Cardiovascular Risk Genes in Prevention and Treatment Response

Hamrefors, Viktor

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Viktor Hamrefors, MD

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Pennington Biomedical Research Center
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Abstract

GENERAL AIM: To investigate how common single-nucleotide-polymorphisms (SNPs) that associate with cardiovascular disease (CVD) could be used in prevention and treatment of CVD.

SUBJECTS: Subjects from the population-based Malmö-Diet-and-Cancer-(MDC)-Study (n=30447) and hypertensives from the Nordic-Diltiazem-(NORDIL)-Study (n=10881).

METHODS AND RESULTS: A nine-SNP-lipid-genetic-risk-score was related to fluvastatin treatment-response in 395 MDC-subjects with asymptomatic carotid atherosclerosis. In women, a higher score (conferring unfavorable baseline-lipid-levels) correlated with HDL-increase (P=0.001), explaining 11.6-12.9% of the variance in HDL-change.

A 13-SNP-myocardial-infarction-(MI)-genetic-risk-score was related to carotid atherosclerosis-markers in 4022 MDC-subjects. The MI-gene-score associated with carotid-bulb-intima-media-thickness (IMT) (beta=0.038 standard deviations of IMT per MI-gene-score-quintile; P-trend=0.005) and plaque (odds-ratio per MI-gene-score-quintile=1.11; 95% confidence interval [CI]:1.04-1.18; P=0.001) in multivariable models.

It was tested if eight blood-pressure-associated SNPs affected antihypertensive treatment-response in 3863 Swedish hypertensives from NORDIL. No robust associations were identified.

Finally, interactions between life-style-factors and the CVD-SNP rs4977574 on chromosome 9p21 were evaluated in 24944 MDC-subjects during 15 years follow-up. There were interactions between rs4977574 and smoking on incident CAD (P=0.035) and CVD-mortality (P=0.012). The risk conferred by rs4977574 in never-smokers (n=9642; Hazard-ratio[HR] per risk-allele[CAD]=1.26; 95%CI:1.13-1.40; HR per risk-allele[CVD-mortality]=1.40; 95%CI:1.20-1.63) was attenuated in smokers (n=7000; HR per risk-allele[CAD]=1.05; 95%CI:0.95-1.16; HR per risk-allele[CVD-mortality]=1.08; 95%CI:0.94-1.23).

CONCLUSIONS: CVD-genetics identifies subjects with markers of subclinical atherosclerosis, suggesting that early atherosclerosis-prevention may be targeted to such individuals. Smoking attenuates the relative influence of the thus far strongest identified polygenic CVD-risk-locus, implying potential utility of common CVD-genetics in mainly conventional lower-risk-subjects. Lipid-polymorphisms may predict statin-induced HDL-increase in women, but eight blood-pressure-SNPs did not affect antihypertensive treatment-response.

Key words
Coronary artery disease, stroke, cardiovascular genetics, cardiovascular prevention, dyslipidemia, statins, hypertension, blood pressure treatment, chromosome 9p21, pharmacogenetics, pharmacogenomics

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Viktor Hamrefors, MD

LUND
UNIVERSITY

Department of Clinical Sciences in Malmö
Faculty of Medicine
Lund University, Sweden

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To Emma and Hugo

Contents

List of papers

This thesis is based on four studies presented in four papers, which are referred to in the text by their corresponding Roman numerals.

I. Hamrefors V, Orho-Melander M, Krauss RM, Hedblad B, Almgren P, Berglund G, Melander O. A gene score of nine LDL and HDL regulating genes is associated with fluvastatin-induced cholesterol changes in women. J Lipid Res 2010;51:625-634.

II. Hamrefors V, Hedblad B, Engström G, Almgren P, Sjögren M, Melander O. A myocardial infarction genetic risk score is associated with markers of carotid atherosclerosis. J Intern Med 2012;271:271-281.

III. Hamrefors V, Sjögren M, Almgren P, Wahlstrand B, Kjeldsen S, Hedner T, Melander O. Pharmacogenetic implications for eight common blood pressure-associated single-nucleotide polymorphisms. J Hypertens 2012;30:1151-1160.

IV. Hamrefors V, Hedblad B, Hindy G, Smith JG, Almgren P, Engström G, Sjögren M, Gränsbo K, Orho-Melander M, Melander O. Smoking modifies the associated increased risk of future cardiovascular disease by genetic variation on chromosome 9p21. PLoS One 2014: In press.

Reprints of the papers are enclosed at the end of the thesis with permissions from the publishers.

Abbreviations

95 % CI	95 % confidence interval
ACS	acute coronary syndrome
ARIC	Atherosclerosis Risk in Communities Study
ASE	American Society of Echocardiography
BP	blood pressure
CA	coronary angiography
CABG	coronary artery by-pass graft surgery
CAD	coronary artery disease
CCA	common carotid artery
CCTA	coronary computed tomography angiography
CMRI	cardiac magnetic resonance imaging
CNV	copy number variation
CRP	C-reactive protein
CT	computed tomography
CVD	cardiovascular disease
DBP	diastolic blood pressure
EC	endothelial cell
ECA	external carotid artery
FRS	Framingham risk score
GRS	genetic risk score
GWAS	genome wide association study
HDL	high density lipoprotein
HR	hazard ratio

ICA	internal carotid artery
IMT	intima media thickness
IVUS	intravascular ultrasound
LD	linkage disequilibrium
LDL	low density lipoprotein
MDC study	Malmö Diet and Cancer study
MI	myocardial infarction
NSTEMI	non-ST-elevation myocardial infarction
OCT	optical coherence tomography
OR	odds ratio
oxLDL	oxidized low density lipoprotein
PCI	percutaneous coronary intervention
PAD	peripheral artery disease
PET	positron emission tomography
SBP	systolic blood pressure
SCAD	stable coronary artery disease
SCD	sudden cardiac death
SMC	smooth muscle cell
SNP	single nucleotide polymorphism
STEMI	ST-elevation myocardial infarction
TG	triglyceride
UA	unstable angina

Introduction

Cardiovascular disease (CVD) - broadly defined as any disease that affects the heart or the blood vessels - is the leading cause of death globally, causing approximately one third of all annual deaths (1). Although CVD encompasses a wide spectrum of diseases the great majority of the global CVD burden stems from atherosclerosis, of which coronary artery disease (CAD) and stroke are the major clinical manifestations (2). In Europe, CAD and stroke are responsible for more than 25 % of deaths under the age of 75 (3), emphasizing that CVD is a major contributor also to premature mortality.

Naturally, the huge disease burden that stems from CVD has fueled extensive research efforts, which have in turn resulted in the identification of a number of important modifiable risk factors for atherosclerotic CVD. These risk factors include lifestyle factors such as smoking, metabolic factors such as dyslipidemia and high blood pressure (BP) and psychosocial factors (2,4-6). Efforts that target such modifiable risk factors, in combination with improved medical therapies, have successfully reduced the atherosclerotic CVD morbidity and mortality in developed countries (7-13). Nevertheless, the incidence as well as the prevalence of CVD is still very high and estimates suggest that CVD will remain the major cause of death by 2030 (14). Clinically, a substantial fraction of high risk subjects do not reach treatment goals for major risk factors (15-17), highlighting the problem of markedly varied treatment response among individuals. Also, finding the "concealed" high risk subjects that are missed by current risk assessments (18) constitutes another challenge if CVD is to be further reduced.

As is the case for most diseases, family history is a well-established "fixed" risk factor for atherosclerotic CVD (19,20) suggesting a substantial genetic component. The search for common genetic variants with impact on CVD in the general population has however been difficult, and it was not until quite recently that our knowledge of common CVD genetics started to increase substantially.

Using the new genetic knowledge for potential improvements in prevention and treatment of CVD and its risk factors may be a strategy that could further reduce CVD morbidity and mortality. The purpose of the current thesis was to address this topic and to investigate how a number of recent genetic discoveries could potentially be implemented in aspects of prevention and treatment of atherosclerotic CVD, with emphasis on CAD and risk factors for CAD in the population.

Pathophysiology of atherosclerotic CVD

Atherogenesis

The normal artery

The normal artery is composed of three distinct layers. Lining the lumen and comprising the contact surface with the blood is the intima, which consists of a layer of endothelial cells (ECs) on a layer of sub-endothelial connective tissue. The ECs, collectively forming the endothelium, are cells with structural, metabolic and synthetic properties. Separated from the intima by the internal elastic lamina, the thicker media layer consists of varying amount of connective tissue fibers, extracellular matrix components such as proteoglycans and smooth muscle cells (SMCs). SMCs affect the tonus of the artery and synthesize major structural fibers of the connective tissue as well as growth factors and cytokines. Outermost is the adventitia which consist of connective tissue, nerve fibers and the vasa vasorum, small arterioles nourishing the other layers of the artery (21).

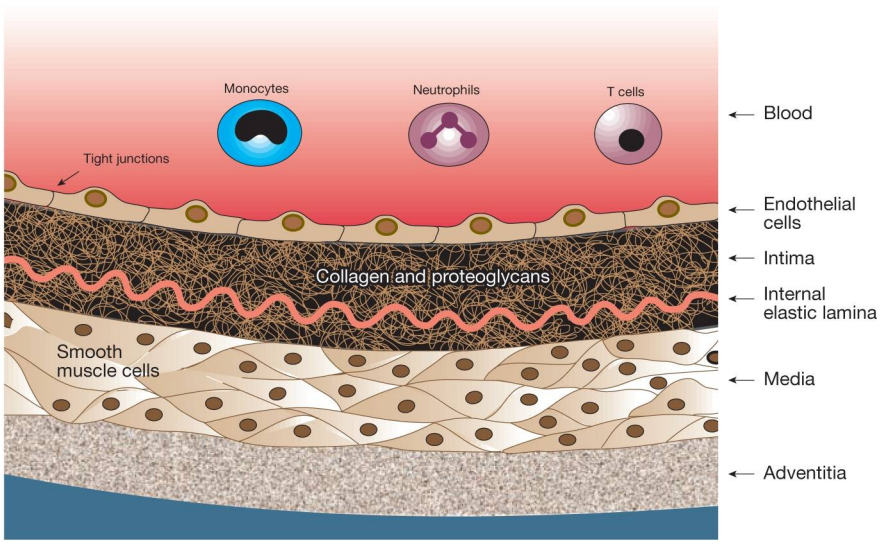

Figure 1
The basic structure of a normal artery. Reprinted from (22) with permission from the publisher.

Arteries are generally classified into three categories based on size and composition: large elastic arteries such as the aorta and its major branches; medium sized muscular arteries such as the coronary arteries and small sized arteries and arterioles within the organs. As could be interpreted from their names, the media of the elastic arteries is largely composed of elastic connective tissue whereas muscular arteries have a large component of SMCs (21).

Development of atherosclerosis

Atherosclerosis, which is one of the three forms of the broader term arteriosclerosis, is a pathology striking primarily the elastic and large to medium sized muscular arteries. It is characterized by intimal lesions – atheromas or fibrofatty plaques – that may obstruct the blood flow, make the media underneath weaker and promote thrombus formation (21).

The development of atherosclerosis – atherogenesis - is a complex process that is yet to be fully understood. A number of hypotheses covering various parts of the atherosclerotic process have been formulated throughout the years. "The response-to-injury-hypothesis" originally postulated forty years ago (23) and thereafter somewhat modified (24), emphasizes endothelial dysfunction and subsequent inflammation as central characteristics in development of atherosclerosis. This hypothesis describes a situation in which a chronic "injury" to the endothelium caused by factors such as dyslipidemia with increased numbers of and/or modified low density lipoprotein (LDL) particles, free radicals by cigarette smoking, local flow disturbances, infectious agents, raised plasma homocysteine and local genetic alterations in response to various stimuli will result in a dysfunction of the endothelium. This will in turn cause a number of compensatory responses of the endothelium, altering its normal homeostasis and provoking increased permeability (including for potentially atherogenic LDL-particles), increased numbers of adhesive molecules for platelets and leukocytes, a pro-coagulative rather than anticoagulative state and the formation of various vasoactive molecules, cytokines and growth factors. Loss of the endothelial vasodilator response, caused by loss of endothelial-derived nitric oxide (EDNO) is thought be of specific importance for the endothelial dysfunction (25).

According to a second major hypothesis of atherogenesis – termed "The response-to-retention hypothesis" (26) LDL plays a pivotal role in atherosclerosis, being a necessary as well as a sufficient cause. This hypothesis emphasizes the process in which LDL-particles from the blood enter the intima through the endothelium, where they will adhere to extracellular matrix proteoglycans. More LDL-particles may enter through a dysfunctional and more permeable endothelium (27), an assumption that would link the hypotheses of response-to-retention and response-to-injury. However, as the name implies, the response-to-retention-hypothesis suggests that factors primarily affecting the retention of LDL in the arterial wall (rather than the entry of LDL into the artery wall) is the key process of

atherosclerosis (26). Within the intima, LDL particles may undergo modification such as oxidation, generating oxidized LDL (oxLDL), which is a process emphasized in a third major hypothesis – "The oxidation hypothesis" (28). Whereas LDL is normally cleared by monocytes that have differentiated into macrophages in an ordered fashion, oxLDL cannot be recognized by the normal receptors of these cells, and will instead be cleared by means of their "scavenger receptors". The uptake by scavenger receptors lacks the normal negative feedback for LDL uptake, and if the cholesterol contained in the LDL-particles cannot be mobilized from the cells, it will accumulate as cytosolic lipid droplets. Ultimately, the macrophage is transformed into a so called foam cell. Although being initially an adaptive and protective response, the uncontrolled accumulation of cholesterol in the foam cell may cause mitochondrial dysfunction, apoptosis and necrosis, leading to subsequent tissue damage and promoting an inflammatory response (21,29,30). Additionally, oxLDL is in itself inflammatory, leading to activation of endothelial cells, monocytes/macrophages and T cells. The release of various inflammatory lipid components from oxLDL, such as lysophospatidylcolins generated from lipoprotein-associated phospholipase A2 (Lp-PLA2) has gained much attention as a potential mechanism, although there might be multiple ways for oxLDL to induce inflammation in the arterial wall (reviewed in (31)).

By integrating the three major hypotheses described in the former section, the generation of an atherosclerotic plaque can be seen as the result of a viscous cycle. With endothelial dysfunction and the inflammatory response that results from LDL retention and the subsequent generation of oxLDL (and other modifications of LDL), SMCs are stimulated to migrate from the media to the intima. These SMCs will undergo a phenotype switch from a contractile to a proliferative phenotype with an enhanced capacity for extracellular matrix protein synthesis. The altered endothelium will also attract and display adhesion molecules for monocytes and lymphocytes, that emigrate from the blood into the arterial intima, where they will proliferate, differentiate and become active. This activation of the recruited inflammatory cells within the lesion will lead to release of number of inflammatory agents such as hydrolytic enzymes, cytokines and growth factors, which will further sustain and amplify the inflammatory reaction. The continuous process of accumulating LDL-particles, oxidation/modification and uptake in foam cells, accumulation of monocytes and lymphocytes, migration and proliferation of smooth muscles cells and deposition of fibrous tissue by these cells will eventually lead to development of an advanced lesion that constitute the atherosclerotic plaque. This consists of a central core of lipid and necrotic tissue of various size, which is covered by a fibrous cap of predominantly SMCs and collagens (21,22,24,32).

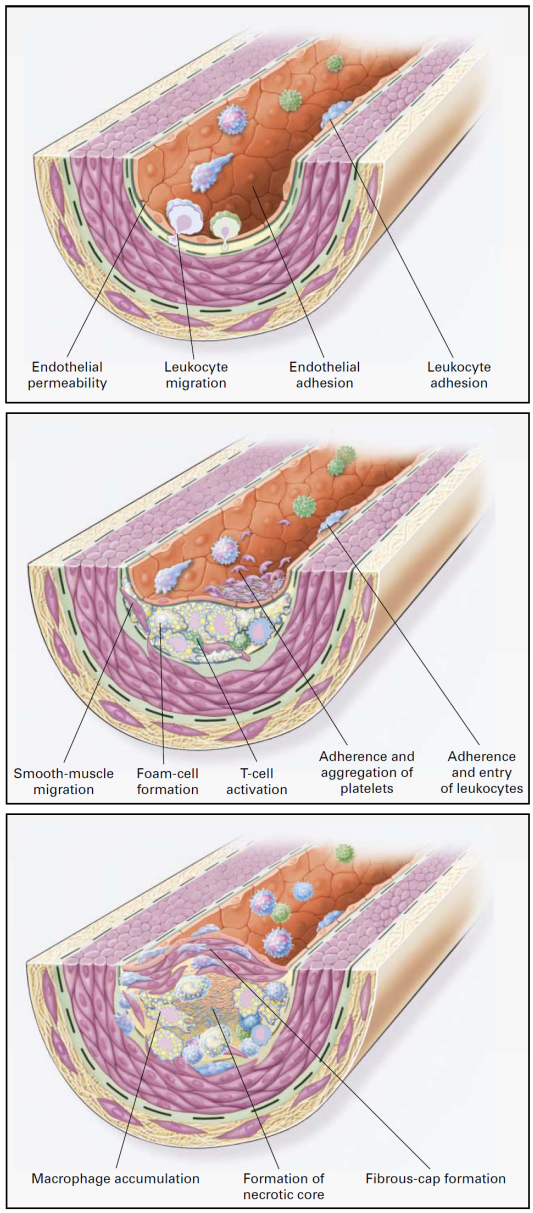

Figure 2
The generation of an atherosclerotic plaque. Reproduced with permission from (24). Copyright Massachusetts Medical Society.

In theory, any part of an artery could be affected by atherosclerosis, however sites where the flow of blood becomes more turbulent rather than laminar, such as osties and branches, show a clear predominance for atherosclerotic lesions. The altered flow at these sites may cause locally altered gene expression in the endothelium, thereby increasing the display of adhesive molecules that act in recruitment of leukocytes (21,24,33,34). It has also been suggested that LDL is retained to a larger extent within the intima at these sites (26).

Consequences of atherosclerosis and the vulnerable plaque

Whereas a focal atherosclerotic lesion could initially be compensated by dilatation of the artery, a process known as outward remodeling (35), a growing atherosclerotic plaque will eventually protrude into the arterial lumen. As lesions become larger and more numerous, potentially also coalescing into large aggregates along the vessel wall, there will eventually be a stenosis that disrupt blood flow. If collateral circulation is insufficient this will lead to (chronic) ischemia of organ that is supplied by the artery. However, acute vascular events often follow from much less prominent plaques (21). “The vulnerable plaque” is a term used for an atherosclerotic plaque that is prone for acute thrombus formation and thus acute total or partial occlusion of the artery with subsequent acute ischemia of the affected organs (36). Three situations predispose for thrombus formation: plaque rupture, plaque erosion and plaque calcified nodules (36,37). Plaques prone to rupture generally consist of a large lipid and necrotic core, overlaid by a thin fibrous cap, which is further weakened by proteolytic enzymes and insufficient repair mechanisms. Thus, a plaque prone to rupture has an increased number of macrophages secreting proteolytic enzymes and a decreased number of SMCs producing components of stabilizing extracellular matrix (36,38). Of note, plaque rupture could occur simultaneously as multiple sites, an observation that highlights the systemic nature of atherosclerosis (36). Plaque erosion is the abrasion of the endothelium in the absence of plaque rupture (39). Contrary to the ruptured plaque, plaques prone to erode are richer in extracellular matrix and SMCs and have fewer inflammatory cells (36). The third and most rare form of vulnerable plaque is that in which calcified nodules protrude into the lumen (37).

Regardless of the underlying histology, thrombus formation is the common consequence of a vulnerable plaque. Formation of a thrombus occurs by means of thrombogenic factors exposed to the blood from the plaque in combination with an interaction between blood, platelets and endothelium (36). In addition to the consequences of acute occlusion, the thrombus may be organized into the plaque which further increases the size of the lesion. Finally, the disruption of a plaque may also cause discharge of small emboli into the circulation where acute manifestations could be caused at sites distant to the lesion (21).

Clinical manifestations of atherosclerosis

Coronary artery disease (CAD)

CAD is the atherosclerotic process in the three major arteries of the heart and their branches. CAD constitutes the most important clinical manifestation of atherosclerosis, being the single leading cause of death worldwide as well as an extensive cause of morbidity (2,40).

Traditionally, CAD is divided into a chronic, stable form (SCAD) and an acute form (Acute coronary syndrome; ACS), although the two forms commonly alternate in a patient. According to the definition from the European Society of Cardiology, SCAD "…is generally characterized by episodes of reversible myocardial demand/supply mismatch, related to ischemia or hypoxia, which are usually inducible by exercise, emotion or other stress and reproducible—but, which may also be occurring spontaneously…", however "…excluding the situations in, which coronary artery thrombosis dominates clinical presentation…" (41). The pathophysiologic substrate of SCAD is most commonly that of atherosclerotic stenoses of the coronary arteries, but microvascular dysfunction and vasospasm may be additional components. Classically, SCAD present itself as chest discomfort (angina pectoris) during episodes of myocardial ischemia; however the symptoms might also be more diffuse, including as dyspnea and fatigue. Importantly, SCAD could also be a completely silent disease. Repeated episodes of myocardial ischemia and/or chronic ischemia may impair left ventricle function and cause ischemic cardiomyopathy, due to myocardial necrosis and/or hibernation of ischemic myocytes. SCAD is diagnosed by patient history in combination with CVD risk assessment, and could be aided by a number of diagnostic tests that are to be used differently in different contexts. Most tests provide both diagnostic and prognostic information (41). The prevalence of SCAD in the population increases with age, from about 5 % in age groups 45-64 years, to 10-14 % in ages 65-84 years. The annual incidence is approximately 1 % for subjects aged less than 65 years, however the incidence increases to about 4 % in older subjects (42,43).

