathogen-free context, which is not representative of human pathogen exposure. Thus, future studies must assess the risk of long-term exposure to such chemokine targeting therapies. The complexity of the chemokine network, the lack of data concerning the toxicity of their targeting agents, and the availability of other, safer therapeutic approaches have drastically slowed down the translation of chemokine targeted therapies to human cardiovascular disease. Future studies should include a parallel assessment of risks and benefits provided by molecules targeting chemokine interactions to better appreciate the net effect on patients with cardiovascular diseases.

3 Modulation of the Adaptive Immune Response

Adaptive immunity plays a key role in driving and modulating the pathogenesis of atherosclerosis (Sage et al. 2018; ; Wolf and Ley 2019; Libby 2021). The adaptive branch of immunity consists primarily of T and B cells, both of which have the capacity to induce immune responses that are specifically targeted to disease-associated antigens. Adaptive immunity therefore represents an attractive therapeutic target as its modulation could allow for a more specific response, potentially reducing the risks of weakened host defenses associated with broad anti-inflammatory therapies (Fig. 2).

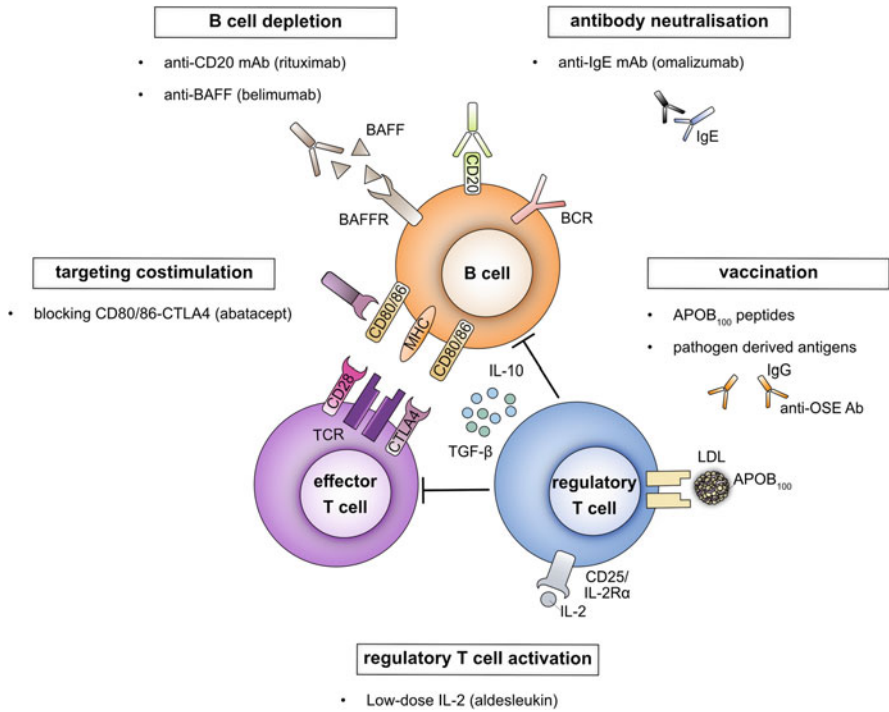


Fig. 2 Targeting adaptive immune responses in atherosclerosis. Regulatory T cells (T_{regs}) play a protective role in atherosclerosis via their immunosuppressive capacities such as the secretion of IL10 and TGF β . They can suppress the activity of proatherogenic effector T cells such as TH1 cells. T_{reg} numbers can be increased in a non-specific manner via the use of low-dose IL2 or in an antigen-specific manner by immunization with ApoB100-derived peptides. Furthermore, blocking effector T cell activation by inhibiting the interaction between co-stimulatory CD80/CD86 on antigen-presenting cells such as B cells and CD28 on T cells has been shown to reduce atherosclerosis in mice. B2 cells and many of their effector functions including some proatherogenic antibody isotypes such as IgE have been shown to promote atherogenesis. Thus, B cell depleting antibodies such as anti-CD20 or anti-BAFF antibodies have been shown to protect mice from atherosclerosis. Additionally, the depletion of proatherogenic antibodies such as IgE may have beneficial effects. On the other hand, immunization with modified LDL has been shown to induce protective antibody responses (both IgG and IgM) against oxidation-specific epitopes (OSE) that ameliorate atherosclerosis in preclinical studies

3.1 Strategies Targeting the Homeostatic Balance Between Regulatory and Effector T Cells

Although no therapies targeting T cells are currently in clinical use for the treatment and prevention of cardiovascular disease, some therapeutic approaches have shown promise in preclinical models and are being evaluated in clinical trials. In atherosclerosis-prone mice, regulatory T cells (T_{regs}) have been shown to be beneficial both in the progression and regression of atherosclerosis (Ait-Oufella et al. 2006; Mor et al.

2007; Klingenberg et al. 2013; Sharma et al. 2020). In contrast, many effector T cells are thought to be proatherogenic, particularly IFN γ -secreting T helper 1 (Th1) (Saigusa et al. 2020; Buono et al. 2005). In humans, cardiovascular disease is associated with an imbalance in T_{reg} and effector T cells, where T_{regs} are decreased in patients with acute coronary syndrome and low numbers are associated with increased rates of acute coronary events (Mor et al. 2006; Han et al. 2007; Cheng et al. 2008; Ammirati et al. 2010; Wigren et al. 2012).

Therefore, the aim of T cell-targeted therapeutic approaches in atherosclerosis is to change the homeostatic balance of different T cell subsets by expanding atheroprotective, immunosuppressive T_{regs} while suppressing putatively proatherogenic effector T cell populations (Foks et al. 2015). This could be achieved on the one hand by the stimulation of non-specific T_{regs} by targeting pathways such as interleukin-2 (IL-2). On the other hand, specific T_{regs} can be expanded with the use of tolerogenic vaccines against atherosclerosis-relevant antigens.

3.1.1 Low-Dose IL-2 Therapy

Interleukin-2 is a cytokine that plays a key role in the regulation of T cell activity and survival. Although IL-2 has the ability to regulate all T cells, it appears to be particularly important in the development and survival of T_{regs} (Boyman and Sprent 2012). The activation threshold for T_{regs} is lower than for other effector T cells (Yu et al. 2009), which may explain the somewhat paradoxical effect of IL-2 therapy. While high-dose IL-2 therapy is thought to promote effector immunity, low-dose IL-2 stimulates immunosuppressive T_{regs} (Klatzmann and Abbas 2015). A potential added benefit of this therapy could be the IL-2 mediated expansion of innate lymphoid cells 2 (ILC2) (Van Gool et al. 2014). ILC2 cells represent a rare subset of immune cells that likely exert atheroprotective functions via the secretion of type-2 cytokines such as IL-5 and IL-13 (Binder et al. 2004; Cardilo-Reis et al. 2012; Newland et al. 2017). Preclinical studies have shown that administration of recombinant IL-2 complexed with monoclonal antibodies against IL-2 increases the levels of T_{regs} and reduces atherosclerotic plaque burden in mice (Foks et al. 2011; Dinh et al. 2012; Proto et al. 2018).

The IL-2 analogue aldesleukin is currently approved for the treatment of advanced renal cell carcinoma and metastatic melanoma. In cardiovascular disease, low-dose IL-2 therapy is being evaluated in clinical trials with the aim to increase T_{reg} numbers, thereby limiting post-ischemic inflammatory responses and promoting myocardial healing in the setting of ischemic heart disease and acute coronary syndrome. The low-dose Interleukin-2 in patients with stable ischemic heart disease and acute coronary syndromes (LILACS) study is a randomized, placebo-controlled, double-blind phase I/IIa clinical trial that aims to assess the safety and tolerability of escalating doses of recombinant IL2, as well as its ability to alter T_{reg}, effector T cell and other immune cell populations (NCT03113773; expected completion date 06/2021; Zhao et al. 2018). Preliminary results indicate the treatment is well tolerated and induces robust increases in T_{regs} without affecting effector T cells. Interestingly, a dose-dependent reduction in B cells was also seen, which may have further beneficial effects on atherosclerosis (Zhao et al. 2020a). The subsequent

low-dose IL-2 for the Reduction of Vascular Inflammation In Acute Coronary Syndromes (IVORY) phase II trial will assess changes in vascular inflammation in patients with acute coronary syndromes (NCT04241601; expected completion date 01/2024; Zhao et al. 2020b).

One limitation of low-dose IL-2 therapy are potential off-target effects, which include mild increases in NK cells and eosinophils (Zhao et al. 2020a). Furthermore, initially protective T_{regs} may undergo a phenotypic switch toward a proinflammatory T_{H1} -like phenotype with progressing disease in mice (Li et al. 2016; Butcher et al. 2016; Wolf et al. 2020). It remains to be addressed whether this switch can occur in humans.

3.1.2 Alternative Means to Expand Regulatory T Cells

Alternative options to harness the immunosuppressive capacity of T_{regs} is by ex vivo expansion of T_{regs} , where autologous naïve Tregs are isolated from the blood and expanded in vivo using a cytokine cocktail or antigenic stimuli. The adoptive transfer of T_{regs} has been tested in humans in the setting of transplantation (Trzonkowski et al. 2009; Brunstein et al. 2011; Di Ianni et al. 2011; Mathew et al. 2018) and type 1 diabetes (Marek-Trzonkowska et al. 2014), but not cardiovascular disease. Another way to expand T_{regs} is using tolerogenic non-FcR binding anti-CD3 antibodies such as teplizumab, which have shown promise in clinical trials for the treatment and prevention of type 1 diabetes (Herold et al. 2013, 2019). However, the effect of anti-CD3 on atherosclerosis has so far only been addressed in preclinical murine studies alone (Steffens et al. 2006; Kita et al. 2014) or in combination with IL-2 complex (Kasahara et al. 2014), where it was associated with reduced atherosclerosis progression or increased T_{reg} -dependent plaque regression.

3.2 Strategies Targeting B Cells and Humoral Immunity

B cells play a well-established role in the pathogenesis of atherosclerosis, with different B cell subsets having distinct effects on the pathogenesis of atherosclerosis (Sage et al.). B cells exert their function primarily via the generation of humoral immunity in the form of highly specific antibodies, but they also have the capacity to act as antigen-presenting cells to T cells and to secrete cytokines. The most abundant B cell type, follicular B2 cells, as well as B cell-derived germinal center B cells that arise upon interaction with follicular T helper cells, are generally considered proatherogenic (Clement et al. 2015; Tay et al. 2018; Centa et al. 2019; Bagchi-Chakraborty et al. 2019). In contrast, marginal zone B2 cells may protect from atherosclerosis by suppressing T follicular helper cells (Nus et al. 2017) and potentially via the secretion of atheroprotective IgM (Grasset et al. 2015). B1 cells, which can be further subdivided into B1a and B1b cells, represent an innate-like B cell type that can secrete germline-encoded natural antibodies that are typically of the IgM class in a T cell-independent manner. Their atheroprotective function, which is dependent on their ability to secrete IgM, is well-established in mice (Lewis et al. 2009; Kyaw et al. 2012; Rosenfeld et al. 2015; Gruber et al. 2016; Tsiantoulas et al.

2017). Other minor B cell subsets include B1 cell derived innate response activator (IRA) B cells that can promote atherosclerosis via the secretion of GM-CSF and the subsequent induction of Th1-differentiation (Hilgendorf et al. 2014) and IL-10-secreting B regulatory cells (Bregs), whose role in atherosclerosis remains controversial (Strom et al. 2015; Sage et al. 2015; Douna et al. 2019).

Although most evidence on the role of B cells in atherosclerosis stems from preclinical studies, B cells also appear to be key players in human cardiovascular disease. Patients suffering from autoimmune diseases such as systemic lupus erythematosus (SLE) and rheumatoid arthritis have an increased risk of cardiovascular disease independently of other risk factors (Hollan et al. 2013). A genome wide association study (GWAS) and transcriptome analysis of the Framingham Heart Study cohort recently revealed genes associated with B cell activation and differentiation to be the most deregulated genes between patients with and without coronary heart disease, suggesting a key function for B cells in modulating atherosclerosis (Huan et al. 2013). B cells are present in the vascular wall of human atherosclerotic plaques (Hamze et al. 2013; Kortelainen and Porvari 2014), where they may undergo antigen-driven clonal expansion ((Burioni et al. 2009). However, it is currently not clear how findings from murine B cell subsets and their functions in cardiovascular disease translate to human B cells. For example, although B1 cells are a well-described atheroprotective subset in mice, the nature and existence of the human equivalent of murine B1 cells remain controversial (Griffin et al. 2011; Upadhye et al. 2020). McNamara and colleagues have recently found a subset of CXCR4-expressing B1 cells that correlated with the levels of IgM directed against MDA-LDL and that inversely correlated with plaque volume and stenosis (Upadhye et al. 2019). Furthermore, increased levels of CD86-expressing CD19+ B cells were found to associate with an increased risk of stroke, while CD40-expressing CD19+ B cells showed a negative association (Mantani et al. 2014).

Overall, the aim of B cell-targeted therapy is to reduce the proatherogenic effects of follicular B2 cells, ideally while preserving atheroprotective IgM-secreting B1 cells (Porsch and Binder 2019). Currently, B cell depletion therapy is primarily used in the treatment of B cell malignancies and in the treatment of autoimmune diseases. An alternative therapeutic target is humoral immunity. On the one hand, putatively proatherogenic antibody classes such as IgE may be neutralized. On the other hand, passive and active immunization strategies may be harnessed to specifically target atherosclerosis-associated antigens such as oxidation-specific epitopes.

3.2.1 Anti-CD20 Mediated B Cell Depletion (Rituximab)

Given the strong evidence for a proatherogenic role of follicular B2 cells, the major B cell type in the body, some high-risk patients may benefit from their depletion. Indeed, preclinical studies suggest that anti-CD20 mediated B cell depletion could ameliorate the development of several cardiovascular pathologies including atherosclerosis (Ait-Oufella et al. 2010; Kyaw et al. 2010), myocardial infarction (Zougari et al. 2013), myocardial infarction-accelerated atherosclerosis (Kyaw et al. 2021), abdominal aortic aneurysm (Schaheen et al. 2016), and hypertension (Chan et al. 2015).

In mice, anti-CD20 treatment preferentially depletes B2 cells, while preserving atheroprotective B1 cells (Hamaguchi et al. 2005). In line with that, anti-CD20 treatment of *Ldlr*^{-/-} and *Apoe*^{-/-} mice potently decreases B2 cell numbers and IgG levels and induces significant reductions in atherosclerosis (Ait-Oufella et al. 2010; Kyaw et al. 2010). In the context of acute myocardial infarction, Zougari et al. could show that anti-CD20 mediated B2 cell depletion was associated with reduced inflammation and CCL7-mediated monocyte mobilization while improving cardiac function, reducing infarct size and fibrosis (Zougari et al. 2013). Furthermore, anti-CD20 mediated B2 cell depletion significantly reduced myocardial infarction-accelerated atherosclerosis (Kyaw et al. 2021).

Rituximab is a CD20-targeted monoclonal antibody that has been approved for the treatment of some B cell malignancies (NHL, CLL) and autoimmune diseases (rheumatoid arthritis, granulomatosis with polyangiitis, pemphigus vulgaris) for more than two decades. Given its long clinical use, there is increasing evidence on its effect on cardiovascular parameters. Although a recent meta-analysis could not show an effect on short-term cardiovascular adverse events in randomized placebo-controlled trials involving rituximab, long-term follow-up data is lacking (Morris-Rosenfeld et al. 2014). Similarly, a study assessing the effects of biologic and conventional disease-modifying antirheumatic drugs on incident cardiovascular disease in rheumatoid arthritis patients with a mean follow-up of 4 years could find no beneficial effects of rituximab on cardiovascular risk, although the number of patients on rituximab in this study was relatively low (Ozen et al. 2020). However, several small human studies could show reductions in carotid intima-media thickness (Kerekes et al. 2009; Benucci et al. 2013; Novikova et al. 2016), improved flow-mediated dilation (Kerekes et al. 2009; Benucci et al. 2013; Hsue et al. 2014) and decreased arterial thickness (Provan et al. 2015) in patients receiving rituximab.

The Rituximab in patients with acute ST-elevation myocardial infarction (RITA-MI) trial was a prospective, open-label, single-arm phase I/IIa trial testing a single intravenous injection of rituximab in patients with STEMI within 48 h of symptom onset with a 6-month follow-up. Preliminary data suggests the treatment is well tolerated at all doses tested and resulted in a rapid, robust reduction of circulating B cells by a mean 96.3% that persisted throughout the follow-up period in a dose-dependent manner. Interestingly, immunoglobulin levels were not affected at that point. Additionally, clinical cardiac parameters including ejection fraction and (cardiac) biomarkers such as C-reactive protein (CRP) and B-type natriuretic peptide (BNP) were improved at follow-up (NCT03072199; Zhao et al. 2021). A subsequent phase IIb trial, RITA-MI 2, will be carried out in the form of a placebo-controlled randomized trial, which will allow further assessment of whether anti-CD20-mediated B cell depletion provides clinical benefits compared to placebo treatment (Zhao et al. 2021). The ICFER-RITU2 trial is another phase II clinical trial assessing the safety of rituximab and the effect of rituximab on cardiac fibrotic remodeling in patients with chronic heart failure with reduced ejection fraction (HFrEF; NCT03332888; Sánchez-Trujillo et al. 2019). Furthermore, the risk of atherosclerotic cardiovascular disease was reduced in patients receiving rituximab following

kidney transplantation (Kim et al. 2019), further supporting the potential of anti-CD20 mediated B cell depletion in the treatment of cardiovascular disease.

3.2.2 Other B Cell Depleting Antibodies

In addition to rituximab, there are emerging targets on B cells such as CD19 (inebilizumab, blinatumomab) and CD22 (inotuzumab ozogamicin) that are being targeted to deplete B cells in cancer and autoimmune disease (Hofmann et al. 2018). However, these receptors are also expressed on B1 cells, and may thus interfere with atheroprotective B cell effects. Additionally, there are therapies targeting receptors expressed on antibody-secreting plasma cells such as CD38 (daratumumab) and SLAMF7 (elotuzumab), which are used in the treatment of multiple myeloma (Radocha et al. 2021). However, the effect of such B cell depleting agents on cardiovascular disease has not been assessed directly.

3.2.3 B Cell Depletion via Targeting of the BAFF/APRIL System

An alternative means to deplete B cells is via the targeting of the B cell activating factor (BAFF)/A Proliferation-Inducing Ligand (APRIL) system, which is made up of soluble mediators that are required for B cell survival. BAFF appears to be particularly important in the survival of B2 cells (Rauch et al. 2009). In preclinical studies, BAFFR deficiency and anti-BAFFR mAb administration have been shown to robustly and selectively deplete B2 cells, thus decreasing atherosclerotic lesion formation (Kyaw et al. 2012; Sage et al. 2012) and improving recovery from myocardial infarction (Zougari et al. 2013). However, there is also evidence that BAFF may promote Breg differentiation (Yang et al. 2010) and that BAFF overexpression might protect from atherosclerosis (Jackson et al. 2016). BAFF may also exert atheroprotective functions via inhibition of TLR9-IRF7-dependent proinflammatory signaling in macrophages. Indeed, BAFF neutralization promoted lesion formation in atherosclerosis-prone mice and increased lesional CXCL10 content, suggesting potential adverse effects of BAFF-targeted therapies (Tsiantoulas et al. 2018). Belimumab is a BAFF-targeting antibody that is approved for the treatment of SLE, which robustly depletes B cells, plasma cells, and immunoglobulins. So far, the effect of belimumab on cardiovascular disease has not been assessed in human patients.

3.2.4 IgE Neutralization (Omalizumab)

The primary effector function of B cells is the generation of humoral immunity in the form of antigen-specific antibodies. There is ample epidemiological and preclinical experimental evidence suggesting an important role for humoral immunity and different antibody classes in atherosclerosis (Khamis et al. 2016; Sage et al. 2017; Tay et al. 2018; Centa et al. 2019; van den Berg et al. 2019). IgE is a class-switched, typically follicular B cell-derived antibody class that is associated with allergic responses. It can trigger proinflammatory responses via interaction with FcεRI receptors on mast cells, basophils, macrophages, and vascular cells (Wu and Zarrin 2014). Plasma IgE levels correlate with coronary heart disease in humans (Kounis

and Hahalis 2016). Interestingly, patients suffering from asthma are also at increased risk for cardiovascular disease (Tattersall et al. 2015). Preclinical studies suggest a proatherogenic role for IgE in Apoe^{-/-} and Ldlr^{-/-} mice (Wang et al. 2011; Wezel et al. 2015; Tsiantoulas et al. 2017; Zhang et al. 2020). Antibody-mediated IgE neutralization was found to reduce atherosclerotic lesion formation in Ldlr^{-/-} mice in the context of secreted IgM deficiency (Tsiantoulas et al. 2017).

Omalizumab is an IgE-neutralizing antibody that interferes with the interaction of IgE and its receptors, resulting in the reduction of IgE levels. It is approved for the treatment of severe asthma and chronic idiopathic urticaria. Although a clinical trial suggested potential cardiovascular side effects of omalizumab treatment, this clinical trial was biased by the dramatically increased cardiovascular disease burden at baseline in the patients receiving omalizumab (Iribarren et al. 2017a). A subsequent pooled analysis of randomized, double-blind placebo-controlled trials involving omalizumab did not show an increased cardiovascular risk (Iribarren et al. 2017b). Therefore, the effect of IgE neutralization on cardiovascular disease remains to be evaluated in a clinical trial setting.

3.3 Strategies Targeting the Interaction Between T Cells and Antigen-Presenting Cells

Efficient T cell activation is crucially dependent on the engagement of co-stimulatory pathways. It has been hypothesized that T cells require two signals in order to become fully activated, one being antigen-presentation via MHC-molecules, the other being co-stimulation by receptors expressed on the surface of antigen-presenting cells such as dendritic cells, macrophages, and B cells. Inhibition of such co-stimulatory pathways may therefore interfere with the activation, proliferation, and differentiation of T cells, which may have beneficial effects on atherosclerosis. In contrast, targeting co-inhibitory pathways with the use of immune checkpoint inhibitors in cancer therapy may enhance T cell activity, which could have detrimental effects on cardiovascular disease.

3.3.1 Targeting Co-stimulatory Pathways

Abatacept

CD80 (B7-1) and CD86 (B7-2) are expressed on activated antigen-presenting cells, where they can interact with both the co-stimulatory CD28 receptor and the inhibitory CTLA4 receptor on T cells (Sharpe and Freeman 2002).

Abatacept is a recombinant biologic consisting of the Ig-domain of CTLA4 and a human IgG1 Fc region. It binds CD80/CD86 with high affinity, thus blocking the interaction of CD80/CD86 with the co-stimulatory CD28 receptor and activation of T cells. Preclinical studies suggest a primarily proatherogenic role for CD80/CD86. However, CD80/CD86 may also be required for the efficient induction of T_{regs} (Ait-Oufella et al. 2006). However, abatacept was shown to reduce atherosclerosis in different murine models of atherosclerosis (Ma et al. 2013; Ewing et al. 2013).

Abatacept is currently clinically approved for the treatment of rheumatoid, juvenile idiopathic, and psoriatic arthritis, while the highly similar belatacept is used in patients receiving kidney transplants. Some clinical trials suggest slightly reduced cardiovascular disease risk in patients receiving abatacept compared to TNF inhibitors (Zhang et al. 2016; Kang et al. 2018; Jin et al. 2018; Hsieh et al. 2020).

CD40-CD40L

Another important co-stimulatory signal is provided by the CD40-CD40L interaction, a member of the tumor necrosis factor receptor (TNFR) family. Targeting this pathway reduced atherosclerosis in mice (Lutgens et al. 2000, 2010; Schönbeck et al. 2000), but not in others (Zirlik et al. 2007). Additionally, CD40-signaling intermediates, the so-called TNF-receptor associated factors (TRAFs), have been targeted successfully by a small molecule inhibitor in mouse models (Lutgens et al. 2010; Chatzigeorgiou et al. 2014). Importantly, no immunosuppressive side effects were observed with this inhibitor (Seijkens et al. 2018) and a newly developed delivery system, linking a TRAF6 inhibitor to a HDL particle, was found to be safe in non-human primates (Lameijer et al. 2018). Based on these initial findings, further assessment of CD40-TRAF inhibition needs to be carried out to verify its role in human atherosclerosis. While CD40-CD40L targeting therapeutics have not been approved for clinical use yet, several antagonistic antibodies are currently being evaluated in clinical trials for the treatment of inflammatory conditions (Karnell et al. 2019).

Co-inhibitory Pathways

Recent years have seen the emergence of cancer immunotherapy and the widespread use of immune checkpoint inhibitors. Immune checkpoint inhibitors are used clinically with the aim to enhance anti-tumor immunity by T cells. Many of these drugs target pathways that have been described to play a proatherogenic role. Given the prominent role of effector T cells and these immunomodulatory pathways in cardiovascular disease, such T cell-stimulating therapy is expected to have detrimental effects on atherosclerosis (Simons et al. 2019). Importantly, a recent study by Drobni et al. could demonstrate a threefold increased risk for cardiovascular events and more than threefold increased aortic plaque volumes in patients receiving immune checkpoint inhibitor therapy (Drobni et al. 2020). Additionally, atherosclerotic mice which underwent short-term check point inhibition therapy developed a more vulnerable, proinflammatory atherosclerotic phenotype due to an excess of effector T cells, endothelial activation, and increased CD8+ T cell recruitment (Poels et al. 2020). This study highlights the importance of cardiovascular risk awareness in cancer patients receiving checkpoint inhibitor immunotherapy.

Vaccination Strategies

Vaccines represent an attractive future therapeutic avenue, as they may provide a durable, antigen-specific approach, thus limiting adverse effects on the host immune response. However, vaccination approaches remain challenging, as they require the targeting of specific self-antigens that may trigger unwanted autoimmune responses.

Unlike traditional vaccines that aim at enhancing the activity of the adaptive immune system against pathogens, the idea behind immunization strategies against atherosclerosis is either to induce immunological tolerance or to neutralize atherosclerosis-specific antigens without inducing a considerable inflammatory response. Additionally, passive immunization strategies offer the opportunity to directly administer antibodies that may modulate inflammatory and metabolic pathogenic mechanisms in atherosclerosis, although their effect is transient.

3.3.2 Immunization Against LDL-Related Antigens

LDL and particularly its modified forms represent well-established immunogenic epitopes that have previously been shown to play a role in atherosclerosis and to be recognized by various components of the immune system. LDL can undergo oxidative modifications that result in the formation of a variety of lipid peroxidation-derived oxidation-specific epitopes (OSE), including phosphorylcholine (PC)-containing oxidized phospholipids and malondialdehyde (MDA), which are highly immunogenic and trigger inflammation in the vascular wall. Notably, OSE are targeted by specific circulating antibodies that have been shown to neutralize their proinflammatory activities. Thus, several experimental studies have addressed the effect of immunization with modified LDL or parts of its main lipoprotein component, Apolipoprotein B (ApoB).

Palinski and colleagues were able to show that immunization with malondialdehyde (MDA)-modified LDL reduced atherogenesis in LDLR-deficient rabbits (Palinski et al. 1995), which was further confirmed by several groups using modified LDL in rabbits (Ameli et al. 1996; Nilsson et al. 1997; Asgary et al. 2007) and mice (George et al. 1998; Freigang et al. 1998; Zhou et al. 2001; Binder et al. 2004; Van Puijvelde et al. 2006). Specific advanced MDA adducts have been shown to be particularly immunogenic, which may further impact the effect of vaccination on atherogenesis (Gonen et al. 2014).

Although the mechanisms responsible for the atheroprotective effects observed in these studies have not been fully elucidated, several pathways play a role, including the induction of neutralizing antibodies that interfere with the proinflammatory effects of these OSE. However, even though several preclinical studies have shown a prominent induction of humoral immunity upon immunization (George et al. 1998; Freigang et al. 1998; Zhou et al. 2001; Binder et al. 2004) and the inverse association between plaque size and antibody levels against OSEs (Zhou et al. 2001), it is currently not clear how these findings translate to the clinic.

Passive immunization via administration of antibodies against OSE has shown promise in preclinical models (Schiopu et al. 2004, 2007; Poulsen et al. 2016). The phase II GLACIER trial failed to show reduced vascular inflammation following administration of a recombinant IgG1 antibody against MDA-modified ApoB100 (Lehrer-Graiwer et al. 2015). However, the relatively short follow-up period, the use of FDG-PET instead of coronary artery assessments, and the patient selection criteria may have been a limitation of this study. Given the proposed atheroprotective role of IgM against oxidation-specific epitopes, it will be interesting to assess the effect of passive administration of IgM antibodies (or single chain and other class switch

variants thereof) on human atherosclerosis in the future. One attractive candidate is the IgM antibody E06, which binds the phosphocholine (PC) headgroup of oxidized phospholipids (oxPLs), thus blocking the proinflammatory properties of oxPL and the uptake of oxLDL by macrophages (Shaw et al. 2000; Que et al. 2018).

3.3.3 Tolerogenic Vaccination

Another proposed atheroprotective mechanism is tolerization, which is associated with the induction of immunosuppressive T_{regs} that may limit inflammation via IL10, TGF β and the suppression of T effector functions. Several preclinical studies found the reduction in atherosclerotic lesion formation upon administration of modified LDL or ApoB100-derived peptides to be associated with significant increases in T_{reg} numbers (Fredrikson et al. 2008; Wigren et al. 2011; Herbin et al. 2012).

In addition to lipid-derived antigens, peptide-based vaccination strategies based on ApoB100-derived peptides, many of which are recognized by autoantibodies from sera of patients with coronary heart disease, have also shown promise in preclinical studies (Fredrikson et al. 2003). Administration of several ApoB100 peptides, including P210, P45, P74, (Fredrikson et al. 2003, 2005, 2008; Dunér et al. 2021), P2 (Chyu et al. 2005), P3 (Tse et al. 2013), P18 (Kimura et al. 2018), P265 and P295 (Gisterå et al. 2017), has been shown to reduce lesion formation in murine atherosclerosis

However, several challenges remain before the clinical implementation of inflammation-restraining tolerogenic T cell vaccine is possible. Given that the interaction of TCRs with their cognate peptide is dependent on the efficient presentation on MHC type II molecules, HLA types need to be matched. The necessity for HLA matching has resulted in tetramer-based approaches to allow the identification of antigen-specific T cell populations (Kimura et al. 2017, 2018). The considerable heterogeneity of the human HLA locus and the need to adapt peptides to individual HLA types present a major challenge in the design of T-cell based peptide vaccines. Furthermore, the efficient induction of tolerance critically depends on dose, choice of adjuvant, and the route of administration (Van Puijvelde et al. 2006, 2007; Klingenberg et al. 2010; Czerkinsky and Holmgren 2012).

3.3.4 Immunization Against Pathogen-Derived Antigens

Some studies suggest that vaccination against common diseases such as influenza and pneumonia can reduce cardiovascular risk (Ren et al. 2015; Barnes et al. 2015). A double-blind randomized placebo-controlled clinical trial (Influenza Vaccination After Myocardial Infarction, IAMI; NCT02831608) is currently underway to assess the effect of influenza vaccination on cardiovascular outcomes in patients with preexisting cardiovascular disease (Fröbert et al. 2017). While it cannot be excluded that inflammation associated with infections may affect the plaque, the potential beneficial effect of pneumococcal vaccination may be due to its ability to induce antibodies that react with the oxidation-specific epitope, PC (Binder et al. 2003; Caligiuri et al. 2007). Initial studies have evaluated the consequences of the molecular mimicry between oxidized LDL and the CPS of pneumococci and demonstrated that immunization of $Ldlr^{-/-}$ mice with pneumococcal extracts induced PC-specific

IgM and reduced lesion formation (Binder et al. 2003). A similar effect has been observed when Apoe^{-/-} mice were immunized with PC-conjugated KLH (Caligiuri et al. 2007).

These studies suggested the possibility that existing pneumococcal vaccines could be repurposed to trigger anti-atherogenic immunity in humans. However, PC is not a major antigenic component of pneumococcal vaccines due to poor immunogenicity, and several studies indicated that unlike natural infections vaccination with existing vaccines does not trigger robust anti-OxLDL antibody titers. Small-scale clinical trials of the 13-valent conjugated pneumococcal vaccine Prevnar-13 did not find an induction of antibodies against oxLDL or phosphorylcholine in a small cohort of patients with metabolic diseases (Grievink et al. 2020; Shiri-Sverdlov et al. 2021), which may be due to the fact that PC is only a minimal constituent of Prevnar-13. The AUSPICE trial to assess the effect of the pneumococcal polysaccharide vaccine Pneumovax 23 on antibody levels against oxidation-specific epitopes and several cardiovascular disease markers in 4725 patients is currently underway (ACTRN12615000536561; Ren et al. 2016).

Heat shock proteins (HSPs) represent a group of highly conserved stress-associated proteins that can act as autoantigens either due to cross-reactivity against microbial-derived HSPs or following their expression during endothelial injury (Wick et al. 2014). Preclinical studies show that administration of microbial HSPs or HSP-related peptides protects mice from atherosclerosis (Maron et al. 2002; Van Puijvelde et al. 2007; Jing et al. 2011; Klingenberg et al. 2012; Grundtman et al. 2015). The atheroprotective mechanism may involve induction of cross-reactive antibodies against MDA-LDL (Kyrklund et al. 2020) or tolerogenic activation of T_{regs} (Van Puijvelde et al. 2007).

4 Conclusion

The immune system represents a versatile and essential part of human homeostasis which harbors great potential for the treatment and prevention of atherosclerosis. While the identification of relevant therapeutic targets remains challenging, it has greatly improved over the last decade as various anti-inflammatory and immunomodulatory approaches have been studied. These range from modulation of the innate immune system by interfering with cytokines and their production, chemokines and general inflammation, to the adaptive immune system where B cells, T cells, their interaction and their potential in immunization are utilized. While these approaches have proven promising in multiple preclinical studies, with some translation into human trials, a wider application and breakthrough into the clinic is yet to occur. One factor which slows the translation of preclinical findings into the clinic is the immune system's essential role in fighting infections. Unspecific modulation or downregulation of the immune system can lead to unwanted side effects such as increased infections. It is thus important to conduct further research in order to harvest the positive potential while minimizing side effects. Research into potential biomarkers such as hsCRP and IL-6 – but with even greater specificity for

the atherogenic disease process – represents an important step toward solving this issue, as they allow the identification of patients who are most likely benefiting from immunomodulatory treatment. Moreover, highly specific biomarkers would also be needed to monitor a successful response to anti-inflammatory or immunomodulatory treatments. Additionally, further research into the immune system's role in human atherosclerosis, combined with large-scale clinical trials will pave the way for immunomodulatory treatments to become a realistic addition to standard atherosclerosis therapies.

