Genetic and biochemical risk factors in coronary artery disease
Boekholdt, S.M.
General introduction and outline of the thesis

S. Matthijs Boekholdt, Ron J.G. Peters, John J.P. Kastelein

Departments of Cardiology and Vascular Medicine,
Academic Medical Center, Amsterdam, The Netherlands

General introduction

Cardiovascular disease is by far the most common cause of morbidity and mortality worldwide.¹ In the Western World, this has been the case for decades but the burden of cardiovascular disease is not limited to the developed world. In developing countries, cardiovascular disease outnumbers all other causes of morbidity and mortality, including infectious disease.²,³ However, in many developing countries, a transition is taking place towards urbanization, industrialization, and more Western life styles which will inevitably lead to a further increase in cardiovascular morbidity and mortality.²,³ The term cardiovascular disease comprises clinical manifestations of arterial atherosclerosis or atherothrombosis, such peripheral artery disease, cerebrovascular disease, and coronary artery disease (CAD). Well-established risk factors are dyslipidemia, smoking, diabetes, hypertension, and abdominal obesity.⁴ The pathogenesis of atherosclerosis is considered to be multifactorial, i.e. caused by a combination of genetic predisposition, lifestyle, and environmental influences.

An analysis of the Swedish Twin Registry, which contains data from 21,004 twins born in Sweden between 1886 and 1925, showed that men whose monozygotic twin died of CAD, had an 8-fold increased risk of dying from CAD.⁵ Among women, this relative risk was 15 times higher. Similarly, people who had a parent who died of CAD before the age of 55, had a 2-fold increased risk to develop CAD.⁶ These results suggest that hereditary predisposition plays an important role in determining the risk of CAD, even after adjustment for traditional risk factors such as cholesterol level, hypertension and smoking. However, genetic predisposition is but a part of the puzzle. Migration studies have shown that within one generation, the risk of cardiovascular disease can change dramatically by a change of lifestyle, suggesting that besides genetic predisposition, environmental factors play a pivotal role in determining this risk. For instance, Japanese who migrated to Hawaii and California had a striking increase in the incidence of myocardial infarction compared to Japanese living in Japan.⁷ Similarly, within 2 years, people from a rural Kenyan tribe who migrated to urban areas developed higher blood pressure than their tribesmen who did not migrate.⁸ The current consensus is that atherosclerosis and its clinical manifestations result from a dynamic, lifelong interaction among genetic, environmental, and behavioural factors.

Pathogenesis of atherosclerosis

For decades, atherosclerosis has been considered a disease of the arteries caused by excessive cholesterol storage. After their Nobel Prize-winning discovery of the LDL receptor as a modulator of cholesterol metabolism,⁹ Brown and Goldstein declared that their discovery would lead to the eradication of myocardial infarction by the year 2000. Unfortunately, this was not the case. Part of this may be attributable to the fact that available treatment modalities are not being used to their full potential, for instance
by suboptimal use of medication and other preventive strategies. On the other hand, atherosclerosis has proved to be a disease that is considerably more complicated than suspected by Brown and Goldstein, and certainly not explained by elevated cholesterol levels alone. Accumulating evidence suggests that the atherosclerotic disease process in the arterial wall is the consequence of an intricate interplay between numerous related and interacting processes and pathways, such as the transport of cholesterol into the arterial wall, reverse cholesterol transport from peripheral tissues back to the liver, oxidation of constituents of these lipoproteins which may trigger an inflammatory response in the vessel wall, pro-inflammatory mediators which may enhance lipoprotein uptake by macrophages and enhance their pro-inflammatory response, protection against oxidation and inflammation by enzymes on some lipoproteins, and pro-oxidative enzymes which may interfere with reverse cholesterol transport. Finally, clinical manifestations of atherosclerosis, which are often caused by atherosclerotic plaque rupture and subsequent superimposed thrombotic occlusion of the artery, may depend on interplay between pro- and anti-inflammatory pathways and pro- and antithrombotic mechanisms. Any classification of all these interrelated and interacting pathophysiological pathways into separate categories is arbitrary and inadequate. However, for reasons of clarity and readability, I have classified them into three sections.