At the opposite side of the CAD spectrum is ACS, which denotes acute manifestations of CAD and includes unstable angina (UA), myocardial infarction (MI) and sudden cardiac death (SCD). The formation of acute thrombosis at an atherosclerotic plaque leading to acute ischemia of the myocardium constitutes the central underlying cause of ACS (44). Importantly, ACS is not seldom the first presentation of CAD (45-47), which highlights the importance of correct risk stratification and accurate preventive therapy also in subjects free of symptoms for CAD.

UA is characterized by either newly diagnosed severe angina pectoris or by a formerly stable angina pectoris that is increasing in severity. UA also involves

angina within a month after an MI. By definition, there is no evidence of myocardial necrosis in UA, however UA often precedes an MI. MI is diagnosed when there is myocardial necrosis in the setting of myocardial ischemia; the evidence of myocardial necrosis could be diagnosed by biochemical markers or imaging modalities (in addition to autopsy.). MIs are classified into five different subtypes based on the underlying pathology (48). For clinical treatment decisions, a distinction is made between patients with acute MI based on whether or not they present with ST-elevation on the electrocardiogram, termed ST-elevation myocardial infarction (STEMI) and non-STEMI (NSTEMI) respectively. STEMI, for which the incidence has decreased with improved prevention and treatments (49) is considered to be caused by a fixed total blockage of an artery and subsequent transmural ischemia. Contrary, NSTEMI is considered to be caused by partial or intermittent blockage. Importantly, a substantial minority of patients with MI show no signs of CAD when conventional imaging modalities are used. The underlying pathology in these patients might be diverse, including embolization, dissection and ”pure” vasospasm, however rupture of a non-occlusive plaque, that is undetected by current routine imaging modalities, is common (50). SCD could often be attributed to a lethal ventricular arrhythmia secondary to acute myocardial ischemia, however SCD could also be caused by other manifestations of CAD as well as other underlying cardiac pathologies (44).

The annual incidence of hospital admissions due to acute MI (both STEMI and NSTEMI) varied between 90–312/100 000 in 30 European countries during the second half of the previous decade (51). In Sweden, the incidence of acute MI during 2012 was estimated to be 522/100 000 men and 344/100 000 women. The 28 day case fatality for acute MI was 26 % for men and 30 % for women, however in subjects admitted to an hospital the 28-day case-fatality was only 13 %, which is half the case fatality rates observed in the early 90s (52).

Stroke and other clinical manifestations of atherosclerosis

Stroke is classically characterized as a persisting (> 24 hours) neurological deficit that is caused by an acute injury of the central nervous system on a vascular basis (53). About 80 percent of all strokes in developed countries are of arterial ischemic origin whereas 10-15 % are caused by intracerebral hemorrhage and 5 % are caused by subarachnoid hemorrhage and a smaller percent of other (at times unknown) ethology (54).

An arterial ischemic stroke could be caused by either a thrombosis or embolization. A thrombotic stroke in the large extra and intracranial arteries is most often caused by atherosclerotic plaques, with a similar underlying pathology as in the coronary circulation. The plaques could also serve as sources of embolization to a more distant location, so called artery-to-artery embolization. Occlusion of the smaller arteries and arterioles of the brain (“small vessel disease”) is often caused by arteriolar sclerosis, which is mainly a consequence of

hypertension. Embolic strokes are usually caused by cardiac or arterial emboli although the source might be unknown (55). A classification widely used for ischemic stroke is the TOAST-classification which is based on the above pathologies (56).

Primary hemorrhagic strokes are intracerebral or subarachnoid hemorrhages respectively. Hypertension and its consequences on the arteries and arterioles is the major risk factor for the first one, whereas subarachnoid hemorrhages are usually caused by saccular aneurysms, for which the precise ethology is unknown (55).

As is the case for MI, there is a large increase in the incidence of stroke with increasing age. In Europe, the estimated annual stroke incidence per 100 000 in the middle of the last decade varied from approximately 10-20 cases in ages 20-35 to 500-2000 cases in ages over 65 years (57).

As a systemic disease atherosclerosis might develop in any arterial bed and the clinical consequences will thus depend on the location and the organs supplied by the affected arteries. Even though the major clinical manifestations of atherosclerosis are CAD and ischemic stroke, peripheral atherosclerotic disease (PAD) in the arteries supplying the lower limbs and aortic atherosclerosis with aortic aneurysms are clinically relevant manifestations as well. Whereas the current thesis is primarily focused on CAD and the risk factors for CAD, different manifestations of atherosclerotic CVD commonly co-exist and the underlying pathophysiology and many risk factors are shared between the different forms of atherosclerotic CVD.

Prevention of atherosclerotic CVD

Modifiable cardiovascular risk factors and their treatments

As pointed out in the introduction, the huge research efforts taken in previous decades have resulted in identification of a number of risk factors that are of major importance for atherosclerotic CVD. Although some of these risk factors are fixed, such as age and sex, the majority of current known CVD risk factors are modifiable to a various extent (58-60). Their importance could be emphasized by marked differences in lifetime risk of CVD among subjects depending on the occurrence of these risk factors (61) and by the decline in CVD (which is most evident for CAD) in the population that could attributed to better control of them (11-13).

Lipids

Cholesterol and triglycerides (TGs) are transported in the circulation in specific lipoprotein complexes. These lipoproteins are composed of varied proportions of cholesterol, TGs, phospholipids and surface proteins, the latter known as apolipoproteins. Apolipoproteins take the role of receptor ligands and cofactors for various enzymes in the lipid metabolism.

Table 1
Major lipoprotein classes. Based on text and supplementary in (62).

Lipoprotein	Major component	Apolipoproteins	Main known function
Chylomicrons	Triglycerides	A-I, A-IV, A-V; B-48,C-I, C-II, C-III, E	Exogenous lipid transport from the intestines
Very low density lipoprotein (VLDL)	Triglycerides	B-100, E, C-I, C-II, C-III	Endogenous lipid transport
Low density lipoprotein (LDL)	Cholesterol	B-100	Cholesterol transport
High density lipoprotein (HDL)	Cholesterol, proteins	A-I, A-II, E	Surface component turnover, reverse cholesterol transport

Most of the cholesterol in the blood is carried in LDL. Total and LDL-cholesterol levels constitute a major risk factor for development of CVD, as can be suspected from pathophysiological (29,30), observational (63-66) and early genetic studies of rare forms of inherited hypercholesterolemia (67,68). However more substantial evidence linking in particular LDL cholesterol to CAD stems from interventional

studies showing a clear benefit of lowering LDL by statins in both primary (69,70) and secondary prevention (71,72) with greater risk reduction with more LDL reduction (73,74). In addition, modern genetic studies in which the impact of LDL-cholesterol concentrations from birth could be estimated by genetic variations that affect LDL-levels (called Mendelian randomization), strongly support the role of LDL as a causative factor in CAD (75,76).

In contrast to LDL, high HDL levels have been found to be associated with lower risk of CVD (77-79), including in individuals with low LDL-levels (80). Accounting for this and the potential pathophysiological role for HDL in reverse cholesterol transport from arterial walls and other potentially atheroprotective effects (81), development drugs that increase HDL levels has been a desirable goal. However, pharmacological efforts of increasing HDL levels have yet showed disappointing results (82-84). Additionally, in contrast to LDL, there are no convincing results from modern genetic studies that would imply HDL as a true causative protective factor in CAD (85-88). Thus, the "HDL hypothesis" could be challenged (81), and the effects of HDL on the vasculature are likely to be heterogeneous and complex (89).

There seems to be a moderate association between TGs and risk of CVD in the form of CAD, however considering the strong relationship between TGs and the general lipid profile as well as other cardiovascular risk factors, the specific role of TGs is not clear (90). The atherogenic lipid profile that consists of a combination of high TG, low HDL and small potentially atherogenic LDL particles has been emphasized in diabetes and in the metabolic syndrome (91).

Beyond the assessment of LDL, HDL and TGs, there has been an intention to further improve lipid diagnostics. Such studies have included measurements of oxidized LDL (92), the specifically atherogenic lipoprotein termed lipoprotein(a) (92-94), lipoprotein subfractions (95,96) and specific lipid patterns of such subfractions (97). The apolipoproteins can also been measured directly, with the ratio of ApoB/ApoA1 showing a strong association with CAD (98-100). However, the treatment goals for this variable have not been determined (5).

Treatment of dyslipidemia in order to reduce the risk of CVD is largely focused on reducing LDL, by the use of statins. Statins inhibit 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA-reductase). HMG-CO-reductase is an enzyme in the production of cholesterol, and by inhibiting this essential step in cholesterol production LDL-receptors are unregulated, which in turn results in increased clearance LDL from the circulation. Whereas most of the effect of statins on risk of CVD is likely to be mediated by their LDL-lowering mechanisms, statins also show other potentially anti-atherosclerotic effects, including favorable effects on the endothelium, reduced SMC proliferation and production of cytokines, and a possible effect on hemostasis (101). Accounting for their robust evidence in secondary and high-risk primary prevention of CAD, a statin is the primary drug

of choice for treating hyperlipidemia in such patients according to current guidelines (102,103). Additionally, whereas studies for LDL reduction with statins have primarily focused on CAD, statins have been shown to be beneficial also for reducing ischemic stroke (73,74,104). Recent studies suggest a potential benefit of statin therapy also in patients at low risk for CVD (74,105) however the interpretation of these results has been debated (106).

Despite the extensive use of statins for decreasing LDL cholesterol, only approximately half of patients with established CAD have been shown to reach the increasingly strict set treatment goals (15) and these numbers are even lower in high risk patients without overt CVD (16).

Hypertension

Hypertension is defined as a resting BP ≥ 140/90 mmHg (107). However, in similar to LDL levels, BP is a continuous trait and the risk of CVD increases continuously already from normal levels (108).

The burden of hypertension worldwide is massive, with an estimated prevalence ranging from approximately one third to around 50 % in some countries (1). Hypertension is the single leading cause of mortality world-wide, estimated to be the cause of 12.8 % of all annual deaths (1,4). Roughly 90 % of the cases of hypertension are classified as essential hypertension – for which the causes are multifactorial. The remaining cases of hypertension could be attributed to more specific causes and are classified as secondary hypertension; such cases include diseases of the kidneys and endocrine disorders such as primary hyperaldosteronism, Cushing disease and disorders of the thyroid (21).

Hypertension is a risk factor for atherosclerotic CVD (6,59,60,108-110) and the causal role is supported by results from recent genetic studies (111,112). Hypertension is also a risk factor for CVD where atherosclerosis is usually not the primary underlying pathology, such as atrial fibrillation (113) and heart failure in the absence of detectable CAD (114).

Treatment of hypertension is beneficial for reducing major CVD events in both older and younger subjects (115). Hypertension correlates with additional CVD risk factors (116,117) and the management of hypertension is based on the total risk assessment of the patient. In addition to conventionally assessed CVD risk factors, the presence of subclinical organ damage in the form of microalbuminuria, left ventricular hypertrophy, increased pulse wave velocity and carotid plaques is considered when deciding if and how treatment should be started (107,118). Generally, treatment goals are set to a resting BP of less than 140/90 mmHg, however with some modifications in elderly and diabetic subjects (107).

As for CVD prevention in general, life style modifications constitute an important base for treatment of hypertension, including (no more than) moderate alcohol

intake, regular exercise, cessation of smoking, weight loss, salt restriction and increased intake of vegetables (107). There are numerous pharmacological alternatives for treating hypertension, the major drug classes being thiazide diuretics, angiotensin converting enzyme (ACE)-inhibitors/angiotensin-II-antagonists, calcium antagonists and beta-blockers. Depending on the patient the recommendations for using a specific drug differs and different drugs can be used in different combinations in order to reach the treatment goals (107). Newer drugs include direct renin-inhibitors (119), and there are also less frequently used classes of drugs for hypertension such as alpha-adrenergic receptor blockers.

Despite numerous drugs with potent effects, the proportion of CVD risk patients that reach BP treatment goals is only around 50 percent (15,16).

Other risk factors of importance

The risk of CVD is increased in subjects with diabetes (120). Diabetic subjects generally show an adverse CVD risk factor profile and treating hypertension (121-123) and dyslipedmia (124) is of major importance for reducing CVD in this group of patients. In contrast, meta-analyses and systematic reviews of major diabetes treatment trials, have not shown that improved glucose control associates with reduction in CVD mortality (125,126), implying that the pathophysiological links between DM and CVD are likely to be complex (127).

Obesity is strongly associated with other modifiable risk factors, but body-mass-index (BMI) has been shown to predict CVD also after accounting for these risk factors (128). Chronic kidney disease is a major risk factor for CVD (129). Patients with autoimmune inflammatory diseases such as rheumatic arthritis (RA) show signs of increased atherosclerosis (130), and reducing inflammation in RA has proven beneficial for reducing CVD risk, highlighting the possible role of inflammation as an underlying cause of CVD (131). Inflammatory markers, most notably C-reactive protein (CRP) has been found to associate with CVD (132), however the role of CRP as a causative risk factor (rather than a marker of the underlying inflammatory process) has been largely questioned, accounting for the lack of association between CRP and CVD in Mendelian randomization studies (133).

Lifestyle risk factors

A number of important lifestyle risk factors for atherosclerotic CVD have been identified. Cigarette smoking is perhaps the most widely acknowledged behavioral risk factor for CVD (4), including MI (59,134,135) and stroke (60,136). The risk of death from CVD within 10 years is approximately twice as high in smokers compared to non-smokers (137). With smoking cessation, the risk of CVD rapidly decreases (138). The detrimental effects of smoking on the cardiovascular system and the subsequent risk of CVD could be attributed to multiple potential pathophysiological mechanisms, including free radical-mediated oxidative stress

on the endothelium and oxidative modification of LDL, promotion of inflammation and alteration of antithrombotic and prothrombotic factors (reviewed in (139)). Smoking also causes increased insulin resistance, raised catecholamine levels, and unfavorable effects on the lipid profile (140).

Beyond smoking, a number of additional life style related risk factors associate with CVD. Regular physical activity has favorable effects on conventional CVD risk factors and has been shown to associate with reduced CVD mortality (141,142). A low fitness level in midlife is predictive of CVD mortality (143). Socioeconomic factors such as education level and income (144), psychological factors such as depression (145) and other socio-psychological measurements such as marital status and strain (146) have been found to associate with risk of CVD as well.

Risk prediction and assessment of atherosclerosis

CVD preventive strategies and risk prediction

Traditionally, preventive cardiology has been divided into two distinct strategies. "The population strategy", advocated by Geoffrey Rose in a widely cited paper from 1985 (147) aims at reducing a known CVD risk factor in the whole population. Such efforts could include actions such as increasing taxes on cigarettes with the aim of reducing smoking in general. A successful population strategy would shift a specific risk factor in the population to lower average levels, resulting in a potentially large decrease in total disease incidence in the population, even though the individual benefit for most subjects may be small. Contrary, the "high risk strategy" emphasizes the detection and treatment of high risk subjects in the population. The benefit for this particular group of subjects could be large, whereas the effect on the total incidence in the population may be small, accounting for the fact that only a minority of the population would be affected by the efforts and that most CVD cases would still occur in subjects with modest risk factor levels (5).

For CVD prevention there is a general consensus that the two strategies described in the former section complement each other (5). Whereas life-style interventions such as reducing smoking is beneficial for everyone, effective medical interventions often have potent side-effects as well as high costs, emphasizing the importance of identifying subjects where such interventions could have the most benefit in relation to the downsides (cost-benefit ratio). Assessing the total baseline CVD risk (rather than just the highest level of a specific risk factor such as cholesterol or blood pressure) has been suggested to an effective CVD preventive strategy (148), and this approach is encouraged in clinical guidelines for CVD prevention (5).

As tools for assessment of CVD risk, a number of statistical prediction models that estimates the absolute CVD risk in an individual patient have been developed (however, naturally these risk prediction models are based on population statistics). Examples of common risk prediction equations include the European SCORE-model that estimates the absolute 10 year risk of fatal CVD (137) and the risk scores derived from the Framingham cohort (Framingham risk score, FRS) (149,150). Inevitably, a substantial minority of high risk subjects are missed by current risk algorithms (18) and the search for novel risk factors and risk markers that could aid further in CVD risk prediction is a field with an intensive activity (151). Common metrics usually applied to evaluate the added clinical utility of new risk factors and risk markers include discrimination using the C-statistic (the ability of to distinguish between future cases and non-cases), calibration (the correlation between predicted and observed risk) and reclassification, such as the net reclassification improvement and the integrative discrimination index, assessing if and how subjects will be correctly reclassified into a more appropriate risk category (152).

Visualizing atherosclerosis in the coronary circulation

The insidious nature of atherosclerosis, being a subclinical disease that evolves during many decades before ultimately culminating in a clinical event (153), highlights the need for effective methods that could assess the degree of (subclinical) atherosclerosis. Today, a number of methods could visualize the status of the arteries, providing further guidance for risk assessment and treatment decisions.

Coronary angiography (CA) remains the "gold standard" for diagnosing obstructive CAD (41,154). However, in addition to the invasive nature of this procedure and the risks that stem from it, CA visualizes intraluminal atherosclerotic plaques, and may miss a substantial number of non-occlusive plaques. Additionally, microvascular disease could not be assessed. Aided by intravascular ultrasound (IVUS) and more recently optical coherence tomography (OCT) plaques and their potential vulnerability may be further classified (155). With technical advancements, computed tomography (CT) has emerged as a potential diagnostic modality for assessing CAD. Non-contrast CT for Agatson calcium-scoring approximates the amount of calcium in the coronary vasculature, which, in the absence of calcific media sclerosis, would equal calcified atherosclerotic lesions. Coronary artery calcium scoring has been shown to predict the risk of CAD events and is considered to be of value of risk stratification in intermediate risk patients (156,157). Coronary CT angiography (CCTA) can visualize the coronary lumen and also non-significant atherosclerotic plaques, whether calcified or not. This technique offers a very high negative predictive value for ruling out significant stenoses in patients with suspected CAD (158) and the absence of CAD on CCTA implies a good prognosis concerning CAD events

and mortality (159,160). Clinically, CCTA could be of value for excluding CAD in low intermediate risk subjects presenting with chest pain (41). There is however no consensus regarding the value of CCTA for assessing the total atherosclerotic burden, such as plaque stability and extent (157,161), although with further improvements CCTA may prove valuable in such settings (162). Cardiac magnetic resonance imaging (CMRI) constitutes a technique for determining myocardial perfusion, viability and coronary flow as well as for assessment of plaque anatomy and morphology. With the use of hybrid imaging, also incorporating functional radionuclide techniques such as positron emission tomography (PET) and single photon emission computed tomography (SPECT) (reviewed in (163)) further preventive diagnostic opportunities within the field of atherosclerosis and CVD risk prevention may arise.

Intima-Media-Thickness and carotid plaques as makers of atherosclerosis

The fact that atherosclerosis is a systemic disease would mean that the assessment of atherosclerosis in one territory could potentially determine the occurrence of atherosclerosis also in other arterial territories, as suggested by autopsy studies (164,165). The carotid artery is well suited for assessing vascular status, being a representative artery for atherosclerosis development with a superficial location.

By using B-mode ultrasound the combined thickness of the intima and media layers of the carotid artery, termed carotid Intima-Media-Thickness (carotid IMT) can be measured. Increased carotid IMT is considered a surrogate marker not just for carotid but also for general atherosclerosis, meaning that non-invasive ultrasound could be used as a tool for identifying subclinical vascular disease (166). The use of carotid IMT for assessing subclinical atherosclerosis can be justified primarily by a number of epidemiological observations. First, increased carotid IMT has been shown to correlate with occurrence atherosclerosis in coronary and other vascular beds (167-170). Second, carotid IMT predicts incident CVD manifestations such as CAD, stroke and death from CVD (171). Third, particularly in young subjects (which are most often free of overt CVD) there is a strong association between increased carotid IMT and occurrence and severity of conventional risk factors for atherosclerosis (172-175).

Carotid IMT has been shown to improve risk prediction over traditional risk factors in the general population (176) and in asymptomatic subjects with multiple CVD risk factors (177,178). However, in terms of actual clinical utility, the improvement in prediction of carotid IMT over conventional risk factors has been questioned (179).

As shown in Figure 3, carotid IMT can be measured in the internal (ICA), external (ECA) or common carotid artery (CCA) using mean or maximum values in a segment. The mean IMT of the far wall in the CCA is usually that of the carotid IMT measurements that is most easy to obtain, and consequently this is also the

IMT measurement that has been used in the majority of carotid IMT studies (179). Mean CCA-IMT is also the primary measurement recommended for clinical risk evaluation in the context of subclinical vascular disease (166).