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Neutrophil Extracellular Traps in Atherosclerosis and Thrombosis

Thomas M. Hofbauer, Anna S. Ondracek, and Irene M. Lang

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Abstract

Despite effective therapeutic and preventive strategies, atherosclerosis and its complications still represent a substantial health burden. Leukocytes and inflammatory mechanisms are increasingly recognized as drivers of atherosclerosis. Neutrophil granulocytes within the circulation were recently shown to undergo neutrophil extracellular trap (NET) formation, linking innate immunity with acute complications of atherosclerosis. In this chapter, we summarize mechanisms of NET formation, evidence for their involvement in atherosclerosis and thrombosis, and potential therapeutic regimens specifically targeting NET components.

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Keywords

Atherosclerosis · Cl-amidine · Deoxyribonuclease · Heparin · Myocardial infarction · Neutrophil extracellular traps · Neutrophils · Thrombosis

Abbreviations

AAA	Abdominal aortic aneurysm
citH3	Citrullinated histone H3
DNase	Deoxyribonuclease
dsDNA	Double-stranded DNA
DVT	Deep vein thrombosis
ET	Extracellular traps
HIT	Heparin-induced thrombocytopenia
IL	Interleukin
MI	Myocardial infarction
MPO	Myeloperoxidase
NE	Neutrophil elastase
NETs	Neutrophil extracellular traps
PAD	Peripheral artery disease
PAD-4	Peptidylarginine deiminase 4
PE	Pulmonary embolism
PMA	Phorbol myristate acetate
ROS	Reactive oxygen species
tPA	Tissue plasminogen activator
VTE	Venous thromboembolism

1 Introduction

Atherosclerosis accounts for a substantial global disease burden. In recent decades, significant progress in understanding atherosclerosis was made. The identification of modifiable risk factors (Catapano et al. 2016), e.g. arterial hypertension or hypercholesterolemia, and the advent of percutaneous primary coronary intervention for treating acute complications of atherosclerosis like acute myocardial infarction (MI) (Beran et al. 2002) have led to a further reduction in incidence, cardiovascular morbidity, and markedly improved prognosis (Herrington et al. 2016). Still, due to the growing global population, the absolute number of deaths attributed to atherosclerotic disease is rising (Barquera et al. 2015) and increasing the need for novel therapies.

Besides traditional risk factors, inflammation and leukocytes are increasingly recognized as contributors to vascular disease and its complications. Pathomechanisms are manifold, with T cells (Bullenkamp et al. 2016), B cells

(Sage et al. 2019), dendritic cells (Gil-Pulido and Zernecke 2017), monocytes, and macrophages (Moroni et al. 2019) being implicated. Recently, neutrophils were suggested to significantly promote atherosclerosis (Doring et al. 2015), particularly its acute vascular syndromes (Mangold et al. 2015). Upon activation, neutrophils are able to undergo drastic morphological changes, leading to cellular disintegration and release of intracellular content into the extracellular space, in a process called neutrophil extracellular trap (NET) formation (Brinkmann et al. 2004). Besides atherosclerosis (Doring et al. 2017), NETs have emerged as important drivers of disease (Papayannopoulos 2018), including auto-immunity, sepsis, and cancer.

In this chapter, we aim to give a broad overview of the role of vascular NETs in atherosclerotic disease and its specific manifestations. Finally, we discuss potential therapeutic regimens targeting NETs and their components.

2 Neutrophils and Neutrophil Extracellular Traps

Under physiological conditions, neutrophil granulocytes comprise approximately 60% of total leukocytes in humans (Bainton et al. 1971). Neutrophils have a rather short lifespan and are released from the bone marrow into the circulation (Ley et al. 2018). Neutrophils are crucial fighters of the innate immune system, being the first cell population recruited to sites of inflammation and injury (Distelmaier et al. 2014). Neutrophils are critical for host defense (Wang and Arase 2014).

Their abundance and cellular properties enable them to effectively fight pathogens by phagocytosis, degranulation, or cytokine secretion (Witko-Sarsat et al. 2000). Neutrophils are equipped with numerous types of granules (Cowland and Borregaard 2016) containing catalytic enzymes, such as myeloperoxidase (MPO) and serine proteases. Upon phagocytosis of pathogens, neutrophils can fuse their granules with phagolysosomes, resulting in intracellular degradation of pathogens. Alternatively, neutrophils can release their granular content into the extracellular space (Cowland and Borregaard 2016).

Another effector mechanism of neutrophils was identified only 15 years ago, although first evidence had already emerged in 1996: It was reported that phorbol myristate acetate (PMA) could rapidly induce cell death of neutrophils. Thereby, neutrophils underwent substantial morphological changes different from apoptosis or necrosis, including signs of nuclear decondensation (Takei et al. 1996). Extending this finding, the group of Arturo Zychlinsky demonstrated that not only PMA, but also lipopolysaccharide (LPS) or interleukin (IL)-8 led to the appearance of extracellular structures described as fragile fibers of decondensed DNA covered in histones and granule proteins. Gram-positive as well as gram-negative bacteria could be ensnared by these NETs, and presence of neutrophil elastase (NE) or MPO promoted degradation of important bacterial virulence factors (Brinkmann et al. 2004).

Using live-cell imaging, NET formation was found to be an active process, different from apoptosis and necrosis. After stimulation, isolated neutrophils first flattened, forming intracellular vacuoles, followed by loss of the nuclear lobular

shape and further expanding in the cytoplasm. In this stage, neutrophils were still viable, containing the vital dye calcein blue but were not positive for Annexin V. This was reversed upon ultimate rupture of the plasma membrane. Furthermore, and in contrast to apoptosis, chromatin was decondensated. Intracellular membranes were fragmented, enabling mixing of nuclear, granular, and cytoplasmic components, which was not a feature of necrosis (Fuchs et al. 2007).

An important pre-requisite for NET formation is the presence of reactive oxygen species (ROS) (Fuchs et al. 2007; Hakkim et al. 2011). Inhibiting ROS production diminished NET formation, and neutrophils isolated from chronic granulomatous disease patients carrying mutations in the phagocyte NADPH oxidase did not produce NETs in response to PMA. However, when hydrogen peroxide was added to the system, the ability to form NETs was restored. These experiments indicated that NETosis is dependent on assembly and activation of the NADPH oxidase (Fuchs et al. 2007) and can be triggered by protein kinase C signaling via the raf-MEK-ERK pathway (Hakkim et al. 2011).

ROS serve as substrate for MPO, but presence of both is an essential stimulus for activation of NE (Metzler et al. 2014). Upon nuclear translocation, NE degrades core histones, facilitating decondensation of chromatin in synergy with histone citrullination by the calcium-dependent enzyme peptidyl arginine deiminase 4 (PAD-4) (Wang et al. 2004). Subsequent disintegration of intracellular membranes enables adsorption of granular proteases and antimicrobials onto chromatin. Rupture of the outer cell membrane finally leads to expulsion of cellular meshwork resulting in formation of NETs (Remijnsen et al. 2011). Many of these released NET-associated proteins were shown to be degraded by neutrophil proteases *in vitro*, probably reducing their capacity to act as autoantigens *in vivo* (de Bont et al. 2020).

Since their discovery, pathways of NET formation are under thorough debate. Numerous triggers were reported; however, key events essential for NETosis could hardly be connected by signaling molecules to describe defined intracellular cascades. Even the absolute necessity of PAD-4 activity for NET formation is in question. Knockout or inhibition of PAD-4 was, on the one hand, reported to disrupt mouse and human NET formation (Lewis et al. 2015; Li et al. 2010; Martinod et al. 2013) while, on the other hand, other groups still observed NETs in response to the same stimuli independent of PAD-4 activity (Claushuis et al. 2018; Kenny et al. 2017). These conflicting data highlight the problems regarding different methods and also different interpretations of results. Nevertheless, citrullinated histone H3 (citH3) is still considered the most specific marker for NET formation. Differentiation between “vital” and “suicidal” NETosis even questions terminology itself by indicating that NET formation does not have to result in immediate cell death (Desai et al. 2016; Madhusoodanan 2017). Despite uncertainty regarding NET formation, it is increasingly recognized that presence of NETs fundamentally influences disease, including atherosclerosis and thrombosis.

Recently, the exclusivity of extracellular trap (ET) formation to neutrophils came into debate. Among granulocytes, mast cells (Campillo-Navarro et al. 2017) and eosinophils (Mukherjee et al. 2018) were reported capable of forming ETs. Another group even found monocytes to release ETs containing myeloperoxidase and citH3

(Granger et al. 2017). Furthermore, evidence implicated macrophages to equally expel their intracellular content in a process of ET formation (Doster et al. 2018). However, the significance of these non-neutrophil-associated ETs so far remains incompletely understood.

3 NETs in Venous Thrombosis

Deep vein thrombosis (DVT) and its major complications are prevalent in Europe and associated with high morbidity and mortality. Virchow's triad (Kumar et al. 2010) serves as an excellent framework for understanding risk factors of thrombosis, which are hypercoagulability, vascular dysfunction, and stasis. Recently, however, it was proposed to extend this triad to a tetrad, taking into account the paramount influence of the immune system and its dysregulation on thrombosis (Kapoor et al. 2018). In a rat model of inferior vena cava ligation, pro-inflammatory neutrophils were observed in emerging thrombi and vein walls (Wakefield et al. 1995). In humans, the pro-inflammatory markers interleukin (IL)-6 and C-reactive protein in plasma were increased in DVT and gradually declined after disease onset (Roumen-Klappe et al. 2002), while C-reactive protein predicted post-thrombotic syndrome (Roumen-Klappe et al. 2009).

The potential role of NETs in venous thrombosis was indicated by a flow chamber experiment, where NETs provided a fibrous scaffold for fibrin, von Willebrand factor (vWF) and platelets. NETs were then observed in thrombi of baboons subjected to experimental DVT (Fuchs et al. 2010). In a mouse model of DVT, large amounts of DNA were observed in thrombi, forming NET-like structures (von Bruhl et al. 2012). These observations were complemented by another group showing that fresh parts of thrombi were rich in the NET-specific marker citH3, which co-localized with vWF (Brill et al. 2012).

The first observation of NETs in human venous thrombosis was presented in a case report of a patient suffering from microscopic polyangiitis and DVT: both in kidney and thrombus samples, NETs were abundantly present (Nakazawa et al. 2012). Characterizing thrombi based on histological methods, DNA webs and citH3 were concentrated in organizing sections of thrombi, but not in already organized parts (Savchenko et al. 2014). Extending these findings, plasma DNA levels were found increased, diagnosing DVT with a sensitivity of 81%. Also, thrombus DNA positively correlated with D-dimer, vWF activity, the clinical Wells score, and neutrophil-derived MPO (Diaz et al. 2013). Similar results were obtained with concentrations of nucleosomes, which were elevated in DVT patients and positively correlated with neutrophil activation (van Montfoort et al. 2013).

Venous thromboembolism, mainly presenting as pulmonary embolism (PE), is a major complication of DVT (Di Nisio et al. 2016). Levels of nuclear DNA were shown to be elevated in PE (Arnalich et al. 2013) and independently predictive of mortality (Jimenez-Alcazar et al. 2018). In chronic thromboembolic pulmonary hypertension, a long-term sequela of PE (Lang 2004) characterized by the apposition of non-resolving, organized clots (Galie et al. 2016), neutrophils were shown to be

hyperresponsive (Rose et al. 2003) and present in superficial areas of thrombi (Quarck et al. 2015), while soluble NET surrogates were increased compared to healthy controls (Aldabbous et al. 2016).

4 NETs in Atherosclerosis and Arterial Thrombosis

4.1 Atherosclerosis

First evidence for the importance of neutrophils in human atherosclerosis arose indirectly, when increased MPO levels predicted risk of coronary artery disease independently of traditional risk factors (Zhang et al. 2001). High numbers of circulating neutrophils as important source of MPO were linked to both formation and severity of atherosclerotic lesions (Huang et al. 2001) and chronic stable angina pectoris (Avanzas et al. 2004). Direct assessment of human atherosclerotic lesions revealed presence of MPO (Daugherty et al. 1994) and neutrophils (Tavora et al. 2009) producing pro-inflammatory IL-8 (Marino et al. 2015). The extent of neutrophil infiltration was associated with a pro-inflammatory state and rupture-prone lesions (Ionita et al. 2010). Furthermore, levels of the NET surrogate markers dsDNA and chromatin were independently associated with the severity of coronary atherosclerosis and occurrence of adverse cardiovascular events (Borissoff et al. 2013).

Experimental models to identify mechanistic pathways of atherosclerosis typically rely on mice deficient for Apo E or low-density lipoprotein receptor, which are fed with a high-fat diet to develop atherosclerotic lesions. This led to the identification of a plethora of contributors underlying lesion formation and progression and confirmed a significant role for neutrophils in plaque development.

MPO-positive neutrophils were predominantly found in lesional caps of plaques (van Leeuwen et al. 2008) and plaque regions with already high inflammatory activity outnumbering present macrophages (Rotzius et al. 2010). Depletion of neutrophils was shown to attenuate lesion formation (Zernecke et al. 2008); however, protective effects were only apparent if depletion was performed within the first weeks, confining the effect of neutrophil activity to early stages of plaque development (Drechsler et al. 2010).

In *Apo E* knockout mice, neutrophils adhered to the luminal site of carotid atherosclerotic lesions and released DNA, indicative of NET formation, an interpretation which was supported by visualization of NETs in human endarterectomy samples (Megens et al. 2012). Presence of NETs was further verified in atherosclerotic lesions of mice in conjunction with an increase of the pro-inflammatory markers IL-1 α , IL-1 β , and IL-6 (Warnatsch et al. 2015). NETs, via histones, induced lytic cell death of smooth muscle cells in atherosclerotic lesions, leading to decreased plaque stability (Silvestre-Roig et al. 2019).

Recently, atherosclerotic lesions were classified into rupture-prone or erosion-prone phenotypes (Quillard et al. 2017). A growing body of evidence suggests that pathomechanisms are profoundly different in these two entities. Rupture-prone

lesions typically contain many macrophages, harbor large lipid pools but have low interstitial collagen and few smooth muscle cells covered by thin fibrous caps. Disruption of fibrous caps makes up about two thirds of coronary events (Prati et al. 2013; Virmani et al. 2000). Conversely, eroded plaques typically present with a thick or even intact fibrous cap with a discontinuous endothelial layer (Libby 2017). Neutrophil infiltration appears to be critical for erosion as shown by an optical coherence tomography study to distinguish between plaque rupture and erosion in acute coronary syndrome. Of 25 included patients, seven exhibited erosion, and levels of systemic MPO were strikingly increased compared to patients with plaque rupture (Ferrante et al. 2010). Characterization of human endarterectomy samples demonstrated that presence of NETs was positively correlated with endothelial cell apoptosis, a hallmark feature of eroded plaques. Yet, the extent of NET burden was not different in both lesion types, emphasizing the power of neutrophil effector function (Quillard et al. 2015).

4.2 Arterial Thrombosis

The role of NETs in arterial thrombosis was mostly studied in two major conditions, acute myocardial infarction and ischemic stroke.

4.2.1 Acute Myocardial Infarction

Apart from atherosclerosis itself, neutrophils are critically involved in myocardial infarction, an acute manifestation of stable disease. Naruko et al. discovered that neutrophils were abundantly present in both ruptured and eroded plaques of patients who have died from MI (Naruko et al. 2002). The emergence of catheter-based thrombus aspiration in MI (Beran et al. 2002) made a detailed examination of thrombi in a high number of patients possible, and enabled a new view on atherosclerosis outside of autopsy specimens. This provided crucial insights into pathomechanisms underlying coronary thrombosis. Analyses revealed that leukocytes are a major component of fresh thrombi (Rittersma et al. 2005). The majority of thrombus leukocytes were neutrophils which co-localized with large quantities of endothelin-1 (Adlbrecht et al. 2007), a potent vasoconstrictor and pro-inflammatory mediator associated with left ventricular dysfunction after MI (Taylor et al. 2004). Neutrophil accumulation at the culprit site was associated with a local increase in pro-thrombotic complement factors and infarct size (Distelmaier et al. 2009). This was corroborated by the observation that thrombus neutrophil count was associated with impaired coronary microcirculation and reduced left ventricular function at six-month follow-up (Arakawa et al. 2009).

Ultimately, the presence of NETs was demonstrated in culprit site thrombi (de Boer et al. 2013). NETs were decorated with pro-inflammatory interleukin-17, which drives neutrophil accumulation (Liao et al. 2012) and is suggested to be important in the pathogenesis of MI (Mora-Ruiz et al. 2019). NETs in culprit site thrombi were confirmed by another group, which demonstrated NET formation to be induced by high mobility group box 1, an important danger-associated molecular

pattern (Maugeri et al. 2014). In comparison with venous thrombi, NET burden was significantly higher in coronary thrombi and positively correlated with infarct size (Mangold et al. 2015). Recently, levels of dsDNA measured one day after MI were also associated with microvascular obstruction, myocardial salvage index, and left ventricular ejection fraction at four months (Helseth et al. 2019). Furthermore, neutrophils isolated from the culprit lesion site were more prone to undergo NETosis *ex vivo* in comparison with neutrophils harvested from a non-infarct related coronary artery (Stakos et al. 2015). The same group also found NETs to be decorated with tissue factor, an important mediator of coagulation. NETs were also shown to contribute to myocardial fibrosis by leading to increased activation and differentiation of fibrocytes at the culprit site (Hofbauer et al. 2019).

Circadian rhythms and neutrophil aging were recently proposed to modulate neutrophil migratory properties into tissues with substantial influence on vascular health and thrombo-inflammatory reactions in ischemia reperfusion (Adrover et al. 2019; Steffens et al. 2017).

4.2.2 Acute Ischemic Stroke

NETs were also shown to be associated with acute ischemic stroke. Concentrations of plasma DNA identified patients at risk of death at follow-up (Rainer et al. 2003), while nucleosomes were correlated with neurological dysfunction and infarction volume (Geiger et al. 2006). Another group demonstrated that DNA was increased after stroke (Tsai et al. 2011). Correspondingly, immunohistological analysis of thrombectomy samples revealed large numbers of neutrophils positive for citH3 (Laridan et al. 2017). NET burden in ischemic stroke thrombi retrieved via endovascular therapy was associated with the complexity of intervention, measured as duration of procedure and number of required wire passes (Ducroux et al. 2018). With respect to prognosis and outcome, soluble NET markers were associated with a higher NIHSS score, an indicator of stroke severity. Increased all-cause mortality was reported in patients with citH3 levels ranging in the upper quartile (Valles et al. 2017). Mechanistically, a mouse model of cerebral artery ischemia/reperfusion revealed significant exacerbation of brain injury after infusion of exogenous histones, highlighting the cytotoxic properties of NET components and their devastating influence on vascular tissues (De Meyer et al. 2012).

4.2.3 Other Conditions Associated with Arterial Thrombosis

In other diseases associated with arterial thrombosis, most evidence is available in *abdominal aortic aneurysm* (AAA). AAA, characterized by vessel dilation and formation of multilayered intraluminal thrombi (Delbosc et al. 2011), is increasingly being recognized as an inflammatory condition (Piechota-Polanczyk et al. 2015). Neutrophils appear to be crucial, as their depletion using a specific, cytotoxic antibody resulted in drastically reduced AAA formation (Eliason et al. 2005). NETs were shown to be abundantly present in the luminal part of human AAA thrombi and adventitia, and to be induced by periodontal pathogens (Delbosc et al. 2011) associated with AAA progression (Nakano et al. 2011). Finally, NETs co-localized with IL-1 β in AAA thrombi (Meher et al. 2018), a pro-inflammatory

mediator that drives AAA (Johnston et al. 2013) and NET (Keshari et al. 2012) formation.

In other pathologies associated with arterial thrombosis, evidence for the influence of NETs is scarce. *Peripheral artery disease* thrombi contain NETs to a similar extent as coronary and stroke thrombi (Farkas et al. 2019). Comparing plasma samples of DVT and PAD patients, neutrophil elastase alpha1 anti-trypsin complex, a specific marker for NETs, was increased in PAD (Kremers et al. 2019). Increased NET markers were also observed in *thrombotic microangiopathies* like thrombotic thrombocytopenic purpura or hemolytic uremic syndrome, with plasma levels being reflective of disease activity (Fuchs et al. 2012).

5 Neutrophil Extracellular Traps as a Therapeutic Target

Given their importance in the pathogenesis of atherosclerotic vascular disease, there is an interest in finding therapeutic compounds to inhibit NETs and block their detrimental effects. Several pathways have been suggested, targeting various NET components.

5.1 PAD-4 Inhibitors: Cl-Amidine

Since activation of PAD-4 is regarded critical for efficient uncoiling of chromatin in NETosis (Wang et al. 2009), inhibition of this key enzyme was suggested as a potential therapeutic regimen. Initially, synthetic PAD-4 inhibitors such as Cl-amidine were envisioned for treatment of rheumatoid arthritis (Kearney et al. 2005; Luo et al. 2006), a disease exacerbated by PAD-mediated excessive formation of citrullinated proteins that promote auto-immunity (Turunen et al. 2016). As Cl-amidine was characterized to irreversibly block PAD4, it critically interferes with NET formation (Wang et al. 2009). Cl-amidine was shown to attenuate disease severity in mouse models of sepsis (Biron et al. 2017), collagen-induced arthritis (Willis et al. 2011) and systemic lupus erythematosus, where it decreased NET formation and reduced deposition of inflammatory immunoglobulin and complement factors in the kidney (Knight et al. 2013). Daily subcutaneous treatment of ApoE knockout mice with Cl-amidine could alleviate atherosclerotic lesions under high-fat diet, while accumulation of neutrophils and macrophages into lesions was reduced (Knight et al. 2014). Thrombus formation induced by photochemical injury of the carotid artery could be significantly delayed by pre-treatment with Cl-amidine (Knight et al. 2013). It was demonstrated that Cl-amidine treatment reduces infarct size in a mouse model of coronary artery ligation and was associated with improved cardiac function (Novotny et al. 2018). In a model of ischemic stroke, administration of Cl-amidine prevented thrombotic occlusions (Pena-Martinez et al. 2019). These observations emphasize a certain dependency of NETosis on enzymatic PAD4 activity despite conflicting results and render pharmaceutical PAD-4 inhibition, a potentially promising target for treatment of human atherosclerotic and thrombotic disease.

5.2 Deoxyribonuclease

Deoxyribonuclease (DNase) degrades NETs by hydrolysis of the DNA backbone (Fuchs et al. 2010). Two isoforms of different cellular origins target DNA strands *in vivo*. DNase 1, secreted by the non-hematopoietic compartment, preferentially degrades protein-free DNA, while leukocyte-derived DNase 1 like 3 (DNase 1L3) cleaves DNA:protein complexes (Napirei et al. 2009). Adequate DNase activity was suggested to be crucial for a homeostatic balance between NET formation and degradation. Indeed, neutrophilic mice deficient in both plasmatic DNases developed severe disseminated thrombosis. However, reconstitution with or presence of just one functional DNase type was sufficient to protect mice from vascular occlusion (Jimenez-Alcazar et al. 2017). Targeting chromatin and NETs by DNase 1 was shown to be beneficial in experimental DVT (Brill et al. 2012; von Bruhl et al. 2012) and ischemic injury, including intestinal ischemia (Boettcher et al. 2017a), testicular torsion (Boettcher et al. 2017b), and ischemic stroke (Pena-Martinez et al. 2019).

In atherosclerotic mice, injection of DNase reduced lesion size and attenuated lesional NET burden as well as pro-inflammatory cytokines (Warnatsch et al. 2015). Furthermore, DNase 1 was used in rodent models of cardiac ischemia. Although one study reports reduction of neutrophil infiltration by DNase 1 alone in a model of ischemia/reperfusion, the authors could only show improved cardiac function by co-administration of tissue plasminogen activator (tPA) (Ge et al. 2015). In contrast, DNase 1 treatment improved cardiac function in mice after coronary artery ligation, without any reduction in neutrophil infiltration to the ischemic myocardium (Vogel et al. 2015). These apparently contradictory results may be due to variations in methodology of ligation, duration of ischemia, and timing of DNase application. *In vitro*, DNase 1 was shown to accelerate tPA-mediated thrombolysis of human coronary (Mangold et al. 2015) and cerebral (Laridan et al. 2017) thrombi in comparison with tPA alone. Importantly, in MI patients, low DNase activity was associated with increased infarct size (Mangold et al. 2015).

Considering the mounting evidence on the benefits of DNase application in different well-established disease models, these data raise the possibility of DNase 1 treatment of patients in neutrophil-driven disease settings in which NET formation plays a pathogenic role.

5.3 Heparin

Due to their anticoagulant properties, unfractionated heparin and low molecular weight heparins are long recognized therapeutic cornerstones for deep vein thrombosis (Mazzolai et al. 2018) and MI (Neumann et al. 2019). Given the inflammatory component of thrombotic diseases, it is intriguing that heparins have a variety of anti-inflammatory effects (Mulloy et al. 2016; Rao et al. 2010) which seem to be unrelated to their anticoagulant activity (Rao et al. 2010). In a model of acute inflammation, diminished accumulation of neutrophils was at least in part ascribed to the ability of heparin oligosaccharides to block L- and P-selectin (Nelson et al.

1993). Likewise, CD11b-dependent adhesion could be attenuated by interaction with heparin (Salas et al. 2000; Wang et al. 2002). Furthermore, heparins can not only limit NET formation itself as shown by treatment in vitro and in vivo (Manfredi et al. 2017), but can also target existing NETs in various ways: heparin was shown to inhibit enzymatic activity of neutrophil elastase and Cathepsin G (Fryer et al. 1997), two major enzymes present in NETs (Folco et al. 2018). Binding and displacement of histones by heparin promotes NETs disassembly, limiting their pro-thrombotic properties in vitro (Fuchs et al. 2010) and in vivo (von Bruhl et al. 2012). At the same time, excessive release of histones during cell death and NETosis was suggested to counteract the anti-thrombotic function of heparin, potentially explaining non-responsiveness to heparin (Longstaff et al. 2016).

These observations stimulated the development of heparinoids that lack anticoagulant properties while keeping their anti-inflammatory effects (Rao et al. 2010). Indeed, sevuparin, a low-anticoagulant heparin analog, inhibited NE and histone H4, proteins associated with NETs (Rasmuson et al. 2019). In another study, non-anticoagulant heparin prevented histone-mediated cytotoxicity and improved survival in a murine sepsis model (Wildhagen et al. 2014). Thus, it might be a crucial therapeutic add-on to reduce NET burden without increasing risk of bleeding complications.

6 Summary

A summary of the content of this chapter is provided in the central Fig. 1. Taken together, NETs are important for the initiation and progression of thrombosis in the setting of atherosclerosis, including VTE, MI, and stroke. For these reasons, NETs

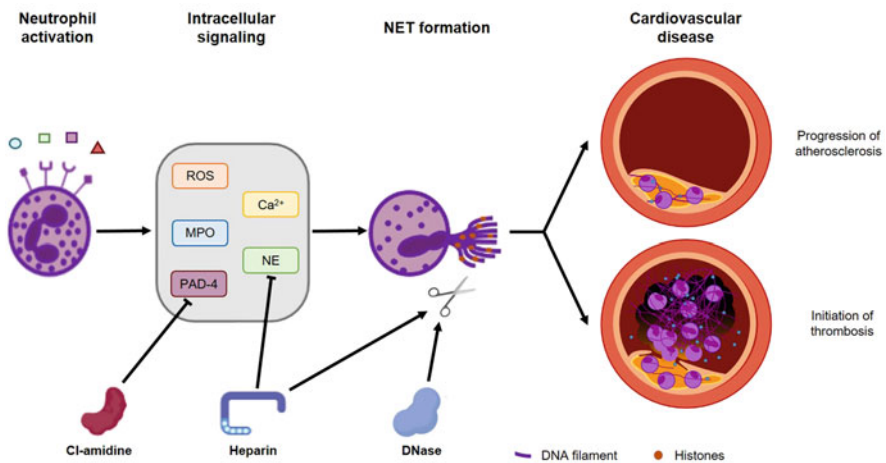


Fig. 1 The central figure summarizes mechanisms of NET formation, its pathways and consequences in atherosclerosis and thrombosis. The figure was constructed using Biorender and Microsoft PowerPoint

have emerged as potential targets for treatment. Despite promising in vitro and experimental in vivo data, adequately powered clinical trials are required to assess clinical benefits and safety of anti-NETotic regimens.

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Part IV

Hypothesis-free Approaches to Unravel Novel Targets



Genomic Strategies Toward Identification of Novel Therapeutic Targets

Thorsten Kessler and Heribert Schunkert

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Abstract

Coronary artery disease, myocardial infarction, and secondary damages of the myocardium in the form of ischemic heart disease remain major causes of death in

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Western countries. Beyond traditional risk factors such as smoking, hypertension, dyslipidemia, or diabetes, a positive family history is known to increase risk. The genetic factors underlying this observation remained unknown for decades until genetic studies were able to identify multiple genomic loci contributing to the heritability of the trait. Knowledge of the affected genes and the resulting molecular and cellular mechanisms leads to improved understanding of the pathophysiology leading to coronary atherosclerosis. Major goals are also to improve prevention and therapy of coronary artery disease and its sequelae via improved risk prediction tools and pharmacological targets. In this chapter, we recapitulate recent major findings. We focus on established novel targets and discuss possible further targets which are currently explored in translational studies.

Keywords

Coronary artery disease · Exome sequencing · Genomics · Genome-wide association studies · Myocardial infarction

1 Introduction

Coronary artery disease (CAD) and myocardial infarction (MI) are the main causes of morbidity and mortality. The identification of risk factors is a prerequisite to improve prevention and therapy of the disease via gaining knowledge about the underlying pathophysiological processes as well as the identification of therapeutic targets. Hyperlipidemia, hypertension, and diabetes mellitus are examples for risk factors which can be treated via pharmacological intervention, whereas smoking and obesity can be addressed by lifestyle interventions. Age and male gender, which are also major risk factors, cannot be addressed therapeutically. In the past, a positive family history has also been regarded as a non-modifiable risk factor. However, the underlying risk factors were not known for decades. In this chapter, we summarize the developments in the past years which led to the identification of a plethora of genomic loci which are associated with CAD with high statistical certainty.

2 Methodological Aspects

Over the past decades, the methodological spectrum has been vastly expanded to identify novel genetic risk factors of CAD and MI. Details on these methods have been discussed elsewhere (Kessler et al. 2016). In this section, we aim to briefly mention some important points.

2.1 Techniques

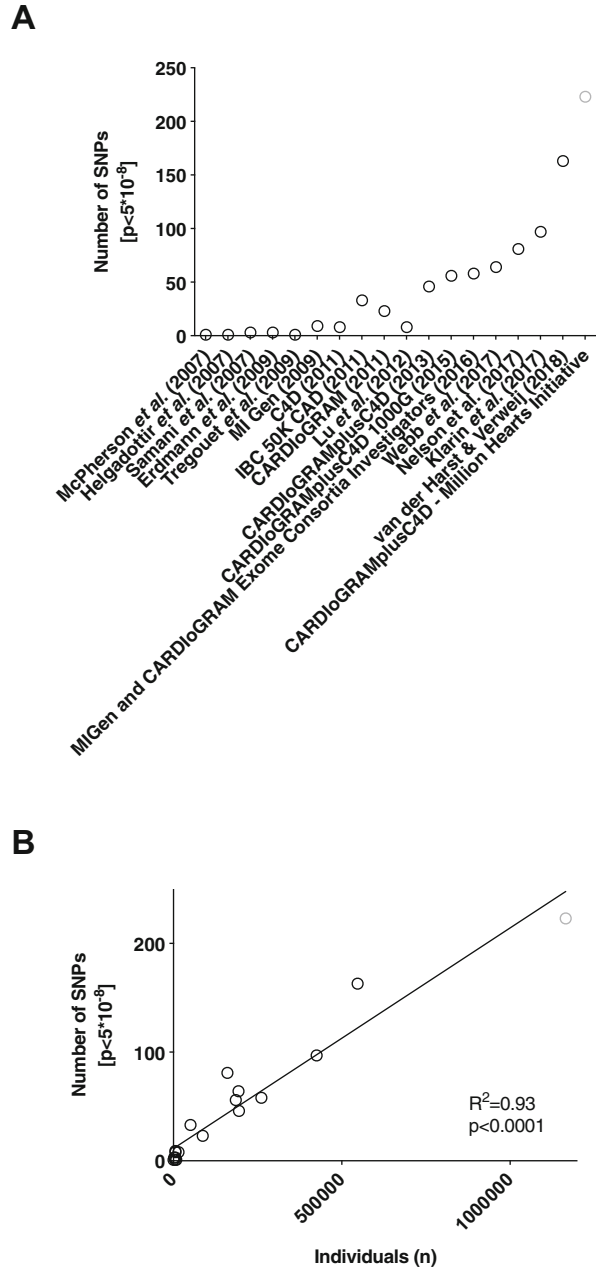
The development of arrays with hundred thousands of common single nucleotide polymorphisms (SNPs) distributed all over the genome of an individual enabled researchers to deeply genotype large numbers of individuals. An important prerequisite was knowledge of the human genome which has been mainly gained via projects as the human genome project (Lander et al. 2001; Sachidanandam et al. 2001). Subsequent projects as the *1000 Genomes Project* (1000 Genomes Project Consortium et al. 2012, 2015) raised further possibilities with the imputation of SNPs which had not been genotyped directly. As a consequence genome-wide association studies investigating millions of SNPs have been performed, and mostly common variants were found to be associated with the disease and lend support to the *common disease-common variant* hypothesis (Reich and Lander 2001). Thereby, since 2007, mostly genome-wide association studies and subsequent analyses led to the identification of genetic risk variants for various traits including CAD and traditional risk factors as blood pressure or lipids. Subsequently, large international consortia were formed that were responsible for the identification of most of the currently known CAD risk loci. Figure 1 illustrates the evolution of genome-wide association studies focusing on the CARDIoGRAMplusC4D consortium, a coalition of several individual studies to facilitate the identification of CAD risk factors (Schunkert et al. 2019) and further studies using data from UK Biobank (Littlejohns et al. 2019). The idea of exome-wide association studies is comparable to that of a genome-wide association study with the exception that the investigated SNPs are enriched for coding variants distributed over the genome (Myocardial Infarction Genetics and CARDIoGRAM Exome Consortia Investigators et al. 2016). Most of the currently known genetic CAD risk factors have been identified using these methods (Kessler et al. 2016; Khera and Kathiresan 2017a; Erdmann et al. 2018).

Apart from common variants, the association of rare variants and private mutations has also been extensively studied. To identify such genetic variation, mostly exome sequencing has been used in the past (Erdmann et al. 2013). As the name says, this next-generation sequencing method is able to determine the genomic sequence of an individual enriched for coding regions and thus renders the identification of missense variants co-segregating with a certain disease in, e.g., a family with high prevalence possible. Whole-genome sequencing is not restricted to coding sequences. However, due to costs and computational challenges of the different methods, whole-genome sequencing has not yet contributed to the current knowledge of genetic variation in CAD.