**Lipids, lipoproteins, and apolipoproteins**

Cholesterol is assumed to be the driving force in atherosclerosis because populations with low levels of low-density lipoprotein cholesterol (LDL-c) have a very low incidence of cardiovascular disease, despite having similar levels of other risk factors. The majority of cholesterol in plasma is transported in LDL particles, which carry one apolipoprotein B (apoB) molecule each. Historically, quantification of this plasma component is done by measurement of the amount of cholesterol carried in LDL particles. However, evidence is now accumulating that the number of LDL particles may be a more accurate predictor of CAD risk. Passive diffusion drives entry of the LDL particle into the vessel wall, and exit from it. The balance between these processes determines the amount of LDL-c present in the arterial wall, and the length of time lipoproteins spend there. Increased retention of LDL particles in the arterial wall is an early hallmark of atherosclerotic lesion progression. Consequently, factors that affect either of these processes may result in increased or decreased cardiovascular risk. For instance, it was recently noted that the atherogenicity of apoB-containing lipoproteins is associated with their affinity for proteoglycans in the arterial wall. Mice expressing proteoglycan-binding-defective LDL developed significantly less atherosclerosis than mice expressing control LDL. A pathway also exists to transport cholesterol from peripheral tissues back to the liver for excretion into the bile, a mechanism known as reverse cholesterol transport. In short, nascent HDL is formed by the lipidation
of apolipoprotein A-I (apoA-I), the principal protein component of HDL particles which is synthesized in both the intestine and the liver. These pre-beta particles acquire free cholesterol from peripheral cells avidly through an export process facilitated by ATP binding cassette transporter 1 (ABCA1). A genetic deficiency of ABCA1 results in Tangier disease, which is characterized by absence of HDL-c and accelerated CAD.\textsuperscript{21,22} Similarly, partial genetic ABCA1 deficiency results in defective cholesterol efflux from peripheral tissues, and increased progression of atherosclerosis.\textsuperscript{24} Free cholesterol is subsequently esterified to cholesteryl ester (CE) by lecithin: cholesterol acyltransferase (LCAT), an enzyme that is associated with HDL particles and uses apoA-I as a cofactor.\textsuperscript{25} CE can then be transported to the liver directly, and taken up selectively by the scavenger receptor class B type 1 (SRB1), at least in mice. Endocytosis of the entire HDL particle by the \(\beta\)-chain of ATP synthase may also play a role in this process.\textsuperscript{26} Alternatively, CE can be transported to the liver indirectly, after transfer from HDL particles to apoB-containing lipoproteins which in turn interact with the liver LDL receptor. This transfer process of CE between HDL and apoB-containing lipoproteins is facilitated by cholesteryl ester transfer protein (CETP), and is reciprocated by triglyceride transfer from very low density lipoproteins (VLDL) to LDL and HDL.\textsuperscript{27} The distribution of CE among LDL and HDL particles is crucial for the development of atherosclerosis, and may be partially determined by the plasma concentration of CETP. Therefore, various recent studies have focused on the hypothesis that a genetic variant in the CETP gene which is associated with slightly lower CETP plasma levels, is also associated with a decreased risk of CAD, but the results have been inconsistent.\textsuperscript{28-30}