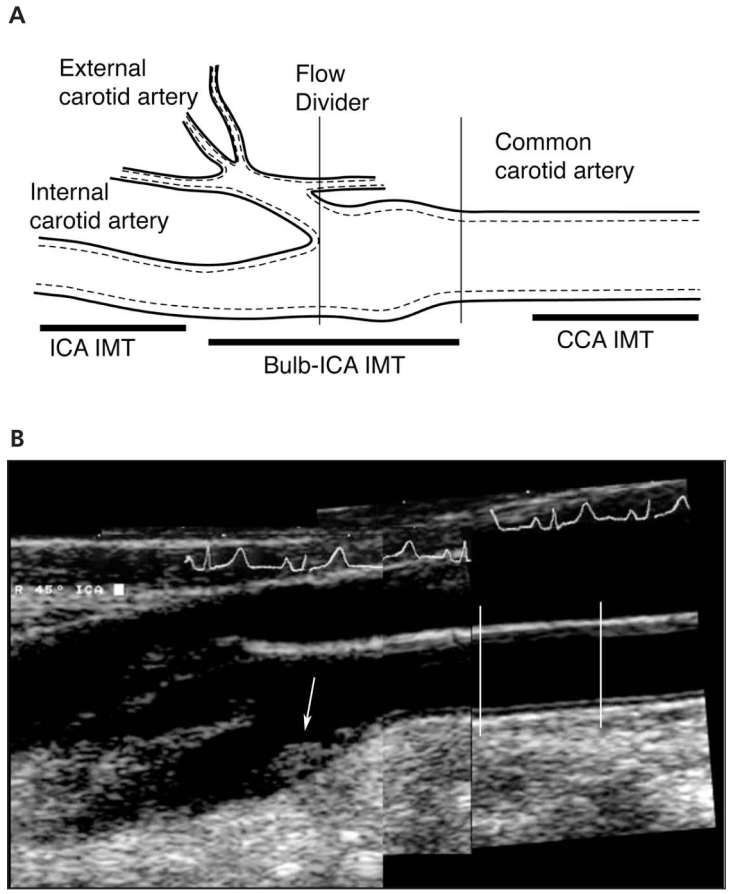

Figure 3
Drawing of the carotid artery (A) and composite carotid sonograms used as part of the Framingham IMT protocol (B). Displaying measurement sites for maximum IMT in the bifurcation (arrow) and the mean IMT along a 1 cm distance in the common carotid artery (vertical lines). Reprinted from (180) with permission from the publisher.

A carotid plaque is defined by the American Society of Echocardiography (ASE) as a focal wall thickening that is at least 50 percent greater than that of the surrounding vascular wall or a focal IMT greater than 1.5 mm, with distinct

boundaries (166). Being atherosclerotic lesions, substantial evidence exists for an association between occurrence of carotid plaques and incident CVD events and death (181-186). In the context of risk prediction, the occurrence of carotid plaque also has an additive value to IMT (176).

In the clinic carotid ultrasound is frequently used for assessing carotid stenosis in the setting of primary and secondary prevention of ischemic stroke. However, carotid ultrasound with measurements of carotid IMT in combination with assessment of plaques is also suggested to be a potential tool for risk classification in asymptomatic subjects with intermediate risk for CVD (5,157,166,187). Nevertheless, the possible clinical benefit over traditional risk factors in terms of reduction of CVD by the use of carotid ultrasound is yet to be determined (188).

Genetics of atherosclerotic CVD

General genetic concepts

Human genetic variation

The human DNA sequence consists of approximately three billion base pairs of which roughly 1 % is highly conserved protein coding sequences and 4 % consists of non-protein coding DNA-sequences such as for functional RNA. The remaining 95 percent of DNA is various forms of non-coding sequences (189). The numerically most abundant genetic variants are polymorphisms in single base pairs, which occur at approximately 1 in 300 base pairs at highly variable genotype frequencies. These variants, termed single nucleotide polymorphisms (SNPs), usually show two alternate forms (alleles) in the population. Copy number variations (CNVs) constitute variations in the number of copies of a particular DNA sequence and are responsible for the greatest amount of nucleotides differing between individuals (190).

Inheritance of disease

The simplest form of inheritance is that in which a trait or disease depends upon a single genotype at a specific locus – monogenic or Mendelian inheritance. Whereas there is a large number of diseases that show a mendialian type of inheritance with single genetic variants having a strong effect on disease (in the majority of cases via altered protein coding), pure monogenic diseases are rare in the population. Instead the vast majority of disease in the population depends on several genetic variants at various loci which likely act in combination with environmental factors on development of disease and as such is usually referred to as complex or multifactorial disease (191). "The polygenic theory", described by RA Fisher nearly 100 years ago (192), implies that a continuous trait in an individual is the result of a combination of many different independent genetic variants. With increasing number of contributing genetic variants and thus increasing combinations of genetic polymorphisms for a trait, the distribution in the population takes the form of a Gaussian normal distribution. For a disease, which is a dichotomous manifestation, "the polygenic threshold theory" emphasizes the concept of susceptibility – the "trait" is in this case the susceptibility for a disease. The individuals in a population who exceed a specific threshold (i.e. with a sufficient number and / or adequately strong genetic variants) will develop disease. Since complex disease (and traits) in addition to genetics is the result of environmental factors as well as possible gene-gene and gene-environment interactions, the models described are highly simplified (191). In addition, the concept of epigenetics, cellular inheritance not conveyed by changes

in the DNA sequence, adds further to the complexity of genetic basis for multifactorial disease such as CVD (193).

The genetic variants accounting for complex disease might be either preserved common variants that each show a relatively weak effect ("common disease – common variant hypothesis") (194) or a heterogeneous set of rare more recently acquired genetic variants that individually show a moderate effect ("mutation selection hypothesis") (both theories are reviewed in (195)). These two hypotheses are not necessary incompatible; rather it has been suggested that complex disease such as CVD in the population is the result of both common variants with small effects and heterogeneous rare variants with individually moderate or strong effects (196). In this context it should also be added that a few common strong effect variants have been detected (such as genetic variation in APOE in association with Alzheimer's disease), and likewise one could assume existence of a substantial number of low frequency small effect genetic variants that are not detected by current methods (197). A locus that is known to be involved in a specific or disease might contain variants with both strong and small effects (196).

Methods for assessing genetic variation in association with disease

The methods that have been used for finding the genetic variants underlying a disease have differed according to whether the aim has been to find rare variants with a Mendelian type of inheritance or more common genetic variants for complex disease. For rare Mendelian-type diseases the traditional method has been linkage analysis, in which the transmission of adjacent chromosomal segments in affected family members is mapped, giving the approximate chromosomal location of the responsible genetic variant. Subsequent genetic refinement (such as positional cloning) can then pinpoint the genetic variant responsible for disease. This approach has been successful for finding numerous Mendelian-type genetic disorders (197-199).

Linkage analysis has also been utilized as a method for finding genetic associations with complex disease and quantitative traits (195) including CVD (200) and CVD risk factors such as BP (201). In the context of CVD risk factors, a successful and today widely acknowledged example is the associated risk of type 2 diabetes conferred by variation the gene encoding the transcription factor 7-like 2 (TCF7L2), which was originally identified by linkage analysis (202).

During the latest years the mainly used method for discovery of common disease susceptibility variants has been based on "association". Association studies search for frequency differences between subjects with and without disease. Early genetic association studies focused on polymorphisms situated within or near genes that were suggested from linkage analysis studies and/or near genes with a plausible biological role in the disease of interest. Unfortunately, although these candidate-

gene based studies yielded numerous results and in some cases provided important findings, many of the findings could not be replicated (203,204).

In the last decade rapid technical advancements in combination with a more adequate statistical approach for genetic associations has enabled a new form of genetic association studies that do not require a priori hypotheses. In these studies, termed Genome Wide Association Studies (GWASs) (205) an array of hundreds of thousands of common SNPs (and sometimes CNV:s), are tested against disease or quantitative phenotypes in case-control studies or in in population based samples. Given the absence of a priori hypotheses and the large number of statistical tests, strict significance thresholds are needed for minimizing type 1 errors, and replication of the associated signals in a separate population is usually required in order for an association to be considered solid (206). An important fundament that enabled the GWAS design was the HapMap-project (207). HapMap created a detailed mapping of ancestral chromosome segments in four populations, resulting in a catalogue of 10 million SNPs from which certain SNPs can serve as markers ("tag-SNPs") for specific haplotypes (SNPs inherited together). Thus, a few hundred thousand SNPs could be used as markers for most of the common variation of the human genome (208).

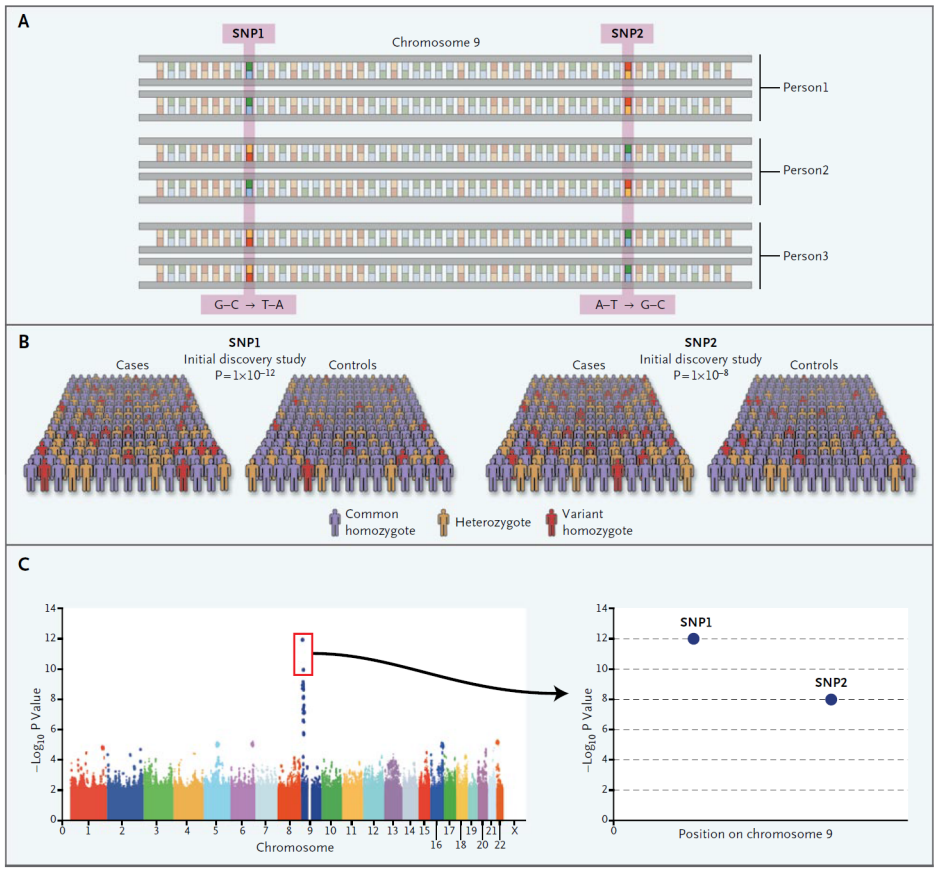

Figure 4
Design and presentation of a Genome-Wide Association Study (GWAS). Two SNPs on chromosome 9 (A) show an association with disease in a case control study with P-values of 1 x 10^{-12} and 1 x 10^{-8} respectively, indicating genome-wide significance (B). The results for all SNPs tested in the GWAS are displayed in a so called Manhattan plot, highlighting the two SNPs on chromosome 9 (C). Reproduced with permission from (205). Copyright Massachusetts Medical Society.

Previous GWAS:s, which have largely focused on common SNPs (minor allele frequency > 5 % in the population) and CNVs, have identified numerous variants in association with disease and traits. Currently, the Catalog of Published Genome-Wide Association Studies from the National Human Genome Institute (209) includes 1771 publications and 12076 SNPs, and the number has probably further increased as this thesis is being read. Interestingly, the vast majority of disease-associated SNPs are not located in protein coding regions, highlighting the importance of the non-protein-coding part of the DNA in accordance with the small effect sizes conferred by these SNPs. Also, a number of SNPs have shown associations for various types of disease (i.e. pleiotropic effects) suggesting a common underlying cause for these diseases. Importantly, rather than being the

causative variants, the SNPs showing disease association might well be markers for other co-inherited SNPs (i.e. SNPs in linkage disequilibrium, LD) that constitute the true causative variants (205).

Lately, the improved and cheaper technique for exome and whole genome sequencing has opened the door for finding rare variants in association with not yet discovered Mendelian diseases and for finding rare variants that contribute to common disease. In this technique the DNA in the protein coding segments (exomes) or the whole genome is sequenced and directly compared between cases and controls, making it possible to find rare variants not captured by former common-type-variants designed GWAS arrays (210,211). This approach may enable finding heterogeneous rare variants with moderate effects that possibly explain the "missing heritability" that exists for discovered common variants explaining common disease (197).

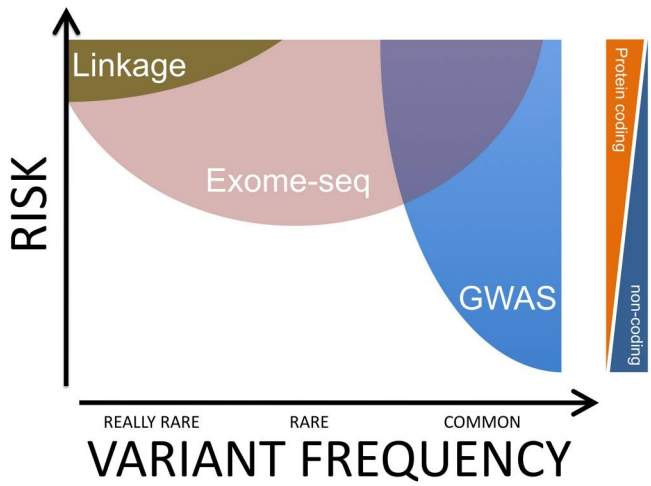

Figure 5
Hypothesis of rare and common genetic variants in association with disease and the potential approaches for identifying them. Reprinted from (197) with permission from the publisher.

Genetic findings for CVD

The discovery of common genetic variants that associate with CVD

Family history is a well-established risk factor for CVD, most notably early onset CAD (212), and this heritability has been shown to hold true independently of other known CVD risk factors (19,20). As is the case for most complex disease such findings highlight a substantial underlying genetic component. A number of rare Mendelian forms of CVD, perhaps best exemplified by the LDL-receptor mutations and other mutations that cause familial monogenic hypercholesterolemia have previously been identified by linkage analysis and subsequent genetic refinement (213). More lately, direct exome sequencing has been utilized for finding genetic variants for other Mendelian-type CVD such as rare forms of genetic hypertension (214).

For common multifactorial forms of CVD in the general population, a breakthrough came in 2007 when the first GWASs for CAD were published. In these studies, SNPs at chromosome 9p21 with risk allele frequencies of approximately 50 % in Caucasian populations were found to strongly associate with MI and CAD, with odds ratios (OR) of about 1.30 per risk allele (215-218). Since then, numerous GWASs involving large consortia such as Myocardial Infarction Genetics Consortium (MIGen) (219), Coronary Artery Disease Genome Wide Replication and Meta-Analysis (CARDIoGRAM) (220,221), and the Coronary Artery Disease Genetics consortium (C4D) (222) have succeeded in additional identifications. The most recent large GWAS identified 15 new SNPs that associate with CAD (223), meaning that 50 common SNPs associating with CAD and/or MI have been reported to date (224). Whereas some of these SNPs additionally associate with traditional CVD risk factors such as lipids and hypertension, the majority of the reported CAD- and MI-SNPs show no such associations, and many are located in DNA regions not previously thought be involved in CVD. Together, the common genetic variants discovered to date explain around 10 % of the heritability for CAD (223).

Additionally to the SNPs with direct association with CAD and/or MI there are numerous SNPs in association with risk factors for CAD (of which some but not all also show direct associations with hard endpoints). As is the case for CAD and MI, a number of large consortia have been formed in order to increase study sample sizes and achieve sufficient power for detecting genetic associations. The Global Lipids Genetics Consortium recently reported 157 loci in association with lipid traits, 62 of which were novel discoveries (225). The BP-associated SNPs reported by consortia such as the Global Blood Pressure Genetics (Global BPgen) consortium (226), The Cohorts for Heart and Aging Research in Genomic Epidemiology (CHARGE)-BP consortium (227) and subsequently the International Consortium for Blood Pressure Genome-Wide Association Studies (ICBP-GWAS) (228) are less numerous. Nevertheless, the investigators of a recent

BP-genetic study concluded that a total of 32 common SNPs have been shown to be associated with BP levels in the population (112). Most notable for lipid levels, even though the majority of the novel GWAS - derived SNPs are not located within loci previously involved in Mendelian-type dyslipidemia, this is the case for a substantial minority of the SNPs, supporting the hypothesis that both common and rare variants within the same loci contribute to CVD traits in the population (196).

Chromosome 9p21

The SNPs on chromosome 9p21 that were initially discovered in 2007 are still by many considered the best validated polygenic CVD risk variants (229). The association between these SNPs (which are all in strong LD) and CAD and MI has been replicated several times in multiple populations (219,230-233). Also, the same SNPs have been found to associate with ischemic stroke (234), PAD (235) and also arterial aneurysms (236) suggesting a potential common pathophysiology. Interestingly, the SNPs on Chromosome 9p21 do not show any association with any of the traditional CVD risk factors (215-218), suggesting that the underlying pathophysiological mechanisms are novel. The SNPs are located in a "gene desert" near the known tumor suppressor genes that encode cyclin-dependent kinase inhibitors 2A and 2B (CDKN2A/CDKN2B). Molecular studies have shown that the chromsome 9p21 CVD risk locus involves a specific non-coding RNA, termed antisense non-coding RNA in the INK4 locus (ANRIL). ANRIL has been proposed to regulate epigenetic modification and expression of other genes potentially involved in the CVD process (discussed in (229,237)). Nevertheless, the exact molecular mechanisms are unknown.

Genetic risk scores

In accordance with the "common disease – common variant hypothesis", the SNPs in association with CVD that have been identified in GWASs show individually small effect sizes on risk of disease. The concept of adding individual genetic effects to a summed genetic risk score (GRS) have been used in a number of studies in order to assess the aggregated genetic effects of a specific combination of risk alleles. Usually, an additive model for genetic effects is assumed (206), and the GRS can be constructed either by simple addition of number of risk alleles or by weighting the contribution of the different risk alleles by their individual effect sizes. Successful studies utilizing the concept of a GRS include trait-specific GRSs for lipids (238), BP-levels (111) and hard-end-point GRSs for MI and CAD (239-241). Although a CAD-genetic-score acts through a number of different pathways, including a combination of thus far unknown ones and known risk factors (e.g. LDL cholesterol and blood pressure), its aggregate effect on CAD seems more relevant regarding risk prediction than the use of single gene variants. On the other hand, it may be too heterogeneous to be used to identify responders to a pathway- or molecule-specific preventive intervention.

Pharmacogenetics and pharmacogenomics in CVD

As highlighted in the introduction the response to CVD preventive treatments varies considerably among individuals. In Figure 6 this is exemplified by variations in LDL-reduction after six weeks' daily treatment with 40 mg simvastatin (242), a widely prescribed lipid-lowering drug.

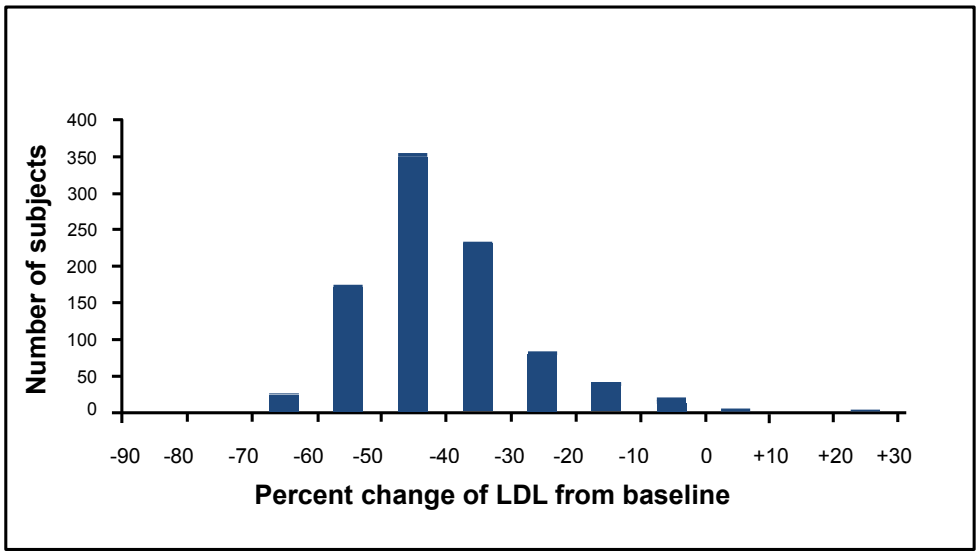

Figure 6
Variation in LDL-reduction among individuals treated with simvastatin 40mg/day for six weeks. Figure adapted from (242).