2.2 Selection of Individuals

Most large-scale genomic studies use a case-control design, i.e., a cohort of patients suffering from the trait of interest is compared to a group of healthy individuals free of the disease. Obviously, clear definitions of phenotypes are important to gain reliable results. Most GWAS on CAD/MI focused on cases suffering from the

Fig. 1 *Discovery of genomic variants associated with CAD/MI using genome-/exome-wide association studies* (modified after Kessler et al. 2016). **(a)** Over the past years, ongoing research led to the identification of increasing numbers of SNPs which are associated with CAD/MI at the genome-wide level of significance. Results from the CARDIoGRAMplusC4D-Million Hearts Initiative are awaited for 2019/2020. **(b)** The number of investigated individuals correlates with the number of identified risk variants which highlights the importance of international collaborations



disease at an early age. This increased the possibility of identification of the genetic predisposition which, together with gender, still represents the strongest risk factor. For controls, population-based samples as well as individuals which have been

diagnosed to not to suffer from CAD/MI have been included. An important resource that has increasingly been used in recent projects is the UK Biobank (<https://www.ukbiobank.ac.uk/>). It includes health information as well as genetic data for 500,000 individuals which can be accessed by researchers after approval of specific projects (Littlejohns et al. 2019). In the USA, the Million Veteran Project has formed and stated to provide likewise genome-wide data (Klarin et al. 2018).

2.3 Statistical Analysis

To reduce the possibility of a false-positive finding, rigorous statistical thresholds have been determined to identify a certain variant to be associated with a trait as CAD/MI. Currently, a p -value below 5×10^{-8} is commonly accepted as a genome-wide significant finding. The strict p -value is a result of the large number of statistical tests which are performed in parallel on one dataset in a genome-wide association study (Pe'er et al. 2008). However, it cannot be excluded that true positive signals are discarded because they do not reach the stringent p -value. Recent studies also reported loci below certain false discovery rate thresholds to address this issue.

2.4 Mendelian Randomization Studies

Mendelian randomization studies (for an overview see Jansen et al. 2014) enable to investigate whether a biomarker or a trait is causal in the development of a disease. In epidemiological research, associations between biomarkers and diseases are frequently observed. However, the association can be influenced by unknown factors. On the other hand, the investigated disease itself can influence a given biomarker. In Mendelian randomization studies, causality between a biomarker and development of a disease can be assumed if a genetic variant influences this biomarker in the same direction as the biomarker is associated with the disease. Important examples in CAD are biomarkers such as LDL-cholesterol or C-reactive protein as well as behavioral traits such as educational attainment (Zeng et al. 2019a) which are discussed in more detail below.

3 Genetic Risk Factors Associated with Coronary Artery Disease

3.1 Genome-Wide Association Studies

The discovery of genetic CAD/MI risk factors was launched in 2007 by three independent studies which reported the chromosome 9p21 locus as the first and, until today, strongest genetic risk locus (Helgadottir et al. 2007; McPherson et al. 2007; Samani et al. 2007). Since then, with the inclusion of more individuals and the

possibility of analyzing a larger number of SNPs, more and more variants associated with CAD/MI have been reported (for an overview see Kessler et al. (2016), Khera and Kathiresan (2017b), and Erdmann et al. (2018)). The currently known loci are depicted in Table 1. Surprisingly, most of the SNPs tag genes which have not been studied in the pathophysiology of coronary atherosclerosis before. Another surprising finding was that only the minority of genes is also associated with traditional risk factors as hypertension or lipid metabolism rendering the involvement of other cellular mechanisms likely. Additionally, almost every lead SNP is located in a non-coding region of the genome. The most prominent example is again the chromosome 9p21 locus. Here, it is still unclear which gene might be responsible for the strong signal. Rather, there is much evidence that the effect might be mediated by the circular non-coding RNA *ANRIL* (Holdt et al. 2010, 2013, 2016). Overall, it has been estimated that the 163 so far known CAD risk loci explain between 30% and 40% of CAD heritability (Nelson et al. 2017). It has also been found that a large number of the reported loci also harbor multiple independent signals (van der Harst and Verweij 2018). Additionally, most of the loci show pleiotropy, i.e., a risk variant is associated with several phenotypes. About half of the currently known CAD risk loci have been reported to be associated with other traits (Webb et al. 2017).

The studies which led to the discovery of the currently known CAD/MI risk variants as well as studies which gave first insights into their involvement in CAD/MI are referenced in Table 1. Specific genes and pathways are further discussed below.

3.2 Exome-Wide Association Studies

As mentioned above, GWAS mainly led to the identification of non-coding variation associated with the disease. To specifically investigate the role of coding variants, dedicated arrays enriched for exonic variants were used. In an international effort, the Myocardial Infarction Genetics and CARDIoGRAM Exome Consortia Investigators performed the largest exome-wide association study so far (Myocardial Infarction Genetics and CARDIoGRAM Exome Consortia Investigators et al. 2016). The results are depicted in Table 2. In summary, only four variants were identified. First, it could be replicated that coding variation in the *LPA* and *PCSK9* genes is associated with CAD/MI. Second, two novel coding variants in *ANGPTL4* and *SVEP1* were found. Whereas *LPA*, *PCSK9*, and *ANGPTL4* are associated with lipid metabolism (see below), *SVEP1* only displayed association with blood pressure (Myocardial Infarction Genetics and CARDIoGRAM Exome Consortia Investigators et al. 2016). However, this effect cannot fully explain the association signal for CAD/MI.

Table 1 Loci associated with CAD/MI identified by GWAS (modified after Erdmann et al. (2018))

Chr.	Lead SNP	EA (EAF)	OR	Gene(s)	Association with traditional risk factors		Ref.
					HTN	Lipids	
1	rs36096196	T (0.15)	1.05	<i>MORNI, SKI</i>			Van der Harst and Verweij (2018)
	rs2493298	A (0.14)	1.06	<i>PRDM16, PEX10, PLCH2, RERI</i>			Van der Harst and Verweij (2018)
	rs61776719	A (0.53)	1.04	<i>FHL3, UTP11, SF3A3, MANEAL, INPP5B</i>			Van der Harst and Verweij (2018)
	rs11206510	T (0.82)	1.08	<i>PCSK9</i>	+		Abifadel et al. (2003), Cohen et al. (2006), Myocardial Infarction Genetics Consortium et al. (2009), and Teslovich et al. (2010)
	rs17114036	A (0.91)	1.17	<i>PPAP2B</i>			Teslovich et al. (2010), Schunkert et al. (2011)
	rs599839	A (0.78)	1.11	<i>SORT1, PSCRI, CELSR2</i>	+		Samani et al. (2007), Teslovich et al. (2010), and Schunkert et al. (2011)
	rs11806316	G (0.66)	1.04	<i>NGF, CASQ2</i>			Van der Harst and Verweij (2018)
	rs11810571	G (0.79)	1.07	<i>TDRKH, RPI1-98D18.9</i>			Verweij et al. (2017), Nelson et al. (2017)
	rs4845625	T (0.47)	1.06	<i>IL6R, AQP10, ATP8B2, CHTOP, UBAP2L</i>			CARDIoGRAMplusC4D Consortium et al. (2013)
	rs1892094	C (0.50)	1.04	<i>ATPIB1, BLZF1, CCDC181, F5, NME7, SELP, SLC19A2</i>			Howson et al. (2017)
	rs6700559	C (0.53)	1.04	<i>DDX59, CAMSAP2, KIF14</i>			
	rs2820315	T (0.30)	1.05	<i>LMOD1, IPO9, NAV1, SHISA4, TIMM17A</i>			
	rs60154123	T (0.15)	1.05	<i>HHAT, SERTAD4, DIEXF</i>			Van der Harst and Verweij (2018)
	rs17465637	C (0.74)	1.14	<i>MIA3, AIDA, C1orf58</i>			Samani et al. (2007), Schunkert et al. (2011)
	rs699	G (0.42)	1.04	<i>AGT, CAPN9, GNPAT</i>			Van der Harst and Verweij (2018)

(continued)

Table 1 (continued)

Chr.	Lead SNP	EA (EAF)	OR	Gene(s)	Association with traditional risk factors		Ref.
					HTN	Lipids	
2	rs515135	G (0.83)	1.07	APOB		+	Teslovich et al. (2010), CARDIoGRAMplusC4D Consortium et al. (2013)
	rs6544713	T (0.30)	1.06	ABCG5, ABCG8		+	Teslovich et al. (2010), Schunkert et al. (2011), and IBC 50K CAD Consortium (2011)
	rs582384	A (0.53)	1.03	PRKCE, TMEM247			Van der Harst and Verweij (2018)
	rs1561198	A (0.45)	1.06	VAMP5, VAMP8, GGCX			CARDIoGRAMplusC4D Consortium et al. (2013)
	rs2252641	G (0.46)	1.06	ZEB2, TEX41			CARDIoGRAMplusC4D Consortium et al. (2013)
	rs12999907	A (0.82)	1.06	FIGN			Van der Harst and Verweij (2018)
	rs840616	C (0.65)	1.04	CALCRL, TFPI			Van der Harst and Verweij (2018)
	rs6725887	C (0.15)	1.14	WDR12, CARF, FAM117B, ICAIL, NBEAL1			Myocardial Infarction Genetics Consortium et al. (2009), Schunkert et al. (2011)
	rs1250229	T (0.26)	1.07	FN1, AT1C, LOC102724849, ABCA12, LINC00607		+	Klarin et al. (2017), Nelson et al. (2017)
	rs2571445	A (0.39)	1.04	TNSI, CXCR2, RUFY4			Howson et al. (2017)
	rs2972146	T (0.65)	1.07	LOC646736, IRS1, MIR5702		+	Klarin et al. (2017)
	rs1801251	A (0.35)	1.05	KCNJ13, GIGYF2			Webb et al. (2017)
	rs11677932	G (0.68)	1.03	COL6A3			Van der Harst and Verweij (2018)

3	rs748431	G (0.36)	1.04	FGD5				Klarin et al. (2017)
	rs7633770	A (0.41)	1.03	ALS2CL, RTP3				Van der Harst and Verweij (2018)
	rs7617773	T (0.67)	1.04	CDC25A, SPINK8, MAP4, ZNF589		+		
	rs7623687	A (0.86)	1.07	RHOA, AMT, TCTA, CDHRA, KLHDC8B, etc.				Verweij et al. (2017), Klarin et al. (2017), Nelson et al. (2017), and Howson et al. (2017)
	rs142695226	G (0.14)	1.08	UMPS, ITGB5				Verweij et al. (2017), Klarin et al. (2017), and Nelson et al. (2017)
	rs10512861	G (0.86)	1.04	DNAJC13, NPHP3, ACAD11, UBA5		+		Van der Harst and Verweij (2018)
	rs667920	T (0.78)	1.05	STAG1, MSL2, NCK1, PPP2R3A		+		
	rs2306374	C (0.18)	1.12	MRAS, CEP70				Erdmann et al. (2009), Schunkert et al. (2011)
	rs12493885	C (0.85)	1.07	ARHGEF26				Verweij et al. (2017), Klarin et al. (2017), and Nelson et al. (2017)
	rs4266144	G (0.32)	1.03	CCNLI, TIPARP				Van der Harst and Verweij (2018)
	rs12897	G (0.41)	1.04	FND3B				
	rs16844401	A (0.07)	1.07	HGFAC, RGS12, MSANTD1		+		
	4	rs17087335	T (0.21)	1.06	REST, NOA1			
rs12500824		A (0.36)	1.04	SHROOM3, SEPT11, FAM47E, STBD1				Van der Harst and Verweij (2018)
rs10857147		T (0.27)	1.06	PRDM8, FGF5			+	Verweij et al. (2017), Klarin et al. (2017), and Nelson et al. (2017)
rs11099493		A (0.69)	1.04	HNRNPD, RASGEF1B				Van der Harst and Verweij (2018)
rs3775058		A (0.23)	1.04	UNC5C				
rs11723436		G (0.31)	1.05	MAD2L1, PDE5A				Verweij et al. (2017), Klarin et al. (2017), and Nelson et al. (2017)
rs35879803		C (0.70)	1.05	ZNF827				Verweij et al. (2017)
rs1878406		T (0.15)	1.10	EDNRA				CARDIoGRAMplusC4D Consortium et al. (2013)
rs7692387		G (0.81)	1.08	GUCY1A1		+		International Consortium for Blood Pressure Genome-Wide Association Studies et al. (2011), CARDIoGRAMplusC4D Consortium et al. (2013), and Erdmann et al. (2013)
rs7696431		T (0.51)	1.04	PALLD, DDX60L				Van der Harst and Verweij (2018)

(continued)

Table 1 (continued)

Chr.	Lead SNP	EA (EAF)	OR	Gene(s)	Association with traditional risk factors		Ref.
					HTN	Lipids	
5	rs1508798	T (0.81)		SEMA5A, TAS2R1		+	
	rs3936511	G (0.18)	1.04	MAP3K1, MIER3		+	
	rs1800449	T (0.17)	1.09	LOX			Klarin et al. (2017)
	rs273909	C (0.14)	1.07	SLC22A4			CARDIoGRAMplusC4D Consortium et al. (2013)
	rs2706399	G (0.51)	1.07	IL5, RAD50			IBC 50K CAD Consortium (2011)
6	rs246600	T (0.48)	1.05	ARHGAP26			Howson et al. (2017)
	rs9501744	C (0.87)	1.05	FOXC1			Van der Harst and Verweij (2018)
	rs12526453	C (0.67)	1.10	PHACTR1, EDN1			Myocardial Infarction Genetics Consortium et al. (2009), Schunkert et al. (2011)
	rs35541991	C (0.31)	1.05	HDGFL1			Verweij et al. (2017), Nelson et al. (2017)
	rs3130683	T (0.86)	1.09	C2, C4A, etc.			Webb et al. (2017)
	rs17609940	G (0.75)	1.07	ANKS1A, UHRF1BP1			Schunkert et al. (2011)
	rs1321309	A (0.49)	1.03	CDKN1A, PI16		+	Van der Harst and Verweij (2018)
	rs10947789	T (0.76)	1.07	KCNK5			CARDIoGRAMplusC4D Consortium et al. (2013)
	rs6905288	A (0.57)	1.05	VEGFA, MRPL14, TMEM63B		+	Van der Harst and Verweij (2018)
	rs9367716	G (0.68)	1.04	PRIM2, RAB23, DST, BEND6			
rs4613862	A (0.53)	1.03	FAM46A				
rs1591805	A (0.49)	1.04	CENPW				
rs12190287	C (0.62)	1.08	TCF21, TARID (EYA4-AS1)			Schunkert et al. (2011)	
rs17080091	C (0.92)	1.05	PLEKHG1, IYD		+	Van der Harst and Verweij (2018)	
rs3798220	C (0.02)	1.51	LPA, SLC22A3, LPAL2		+	Tregouet et al. (2009), Teslovich et al. (2010), and Schunkert et al. (2011)	
rs4252120	T (0.73)	1.07	PLG, LPAL2		+	CARDIoGRAMplusC4D Consortium et al. (2013)	

7	rs10267593	G (0.8)	1.04	MAD1L1				Van der Harst and Verweij (2018)
	rs7797644	C (0.77)	1.04	DAGLB, RAC1, FAM220A, KDELR2				
	rs11509880	A (0.36)	1.04	TMEM106B, THSD7A				
	rs2023938	G (0.10)	1.08	HDAC9				CARDIoGRAMplusC4D Consortium et al. (2013)
	rs2107732	G (0.91)	1.06	CCM2, MYO1G			+	Van der Harst and Verweij (2018)
	rs10953541	C (0.80)	1.08	BCAP29, GPR22				Coronary Artery Disease C4D Genetics Consortium (2011a)
	rs975722	G (0.4)	1.03	CTTNBP2, CFTR, ASZ1				Van der Harst and Verweij (2018)
	rs11556924	C (0.62)	1.09	ZC3HC1, KLHDC10				Schunkert et al. (2011)
	rs10237377	G (0.65)	1.05	PARP12, TBXAS1				Howson et al. (2017)
	rs3918226	T (0.06)	1.14	NOS3			+	Nikpay et al. (2015)
	rs6997340	T (0.31)	1.04	NAT2			+	Van der Harst and Verweij (2018)
	rs264	G (0.86)	1.11	LPL			+	Testovich et al. (2010), CARDIoGRAMplusC4D Consortium et al. (2013), and Myocardial Infarction Genetics and CARDIoGRAM Exome Consortia Investigators et al. (2016)
	rs6984210	G (0.06)	1.08	BMP1, SFTPC, DMTN, PHYHIP, DOK2, XPO7				Van der Harst and Verweij (2018)
	rs10093110	G (0.58)	1.03	ZFPM2				
rs2954029	A (0.55)	1.06	TRIB1			+	Testovich et al. (2010), IBC 50K CAD Consortium (2011), and CARDIoGRAMplusC4D Consortium et al. (2013)	
9	rs1333049	G (0.46)	1.29	ANRIL, CDKN2B-AS				Helgadottir et al. (2007), McPherson et al. (2007), Samani et al. (2007), Holdt et al. (2010), and Schunkert et al. (2011)
	rs944172	C (0.28)	1.04	KLF4				Van der Harst and Verweij (2018)
	rs885150	C (0.27)	1.03	DAB2IP				
	rs579459	C (0.21)	1.10	ABO, SURF6, GBGT1			+	Testovich et al. (2010), Reilly et al. (2011), and Schunkert et al. (2011)

(continued)

Table 1 (continued)

Chr.	Lead SNP	EA (EAF)	OR	Gene(s)	Association with traditional risk factors	Ref.
10	rs61848342	C (0.36)	1.04	CDC123, NUDT5, OPTN		Van der Harst and Verweij (2018)
	rs2505083	C (0.38)	1.07	KIAA1462		Coronary Artery Disease C4D Genetics Consortium (2011a), Erdmann et al. (2011)
	rs1746048	C (0.87)	1.09	CXCL12		Samani et al. (2007), Schunkert et al. (2011)
	rs17680741	T (0.72)	1.05	TSPAN14, MAT1A, FAM213A		Van der Harst and Verweij (2018)
	rs1412444	T (0.42)	1.09	LIPA		Coronary Artery Disease C4D Genetics Consortium (2011a)
	rs12413409	G (0.89)	1.12	CYP17A1, CNNM2, NT5C2	+	Levy et al. (2009), Newton-Cheh et al. (2009), and Schunkert et al. (2011)
11	rs4918072	A (0.27)	1.04	STN1, SH3PXD2A		Van der Harst and Verweij (2018)
	rs4752700	G (0.45)	1.03	HTRA1, PLEKHA1		
	rs11601507	A (0.07)	1.09	TRIM5, TRIM22, TRIM6, OR52N1, OR52B6		
	rs10840293	A (0.55)	1.06	SWAP70		Nikpay et al. (2015)
	rs11042937	T (0.49)	1.03	MRV1, CTR9		Webb et al. (2017)
	rs1351525	T (0.67)	1.05	ARNTL		Verweij et al. (2017), Nelson et al. (2017)
	rs7116641	G (0.31)	1.03	HSD17B12		Van der Harst and Verweij (2018)
	rs12801636	G (0.77)	1.05	PCNX3, POLA2, RELA, SIPA1, etc.	+	Howson et al. (2017)
	rs590121	T (0.30)	1.05	SERPINH1		Howson et al. (2017)
	rs7947761	G (0.28)	1.04	ARHGAP42		Van der Harst and Verweij (2018)
	rs974819	T (0.32)	1.07	PDGFD		Coronary Artery Disease C4D Genetics Consortium (2011a)
rs964184	G (0.13)	1.13	APOA1-C3-A4-A5		Schunkert et al. (2011), Do et al. (2015)	
rs11838267	T (0.87)	1.05	C1S	+	Van der Harst and Verweij (2018)	

12	rs10841443	G (0.67)	1.06	RP11-664H17.1, PDE3A				Klarin et al. (2017)
	rs11170820	G (0.08)	1.10	HOXC4				Verweij et al. (2017)
	rs11172113	C (0.41)	1.06	LRP1, STAT6				Webb et al. (2017)
	rs7306455	G (0.9)	1.05	NDUFA12, FGD6				Van der Harst and Verweij (2018)
	rs3184504	T (0.44)	1.07	SH2B3, FLJ21127, ATXN2, etc.	+			Gudbjartsson et al. (2009), Levy et al. (2009), Newton-Cheh et al. (2009), Teslovich et al. (2010), and Schunkert et al. (2011)
	rs11830157	G (0.36)	1.12	KSR2				Nikpay et al. (2015)
	rs2244608	G (0.35)	1.06	HNF1A, OASL, C12orf43, and others	+			Verweij et al. (2017), Klarin et al. (2017), Nelson et al. (2017), and Howson et al. (2017)
	rs11057401	T (0.69)	1.08	CCDC92				Klarin et al. (2017)
	rs11057830	A (0.15)	1.07	SCARB1				Webb et al. (2017), Howson et al. (2017)
	rs9319428	A (0.32)	1.06	FLT1				CARDIoGRAMplusC4D Consortium et al. (2013)
13	rs9591012	G (0.66)	1.04	N4BP2L2, PDS5B				Van der Harst and Verweij (2018)
	rs4773144	G (0.44)	1.07	COL4A1, COL4A2				Schunkert et al. (2011)
	rs1317507	A (0.26)	1.04	MCF2L, PCID2, CUL4A				Van der Harst and Verweij (2018)
	rs2145598	G (0.42)	1.03	ARID4A, PSMA3				
	rs3832966	I (0.46)	1.05	TMED10, ZC2HC1C, RPS6KLI, NEK9, EIF2B2e, ACYP1				Verweij et al. (2017)
	rs112635299	G (0.92)	1.13	SERPINA2, SERPINA1				Van der Harst and Verweij (2018)
	rs2895811	C (0.43)	1.07	HHPL1, YY1				Schunkert et al. (2011)
15	rs6494488	A (0.82)	1.05	OAZ2, RBPMS2, TRIP4, etc.				Howson et al. (2017)
	rs56062135	C (0.79)	1.07	SMAD3				Nikpay et al. (2015)
	rs3825807	A (0.57)	1.08	ADAMTS7				Reilly et al. (2011), Schunkert et al. (2011), and Coronary Artery Disease C4D Genetics Consortium (2011a)
	rs8042271	G (0.9)	1.10	MFG8, RPI1-326A19.4, ABHD2				Nikpay et al. (2015)
	rs17514846	A (0.44)	1.07	FURIN, FES	+			International Consortium for Blood Pressure Genome-Wide Association Studies et al. (2011), CARDIoGRAMplusC4D Consortium et al. (2013)
rs17581137	A (0.75)	1.04	Gene desert				Van der Harst and Verweij (2018)	

(continued)

Table 1 (continued)

Chr.	Lead SNP	EA (EAF)	OR	Gene(s)	Association with traditional risk factors	Ref.
16	rs1800775	C (0.51)	1.03	CETP	+	Webb et al. (2017)
	rs1050362	A (0.38)	1.04	<i>DHX38</i> , HP , <i>DHODH</i>	+	Howson et al. (2017)
	rs3851738	C (0.60)	1.07	CFDP1 , BCARI		Verweij et al. (2017), Klarin et al. (2017)
	rs7199941	A (0.4)	1.04	PLCG2 , <i>CENPN</i>		Van der Harst and Verweij (2018)
	rs7500448	A (0.77)	1.07	CDH13		Verweij et al. (2017), Klarin et al. (2017), and Nelson et al. (2017))
17	rs216172	C (0.37)	1.07	SMG6 , <i>SRR</i>		Schunkert et al. (2011)
	rs12936587	G (0.56)	1.07	Rai1 , PEMT , <i>RASD1</i> , <i>SMCR3</i> , <i>TOM1L2</i>		Schunkert et al. (2011)
	rs13723	G (0.49)	1.04	CORO6 , BLMH , ANKRD13B , GIT1 , SSH2 , EFCAB5		Van der Harst and Verweij (2018)
	rs76954792	T (0.22)	1.04	COPRS , RAB11FIP4		
	rs2074158	C (0.18)	1.05	DHX58 , KAT2A , RAB5 , NKIRAS2 , DNAJC7 , KCNH4 , HCRT , GHDC	+	
18	rs17608766	C (0.14)	1.07	GOSR2 , <i>MYL4</i> , <i>ARL17A</i> , etc.	+	Howson et al. (2017)
	rs46522	T (0.53)	1.06	UBE2Z , GIP , <i>ATP5G1</i>		Schunkert et al. (2011)
	rs7212798	C (0.15)	1.08	BCAS3		Nikpay et al. (2015)
	rs1867624	T (0.61)	1.04	PECAM1 , DDX5 , TEX2 , etc.		Howson et al. (2017)
	rs9964304	C (0.38)	1.04	ACAA2 , RPL17		Van der Harst and Verweij (2018)
	rs663129	A (0.26)	1.06	PMAIP1 , MC4R	+	Nikpay et al. (2015)

19	rs1122608	G (0.77)	1.14	LDLR, SMARCA4	+	Myocardial Infarction Genetics Consortium et al. (2009), Teslovich et al. (2010), Schunkert et al. (2011), and Do et al. (2015)
	rs73015714	G (0.2)	1.06	FCHO1, COLGALTI		Van der Harst and Verweij (2018)
	rs12976411	A (0.91)	1.33	ZNF507, LOC400684		Nikpay et al. (2015)
	rs8108632*	T (0.48)	1.05	HNRNPUL1, CCDC97, TGFB1, B9D2		Verweij et al. (2017), Klarin et al. (2017), and Nelson et al. (2017)
	rs2075650	G (0.14)	1.14	APOE, APOC1, TOMM40, PVRL2, COTL1	+	Teslovich et al. (2010), IBC 50K CAD Consortium (2011)
	rs1964272	G (0.51)	1.04	SNRPD2, GIPR		Nelson et al. (2017)
20	rs867186	A (0.89)	1.07	PROCR, ASIP, NCOA6, ITGB4BP, EIF6, etc.		Howson et al. (2017)
	rs6102343	A (0.25)	1.04	ZHX3, PLCG1, TOPI		Van der Harst and Verweij (2018)
	rs7270354	A (0.15)	1.06	PCIF1, ZNF335, NEURL2, PLTP, MMP9	+	Braenne et al. (2017)
	rs260020	T (0.13)	1.04	ZNF831		Van der Harst and Verweij (2018)
	rs2832227	G (0.18)	1.04	MAP3K7CL, BACHI		
21	rs9982601	T (0.15)	1.18	MRPS6, SLC5A3, KCNE2		Myocardial Infarction Genetics Consortium et al. (2009)
22	rs180803	G (0.97)	1.20	ADORA2A		Nikpay et al. (2015)

The most likely candidate gene at the locus is marked in bold (after McPherson and Tybjaerg-Hansen (2016)). *Chr* chromosome, *EA* effect allele, *EAF* effect allele frequency, *HTN* hypertension, *I/D* Indel/Deletion, *OR* odds ratio, *Ref* reference, *SNP* single nucleotide polymorphism

Table 2 Coding variants associated with CAD/MI risk identified by exome-wide association studies

Chr.	SNP (AA change)	EA (EAF)	OR	Gene(s)	Association with traditional risk factors		Ref.
					HTN	Lipids	
1	rs11591147 (p. R46L)	T (0.0152)	0.78	PCSK9		+	Myocardial Infarction Genetics and CARDIoGRAM Exome Consortia Investigators et al. (2016)
6	rs3798220 (p. I4399M)	C (0.019)	1.54	LPA		+	
9	rs111245230 (p. D2702G)	C (0.036)	1.14	SVEP1	+		
19	rs116843064 (p. E40K)	A (0.02)	0.86	ANGPTL4		+	

AA amino acid, Chr chromosome, EA effect allele, EAF effect allele frequency, HTN hypertension, OR odds ratio, Ref reference, SNP single nucleotide polymorphism

3.3 Exome and Whole-Genome Sequencing

As discussed above, due to still comparatively high costs, whole-genome sequencing has not yet significantly contributed to the knowledge of genetic CAD risk factors. In contrast, several studies made use of exome sequencing to identify variation in the coding sequence which is associated with CAD/MI. One scope of application the investigation of members of families with a high disease prevalence. Using this approach and subsequent co-segregation analyses genetic factors underlying the development of CAD/MI or its risk factors was possible. An important example is a mutation in the *GUCY1A3* gene, which has been – together with a coding mutation in the *CCT7* gene – shown to be responsible for the phenotype of premature CAD/MI in a family (Erdmann et al. 2013). As shown in Table 1, the *GUCY1A3* locus also harbors common, non-coding variants associated with CAD/MI. Thus, an allelic series has been shown at the locus with a mutation and common variants which lead to a strong or only moderate risk increase, respectively. Further loci have been identified by analyzing large cohorts of cases and controls comparable to the GWAS approach. Most of the genes that were thereby identified play a role in lipid metabolism. Some of the genes, e.g., *LDLR* or *PCSK9*, also demonstrate allelic series. A selection of genes which have been identified using exome sequencing is depicted in Table 3 and discussed below in more detail.

4 Genetic Overlap and Demarcation with Other Atherosclerotic Diseases

An increased prevalence of CAD risk alleles can be traced in a large number of cardiovascular conditions including heart failure, peripheral arterial disease, or atrial fibrillation (Ntalla et al. 2019). Large-scale genomic studies have also been published in particular for stroke (for a review see Dichgans et al. (2019)). Despite there is genetic overlap between CAD and atherosclerotic stroke (Dichgans et al. 2014; Kessler et al. 2015a), the genetic risk factors of CAD and stroke are not similar. The formation of large, international consortia has advanced the identification of risk genes in the fields of CAD and stroke genetics. In other atherosclerotic diseases as peripheral artery disease, genetic research will be facilitated by publicly available data from large-scale biobanks. Nevertheless, the identification of common genetic risk factors will be important to evaluate novel therapeutic strategies. Other genetic risk factors for CAD might also not play an important role in related diseases: as such, the strongest genetic risk factor reported so far, chromosome 9p21, is not associated with calcified aortic stenosis; in contrast, the *LPA* gene is associated with both diseases (Trenkwalder et al. 2018).

Table 3 Genes associated with CAD/MI which have been identified using exome sequencing

Chr.	Gene(s)	Mechanism	Ref.
1	<i>ANGPTL3</i>	<ul style="list-style-type: none"> • Angiopoietin-like 3 inhibits lipoprotein lipase • <i>ANGPTL3</i> loss-of-function mutations are associated with reduced LDL-cholesterol and triglycerides as well as reduced CAD risk 	Stitzel et al. (2017)
2	<i>APOB</i>	<ul style="list-style-type: none"> • Apolipoprotein B is a main component of LDL-cholesterol and triglyceride-rich lipoproteins • Truncating APOB mutations are associated with reduced LDL-cholesterol and reduced CAD risk 	Peloso et al. (2019)
4/2	<i>GUCY1A3/CCT7</i>	<ul style="list-style-type: none"> • Premature stop codon in <i>GUCY1A3</i> leads to loss of α_1-subunit of the soluble guanylyl cyclase (sGC); missense mutation in <i>CCT7</i> which encodes a chaperone protein stabilizing the sGC • Carriers of the <i>GUCY1A3</i>+<i>CCT7</i> mutation show reduced sGC-dependent cGMP formation in platelets • Coding variants in <i>GUCY1A3</i> are overrepresented in young MI patients 	CARDIoGRAMplusC4D Consortium et al. (2013), Erdmann et al. (2013), and Wobst et al. (2016)
7	<i>NPC1L1</i>	<ul style="list-style-type: none"> • Niemann-Pick C1-like protein 1 is responsible for the uptake of cholesterol from the intestine • <i>NPC1L1</i> loss-of-function mutations are associated with reduced LDL-cholesterol and reduced risk of CAD/MI 	Myocardial Infarction Genetics Consortium Investigators et al. (2014)
8	<i>LPL</i>	<ul style="list-style-type: none"> • Lipoprotein lipase reduced triglyceride levels • Loss-of-function variants are associated with increased triglyceride levels and CAD/MI 	Myocardial Infarction Genetics and CARDIoGRAM Exome Consortia Investigators et al. (2016) and Khera et al. (2017)
11	<i>APOA5</i>	<ul style="list-style-type: none"> • Apolipoprotein A-V increases lipoprotein lipase activity • <i>APOA5</i> loss-of-function mutations are associated with high triglyceride levels and CAD/MI 	Do et al. (2015)
	<i>APOC3</i>	<ul style="list-style-type: none"> • Apolipoprotein C-III reduced lipoprotein lipase activity • <i>APOC3</i> loss-of-function mutations are associated with 	The TG and HDL Working Group of the Exome Sequencing Project, National Heart, Lung, and Blood Institute (2014)

(continued)

Table 3 (continued)

Chr.	Gene(s)	Mechanism	Ref.
		reduced triglyceride levels and CAD/MI risk	
16	<i>CETP</i>	<ul style="list-style-type: none"> • Cholesteryl ester transfer protein reduces HDL-cholesterol levels • Carriers of truncating <i>CETP</i> mutation displayed higher levels of HDL-cholesterol, lower levels of LDL-cholesterol, and a lower CAD risk 	Nomura et al. (2017)
19	<i>LDLR</i>	<ul style="list-style-type: none"> • Loss of LDL-receptor function leads to reduced uptake of LDL-cholesterol and increased plasma LDL-cholesterol • High levels of LDL-cholesterol increase risk of CAD/MI 	Do et al. (2015)

Vascular tone/ blood pressure

e.g., *NOS3*, *GUCY1A3*,
SH2B3, *CYP17A1*,
FURIN

Plaque formation/ plaque progression

lipids (e.g., *LDLR*, *PCSK9*, *SORT1*), inflammation-related variants
(e.g., *IL6*, *CXCL12*), vascular remodeling (e.g., *ADAMTS7*)

Atherothrombosis/ platelet function

e.g., *GUCY1A3*, *REST*,
TCF21

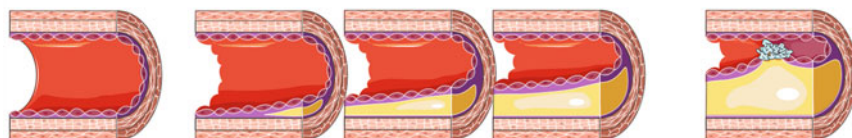


Fig. 2 Examples of CAD risk genes involved in the sequence of atherosclerotic plaque formation and rupture. Details see text. Contains modified image material available at Servier Medical Art under a Creative Commons Attribution 3.0 Unported License

5 Pharmacological Targets Identified by Genomic Studies

Genomic studies identified putative pharmacological targets at every stage of plaque formation, progression, and rupture (Fig. 2) which are discussed in this section.