\textit{Oxidation and inflammation}

As described above, a longer residence time of LDL particles in the arterial wall increases the likelihood of chemical modifications, which may enhance their atherogenicity. For instance, type II secretory phospholipase A2 (sPLA2) is an enzyme that hydrolyzes the sn2 ester bond of phospholipids, which form the outer layer of lipoproteins. This sPLA2-mediated modification yields LDL particles with a higher affinity for proteoglycans, thus increasing their residence time in arterial wall. Some small-scale evidence suggests that higher circulating levels of sPLA2 are associated with an increased risk of CAD.\textsuperscript{31} In addition to enhancing the residence time of LDL particles, sPLA2 may also have direct pro-atherogenic effects on these lipoproteins, for instance by hydrolizing phospholipids, which yields lysophospholipids and arachidonic acid, known precursors of various proinflammatory mediators such as leukotrienes and prostaglandines.\textsuperscript{32} Also, sPLA2 has been shown to modify lipoproteins such that they are more susceptible to lipid peroxidation.\textsuperscript{33} Such oxidative modifications of LDL lipoproteins are essential in atherogenesis because uptake of LDL particles by macrophages in the vessel wall is a first step towards the transformation of these macrophages into foam cells, a cell
type that characterizes advanced atherosclerosis. Yet, paradoxically, native LDL particles are recognized poorly by macrophages and do not produce CE accumulation in these cells in culture. Biological modification converts native LDL particles into a form that can be recognized by macrophage receptors and that leads to greatly enhanced cellular uptake and promotion of CE accumulation. This modification is characterized by oxidative changes of the apoB molecule present on each LDL particle, and also of the phospholipid outer membrane of these particles. However, oxidative modification of LDL particles may not only play a crucial role in the uptake by macrophages which is important in foam cell formation, but may also be involved in conformational changes of these LDL particles which may be crucial in triggering the innate immune system to initiate an inflammatory response. These oxidative modifications comprise a wide range of molecular alterations. Importantly, it is now well-recognized that certain modifications of phospholipids lead to the exposure of the phosphorylcholine (PC) subgroup, which remains cryptic in native LDL. A similar exposure of PC groups occurs when viable cells turn apoptotic. These PC subgroups are structurally identical to molecular patterns in the cell membrane of gram-negative bacteria, including S. pneumoniae. This molecular mimicry has led to the hypothesis that mediators of the innate immune system mount an inflammatory reaction against newly exposed auto-antigens which are recognized as molecular patterns associated with true pathogens. Several mediators of the innate immune response have been identified as recognizing these PC groups, including the scavenger receptor toll-like receptor-4 (TLR-4), C-reactive protein (CRP), and a natural anti-oxLDL antibody of the E06 idiotype. Interestingly, this E06 antibody was shown to be 100% homologous with an antibody directed against S. pneumoniae. Several studies have shown that oxidized phospholipids bearing the PC subgroup as ligands on oxLDL mediated the uptake by macrophages. In addition, ligation of TLR-4 with certain oxidized phospholipids may lead to the release of pro-inflammatory cytokines, including interleukin-8 (IL-8). Similarly, CRP binds to oxLDL and to apoptotic cells by ligation with PC. Thus, PC exposure, which occurs in oxLDL and apoptotic cells, generates an antigen that is recognized by CRP, the natural antibody EO6 and certain macrophage scavenger receptors, including TLR-4, and may mediate highly conserved and concerted innate responses.

The interaction between PC subgroups exposed on apoptotic cells and CRP may not only be relevant in the initiation and progression of atherosclerosis, but also in the setting of clinical cardiovascular events caused by myocardial ischemia. Evidence exists to support the hypothesis that CRP can bind to ischemic cardiomyocytes and initiate the complement cascade which aggravates the inflammatory reaction ensuing in ischemic myocardium and enhances myocardial damage. For instance, experimental myocardial infarction models have shown that raising CRP levels before coronary occlusion resulted in increased
infarct size.\textsuperscript{47,48} This effect was inhibited by complement depletion. In humans, immunohistochemical studies have shown co-localization of CRP and complement in the hearts of patients who died from myocardial infarction.\textsuperscript{49,50}

Finally, oxidation may also have atherogenic effects that are not related to the inflammatory processes in the arterial wall, but to their effect in plasma. Circulating levels of myeloperoxidase, an oxidizing enzyme secreted by activated neutrophils, have been shown to predict cardiovascular events in people with unstable angina.\textsuperscript{51} Myeloperoxidase-catalyzed oxidation has been shown to induce functional impairment of apoA-I.\textsuperscript{52,53} Because interaction between apoA-I and ATP binding cassette transporter-1 (ABCA1) is essential for the uptake of cholesterol from peripheral cells by avid HDL particles, functional impairment of apoA-I leads to accumulation of cholesterol in peripheral tissues.