The hypothesis that part of the variation in individual treatment response is due to genetic causes have traditionally been based on logical reasoning rather than study results, accounting for the fact that twin and family studies are rarely feasible to perform in such pharmacological settings. However, pharmocogenetics – utilizing genetic information for tailored and improved use of medications concerning efficacy and adverse reactions – has been a major research area for a number of decades. Strictly, "pharmacogenetics" is the study of how single genes affect the drug response whereas the term "pharmacogenomics" denotes how the "whole" genome can influence the response to drugs. With the possibility of using a hypothesis-free GWAS approach in order to search the genome for polymorphisms that associate with pharmacological responses, the latter term has been increasingly used in the literature. However, the terms pharmacogenetics and pharmacogenomics are often used interchangeably (243) and the meaning is usually obvious from the context in which the words are used.

Pharmacology is concerned with two distinct aspects of the effect of drugs. Pharmacokinetics refers to the uptake, metabolism, distribution and excretion of a

substance, which would affect the amount of substance available for action on the body. Pharmacodynamics is the effect of a specified amount of drug on the target organ(s), i.e. describing the action of the specific drug on the body (244). Naturally, the genetic impact on drug responses could be the consequence of genetic variation affecting any of the steps involved within the pharmacokinetic or pharmacodynamic pathways. Also, there might be genetic variations in pathways of the specific disease outside the pharmacodynamic pathways that could still have important impact on treatment response (243,245). As is the case for genetic associations in general, there might be common variations individually affecting treatment response to a less extent and rare genetic variations with individually more substantial effects (243).

Pharmacogenetic studies have traditionally used a candidate-gene based approach, focusing on genetic variations in pathways known to be of relevance for the effect of a drug. In contrast to the candidate gene approach for finding associations with CVD in general, this approach has been quite successful in the setting of CVD pharmacogenetics (245). Much effort has been concentrated on genetic variation affecting pharmacokinetic pathways, most notably the metabolization of drugs by the phase 1-reactions by the CYP450 enzymes. A known example within CAD pharmacogenetics is the associations between genetic variations in CYP2C19 affecting the conversion of the antiplatelet drug clopidogrel to its active form, with loss-of-function variants resulting in clopidogrel resistance and worse clinical outcomes (246,247). The candidate-gene based approach has also yielded genetic variations in association with pharmacodynamic pathways, including the drug targets for beta-blockers (248), angiotensin converting enzyme-inhibitors (249) and statins (250), although the clinical implications of these findings have remained unclear (reviewed in (251)).

With the success of using GWASs for common disease a natural step has been to use a similar hypothesis-free design also for testing common SNPs in association with drug treatment response. Such GWASs have confirmed many of the previous discovered associations from candidate gene based studies, exemplified by genetic polymorphisms affecting the response to clopidogrel and warfarin (summarized in (252)) and statins (253), the latter however suggesting no actual clinical relevance for the individual SNPs. As for statins, a recent GWAS also suggested a common polymorphism associating with a reduction in statin efficacy for hard end points (254) emphasizing another important aspect in pharmacogenomics.

Warfarin is an anticoagulant with a markedly varied treatment response among individuals as well as a narrow therapeutic interval, making it an ideal drug for pharmagogenetic studies. As shown in Figure 7, warfarin also constitutes an illustrative example of how the underlying genetic architecture could possibly affect the response to a drug, and where findings from previous candidate based gene studies have been confirmed in subsequent GWASs (255). In addition, two small randomized controlled trials have suggested potential benefit for genetic

assessment compared to non-genetic algorithms for the dosage of warfarin (256,257) highlighting a potential clinical benefit that may be translated to clinical use if similar results are found in larger studies (258).

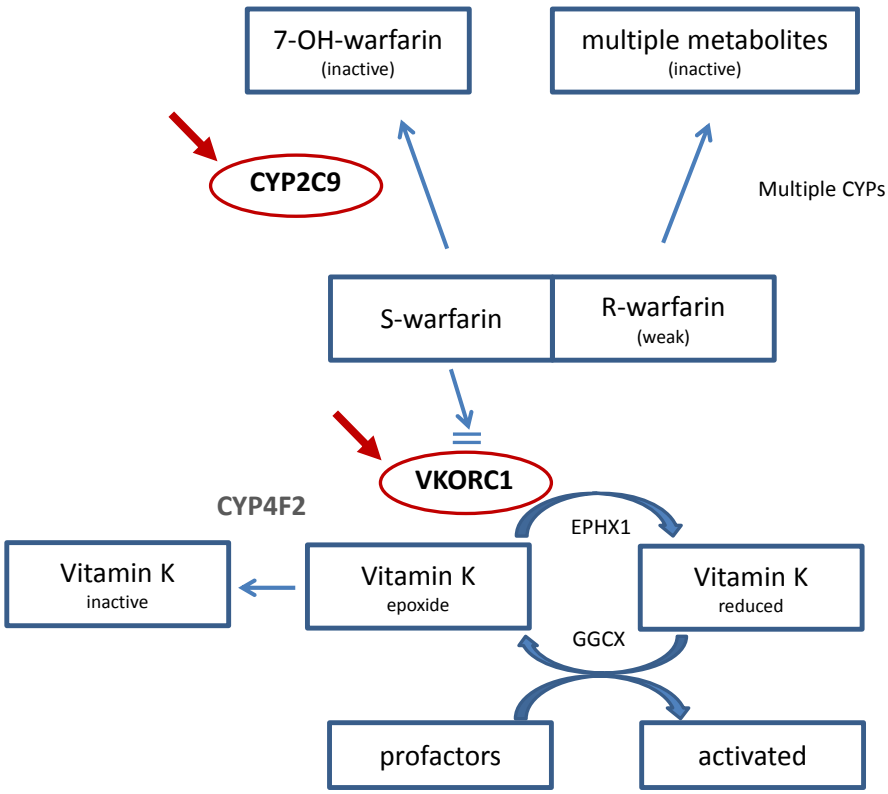

Figure 7
Warfarin as an illustrative example of CVD pharmacogenetics/pharmacogenomics. Genetic variation in both pharmacokinetic (CYP2C9) and pharmacodynamic (VKORC1) pathways affect the treatment response (highlighted in circles with arrows). These genetic variations were first identified in candidate-based studies after which they have been confirmed in GWASs. Some studies have shown that genetic variation in CYP4F2 affects the response to warfarin, however these results have not been consistent across studies. Additional genes that could potentially influence warfarin treatment response are also shown. Figure adapted from (245).

An approach that constitutes an intermediate between the traditional candidate-gene approach and the recent GWAS-approach is to take forward GWAS-identified SNPs that associate CVD and CVD-traits and perform pharmacogenetic studies for these SNPs (245). This "intermediate design" may combine the benefits from the candidate gene based approach (i.e. reducing number of tests, thus requiring smaller study samples for adequate power) with the advantage of a

GWAS-based approach (i.e. also studying variants that would at first not have been considered to be of importance for CVD).

Whereas knowledge of how genetic variation influences drug responses is of major importance for improved and more individualized use of CVD preventive medications, gene-treatment effects should naturally also be evaluated from the opposite direction. That is, with an increasing number of genetic polymorphisms that associate with CVD and CVD traits, we are faced with the challenge of assessing how subjects that are genetically susceptible to CVD should be treated – ideally in order to eliminate the genetic risk increase. Not least the fact that many CVD-associated polymorphisms seem to mediate their risk increase independently of traditional risk factors makes this question highly relevant.

Aims

The general aim of the current thesis was to investigate how common genetic polymorphisms that associate with CVD and CVD risk factors in the population could possibly be implemented in a number of aspects concerning prevention and treatment of CVD.

The specific aims were:

I. To test if a lipid genetic risk score, based on nine SNPs with validated effects on lipid levels in the population, also influences the response to lipid-lowering therapy with statins in subjects with asymptomatic carotid atherosclerosis.

II. To investigate if a genetic risk score of 13 SNPs, strongly associated with risk of CAD and MI, is associated with markers of carotid atherosclerosis in the general population.

III. To evaluate if eight common SNPs with well-validated effects on blood pressure levels in the population also affect the response to different antihypertensive medications in hypertensive subjects.

IV. To assess if the future risk of CVD and CVD-mortality conferred by genetic variation on chromosome 9p21 is modified by lifestyle factors in the general population.

Methods

Study population

The hypotheses of this thesis were tested in subjects from two study populations (Figure 8). The study that evaluated lipid-associated genetic polymorphisms in association with statin treatment response (Study I), the study of CAD- and MI-polymorphisms in association with markers of carotid atherosclerosis (Study II) and the study evaluating interactions between chromosome 9p21 genetic variation and lifestyle factors on the risk of CVD (Study IV) were all based on the cohort of 30 447 subjects from the population based Malmö Diet and Cancer (MDC) Study (259). The study evaluating associations between common genetic variations and BP reduction by different antihypertensive drugs (Study III) was done in subjects from The Nordic Diltiazem (NORDIL) study, a randomized antihypertensive treatment study of 10 881 middle-aged hypertensive Swedish and Norwegian subjects (260).

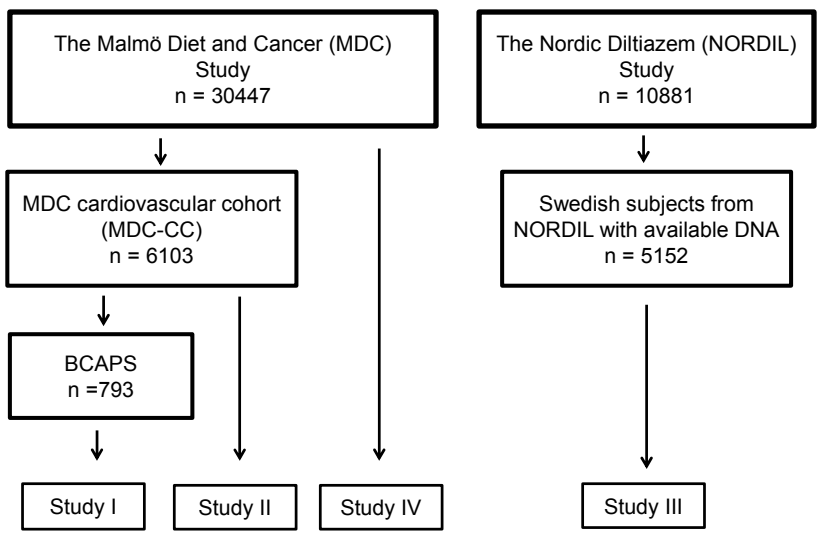

Figure 8
Overview of the study population in the current thesis. More detailed descriptions of the selection of subjects for Studies I-IV are found in the section "Specific methods".

The Malmö Diet and Cancer cohort

The MDC study

The MDC study is a population-based prospective cohort study that recruited subjects between 1991 and 1996, with the aim of investigating the relation between diet, life-style factors and cancer in the population (259). All men born between 1923 and 1945 and all women born between 1923 and 1950 that were living in the city of Malmö in Sweden (a total of 74 138 subjects) were invited. Invitations were sent by letter and participants were also recruited by advertisements in newspapers and public places. Of those invited 41 % attended, yielding a total of 30 447 subjects that were included in the cohort.

Invited subjects that did not participate in MDC had higher mortality during and after the recruitment period, implying a selection towards better health. These and other differences between participants and non-participants have been previously described (261).

Approximately 60 % of the participants in MDC were women, which stems from the intention of the investigators to increase the sample size of young women in order to study breast cancer. Approximately 13 % of the participants in the cohort were born outside Sweden, the majority of whom had immigrated from Denmark. As has been previously described the MDC cohort is not representative of the present population in Malmö (of which around 40 % of the inhabitants were born or have both parents born outside Sweden) but represents better the endogenous Swedish population in Malmö (262).

At baseline, participants in MDC underwent assessment of anthropometric variables, had their BP measured and they provided non-fasting blood samples for storage in a biological bank. Subjects were also asked to complete a questionnaire of health and lifestyle related factors, including current and previous disease, medication, smoking, level of education, and physical activity. Additionally, participants were given a dietary assessment, including a self-completed seven-day menu book, a questionnaire and a one hour personal interview. The self-reported questionnaires were collected and checked by study assistants at a second visit approximately 2 weeks after the first visit. A total of 1998 subjects failed to complete the baseline examination, yielding 28 449 subjects with complete baseline data (of whom 28 098 subjects also had complete dietary data (263)).

The MDC study protocols were approved by the ethical committee at Lund University. All participants provided written informed consent.

The MDC cardiovascular cohort (MDC-CC)

Between October 1991 and February 1994 a random 50 % of enrolled subjects in MDC were also invited to take part in a study of the epidemiology of carotid artery disease, involving an ultrasound examination of the right carotid artery (further

described in the specific methods for Study II). This sub-cohort, which is referred to as the MDC Cardiovascular Cohort (MDC-CC) consists of 6103 subjects (60% women). Of these, 5540 subjects also agreed to an additional extended battery of blood tests provided under standardized fasting conditions at a later visit, as previously described in detail (264).

The BCAPS study

Of the subjects that were included in MDC-CC, 2585 participants (44% of examined subjects) had plaque in the right carotid artery. Of these, subjects free of symptoms suggestive of carotid artery disease were eligible for participation in the Beta-Blocker Cholesterol-Lowering Asymptomatic Plaque Study (BCAPS). BCAPS is a randomized, double blind, placebo-controlled study that tested if treatment with low dose metoprolol (25 mg) and/or fluvastatin (40 mg) could reduce carotid IMT progression in comparison with placebo. The first subject in BCAPS was randomized in November 1994, and the treatment period of 36 months was completed for all participants by February 1999. Exclusion criteria in BCAPS were a history of MI, angina pectoris or stroke within the preceding three months, a history of surgical intervention in the right carotid artery, beta-blocker or statin use, BP >160/95 mmHg, total cholesterol > 8.0 mmol/L, hyperglycemia suspected to require insulin treatment and conditions that in the opinion of the investigator suggested that the subject was unsuitable for the trial. In total, 793 subjects from MDC-CC met all criteria and participated in BCAPS (265).

The NORDIL study population

The Nordic Diltiazem (NORDIL) study (260) is a prospective, randomized, open, blinded endpoint study that compared the effect of the calcium channel blocker diltiazem with that of diuretics, beta-blockers, or both, on cardiovascular morbidity and mortality in hypertensive patients. Between 1992 and 1999 a total of 10 881 middle-aged Swedish and Norwegian hypertensive subjects from 1032 primary health care centers or hospital based hypertension units in Norway and Sweden were recruited. Subjects aged 50–69 years (extended to 74 years during the study period), with no previous antihypertensive medication and who had diastolic BP of at least 100mmHg on two separate occasions were eligible for inclusion. Subjects previously treated with antihypertensive medications were included if they had a diastolic BP of 100 mmHg or more on two following visits, separated by a week when no antihypertensive drugs were given. Exclusion criteria in NORDIL have been previously described (266) and included clinically relevant bradycardia, secondary hypertension, atrial fibrillation, WPW-syndrome, stroke or MI within the preceding six months and present congestive heart failure. Also, subjects with contraindications to any study medication were excluded from

participation as were also subjects that specifically required any of these medications, or other antihypertensive medications for some other reason.

The participants in NORDIL were randomized to antihypertensive treatment with either diltiazem or to treatment with beta-blockers and/or diuretics, with the aim of reducing diastolic BP to less than 90 mmHg. During the first six months, a majority of the subjects were given only one antihypertensive medication. Baseline characteristics of the full NORDIL study population have been previously described in detail (260,266).

Specific methods

Lipid genetics and statin treatment response (Study I)

Study population specification, measurement of lipids and follow-up

The study that investigated the potential influences of common lipid-associated SNPs on statin treatment response (Study I) was conducted in a subset of the BCAPS study population. Subjects in BCAPS that had been randomized to receive either a daily dose of 40 mg fluvastatin (n = 198) or 40 mg fluvastatin plus 25 metoprolol daily (n = 197) was included in this study, yielding a total of 395 subjects.

HDL and TGs were measured under standardized fasting conditions by a direct standard laboratory method, whereas LDL-levels were calculated from the Friedewald formula (267), meaning that subjects with TG levels > 4.5 mmol/l were excluded from LDL analyses. The BCAPS protocol included measurements of the fasting lipid profile at randomization and after 12, 24 and 36 months of treatment. Weight was measured every six months and was thus also available at the same time as the lipid profiles. Since the cholesterol reduction of statins is usually rapid and since dropout increases and compliance may decrease with time, the difference between baseline and the 12 months lipid profile was chosen as the treatment response measurement.

Genetic polymorphisms and genetic score construction

Study I included nine SNPs that were previously shown to be associated with levels of LDL or HDL in the population (238). These were for LDL rs693 in APOB, rs4420638 in APOE, rs12654264 in HMGCR, rs1529729 in LDLR, and rs11591147 in PCSK9 and for HDL rs3890182 in ABCA1, rs1800775 in CETP, rs1800588 in LIPC (hepatic lipase) and rs328 in LPL.

A previously used lipid genetic score (238) was constructed by adding the number of unfavorable alleles (i.e. alleles associated with higher LDL-levels or lower HDL-levels), yielding a lipid-genetic risk score ("Score LDL + HDL"; 0-18 points). In MDC-CC this score was previously shown to strongly associate with lipid levels (bottom to top score LDL increase 3.9 - 4.4 mmol/L [P-trend = 2×10^{-18}] and bottom to top score HDL decrease 1.6-1.3 mmol/L [$P_{trend}= 3\times10^{-24}$]) as well as with incident CVD (OR per risk allele 1.15 [95% CI 1.07–1.24; P <0.001]) (238). In addition to the main score, two sub-scores were constructed for the five SNPs in association with LDL ("Score-LDL"; 0-10 points) and the four SNPs in association with HDL ("Score-HDL"; 0-8 points) respectively.

Genotyping was performed by a matrix-assisted laser desorption–ionization time of- flight mass spectrometry on a MassARRAY platform (Sequenom). This process and genotype quality control has been previously described (238).

CAD and MI genetics and markers of atherosclerosis (Study II)

Study population specification and assessment of CVD risk factors

The cross-sectional study of SNPs associated with CAD and MI (Study II) was done in a total of 4022 subjects from MDC-CC. These subjects were selected on the basis of complete data of right carotid artery measurements (bulb-IMT or CCA-IMT), CVD risk factors including age, sex, smoking, systolic blood pressure (SBP), diastolic blood pressure (DBP), antihypertensive medication, diabetes mellitus (DM), LDL, HDL, CRP, and waist circumference, in addition to genetic data for at least 12 of the 13 SNPs.

The assessment and definitions of CVD risk factors in MDC-CC have been previously described in detail (183,268). In summary, smoking status was based on the MDC baseline questionnaire, and was classified as smoking versus non-smoking (in Study II). Blood pressure was measured in supine position after 10 minutes of rest. The use of antihypertensive medication was assessed from the questionnaire. DM was defined as either self-reported diagnosis in the questionnaire, use of anti-diabetic medication or a fasting blood glucose $\geq$ 6.1 mmol/L. Lipoprotein levels and CRP levels were analyzed according to standard procedures at the Department of Clinical Chemistry, Malmö University Hospital. LDL was calculated from the formula of Friedewald (267). Waist circumference was measured at the umbilical level.

Ultrasound examination of the carotid artery

In MDC-CC a carotid ultrasound examination (Acuson 128 CT system) of the right carotid artery was performed by a trained and certified sonographer (269). The methods have been previously described (183,268). Using B-mode ultrasound the right carotid artery was scanned within a pre-defined window of three cm of the distal common carotid artery, the bulb and one cm of the internal and external carotid arteries, for assessment of plaques (defined as a focal IMT > 1.2 mm). Carotid plaques were assessed "online" and quantified based on the number and size of plaques in a semi-quantitative carotid plaque score that were previously described in detail (270).

IMT was measured "off-line" in the far wall according to the leading edge principle, using a computer-assisted image analyzing system, with the possibility of manual correction. In the bulb the maximum IMT was recorded (bulb-IMT), whereas in the CCA the mean IMT along one cm immediately proximal to the bulb was recorded (CCA-IMT). The intra- and interobserver variability for these

IMT measurements and for the assessment of plaques has been previously described (264,270).

Genetic polymorphisms and genetic score construction

Study II included a total of 13 SNPs with previously identified solid associations with MI and/or CAD: chromosome9p21-rs4977574, SORT1-rs646776, MIA3-rs17465637, CXCL12-rs1746048, KCNE2-rs9982601, PHACTR1-rs9349379, WDR12-rs6725887, LDLR-rs1122608, PCSK9-rs11206510 (219); MRAS-rs9818870, HNF1A-rs2259816 (271); SH2B3-rs3184504 (272) and LPA-rs3798220 (273).