5.1 Lipid Metabolism

While less than half of the identified variants tag genes which are associated with traditional risk factors, lipid metabolism represents a cluster of such variants.

LDL-Cholesterol Metabolism High levels of LDL-cholesterol are an established risk factors for CAD. Not surprisingly, the most prominent genes which are directly

involved in LDL-cholesterol metabolism and have been tagged by GWAS are *LDLR*, *PCSK9*, and *SORT1*. In line, mutations in the *LDLR* gene leading to reduced hepatic uptake of LDL-cholesterol have been shown to underlie familial hypercholesterolemia which itself increases CAD risk (Tolleshaug et al. 1983; Brown and Goldstein 1986), and common variants at the *LDLR* locus have been associated with both phenotypes (Myocardial Infarction Genetics Consortium et al. 2009; Teslovich et al. 2010; Schunkert et al. 2011; Do et al. 2015). *PCSK9* has been identified as an interaction partner of the LDL-receptor. If *PCSK9* binds to the LDL-receptor, it is internalized and degraded resulting in reduced hepatic uptake and high plasma levels of LDL-cholesterol (Cameron et al. 2006). Gain-of-function mutations in *PCSK9* have been shown to increase LDL-cholesterol (Abifadel et al. 2003), whereas loss-of-function variants have been shown to reduce LDL-cholesterol and CAD risk (Cohen et al. 2006). *PCSK9* has been targeted pharmacologically via different approaches. The inhibition of *PCSK9* using, e.g., neutralizing antibodies resulted in a marked reduction of LDL-cholesterol and reduced risk of cardiovascular events in CAD patients (Sabatine et al. 2015, 2017). Sortilin 1 encoded by *SORT1* has also been shown to affect LDL-cholesterol (Samani et al. 2007; Musunuru et al. 2010; Teslovich et al. 2010; Schunkert et al. 2011). However, as it seems to also play a role in other processes and as the mechanism involving sortilin 1 in LDL-cholesterol metabolism has still not been fully understood, it might not represent an ideal drug target. *NPC1L1*, a membrane transporter leading to the uptake of cholesterol from the intestine encoded by the *NPC1L1* gene, also revealed a signal. Using exome sequencing, loss-of-function mutations were identified to be associated with reduced LDL-cholesterol and protection from CAD (Myocardial Infarction Genetics Consortium Investigators et al. 2014). In parallel, it has been shown that pharmacological targeting of *NPC1L1* with ezetimibe also reduces the incidence of cardiovascular events in addition to a statin (Cannon et al. 2015). Figure 3 summarizes the multiple lines of evidence for an involvement of LDL-cholesterol metabolism in CAD/MI from a genetic point of view.

Triglyceride Metabolism Lipoprotein lipase has been identified as a central enzyme regulating triglyceride levels. In line, genetic variants in *LPL* have been associated with both triglyceride levels and CAD risk (Khera et al. 2017). Additionally, the genes encoding several modulators of lipoprotein lipase activity were found to be associated with CAD/MI risk: (1) apolipoprotein C-III reduces lipoprotein lipase activity, and mutations in *APOC3* are associated with both increased triglyceride levels and CAD risk (The TG and HDL Working Group of the Exome Sequencing Project, National Heart, Lung, and Blood Institute 2014; Klarin et al. 2018); (2) apolipoprotein A-V increases lipoprotein lipase activity, and mutations in *APOA5* increase triglyceride levels and CAD risk (Do et al. 2015); and (3) angiopoietin-like 4 inhibits lipoprotein lipase activity, and mutations in *ANGPTL4* reduce triglyceride levels and CAD risk (Myocardial Infarction Genetics and CARDIoGRAM Exome Consortia Investigators et al. 2016). Angiopoietin-like 3 seems to have comparable effects as deficiency was also associated with reduced triglycerides and CAD risk (Stitzel et al. 2017). Mainly apolipoprotein C-III but also

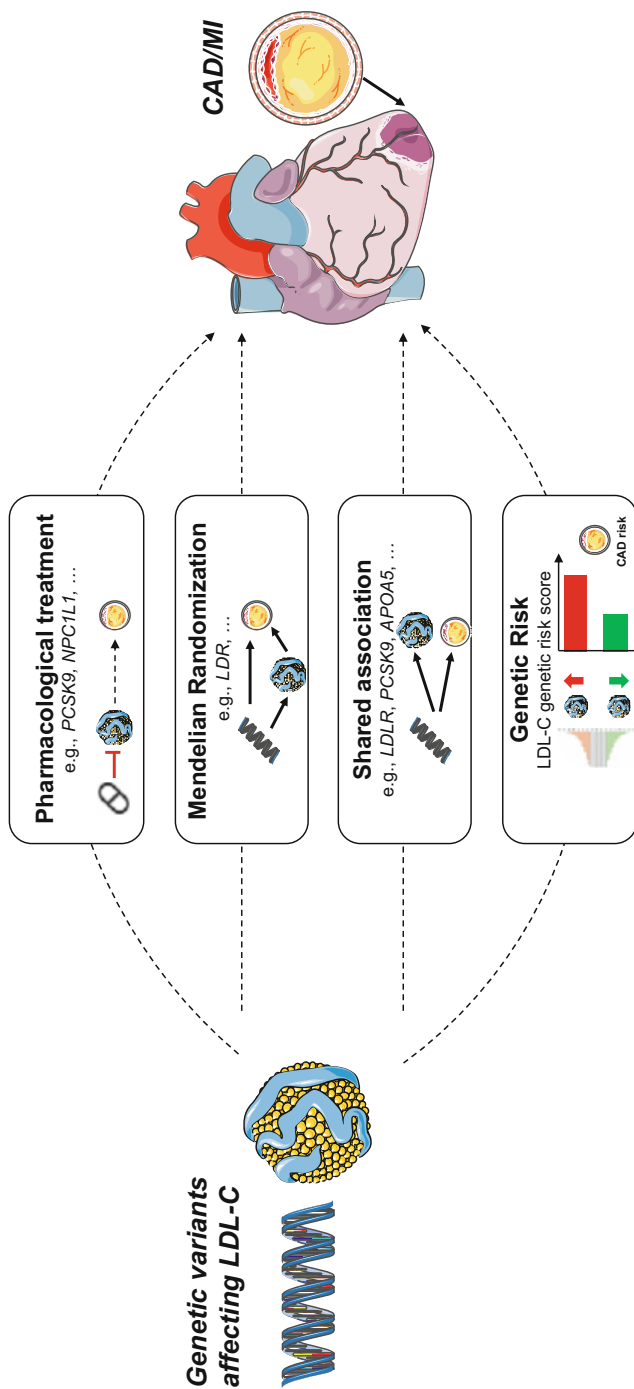


Fig. 3 Multiple lines of genetic evidence for an involvement of LDL-cholesterol metabolism in CAD/MI. 1. Pharmacological treatments targeting genes which have been associated with LDL-cholesterol reduce risk of cardiovascular events. 2. Mendelian randomization studies have proven the causal influence of LDL-cholesterol metabolism in CAD/MI. 3. CAD/MI and LDL-cholesterol show shared genetic risk variants which are associated with both traits at the genome-wide level of statistical significance. 4. Genetic risk scores for LDL-cholesterol including genome-wide significant variants are also associated with CAD/MI risk, i.e., individuals with a high genetic LDL-cholesterol risk score are also at higher risk for CAD/MI and vice versa. Contains modified image material available at Servier Medical Art under a Creative Commons Attribution 3.0 Unported License

angiopoietin-like 3 are subjects of research for novel strategies targeting lipoprotein lipase metabolism (Graham et al. 2013; Gaudet et al. 2015; Ahmad et al. 2019).

5.2 Inflammation

Vascular inflammation and the recruitment of leukocytes are the hallmarks of atherosclerosis (for reviews see Lusis (2000), Libby et al. (2011), and Swirski and Nahrendorf (2013)). Several clinical studies already in the past decades thus targeted inflammatory parameters. A prominent example is C-reactive protein (CRP), which has been regarded for a long time as a pathophysiological player. This was mainly due to the observation of elevated CRP levels in individuals suffering from CAD in epidemiological studies (Koenig et al. 1999; Danesh et al. 2004) making CRP a promising therapeutic target (Pepys et al. 2006). Indeed, clinical trials, e.g., the JUPITER trial which investigated the effect of rosuvastatin in individuals with elevated CRP levels, showed a benefit (Ridker et al. 2008). Genetic studies were, however, able to dissect this association and trial outcomes. As such, LDL-cholesterol could be clearly proven as a causal risk factor for CAD as a genetic risk score for LDL-cholesterol elevating variants was also associated with CAD risk (Kathiresan et al. 2008). In contrast, several variants found to increase CRP levels did not show an association with CAD risk (Lange et al. 2006; Zacho et al. 2008; Linsel-Nitschke et al. 2008; Schunkert and Samani 2008; C Reactive Protein Coronary Heart Disease Genetics Collaboration (CCGC) et al. 2011). While these studies on CRP remained disappointing, targeting inflammation has indeed lately been proven to be effective in CAD. In the CANTOS trial, the administration of an interleukin-1 β neutralizing antibody was able to reduce the incidence of cardiovascular events ultimately proving the inflammation hypothesis (Ridker et al. 2017). This is in line with Mendelian randomization studies that revealed evidence for a causal role of interleukin-6/interleukin-6 receptor signaling, which is downstream of interleukin-1 β , in CAD (Interleukin-6 Receptor Mendelian Randomisation Analysis Consortium et al. 2012).

GWAS led to the identification of a number of variants tagging genes which play a role in inflammatory processes to be associated with CAD/MI.

Autoimmune Processes CAD/MI and autoimmune disease share genetic risk factors. As such, the *SH2B3* locus has been identified to be associated with CAD/MI (Coronary Artery Disease C4D Genetics Consortium 2011b; Schunkert et al. 2011) but also, e.g., type 1 diabetes mellitus (Barrett et al. 2009) and celiac disease (Hunt et al. 2008). A role for the encoded protein, SH2B adaptor protein 3 (SH2B3), has been shown in post MI remodeling: lack of Sh2b3 in rats was associated with increased fibrosis, increased leukocyte infiltration, and decreased cardiac function (Flister et al. 2015). Additionally, it is involved in dendritic cell function leading to T-cell interferon signaling (Mori et al. 2014). Nevertheless, uncertainties remain as the locus was also found to be associated with blood pressure (Levy et al. 2009; Newton-Cheh et al. 2009) and hematologic parameters (Soranzo

et al. 2009). The latter observation might be important as an involvement in megakaryopoiesis and stabilization of thrombi has also been shown experimentally (Tong and Lodish 2004; Takizawa et al. 2010). In a recent study, the *SH2B3* risk allele was found to be associated with decreased SH2B3 expression which, at least for the murine counterpart *Sh2b3/Lnk*, together with cholesterol loading promoted platelet production and activation (Wang et al. 2016).

Innate Immunity CXCL12 is a chemokine which is encoded by the CAD risk gene *CXCL12* (Myocardial Infarction Genetics Consortium et al. 2009; Coronary Artery Disease C4D Genetics Consortium 2011b) and has a function in various cellular processes. In atherosclerosis the CXCL12/CXCR4 axis has initially been described to have a rather protective role (Döring et al. 2014). Disruption of this axis led to increased plaque formation and proinflammatory plaque phenotypes in vivo (Zernecke et al. 2008). In line, CXCL12/CXCR4 signaling was shown to be important in endothelial cells and smooth muscle cells via maintaining barrier function and contractile responses, respectively (Döring et al. 2017). However, the interaction of CXCL12 and CXCR4 in atherosclerosis seems to be more complex. Whereas the atheroprotective findings were mainly derived from studies in which the interaction was inhibited by small molecules or genetic deletion of CXCR4, endothelial cell-derived CXCL12 was found to promote atherosclerosis (Döring et al. 2019). CXCR4 in contrast seems to be atheroprotective: in addition to the mentioned in vivo studies, the rs2322864 C-allele, which was found to be associated with CAD risk in a candidate gene study, was also associated with CXCR4 expression in plaques (Döring et al. 2017). The double-edged effects of CXCL12/CXCR4 signaling need to be considered to successfully target this pathway.

5.3 Platelet Function and Nitric Oxide Signaling

Genetic studies led to the identification of several genes which play a role in nitric oxide (NO) signaling leading to the formation or degradation of the second messenger cyclic guanosine monophosphate (cGMP), an endogenous inhibitor of platelet aggregation (Moro et al. 1996). Whereas several genes (e.g., *NOS3*, *PDE5A*, *MRVII*, *PDE3A*) fulfill important functions in this pathway, most is known about *GUCY1A3* which encodes the α_1 -subunit of the soluble guanylyl cyclase (α_1 -sGC). As discussed above, a digenic mutation in *GUCY1A3* and the *CCT7* gene, which encodes the chaperone protein, was identified in a family by exome sequencing (Erdmann et al. 2013). At the same time, also a common non-coding variant (rs7678555) was identified in Europeans to be associated with CAD by GWAS (CARDIoGRAMplusC4D Consortium et al. 2013). Whereas the digenic mutation led to loss of α_1 -sGC due to premature stop of translation (Erdmann et al. 2013), the common variant has been shown to influence *GUCY1A3* expression, i.e., the risk allele G was linked to reduced expression (Kessler et al. 2017). Both the digenic mutation and the common risk variant led to reduced cGMP formation in platelets (Erdmann et al. 2013; Kessler et al. 2017). As a consequence, platelets of carriers of

the common risk variants showed impaired inhibition of platelet aggregation secondary to NO stimulation (Kessler et al. 2017). The sGC is a known pharmacological target. Specific stimulators and activators of the sGC are available (for an overview see Stasch et al. (2011)) and approved for other traits, e.g., pulmonary hypertension (Ghofrani et al. 2013a, b). First preclinical data also render a positive influence of sGC stimulators on atherosclerotic phenotypes possible (Tsou et al. 2014). Additionally, the impaired response to nitric oxide in carriers of the common risk allele G in platelets might also be targeted via unspecific inhibitors of platelet aggregation. Recently, we showed that homozygous risk allele carriers might benefit from aspirin treatment in the primary prevention of cardiovascular diseases, whereas homozygous or heterozygous carriers of the non-risk allele A seem to even display increased risk (Hall et al. 2019). Additionally, homozygous carriers of the risk allele G are at an increased risk of ischemic events after the implantation of coronary stents, at least in part via higher on-aspirin platelet reactivity (Kessler et al. 2019). While platelets, in addition to their well-known role in atherothrombosis, are also involved in atherosclerotic plaque formation (Gawaz et al. 2005) and despite the known influence of sGC function on inflammatory phenotypes (Ahluwalia et al. 2004), the exact mechanisms are still unknown. This is complicated by the fact that a complete knockout of the murine counterpart *Gucyl1a3* led to the unexpected finding of reduced atherosclerotic plaque formation (Segura-Puimedon et al. 2016), whereas genetically determined reduced but not lacking expression of *Gucyl1a3* was also associated with increased plaque formation (Kessler et al. 2017). The genetic findings at the further mentioned NO-cGMP-signaling loci associated with CAD/MI have been extensively discussed elsewhere (Wobst et al. 2018). A promising target in addition to sGC is phosphodiesterase 5A encoded by the *PDE5A* gene (Nelson et al. 2017) which leads to degradation of cGMP. However, there is currently no evidence for a beneficial effect of PDE5A inhibition in atherosclerosis.

5.4 Vascular Phenotypes

Several genes identified by GWAS have been linked to vascular phenotypes including the regulation of vascular tone and vascular remodeling.

Vascular Tone and Blood Pressure Hypertension is a known risk factor for CAD (Yusuf et al. 2004). Some of the CAD risk genes also display genome-wide association with blood pressure. In particular, *NOS3*, encoding endothelial NO synthase (eNOS), and *GUCY1A3* (see above) have an established role also in smooth muscle cells leading to vasodilatation after production of cGMP (Moro et al. 1996). Both have been identified as blood pressure genes (International Consortium for Blood Pressure Genome-Wide Association Studies et al. 2011; Salvi et al. 2012). In line, a genetic risk score for *NOS3* and *GUCY1A3* was associated with increased CAD risk. However, the effect could only in part be explained by effects on blood pressure (Emdin et al. 2018). Further genes associated with both CAD and blood pressure

include *SH2B3*, *CYP17A1*, *FURIN*, *AGT*, and *ARHGAP42*. Profound knowledge about the underlying mechanisms is still lacking. However, a genetic risk score for hypertension was strongly associated with CAD/MI and vice versa highlighting the importance of this risk factor which also has a strong heritability (Ntalla et al. 2019).

Vascular Remodeling A large number of genes have been linked to vascular remodeling (for an overview see Erdmann et al. (2018)). One example is the *REST* gene which has been identified as a CAD risk gene in 2017 (Nelson et al. 2017). *REST* encodes the RE-1 silencing transcription factor (REST) which has been mainly studied in neuronal diseases as Huntington disease (Zuccato et al. 2003) or seizures (McClelland et al. 2014). However, there is also evidence for a role in cardiac development where REST led to increased proliferation (Zhang et al. 2017). Additionally, REST has been described to inhibit microRNA-21 and to inhibit proliferation in vascular smooth muscle cells. As a consequence, REST might influence the formation of vulnerable atherosclerotic plaques (Jin et al. 2018). As it acts as a transcriptional regulator, several downstream transcripts might play important pathophysiological roles and represent novel targets. Another novel target is a disintegrin and metalloproteinase with thrombospondin motifs 7 (*ADAMTS-7*) which has been identified as a CAD risk gene (*ADAMTS7*) in 2011 (Reilly et al. 2011; Schunkert et al. 2011; Coronary Artery Disease C4D Genetics Consortium 2011a). It has been shown that *Adamts7* deficiency leads to reduced neointima formation after vascular injury (Bauer et al. 2015; Kessler et al. 2015b) as well as reduced atherosclerotic plaque formation under proatherogenic conditions (Bauer et al. 2015). Whereas the influence on vascular remodeling is influenced by *ADAMTS-7*-dependent degradation of cartilage oligomeric matrix protein (COMP) (Wang et al. 2009) and thrombospondin-1 (TSP-1) (Kessler et al. 2015b) with effects on vascular smooth muscle cells and endothelial cells, respectively, the mechanism underlying reduced atherosclerotic plaque formation in mice lacking *Adamts7* remains unknown so far. Of note, the *ADAMTS7* locus is the only locus that has been identified to exert a gene-environment interaction with smoking. In smokers, the protective effect of the *ADAMTS7* non-risk allele was outweighed in smokers, presumably via upregulation of *ADAMTS7* expression secondary to exposure to tobacco ingredients (Saleheen et al. 2017).

5.5 Further Directions

Other CAD risk genes have been clustered into similarly interesting pathways which could in principle be targets of therapeutic interventions. Genes have, for example, also been annotated to *transcriptional gene regulation, mitosis and proliferation, or neovascularization and angiogenesis* (Lempiäinen et al. 2018; Zeng et al. 2019b). The majority of variants and genes has nevertheless not yet been classified to such pathways (Erdmann et al. 2018). Also, the role of the first and strongest risk locus chromosome 9p21 is still not fully understood. Whereas studies initially focused on the role of the cyclin-dependent kinase inhibitors 2A/2B (*CDKN2A/B*) (Harismendy

et al. 2011), the circular form of the non-coding RNA *ANRIL*, which is also located at the locus (Pasmant et al. 2011), was found to have an atheroprotective role through balanced regulation of vascular cells. In contrast, the linear form which is increased in carriers of the risk allele increases atherosclerosis risk (Holdt et al. 2016).

6 Risk Scores and Risk Prediction

Apart from identifying novel pharmacological targets, GWAS raised hope to identify patients at risk early on to improve prevention of CAD. Whereas initial studies including some of the identified variants only led to modest success in this regard (Hughes et al. 2012; for an overview see Kessler and Schunkert (2012)), recent studies using polygenic risk scores changed the scenario. A polygenic risk score for CSD including more than six millions of variants was able to identify individuals with a substantially elevated CAD risk. Strikingly, the score identified 20 times more individuals than familial hypercholesterolemia patients, while the carriers of a high genetic risk score were at even higher CAD risk (Khera et al. 2018). Even in a scenario in which high polygenic risk score and the presence of familial hypercholesterolemia lead to a same increase in CAD risk, the prevalence of a high polygenic risk score was ten times higher than that of familial hypercholesterolemia (Khera et al. 2019) illustrating the potential of including such scores in prevention programs.

Another field in which polygenic risk scores can be used is to investigate associations with other phenotypes. In particular, as discussed above it has been shown that CAD/MI and other cardiovascular phenotypes, e.g., peripheral arterial disease, stroke, or abdominal aneurysm, share genetic predisposition. In contrast, risk of migraine was reduced with increased genetic CAD/MI risk (Ntalla et al. 2019). Risk scores were also able to dissect an interrelationship between educational attainment, which is regarded as a determinant of lifestyle factors, and CAD/MI risk. Here, a genetic risk score including variants which are known to be associated with educational attainment was also associated with CAD risk. As the signal was lost after adjusting for body mass index and smoking, it can be hypothesized that a genetic predisposition to educational attainment might influence a healthier lifestyle and, subsequently, reduce CAD/MI risk (Zeng et al. 2019a).

Furthermore, there is hope that knowledge of particular genetic risk factors can be used to design individualized treatment strategies. *GUCY1A3* as an example has been discussed above. Here, knowledge of genotype could inform aspirin therapy in the setting of primary prevention (Hall et al. 2019) and ischemic risk in CAD patients after PCI (Kessler et al. 2019). Genetic risk scores have also been able to identify individuals who could have a larger benefit from statin treatment (Mega et al. 2015; Natarajan et al. 2017). In the future, polygenic risk scores might therefore also be used in the design of clinical trials. A recent post hoc study of the ODYSSEY trial which investigated the benefit from the PCSK9 inhibitor alirocumab in CAD (Schwartz et al. 2018) revealed that individuals with a higher polygenic risk score particularly benefited from treatment (Damask et al. 2020). Whereas this clearly

indicated that polygenic risk scores might provide an excellent tool for risk stratification, a prospective benefit in the design of clinical trials needs to be demonstrated.

7 Summary

Genomic studies led to the identification of a large and still growing number of genes which play a role in the pathophysiology of CAD/MI. While only a few have been functionally investigated so far, novel therapeutic strategies have been developed in, e.g., LDL-cholesterol metabolism, and further promising targets might be identified. In addition, knowledge of genetic risk factors might facilitate prevention of the disease through early identification of individuals at risk and therapy via individualized treatment strategies.

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Regulatory Non-coding RNAs in Atherosclerosis

Andreas Schober, Saffiyeh Saboor Maleki, and Maliheh Nazari-Jahantigh

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Abstract

Regulatory RNAs like microRNAs (miRNAs) and long non-coding RNAs (lncRNAs) control vascular and immune cells' phenotype and thus play a crucial role in atherosclerosis. Moreover, the mutual interactions between miRNAs and lncRNAs link both types of regulatory RNAs in a functional network that affects lesion formation. In this review, we deduce novel concepts of atherosclerosis from the analysis of the current data on regulatory RNAs' role in endothelial cells

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(ECs) and macrophages. In contrast to arterial ECs, which adopt a stable phenotype by adaptation to high shear stress, macrophages are highly plastic and quickly change their activation status. At predilection sites of atherosclerosis, such as arterial bifurcations, ECs are exposed to disturbed laminar flow, which generates a dysadaptive stress response mediated by miRNAs. Whereas the highly abundant miR-126-5p promotes regenerative proliferation of dysadapted ECs, miR-103-3p stimulates inflammatory activation and impairs endothelial regeneration by aberrant proliferation and micronuclei formation. In macrophages, miRNAs are essential in regulating energy and lipid metabolism, which affects inflammatory activation and foam cell formation.

Moreover, lipopolysaccharide-induced miR-155 and miR-146 shape inflammatory macrophage activation through their oppositional effects on NF- κ B. Most lncRNAs are not conserved between species, except a small group of very long lncRNAs, such as MALAT1, which blocks numerous miRNAs by providing non-functional binding sites. In summary, regulatory RNAs' roles are highly context-dependent, and therapeutic approaches that target specific functional interactions of miRNAs appear promising against cardiovascular diseases.

Keywords

Endothelial cell · Long non-coding RNA · Macrophage · MicroRNA

1 Introduction

RNA molecules have a great variety of functions in the cell, including the transfer of protein-coding information in the primary nucleotide sequence of the mRNA, reading the codons and matching them to amino acids by tRNA, catalysis of peptide bond formation by rRNA, and splicing of pre-mRNA by small nuclear RNAs in the spliceosome. Besides, regulatory non-coding RNAs, which have been grossly grouped into microRNAs (miRNAs) and long, non-coding RNAs (lncRNAs), play a critical role in the control of gene expression (Kopp and Mendell 2018). miRNAs have a comparatively confined length of 20–24 nucleotides and are processed from a primary transcript that contains a secondary hairpin structure with an imperfectly paired stem by endonucleases, such as Drosha and Dicer. By contrast, lncRNAs, linear and circular, are 200 to several thousand nucleotides long, which often adopt a complex secondary structure, and are generated in many different ways, such as RNaseP cleavage or back-splicing (Wu et al. 2017).

miRNAs play an essential role in nematodes, like miRNA let-7, the second described miRNA, which is critical in the development of *C. elegans* (Roush and Slack 2008). The nucleotides 2–7 at the 5' end of the miRNA, also known as the “seed sequence,” bind via Watson-Crick-based complementary base pairing to the recognition element mostly located in the 3'-UTR of mRNAs (Bartel 2018). However, also non-canonical interactions, including, e.g., G:U wobbles or bulges, can occur and mediate the effect of miRNAs (Chipman and Pasquinelli 2019). Thus, the

seed sequence is essential for the function of miRNAs. Notably, some miRNAs share the same seed sequence but differ in one or two nucleotides outside the seed sequence, establishing a seed family of miRNAs. Moreover, multiple genomic loci may exist for a specific miRNA. For example, the human let-7 seed family, e.g., comprises 12 miRNAs, such as let-7a, let-7b, let-7c, etc., which share the same seed sequence (Lee et al. 2015). Moreover, three genomic loci encode the let-7a miRNA (let-7a-1, let-7a-2, and let-7a-3). Because of the same seed sequence, miRNA families may form a redundant system resistant to genetic losses and mutations.

Dicer cleaves the apical loop of the precursor hairpin and produces a miRNA duplex that contains the 5p (miRNA from the 5' end of the precursor miRNA) and the 3p strand of the mature miRNA. Following cleavage of the precursor miRNA hairpin by Dicer in the cytoplasm, a miRNA duplex is loaded to Argonaute proteins constituting the RNA-induced silencing complex (RISC). The two miRNA strands are separated, and one of the two strands exits the RISC. Argonaute proteins present the seed sequence of the retained miRNA strand in the mature RISC, and thus greatly enhance the interaction with the mRNA target sequence. Following binding of the target, miRNAs mediate either translational inhibition or degradation of the target mRNA through GW182 proteins (Schober and Weber 2016). Many miRNAs target hundreds of mRNAs in a cell, but the level of suppression rarely exceeds 50%. An increase in miRNA genes parallels the development of vertebrates (Fromm et al. 2015). In humans, the number of miRNA genes expanded by 179 genes after the split from mouse, peaking at 585 miRNA genes with even many more mature miRNAs (Fromm et al. 2015). Therefore, miRNAs may play a role in the evolution of morphological complexity, probably by shaping specific cell phenotypes through buffering variations in gene expression due to environmental cues and internal noise (Kosik 2010; Siciliano et al. 2013). Thus, regulation by miRNAs confers robustness to differentiation processes during development (Ebert and Sharp 2012).

lncRNAs are not a homogenous class of molecules, but rather a mixture of different functional types with distinct biological mechanisms (Chen et al. 2016a). For example, a lncRNA can affect gene expression by the transcription of its genomic locus, by regulating chromatin states, influencing nuclear structure and organization, and interacting with and controlling proteins or other RNA molecules (Kopp and Mendell 2018; Yao et al. 2019). Many lncRNAs are biochemically identical to mRNAs, harboring a 5' cap and a 3' polyadenylated tail, and alternative splicing can produce numerous lncRNA transcripts. Whereas many miRNAs are highly conserved between species, most lncRNAs are evolutionary "novel" (< 50 Million years), and conservation even between humans and mice is rare (Ulitsky 2016). In humans, around 50–90,000 genomic lncRNA loci are annotated (Ulitsky 2016; Uszczynska-Ratajczak et al. 2018), the majority is expressed at a very low level and may not play a functional role. However, a small group of very long, highly expressed, and evolutionarily conserved lncRNAs exists, including, e.g., metastasis-associated lung adenocarcinoma transcript (MALAT)1 and myocardial infarction associated transcript (MIAT). Notably, these lncRNAs contain binding sites for a large number of miRNAs and interact with those in the RISC, the so-called sponge effect. Numerous reports have shown that the functional effect of sponging lncRNAs

is due to the inhibition and suppression of the bound miRNAs. Thus, sponging miRNAs appears to be an evolutionarily conserved function of this group of lncRNAs, which closely links the miRNA and the lncRNA world in a complex interaction network.

2 The Role of Non-coding RNAs in Arterial Endothelium

The arterial system is inherently vulnerable to chronic, low-grade endothelial injury at branching sites and curvatures due to disturbed laminar blood flow (Chen et al. 1997). Considering the specifications of ECs in different vascular regions and organs, the response of arterial ECs to non-laminar blood flow appears as if they are maladapted to this physiologic condition at branching arteries. Whereas the low shear stress generated by non-laminar blood flow is stable, the direction of the shear stress and blood flow near the arterial wall in these regions changes randomly, indicating complex vortices. Accordingly, ECs do not align to the direction of the blood flow at branching sites like at arterial areas exposed to laminar blood flow (Gau et al. 1980).

Interestingly, the multidirectionality of near-wall shear stress better predicts the effect of non-laminar blood flow on the localization of atherosclerotic lesions than low shear stress (Peiffer et al. 2013; Mohamied et al. 2015, 2017). Because cellular adaptation to chaotic conditions is impossible, the unpredictable changes of the shear stress direction prevent the adaptation of arterial ECs to non-laminar blood flow and trigger a heterogeneous stress response (Fig. 1). Although at a low frequency, the non-laminar blood flow induces apoptosis of ECs by activating the endoplasmatic reticulum stress response and SUMOylation of p53 (Kim and Woo 2018; Bjorkerud and Bondjers 1972; Zeng et al. 2009; Pan et al. 2017; Hansson et al. 1985; Heo et al. 2011, 2013). Apoptotic ECs are rapidly replaced by the migration and proliferation of adjacent ECs. The endothelial dysadaptation to disturbed laminar blood flow also triggers inflammatory activation and increased adhesiveness to leukocytes, which may play a role in endothelial repair (Tsao et al. 1995). Stress-induced transcription factors, such as NF- κ b and hypoxia-inducible factor (HIF)-1 α , shape the inflammatory phenotype of dysadapted arterial ECs (Akhtar et al. 2015).

Moreover, chronic endothelial healing in arterial regions exposed to disturbed laminar blood flow impairs endothelial barrier function and increases the permeability to macromolecules, such as lipoproteins (Mundi et al. 2018). Low-density lipoproteins (LDL) are trapped in the subendothelial space and become oxidized by ECs (van Hinsbergh et al. 1986). Oxidized LDL constitutes the second cause of endothelial death (Salvayre et al. 2002) and drives the accumulation of monocyte-derived macrophages in the vessel wall. In arterial regions exposed to laminar blood flow, which stimulates the expression of the transcription factors Krüppel-like factor (KLF) 2 and KLF4, endothelial turnover and vascular permeability are low (Gerrity et al. 1977). KLF2 and KLF4 contribute to a quiescent endothelial phenotype by regulating up to 15% of the flow-regulated genes in a partially redundant manner resulting in the reduction of vascular permeability, inflammatory gene expression, and thrombogenicity (Schober et al. 2015). Moreover, high shear stress-exposed

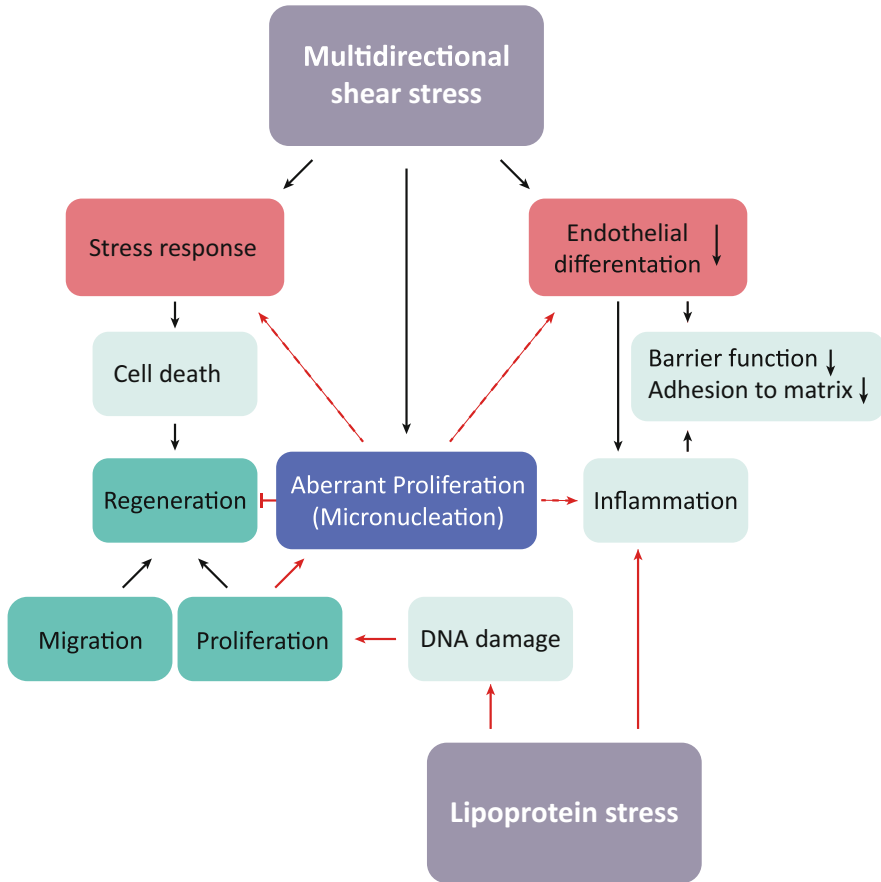


Fig. 1 Dysadaptation of arterial ECs sensitizes to lipoprotein stress. The unpredictable changes in the direction of near-wall shear stress and blood flow at arterial bifurcations make the adaptation of arterial ECs impossible. The dysadaptation induces a stress response characterized by EC death and a lack of endothelial differentiation. The damaged endothelium is regenerated by migrating and proliferating ECs; however, the chronic endothelial wound healing poses a risk for aberrant proliferation due to DNA damage of mitotic ECs by oxidative lipoprotein stress. Dashed lines indicate hypothetical effects

ECs can rapidly proliferate and regenerate the endothelium in response to acute injury (Hansson et al. 1985).