The inflammatory processes that evolve in the arterial wall are initiated and perpetuated by a concert of cytokines, interleukins and other signalling molecules. Circulating levels of these inflammatory mediators may be used as predictors of cardiovascular events; first, because they reflect the extent of atherosclerosis in the arterial wall, and second, because atherosclerotic plaque rupture occurs predominantly at sites of plaque inflammation.\textsuperscript{54} As described above, IL-8 is one of the messengers that are expressed by macrophages upon contact with oxLDL. IL-8 plays an important role in the recruitment of leukocytes into the subendothelial space, a process known to occur in early stages of atherosclerosis. Evidence from murine atherosclerosis models suggests that initial leukocyte adhesion to the endothelium is mediated by KC, the murine equivalent of IL-8, whereas subsequent interaction between monocyte chemoattractant protein-1 (MCP-1) is and its receptor CCR1 is essential for transendothelial diapedesis and recruitment into the arterial wall.\textsuperscript{55} In mice, atherosclerosis can be largely prevented by eliminating the genetic expression of IL-8, MCP-1 or their leukocyte receptors.\textsuperscript{56-58} Another inflammatory marker that has received extensive attention is CRP. Numerous epidemiological studies have established CRP as a predictor of future cardiovascular events in people with stable CAD\textsuperscript{59} and unstable CAD,\textsuperscript{60,61} and even in apparently healthy individuals.\textsuperscript{62,63} A recent clinical guideline\textsuperscript{64} has suggested that CRP measurement could be used in clinical practice at the discretion of the physician to improve risk assessment in people with prevalent CAD, and in people without prevalent CAD at intermediate risk of cardiovascular events (10-20\% over the next 10 years) as estimated by established risk scores.\textsuperscript{65,66}

This recommendation has recently been challenged by a very large prospective study and updated meta-analysis\textsuperscript{67} which suggested that the risk estimate for CRP was importantly lower than described in a previous meta-analysis,\textsuperscript{68} and that CRP levels added only marginally to the predictive value of traditional cardiovascular risk factors. This conclusion has caused an intense and ongoing debate.\textsuperscript{69-73}
Coagulation and fibrinolysis

The most common cause of acute coronary events is atherosclerotic plaque rupture with superimposed thrombosis, leading to arterial occlusion. This hypothesis implicates the haemostatic system as a potentially relevant determinant of cardiovascular events. In addition, the haemostatic system may be relevant in the initiation and progression of atherosclerosis as well. Platelet adhesion and mural thrombosis are ubiquitous in the initiation and generation of the lesions of atherosclerosis in animals and humans. Platelets can adhere to dysfunctional endothelium, exposed collagen, and macrophages. When activated, platelets release their granules, which contain cytokines and growth factors that may contribute to the migration and proliferation of smooth-muscle cells and monocytes. Activation of platelets leads to the formation of free arachidonic acid, which can be transformed into prostaglandins such as thromboxane A2, a potent vasoconstricting and platelet-aggregating substance, or into leukotrienes, which can amplify the inflammatory response. This has led to the hypothesis that variations in the coagulation and fibrinolytic systems can predispose carriers to accelerated atherosclerosis, or to an increased risk of its clinical manifestation including coronary events. The limited evidence about people with hypocoagulable disorders suggests that these people are at least not protected against the development of atherosclerosis.

Levels of various coagulation factors have been studied in relation to the risk of cardiovascular events. A strong relationship is consistently observed for fibrinogen, but most likely this is not explained by its role in the coagulation cascade but by its expression pattern as an acute phase reactant. Thus, high fibrinogen levels may be associated with an increased risk of CAD not via hypercoagulability but because they reflect low-grade inflammation of the arterial wall, which is associated with an increased risk of atherosclerotic plaque rupture. No strong or consistent relationship has been observed for factor VII levels, whereas the associations with von Willebrand factor (vWF), factor VIII, tissue-type plasminogen activator (t-PA), and plasminogen activator inhibitor-1 (PAI-1) are moderate, and dependent on modifying factors. Thus, the observed relationships are moderate and probably not causal in the development of cardiovascular events. Instead, they may reflect low-grade inflammation of the arterial wall or endothelial cell activation. In recent years, numerous genetic association studies have explored the relationships between variants in genes for haemostatic proteins and the risk of coronary events. A limited number of variants has received extensive attention, including the factor V Leiden mutation and the prothrombin G20210A mutation which both predispose to venous thromboembolism, the fibrinogen G(-455)A variant which is associated with increased fibrinogen plasma levels, the PAI-1 4G/5G polymorphism, and the Leu33Pro variant in glycoprotein IIIa, which is one of the subunits of the platelet IIb/IIIa receptor. All these variants have no, or only a very limited effect on the risk of cardiovascular events.
In summary, the initiation of atherosclerosis, its progression over time, and the development of its clinical manifestations are multifactorial processes that result from an intricate interplay of related and interacting pathophysiological pathways. Some players in these pathways may have a substantial effect on the ultimate risk of cardiovascular events, others may have a limited effect or none at all, and yet others may have effects that vary depending on other genetic or environmental factors.