A CAD/MI genetic risk score ("Score-MI") was constructed by summing the total number (maximum 26) of risk alleles, assuming an additive genetic model. The contribution of every added risk allele to the score was weighted based on the effect size for CAD/MI for that allele in discovery studies. Cohort specific averages were used for imputation in case of missing data for one of the 13 SNPs. The same genetic risk score was previously shown to strongly associate with incident CAD events (HR for top versus bottom quintile of score 1.66 [95 % CI 1.35–2·04; P-trend = 7.3×10^{-10}]) (239). In addition to the main score, two sub-scores were constructed; "Score-MI-LDL" included the five SNPs that were formerly shown to be associated with LDL (LDLR-rs1122608, SORT1-rs646776, PCSK9-rs11206510, HNF1A-rs2259816 and LPA-rs3798220) whereas "Score-MI-non-LDL" included the remaining eight SNPs.

The SNPs were genotyped using IPLEX on a MassARRAY platform (Sequenom, San Diego, CA, USA) according to the manufacturer's standard protocols. Fifteen per cent of the samples were run in duplicate without any inconsistencies. All genotypes were called by two different investigators. No SNP failed the Hardy-Weinberg equilibrium at a P-value of 0.001.

BP genetics and antihypertensive therapy (Study III)

Study population specification and definition of BP treatment response

The study of SNPs associated with BP-levels in the population and their potential impact on antihypertensive treatment response (Study III) was conducted in the Swedish subset of the NORDIL study population. As has been previously described (274) DNA could be extracted in 5152 subjects (72.4 % of the Swedish subset in NORDIL), making these subjects eligible for genetic studies. This study protocol was approved by the ethics committee at Lund University and Gothenburg University. All patients gave their informed consent.

In the NORDIL study, BP levels were measured in recumbent position every six months. Aiming for a reduction of diastolic BP to less than 90 mmHg there was a

stepwise approach of adding antihypertensive treatments at the follow-ups, as previously described in detail (260). Accounting for the fact that during the first six months of the NORDIL-study a majority of participants had only one antihypertensive drug (diltiazem, a beta-blocker or a thiazide diuretic respectively), this time period was selected for studying the genetic influences on BP treatment response. Accordingly, subjects that were on mono therapy during this period (n = 4052) and who had complete data for covariates of interest including age, sex, DM, smoking, serum creatinine, BMI and previous CVD (n = 3863) were included in Study III.

Treatment response was defined as the absolute and percent decrease (positive direction) in systolic BP (SBP) and diastolic BP (DBP) between baseline and six months (mean of BP levels at inclusion and randomization - BP level at six months).

Genetic polymorphisms

Eight SNPs previously found to be strongly associated with SBP and/or DBP levels in the population were selected for Study III (226). These were:

- rs16998073 near PRDM8/FGF5/c4orf22
- rs1378942 near CYP1A1/CYP1A2/CSK/LMAN1L/CPLX3/ARID3B
- rs3184504 near SH2B3/ATXN2
- rs1530440 near c10orf107/TMEM26/RTKN2/RHOBTB1/ARID5B
- rs16948048 near ZNF652/PHB
- rs17367504 near MTHFR/CLCN6/NPPA/NPPB/AGTRAP
- rs12946454 near PLCD3/ACBD4/HEXIM1/HEXIM2
- rs11191548 near CYP17A1/AS3MT/CNNM2/NT5C2

The effect sizes of the eight SNPs in Study III are in the range of 0.5-1 mmHg higher BP per allele (226). As in Studies I and II we additionally constructed a genetic risk score ("Score-BP") for participants in the study population who had data of at least seven of the eight SNPs (n = 3647). This score was constructed by summing the total number of BP-elevating alleles (maximum 16), assuming an additive genetic model. The contribution of every added BP-elevating allele to the score was weighted based on the beta-coefficient for association with BP levels in the discovery study (226). Cohort-specific averages for alleles were imputed in the case of missing data for one of the eight SNPs.

The SNPs in Study III were genotyped by IPLEX on a MassARRAY platform (Sequenom, San Diego, CA, USA) according to standard protocols from the manufacturer. Fifteen percent of the samples were run in duplicate without any inconsistencies. All genotypes were called by two different investigators. No

included SNP deviated from the Hardy–Weinberg equilibrium at a P-value of 0.05.

Gene-lifestyle interactions and risk of CVD (Study IV)

Study population specification

The interaction study that examined whether the future risk of CVD and CVD-mortality conferred by genetic variation on chromosome 9p21 is modified by lifestyle factors (Study IV) was done in the MDC cohort. DNA was extracted and the SNP rs4977574 on chromosome 9p21 was previously successfully genotyped in 27885 subjects in MDC. After excluding subjects with previous CVD at baseline (i.e. a history of MI, coronary-artery-by-pass graft surgery (CABG), percutaneous coronary intervention (PCI), or stroke) a total of 26855 subjects remained. Of these, subjects that had complete baseline data for all variables and covariates of interest including smoking status, education level, physical activity, SBP, use of antihypertensive medication and BMI were selected, yielding a total of 24944 subjects that were included in the analyses for Study IV.

Lifestyle related factors at baseline

Data of life-style related health factors including smoking, education and physical activity levels at baseline were obtained from the self-reported questionnaires from the MDC baseline examination.

The status of smoking was self-reported and coded as never, former or current (i.e. smoking within the past year) in a categorical variable. Passive smoking was defined as exposure to smoking either at home ("Do the persons you live with smoke indoors, or have they done so previously?") or at work ("Do you regularly stay in places of work [apart from your home] where people smoke, or have you previously been staying in such places regularly?") and was dichotomously coded.

Education was defined as the self-reported highest level of education and coded as a six-graded categorical variable (0 = did not complete elementary school, 1 = elementary school (6-8 yrs), 2 = junior secondary school (9-10 yrs), 3 = education at advanced level (12 yrs); 4 = at least one additional year, 5 = university degree).

Data of leisure-time physical activity was obtained from a comprehensive physical activity questionnaire. This questionnaire, adapted from a modified Minnesota leisure time physical activity questionnaire (275) contained questions covering various activities in the four seasons. By combining the intensity factors for the reported activities with the time spent on each activity, a physical activity score (PA-score) was previously calculated in MDC. For Study IV, this PA-score was categorized into study population-specific quintiles. The PA-score has been

previously validated with an accelerometer monitor in a random sample of 369 subjects from MDC (276).

Genotyping

In MDC, DNA was extracted from frozen granulocyte or buffy coat samples from blood from the baseline examination (1991–1996) using QIAamp 96 spin blood kits (QIAGEN, VWR, Gaithersburg, MD, USA).

The rs4977574 SNP (A/G) on chromosome 9p21 was used as a "tag-SNP" for denoting the CVD risk locus on Chromsome 9p21. The alleles of this SNP were determined by "Assay by design" TaqMan probes with a real time polymerase chain reaction assay on an ABI-7900HT equipment (Applied Biosystems, Foster City, CA) according to the manufacturer's standard protocols. For quality control 20% of the samples were run in duplicate and the concordance was > 99.9%.

The number of rs4977574 risk alleles (G) for each subject was assessed and coded as a linear variable assuming an additive effect (0-1-2 alleles).

Follow-up and definitions of endpoints

The three primary endpoints of study IV were CAD, ischemic stroke and CVD-mortality respectively (all endpoints are defined below). These were identified by linking the 10-digit Swedish personal identification number of each subject with four registers: the Swedish Hospital Discharge Register, Swedish Coronary Angiography and Angioplasty Registry (SCAAR), the Stroke Register of Malmö and the Swedish Cause of Death Register. These registers have been previously described in detail and they have been validated for outcome classification (277-280). Follow-up extended to June 30, 2009.

The definition of CAD included fatal or non-fatal MI, death from ischemic heart disease, CABG or PCI. MI was defined on the basis of International Classification of Diseases 9th and 10th Revisions (ICD9 and ICD10) codes 410 and I21, respectively. Death due to ischemic heart disease was defined on the basis of codes 412 and 414 (ICD9) or I22–I23 and I25 (ICD10). CABG was identified from national Swedish classification systems of surgical procedures, the KKÅ system from 1963 until 1989 and the Op6 system after that. CABG was defined as a procedure code of 3065, 3066, 3068, 3080, 3092, 3105, 3127, 3158 (Op6) or FN (KKÅ97). PCI was defined based on the operation codes FNG05 and FNG02.

Fatal or nonfatal stroke was assessed using codes 430, 431, 434 and 436 (ICD9) and I60, I61, I63, and I64 (ICD10). Hemorrhagic strokes were however censored in the analyses, meaning that only cerebral infarctions (code 434 for ICD9 / I63 for ICD 10) were included in the endpoint definition.

CVD-mortality was defined as the underlying cause of death classified by ICD-9 diagnoses 390-459 and ICD-10 diagnoses I00-199.

Statistics

General statistics

Statistical analyses in the current thesis were done by using SPSS Statistics versions 16.0-21.0 (IBM Corp., Armonk, NY, USA) and Stata 11.0 (StataCorp LP, College Station, TX, USA). Power calculations were performed by using the software PS 3.0 by WD Dupont and WD Plummer Jr from the Department of Biostatistics, Vanderbuilt University (Nashville, TN, USA).

Generally, a P-value < 0.05 was considered significant however with correction for multiple testing taken into account when appropriate.

Statistical analyses in Studies I-IV

In Study I we related the lipid genetic risk scores to the change of LDL and HDL levels (absolute and percent change) between baseline and 12 months of treatment in linear models. LDL-change was defined as baseline LDL – 12 months LDL and HDL change was defined as 12 months HDL – baseline HDL. We tested unadjusted models and models including covariates age, blood glucose and percent BMI-reduction during the study period. Applying a sex-specific approach, we tested for interaction between genotype score and sex (genotype score × sex, genotype score and sex as independent variables) on the outcome of LDL and HDL change.

For Study II the relationships between the genetic risk scores, bulb-IMT and CCA-IMT were assessed in linear models in which the genetic scores were entered as the independent variables and IMT as the dependent variables. Because of positive skew distribution, IMT-values were log-transformed in all analyses. Both unadjusted and adjusted models were used. In the adjusted models, residuals of bulb- and CCA-IMT, adjusted for age, sex, smoking, DM, SBP, use of antihypertensive medication, LDL, HDL, CRP and waist circumference, were entered as dependent variables and the genotype scores as the independent variables. Additionally to IMT, the genotype scores were related to the occurrence of at least one carotid plaque ≥ 10 mm^2 in logistic regression analyses. Accounting for multiple testing (three genotype scores on three outcomes: bulb-IMT, CCA-IMT and carotid plaque) a P-value of $0.05/9 = 0.0056$ was considered significant in Study II.

In Study III we related each of the eight BP-SNPs to the BP reduction (absolute and percent) achieved after six months, using linear models with number of BP-elevating alleles as the independent variables. Additive genetic models were

assumed. We used unadjusted models and models including covariates age, sex, DM, smoking, serum creatinine, BMI and previous CVD. Power at an alpha of 0.05 was calculated based on a detectable difference in linear regression slope (i.e. beta-coefficient) of 2/1 mmHg SBP/DBP per allele, as these are BP reductions suggested to translate into reductions in CVD in the population (281). For the genetic risk score (Score-BP), the detectable difference was set to 1/0.5 mmHg SBP/DBP per risk allele. A power ≥ 80% was considered adequate to correctly reject a false null hypothesis stating that there are no differences in BP reduction depending on number of BP-elevating alleles.

We primarily aimed at detecting genetic effects affecting either renal sodium retention or genetic effects on vascular smooth muscle, and in order to achieve sufficient statistical power analyses were performed separately in two (i.e. not three) groups: group 1 ("BB/diuretics-group") included subjects treated with either beta-blockers or diuretics (i.e. drugs that directly or indirectly affect renal sodium retention) and group 2 ("Diltiazem-group") included subjects treated with diltiazem (a non-selective calcium channel blocker affecting cardiac muscle, chronotropy and vascular smooth muscle, however with no obvious effects on renal sodium retention).

For Study IV we constructed proportional-hazards models and used Cox regression analysis to test associations between the independent variables and time to the first event of each of the three end-points. The proportional-hazards assumption was confirmed by visual inspection of survival curves for all endpoints. Evidence of (multiplicative) interaction between the number of rs4977574 risk alleles and smoking, educational level and physical activity on the end-points was assessed in Cox regression models that included the respective interaction terms (rs4977574 x smoking status; rs4977574 x educational level; rs4977574 x quintile of physical activity score) in addition to the main effect terms. We then used the likelihood ratio (LR) test to compare model fit with and without the interaction terms in order to test for significant interaction. We compared the fit of models adjusted for age and sex only, as well as models including the additional covariates BMI, SBP and use of antihypertensive medication in addition to the three main effect terms.

For incident CAD we also designed a meta-analysis including additional results from a study of interaction analyses between chromosome 9p21 variation and a number of environmental factors in 9877 subjects from the Atherosclerosis Risk in Communities (ARIC) Study (282). The meta-analysis was performed on the study level, by pooling the effect estimates for the associated risk of incident CAD by the chromosome 9p21 risk locus in smokers and non-smokers (i.e. never or former smokers) respectively.

Results

Lipid genetics and statin treatment response (I)

Study population characteristics

The baseline characteristics of the subjects that were included in Study I are shown in Table 2. Age and BMI did not differ between sexes. Women had higher total cholesterol and HDL and lower DBP and blood glucose than men, whereas a history of CVD was more common in men. There were no differences in baseline variables between subjects treated with fluvastatin and subjects treated with both fluvastatin and metoprolol (data not shown).

Table 2
Baseline characteristics of the subjects included in Study I.

	Men (n = 180)	**Women (n = 215)**
Age, years	62.3 (5.3)	61.9 (5.1)
Total cholesterol, mmol/L	6.03 (0.86)	6.22 (1.01) [a]
LDL-cholesterol, mmol /L	4.12 (0.80)	4.20 (0.89)
HDL-cholesterol, mmol/L	1.27 (0.31)	1.47 (0.35) [c]
Cholesterol > 5 mmol/L	164 (91.1 %)	193 (89.8 %)
Triglycerides, mmol/L [d]	1.18 (0.86)	1.05 (0.71)
Body mass index, kg /m2	25.8 (3.4)	25.3 (3.5)
Systolic blood pressure, mmHg	139.3 (12.8)	139.4 (14.9)
Diastolic blood pressure, mmHg	86.0 (6.2)	84.0 (7.1) [b]
Blood glucose, mmol/L	5.29 (0.63)	4.98 (0.73) [c]
Mean CCA-IMT, mm	0.90 (0.16)	0.89 (0.21)
History of Diabetes	4 (2.2 %)	4 (1.7 %)
Smokers	60 (33.3 %)	64 (29.8 %)
History of CVD	16 (8.9 %)	5 (2.3 %) [e]

Data presented as mean (SD) or number (%) unless otherwise specified.
For Student's T-test:
[a] $P < 0.05$
[b] $P < 0.01$
[c] $P < 0.001$
[d] Data shown as median (interquartile range)
[e] P Chi2 (Pearson) = 0.004

Sex-specific genetic associations with treatment response

Genetic influence on LDL-reduction

Fluvastatin treatment for 12 months significantly decreased LDL levels in the study population (mean [SD] reduction: 0.91 [0.76] mmol/L or 21.1 [16.8] %; $P < 0.001$ for both). There was no significant difference in LDL-reduction between men and women (data not shown). However, interaction analyses revealed an interaction between sex and Score LDL + HDL on fluvastatin-induced LDL change ($P_{interaction} = 0.012$ for absolute and $P_{interaction} = 0.033$ for percent reduction). Accordingly, a higher Score LDL + HDL was associated with less prominent reduction of LDL in women (Figure 9), whereas no such genetic-treatment association was identified in men ($P > 0.05$). The LDL-related SNPs seemed to explain the association found in women, as revealed by sub-score analyses (Table 3). Results were similar in models adjusted for age, blood glucose and percent BMI change during treatment (the results of the adjusted models are shown in Paper I).

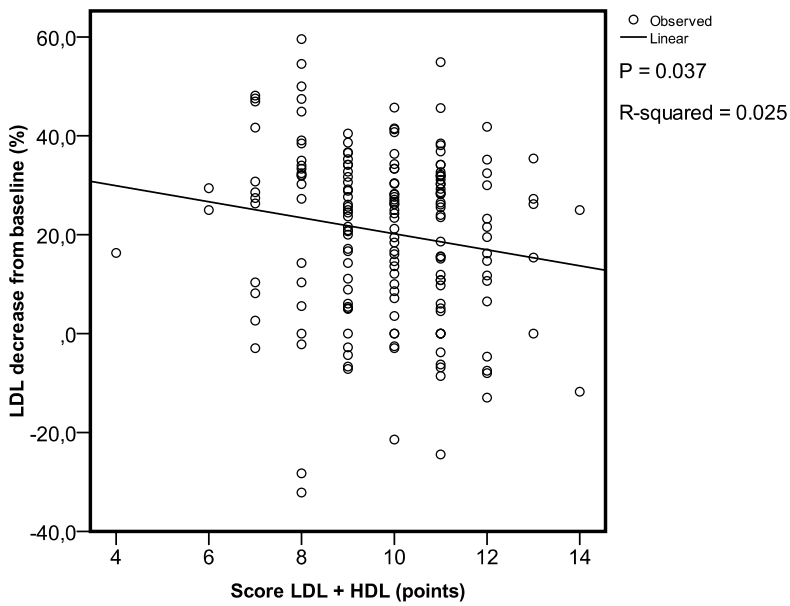

Figure 9
The relationship between Score LDL + HDL and the LDL decrease (positive direction) among women after 12 months of fluvastatin treatment.

Table 3
Influence of the genetic risk scores on LDL-reduction (positive direction) in women.

Score	Points		Beta	Variance explained	P
Possible points	Min-max		mmol/L per point (% per point)	%	
Score LDL + HDL (0-18 p)	4-14	Absolute Percent	-0.0800 -1.62	2.66 2.50	**0.031** **0.037**
Score LDL (0-10 p)	2-8	Absolute Percent	-0.101 -2.11	2.92 2.92	**0.023** **0.023**
Score HDL (0-8 p)	0-8	Absolute Percent	-0.014 -0.132	0.0400 0.00810	0.789 0.908

Genetic influence on HDL-increase

There was a difference in fluvastatin-induced HDL change between subjects treated with metoprolol (n = 188; mean HDL-change [SD] -0.03 [0.18] mmol/L or -0.9 [11.9] %) and subjects without simultaneous metoprolol treatment (n = 187; mean HDL-change [SD] +0.03 [0.20] mmol/L or +2.6 [13.8] %) after 12 months (P = 0.007 for absolute and P = 0.009 for percent change differences between the groups). Thus, in order to avoid any influence of metoprolol on HDL levels, the genetic treatment-response analyses for HDL change were restricted to subjects that were treated exclusively with fluvastatin.

There was no difference in HDL change between sexes (data not shown). However, as was the case for fluvastatin-induced LDL change, there was an interaction between sex and Score LDL + HDL on the HDL change in subjects treated exclusively with fluvastatin ($P_{interaction}$ = 0.015 for absolute and $P_{interaction}$ = 0.047 for percent change). Accordingly, an increased Score LDL + HDL was associated with a more pronounced HDL increase in women (Figure 10), whereas there was no such treatment association in men (P >0.05). In women, Score LDL+ HDL explained 11.6-12.9 % of the variance in fluvastatin-induced HDL change (Table 4). Results were very similar in models adjusted for age, blood glucose and percent BMI change during treatment (the results of the adjusted models are shown in Paper I).

Analysis of sub-scores revealed that the genetic treatment response association for HDL levels in women could mainly be attributed to the HDL-related SNPs (Table 4).

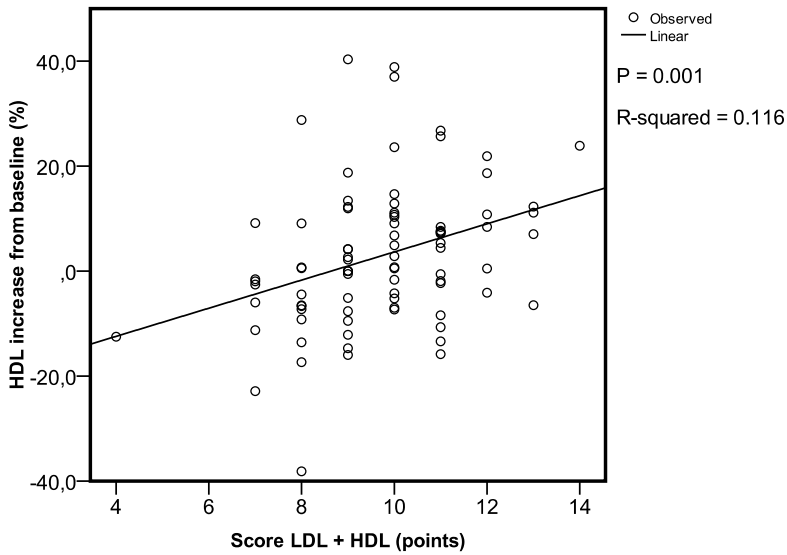

Figure 10
The relationship between Score LDL + HDL and the HDL increase (positive direction) among women after 12 months of fluvastatin treatment.

Table 4
Influence of the genetic risk scores on HDL increase (positive direction) in women.