2.1 miRNAs Drive Inflammatory Activation in Dysadapted Arterial ECs

ECs express 164 to 315 miRNAs, and the miRNA expression profile differs significantly between ECs from different vascular regions (McCall et al. 2011; Voellenkle

et al. 2012). miR-21-5p (32% of all endothelial miRNAs) is by far the most abundant miRNA in human umbilical vein ECs, followed by miR-126-3p (7%) (Voellenkle et al. 2012). Surprisingly, *Mir21* knockout mice do not have any gross phenotypic changes and develop normally (Patrick et al. 2010). By contrast, 50% of all *Mir126* knockout mice die during development due to vascular leakage, but the surviving mice develop normally (Kuhnert et al. 2008). Notably, specific miRNA families and clusters are primarily expressed in ECs, such as the miR-221/222 cluster, the let-7 miRNA family, and the miR-17-92 cluster. Endothelial Dicer is not essential in embryonic development, and, surprisingly, Dicer suppresses endothelial differentiation in vitro by downregulating *KLF2* and by upregulating inflammatory genes, such as *CXCL1* and *IL-8* (Suarez et al. 2007, 2008; Wu et al. 2011). Accordingly, knockout of endothelial Dicer in *ApoE*^{-/-} mice limits endothelial inflammation and atherosclerotic lesion formation (Hartmann et al. 2016; Natarelli et al. 2018), indicating that the whole miRNA system counter regulates high shear stress-induced endothelial differentiation. This effect was associated with the downregulation of a relatively small subset of miRNAs, including miR-103-3p, miR-652-3p, and miR-433-3p, whereas miR-126-3p was not reduced, indicating substantial differences in the turnover rates of these miRNAs. In general, the stability of miRNAs is more than ten times higher than that of mRNAs; however, turnover rates significantly differ between individual miRNAs (Hartmann et al. 2016; Gantier et al. 2011). Thus, the role of endothelial Dicer in atherosclerosis and endothelial differentiation may depend on the generation of miRNAs with a short half-life. Similar to the in vitro results, endothelial Dicer increases inflammatory gene expression, such as *Cxcl1* and *Ccl2*, and reduces the expression of endothelial genes such as cadherin 5 and SRY-box transcription factor (*Sox*) 17, in murine atherosclerotic arteries. Also, Dicer significantly regulates *KLF4*, Notch, and β -catenin signaling pathways in atherosclerotic ECs (Hartmann et al. 2016).

miR-103-3p mediates many of the inflammatory effects of Dicer in ECs (Hartmann et al. 2016). The *Mir103* gene is expressed from two different genomic loci located in the intronic sequences of the pantothenate kinase (*Pank*) 2 and *Pank3* genes. These genomic loci and the sequence of the mature miR-103-3p are highly conserved within deuterostomes (Finnerty et al. 2010). Notably, miR-103-3p is abundant in ECs at branching sites of arteries, probably due to disturbed flow-induced NF- κ B activation (Hartmann et al. 2016; Tzima et al. 2005). Hyperlipidemia and mildly oxidized LDL also upregulate miR-103-3p expression in arterial ECs (Hartmann et al. 2016). miR-103-3p promotes dedifferentiation by targeting *KLF4* and increases *Cxcl1*-dependent monocyte recruitment to atherosclerotic arteries. Accordingly, inhibiting the interaction between miR-103-3p and *KLF4* with a sequence-specific oligonucleotide ameliorates atherosclerosis (Hartmann et al. 2016). Moreover, inhibition of miR-103-3p also reduces atherosclerosis, endothelial inflammation, and endoplasmic reticulum stress in *ApoE*^{-/-} mice (Jiang et al. 2020). In human aortic ECs, miR-103-3p targets phosphatase and tensin homolog (*PTEN*) through a canonical binding site, which is not conserved in mouse (Jiang et al. 2020).

In addition to miR-103-3p, the expression of miR-652-3p in ECs promotes atherosclerosis (Huang et al. 2019). The development of atherosclerosis is reduced

in *Mir652*^{-/-}/*ApoE*^{-/-} mice, probably due to increased endothelial regeneration, because miR-652-3p inhibits endothelial proliferation and repair following mechanical injury (Huang et al. 2019). miR-652-3p suppresses endothelial proliferation by targeting the G1 cyclin, cyclin D2 (*Ccnd2*) in human and mouse via different non-canonical sites, indicating functional conservation of this interaction (Huang et al. 2019). *Ccnd2* is targeted by two other miRNAs, miR-494, and the let-7 family member miR-98, in ECs, which thereby inhibit endothelial proliferation (Li et al. 2016; Wu et al. 2016). Besides, the expression of miR-652-3p inversely correlates with the endothelial expression of *CCND2* and EC proliferation in human atherosclerosis, suggesting that miR-652-3p plays a vital role in endothelial dysadaptation (Huang et al. 2019).

miR-92a-3p is another important miRNA in endothelial dysadaptation and inflammation, which is, like miR-103-3p, upregulated in ECs by low shear stress, oxidized LDL, and oxidative stress (Chen et al. 2015; Fang and Davies 2012). miR-92a-3p cooperates with miR-103-3p in suppressing *KLF4* and enhancing inflammatory gene expression through activation of NF- κ B. Moreover, miR-92a-3p targets several other transcripts in ECs, like *KLF2*, *SOCS5*, and *SIRT1* (Chen et al. 2015), which also contributes to the inflammatory phenotype of dysadapted ECs. Accordingly, inhibition of miR-92a-3p by antisense oligonucleotides in *ApoE*^{-/-} and *Ldlr*^{-/-} mice reduced atherosclerosis and endothelial inflammation (Chen et al. 2015; Loyer et al. 2014). However, after subtotal nephrectomy in *ApoE*^{-/-} mice on a regular diet, as a model of atherosclerosis triggered by chronic renal failure, locked nucleic acid-based inhibitors of miR-92a-3p applied together with HDL did not reduce lesion formation, despite a strong decrease in endothelial miR-92a-3p expression (Wiese et al. 2019). This result indicates that the role of endothelial miR-92a-3p in atherosclerosis may be model dependent. ECs also release miR-92a-3p in microvesicles, which increases the level of miR-92a-3p in the circulation of patients with coronary atherosclerosis (Liu et al. 2019). miR-92a-3p is processed from the 7-kb long lncRNA *MIR17HG* together with five other miRNAs, including miR-17-5p, miR-18a-5p, miR-20a-5p, miR-19a-3p, and miR-19b-3p (Mogilyansky and Rigoutsos 2013). Whereas miR-92a and miR-19b are additionally processed from the miR-106a-363 cluster transcript, miR-19a is only expressed from the miR-17-92a locus. Activation of HIF-1 α in arterial ECs by turbulent flow and lipoprotein-derived lysophosphatidic acid selectively upregulates miR-19a-3p (Akhtar et al. 2015). Because HIF-1 α -mediated miR-19a-3p expression in ECs increases monocyte adhesion by activating NF- κ B, this mechanism may mediate the atherogenic effect of endothelial HIF-1 α activation (Akhtar et al. 2015). Accordingly, blocking miR-19a-3p by systemic injection of antisense oligonucleotides decreases atherosclerosis in *ApoE*^{-/-} mice (Chen et al. 2017).

The miR-106b-25 cluster contains three miRNAs, including miR-106b, miR-93, and miR-25, and closely relates to the miRNAs of the miR-17-92a locus. Whereas miR-106b and miR-25 belong to the miR-17 family of miRNAs, miR-25 shares the same seed sequence with miR-92a (Mogilyansky and Rigoutsos 2013). Knockout of the miR-106b-25 cluster in *ApoE*^{-/-} mice decreases atherosclerosis, reduces the cholesterol content in VLDL and LDL in the blood, and increases VLDL and LDL

receptor expression in splenocytes (Semo et al. 2019). These findings indicate that the miRNAs of the miR-106b-25 cluster elevate plasma lipid levels by suppressing lipoprotein clearance by the spleen (Semo et al. 2019).

2.2 The miR-126 Strands in Apoptosis and Regeneration of Dysadapted ECs

MiR-126-3p is one of the most highly expressed miRNAs in ECs. Apoptosis triggers the packaging of miR-126-3p into apoptotic bodies, which are released to the extracellular space, and adjacent ECs take up the apoptotic vesicles. The paracrine transfer of miR-126-3p from apoptotic ECs upregulates the chemokine CXCL12 in the recipient ECs and reduces atherosclerosis (Zernecke et al. 2009). However, the role of CXCL12 and its receptor CXCR4 in atherosclerosis is ambiguous. Whereas endothelial knockout of CXCL12 decreases lesion formation, deletion of its receptor CXCR4 in ECs promotes atherosclerosis (Döring et al. 2017, 2019). These findings suggest that the absence of endothelial CXCL12 expression, which reduces circulating CXCL12 levels, limits lipid deposition in the arterial wall, probably due to its inhibitory effect on cholesterol efflux from macrophages (Gao et al. 2019a). By contrast, local delivery of miR-126-3p in apoptotic vesicles to ECs may support endothelial function by CXCR4-mediated activation of the AKT pathway (Döring et al. 2017). Interestingly, miR-126-3p increases AKT signaling in ECs (Chen et al. 2016b; Fish et al. 2008), reduces endothelial apoptosis, and supports endothelial barrier function (Döring et al. 2017; Cheng et al. 2017a). Several miR-126-3p targets, such as RGS16, phosphatidylinositol 3-kinase regulatory subunit beta, and transforming growth factor- β may play a role in the protection of ECs from apoptosis.

Processing of the precursor miR-126 by Dicer also produces a mature miRNA from its 5p end. Although the miR-126-5p expression level is lower than that of miR-126-3p, it is still one of the most highly expressed miRNAs in ECs. Disturbed laminar flow suppresses miR-126-5p expression in dysadapted ECs and limits, thereby their capacity to compensate additional hyperlipidemia-induced injury by increased proliferation (Schober and Weber 2016). Conversely, higher miR-126-5p levels in ECs exposed to laminar flow increase their proliferative reserve compared with dysadapted ECs (Schober et al. 2014). miR-126-5p generates an endothelial proliferative reserve by suppressing delta like non-canonical notch ligand (DLK) 1, an inhibitor of the Notch1 signaling pathway (Schober et al. 2014). Derepression of DLK1 in dysadapted ECs limits endothelial regeneration in response to hyperlipidemic stress (Fig. 2). Thus, inhibition of miR-126-5p expression in ECs by disturbed flow promotes atherosclerosis (Schober et al. 2014). The inhibition of Notch1 may at least partially mediate the effects of DLK1. Accordingly, disturbed flow reduces endothelial Notch1 activation, and knockout of Notch1 in ECs increases lesion formation (Mack et al. 2017). Moreover, high shear stress reduces endothelial apoptosis by miR-126-5p-mediated inhibition of caspase 3 in the nucleus (Santovito et al. 2020). These results indicate a dual role of miR-126-5p in ECs; in

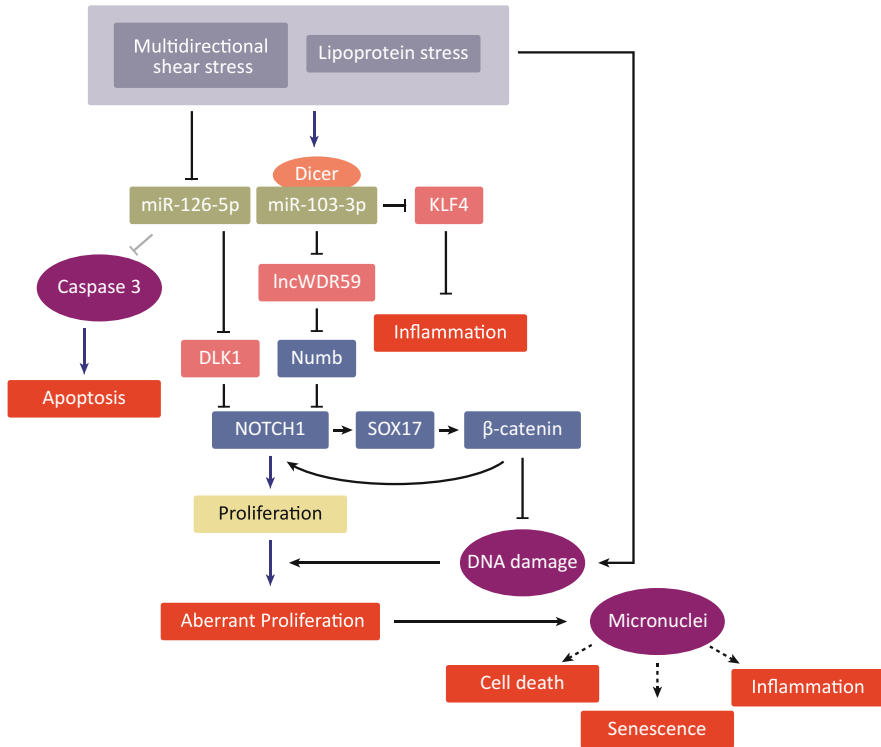


Fig. 2 miRNAs modulate endothelial dysadaptation. The protective miR-126-5p is downregulated in dysadapted ECs, which limits NOTCH1-mediated endothelial regeneration and increases EC apoptosis through a non-canonical mechanism. By contrast, Dicer produces increased levels of miR-103 in dysadapted ECs, which promotes inflammation by targeting KLF4. Moreover, miR-103 inhibits EC proliferation and promotes DNA damage, thus impairing the chronic wound healing response of dysadapted ECs

the cytoplasm, it facilitates Notch1 signaling and thus promotes endothelial regeneration; in the nucleus, it limits apoptosis through a non-canonical mechanism.

2.3 The miR-103–IncWDR59 Axis Limits Endothelial Regeneration and Promotes Aberrant Proliferation

In arteries, endothelial repair by proliferation is a delicate process because blood flow can detach mitotic ECs and increase vascular permeability (Wechezak et al. 1994). Moreover, mitotic cells are incredibly vulnerable to DNA damage because double-strand breaks cannot be repaired and may lead to errors in chromosomal segregation and the formation of micronuclei consisting of chromatin surrounded by its nuclear membrane (Blackford and Stucki 2020). Chromosomes in micronuclei accumulate DNA damage and replicate imperfectly, leading to genomic instability,

which can induce growth arrest, senescence, apoptosis, and inflammation (Blackford and Stucki 2020; Zhang et al. 2015; Shah and Bennett 2017). Atherogenic LDL triggers DNA damage in aortic ECs in a LOX-1-dependent manner, probably by increasing mitochondrial ROS production (Wang et al. 2018). Moreover, chronic inflammation may enhance DNA damage due to environmental exposures in tissues that are continuously regenerating (Kiraly et al. 2015). Thus, aberrant proliferation characterized by DNA damage and micronuclei formation may impair chronic regeneration of dysadapted arterial ECs and promote atherosclerosis. Accordingly, high-fat diet (HFD) feeding enhances micronuclei formation and DNA damage in ECs, primarily at predilection sites of atherosclerosis (Natarelli et al. 2018; Thum and Borlak 2008). This finding indicates that disturbed laminar blood flow and oxidized LDL synergistically promote genomic instability (Fig. 1).

In addition to inflammation, Dicer increases DNA damage and micronuclei formation in ECs and inhibits endothelial proliferation at predilection sites of atherosclerosis (Natarelli et al. 2018). These effects are due to the suppression of the lncRNA lncWDR59 by miR-103-3p. lncWDR59 promotes Notch1 activation and endothelial proliferation by competitive binding to the Notch1 inhibitor Numb (Natarelli et al. 2018). Moreover, Notch1 induces SOX17 expression and thus increases β -catenin activity, which inhibits oxLDL-mediated DNA damage and micronuclei formation in ECs. Therefore, the interaction between miR-103-3p and lncWDR59 promotes aberrant EC proliferation by abolishing β -catenin-mediated protection against DNA damage. Accordingly, blocking the targeting of lncWDR59 by miR-103-3p in Apoe^{-/-} mice reduces atherosclerosis and endothelial DNA damage and increases endothelial proliferation (Fig. 2). Notably, a human lncWDR59 homolog with a conserved genomic location exists. In human lesions, ECs express lncWDR59, and its expression level correlates with endothelial proliferation, indicating that the targeting of lncWDR59 by miR-103-3p also plays a role in humans (Natarelli et al. 2018).

3 Non-coding RNAs in Macrophage Function

Macrophages drive the progression of atherosclerosis from a clinically silent condition to advanced lesions with a thrombogenic core. Uncontrolled uptake of modified lipoproteins from the extracellular space by macrophages promotes their transformation into foam cells characterized by the accumulation of cholesterol esters and triglycerides in lipid droplets, similar as in adipocytes. Whereas cholesterol is removed from foam cells by ATP binding cassette subfamily A member (ABCA) 1 and ATP binding cassette subfamily G member (ABCG) 1 transporters, triglycerides and fatty acids can be degraded by foam cells and fuel mitochondrial energy production. However, the ongoing influx of lipoproteins exceeds the capacity of macrophages to store cholesterol intracellularly or transfer it to HDL, which causes cell death and the formation of cholesterol crystals. Accordingly, macrophages death by apoptosis or necroptosis increases during the progression of atherosclerosis (Lin et al. 2013). Besides, the phagocytic removal of dying foam

cells is impaired or insufficient in advanced atherosclerosis resulting in the formation of a lipid-rich, necrotic core with cholesterol crystals encapsulated by macrophages.

In contrast to arterial ECs, which have a more stable phenotype, macrophages undergo considerable phenotypic changes during inflammatory activation. Bacterial products such as lipopolysaccharide (LPS) and the T_H1 cell cytokine IFN γ activate the NF- κ B and HIF-1 α pathway and thus shift mitochondrial function from energy production to the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) to combat microbes (Cameron et al. 2019). In turn, energy production is shifted to aerobic glycolysis, which reduces ATP supply. By contrast, stimulation with IL-4 evokes a different response characterized by signal transducer and activator of transcription (STAT) 6 and peroxisome proliferator-activated receptor (PPAR) activation, which reduces nitric oxide (NO) and promotes mitochondrial ATP production by oxidative phosphorylation (OXPHOS) of fatty acids. The latter effect may support phagocytosing macrophages, which need more energy and are challenged by the influx of lipids.

3.1 Regulatory RNAs in Macrophage Energy and Lipid Metabolism

miR-10a-5p, miR-146a-5p, and let-7 family members, and miR-21a-5p are among the most abundant miRNAs in murine macrophages (Canfran-Duque et al. 2017). Dicer knockout reduces the expression of a great majority of miRNAs in macrophages, including miR-21a-5p, miR-342-5p, miR-10a-5p, and miR-503-5p (Wei et al. 2018). In contrast to ECs, knockout of Dicer in macrophages increases the development of atherosclerosis, macrophage apoptosis, and the expression of NOS2 and inflammatory cytokines, such as IL-1 β (Wei et al. 2018). Moreover, Dicer knockout impairs OXPHOS in IL-4 stimulated but not in unstimulated or LPS/IFN- γ -stimulated macrophages, suggesting a central role of miRNAs in the energy metabolism of IL-4-stimulated, anti-inflammatory macrophages (Wei et al. 2018). Notably, Dicer also increases oxygen consumption and mitochondrial oxidation in macrophage-derived foam cells, and thus limits the accumulation of lipids probably by the oxidation of fatty acids (Wei et al. 2018). Ligand-dependent corepressor (LCOR) is a crucial target of miRNAs in macrophages and contains numerous highly conserved miRNA-binding sites in its 3'-UTR. LCOR inhibits retinoid X receptor alpha (RXRA), which promotes the expression of OXPHOS-related genes and increases mitochondrial function through interaction with peroxisome proliferator-activated receptor-gamma coactivator (PGC)-1 (Chae et al. 2013). Among the miRNAs predicted to target LCOR, miR-10a-5p most strongly promoted OXPHOS in IL-4-stimulated macrophages and foam cells through targeting LCOR (Wei et al. 2018). Moreover, miR-10a-5p increases OXPHOS also by interacting with another corepressor of nuclear receptors, nuclear receptor corepressor 2 (NCOR2), which inhibits the activity of the PPAR α (Wei et al. 2018). Blocking the interaction between miR-10a-5p and LCOR in macrophages by target site-specific antisense oligonucleotides increases atherosclerosis in mice, suggesting

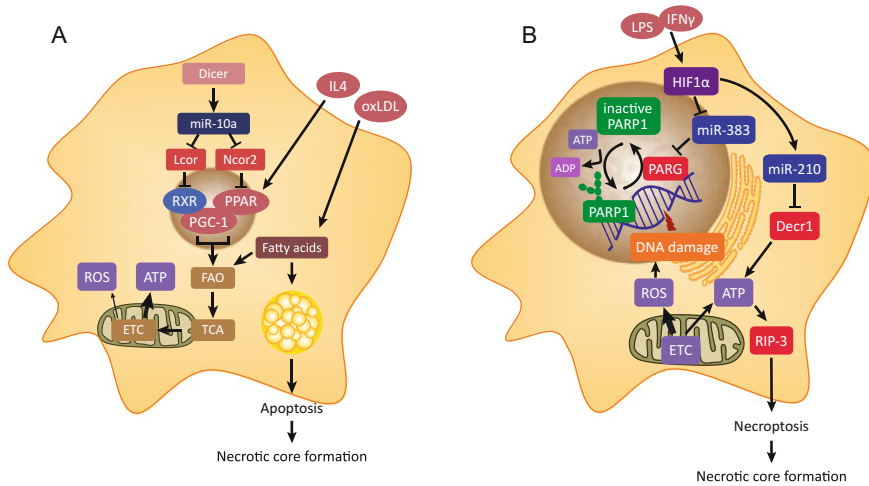


Fig. 3 Effects of miRNAs in macrophages on energy metabolism and cell death. **(a)** In anti-inflammatory macrophages, Dicer promotes mitochondrial energy production by generating miR-10a, which targets the corepressors of nuclear receptors, such as LCOR and NCOR2. miR-10a improves fatty acid oxidation (FAO) and reduces foam cell formation, thus enhancing cell survival. **(b)** In inflammatory macrophages, HIF-1 α activation switches the mitochondrial metabolism from ATP to ROS production, thus generating a pseudo-hypoxic state. This effect of HIF-1 α is mediated by the suppression of Decr1 by miR-210, which reduces ATP synthesis and necroptotic cell death. Moreover, HIF-1 α activation lowers cellular ATP levels by hyperactivating PARP-1 due to the derepression of the miR-383 target PARG. ETC, electron transport chain; TCA, tri-carboxyl acid

that miRNA-mediated OXPHOS in macrophages protects from atherosclerosis (Wei et al. 2018). Notably, RXR α agonists upregulate miR-10a-5p in ECs, indicating a positive forward loop in which RXR α increases its activity by miR-10a-5p-mediated suppression of LCOR (Lee et al. 2018). Moreover, treatment of *ApoE*^{-/-} mice with miR-10a-5p mimics reduces atherosclerosis (Lee et al. 2018). Besides let-7b-5p, but not other members of the let-7 family increases mitochondrial function, indicating that the functional role between the members of a miRNA family can differ despite having the same seed sequence (Fig. 3).

Hypoxia-inducible factor 1 α (HIF-1 α) is an essential transcription factor in the regulation of mitochondrial energy metabolism in inflammatory macrophages. Stimulation of macrophages with LPS and IFN γ activates HIF-1 α , which generates a pseudo-hypoxic state by shutting down OXPHOS and increasing ROS and RNS production. Although this metabolic switch aids to fight off infections, the concomitantly reduced energy supply and enhanced exposure to oxidizing agents can lead to macrophage necroptosis (Pajuelo et al. 2018; He et al. 2011). In atherosclerotic lesions, HIF-1 α activation increases receptor-interacting serine/threonine kinase 3 (RIP3)-mediated macrophage necroptosis and necrotic core formation (Lin et al. 2013; Karshovska et al. 2020). This effect is probably mediated by HIF-1 α -induced

upregulation of miR-210, which lowers ATP levels by suppressing OXPHOS and increasing ROS through targeting 2,4-dienoyl-CoA reductase1 (*Decr1*) (Karshovska et al. 2020). *Decr1* is an essential enzyme in the β -oxidation of unsaturated fatty acids and in the adaptation to metabolic stress during fasting (Miinalainen et al. 2009). Thus, β -oxidation of unsaturated fatty acids may be critical for the survival of energy-depleted inflammatory macrophages by maintaining a basal level of oxidative ATP production. miR-210 is highly abundant in human atherosclerosis, and HIF-1 α activation in macrophages increases lesional miR-210 expression in *ApoE*^{-/-} mice (Karshovska et al. 2020; Raitoharju et al. 2011). However, the functional role of miR-210 in atherosclerosis has not yet been studied.

Similar to *LCOR*, the 3'-UTR of the *ABCA1* transcript contains numerous conserved binding sites for miRNAs, indicating a significant role of miRNAs in foam cell formation, efferocytosis, and inflammatory activation by regulating *ABCA1* expression (Zannis et al. 2006; Wei and Schober 2016). In addition to the transcriptional regulation by the nuclear sterol-activated liver-X-receptors (LXRs), post-transcriptional targeting of the *ABCA1* transcript by at least 15 miRNAs, such as miR-33-5p, miR-302a-3p, and miR-23a-3p, has been experimentally confirmed (Yang et al. 2018; Rayner et al. 2010; Meiler et al. 2015).

miR-33a-5p (only miR-33-5p in mice) is co-transcribed from an intronic region of the transcription factor sterol regulatory element-binding protein-2, and both synergistically promote cellular cholesterol accumulation in macrophages (Schober and Weber 2016). miR-33-5p is one of the most extensively studied miRNA in atherosclerosis, initially because blocking miR-33-5p raised HDL levels, an effect that is likely due to the targeting of *ABCA1* in the liver. The role of miR-33-5p in atherosclerosis has been controversially discussed. Studies in genetically modified mice show conflicting results. In *ApoE*^{-/-} mice, the whole-body knockout of the *Mir33* gene reduces atherosclerosis, whereas the absence of miR-33-5p expression in bone marrow cells does not alter lesion formation (Horie et al. 2012). However, in *Ldlr*^{-/-} mice, only deletion of *Mir33* in bone marrow cells slightly reduces lesion formation, whereas the whole-body knockout of *Mir33* surprisingly does not affect atherosclerosis (Price et al. 2017). These results indicate that the expression of miR-33-5p in bone marrow cells promotes atherosclerosis in *Ldlr*^{-/-} mice, whereas its expression in non-bone-marrow cells is athero-protective. Interestingly, *Ldlr*^{-/-} mice with bone marrow cells that harbor a mutation of the miR-33-5p binding site in the *ABCA1* 3'-UTR develop less atherosclerosis, similar to the effect of the *Mir33* knockout in bone marrow cells (Price et al. 2019). Although this mechanism can explain not all impacts of miR-33-5p, targeting of *ABCA1* in macrophages appears to play a crucial role in atherogenesis in *Ldlr*^{-/-} mice.

miR-34a-5p is highly abundant in atherosclerotic lesions and targets *ABCA1* and *ABCG1* in macrophages (Xu et al. 2020). Interestingly, the *ABCA1* and *ABCG1* binding sites of miR-34a-5p are not conserved between human and mouse, and only the site in the human *ABCA1* 3'-UTR is canonical (Xu et al. 2020). This finding suggests that non-canonical sites are functional and that miRNA sites can have the same effect in mouse and human despite the lack of a conserved binding site. Consistently, the conditional knockout of *Mir34a* in myeloid cells and the knockout

of *Mir34a* in bone marrow cells reduces atherosclerosis in *Apoe*^{-/-} and *Ldlr*^{-/-} mice, respectively (Xu et al. 2020). This effect has been attributed to improved cholesterol efflux from macrophages due to increased expression of ABCA1, ABCG1, and LXR α .

Notably, several ABCA1-targeting miRNAs promote lesion formation in atherosclerotic mouse models, such as miR-302a-3p (Meiler et al. 2015), miR-23a-5p (Yang et al. 2018), miR-17-5p (Tan et al. 2019), miR-20a/b-5p (Liang et al. 2017), and miR-19b-5p (Lv et al. 2014). However, it is unclear whether the effects of these miRNAs on lesion formation are due to ABCA1 suppression.

LXR activation induces not only ABCA1 expression but also upregulates several lncRNAs, which further increase ABCA1 levels. For example, the primate-specific lncRNA cholesterol homeostasis regulator of miRNA expression (*CHROME*) is upregulated in atherosclerotic lesions and derepresses ABCA1 expression by inactivating several ABCA1-targeting miRNAs, such as miR-27b, miR-33a/b, and miR-128 (Hennessy et al. 2019). Another LXR-induced lncRNA is macrophage-expressed LXR-induced sequence (MeXis), which is transcribed from a gene located near the ABCA1 gene (Sallam et al. 2018). MeXis acts as a nuclear RNA that influences chromatin architecture and facilitates the accessibility of the nuclear receptor coactivator DDX17 at the ABCA1 locus, thus enhancing LXR-mediated ABCA1 expression in response to cholesterol loading in macrophages (Sallam et al. 2018). Knockout of MeXis in bone marrow cells reduces atherosclerosis and increases ABCA1 expression in *Ldlr*^{-/-} mice (Sallam et al. 2018). Notably, a genomic region surrounding the MeXis/ABCA1 locus revealed some degree of conservation between species. In humans, a non-coding RNA transcript in this region was identified as TCONS00016111 with some sequence conservation with MeXis (Sallam et al. 2018). However, its role in lipid metabolism in human lesional macrophages is not fully understood.

3.2 miR-155-5p and miR-146a/b: The LPS Mediators in Atherosclerosis

miR-155-5p is a highly conserved miRNA and predominantly expressed in hematopoietic cells, such as lymphocytes and macrophages. The precursor miR-155 hairpin is encoded in the third exon of a lncRNA called MIR155HG. The expression of MIR155HG is driven by a promoter that is strongly activated by NF- κ B (Schober and Weber 2016; Thompson et al. 2013). Thereby, LPS selectively induces miR-155-5p in macrophages together with a small number of other miRNAs, such as miR-147-5p and miR-210-5p (Androulidaki et al. 2009; Nazari-Jahantigh et al. 2012; Dueck et al. 2014), and miR-155-5p mediates many pro-inflammatory effects of LPS by targeting inhibitors of NF- κ B signaling, such as suppressor of cytokine signaling 1 and brain and muscle ARNTL-like 1 (Curtis et al. 2015; Wang et al. 2016). Interestingly, low amounts of LPS continuously leak from the intestine to the blood, enhanced by an HFD, where LPS is inactivated by binding

to lipoproteins, such as LDL and VLDL. However, chemical modification of LDL leads to reactivation of LPS (Zhu et al. 2017) and upregulation of miR-155-5p in macrophages via toll-like receptor 4 (TLR4) (Du et al. 2014). Accordingly, LPS is detectable in human atherosclerotic lesions, and patients with carotid stenosis have higher blood levels of LPS (Carnevale et al. 2018). Moreover, miR-342-5p, which is abundantly expressed in atherosclerotic lesions, promotes LPS-induced inflammatory macrophage activation by targeting Akt1 and thus upregulates miR-155-5p expression (Wei et al. 2013) (Fig. 4). Blocking miR-342-5p in *ApoE*^{-/-} mice reduces atherosclerosis and miR-155-5p expression in atherosclerotic vessels (Wei et al.

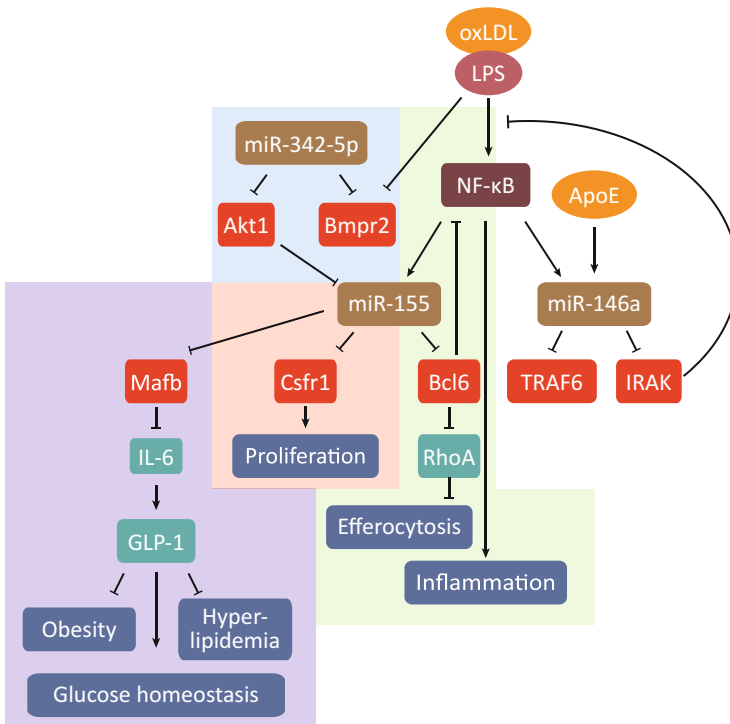


Fig. 4 The complex roles of the LPS mediators miR-155 and miR-146a in atherosclerosis. The stimulation of macrophages with LPS upregulates miR-155 and miR-146a via NF-κB activation. Whereas miR-155 promotes the NF-κB-mediated inflammatory response in macrophages by targeting Bcl6, miR-146a suppresses mediators of LPS signaling and NF-κB activation. MiR-155 increases advanced atherosclerosis by targeting Bcl6, which drives inflammation and inhibits efferocytosis, thus resulting in necrotic core formation in *ApoE*^{-/-} mice (green). In early atherosclerosis, however, miR-155 decreases lesion growth by blocking macrophage proliferation (pink). Moreover, miR-155 improves glucose homeostasis and reduces atherosclerosis in obese *Ldlr*^{-/-} mice by targeting *Mafb* in pancreatic β-cells (violet). The effect of miR-146a in macrophages on atherosclerosis is less clear. Although treatment with miR-146a mimics reduces atherosclerosis and macrophage activation, the anti-inflammatory role of miR-146a in ECs may also protect from lesion formation

2013). The expression level of miR-342-5p and inflammatory cytokines is increased in peripheral leukocytes from patients with coronary artery disease (Ahmadi et al. 2018). However, the effect of miR-155-5p on macrophages during lesion formations differs considerably between early and advanced lesions (Nazari-Jahantigh et al. 2012; Wei et al. 2015). Whereas miR-155-5p expression in early atherosclerosis limits lesion formation by reducing macrophage proliferation, the formation of advanced lesions is promoted by miR-155-5p through inflammation-induced impairment of efferocytosis (Wei et al. 2015). The latter effect is mediated by the targeting of *B cell leukemia/lymphoma 6* (BCL6), a transcriptional repressor of NF- κ B-induced genes, which improves efferocytosis through inhibition of *Ras homolog family member A* (RhoA) (Fig. 4) (Wei et al. 2015).