*Individual predisposition to atherosclerosis*

Today, a person's genetic background is considered in every aspect of clinical medicine, ranging from susceptibility to diseases, pathogenesis, and clinical outcome to diversity in responses to drug treatment (pharmacogenomics). The new panoramic look at the human genome has stimulated a massive search for clinically relevant genomic information, including single-nucleotide polymorphisms (SNPs), which consist of substitutions of one nucleotide for another in a DNA sequence. Individual genomes are 99.9 percent identical, with only 0.1 percent of the genome showing polymorphisms. About 2 to 3 million SNPs have been found in exonic, intronic, regulatory, and intergenic regions. Almost all genes contain SNPs, but only a minority may have functional consequences because they either affect the expression level of a protein or predict an alteration of the amino acid sequence. Complexity increases at the protein level, since one human gene may produce up to five different proteins as a result of alternative splicing. Posttranslational modifications, such as assembly or glycosylation, further increase the diversity of proteins. Furthermore, environmental and other genetic factors may alter the phenotype that results from genetic abnormalities. Such an effect has recently been found in a family with hypertrophic cardiomyopathy caused by a single mutation in the gene for myosin-binding protein C. SNPs in five components of the renin-angiotensin-aldosterone system were found to determine the degree of left ventricular hypertrophy. Also, data from the Framingham Study have suggested that the effect of the common C(-514)T variant in the promoter of the hepatic lipase gene is modified by nutrient intake. Subjects with the TT genotype had low concentrations of HDL-c, but only if their fat intake was at least 30 percent of their total consumed energy. Similarly, the factor V Leiden mutation may become a stronger risk factor for thromboembolism in women who use oral contraceptives. Such gene-gene and gene-environment interactions are usually not taken into account in genetic association studies because they are poorly understood. Better understanding of the genetic and environmental context in which a genetic variant may have an effect, is therefore warranted. As a consequence of these biological complexities, poor reproducibility is often experienced in the field of genetic association studies. In addition to true biological effects that influence the relationship of interest, a number of methodological and epidemiological factors may distort the results of genetic association studies as well, such
as differences in patient and control definition, population heterogeneity, and limited statistical power. These limitations have cast doubts on this type of study, and some biomedical journals have even adopted a policy of not publishing the results of association studies related to complex diseases.

These difficulties, however, should be weighed against the potential benefits of genetic research. Because genetic predisposition does not fluctuate as do, for instance, plasma levels of a given risk factor, risk prediction based on genetic profiling would be a very effective approach. In addition, studying protein risk factors without their genetic determinants will likely result in an incomplete understanding of the pathophysiology. Recently, recommendations have been made for the design of future genetic association studies. The first recommendation concerned the adequacy of statistical power; recent evidence suggests that the large majority of genetic association studies performed thus far have been underpowered to detect the difference they were designed for. A second important recommendation was that an observed genotype-disease association should be accompanied by supporting evidence. Such evidence could be in the form of replication in an independent cohort. Also, supporting evidence could be supplied by a plausible biological explanation based on the functional consequences of the variant allele. For instance, the factor V Arg506Gln mutation, which is associated with an increased risk of venous thromboembolism, has been shown to be located at the exact site where in the wild-type protein inactivation by activated protein C would occur. As a consequence, this mutation leads to a gain of function caused by defective inactivation, and the genetic abnormality is therefore consistent with our knowledge of the underlying physiology. Also, carriers of the Asp299Gly variant in the TLR4 gene have a higher risk of developing septic shock during gram-negative infections. This observation is consistent with the observation that, upon ligation with endotoxin, the variant receptor initiates a blunted inflammatory response, compared to the wild-type receptor. Finally, support for an observed genotype-disease relationship could be obtained by studying the triangulation of genotype and phenotype associations with disease risk, an approach also known as Mendelian randomization. This approach is based on Mendel’s second law which assumes the random assortment of genes from parents to offspring that occurs during gamete formation and conception, or in Mendel’s words:

“the behaviour of each pair of differentiating characteristics in hybrid union is independent of the other differences between the two original plants, and, further, the hybrid produces just so many kinds of egg and pollen cells as there are possible constant combination forms.”