Score	Points		Beta	Variance explained	P
Possible points	Min-max		*mmol/L per point (% per point)*	%	
Score LDL + HDL (0-18 p)	4-14	Absolute Percent	0.045 2.68	12.9 11.6	**0.001** **0.001**
Score LDL (0-10 p)	2-8	Absolute Percent	0.032 1.98	4.20 4.20	0.056 0.057
Score HDL (0-8 p)	0-7	Absolute Percent	0.048 2.75	7.08 5.90	**0.012** **0.022**

CAD and MI genetics and markers of atherosclerosis (II)

Study population characteristics

Of the 4022 subjects from MDC-CC that were included in Study II a total of 2788 participants had bulb-IMT data whereas CCA-IMT measurements were available in 4016 subjects. The occurrence of carotid plaque could be assessed in a total of 2851 subjects. Subjects from MDC-CC that were excluded from this study because of missing data for carotid IMT, genetics and/or CVD risk factors (n = 2081) had slightly higher BP, waist circumference and CRP and slightly lower HDL than included subjects. Excluded subjects were also more likely to be men, to smoke and to have DM. Carotid IMT and occurrence of plaques $\geq 10mm^2$ did not differ between included and excluded subjects (Table 5).

Table 5
Baseline characteristics of the subjects in Study II

	Study population		Excluded from study [a]
	All included subjects	No IMT bulb data	
Total subjects	4022	1234	2081
Men	1634 (40.6 %)	476 (38.6 %)	938 (45.1 %)
Women	2388 (59.4 %)	758 (61.4 %)	1143 (54.9 %)
Age, years	57.5 (5.9)	57.1 (5.8)	57.4 (5.9)
SBP, mmHg	140.9 (18.8)	140.9 (18.9)	142.2 (19.7)
DBP, mmHg	86.8 (9.4)	87.6 (9.4)	87.4 (9.6)
Antihypertensive medication	668 (16.6 %)	251 (20.3 %)	342 (16.5 %)
Waist circumference, cm	83.5 (13)	85.9 (13)	85.6 (13)
Total Cholesterol, mmol/L	6.15 (1.1)	6.12 (1.1)	6.20 (1.2)
HDL, mmol/L	1.39 (0.37)	1.35 (0.35)	1.34 (0.38)
LDL, mmol/L	4.17 (0.98)	4.15 (0.99)	4.15 (1.0)
CRP, mg/L [b]	1.3 (0.1 – 60.2)	1.5 (0.1-51.4)	1.7 (0.1-51.3)
Diabetes	303 (7.5 %)	103 (8.3 %)	183 (12.3 %)
Smokers	1033 (25.7 %)	266 (21.6 %)	582 (33.5 %)
IMT of bulb, mm [b]	1.23 (0.47-4.94)	NA	1.24 (0.46-5.08)
IMT of CCA, mm [b]	0.714 (0.33-2.03)	0.694 (0.33-1.73)	0.714 (0.41-2.63)
Carotid plaque data	2851	420	1391
At least one plaque ≥ 10mm^2	849 (29.8 %)	65 (15.5 %)	442 (31.8 %)
Score-MI	1.825 (0.33)	1.814 (0.32)	NA

Data presented as mean (SD) or number (%) unless otherwise specified.

[a] Subjects in the Malmö Diet and Cancer Cardiovascular Cohort (total n = 6103) excluded from the current study because of missing IMT, genetic and/or risk factor data. Numbers displayed are based on available data in this group: Sex: n= 2081; Age: n = 2080; SBP/DBP: n= 2080; Antihypertensive mediciation: n = 2072; Waist: n = 2072; Cholesterol: n = 1589; HDL: n = 1431; LDL: n = 1349; CRP: n = 713; Diabetes: n = 1487; Smoking: n = 1739; Bulb-IMT: n = 1358; CCA-IMT: n = 2040; Plaque data: n = 1391.
[b] Displayed as median (range)

Genetic risk scores in association with carotid IMT and plaque

The effect of the main CAD- and MI-genetic risk score

Score-MI was associated with carotid bulb-IMT and this association remained significant also after adjustments for conventional CVD risk factors and after accounting for multiple testing with Bonferroni correction. For CCA-IMT the association with Score-MI remained significant after adjustments for CVD risk factors, but not if also accounting for Bonferroni correction (Table 6).

There was an association between Score-MI and the occurrence of moderate to severe carotid atherosclerosis, defined as the occurrence of at least one carotid plaque ≥10 mm^2. This association remained significant after adjustment for CVD risk factors and if multiple testing was taken into account (Table 6).

Table 6
Association between Score-MI and markers of carotid atherosclerosis

	Unadjusted model			**Adjusted model** [a]		
	Beta [b]	**P-value** [c]	**P-value** [d]	**Beta** [b]	**P-value** [c]	**P-value** [d]
Bulb-IMT	0.043	0.001	<0.001	0.038	0.005	0.003
CCA-IMT	0.033	0.003	<0.001	0.028	0.011	0.008
	OR	**95 % CI; P-value**		**OR**	**95 % CI; P-value**	
Plaque [e]	1.12	1.06-1.18; P <0.001		1.11	1.04-1.18; P =0.001	

Applying Bonferroni correction a P-value of 0.05/9 = 0.0056 was considered significant

[a] Adjusted for age, sex, smoking, diabetes mellitus, systolic blood pressure, antihypertensive treatment, LDL, HDL, CRP and waist circumference.
[b] Beta-coefficients relating to number of standard deviations of log-transformed IMT per quintile of Score-MI in the study population.
[c] P-value for linear regression with quintiles of Score-MI as independent variable
[d] P-value for linear regression with the continuous Score-MI as independent variable
[e] Defined as occurrence of at least one carotid plaque ≥ 10 mm^2. Odds ratios are per quintile of Score-MI in binary logistic regression models.

In order to provide a comprehensible estimation of the effect size of Score-MI on carotid IMT we compared the effect size of Score-MI with the effect sizes of LDL- and SBP-levels on carotid IMT in the study population. The effect size of the upper quintiles of Score-MI on bulb-IMT was similar to that seen for the upper quintiles of LDL-levels in the study population (Figure 11).

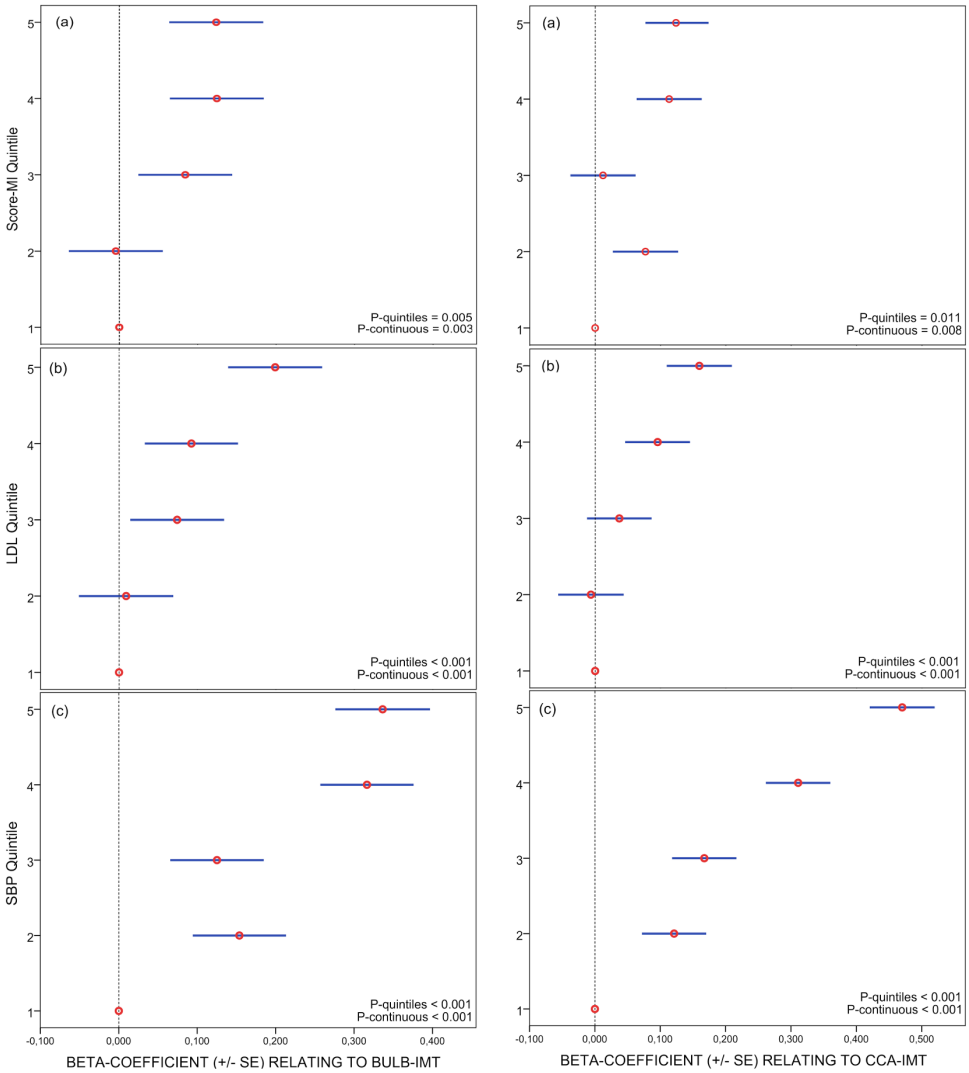

Figure 11
Impact of quintiles of Score-MI (a), LDL-levels (b) and SBP-levels (c) on bulb-IMT (left panel) and CCA-IMT (right panel). Beta-coefficients (circles) ± Standard Error (S.E; vertical lines) displayed as number of standard deviations of log-transformed IMT in relation to the lowest quintile of Score-MI, LDL-levels and SBP-levels in the study population. Models adjusted for age, sex, diabetes, smoking, antihypertensive treatment, CRP levels, HDL-levels (all), LDL-levels (only in a and c), SBP-levels (only in a and b), Score-MI (only in b and c).

The genetic sub-scores and the individual SNPs

After accounting for Bonferroni-correction, there were no significant associations between Score-MI-LDL and bulb-IMT (P = 0.141 for quintiles of Score-MI-LDL; P = 0.157 for continuous Score-MI-LDL), CCA-IMT (P = 0.029 for quintiles of Score-MI-LDL; P = 0.112 for continuous Score-MI-LDL) or occurrence of carotid plaques ≥ 10mm^2 (per Score-MI-LDL-quintile OR = 1.06 [95 % CI 1.00-1.12]; P = 0.066) in the adjusted models. Score-MI-non-LDL associated with bulb-IMT with borderline significance (P = 0.006 for quintiles of Score-MI-non-LDL; P = 0.011 for continuous Score-MI-non-LDL) and was associated with occurrence of carotid plaques ≥ 10mm^2 (per Score-MI-non-LDL quintile OR 1.09 [95 % CI 1.03-1.16]; P = 0.005) but not with CCA-IMT (P = 0.049 for quintiles of Score-MI-non-LDL; P = 0.038 for continuous Score-MI-non-LDL) in the adjusted models.

Analysis of the 13 individual SNPs revealed one SNP, rs4977574 on chromosome 9p21, with evidence of strong association with both bulb-IMT (P = 0.001 for additive model) and CCA-IMT (P = 0.004 for additive model).

Genetic associations with conventional CVD risk factors

There was an association between Score-MI and LDL-levels (beta = 0.041 mmol/L per quintile of Score-MI; P < 0.001). As expected, this association was explained by Score-MI-LDL (P < 0.001) whereas Score-MI-non-LDL did not associate with LDL levels (P > 0.05). The continuous Score-MI showed borderline significant associations with increasing SBP levels (P = 0.037) and with decreasing CRP levels (P = 0.040), but not with any of the other tested conventional CVD risk factors in the study population (P > 0.05).

BP genetics and antihypertensive therapy (III)

Study population characteristics

Clinical characteristics of the subjects that were included in Study III are shown in Table 7. Subjects in the BB/diuretics group had lower baseline BP and achieved a greater reduction in SBP levels during the treatment period than subjects in the diltiazem group. There were no other differences between the treatment groups.

Table 7
Baseline characteristics of subjects in Study III

	All subjects	**BB/Diuretics**	**Diltiazem**
Total subjects	3863	1844	2019
Men	1883 (48.7 %)	872 (47.3 %)	1011 (50.1 %)
Women	1980 (51.3 %)	972 (52.7 %)	1008 (49.9 %)
Age, years	60.3 (6.6)	60.3 (6.7)	60.4 (6.6)
Baseline SBP, mmHg	172.7 (16.2)	171.9 (15.9) [a]	173.3 (16.5) [a]
- ΔSBP, mmHg	17.1 (16.1)	18.1 (16.5) [b]	16.1 (15.7) [b]
- ΔSBP, %	9.6 (8.9)	10.3 (9.1) [b]	8.9 (8.6) [b]
Baseline DBP, mmHg	103.9 (5.0)	103.6 (5.0) [a]	104.1 (5.0) [a]
- ΔDBP, mmHg	14.4 (8.1)	14.2 (8.3)	14.5 (8.0)
- ΔDBP, %	13.7 (7.6)	13.6 (7.8)	13.8 (7.4)
Smokers	780 (20.2 %)	371 (20.1 %)	409 (20.3 %)
Diabetes	324 (8.4 %)	142 (7.7 %)	182 (9.0 %)
BMI, kg/m2	28.0 (4.3)	27.9 (4.4)	28.0 (4.3)
eGFR, ml/min/m^2 [c]			
Men	81.1 (16.7)	81.3 (17.4)	81.0 (16.2)
Women	86.4 (18.3)	86.7 (18.3)	86.1 (18.4)
Previous CVD	146 (3.8 %)	69 (3.7 %)	77 (3.8 %)

Data presented as mean (SD) or number (%).
[a] $P < 0.01$ between BB/diuretics and Diltiazem groups (Student's T-test)
[b] $P < 0.001$ between BB/diuretics and Diltiazem groups (Student's T-test)
[c] Estimated glomerular filtration rate from the Cockcroft – Gault formula and divided by body surface area.

Genetic associations with BP treatment response

Associations of the individual SNPs with BP treatment response

Six of the eight SNPs that were included in Study III showed no associations with BP treatment response in neither the BB/diuretics groups nor the diltiazem group. Power for detecting the desirable 2/1 mmHg reduction per allele in these analyses was > 80 % for four of the SNPs (rs16998073; rs1378942; rs3184504; rs16948048), between 77-87 % for one SNP (rs1530440) and between 65-71 % for one SNP (rs17367504).

Two SNPs associated with BP treatment response. The BP-elevating alleles of the SNP rs12946454 near genes PLCD3/ACBD4/HEXIM1/HEXIM2 were associated with a more pronounced SBP and DBP reduction in the diltiazem group, meaning that more baseline BP-elevating alleles predicted a more pronounced mean SBP and DBP reduction. Contrary, no associations were seen for the same SNP in the BB/diuretics group (Table 8).

The BP-elevating alleles of the SNP rs11191548, near genes CYP17A1/AS3MT/CNNM2/NT5C2, and that of the eight SNPs with strongest effect size on BP levels in the population, were associated with a less pronounced DBP reduction in the BB/diuretics group, meaning that with more baseline BP-elevating alleles there was also less mean DBP reduction during the treatment period. Contrary, no such effect could be detected for rs11191548 in subjects that were treated with diltiazem. Analyses for rs11191548 were underpowered (Table 8).

If multiple testing was taken into account the significance of the associations with BP-treatment response for rs12946454 in the diltiazem group and for rs11191548 in the BB-diuretics group was attenuated.

Associations of the BP genetic risk score with BP treatment response

There were no associations between Score-BP and the magnitude of BP reduction neither in the BB/diuretics group nor in the diltiazem group ($P > 0.05$). Power for detecting the desirable differences in BP reduction per risk allele was > 80 % in all analyses involving Score-BP.

Table 8
Association of two SNPs with BP reduction (positive direction) after six months

SNP rs12946454 (A/T) near genes PLCD3, ACBD4, HEXIM1, HEXIM2.
Main phenotype: SBP.
BP-elevating allele: T (minor allele)

	Unadjusted Model		Adjusted Model [a]		*Power* [b]
Beta-blockers / Diuretics	Beta [c]	P	Beta [c]	P	
- ΔSBP, mmHg	0.761	0.228	0.779	0.218	*88.6 %*
- ΔSBP, %	0.409	0.242	0.417	0.233	
- ΔDBP, mmHg	0.053	0.869	0.054	0.866	*88.0 %*
- ΔDBP, %	0.020	0.947	0.019	0.949	
Diltiazem					
- ΔSBP, mmHg	1.529	**0.010**	1.557	**0.008**	*92.4 %*
- ΔSBP, %	0.767	**0.017**	0.773	**0.016**	
- ΔDBP, mmHg	0.734	**0.014**	0.751	**0.011**	*91.8 %*
- ΔDBP, %	0.661	**0.018**	0.678	**0.014**	

SNP rs11191548 (T/C) near genes: CYP17A1, AS3MT, CNNM2, NT5C2.
Main phenotype: SBP
BP-elevating allele: T (major allele)

	Unadjusted Model		Adjusted Model [a]		*Power* [b]
Beta-blockers / Diuretics	Beta [c]	P	Beta [c]	P	
- ΔSBP, mmHg	-0.665	0.529	-0.719	0.496	*47.4 %*
- ΔSBP, %	-0.366	0.531	-0.418	0.475	
- ΔDBP, mmHg	-1.263	**0.018**	-1.272	**0.017**	*46.5 %*
- ΔDBP, %	-1.161	**0.020**	-1.172	**0.019**	
Diltiazem					
- ΔSBP, mmHg	0.126	0.891	0.128	0.889	*58.5 %*
- ΔSBP %	0.074	0.882	0.063	0.900	
- ΔDBP, mmHg	0.556	0.235	0.561	0.229	*56.9 %*
- ΔDBP, %	0.523	0.231	0.531	0.220	

[a] Age, sex, smoking, s-creatinine, body mass index, diabetes mellitus and previous cardiovascular disease entered as covariates in linear model
[b] Calculated for absolute blood pressure reduction in unadjusted analyses, detecting a beta-coefficient for linear regression slope of 2/1 mmHg per allele
[c] Blood pressure reductions (positive direction) per BP-elevating allele. Linear additive models assumed.

Gene-lifestyle interactions and risk of CVD (IV)

Study population characteristics

The baseline characteristics of the 24944 subjects from the MDC study that were included in Study IV are shown in Table 9. For assessment of the endpoints of CAD (n = 2309), ischemic stroke (n = 1253) and CVD-mortality (n = 1156) the subjects were followed for a median time of 14.5, 14.6 and 14.7 years respectively.

Table 9
Baseline characteristics of the subjects in Study IV according to genotype

	Chromosome 9p21 rs4977574 genotype		
	0 risk alleles (A/A)	*1 risk allele (A/G)*	*2 risk alleles (G/G)*
Total subjects	7609	12311	5024
Men	2881 (37.9 %)	4682 (38.0 %)	1892 (37.7 %)
Women	4728 (62.1 %)	7629 (62.0 %)	3132 (62.3 %)
Age, years	58.0 (7.7)	58.0 (7.6)	57.9 (7.7)
Smoking status			
Never smokers	2896 (38.1 %)	4737 (38.5 %)	2010 (40.0 %)
Former smokers	2537 (33.3 %)	4105 (33.3 %)	1658 (33.0 %)
Current smokers	2176 (28.6 %)	3469 (28.2 %)	1356 (27.0 %)
Highest level of education			
No elementary school	57 (0.7 %)	95 (0.8 %)	51 (1.0 %)
Elementary school (6-8 yrs)	3084 (40.5 %)	5024 (40.8 %)	2015 (40.1 %)
Junior Sec. School (9-10 yrs)	2015 (26.5 %)	3283 (26.7 %)	1260 (25.1 %)
Advanced level (12 yrs)	656 (8.6 %)	1084 (8.8 %)	497 (9.9 %)
At least one additional year	671 (8.8 %)	1086 (8.8 %)	457 (9.1 %)
University degree	1126 (14.8 %)	1739 (14.1 %)	744 (14.8 %)
Low physical activity [a]	1492 (19.6 %)	2355 (19.1 %)	1011 (20.1 %)
Systolic blood pressure, mmHg	141.0 (20.0)	141.0 (20.0)	141.4 (20.2)
Antihypertensive treatment	1321 (17.4 %)	2023 (16.4 %)	821 (16.3 %)
BMI, kg/m^2	25.8 (4.0)	25.7 (4.0)	25.7 (4.0)
Events, n (events/1000 p-ys)			
CAD	633 (6.0 /1000)	1134 (6.7 /1000)	542 (7.9 /1000)
Ischemic Stroke	355 (3.3 /1000)	609 (3.5 /1000)	289 (4.1 /1000)
Cardiovascular mortality	310 (2.8 /1000)	586 (3.4 /1000)	260 (3.6 /1000)

Data displayed as mean (SD) or number (%) unless otherwise specified.
p-ys= person-years

[a] Defined as the lowest quintile of the Physical Activity score in MDC

Compared to included subjects, participants in MDC that were excluded from Study IV (n = 5503) because of incomplete genotype data, covariate data and/or previous CVD at baseline generally had a higher burden of CVD risk factors (more likely to be men [48.4 % versus 37.9 %], less likely to be never-smokers [32.5 % versus 38.7 %], slightly higher SBP [mean SBP 141.5 versus 141.0 mmHg] and BMI [mean BMI 26.3 versus 25.7 kg/m^2]). In accordance with these observations, there was also a higher incidence of the endpoints in excluded subjects (events per 1000 person-years for CAD 13.2 versus 6.7; for ischemic stroke 6.1 versus 3.6; for CVD-mortality 7.8 versus 3.2). Most of the higher CVD risk in excluded subjects could be attributed to subjects with previous CVD at baseline. Compared with included subjects and with excluded subjects without previous CVD, these subjects were much more likely to be men (72.3 %) and less likely to be never smokers (21.0 %); they were older (mean age 62.9 years), had higher SBP (mean 147.2 mmHg) despite an extensive use of antihypertensive medication (60.1 %) and they had higher BMI (mean 27.0 kg/m^2). As expected, the incidence of CAD (39.8 events per 1000 person-years), ischemic stroke (11.4 events per 1000 person-years) and CVD mortality (21.5 deaths per 1000 person-years) was considerably higher in subjects with previous CVD (Complete characteristics of the subjects that were excluded from Study IV are displayed in Tables S1A and S1B in the supporting information for Paper IV).