In contrast to its role in *ApoE*^{-/-} mice, miR-155-5p limits advanced atherosclerosis in *Ldlr*^{-/-} mice (Zhu et al. 2017). This effect is due to the upregulation of miR-155-5p in pancreatic β -cells, which promotes insulin secretion by targeting v-maf musculoaponeurotic fibrosarcoma oncogene family, protein B (MafB). The suppression of MafB by miR-155-5p increases IL-6 secretion from β -cells, which stimulates glucagon-like peptide (GLP)-1 expression in α -cells (Fig. 4). In hyperlipidemic *Ldlr*^{-/-} mice, *Mir155* knockout increases obesity, adipose tissue inflammation, blood lipid levels, and blood glucose levels (Zhu et al. 2017). GLP-1 inhibits glucagon production and increases insulin secretion, which may explain how miR-155-5p in β -cells improves the metabolic status of obese and hyperlipidemic mice. Moreover, miR-155-5p expression increases GLP-1 plasma levels, which may limit vascular inflammation and atherosclerosis (Zhu et al. 2017; Rakipovski et al. 2018). Hyperlipidemia stimulates miR-155-5p expression in β -cells probably due to HFD-induced endotoxemia because LPS bound to oxidized LDL is deposited in pancreatic islets and oxidation of LDL reactivates LPS (Zhu et al. 2017). *ApoE*^{-/-} mice are more sensitive to the pro-inflammatory effects of LPS than *Ldlr*^{-/-} mice (Ali et al. 2005). Thus, the pro-atherogenic role of miR-155-5p in macrophages may predominate in *ApoE*^{-/-} mice (Schreyer et al. 2002, 2003), whereas metabolic effects of miR-155-5p related to glucose hemostasis, obesity, and adipose tissue inflammation are more critical for the development of atherosclerosis in *Ldlr*^{-/-} mice. Thus, hyperlipidemia improves glucose metabolism and adipose tissue inflammation by LPS-mediated upregulation of miR-155-5p in β -cells. This mechanism may explain why patients with familial hypercholesterolemia have a reduced risk for type 2 diabetes (Besseling et al. 2015).

Besides, exosomes transfer miR-155-5p from hematopoietic cells, such as dendritic cells and neutrophils, and smooth muscle cells to other cell types like ECs (Gomez et al. 2020; Zheng et al. 2017). The uptake of exosomal miR-155-5p sensitizes the recipient cells to LPS-induced inflammatory activation by suppressing its target genes (Alexander et al. 2015). In neutrophils, HFD increases the packaging of miR-155-5p into microvesicles and stimulates their release into the circulation, where they are preferentially taken up by ECs exposed to disturbed blood flow (Gomez et al. 2020). The uptake of the neutrophil-derived microvesicles by ECs promotes their inflammatory activation by miR-155-5p-mediated suppression of BCL6 and increases atherosclerosis (Gomez et al. 2020). Thus, the exosomal

transfer of miR-155-5p from neutrophils is essential for the pro-atherogenic role of this miRNA in ECs (Gomez et al. 2020) because the endogenous level of miR-155-5p in ECs is not high enough to drive lesion formation in *ApoE*^{-/-} mice (Wei et al. 2015).

In macrophages, NF- κ B activation by LPS also induces the expression of miR-146a-5p, which antagonizes the pro-inflammatory effects of miR-155-5p by targeting components of the TLR4 signaling pathway, such as *interleukin 1 receptor-associated kinase* (IRAK) and *TNF receptor-associated factor 6* (TRAF6) (Fig. 4) (Mann et al. 2017; Su et al. 2020). Moreover, constitutive NF- κ B activation in *Mir146a* knockout mice drives the development of myeloid malignancies (Zhao et al. 2011). Notably, increased levels of miR-155-5p in macrophages mediate the chronic inflammatory response in *Mir146a*^{-/-} mice by hyper activating NF- κ B (Mann et al. 2017). Treatment of *Ldlr*^{-/-} mice with miR-146a mimics reduces atherosclerosis and inflammatory macrophage accumulation (Li et al. 2015). Notably, the anti-inflammatory effects of ApoE are partially mediated by the upregulation of miR-146a (Li et al. 2015). However, the atherosclerosis phenotype of HFD-fed *Mir146a*^{-/-}/*Ldlr*^{-/-} mice, which develop bone marrow failure and splenomegaly, is unexpected. Although *Mir146a* knockout increases inflammatory cytokine levels in the blood, it decreases lipid levels and the progression of advanced atherosclerosis (Cheng et al. 2017b). This effect was due to the lack of miR-146a expression in bone marrow cells, probably because it leads to bone marrow failure and reduced production of pro-atherogenic hematopoietic cells (Cheng et al. 2017b). By contrast, *Mir146a* knockout in *Ldlr*^{-/-} mice harboring *Mir146a* wildtype bone marrow cells develop increased atherosclerotic lesions. These mice are also more sensitive to arterial inflammation, which may be due to increased NF- κ B activation in ECs (Cheng et al. 2013, 2017b). Although another study using the same mouse model also found lower blood lipid levels, it did not detect an effect of *Mir146a* knockout in bone marrow cells on the development of atherosclerosis (Del Monte et al. 2018).

miR-146b is the second member of the miR-146 seed family and differs from miR-146a in two nucleotides at the 3' end. Similar to miR-146a, miR-146b inhibits NF- κ B activation and the TLR4 pathway (Taganov et al. 2006). *Mir146b*^{-/-} knockout mice also develop hematologic malignancies and splenomegaly, although less frequently than *Mir146a*^{-/-} mice (Mitsumura et al. 2018). It remains unclear whether miR-146b expression, which is not affected by *Mir146a* knockout because it is transcribed from a different genomic locus, can compensate for the lack of miR-146a expression. Notably, murine macrophages stimulated with IL-4 release exosomes that contain preferentially miR-146b together with miR-99a and miR-378a (Bouchareychas et al. 2020). The transfer of each of these miRNAs to LPS-stimulated macrophages reduces NF- κ B activation. Moreover, treatment with exosomes from IL-4-treated macrophages reduces inflammation in atherosclerotic lesions but not lesion size (Bouchareychas et al. 2020).

3.3 Regulatory RNAs Control Macrophage Death and Efferocytosis in Necrotic Core Formation

Necrotic core formation results from the disbalance between cell death and efferocytosis. As reported in the previous chapter, miR-155-5p establishes an essential link between inflammatory activation and impaired efferocytosis during the progression of atherosclerosis, which promotes the formation of a necrotic core (Wei et al. 2015). Besides miR-155-5p, miR-21a-5p, one of the most abundant miRNAs in atherosclerotic lesions, has been implicated in necrotic core formation. miR-21 is an oncomiR that inhibits apoptosis by targeting, e.g., programmed cell death protein 4 (Krichevsky and Gabriely 2009; Hatley et al. 2010). Accordingly, knockout of *Mir21a* in bone marrow cells promotes lesional apoptosis, necrotic core formation, and atherosclerosis in HFD-fed *Ldlr*^{-/-} mice, but it reduces lesions in the aortic sinus in *ApoE*^{-/-} mice fed a regular diet (Canfran-Duque et al. 2017; Chipont et al. 2019). Whole-body *Mir21a* knockout also reduces atherosclerotic lesions, although not in every arterial region studied, in *ApoE*^{-/-} mice fed an HFD and in aged *ApoE*^{-/-} mice fed a regular diet (Chipont et al. 2019; Gao et al. 2019b). Chipont et al. reported the conflicting finding that the inhibition of lesion formation by *Mir21a* knockout is associated with increased apoptosis of lesional macrophages and impaired efferocytosis in the peritoneum (Chipont et al. 2019). Another puzzling finding in this regard is that Jin et al. described increased lesion size by *Mir21a* knockout in *ApoE*^{-/-} mice fed a regular diet (Jin et al. 2018). Unfortunately, the mechanisms of these *Mir21a* effects are incompletely understood.

As discussed in the section “Regulatory RNAs in macrophage energy and lipid metabolism,” HIF-1 α activation in macrophages drives necrotic core formation in part by inducing miR-210, which inhibits OXPHOS and promotes necroptosis. Besides, HIF-1 α activation lowers ATP levels in macrophages by suppressing miR-383-5p expression (Karshovska et al. 2020). miR-383-5p limits ATP consumption by targeting poly(ADP-ribose) glycohydrolase (PARG) (Karshovska et al. 2020), which removes poly(ADP-ribose) polymers from DNA strand breaks and thus antagonizes the ATP-dependent role of poly(ADP-ribose) polymerase (PARP)-1 in response to RNS-induced DNA damage (Ying et al. 2001). The increased activity of PARG following HIF-1 α -mediated suppression of miR-383-5p leads to hyperactivation of PARP-1, and thus to ATP depletion and RIP3-mediated necroptosis (Karshovska et al. 2020). Blocking the interaction between miR-383-5p and PARG in mice reversed the protective effect of *Hif1a* knockout in myeloid cells on the lesion and necrotic core formation, demonstrating the critical role of miR-383-5p in HIF-1 α -mediated lesional macrophage necroptosis (Karshovska et al. 2020).

A genome-wide screen for SNPs associated with myocardial infarction discovered the lncRNA MIAT. Six SNPs in the MIAT gene are strongly associated with myocardial infarction, indicating that they contribute to the genetic risk of cardiovascular disease (Ishii et al. 2006). MIAT expression is upregulated in atherosclerotic lesions, and adenoviral knockdown of MIAT in *ApoE*^{-/-} mice reduced advanced atherosclerosis, necrotic core formation, and efferocytosis (Ye et al.

2019). Notably, MIAT impairs the phagocytic activity of macrophages by binding to miR-149-5p in the RISC, thus upregulating the anti-phagocytic receptor CD47, a target of miR-149-5p (Ye et al. 2019; Kojima et al. 2016).

4 MALAT1: The Master miRNA Sponge

The evolutionary conserved lncRNA MALAT1 (Johnsson et al. 2014) is transcribed from a single exon into a 7-kb long RNA molecule, which adopts a complex secondary structure, including a triple helix and a t-RNA-like cloverleaf at the 3' end (McCown et al. 2019). The triple helix structure is conserved and increases the stability of the poly(A) tail-lacking MALAT1 lncRNA. Cleavage by RNase P releases the cloverleaf structure from the 3' end, which is called *Malat1-associated small cytoplasmic RNA* (mascRNA). In contrast to the processed MALAT1, which is predominantly retained in the nuclear speckles, mascRNA is transferred into the cytoplasm.

The term “miRNA sponge” fits no other lncRNA better than to MALAT1, which contains an extensively high number of canonical miRNA binding sites (Plotnikova et al. 2019). Several studies have shown that MALAT1 is associated with Ago2 in the RISC, where miRNAs, such as miR-22-3p and miR-155-5p, target MALAT1 (Cao et al. 2016; Tang et al. 2015). Surprisingly, the interaction with MALAT1 also reduces the expression of the targeting miRNA (Tang et al. 2015). The functional interaction with more than 50 miRNAs has been established experimentally in various cell types. In ECs, several MALAT1-binding miRNAs have been described, such as miR-19b-3p, miR-216-5p, miR-181b, and miR-155 (Liu et al. 2020; Wang et al. 2019a; Li et al. 2018).

In macrophages, LPS stimulation increases MALAT1 expression in an NF- κ B-dependent manner, whereas IL-4 downregulates MALAT1 (Cui et al. 2019; Zhao et al. 2016). In mice, MALAT1 promotes LPS-induced inflammatory activation of macrophages by upregulating C-type lectin domain family 16, member A, indicating a pro-inflammatory role of MALAT1 (Cui et al. 2019). Moreover, MALAT1 inhibits IL-4-induced anti-inflammatory and pro-fibrotic macrophage polarization by glucose-dependent OXPHOS (Cui et al. 2019). LPS also induces MALAT1 via activating NF- κ B in human THP-1 macrophages. However, in contrast to murine macrophages, MALAT1 reduces inflammatory cytokine expression in THP-1 cells by binding NF- κ B in the nucleus (Zhao et al. 2016).

In patients with atherosclerosis, MALAT1 expression is reduced in lesions (Cremer et al. 2019; Arslan et al. 2017) and increased in the circulation (Wang et al. 2019a; Zhu et al. 2019). By contrast, lesional *Malat1* expression is upregulated in *ApoE*^{-/-} mice (Li et al. 2019). Treatment with an antagomir against *Malat1* for 4 weeks reduces lesion size by almost 50% in *ApoE*^{-/-} mice fed an HFD for 12 weeks (Zhu et al. 2019). This study indicates that *Malat1* promotes atherosclerosis in mice. However, the effect of *Malat1* knockout on atherosclerosis is quite different. Notably, none of the three existing strains of *Malat1*^{-/-} mice has an obvious phenotype (Eissmann et al. 2012; Nakagawa et al. 2012; Zhang et al.

2012). Unexpectedly, crossing *Malat1(LacZ)*^{-/-} mice, generated by insertion of the LacZ gene into the promoter region of *Malat1*, with *Apoe*^{-/-} mice resulted in intrauterine death, whereas *Apoe*^{-/-} mice heterozygous for *Malat1(LacZ)* develop normally (Gast et al. 2019). Interestingly, severe atherosclerosis develops in 8-week-old *Malat1(LacZ)*^{+/-}/*Apoe*^{-/-} mice even without feeding them an HFD (Gast et al. 2019), which is probably related to the hyperinflammatory state of these mice (Gast et al. 2019). Whether this effect is due to lower mascRNA levels, which promotes inflammatory cytokine expression in THP-1 cells (Gast et al. 2019), remains unclear. By contrast, *Malat1*^{-/-} mice produced by Cre-mediated excision of the whole *Malat1* gene could be crossed with *Apoe*^{-/-} mice without having a spontaneous phenotype (Cremer et al. 2019). Moreover, feeding these mice an HFD with only a comparably small amount of cholesterol for 12 weeks did not change lesion size, but slightly increased lesional leukocyte accumulation (Cremer et al. 2019). Besides, *Malat1* knockout in bone marrow cells slightly increased lesion size, and the production of inflammatory leukocytes in *Apoe*^{-/-} after 16 weeks of HFD feeding, probably due to the loss of miR-503-5p sponging by *Malat1* in macrophages (Cremer et al. 2019; Yan et al. 2017). Unfortunately, it is not clear whether also non-irradiated *Malat1*^{-/-}/*Apoe*^{-/-} mice show the same changes in the production of inflammatory leukocytes as irradiated mice. The expression of numerous miRNAs is increased in *Malat1(LacZ)*^{+/-}/*Apoe*^{-/-} mice; however, not that of miR-503-5p (Gast et al. 2019). Taken together, studies in mice with a deletion of the *Malat1* gene indicate a protective role of *Malat1* in macrophages, although the extent of its effect differs considerably between the models. A significant difference between the two *Malat1*^{-/-} mouse strains consists in the regulation of NEAT1, a lncRNA transcribed from a neighboring gene of MALAT1. In *Malat1*^{-/-}/*Apoe*^{-/-} mice, NEAT1 is upregulated in lung tissue (Cremer et al. 2019), whereas *Malat1(LacZ)*^{+/-}/*Apoe*^{-/-} mice express lower levels of NEAT1 in splenocytes than *Malat1* wildtype mice (Gast et al. 2019). NEAT1 enhances oxidized LDL-induced inflammatory activation and foam cell formation in human and mouse macrophage cell lines by the sponging of miRNAs, such as miR-342-3p and miR-128, and by the formation of paraspeckles (Chen et al. 2018; Huang-Fu et al. 2018; Wang et al. 2019b).

5 The Simian lncRNA ANRIL and the Risk Locus for Atherosclerosis

The chromosome 9p21.3 in humans represents a risk locus for many different diseases, including atherosclerosis and coronary artery disease (CAD) (Chen et al. 2014). The 58 kb-long CAD risk region includes 59 single nucleotide polymorphisms and is devoid of protein-coding genes, but encodes the 3' end of the lncRNA ANRIL. Therefore, changes in one of the various ANRIL transcripts by the risk haplotype have been linked to atherosclerosis (Lo Sardo et al. 2018). During rodent evolution, ANRIL was gradually lost and is absent in mouse and rat (He et al. 2013). However, mice have a syntenic region on chromosome 4 ortholog to the 9p21.3 locus, which encodes a lncRNA that is different from ANRIL (Visel et al.

2010). Knockout of the murine 9p21.3 ortholog in *ApoE*^{-/-} mice increases atherosclerosis and lesion calcification but not lesion vulnerability (Kojima et al. 2020). Remarkably, the histological changes in mice were matched with those determined in human lesions from patients carrying the 9p21 risk allele, indicating that it promotes atherosclerosis by lesion calcification independent of ANRIL (Kojima et al. 2020).

6 Conclusions and Therapeutic Perspectives

The world of regulatory RNAs in atherosclerosis has expanded considerably by the combined effects of miRNAs and lncRNAs. Especially the small number of conserved lncRNAs plays an essential role in regulating miRNA activities in atherosclerosis. However, more details about the mechanisms of miRNA sponging and the mutual regulation of miRNAs and lncRNAs are needed. Besides, the fact that the great majority of lncRNAs is species-specific raises the possibility that the often-claimed lack of transferability of mouse data into humans is, at least partly, related to lncRNAs. Whether the conservation of lncRNAs goes beyond pure sequence similarities and could be related to structural conservation or conservation of the genomic locus is unclear. Besides, we think it is also essential to reconsider the conservation of miRNA functions against this background because it might not always be possible to predict that a miRNA in mice and humans only from the sequence conservation of its binding site.

Beyond the species-specific differences of regulatory RNA functions, the chronic course of atherosclerosis passing through various, considerably different stages, which may all be present in one patient simultaneously, is a significant obstacle for any kind of drug therapy of this disease. Therefore, it is essential to identify the stage(s) of atherosclerosis that is/are most suitable for therapeutic interventions and ideally develop a transient treatment with long-term beneficial effects avoiding continuous drug application. According to the regulatory RNA functions described in this article, miRNAs tightly control two critical processes in the pathogenesis of atherosclerosis, which can be targeted by miRNA-based therapies: endothelial dysadaptation and necrotic core formation. In a preventive approach, miRNA-based drugs could reprogram dysadapted ECs to develop an arterial endothelial phenotype without the correct hemodynamic stimulus. By contrast, limiting necrotic core formation and plaque rupture by miRNA-based drugs would make it necessary to define and identify lesions where cell apoptosis begins to exceed the lesional efferocytosis capacity. Although different strategies of miRNA-based therapies are currently tested (Roberts et al. 2020; Herrera et al. 2018), it remains unclear how promising the application of drugs comprising miRNA duplexes and oligonucleotide-based competitive miRNA inhibitors is. One miRNA, such as miR-155, may have opposing effects in different atherosclerosis stages through distinct targets. Thus, tailored miRNA inhibitors, such as target site blockers, which specifically block the interaction of a miRNA with only one of its targets, maybe an alternative approach to reduce unwanted effects. Moreover, more specific

targeting of individual miRNA interactions may enhance efficacy and require lower doses of therapeutic oligonucleotides, thus improving safety.

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Lipidomics in Biomarker Research

Thorsten Hornemann

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Abstract

Lipids are natural substances found in all living organisms and involved in many biological functions. Imbalances in the lipid metabolism are linked to various diseases such as obesity, diabetes, or cardiovascular disease. Lipids comprise thousands of chemically distinct species making them a challenge to analyze because of their great structural diversity.

Thanks to the technological improvements in the fields of chromatography, high-resolution mass spectrometry, and bioinformatics over the last years, it is now possible to perform global lipidomics analyses, allowing the concomitant detection, identification, and relative quantification of hundreds of lipid species. This review shall provide an insight into a general lipidomics workflow and its application in metabolic biomarker research.

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Keywords

Biomarkers · Data analysis · Dyslipidemia · LC/MS · Lipids · Metabolic disease · Metabolites

1 Introduction

Metabolic diseases can present long before becoming clinically apparent. Early predictors of metabolic disease are of particular importance since a delay or prevention of morbidity is possible via pharmacological and behavioral interventions. Thus, biomarkers are essential tools to select patients for appropriate treatment schemes, optimally providing the right treatment to the right patient at the right time.

Cardiovascular diseases (CVDs) and associated mortality have a high prevalence in western societies. For coronary artery disease (CAD), the average annual mortality ranges between 1 and 3% (for fatal and non-fatal myocardial infarctions) and remains a clinical challenge (Morrow 2010). In patients who survived an acute event of a coronary syndrome (ACS), the rate of myocardial infarction and death is markedly increased (Hamm et al. 2012). However, at the individual patient level, the risk may vary considerably and therefore risk estimation tools are needed to better manage such patients. A better risk stratification would also help to identify individuals at risk who require interventions that are more intensive. Conversely, it is equally important to identify patients with a good prognosis, to avoid unnecessary procedures or aggressive drug treatments with associated side effects. Concentrations of cholesterol in total plasma (TC), low density lipoproteins (LDL-C), and high density lipoproteins (HDL-C) as well as triglycerides (TG) have been used for risk prediction. LDL-C has become the main therapeutic target in the management of patients with CAD. However, a number of studies have failed to show any association between LDL-C and outcomes in large series of CAD patients (Puri et al. 2013). There is a clinical need for additional risk markers in CVD as well as a better understanding on how lipids relate with established metabolic risk factors to evaluate their potential as clinical biomarkers.

2 Biochemistry of Lipids

Biological systems are comprised of thousands of chemically distinct lipids. The structural diversity of lipids confers a broad spectrum of functionality. For most lipids, their functions depend on their molecular structure and can be very different for the different lipid classes as well as for different lipid species within the same lipid class (Stahlman et al. 2012). Lipids are found in all living organisms. They are involved in many critical cellular functions such as energy storage, structural plasma membrane integrity, and cell signaling. Imbalances of lipid metabolism are linked to the pathology of various diseases such as diabetes, Alzheimer's, obesity, cancer, and atherosclerosis (Cavojsky et al. 2016; Jung and Choi 2014; Steinberg 2006; Tan et al. 2017; Watson 2006; Wenk 2005). While routine plasma lipid analysis precedes

prescription of lipid-lowering drugs (Quehenberger et al. 2010), the abundance of particular lipid species may be indicative of a specific disease (Quehenberger and Dennis 2011). Abnormal concentrations of lipids are observed in various metabolic disorders.

Moreover, many inborn errors of metabolism are related to alterations in the metabolism of lipids, and particularly that of sphingolipids. Sphingolipidoses are monogenic inherited diseases caused by defects in the sphingolipid degradation pathways (Kolter and Sandhoff 2006; Sandhoff and Harzer 2013), leading to a massive storage of undegraded sphingolipid species in the lysosomes, causing neuroinflammation and neurodegeneration. Sphingolipids also emerged over the last years as significant factors in the pathogenesis of cardiometabolic diseases (Cowart 2009; Summers 2006; Deevska and Nikolova-Karakashian 2011).

Lipids show a large structural diversity that is comprised in the term “lipidome.” According to the comprehensive classification system proposed by the LIPID MAPS consortium (<http://www.lipidmaps.org>), lipids can be classified into eight different classes (Fig. 1): glycerophospholipids (GP), sphingolipids (SL), glycerolipids, sterol lipids, free fatty acids, prenol lipids, saccharolipids, and polyketides (Fahy et al. 2005; Fahy et al. 2009).

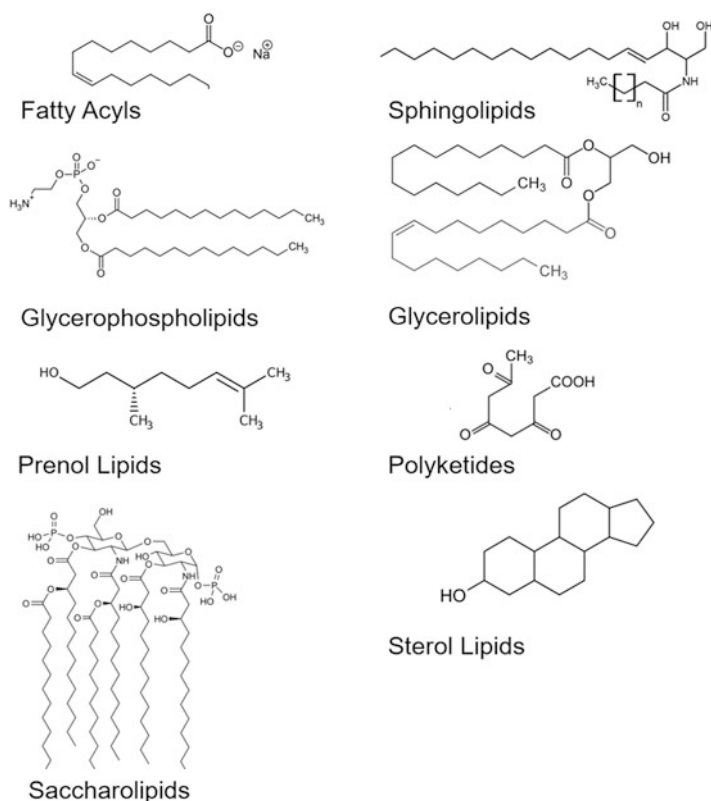


Fig. 1 Structures of the most abundant lipid families

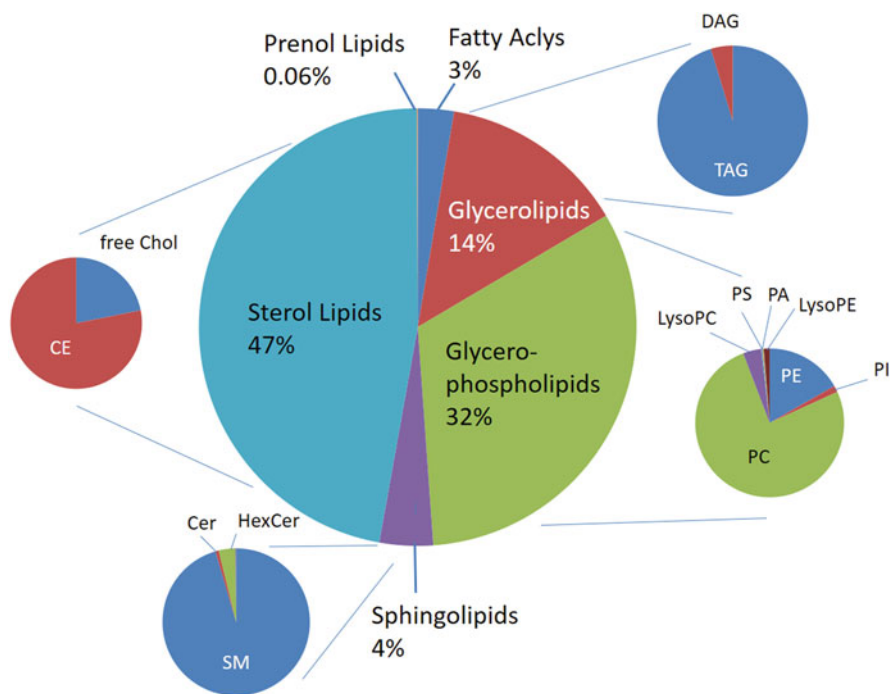


Fig. 2 Distribution of the individual lipid classes in human plasma (reproduced from Quehenberger et al. (2010))

GP constitute the largest lipid class and are derived from sn-glycero-3-phosphoric acid. Nearly 200 GP species were identified in human plasma (Fig. 2). Among them glycerophosphocholines and glycerophosphoethanolamines are most abundant and glycerophosphoserines least abundant (Quehenberger et al. 2010; Quehenberger and Dennis 2011). Lyso-GPs only bear one fatty acid that is esterified to a hydroxyl group either at sn-1 or sn-2 position.

Human plasma contains more than 200 different SL species. The largest SL fraction in human plasma is sphingomyelin, while ceramides are least abundant.

Among sterols cholesterol is most abundant in plasma followed by lathosterol and desmosterol. The largest part of cholesterol in plasma is esterified (Fig. 2). Among the cholesteryl esters (CE) of human plasma, CE(18:2) and CE(20:4) contribute the major fraction (Quehenberger et al. 2010).

Prenol lipids are built from five carbon isoprene units. The two main prenel lipids in plasma are dolichol and ubiquinone (Shiota et al. 2008).

3 Lipidomics

Lipidomics is a relatively young field of science, which aims to identify and quantify all individual lipid species and their functions within a biological system (Han and Gross 2003). Lipidomics technologies are increasingly applied to biomarker

discovery. They offer great promise for new-generation biomarkers in common and complex phenotypes such as dyslipidemia and cardiovascular diseases (CVDs).

3.1 Mass Spectrometry and Chromatography

A lipidomics analysis provides an overall picture of the lipids in a sample, thanks to the combined use of analytical chemistry and data mining tools. However, the comprehensive characterization of a lipidome in biological samples is still challenging. The huge structural diversity of lipids that primarily arises from the combination of various fatty acids and functional head groups makes a complete molecular profiling of the lipidome difficult. In particular, the presence of multiple isobaric lipid species increases the complexity. Also the detection of quantitatively minor lipid species remains a challenge.

The development of mass spectrometry (MS) based technologies over the last decade has rapidly expanded research in the field of lipidomics. In lipidomics, the mass spectrometer can be used without prior separation (“shot-gun lipidomics”) or connected to chromatographic systems in order to provide an additional dimension of separation.

In the shot-gun strategy, the crude lipid extract is directly introduced into the MS system (Han and Gross 1994), which is a fast and simple method to obtain a quantitative lipid profile from biological matrices. However, as the trade-off, the technology suffers from a limited dynamic range and the risk of ion suppression, making the detection of isobaric and low concentrated lipid species difficult. Therefore, recent methodologies are often based on the progress in high-resolution (HR) MS, such as Orbitrap (Ejsing et al. 2009; Schwudke et al. 2011) or Q-TOF detectors (Guo et al. 2012; Li et al. 2013). Using HR-MS, individual lipid species are identified and quantified according to their exact chemical mass. Nevertheless, the major limitation of shot-gun MS is the lack of discrimination between isobaric species. Because of the building block-like nature of many lipid families, isobaric species are frequently found in the lipidome. This is in particular the case for certain glycerol- and sphingolipids, which have a very similar accurate mass (Schwudke et al. 2011).

Thin layer chromatography (TLC) was one of the earliest chromatographic methods applied in lipid analysis. However, it is time-consuming and lacks resolution power and reproducibility. Nevertheless, TLC is still widely used, because of its simplicity and low cost. Also, with gas chromatography (GC), it is possible to obtain information on individual lipid species. Volatile lipid classes, in particular triacylglycerols, can be separated directly and without any chemical modification, whereas the analysis of more polar compounds such as fatty acids, phospholipids, and sphingolipids requires initial derivatization or hydrolysis. Also capillary electrophoresis has been applied for the separation of phospholipids at high data acquisition speed (Jang et al. 2011). However, (ultra) high-performance liquid chromatography ((U)HPLC) is the most versatile method and the majority of lipid

classes including glycerophospholipids, glycerolipids, sphingolipids, sterols, and fatty acids can be separated directly by (U)HPLC (Wenk 2005).

In lipidomics studies, either normal-phase (NPLC) or reversed-phase (RPLC) liquid chromatography is used. In NPLC, lipid species are primarily separated by the polarity of their head groups, while RPLC separates the lipids based on their lipophilicity which is primarily determined by the acyl chain length, the number of double bonds, and hydroxylations (Merrill Jr. et al. 2005). In RPLC, lipids with shorter acyl chains and/or a higher degree of unsaturation elute earlier. Furthermore, RPLC can separate isomeric species based on the type of double bond (*cis* or *trans*) or whether a fatty acid is in sn-1 or sn-2 position (Bird et al. 2012). Other HPLC based separation techniques have been developed recently and show a great potential for lipidomics. In particular, supercritical fluid chromatography-mass spectrometry (SFC-MS) has been used for the separation of lipids over a wide range of polarities depending on the choice of the chromatographic columns (Bamba et al. 2012). In lipidomics, MS instruments are mostly combined with LC systems. This strategy drastically decreases ion suppression and improved the separation of isobaric and low-abundance species (Taguchi and Ishikawa 2010). In addition, ion mobility (IM) separation offers an additional dimension for the separation of isobaric lipid species which can even be combined with LC based separation methods (Kliman et al. 2011). Also, HR-MS is often combined with high- or ultra-performance liquid chromatography (Bird et al. 2011a, b) or ion mobility spectroscopy (Kliman et al. 2011).

In addition to chromatography, technological advancements in MS, particularly in the field of ionization methods, have played a critical role in advancing the lipid analysis from complex matrices. Electrospray ionization (ESI) and atmospheric pressure ion (API) sources, including atmospheric pressure chemical ionization (APCI) and atmospheric pressure photoionization (APPI) are most widely used. Other methods such as matrix-assisted laser desorption ionization (MALDI) are also applied but are not the most established methods for lipid profiling. In contrast to ESI or atmospheric pressure ion sources, MALDI cannot be easily coupled to chromatographic systems. However, MALDI-MS is used in mass spectrometry imaging (MSI) (Aichler and Walch 2015) to analyze individual lipid species directly in tissue sections. Also, the combination of MALDI-MS with TLC provides direct information about the molecular species and the molecular weight (Guittard et al. 1999) as by TLC, lipids are normally separated according to their classes.

The first analysis of a complex lipid mixture using an ESI source was reported by Han and Gross (Han and Gross 1994). Today, ESI is the most widely used method for lipidomic studies, although some important classes, such as cholesteryl esters and glycerolipids (mono-, di- and tri-acylglycerols) are not well ionized with ESI and therefore require the addition of ammonium, lithium, or copper ions to increase signal strength (Murphy and Axelsen 2011; Murphy and Gaskell 2011).