Put simply, this law suggests that the inheritance of one trait is independent of the inheritance of other traits, and provides a method for assessing the causal nature of environmental exposures. The basic idea is that, if genetic variants produce differences in an intermediate phenotype
which in turn alters disease risk, the variants should themselves be related to disease risk to the extent predicted by their influence on the phenotype. For instance, the status of plasma fibrinogen levels as a causal risk factor for CAD remains controversial.\textsuperscript{95,116} Fibrinogen levels certainly predict CAD risk, with the latest meta-analysis reporting a relative risk (RR) of 1.8 (95% CI: 1.6–2.0) for the top to the bottom tertile of the fibrinogen distribution.\textsuperscript{80} However, existing atherosclerosis increases fibrinogen, generating reverse causation between disease and the apparent risk factor, and also there is substantial confounding, with higher fibrinogen levels being seen in smokers, people from less-favourable socioeconomic backgrounds, and non-drinkers.\textsuperscript{117} Recently a large case-control study has examined this issue.\textsuperscript{118} The G(-455)A variant in the β-fibrinogen gene is associated with fibrinogen levels, such that for each A allele there was an increase of 0.12 g/l in fibrinogen. In turn, an increase in fibrinogen levels by 0.12 g/l was associated with an RR of CAD of 1.20 (95% CI: 1.13–1.26). However, when genotype was related to CAD risk, essentially no relationship was seen, with a per allele RR of 1.03 (95% CI: 0.96–1.10). Mendelian randomization provides new opportunities to test causality and demonstrates how investment in the human genome project may contribute to understanding and preventing the adverse effects on human health of modifiable exposures. In the future, well-designed genetic association studies with adequate statistical power may provide new insights into the relationships among genetic variants, intermediate risk factors, and disease risk.

Outline of the thesis
Atherosclerosis can reach the clinical horizon as a range of clinical manifestations. This thesis will focus on coronary artery disease only. It describes a range of factors, both at a genetic and at a plasmatic level, that may be relevant in the development of coronary artery disease. The chapters have been divided into three parts, based on the arbitrary division of pathophysiological themes, as described above.
Part I contains a number of chapters on lipids, lipoproteins and apolipoproteins. Chapter 2 describes a meta-analysis of 3 well-studied genetic variants in apoB, the principal protein component of LDL particles. Chapter 3 describes the role of circulating levels of apoA-I and apoB, in comparison with HDL-c and LDL-c, in the prediction of CAD risk. Chapters 4 through 6 describe the potential relevance of CETP for the distribution of lipid levels and cardiovascular risk. The respective chapters describe the background of genetic research on CETP, a meta-analysis of large studies on the relationship between a genetic variant in the CETP gene, HDL-c levels, and CAD risk, and a study on the relationship between CETP plasma levels and future CAD risk. Chapter 7 describes the potential for therapeutic inhibition of CETP in the primary and secondary prevention of cardiovascular disease in the near future.
Part II addresses oxidation and inflammation. Chapter 8 describes the relationship between circulating levels of sPLA2 and the risk of future...
CAD in apparently healthy individuals. Chapter 9 explores the relationship between genetic variants in the macrophage receptor TLR4 and the risk of future cardiovascular events, and chapters 10 and 11 describe the respective relationships between interleukin-8 and macrophage migration inhibitory factor, two pro-inflammatory cytokines, and the risk of future CAD. Chapter 12 describes the relationship between plasma levels of CRP and the risk of future CAD, with an emphasis on the distinction between fatal and non-fatal CAD.

Part III explores the coagulation and fibrinolytic systems in the development of clinical manifestations of cardiovascular disease, in particular CAD. Chapter 13 describes an interaction between a genetic variant of glycoprotein IIa and fibrinogen levels in determining the risk of cardiovascular events. Chapter 14 is a meta-analysis of published studies on the relationships between genetic variants in 4 haemostatic proteins: factor V, prothrombin, fibrinogen, and plasminogen activator inhibitor-1. Chapter 15 describes our exploration of a potential interaction between fibrinogen levels and a genetic variant in factor XIII in determining the risk of future cardiovascular events.
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