Interactions between chromosome 9p21 and smoking on CVD

Main effects

In Cox regression models adjusted for age and sex rs4977574 associated with all three end-points. Current smoking was strongly associated with all three end-points, whereas former smoking was associated with incident CAD and CVD-mortality, but not with incident ischemic stroke. Highest level of education was associated with incident ischemic stroke, and a significant trend for association also with incident CAD and CVD-mortality could be observed across education categories. Level of physical activity was associated with all three endpoints, however HRs were similar across quintiles 2-5 of the physical activity-score (Table 10).

Table 10
Main effects in Study IV

	CAD HR (95 % CI)	Ischemic Stroke HR (95 % CI)	CVD mortality HR (95 % CI)
rs4977574, per allele	1.16 (1.09-1.23)	1.12 (1.04-1.22)	1.14 (1.05-1.24)
Smoking status [a]			
Former smoker	1.21 (1.09-1.34)	1.02 (0.89-1.17)	1.27 (1.09-1.48)
Current smoker	2.01 (1.8-2.23)	1.65 (1.44-1.89)	2.67 (2.31-3.09)
Education [a]			
Elementary school (6-8 ys)	0.97 (0.63-1.50)	0.53 (0.34-0.82)	0.82 (0.46-1.45)
Junior Sec. School (9-10ys)	0.81 (0.52-1.25)	0.45 (0.29-0.71)	0.68 (0.38-1.22)
Advanced level (12 ys)	0.75 (0.48-1.17)	0.38 (0.23-0.62)	0.58 (0.32-1.07)
At least one additional year	0.75 (0.48-1.18)	0.40 (0.24-0.64)	0.54 (0.29-0.99)
University degree	0.63 (0.40-0.98)	0.35 (0.22-0.57)	0.55 (0.30-1.00)
P for trend	*<0.001*	*<0.001*	*<0.001*
Quintiles of PA score [a]			
Q2	0.68 (0.60-0.78)	0.70 (0.59-0.83)	0.62 (0.52-0.75)
Q3	0.70 (0.62-0.80)	0.70 (0.59-0.83)	0.64 (0.54-0.77)
Q4	0.73 (0.64-0.83)	0.63 (0.53-0.75)	0.63 (0.53-0.75)
Q5	0.75 (0.67-0.85)	0.66 (0.56-0.78)	0.61 (0.51-0.72)

Adjusted for age and sex.
PA score = Physical Activity Score.
[a] Compared to first category in categorical variables (i.e. never smokers, did not complete elementary school, and Q1 of PA respectively**)**

Interaction analyses

There was a significant interaction between rs4977574 and smoking on both incident CAD ($P_{interaction}$ = 0.035) and CVD-mortality ($P_{interaction}$ = 0.012). These interactions remained significant in the fully adjusted models for both incident CAD ($P_{interaction}$ = 0.035) and CVD-mortality ($P_{interaction}$ = 0.029). No interaction was observed between rs4977574 and smoking on incident ischemic stroke ($P_{interaction}$ = 0.702).

As we found significant interactions between rs4977574 and smoking status on incident CAD and CVD-mortality, we tested the associated effect of rs4977574 on these two endpoints according to smoking status. For incident CAD, the associated effect of rs4977574 was found to be attenuated and in current smokers (Figure 12).

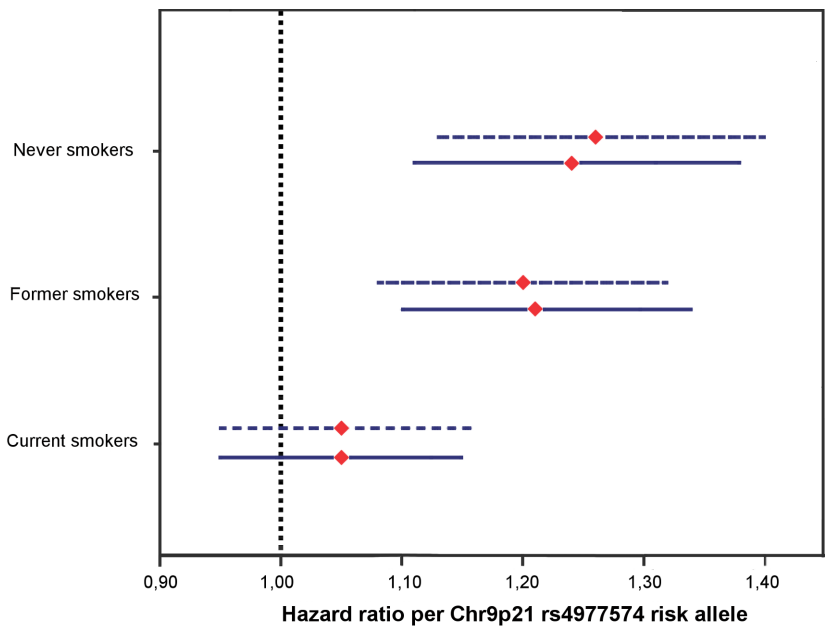

Figure 12
Hazard ratios (HRs) with 95 % Confidence Intervals (CIs) per risk allele of rs4977574 for incident CAD in never (n = 9642) former (n = 8300) and current (n = 7000) smokers respectively. Models adjusted for age and sex (dotted upper lines) and adjusted for additional covariates SBP, antihypertensive medication, BMI, education and physical activity (continuous lower lines).

Since there was additional data also of exposure to passive smoking for 22049 subjects we performed stratification within the groups of never and former smokers according to this variable. In never smokers the significant associated effect of rs4977574 risk alleles on incident CAD was attenuated among subjects that reported passive smoking, whereas the associated effect by rs4977574 on risk of CAD was high in never smokers that were not exposed to passive smoking. In current smokers we had information also on baseline "pack-years" (number of cigarette packs per day x years of smoking; n = 6256) and number of cigarettes smoked per day (n = 6311). Thus, within the group of current smokers we additionally stratified for pack-years and number of daily cigarettes in order to test if there was a suggestive dose-relationship for the modification of the genetic effect by smoking. There was however no such evident pattern of pack-years or number of daily cigarettes further modifying the genetic effect on CAD (Table 11).

Table 11
Risk of incident CAD by rs4977574 stratified by smoking status

	Events (total cases)	rs4977574 HR per allele	95 % CI	P-value
Never smokers	**675 (9642)**	**1.26**	**1.13-1.40**	**<0.001**
No passive smoking	220 (3339)	1.56	1.29-1.88	<0.001
Passive smoking	379 (5069)	1.14	0.99-1.32	0.068
Former smokers	**814 (8300)**	**1.20**	**1.08-1.32**	**<0.001**
No passive smoking	177 (2146)	1.30	1.05-1.60	0.015
Passive Smoking	528 (5221)	1.19	1.06-1.35	0.004
Current smokers	**820 (7000)**	**1.05**	**0.95-1.16**	**0.326**
Pack-years < median	298 (3090)	1.05	0.89-1.23	0.572
Pack-years ≥ median	407 (3165)	1.02	0.89-1.18	0.751
Daily cigs < median	321 (3057)	0.97	0.83-1.13	0.704
Daily cigs ≥ median	390 (3253)	1.08	0.93-1.24	0.317

Adjusted for age and sex
Daily cigs = number of daily cigarettes

For CVD-mortality the associated effect of rs4977574 was found to be highly significant only in the group of never smokers whereas the associated effect was attenuated among both current and former smokers (Figure 13).

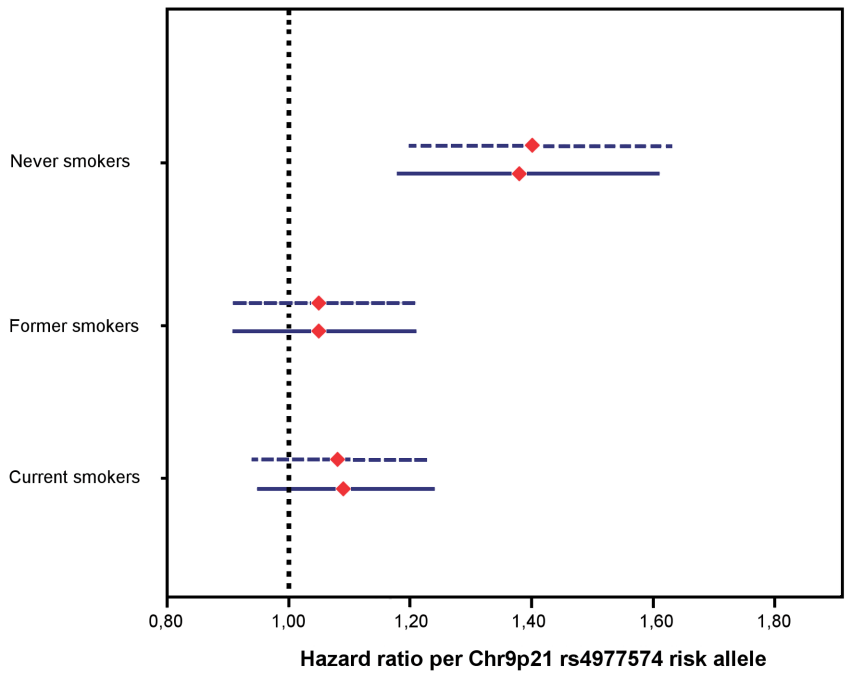

Figure 13
Hazard ratios (HRs) with 95 % Confidence Intervals (CIs) per risk allele of rs4977574 for CVD mortality in never (n = 9642) former (n = 8297) and current (n = 7000) smokers respectively. Models adjusted for age and sex (dotted upper lines) and adjusted for additional covariates SBP, antihypertensive medication, BMI, education and physical activity (continuous lower lines).

The highest associated risk of CVD mortality by rs4977574 was observed in never smokers that were not exposed to passive smoking. In contrast to the results for CAD, there was a suggestive pattern of a dose-response association modifying the genetic effect within current smokers, as the genetic effect seemed to be attenuated to a larger extent in subjects with more extensive smoking habits (Table 12).

Table 12
Risk of CVD mortality by rs4977574 stratified by smoking status

	Events (total cases)	rs4977574 HR per allele	95 % CI	P-value
Never smokers	**327 (9642)**	**1.40**	**1.20-1.63**	**<0.001**
No passive smoking	113 (3339)	1.78	1.37-2.32	<0.001
Passive smoking	174 (5070)	1.27	1.03-1.57	0.025
Former smokers	**383 (8297)**	**1.05**	**0.91-1.21**	**0.525**
No passive smoking	88 (2145)	1.38	1.02-1.85	0.034
Passive smoking	247 (5218)	0.96	0.81-1.15	0.676
Current smokers	**446 (7000)**	**1.08**	**0.94-1.23**	**0.270**
Pack-years < median	154 (3089)	1.20	0.96-1.50	0.11
Pack-years ≥ median	242 (3165)	1.00	0.84-1.21	0.972
Daily cigs < median	179 (3057)	1.14	0.93-1.41	0.214
Daily cigs ≥ median	218 (3252)	1.03	0.85-1.25	0.775

Adjusted for age and sex
Daily cigs = number of daily cigarettes

Smoking as a risk factor for CAD and CVD mortality according to genotype

The interactions between smoking and rs4977574 were evident also from a reverse point of view. That is, although smoking was observed to be a strong risk factor for incident CAD and CVD-mortality regardless of genotype, there was a pattern of smoking conferring a less effect on risk of incident CAD and CVD-mortality in risk allele carriers compared to non-risk allele carriers (Table 13).

Table13
Smoking as a risk factor for incident CAD and CVD mortality according to different genotypes

Smoking as a risk factor for incident CAD

rs4977574 risk alleles	Events (total cases)	Former smoker HR (95 % CI)	P-value	Current smoker HR (95% CI)	P-value
0	633 (7608)	1.24 (1.01-1.52)	0.038	2.21 (1.81-2.70)	<0.001
1	1134 (12309)	1.24 (1.07-1.45)	0.005	2.21 (1.90-2.56)	<0.001
2	542 (5024)	1.12 (0.92-1.38)	0.265	1.48 (1.19-1.84)	<0.001

Smoking as a risk factor for CVD-mortality

rs4977574 risk alleles	Events (total cases)	Former smoker HR (95 % CI)	P-value	Current smoker HR (95% CI)	P-value
0	310 (7604)	1.76 (1.30-2.38)	<0.001	3.35 (2.48-4.52)	<0.001
1	586 (12309)	1.20 (0.96-1.48)	0.106	2.77 (2.26-3.39)	<0.001
2	260 (5024)	1.04 (0.77-1.42)	0.781	2.00 (1.48-2.70)	<0.001

Adjusted for age and sex

Meta-analysis

In the meta-analysis of MDC and ARIC (Figure 14), the associated risk of incident CAD conferred by the chromosome 9p21 risk locus was found to be attenuated in smokers (Pooled HR per allele = 1.07; 95% CI 0.99-1.15). Contrary, when pooling the results from MDC and ARIC in non-smokers (i.e. never and former smokers) there was an increased risk of incident CAD by the risk locus (Pooled HR per allele = 1.23; 95% CI 1.16-1.30).

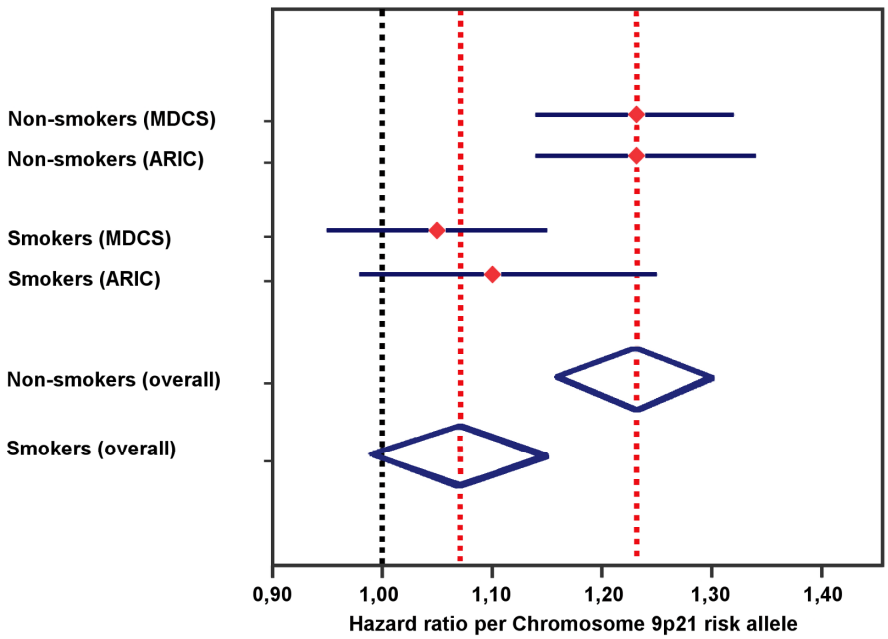

Figure 14
Meta-analysis. HR with 95 % CI per Chromosome 9p21 risk allele in MDCS (SNP rs4977574; n = 24944; 28 % smokers) and ARIC (SNP rs10757274; n = 9877; 25 % smokers). The SNP rs10757274 is in strong LD with rs4977574 (r^2 = 0.94 in data from 1000 genomes pilot 1; value obtained from SNP Annotation and proxy search (SNAP) from Broad Institute; http://www.broadinstitute.org/mpg/snap/)
MDCS = Malmö Diet and Cancer study. ARIC = Atherosclerosis Risk in Communities Study.

Interaction analyses for education and physical activity

No interactions were observed between rs4977574 and education or physical activity on incident CAD ($P_{interaction}$ = 0.082 and $P_{interaction}$ = 0.457 respectively), incident ischemic stroke ($P_{interaction}$ = 0.876 and $P_{interaction}$ = 0.251 respectively) or CVD-mortality ($P_{interaction}$ = 0.681 and $P_{interaction}$ = 0.286 respectively).

Discussion

The overall purpose of this thesis was to investigate how a number of recent genetic discoveries within the field of CVD could possibly be implemented in aspects concerning prevention and treatment of CVD in the population. With focus on a number of well-validated and common CVD risk SNPs from recent GWASs the aim was to assess the possible role of common CVD genetics in general aspects of CVD prevention in the population (covered in Studies II and IV) as well as in pharmacological treatment of dyslipidemia (Study I) and hypertension (Study III), both of which are cornerstones of CVD prevention. Since life-style factors are of equal importance, not least in a primary preventive setting, there was also an intention to assess potential gene-lifestyle interactions for the strong and extensively validated CVD risk locus on chromosome 9p21 (Study IV).

CAD and MI genetics in subclinical atherosclerosis

A central theme in CVD and thus also in CVD genetics is the atherosclerotic process. The key finding in Study II was that a CAD- and MI-genetic risk score that was previously shown to predict incident CAD (239) additionally strongly associates with increased carotid bulb-IMT and with occurrence of carotid plaque in middle-aged subjects from the general population. The effect size of the genetic risk score on carotid bulb-IMT was similar to that of the well-established CVD risk factor LDL in the same population. In general, the results of Study II would suggest that already in a middle-aged and overall healthy population, markers of subclinical atherosclerosis could be detected in individuals that are genetically susceptible to CAD and MI. Nevertheless, these conclusions open up for further discussions, both in terms of what the genetic associations really mean and what the CVD preventive implications of such conclusions might be.

Carotid IMT and plaques as markers of atherosclerosis

Whereas carotid plaques could intuitively be appreciated as valid markers of atherosclerosis this relationship seems less obvious for carotid-IMT. From an epidemiological point of view the use of carotid-IMT as a surrogate marker for not

just carotid but also general atherosclerosis can be justified by the correlations found between increased carotid IMT and occurrence of overt atherosclerosis in other vascular territories (167-170), the observation that carotid IMT predicts a variety of incident atherosclerotic manifestations (171), the association between carotid IMT and conventional atherosclerotic CVD risk factors in young to middle aged subjects (172-175) and the findings that anti-atherosclerotic interventions could also reduce carotid IMT progression (265,283-285). From a pathophysiological point of view the meaning of an increased IMT has however been subject to more debate, as summarized by Stein and colleagues at ASE in a consensus document of the assessment of subclinical vascular disease (166). Rather than manifest atherosclerosis, lower degrees of diffusely increased IMT has been suggested to reflect an adaptive response to altered hemodynamics, (286), changes that may be most obvious in hypertension (287) but that are also seen with increasing age (288). However, many of the histological changes of such increased IMT are also the histological changes seen in development of atherosclerosis, including endothelial dysfunction, increases in procoagulant, vasoconstructive and inflammatory mediators and increased migration and proliferation of smooth muscle cells (288-290). Thus, as stated by Stein and colleagues at ASE (166) it seems reasonable to conclude that a diffusely increased IMT is not in itself equal to atherosclerosis, however the underlying pathophysiological changes and the risk factors are to a large extent the same as for atherosclerosis, meaning that increased IMT is a marker of subclinical vascular disease that is in turn also related to (subclinical) atherosclerosis. From a preventive perspective, this would also mean that increased IMT could serve as a graded risk marker for CVD events (286).