With the advent of high-resolution MS and the capability to perform simultaneous HR-MS and MS/MS analysis, the major challenge for LC-MS based lipidomics is to deal with the vast amount of information generated during data acquisitions. Therefore, bioinformatics tools (Table 1) have been developed to

Table 1 Publicly available software tools for the processing and annotation of MS data

	Application	URL
<i>Databases</i>		
LIPID MAPS	Lipid database and classification	http://www.lipidmaps.org
LipidBank	Official database of Japanese Conference on the Biochemistry of lipids	http://www.lipidbank.jp
LMSD	Structure and annotation of lipid species	http://www.lipidmaps.org/data/structure/index.html
Massbank	High resolution mass spectral database	http://www.massbank.jp
IUPAC	IUPAC lipid nomenclature	http://www.chem.qmul.ac.uk/iupac/lipid
LIPIDAT	Thermodynamic data of lipids	http://www.lipidat.tcd.ie
Cyberlipids	Dedicated site for lipid analysis	http://www.cyberlipid.org
Lipid Library	Dedicated site for lipid analysis	http://lipidlibrary.aocs.org
SphingoMAP	Sphingolipid metabolism	http://www.sphingomap.org
KEGG	Fatty acid, sterol, and phospholipid metabolism	http://www.genome.jp/kegg/pathway.html
METACYC	Lipid metabolism	http://metacyc.org
HMDB	Metabolome database (MS and MS/MS spectra)	http://www.hmdb.ca
METLIN	Metabolome database (MS and MS/MS spectra)	http://metlin.scripps.edu/index.php
mzCloud	High resolution mass spectral database	http://www.mzcloud.org
<i>Free software</i>		
Mzmine 2	Open source software for LC-MS data processing	http://mzmine.github.io/
XCMS	Framework for processing and visualization of LC-MS data	https://bioconductor.org/packages/release/bioc/html/xcms.html
metAlign	Pre-processing and comparison of full scan LC-MS and GC-MS data	https://www.wur.nl/en/show/MetAlign-1.htm
LipidBlast	Tandem mass spectrometry database for lipid identification	https://fiehnlab.ucdavis.edu/projects/LipidBlast
LipidXplorer	Molecular fragmentation query language (MFQL) in shot-gun lipidomics	https://wiki.mpi-cbg.de/lipidx/Main_Page
Lipid Data Analyzer (LDA)	Identifying novel lipid molecular species from mass spectrometry data	http://genome.tugraz.at/lda/lda_description.shtml
LipidIMMS Analyzer	Integrates multidimensional LC-MS/MS spectra and ion mobility data for lipid identification	http://imms.zhulab.cn/LipidIMMS/
Skyline	Powerful open source application for the analysis of proteomics and lipidomics data	https://skyline.ms/project/home/software/Skyline/begin.view
Lipidcreator	Skyline plugin for targeted LC-MS/MS-based lipidomics	https://lifs.isas.de/lipidcreator.html
LIQUID	Open source software for identifying lipids in LC-MS/MS-based data	https://github.com/PNNL-Comp-Mass-Spec/LIQUID

(continued)

Table 1 (continued)

	Application	URL
LIMSA	Integrates and matches MS peaks with a user list of expected lipids, corrects for isotopic patterns, and quantifies the identified lipid species	https://omictools.com/limsa-tool
LipiDex	Unifies LC-MS/MS-based lipid identification using intelligent data filtering	https://github.com/coongroup/LipiDex
LipidHunter	de novo identification of native phospholipids	https://home.uni-leipzig.de/fedorova/software/lipidhunter/
LipidMatch	Rule-based lipid identification using untargeted high-resolution MS/MS data	https://omictools.com/lipidmatch-tool
LipidMS	Lipid annotation in untargeted lipidomics based on fragmentation and intensity rules	https://rdr.io/cran/LipidMS/
<i>Commercial software</i>		<i>Manufacturer</i>
LipidView	LC-MS data processing	AB/Sciex
Marketlynx	LC-MS, LC-MS/MS, GC/MS, and GC-MS/MS data processing	Waters
Metabolic Profiler	NMR and MS data processing	Bruker
Lipid Search	LC-MS/MS data processing	ThermoScientific

handle, process, and interpret large amounts of data generated during lipidomics analysis. Typically, a lipidomics workflow includes four steps.

I. Sample preparation, II. LC-MS/MS analysis, III. Automatic data processing for peak detection and alignment based on commercial or freely available software algorithms, and IV. Feature identification using public or proprietary databases (Fig. 3).

3.2 Pre-analytical Considerations

A key factor for the quality of a lipid profiling study is the integrity of the samples (Ellervik and Vaught 2015). Errors during the pre-analytical phase including sample collection, processing, and storage may severely affect subsequent downstream analyses and resulting data (Ellervik and Vaught 2015). Anticoagulants are commonly used in plasma preparation and the most common anticoagulants such as EDTA and heparin plasma as well as serum are compatible with lipidomics studies. Nonetheless, the peak responses of a number of lipid species can be influenced by the material (Hammad et al. 2010). Therefore, the same anticoagulant should be used throughout a study. In addition, the correct storage of the samples is of importance. Particularly in large clinical cohorts, batches of samples may be stored for a long period of time. Samples are commonly stored at $-80\text{ }^{\circ}\text{C}$ or lower before they undergo a lipidomics profiling. However, certain lipids such as sphingomyelins were lost from plasma samples over 5 years of storage (Haid et al. 2018). Plasma

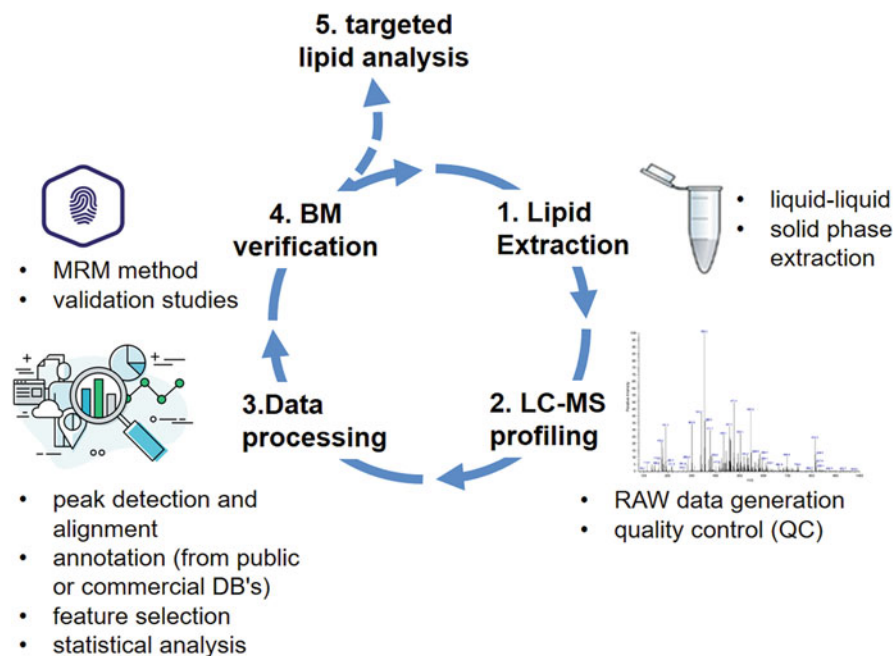


Fig. 3 Typical workflow for lipid biomarker identification

concentrations of cholesterol and triglycerides were shown to decrease in serum samples stored over 7 and 5 years, respectively (Shih et al. 2000) even at -80°C . This should be considered as the time-dependent degradation of certain lipids may lead to biased results. Consequently, the time between sampling and analysis should be kept as short as possible.

3.3 Lipid Extraction

Usually, lipids are extracted from biological sources using liquid-liquid extractions. Lipid extraction is a crucial step in obtaining global coverage of the lipidome. The two most commonly applied methods are using either a 2/1 mixture of chloroform and methanol according to Folch (Folch et al. 1957) or a 1/1 mixture of chloroform and methanol according to Bligh and Dyer (Bligh and Dyer 1959). Based on these traditional protocols, new methods have been developed, although most of them are adaptations of either the one or the other. Chloroform and methanol can be replaced by either dichloromethane (Hu et al. 2008), methyl *tert*-butyl ether (MTBE) or by heptane and ethyl acetate (Lofgren et al. 2012). Comparing the yeast lipidome prepared by three extraction methods (chloroform/methanol 17/1 (v/v), chloroform/methanol 2/1 (v/v), or chloroform/methanol/H₂O 1/1/0.9 (v/v/v)) showed a large heterogeneity in the extraction yield which was also dependent on the

respective lipid classes (Ejsing et al. 2009). Therefore, the appropriate extraction protocol depends on the underlying analytical question and the lipid class of interest (Danne-Rasche et al. 2018).

3.4 Targeted vs. Untargeted Lipidomics

LC-MS is typically applied in two distinct operational modes – targeted or untargeted. A targeted approach is normally chosen for a limited, predefined number of lipid species. It is typically more sensitive and specific than untargeted approaches. In a targeted approach, often low-resolution MS systems are used, such as triple quadrupole or quadrupole linear ion trap instruments, because of their speed, sensitivity, and quantification capabilities (Bielawski et al. 2009). However, the development of HR-MS instruments such as Orbitrap and quadrupole time-of-flight MS also allows untargeted lipidomic approaches, which is independent of a predefined compound list (Junot et al. 2014). HR-MS enables the concomitant analysis of multiple lipid families without the need for extensive fragmentation. Improvements in the duty cycle allow the combination of high-resolution MS and higher energy collisional dissociation (HCD) scans, which provides further information about the accurate mass of precursor and fragment ions of the detected lipids (Bird et al. 2013, 2015). Depending on the model and MS manufacturer, also combined approaches of HR-MS together with ion fragmentation are possible. Many instrument manufacturers offer the possibility to generate HR-MS fragment data using either a data dependent (DDA) or data independent (DIA) acquisition mode.

3.5 Annotation

Lipid species detected by MS-based approaches are typically annotated with the help of dedicated databases. Numerous lipid databases have been developed in the field of lipidomics (see Table 1). The LIPID MAPS consortium has developed the structure database LIPID MAPS (Schmelzer et al. 2007), wherein lipid species are classified by families, class, and subclasses according to their accurate mass and structure. This database is openly available and can be downloaded allowing an automatic annotation. This database consists of compiled databases such as LipidBank, LIPIDAT, or LipidBlast (Cajka and Fiehn 2017). Also databases commonly used in the field of metabolomics, such as HMDB, METLIN, and KEGG, contain data on lipid species as well as other sources such as Cyberlipids and Lipid Library.

However, before the individual lipid species can be annotated, the raw data obtained from an MS analyzer must be pre-processed. Typically, raw MS data are first converted into either a proprietary data format of the respective MS manufacturer or an open data container such as NetCDF, mzXML, or mzData (i.e., XCMS or MZmine). In parallel with advances in instrumentation technology (particularly for high-resolution mass spectrometers), manufacturers have developed commercial

software packages for direct interpretation of the raw data obtained. In contrast to manufacturer specific data formats, which normally can only be read in combination with branded software packages, open data formats are commonly used in freely available software programs such as MZmine (Katajamaa et al. 2006) or XCMS (Smith et al. 2006) (Table 1). Data processing in MZmine is based on algorithms for spectral filtering, alignments according to the retention time, peak picking detection, normalization, and visualization. XCMS in contrast uses non-linear retention time peak alignment, matching by a filter process and peak matching.

Many of these data processing tools allow to perform univariate or multi-variate statistical analysis to identify discriminant variables (potential biomarker lipid species, for example). However, in case of unclear structural definitions, often further MS/MS experiments are needed to identify the lipid metabolite of interest unambiguously.

3.6 Biostatistics

Finally, advanced biostatistical tools are needed to process, analyze, and interpret high-dimensional lipidomics data in the context of clinical information. Based on the typically large and comprehensive dataset, supervised and unsupervised statistical methods are applied. This includes but is not limited to partial least squares discriminant analysis (PLS-DA), orthogonal projection-potential structure analysis (OPLS-DA), principal component analysis (PCA), clustering analysis, linear discriminant analysis, or other stoichiometric methods (Liland et al. 2010). Tools such as MetaboAnalyst can help to identify relevant markers and lipid signatures (Xia and Wishart 2016).

4 Application of Lipidomics in Clinical and Epidemiological Studies

In plasma, lipids are mostly transported and distributed by lipoproteins (e.g., HDL, LDL, VLDL). Several studies have examined the lipid components of lipoproteins among healthy participants (Quehenberger et al. 2010; Christinat and Masoodi 2017; Kontush et al. 2013). However, the lipid composition of lipoproteins varies (Cardner et al. 2020). Triacylglycerols (TAGs) are the predominant core lipid in VLDL, while CEs predominate in the core of LDL and HDL. Compared to LDL, HDL contains a higher amount of phospholipids, particularly PC and LPC. The fatty acid composition of phospholipids, TAGs, and CE is similar in HDL, LDL, and VLDL.

CVD is mostly associated with increased blood levels for one or more lipid classes. These hyperlipidemias (HL) can be linked to familial or non-familial reasons. Familial HL are caused by genetic alterations in lipoprotein metabolism but the penetrance can vary considerably depending on the condition (De Castro-Oros et al. 2010). Non-familial HL occur due to adverse lifestyle behavior such as physical inactivity, intake of lipid-rich diet, smoking, and alcohol consumption or

due to underlying diseases such as obesity, liver diseases, and diabetes. Urban diets, stress, and unhealthy eating pattern have made non-familial HL highly prevalent and a focal topic in health prevention. HL affects a large proportion of the human lipidome, resulting in changes in plasma levels of saturated diacylglycerols (DAGs), TAGs, SM, and phospholipids in obese individuals (Graessler et al. 2009; Hanamatsu et al. 2014; Kim et al. 2010). Plasma levels of PC, phosphatidylethanolamine, ether-linked lipids, phosphatidylinositol (PI), LPC, and CE (Barber et al. 2012; Donovan et al. 2013; Eisinger et al. 2014; Graessler et al. 2009; Hanamatsu et al. 2014; Kim et al. 2010; Samad et al. 2006) were also altered while LPCs showed a mixed behavior. At present, hyperlipidemic conditions are monitored routinely by the measurement of cholesterol and TAG in total plasma, LDL, and HDL. However, lipidomics demonstrated that besides cholesterol also other lipid classes and lipid species of the plasma lipidome are associated with the risk for CVD (Razquin et al. 2018). In particular plasma levels of ceramides show a positive association with the risk of CVD (Laaksonen et al. 2016). Ceramides are primarily present in LDL and may influence the function and atherogenicity of LDL. In fact, LDL extracted from human atherosclerosis lesions are highly enriched in ceramides (Schissel et al. 1996), and animal studies showed a decrease in atherosclerotic lesion size when ceramide synthesis was inhibited (Hojjati et al. 2005).

Relatively few studies have compared the lipidomics profile of lipoproteins in relation to CVD outcomes. Higher TAGs, lower PUFAs, lower phospholipids, and lower sphingomyelin (SM) in HDL may be associated with higher risks of CVD and type 2 diabetes (T2D), although it is currently unknown whether these associations are confounded by HDL-C. In two studies, a low HDL-C has been associated with higher TAGs, lower PUFAs, lower LPC, and lower SM levels (Kontush et al. 2013). Another study found that, compared to participants without CVD or at early stages of CVD, participants with severe CVD had higher levels of short chain FAs in both HDL and non-HDL particles. PC and SM levels were lower in HDL, and PUFAs reduced in non-HDL particles. Additionally, two studies showed that lower levels of PC-plasmalogens were associated with a higher risk of CVD, particularly of acute CVD (Meikle et al. 2019; Sutter et al. 2015).

Obesity also leads to increased lipolysis in adipose tissues and thus increases plasma level of FFAs (Haus et al. 2009). There is a direct relationship between increased intake of saturated fatty acids (e.g., lauric, myristic, or palmitic acid) and an increase in TAGs (Fernandez and West 2005; Steinberg 2005). Increased FFAs and MAGs in plasma can be attributed to an increased lipolysis of TAGs obtained from high fat diet (Ho and Storch 2001). Dietary saturated fatty acids have a tendency to increase TAG while polyunsaturated fatty acids have the ability to lower TAG and LDL-C (Siri-Tarino et al. 2010; Williams and Salter 2016). On the other hand, diets rich in SM were shown to specifically increase HDL-C without affecting other lipids (Ramprasath et al. 2013).

Mouse models of diet-induced obesity showed elevation in plasma ceramide level and alterations in PCs, LPCs, and SMs (Barber et al. 2012; Samad et al. 2006). Perturbation in lipid levels is also associated with both type 1 (T1D) (Fox et al. 2011; Sorensen et al. 2010) and type 2 diabetes (T2D) (Graessler et al. 2009; Barber et al.

2012; Samad et al. 2006). In diabetes, plasma levels of most LPC decrease, while PCs showed a mixed behavior. An important hallmark of T2D is an increase in plasma concentration of FFA (Barber et al. 2012; Samad et al. 2006), ceramides (Samad et al. 2006; Haus et al. 2009; Kang et al. 2013), and TAGs enriched with short chain saturated fatty acids (Rhee et al. 2011). Moreover, atypical 1-deoxy-Sphingolipids (1-deoxySL) were found to be elevated in T2DM and showed a similar or even closer association with T2DM or metabolic syndrome than established markers such as waist circumference, glucose, triglycerides, HDL cholesterol, and blood pressure (Bertea et al. 2010; Othman et al. 2012; Othman et al. 2015a). Further studies showed that elevated plasma 1-deoxySL levels are strong and independent risk predictors of future T2DM, especially for non-obese individuals in the general population (Mwinyi et al. 2017). Plasma C20-Sphingolipids were also shown to indicate cardiovascular events independently from conventional cardiovascular risk factors in patients undergoing coronary angiography (Othman et al. 2015b).

High-throughput lipidomics analyses were recently applied to plasma samples of 10,339 participants from the Australian Diabetes, Obesity and Lifestyle Study (AusDiab) (Huynh et al. 2019; Beyene and Olshansky 2020). The data were validated in a second cohort with 4,207 participants (the Busselton Health Study) (Beyene and Olshansky 2020). The studies showed differences in the plasma lipidome related to metabolic disease and gender. A class of specific ether-phospholipids and lysophospholipids were inversely associated with age in men. The comparison of post- and premenopausal women showed higher TAG and lower lysoPC species in the postmenopausal group. Lysophospholipids were negatively associated with BMI in both sexes (with a larger effect size in men). Based on specific lipid ratios the authors identified the lipid metabolizing enzymes stearoyl CoA desaturase (SCD-1), fatty acid desaturase 3 (FADS3), and plasmanylethanolamine Δ 1-desaturase, as well as the sphingolipid metabolic pathway as relevant factors associated with cardiometabolic phenotypes.

In conclusion, MS-based lipid analysis combined with bioinformatics tools have revolutionized the field of lipidomics. Thanks to commercial and freely available software packages that allow automatic peak detection, alignment, and feature annotation using public or proprietary databases to study large cohorts of patients and identify novel biomarkers which are getting increasingly important particularly in the growing field of precision and personalized medicine.

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The Epigenome in Atherosclerosis

Sarah Costantino and Francesco Paneni

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Abstract

Emerging evidence suggests the growing importance of “nongenetic factors” in the pathogenesis of atherosclerotic vascular disease. Indeed, the inherited genome determines only part of the risk profile as genomic approaches do not take into account additional layers of biological regulation by “epi”-genetic changes. Epigenetic modifications are defined as plastic chemical changes of DNA/histone complexes which critically affect gene activity without altering the DNA

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sequence. These modifications include DNA methylation, histone posttranslational modifications, and non-coding RNAs and have the ability to modulate gene expression at both transcriptional and posttranscriptional level. Notably, epigenetic signals are mainly induced by environmental factors (i.e., pollution, smoking, noise) and, once acquired, may be transmitted to the offspring. The inheritance of adverse epigenetic changes may lead to premature deregulation of pathways involved in vascular damage and endothelial dysfunction. Here, we describe the emerging role of epigenetic modifications as fine-tuners of gene transcription in atherosclerosis. Specifically, the following aspects are described in detail: (1) discovery and impact of the epigenome in cardiovascular disease, (2) the epigenetic landscape in atherosclerosis; (3) inheritance of epigenetic signals and premature vascular disease; (4) epigenetic control of lipid metabolism, vascular oxidative stress, inflammation, autophagy, and apoptosis; (5) epigenetic biomarkers in patients with atherosclerosis; (6) novel therapeutic strategies to modulate epigenetic marks. Understanding the individual epigenetic profile may pave the way for new approaches to determine cardiovascular risk and to develop personalized therapies to treat atherosclerosis and its complications.

Keywords

Atherosclerotic plaque · Epigenetic therapies · Epigenome · Inflammation · Vascular disease

1 Discovery and Impact of Epigenetics

Although inborn genetic variation can influence disease susceptibility, accumulating evidence supports the notion that cardiovascular diseases (CVD) are heavily affected by non-genetic factors. Indeed, the inherited genome determines only part of the risk profile as genomic approaches do not take into account additional layers of biological regulation by “epi”-genetic changes – defined as acquired modifications to the genome subject to influence by the environment (Handy et al. 2011). The discovery of epigenetic modifications dates back to 1956, when the British developmental biologist Conrad Waddington demonstrated for the first time the inheritance of a characteristic acquired in a population in response to an environmental stimulus (Slack 2002). Waddington’s experimental work showed that embryo fruit flies carrying an identical genetic background displayed different thorax and wing structures when exposed to different temperature or chemical stimuli (Noble 2015). While the term “epigenetics” initially embraced the process by which a fertilized zygote develops into a complex organism, the concept was refined over the next years by the observations that cells sharing the same genetic information can exhibit clear differences in gene expression. Waddington’s work has contributed to understand that heritable traits can associate not only with changes in nucleotide sequence, but also with chemical modifications of DNA or proteins interacting with DNA. Later experiments in 1975 showed that epigenetic changes could be

transmitted to daughter cells, thus regulating gene expression across multiple generations (Gonzalez-Recio et al. 2015). There are several examples outlining the importance of epigenetic processing in health and disease. First, epigenetic regulation of gene expression is a major determinant of cell fate. Epigenetic signals occurring at the levels of DNA and histones have the ability to license regions of the genome while shutting down others, thus favoring specific transcriptional programs which enable the differentiation of genetically identical pluripotent stem cells toward different cell types (i.e. myocytes, endothelial cells, adipocytes) (Brunet and Berger 2014). Along the same line, monozygotic twins who share an identical genetic make-up may display different physical and behavioral traits when raised under different environmental conditions (Fraga et al. 2005). Chronic exposure to different stimuli (i.e., pollution, noise) will enable the induction of specific epigenetic programs eventually leading to different gene expression profiles over the lifetime (Baccarelli and Ghosh 2012). Of note, epigenetic-induced deregulation of longevity genes was shown to accelerate cellular senescence and vascular aging (Sen et al. 2016). On the other hand, specific interventions such as caloric restriction have shown to delay age-dependent onset of diseases mostly via an epigenetic reprogramming. Taken together, epigenetic regulation of gene transcription represents a pivotal mechanism of adaptation to different environmental conditions and is among the most important mechanisms underlying cardiovascular diseases. A deep understanding of the epigenetic machinery may enable the characterization of cell-specific transcriptional programs in patients with atherosclerosis and could offer the tools for personalized therapeutic approaches to prevent cardiovascular diseases. The availability of new technologies for the study of our genome has indeed allowed a deep characterization of chromatin structure and function thus unveiling an array of epigenetic changes at the level of DNA and histones. This important information has enabled to understand inter-individual diversity and has prompted us to strive for an ever greater level of personalization in cardiovascular medicine.

2 Classification of Epigenetic Modifications

Epigenetic modifications fall into three main categories: (1) chemical modifications of DNA (i.e., methylation); (2) posttranslational modifications of histone tails; (3) regulation of gene expression by non-coding RNAs [i.e., microRNAs, PIWI-interacting RNAs, endogenous short interfering RNAs, long non-coding RNAs (lncRNAs)] (Fig. 1). The present chapter will focus on the role of chromatin modifications in atherosclerosis. The function of ncRNAs in this setting is described extensively in a different chapter.

2.1 DNA Methylation

Nuclear DNA is tightly wrapped around a core of eight histone proteins (two copies each of H2A, H2B, H3, and H4), generating repetitive units known as nucleosomes.

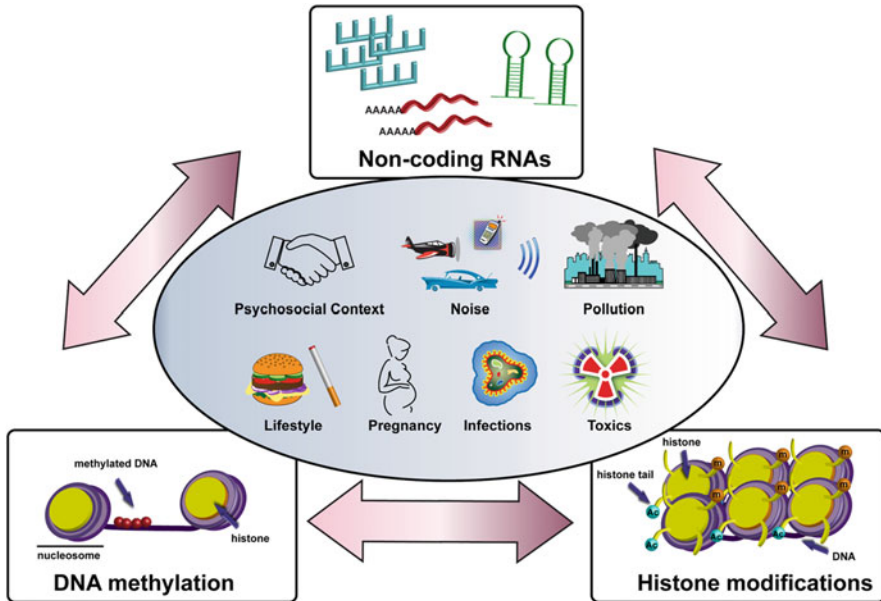


Fig. 1 Role of epigenetic inheritance in vascular disease. Environmental factors induce epigenetic changes which are transmitted to the offspring, thus leading to maladaptive transcriptional programs responsible for early vascular damage

These structures enable a very efficient packaging of DNA within the cell nucleus, through the formation of chromatin. Despite its complex three-dimensional structure, chromatin is a very dynamic entity where DNA and histones can be chemically modified. This alters the accessibility of transcriptional factors to genes and, thus, modulates transcription (Goldberg et al. 2007).

DNA methylation plays an important role in the regulation of chromatin structure and gene expression, and therefore, participates in a variety of biological processes (Hamidi et al. 2015), including tissue-specific regulation of gene expression, genomic imprinting, X chromosome inactivation, silencing of transposable elements, and defense against viral sequences (Prasher et al. 2019). This epigenetic modification is relatively stable, it is tissue-specific and can be transmitted to the offspring (Izquierdo and Crujeiras 2019). Methylation of DNA mainly occurs through attachment of methyl group (CH₃) from S-adenosyl methionine (SAM) to the C5 position in the cytosine-paired-with-guanine (CpG) dinucleotide sequences thus forming 5-methylcytosine (5mC) (Davis and Gallagher 2019). Furthermore, non-CpG methylation (i.e., CHG or CHH region) may also occur and this signature is usually observed in embryonic stem cells (Bernstein et al. 2007).

CpG sequences are generally located into promoter regions of genes, however, they can also be located within gene bodies (Costantino et al. 2015). Promoter methylation is generally associated with transcriptional repression, while gene

body methylation is associated with enhanced transcription (Maunakea et al. 2010). Although both transcriptional repression and activation have been reported, DNA methylation leads – in >95% of cases – to gene silencing (Xiao et al. 2019). Specifically, methylated cytosines are recognized by DNA methyl-binding proteins (MBPs) that prevent the binding of transcription factors to DNA (Prasher et al. 2019). Alternatively, DNA methylation may recruit specific proteins, such as the methyl-CpG binding protein 2 (MeCP2), that specifically bind to methylated regions to foster transcriptional repression (Jones et al. 1998). Besides methylation, other chemical modifications of cytosine have been identified, such as hydroxymethylation, formylation, and carboxylation, but their interrelation with methylation is not completely understood yet (Elia and Condorelli 2019).

DNA methylation is catalyzed by the DNA methyltransferase 1 (DNMT1), 3a (DNMT3a), and 3b (DNMT3b) and is reversed by Tet methylcytosine dioxygenases (TET1, 2, and 3) (Xu et al. 2018). Among these enzymes, Dnmt1 is responsible for the maintenance of methylation patterns in the genome by replicating the hemimethylated CpG sites (Vilkaitis et al. 2005), whereas Dnmt3a/b are considered de novo methyltransferases (Okano et al. 1999). The activity of DNMT1 has been demonstrated to be dependent on a co-factor that recognizes hemi-methylated DNA. The Ubiquitin-like with PHD and Ring Finger Domains 1 (UHRF1), were shown to be essential for maintenance of the methylation status of DNA by directing the recruitment of DNMT1 to the replication forks (Bostick et al. 2007). DNMT3 can also methylate non-CpG sites (at CpA and CHG) and the presence of methylated non-CpG sites in the human genome indicates that there is ongoing de novo DNA methylation at each round of genome replication (Aavik et al. 2019).

DNA demethylation can be achieved by either passive or active mechanisms (Costantino et al. 2015). Passive demethylation can be the result of Dnmt1 inhibition during cell replication (Wolffe et al. 1999) while active demethylation is modulated by DNA demethylases. DNA demethylation may follow two main pathways: the first is dependent on cytosine deamination (AID, APOBEC3G, FTO) while the second is dependent on the oxidation of methylated cytosines (Aavik et al. 2019). The latter reaction is catalyzed by members of the Ten-eleven translocation (TET) proteins family (TET1-3) which convert 5-methylcytosine (5mC) into 5-hydroxymethylcytosine (5hmC) (Wu and Zhang 2010). TET1 is mostly found in embryonic stem cells, whereas TET2 and TET3 are ubiquitously expressed (Costantino et al. 2015). TET1 is low in normal tissues, whereas TET2 is an abundant protein in most tissues/cell types and TET3 expression is overlapping with TET2 in many tissues (Kohli and Zhang 2013). The observed abundance of TET2 and TET3 enzymes in various tissues indicates that DNA demethylation must have an important role in cellular homeostasis (Aavik et al. 2019). The TET family enzymes represent promising therapeutic targets given their ability to oxidize 5mC thus leading to the reversal of gene repression by DNA methylation (Greco et al. 2016).

2.2 Histone Posttranslational Modifications

DNA is packaged into repeating units called nucleosomes by wrapping around multimeric histone proteins. When nucleosomes are organized into tightly packed bundles (heterochromatin), the transcriptional machinery is hampered by a reduction of chromatin accessibility. Conversely, when chromatin is relaxed (euchromatin), DNA is more accessible to transcription factors and gene transcription may occur. Each histone (H) protein is an octamer comprised of 2 sets of H2A, H2B, H3, and H4 proteins with a single histone H1 linker protein between nucleosomes. Each histone subunit has an N-terminal tail containing a lysine (K) residue that protrudes away from the surface of the histone octamer creating an exposed surface (Davis and Gallagher 2019). Histones are amenable to many posttranslational modifications (PTMs), which include methylation, acetylation, ubiquitination, phosphorylation, SUMOylation, GlcNAcylation, carbonylation, and ADP-ribosylation (Zhang et al. 2018). Histone modifications are induced by specific enzymes that act predominantly at histone N-terminal tails primarily involving the amino acids lysine (K) and arginine (R). These modifications can be present on multiple (but specific) sites on histones (Kouzarides 2007).

In addition to DNA modifications, PTMs of histone tails may serve as a repressive or activating signal depending on the specific histone tail, the number of groups added, and the region of chromatin where the modification occurs (Bauer and Martin 2017). Although the biological significance of many PTMs remains to be elucidated, considerable advances have been made in the understanding of lysine acetylation and methylation (Bernstein et al. 2007). The first PTM reported is histone acetylation, an epigenetic signature characterized by the addition of positively charged acetyl groups to amino acid residues on the histones, which neutralizes the negative charges of DNA. This modification reduces the affinity of the histone for DNA and consequently results in the formation of a more relaxed chromatin structure, eventually increasing chromatin accessibility (Nicorescu et al. 2019). Acetylation occurs mainly on lysine residues on histones H3 and H4; this mark mainly associates with activation of transcription by enhancing chromatin accessibility (Gillette and Hill 2015). In this context, bromodomain and extra-terminal proteins recognize histone acetylation marks and initiate the assembly of the transcriptional machinery (Filippakopoulos and Knapp 2014). Acetylation is modulated by histone acetyltransferases (HATs) and histone deacetylases (HDACs) which are involved in the addition or removal of an acetyl group, respectively (Baccarelli and Ghosh 2012). This modification is driven by recognition and binding of transcription factors able to recruit one of a growing family of HATs, namely CBP/p300, MYST, and GNAT (Kouzarides 2007). Under physiological conditions, lysine residues on histone tails are positively charged and can bind DNA which is a negatively charged molecule due to its phosphate components. The interaction between histone and DNA favors a compact chromatin structure, with scarce accessibility to transcription factors. Conversely, when lysine residues are acetylated they lose their positive charge, thus favoring an open chromatin and, hence, enhanced gene expression (Costantino et al. 2015). HATs catalyze the addition of two-carbon acetyl groups

to lysine residues from acetyl-CoA (Carrozza et al. 2003). On the other hand, removal of acetyl groups from histone residues by HDACs represses gene transcription (Stratton et al. 2019). Specifically, HDACs catalyze removal of acetyl groups from lysine residues and are distributed among four classes (Class I, IIa, IIb, III, and IV) (Haberland et al. 2009).

In contrast to lysine acetylation, a process known to enhance gene expression, histone methylation is a more complex modification which may result in different chromatin states according to the methylated residue and the number of added methyl groups (Cooper and El-Osta 2010). For instance, the lysine residues at positions 4, 9, and 27 on histone H3 can be methylated to different levels (mono-, bi-, and tri-methylation), resulting in different states of chromatin accessibility (Elia and Condorelli 2019). Histone methylation is defined as the transfer of methyl group from S-adenosyl-L-methionine to lysine or arginine residues of histone proteins by histone methyltransferases (HMTs). While lysines may be mono-, di-, or tri-methylated, arginines may be monomethylated or demethylated (symmetrically or asymmetrically) (Bannister and Kouzarides 2011). Histone methyltransferases (HMTs) include the EZH, SETD, PRDM, PRMT, METTL, and MLL enzyme families (Stratton et al. 2019).

Over the last few years, a well-established theory was that histone turnover was the only mechanism regulating histone methylation. More recently, histone demethylases (HDMs) have been reported as a novel mechanism capable of demethylating lysine residues on histone tails (Handy et al. 2011). Indeed, histone methylation is a very dynamic process regulated by histone demethylases, which remove the methyl groups from lysine residues with a high gene specificity (Tsukada et al. 2006). HDMs include members of UTX/Y, JARID1, JMJD, LSD, PHF, and FBXL enzyme families (Stratton et al. 2019).

Two important studies have identified the first HDM, lysine demethylase 1 (LSD1) which is able to remove methyl group from H3K4 and H3K9, suggesting that lysine demethylation is subjected to dynamic modulation (Metzger et al. 2005). Several lysine demethylases specific for diverse histone lysine residues have been identified (Costantino et al. 2015). A better comprehension of how such demethylases are regulated during disease states is of paramount importance.

Interestingly, studies have shown that DNA methylase (DNMTs), histone methyltransferase (HMTs), and histone acetyltransferase (HATs) are closely interconnected to regulate chromatin remodelling under specific stimuli (Li et al. 2007). For example, H3K9 methylation induces DNA methylation while CpG methylation favors H3K9m (Cedar and Bergman 2009). Therefore, chromatin modifications may influence each other and can propagate.

3 Epigenetic Inheritance and Vascular Disease

An important aspect to consider when dealing with epigenetics is that epigenetic modifications are heritable. Epigenetic signals acquired during the lifetime are transmitted to the offspring, where they participate to determine substantial changes

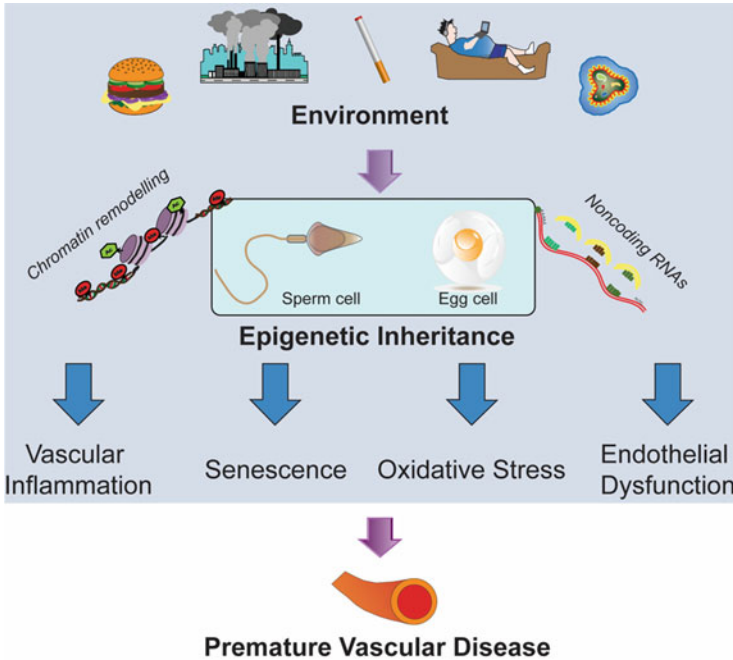


Fig. 2 Epigenetic changes and environmental factors. Exposure to different environmental factors induces several epigenetic modifications. These entail changes of DNA methylation, posttranslational histone modifications, and altered expression of non-coding RNAs. Reproduced with permission from Costantino et al. *Eur Heart J.* 2018;39:4150–4158

in phenotype (Fig. 2) (Heard and Martienssen 2014). In the Överkalix study, conducted in northern Sweden to investigate the physiological effects of various environmental factors on transgenerational epigenetic inheritance, three cohorts born in 1890, 1905, and 1920 were studied until death. The study showed that overeating during a child's slow growth period (SGP), before their prepubertal peak in growth velocity, influences descendants' risk of death from cardiovascular disease and diabetes (Pembrey et al. 2006). If food was not readily available during the father's slow growth period, then cardiovascular disease mortality of the proband was low. By contrast, diabetes mortality increased if the paternal grandfather was exposed to a surfeit of food during his slow growth period (Odds Ratio 4.1, 95% confidence interval 1.33–12.93, $P = 0.01$) (Pembrey et al. 2006). This study was one of the first to demonstrate that a nutrition-linked mechanism through the male line seems to have influenced the risk of developing cardiovascular and metabolic diseases. Periconceptual diet was shown to influence DNA methylation levels with phenotypic consequences. A genome-scale analysis of differential DNA methylation in whole blood after periconceptual exposure to the Dutch Hunger Winter famine revealed changes of differentially methylated regions (Tobi et al. 2014). These changes were preferentially observed at regulatory regions of genes relevant for

metabolic homeostasis, inflammation and longevity, key processes underlying the pathogenesis of atherosclerosis. Specifically, individuals conceived during the Dutch famine showed, six decades later, reduced methylation levels at the promoter of insulin-like growth factor type 2 (IGF-2), a gene regulating of glucose homeostasis, cardiovascular function, and lipid metabolism (Heijmans et al. 2008). Overall, these data suggest that epigenetic modulation of pathways by prenatal malnutrition may promote adverse cardiometabolic phenotypes during childhood. Inheritance of specific epigenetic patterns may contribute to explain the alarming increase in childhood obesity from 32 million in 1990 to around 42 million in 2016 (Brown et al. 2015). Notably, early metabolic alterations in obese children are associated with low-grade inflammation, high oxidative stress levels, impaired nitric oxide bioavailability, endothelial dysfunction, and arterial stiffness (Suglia et al. 2018). Macrophage polarization and adipose tissue inflammation are also important features commonly found in obese children (Singer and Lumeng 2017). Epigenetic modulation of inflammation is supported by the notion that promoter methylation of *TNF- α* , pyruvate dehydrogenase kinase 4 (*PDK4*), and leptin (*LEP*) are all reduced in obese as compared to lean children, while methylation of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (*PGC-1 α*) and proopiomelanocortin (*POMC*) genes are increased (Garcia-Cardona et al. 2014). As a consequence of these alterations, risk of diabetes, dyslipidemia, and silent heart disease are significantly higher in obese as compared to non-obese children (Juonala et al. 2011). Despite emerging evidence indicates the existence of a transgenerational epigenetic inheritance in humans, data still remain inconclusive due to relevant confounders represented by genetic, ecological, and cultural inheritance. Parents and offspring may share the same epigenomic features, but it is challenging to understand whether these features are being transmitted through the germline or are newly established in each generation by the action of shared genes and shared environments (Horsthemke 2018). The most difficult aspect in humans is the demonstration that a specific epigenetic factor in the germ cells is responsible for the phenotypic effect in the next generation. While this phenomenon was clearly described in plants, nematodes, and fruit flies, its occurrence in humans remains controversial. This important aspect of epigenetic regulation is the object of intense investigation and future studies will contribute to clarify the contribution of inheritable epigenetic traits to global vascular risk in humans.

4 Epigenetic Processing in Atherosclerotic Vascular Disease

4.1 DNA Methylation

Reduced DNA methylation is an important signature of atherosclerotic lesions both in humans and in experimental models, such as apolipoprotein E knockout mice (*ApoE*^{-/-}) and neointima of balloon-denuded New Zealand White (NZW) rabbit aortas (Napoli et al. 2012). Several studies have shown profound changes of atherosclerosis-specific methylated CpGs in human atherosclerotic plaques

EPIGENETIC LANDSCAPE IN ATHEROSCLEROSIS

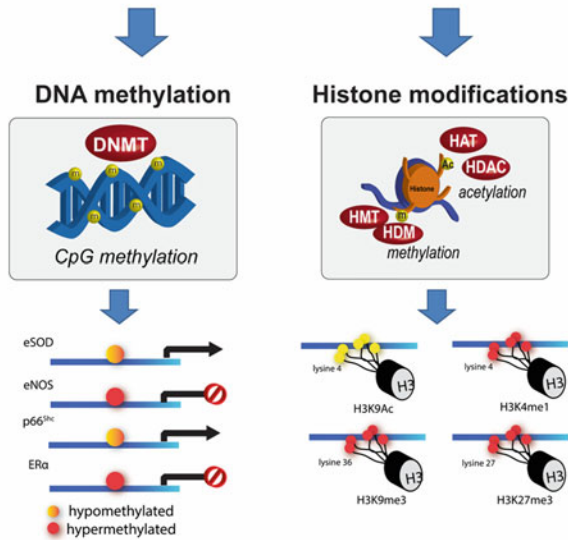


Fig. 3 Alterations of the chromatin landscape in atherosclerosis. *HAT* histone methyltransferase, *HDAC* histone deacetylase, *HMT* histone methyltransferase, *HDM* histone demethylase, *H3* histone 3, *K* lysine residue

(hypomethylation of 3,997 promoter sites = 84%) with a progressive increase in gene methylation as the lesions matured (Aavik et al. 2015). Global hypomethylation of DNA in human aortic lesions was the result of a near-complete demethylation of the subset of CpG islands that were hypermethylated in control aortas (Aavik et al. 2019). Beside global variations in methylation status, alterations in the methylation pattern of specific genes have been causally implicated in the pathogenesis of atherosclerosis. Methylation of genes regulating cellular proliferation, namely genes encoding for estrogen receptor- α (ER α) was found in atherosclerotic plaques from human subjects (Fig. 3). Moreover, ER α gene was also shown to be methylated in vascular smooth muscle cells (SMCs) in vitro during their phenotypic switch (Aavik et al. 2019). Methylation-dependent increase in proliferation of SMCs may represent an important epigenetic mechanism underpinning atherogenesis. Indeed – under physiological conditions – arterial SMCs are terminally differentiated and replication does not occur. This associates with a low activity of the epigenetic machinery. By contrast, during lesion development, changes in DNA methylation affect transcriptional programs fostering SMCs proliferation. The effects of DNA methylation may be the direct result of gene hypomethylation or can be due to methylation-induced alterations of DNA integrity and function. DNA hypomethylation of 15-lipoxygenase and extracellular superoxide dismutase (eSOD) genes were found during SMCs replications (Napoli et al. 2012). Epigenetic remodelling of growth regulatory genes such as platelet-derived growth factor and *c-myc* might also contribute to SMCs proliferation (Hiltunen and Yla-Herttuala

2003). De novo methylation of genes is the most plausible mechanism to explain the dynamic effects of epigenetic processing in atherosclerosis. Intervention studies in cellular models have clearly shown that treatment with the methylation inhibitor 5-azadeoxycytidine heavily affects gene expression in vitro and in vivo (Yang et al. 2010). The importance of de novo methylation is supported by the notion that overexpression of Dnmt, an enzyme which lacks de novo methylation activity, does not reverse genomic hypomethylation in cancer (Zhang and Xu 2017).

The accumulation of reactive oxygen species (ROS) is a key promoter of epigenetic signatures. It has been shown that ROS may foster changes in DNA methylation, and in turn, methylation changes may induce the upregulation of pro-oxidant genes. Reduced methylation of CpG islands at the promoter of the *shc1* gene, encoding for the mitochondrial adaptor p66^{Shc}, has been associated with high oxidative stress levels, mitochondrial insufficiency, apoptosis, and endothelial dysfunction (Paneni et al. 2012). Both diabetic mice and humans display reduced DNA methylation of *shc1* promoter in the vasculature and the heart, and this epigenetic signature persists despite intensive glycemic control (Paneni et al. 2012; Costantino et al. 2017). Interestingly, epigenetic reprogramming by selective targeting of *dnmt3b* restores promoter methylation thus repressing p66^{Shc} transcription (Costantino et al. 2018a). Other genes implicated in oxidative stress and vascular disease, namely the AP-1 transcription factor JunD and the prolyl-isomerase-1 (Pin1) were also shown to be modulated by specific changes of CpG methylation (Paneni et al. 2013, 2015a). DNA methylation was also reported to affect the expression of inflammatory genes including TNF- α , COX-2, and IL-1 β (Stylianou 2019). Genetic deletion of Tet methylcytosine dioxygenase 2 (TET2) in macrophages leads to increased expression of inflammatory cytokines and chemokines in response to native LDL (Peng et al. 2016). A similar increase in plasma interleukin-8 was observed in people with TET2 mutations but not in those without the mutations suggesting that control of DNA methylation by TET2 regulates inflammation in atherosclerosis (Fuster et al. 2017).

Atherosclerosis and ageing are highly interconnected, and age-dependent epigenetic modifications may significantly contribute to vascular disease (Paneni et al. 2017). Aging is generally associated with a progressive decline in global DNA methylation (Hannum et al. 2013). Indeed, DNA methylation content assessed by whole-genome bisulfite is heavily reduced in centenarians as compared with newborns (Heyn et al. 2012). Reduced DNA methylation at the promoter of genes associated with self-renewal was also found in stem cells isolated from aged versus young subjects and associated with functional defects and reduced ability to differentiate (Sun et al. 2014). DNMT3A, TET2, and ASXL1 are key regulators of DNA methylation status and their deregulation is associated with clonal expansion in hematopoiesis and functional defects, a phenomenon known as clonal hematopoiesis of indeterminate potential (CHIP). CHIP was found to be associated with an increased risk of CVD, stroke, coronary calcification, malignancies, and all-cause mortality (Jaiswal et al. 2017). *Dnmt3a* gene was the most recurrently mutated in individuals with CHIP. Hematopoietic tissue-specific conditional *Dnmt3a* deletion in mouse models led to a progressive expansion of long-term HSC with impaired

differentiation by incomplete epigenetic repression of HSC-specific genes (Challen et al. 2011). Hematopoietic tissue-specific Tet2 loss leads to decreased levels of 5hmC, increased stem cell self-renewal, delayed HSC differentiation, and skewed development toward the monocyte/macrophage lineage (Moran-Crusio et al. 2011). Thus, hematopoietic stressors can induce stable epigenetic reprogramming of bone marrow HSCs contributing to clonal hematopoiesis and myeloid skewing. Albeit DNMT3A and TET2 exert opposite functions in terms of DNA methylation, a recent study showed that these chromatin modifiers both repress the erythroid regulator *Klf1* suggesting a model of cooperative inhibition resulting in HSCs transformation (Zhang et al. 2016). Methylation changes at the promoter of eNOS, GATA-2, and GATA-3 during aging were also associated with functional defects and reduced differentiation potential of endothelial progenitor cells (EPCs), whereas reprogramming approaches aimed at restoring promoter methylation of these genes contributed to cell rejuvenation and longevity (Chan et al. 2004). Beside modulating EPCs functionality, DNA methylation directly regulates endothelial cell function mainly via TET2 signaling. TET2 promotes endothelial autophagy and downregulates inflammatory factors such as VCAM1, ICAM1, MCP1, and IL-1 β in lesions of high fat fed *ApoE*^{-/-} mice (Peng et al. 2016). Moreover, TET2 overexpression decreased the methylation levels of Beclin-1 promoter, thus leading to increased endothelial cell autophagy and decreased inflammatory factors in endothelial cells exposed to oxidized LDL (Li et al. 2015). Epigenetic changes of p66^{Shc} and eNOS have also shown to affect endothelial function in vivo as well as in patients with obesity and type 2 diabetes (Costantino et al. 2017, 2019).

4.2 Histone Posttranslational Modifications

Growing evidence indicates that posttranslational modifications of histones, mainly at lysine and arginine residues, significantly affect chromatin accessibility thus enabling cell-specific transcriptional programs implicated in the pathophysiology of atherosclerotic vascular disease (Jiang et al. 2018) (Fig. 3). Chromatin modifications play a pivotal role in regulating vascular inflammation by epigenetic modulation of nuclear factor kappa-B (NF- κ B) signaling. Several cardiovascular risk factors have shown to modulate histone marks and gene expression. In primary human aortic endothelial cells, hyperglycemia induces a specific mono-methylation of histone 3 at lysine 4 (H3K4m1) thus enabling chromatin accessibility at the level of NF- κ B p65 promoter (El-Osta et al. 2008). Hyperglycemia-related H3K4m1 is induced by the mammalian methyltransferase SETD7, which is emerging as a key player in the regulation of vascular inflammation and oxidative stress (Okabe et al. 2012). Several stimuli, including reactive oxygen species are able to increase SETD7 activity. Indeed, overexpression of scavenger enzymes (e.g., uncoupling protein-1) blunted SETD7-dependent H3K4m1 and subsequent activation of NF- κ B p65 and downstream inflammatory genes, namely MCP-1, VCAM-1, and ICAM-1 (El-Osta et al. 2008). Of note, SETD7 expression is increased in peripheral blood monocytes from patients with type 2 diabetes, and correlated with endothelial function and

oxidative stress levels, as assessed by flow-mediated dilation (FMD) of the brachial artery and urinary levels of isoPGF_{2α}, respectively (Paneni et al. 2015b). Moreover, SETD7 induction positively correlated with the expression of proatherosclerotic genes including COX-2, iNOS, and VCAM-1 (Paneni et al. 2015b). Chromatin immunoprecipitation experiments revealed that SETD7 specifically binds a specific region of the human NF-κB p65 promoter thus fostering the expression of inflammatory genes. Beside SETD7, other chromatin modifiers such as the methyltransferase Suv39h1 and LSD1 have shown their ability to modulate NF-κB-dependent inflammatory genes (IL-6 and MCP-1) by reducing the levels of dimethylation (H3K9me2) and trimethylation of H3 at lysine 9 (H3K9me3) in SMCs (Brasacchio et al. 2009). Suv39h1 gain- and loss-of-function approaches in experimental models of type 2 diabetes showed that H3K9me2 and H3K9me3 can be dynamically modulated thus reverting the SMCs phenotype. Histone acetyltransferases, such as CBP/p300 and p/CAF, were found to be critically involved in the NF-κB signaling and upregulation of TNF-α and COX-2, master regulators of the atherosclerotic process (Deng et al. 2004). Mechanistically, p300 interacts with the p65 (RELA) subunit of NF-κB in the nucleus to displace repressive p50–HDAC1 interactions (Ashburner et al. 2001). This results in increased acetylation of both p65 itself and histones in proximity of NF-κB promoter. The p65–p300 interaction has been found to be critical for the inflammatory activation of both macrophages and endothelial cells (Jiang et al. 2018). The relevance of this epigenetic route is supported by the notion that inhibition of p300 by curcumin enhances macrophage cholesterol efflux in human and mouse macrophages in vitro, and exerts an anti-inflammatory effect by suppressing NF-κB activity (Khyzha et al. 2017). Deregulation of histone deacetylases, namely sirtuins, also represents a major underpinning of vascular disease and atherosclerosis via modulation of oxidative stress, endothelial function, inflammation, mitochondrial bioenergetics, and cellular metabolism (Winnik et al. 2015). SIRT1 activity is reduced in patients with obesity and significantly contributes to endothelial dysfunction (Masi et al. 2020). By contrast, pharmacological activation of SIRT1 by resveratrol restores endothelial function and NO availability and attenuates dyslipidemia and obesity-induced metabolic alterations in human subjects (Carrizzo et al. 2013). Overexpression of SIRT1 in endothelial cells increases their sprouting and migration, whereas SIRT1 depletion impairs angiogenic response, mainly via FLT1 and CXCR4 (Potente et al. 2007). SIRT1 was also found to regulate the transcription of the mitochondrial adaptor p66^{Shc}, thus leading to oxidative stress and endothelial dysfunction (Zhou et al. 2011; Mohammed et al. 2020). Furthermore, pharmacological activation of SIRT1 by visfatin has shown to attenuate oxLDL-induced senescence of EPCs through the PI3K/Akt/ERK pathway (Ming et al. 2016). SIRT3 and SIRT6 have also recently emerged as an essential regulator of endothelial cell senescence and angiogenesis, key alterations commonly observed in atherosclerosis (Costantino et al. 2018b). Deregulation of chromatin modifying enzymes SUV39H1, JMJD2C, and SRC-1 in visceral fat arteries from obese patients was associated with increased di-(H3K9me2) and trimethylation (H3K9me3) as well as acetylation (H3K9ac) of histone 3 lysine 9 (H3K9) (Costantino et al. 2019). This epigenetic pattern was found

to promote transcriptional signatures involved in endothelial ROS generation and vascular dysfunction. Interestingly, *in vivo* editing of SUV39H1, JMJD2C, and SRC-1 blunted obesity-related endothelial dysfunction in obese mice (Costantino et al. 2019). Other histone deacetylases have shown to affect the atherosclerotic phenotype, although often with conflicting results. HDAC3, HDAC5, and HDAC7 were shown to repress KLF2 expression in human endothelial cells, while their overexpression fostered a proatherogenic phenotype (Kwon et al. 2014). In mice, HDAC3 and HDAC9 deletion in macrophages favors the upregulation of pro-inflammatory signatures eventually leading to a proatherosclerotic phenotype (Stratton et al. 2019). Consistently, *ApoE*^{-/-} mice with endothelial specific deletion of HDAC3 displayed increased neointimal formation, suggesting an atheroprotective role of endothelial HDAC3 (Zampetaki et al. 2010). Along the same line, *Ldlr*^{-/-} mice treated with the HDAC inhibitor Trichostatin A exhibited increased plaque size and enhanced macrophage infiltration (Choi et al. 2005). In rats, HDACs are upregulated in aortic SMCs under mitogenic stimulation and their inhibition alters the expression of cell cycle genes, resulting in diminished SMC proliferation, which might be expected to be atheroprotective (Findeisen et al. 2011).

Specific chromatin modifications occurring in macrophages, namely increased activity of the demethylase JMJD3, were also found to act as pivotal regulator of pro-inflammatory cytokines (i.e., TNF α), by erasing the H3K27me3 mark (Yan et al. 2014). Further work is needed to test whether chemical inhibition of JMJD3 has a protective effect in atherosclerotic mouse models. Technologies to probe H3K27me3 and other histone PTMs genome-wide in small samples will be vital to fully understand the cause and consequence of epigenetic changes that occur during atherogenesis.

5 Chromatin Signatures as Epigenetic Biomarkers in Atherosclerosis

Epigenetic signals acquired during the life course can be employed as potential biomarkers of cardiovascular disease. Indeed, modifications of the epigenetic landscape may reflect dynamic changes in expression of genes relevant to vascular homeostasis. A global reduction of DNA methylation was reported in patients with coronary atherosclerosis. The evaluation of the methylation status of LINE-1 due to its high representation in the human genome (55%) and its high methylation levels can be used to evaluate global methylation changes (Aavik et al. 2019). Hypomethylation of the IL-6 promoter, leading to its upregulation and systemic inflammation, was also associated with a higher risk of coronary heart disease (Zuo et al. 2016). The promoter of two atheroprotective genes, namely the estrogen receptor α (ESR1) and estrogen receptor β (ESR2), was hypermethylated in atherosclerotic vascular tissues as well as in cultured vascular SMCs (Napoli et al. 2012). Age-related mutations of key epigenetic regulators including DNMT3A, TET2, and ASXL1 in hematopoietic stem cells (HSCs) is emerging as a major mechanism underlying clonal expansion of HSCs, a phenomenon known as clonal

hematopoiesis of indeterminate potential (CHIP) (Li et al. 2018). Notably, the presence of CHIP was associated with an increased risk of CVD and all-cause mortality (Jaiswal et al. 2017). In the northern Sweden population health study, individuals with a history of MI showed differential DNA methylation at 211 CpG-sites representing genes related to CVD, cardiac function, cardiogenesis and recovery after ischemic injury (Rask-Andersen et al. 2016). Hence, epigenetic information may explain the alterations in cardiovascular gene expression trajectories and offer biomarkers for the follow-up of these patients. Overall, available evidence suggests that the epigenome represents a fundamental biological layer in the regulation of gene expression, and epigenetic information may contribute to advance CV risk stratification. The “epigenetic landscape” may provide a real post-genomic snapshot offering the tools to build individual maps of CV risk (Costantino et al. 2018c). Hence, epigenetic changes accumulated during the lifetime may be employed to customize diagnostic and therapeutic approaches in primary and secondary prevention of CVD. Individual epigenetic maps will be invaluable to define new risk scores predicting beyond traditional calculators. This innovative approach will be invaluable for the development of personalized therapies in patients with atherosclerosis (Fig. 4).

6 Epigenetic Drugs

Targeting epigenetic modifications is a highly promising approach to restore gene expression and to rescue or prevent alterations of vascular phenotype (Masi et al. 2020; Landmesser et al. 2020). There are several examples of how specific interventions can be employed to modify the landscape of DNA/histone modifications (Table 1). Emerging evidence indicates that circulating angiogenic cells (CACs), endothelial progenitor cells (EPCs) and, more in general, bone marrow-derived stem cells are critically involved in vascular repair and maintenance of vascular homeostasis via different mechanisms which include local engraftment and paracrine actions. Epigenetic mechanisms substantially contribute to the functional decline of these cells during life (Costantino et al. 2018b). Indeed, DNA methylation is decreased at the promoter of genes associated with self-renewal, whereas promoters of genes regulating differentiation are generally hypermethylated (Sun et al. 2014). Pharmacological blockade of the DNA methyltransferase by RG108 has shown to rejuvenate BM-derived stem cells by resetting the expression of senescence-related genes (Oh et al. 2015). These findings may open perspectives for the *ex vivo* epigenetic reprogramming of BM-derived cells for the treatment of atherosclerosis and ischemic heart failure. However, pharmacological editing of global DNA methylation lacks specificity and may be associated with undesirable effects, such as autoimmune diseases (lupus erythematosus was reported in 25–30% of patients) (Chen et al. 2017). The relevance of epigenetic drugs is outlined by the notion that several of these compounds are being tested in clinical trials for the treatment of cancer, neurological and cardiovascular diseases (Costantino et al. 2015). More than 20 years ago, Decitabine (5-aza-2'-deoxycytidine) was found to

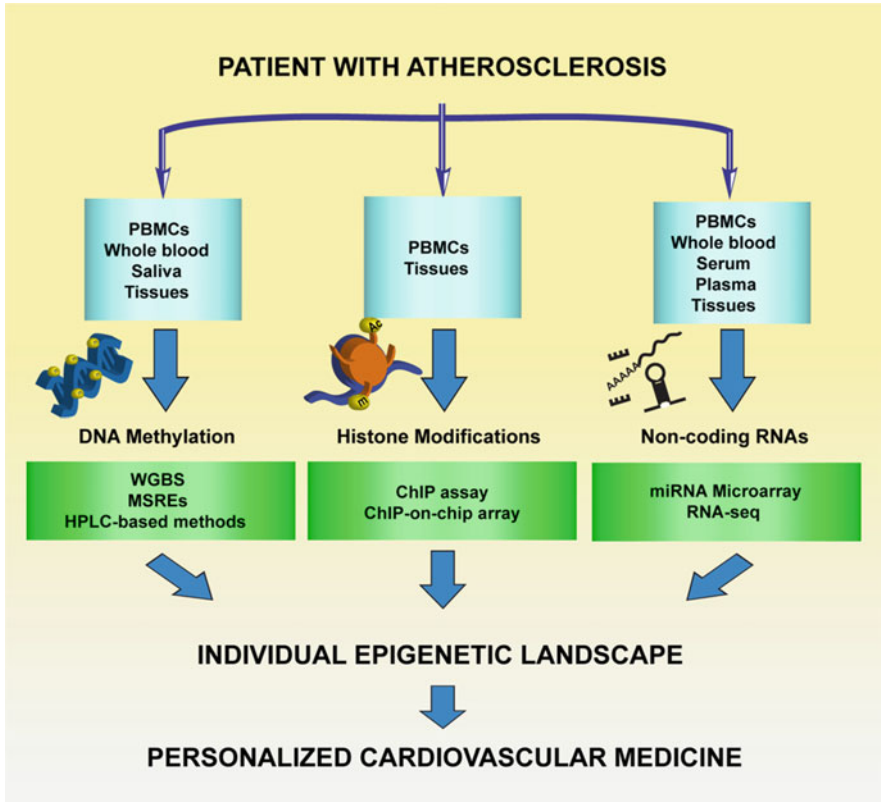


Fig. 4 Approaches to detect epigenetic biomarkers in patients with atherosclerosis. *PBMCs* peripheral blood mononuclear cells, *WGBS* whole-genome bisulfite sequencing, *MSREs* methylation sensitive restriction enzymes, *HPLC* high-performance liquid chromatography, *ChIP* chromatin immunoprecipitation, *miRNAs* microRNAs, *RNA-seq* RNA sequencing. Modified from Costantino et al. Eur Heart J. 2018;39:4150-4158

reactivate *ESR1* and *ESR2* expression in cancer cells and used to treat myelodysplastic syndromes. However almost 20 years later this drug was reported to blunt inflammation in endothelial cells in a mouse model of atherosclerosis (Dunn et al. 2014). The FDA-approved drug Vorinostat (suberoylanilide hydroxamic acid), a histone deacetylase inhibitor, has shown to prevent eNOS uncoupling, NF- κ B signaling, and oxidative stress in experimental diabetes (Advani et al. 2011). Vorinostat also promotes the autophagic flux, a process which is defective in atherosclerosis (Xie et al. 2014). In experimental models of atherosclerosis, the HDAC inhibitor sodium butyrate was shown to blunt NF- κ B signaling and inflammatory molecules, namely TNF- α , IL-6, VCAM-1, and ICAM-1 (Hu et al. 2014). Chronic treatment with the SIRT1 activator resveratrol improves endothelial function and insulin sensitivity in patients with obesity and T2D (Pollack and Crandall 2013). Metformin and glucagon-like peptide 1 (GLP-1), widely-used anti-diabetic

Table 1 Epigenetic drugs with potential application for the prevention and treatment of atherosclerosis

Drug	Epigenetic mechanism	Main finding
RG108	DNMT inhibitor	RG108 resets the expression of senescence-related genes in BM-derived stem cells
Azaticidine (5-AZA)	DNMT inhibitor	Blunts the upregulation of inflammatory genes in endothelial cells
(SAHA)/Vorinostat	HDAC inhibitor	Prevents eNOS uncoupling and activation of NF-kB signalling in the setting of diabetes. Reactivates autophagy
Sodium butyrate	HDAC inhibitor	Suppresses NF-kB signaling and transcription of NF-kB-dependent inflammatory molecules
Resveratrol	HDAC activator	Improves endothelial function in obese and diabetic patients by activating SIRT1
Metformin	HDAC activator	Preserves the expression of genes implicated in beta cell function and insulin signaling via activation of SIRT1
GLP-1	HDAC activator	Modulates SIRT1 activity thus preserving insulin signaling
Curcumin	HAT inhibitor	Reduces the expression of proatherogenic cytokines and attenuates atherosclerotic lesion size in ApoE and LDLR double knockout mice. Curcumin supplementation in diabetic patients ameliorates proteinuria, reduces pro-fibrotic cytokines and improves microangiopathy.
Apabetalone	BET inhibitor	Stimulates reverse cholesterol transport by inducing ApoAI expression and increasing HDL levels. It has also shown to decrease systemic inflammation in humans, as assessed by C-reactive protein as well as to act as an anti-thrombotic agent.

DNMT DNA methyltransferase, *HAT* histone acetyltransferase, *HDAC* histone deacetylase, *HMT* histone methyltransferase

medications, have also shown to modulate SIRT1 activity, thus affecting histone acetylation and transcription of genes implicated in beta cell function and preservation of insulin signaling (Costantino et al. 2015). The histone acetyltransferase p300 inhibitor curcumin has shown to reduce lipopolysaccharide-induced expression of key proatherogenic cytokines such as MCP-1, IL-1 β , and TNF- α in primary human monocytes as well as to reduce macrophage polarization (Moss and Ramji 2016). Chronic treatment (4 months) with curcumin reduced atherosclerotic lesion size in ApoE and LDLr double knockout mice (Hasan et al. 2014). Moreover, a randomized double-blind trial involving 240 individuals with T2D reported a decrease in CVD risk with 6 months of curcumin dietary supplementation, exemplified through a lower pulse wave velocity and improved metabolic profile (Chuengsamarn et al. 2014). In T2D patients, chronic supplementation with curcumin (1–2 months) ameliorated proteinuria, reduced pro-fibrotic cytokines (i.e., TGF- β and IL-8), and improved microangiopathy (Khajehdehi et al. 2011). Current limitations in the use of curcumin include its poor bioavailability due to a very rapid excretion from the body (Lopresti 2018). The use of liposomal curcumin, nanoparticles, and a curcumin phospholipid complex represents current strategies to boost the clinical applicability

of this compound. Several other compounds including folates, apicidin, PPAR γ agonists, and valproic acid have shown the ability to revert chromatin modifications in cardiometabolic states (Napoli et al. 2012). Recent evidence indicates that apabetalone (RVX-208) – an epigenetic regulator targeting bromodomain and extra-terminal (BET) proteins – stimulates reverse cholesterol transport by inducing ApoAI expression and increasing HDL levels (Ghosh et al. 2017). This drug has also shown to decrease systemic inflammation in humans, as assessed by C-reactive protein as well as to act as an anti-thrombotic agent (Schooling and Zhao 2019). Pooled analysis of short-term non-randomized studies showed fewer cardiovascular events among patients treated with apabetalone as compared to placebo (Nicholls et al. 2018). The recent phase III BETonMACE trial, designed to investigate the impact of apabetalone on cardiovascular outcomes in 2425 patients with diabetes after an ACS, failed to meet the primary endpoint (cardiovascular death, non-fatal myocardial infarction, or stroke) (Ray et al. 2020). However, the drug showed to significantly impact on secondary endpoints. Undoubtedly, larger clinical trials are needed to better explore the safety and efficacy of apabetalone for the treatment of cardiovascular disease.

7 Conclusions

Evidence discussed here suggests that epigenetic processing plays a central role in the pathogenesis of atherosclerosis. Specific epigenetic patterns, characterized by modifications of DNA/histone complexes as well as by ncRNAs, have shown to promote transcriptional programs involved in inflammation, oxidative stress, and endothelial dysfunction. Plastic changes may also contribute to the phenotypic switch of SMCs, a key factor involved in the pathophysiology of atherosclerosis. A careful analysis of the individual epigenetic landscape may furnish novel biomarkers of atherosclerotic vascular disease. An important challenge is the demonstration that epigenetic signals are causally linked to vascular disease phenotypes. The causality of presumed epigenetic events and atherosclerotic phenotypes can be assessed by using different strategies. One possibility would be using longitudinal studies whereby the epigenome can be profiled before the onset of the disease and during follow-up. Alternatively, specific tools, such as the framework of Mendelian randomization which explores the causal link between exposure, epigenetic marks and outcome, can be employed to discriminate between epigenetic phenomena and epi-phenomena. Ongoing epigenomic studies will help to unveil the complex relationship among genetics, epigenetics, and CVD, and will contribute to characterize the incremental value of epigenetic information over established clinical and genetic scores. Epigenetic information can also be used to develop new mechanism-based therapies. The growing understanding of chromatin architecture and metabolism has led to the design of specific molecules able to modulate chromatin accessibility by enhancing or repressing epigenetic marks on DNA/histone complexes. Noteworthy, some of these drugs have been already approved for the treatment of several conditions including cancer, neurological and cardiovascular disease. Taken

together, epigenetic information could advance individualized risk assessment and personalized therapeutic approaches in patients with cardiometabolic disturbances.

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Correction to: Blood Pressure-Lowering Therapy

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Correction to:
Chapter “Blood Pressure-Lowering Therapy” in:
Isabella Sudano et al., Handbook of Experimental
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The Open Access chapter ‘Blood Pressure-Lowering Therapy’ was published online unfortunately without the Conflict of Interest statement. The COI statement should appear as:

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FR has been paid for the time spent as a committee member for clinical trials, advisory boards, other forms of consulting and lectures or presentations. These payments were made directly to the University of Zurich and no personal payments were received in relation to these trials or other activities since January 2018.

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Before 2018, FR reports grants and personal fees from SJM/Abbott, Servier, Novartis and Bayer, personal fees from Zoll, Astra Zeneca, Sanofi, Amgen, BMS, Pfizer, Fresenius, Vifor, Roche, Cardioentis and Boehringer Ingelheim, other from Heartware and grants from Mars, outside the submitted work.

These corrections have been updated in the original chapter.