A natural question in this context is why the genetic associations found in Study II differed between bulb-IMT and CCA-IMT, the latter for which the strength of the association with the CAD/MI genetic score was weaker. Most likely, these results reflect the different meaning of increased IMT in the bulb compared to in the CCA. An increased maximal IMT in the carotid bulb might well represent the occurrence of carotid plaques, which are found more often in this location than in the CCA (291). Thus, the associations found between the genetic risk score and bulb-IMT and plaque respectively may to a large extent represent the same associations. The occurrence of carotid plaque is a better predictor of future CAD events than carotid IMT (292) and carotid IMT has been shown to be a more accurate predictor of CVD and CAD when the measurements involve the bulb and ICA (i.e. locations where plaques are often found) rather than just the CCA (180,292,293). Thus, it seems reasonable to assume that a “hard-endpoint” CAD- and MI-genetic risk score would be more strongly associated with maximum bulb-IMT and occurrence of plaque as these measurements would represent overt atherosclerotic lesions. Contrary, a diffusely increased mean IMT in the CCA often represents changes of smooth muscle proliferation in the media and ground

substance accumulation rather than plaque (discussed in (180,292,294,295)) and even in the presence of plaque the mean measurement would be less affected. It is also worth noting that CCA-IMT is more strongly correlated with risk factors for stroke, especially BP levels (294,296) and there is thus reason to believe that such factors could have greater impact on CCA-IMT than a genetic score that is primarily associated with CAD and MI. In line with this reasoning, we also found that BP levels were strongly associated with CCA-IMT in particular (see Figure 11 in the results section).

Is a CAD- and MI-genetic score superior to individual SNPs?

Testing the individual SNPs included in the genetic risk score in Study II revealed that the majority of the association with carotid-IMT could be attributed to rs4977574 on chromosome 9p21, which is by many considered the best validated CVD risk SNP to date (229). Previous studies relating the chromosome 9p21 CVD locus to markers of subclinical atherosclerosis have reported associations with carotid plaques (297,298) but not with CCA-IMT (297-300) or with mean IMT estimated over a number of carotid segments (301). These findings are thus in line with our results.

Accounting for the fact that rs4977574 on chromosome 9p21 was such an evident contributor to effects of the genetic score, the value of the additional CAD- and MI-SNPs included can be questioned. A more recent study that tested an updated genetic risk score including 24 MI and CAD-associated SNPs did not find any association between this score and carotid-IMT (CCA-IMT) or incidence of carotid plaque during a six year follow-up period (302). However, when the included SNPs were examined individually rs4977574 was the only SNP that showed an association with carotid IMT ($P = 0.010$), something that further emphasizes the strong effect size of this SNP in a cross-sectional study setting. However, cumulating genetic effects rather than relying on single SNPs might still be valuable in a predictive setting, as discussed below.

The potential role of genetics in early CAD prevention

Even if rs4977574 on chromosome 9p21 is the main contributor to the effects seen for the CAD- and MI-genetic risk score, the associations with markers of atherosclerosis in the general population do suggest some potentially important preventive implications in the context of early CAD and MI prevention.

First, the 13 included SNPs (with emphasis on rs4977574) likely confer their associated risk of MI mainly by increased atherosclerosis, and this process is likely to start long before the presentation of clinical manifestations. This

conclusion is in line with GWAS findings showing associations for the individual SNPs of the score with CAD, but not with MI on top of CAD (303). Also, a recent GWAS demonstrated substantial overlap in the genetic risk of ischemic stroke (particularly the large-artery subtype) with CAD (304) emphasizing atherosclerosis as the shared underlying pathology. For the CVD risk locus on chromosome 9p21 a recent meta-analysis also further supports the hypothesis that this locus is associated with increased severity of CAD, but not with MI in the presence of underlying CAD (305). The reported additional associations with ischemic stroke (234) and PAD (235) would further suggest that increased general atherosclerosis is the common underlying disease mechanism for this strong CVD risk locus. The association with CAD compared to MI for the chromosome 9p21 risk locus have been discussed (306), and a recent study suggested two different haplotype blocks associating with different disease phenotypes (CAD versus MI among patients with established CAD respectively) (307).

Furthermore, as outlined in the preceding discussion, the results of Study II suggest that already in a middle-aged and overall healthy population signs of atherosclerosis could be detected in subjects that are genetically predisposed to CAD and MI. Of the 4022 subjects that were included, 3921 subjects (97.5 %) had no history CVD (i.e. no history of MI, PCI, CABG or stroke) and the genetic associations with bulb-IMT and occurrence of carotid plaque were nearly identical if analyses were restricted to the 3921 subjects that were free of overt CVD (data not shown, these analyses were done after the publication of Study II). Thus, the results of Study II imply a genetic association also with subclinical atherosclerosis in the general population. Accounting for the fact that atherosclerosis is a disease that starts early in life, this would in turn suggest that subjects with a "CAD genotype" may be identified as potential subjects for primary anti-atherosclerotic preventive therapy already before atherosclerotic markers, such as increased carotid-IMT and occurrence of plaque, could be detected by conventional methods. It should be stressed that we did not investigate whether genotyping is valuable for treatment decisions in the prevention of CAD and other atherosclerotic manifestations, however our results encourage such studies to be performed, particularly in relatively young subjects that are free of overt CVD.

Whereas the results of Study II and the subsequent findings (303-305) converge on the conclusion that increased atherosclerosis is the common denominator mediating the genetically increased MI risk of the genetic score (with the results from Study II also emphasizing the association with subclinical atherosclerosis in the population), the clinical utility of common CVD and CAD polymorphisms in a preventive (predictive) setting has been questioned. This stems from the fact that most studies of genetic risk scores as well as single SNPs (for which studies have been mainly focused on the SNPs on chromosome 9p21) have suggested modest, if any, improvement in discrimination or risk reclassification over traditional risk factors (239,308-312) including in the MDC cohort (313). Accordingly, genetic

screening for common CVD polymorphisms is currently not recommended as a tool for CVD risk estimation (5,314).

As the number of CAD and CVD risk SNPs has increased, new studies testing updated CAD genetic risk scores for potential predictive utility in a primary preventive setting, have been conducted. A recent study of 10 000 Swedish subjects free of CAD at baseline that were followed for a median time of 4.3 years found that an updated 46 SNP CAD/MI-genetic risk score improved discrimination and risk classification over traditional risk factors (C-index improvement 0.004 and net reclassification improvement 4.9% for incident CAD events). In this study it was estimated that if 318 conventionally assessed intermediate risk subjects were to be additionally screened for the 46 SNPs, one CAD event could be prevented during a 10-year period, by the appropriate use of statin therapy in correctly reclassified subjects (240). A concurrent study in 24 000 Finnish subjects that were followed for a median time of 12 years, found a similar improvement in discrimination index (C-index 0.856 versus 0.851 and reclassification (Net reclassification index 5%) for incident CAD events. In this study, it was estimated that genetic screening in intermediate risk subjects would prevent one additional CAD event during a period of 14 years for every 135 people screened, by the correct allocation of statin therapy in the reclassified subjects. For comparison, it was estimated that genetic screening would prevent 2.5 times more events than random allocation of statins in intermediate CVD risk subjects (241). Since the mean age of the subjects in the latter study was lower than in the former, a question that follows is if genetic screening for allocating anti-atherosclerotic therapy would be more beneficial in younger subjects, with the potential of slowing the atherosclerotic process at a much earlier stage. The evidence that the atherosclerotic process starts early in life (315,316) and the suggestion that a life-time rather than the usual 10-year risk perspective might be more relevant for CVD risk predcition, not least in younger subjects (315,317) would support the hypothesis that genetic determination may be of value for detecting subjects at high life-time risk before conventional risk markers can be assessed (318,319).

Using genetics in CVD preventive treatments

Whereas the results of Study II emphasize an association between common CAD/MI genetics and subclinical atherosclerosis in the general population, Studies I and III were designed in order to examine potential genetic influences on specific CVD preventive treatments that affect the atherosclerotic process. Lipid lowering therapy with statins (73) and BP reduction by the use of various antihypertensive therapies (115) are both considered cornerstones in preventive

treatment for atherosclerotic CVD. However, as already pointed out, studies show that only approximately 50 % of treated subjects with established CVD reach the treatment goals for lipids and BP (15), these numbers being even lower in high-risk subjects free of overt CVD (16). This unsatisfactory treatment response is problematic not just for the individual patients but also from a population preventive perspective if CVD is to be further reduced. Even though there might be a number of reasons underlying the lack of adequate treatment response (with compliance likely being a major issue) individual genetic influences probably affect treatment response to a various extent as well. Whereas most pharmacogenetic studies have used either a candidate-gene based approach or more recently a pure unbiased GWAS-design, we used an "intermediate approach" of selecting common GWAS-derived genetic variations that associate with lipid and BP levels, and testing such variants also in a gene-treatment response setting.

A lipid genetic risk score and statin treatment in women

The main finding in Study I was that a nine-SNP lipid genetic risk score, with strong association with lipid levels as well as with incident CVD in the population (238), was additionally associated with variation in fluvastatin treatment response in middle-aged women with carotid plaque. Interestingly, although statins primarily affect LDL-levels, the strongest association of the lipid genetic risk scores was for fluvastatin-induced HDL increase, for which the genetic score showed a variance explained of approximately 12 %. A higher lipid genetic risk score (i.e. a score associated with higher LDL and/ or lower HDL levels at baseline and higher CVD risk) correlated with a more pronounced HDL-increase. Thus, even though the HDL-elevating properties of statins are very modest on average, this genetic score might be able to detect a subset of women that show a substantial HDL increase in response to statin treatment. Although the genetic score additionally associated with LDL-decrease in the opposite, less-beneficial direction (i.e. a higher genetic risk score also associated with less pronounced LDL-lowering), the strength of the associations with LDL response was weak compared to the strength of the associations seen for HDL-response. Conceptually, as is also the case for baseline lipid levels (238), a lipid genetic risk score seems to be more informative than single gene effects. That is, although the unfavorable alleles of all individual HDL (and LDL) SNPs had positive effect estimates in relation to the statin-induced percentage of HDL increase in women, only one of the individual HDL SNPs showed a significant association (LPL rs328; full results are displayed in the supplementary material for Paper I). The genetic risk score showed a variance explained of approximately 12 % for fluvastatin induced percent HDL increase in women, whereas the LPL-polymorphism showed a variance explained of approximately 6 %. Thus, although the distribution of the individual SNPs in the small study population were markedly skewed in some

cases (including LPL rs328 among women) and the individual SNP-treatment results in such a small study sample should be interpreted with caution, it seems reasonable to assume that a genetic risk score may be more informative than individual SNPs also in a gene-treatment response setting.

Regarding preventive aspects, the results of Study I raise a number of questions that would open up for further investigation. Even though the previous view that there is insufficient evidence for using statins in primary prevention of CVD in women (70,320,321) could be questioned (322), the gender-specific genetic associations with statin treatment response still raise the question of whether genetic screening could be valuable for detecting a subset of women that show a specifically beneficial treatment response to statins. Accounting for the fact that the women in the study population of Study I all had atherosclerosis (i.e. asymptomatic carotid plaques), the use of statins is potentially highly relevant in this group. However, the actual beneficial effect of increased HDL in response to statins can also be questioned. Whereas high HDL-levels are epidemiologically associated with a markedly decreased risk of CVD (77,79), a number of issues, including the lack of evidence that genetic increases of HDL affects CVD risk (85-88), have resulted in doubts whether HDL really has a causative role in protection from CVD (81). On the other hand, high HDL levels following three months of statin treatment have been shown to predict lower risk of CVD events (80) and even if not truly causative, elevated HDL may nevertheless be a marker for a beneficial lipid pattern (81).

The analyses of genetic score treatment associations in Study I were restricted to studying the lipid response and we did not assess associations with hard endpoints, such as incident CVD events. Naturally, a hard-end point study could provide further clues to whether the response of increased HDL would also translate into clinically meaningful outcomes. Also, a hard end point study would be able to assess potential genetic associations for reductions in CVD risk that are independent of the (measured) lipid level changes (254).

The SNPs that were included in Study I are all located in genes with known roles in lipid and/or statin metabolism (238). Variations in many of the same genes have been previously found to individually associate with statin treatment response in candidate-gene based pharmacogenetic studies, including variations in the genes for HMGCR (250) and APOE (323) concerning LDL-reduction and LPL (324) concerning HDL increase. With the advent of GWASs, a substantial number of new GWAS-derived variants showing associations with population lipid levels have been discovered (225), most of which are located in loci not previously involved in lipid metabolism (196). Testing an updated GWAS-derived genetic risk score for association with statin treatment response would thus seem relevant. Even if such updated lipid genetic risk scores were recently shown not to improve discrimination over conventional risk factors for predicting coronary events (325,326), these findings do not preclude that such scores would have an

important effect on treatment response. Of note, the lipid genetic risk score used in Study I was found not to improve discrimination in the original study, although it did have a modest effect on reclassification (238).

A number of recent GWAS-approach pharmacogenomics studies for statin treatment response have shown suggestive associations for LDL-reduction for a number of novel loci (253,327-329), most of which have been suggested also from previous candidate based studies. However, findings have been inconsistent and of doubtful clinical relevance (253). Using the aggregate effects of GWAS-derived SNPs (with the additional possibility of including also bordeline-genomewide-significant SNPs into the score) may be a more efficient approach in a gene-treatment response setting.

A final question of clinical aspects raised by the results of Study I is if the genetic associations for treatment response are specific to fluvastatin or whether the associations can be considered a statin class effect. Testing other statins, primarily simvastatin or atorvastatin as these are the statins most widely used today, in a similar setting would naturally be of value.

Genetics of BP treatment response in the GWAS-era

BP levels are complexly regulated by a number of interacting physiological systems, that involve regulation of extracellular fluid volume as well as cardiac and vascular contractility by renal, neural and endocrine systems (330). A substantial number of candidate-gene based pharmacogenetic studies have investigated BP-treatment response and CVD outcomes in relation to genetic variation within known drug target systems such as the renin–angiotensin–aldosterone system (RAAS) system (331), specific renal sodium retention mechanisms (274,332), beta-adrenergic receptors (333-335) and atrial natriuretic peptides (336). Even though some of these gene-treatment associations are, not least from a mechanistic view very interesting, studies for many potential genetic associations (including variation in adrenergic receptors and the ACE Insertion/deletion polymorphism) have not been not consistent, and the evidence of utility for using genetic assessments for predicting BP treatment response is still mediocre (reviewed in (251)). Considerably less research has been conducted in the setting of associating GWAS-derived BP-SNPs with treatment response. Although the eight SNPs included in Study III individually explain only 0.04-0.09 % of the proportion of variance of BP-levels in the population (226), their widespread distribution and the proximity of these polymorphisms to genes encoding possible targets for antihypertensive therapy makes them interesting in a gene-treatment setting.

The results of Study III were however mainly negative, with six of the SNPs showing no associations with treatment response neither with beta-

blockers/diuretics nor with diltiazem-based antihypertensive therapy. Two SNPs (rs12946454 and rs11191548) showed nominally significant associations with treatment response however if multiple testing was taken into account these results should also be interpreted as negative. Mechanistically, the nominal associations for the two SNPs nevertheless merit some discussion. The BP-elevating alleles of the SNP rs12946454 were suggested to associate with more SBP and DBP-reduction in subjects treated with diltiazem, whereas there was no such treatment association with beta-blockers/diuretics. This SNP is located near the gene for PLCD3 which encodes one of the Phospholipase C enzymes, thus affecting calcium release in smooth muscle and vascular tonus. Similarly, the SNP rs11191548, whose BP-elevating alleles were suggested to associate with less pronounced DBP-reduction with beta-blockers/diuretics, is located in a locus involving the gene CYP17A1. This gene encodes the cytochrome P450 enzyme CYP17A1 (also known as p450c17) which is involved in the production of mineralocorticoids and glucocorticoids and is thus highly relevant for renal sodium retention. Thus, even though in the light of multiple testing we would be very cautious about drawing any conclusions from the results for rs12946454 and rs11191548, the associations found for these two SNPs are still interesting from a pharmacological point of view.

Since the design of Study III, a number of additionally BP-associated SNPs have been discovered (112,228,330,337). In a recent study 32 GWAS-derived BP-SNPs were assembled into a BP-genetic risk score which associated strongly with SBP and DBP ($P < 10^{-62}$) and with incident CAD and stroke. However, individually, only 23 of the 32 SNPs could be replicated for association with either SBP or DBP (111). Of note, the SNP rs12946454 could not be replicated neither in this study (111) nor in a preceding GWAS (228). Although the BP-SNPs discovered thus far explain only a small proportion (approximately 2 %) of variance in BP levels (330) many of the newly discovered polymorphisms (similarly to previous loci) are located near genes involving potential targets for antihypertensive therapy, and an obvious follow-up to our results would be to assess these loci with antihypertensive treatment response in a similar manner.

It can be debated if a hypothesis-free GWAS-approach would be more efficient than the “intermediate” design used in Study III for finding loci that influence BP treatment response. Such pure GWASs have suggested loci that associate with opposite treatment effects for angiotensin 2-blockers versus thiazides (338) as well as suggesting loci specifically associated with thiazide response (339). However the potential clinical utility of these findings is yet to be determined.

A main difference of our findings concerning the genetic influences on BP treatment response compared to the genetic associations with statin treatment response is the (lack of) potential utility for a genetic risk score. Thus, whereas the use of a genetic risk cumulating the effects of individual SNPs may have an obvious value when assessing genetic influences on the response to a single agent

(such as fluvastatin in Study I), this potential benefit may be lost in the setting of multiple treatments. We did not find any association between the eight-SNP BP-genetic risk score and treatment response with any antihypertensive medication. Likely, this lack of gene-score treatment response association could be at least partly explained by the diversity of the BP-SNPs included in the score, where the direction of the treatment effect for a particular SNP may go in opposite directions for different treatments. If the concept of a BP-genetic risk score is to be utilized in a BP-treatment response setting, therapy-specific scores involving SNPs with concordant treatment effect directions may be a more effective approach for finding relevant associations (340).

Gene-environment interactions in CVD prevention

Controlling modifiable life-style factors that increase the risk of CVD is considered as important as pharmacological treatments, not least in primary prevention of CVD in the general population (5,341). Such life-style factors include tobacco smoking, a low socioeconomic status, often measured as a low level of education, and physical (in)activity. Accounting for the complex nature of CVD, the knowledge of how these life-style related risk factors may interact with genetic susceptibility variants on CVD risk should be of importance for CVD risk prediction and prevention in the population (342,343).

In Study IV we therefore evaluated gene-life-style interactions for the CVD risk locus on chromosome 9p21, which is one of the strongest, and likely the best validated, polygenic CVD risk locus reported thus far (229). As previously discussed, this locus was also the main contributor to the genetic associations with subclinical atherosclerosis found in Study II.

In a population based cohort free of CVD at baseline, we found a significant interaction between the SNP rs4977574 (which denotes the chromosome 9p21 CVD risk locus) and smoking for incidence of CAD and CVD-mortality. For both incident CAD and CVD-mortality, the associated risk increase by rs4977574 was attenuated in current smokers, contrary to in the group of never smokers in which HRs were high. A meta-analysis of nearly 35 000 subjects, including results from ARIC (282) further supported the findings concerning CAD (see Figure 14 in the results section). The interactions between smoking and rs4977574 on CAD and CVD-mortality were evident also from a reverse point of view. That is, although smoking was a strong risk factor regardless of genotype, we observed that the risk conferred by smoking on incident CAD and CVD-mortality seemed to be lower in the risk allele carriers (see Table 13 in the Results section).

Smoking and genetic susceptibility to CVD

The causes behind the observed interactions between genetic variation on chromosome 9p21 and smoking on risk of CVD could be looked at from both a pathophysiological and genetic-epidemiological point of view. From a pathophysiological view the chromosome 9p21 CVD risk locus has been consistently found not to associate with the traditional CVD risk factors (215-218). Molecular studies have found that the locus involves a specific non-coding RNA, termed antisense non-coding RNA in the INK4 locus (ANRIL), which has been suggested to regulate epigenetic modification and expression of other genes potentially involved in CVD pathophysiology (229,237). As far as the author of this thesis is aware there are no reported associations between ANRIL and smoking or the known pathophysiological pathways of smoking. However, considering that smoking is likely to affect the risk of CVD via multiple pathways, one could of course speculate that smoking and the chromosome 9p21 risk locus might act via at least partially same (yet unknown) pathway(s) on risk of CVD, and that smoking in itself would be a sufficient cause for disease. There was no suggestive dose-response relationship for smoking modifying the associated genetic risk of CAD, however rather than to contradict a pathophysiological explanation, this may of course indicate a threshold effect at which smoking becomes sufficient for attenuating the genetic effect.

The results of Study IV could also be looked at from a more strict genetic-epidemiological view. Smoking is in itself a very strong risk factor for CVD and smokers constitute a high risk group for all cardiovascular events. It is thus tempting to speculate that the relative influence of genetic factors on risk of CVD could be attenuated in such a high risk group. Among individuals with low conventional risk of CVD the relative effect of genetic factors might instead be accentuated. The reported effect size for the chromosome 9p21 risk locus on CAD and MI is larger for early-onset than for latter onset disease (215,221) . A question that arose from the current study results for incident CAD is thus if the observed modification of the genetic effect on CAD by smoking could be further influenced by age. In order to address this question we stratified the group of non-smokers / smokers according to age at baseline. Although no formal interaction tests were done, these analyses did reveal a pattern of smoking seeming to be a more evident modifier of the genetic effect of rs4977574 in older subjects compared to younger subjects (the results are displayed in Table 7 in Paper IV). A possible explanation is that the chromosome 9p21 CVD risk locus might confer a substantial associated risk of CAD in the low-risk group of younger subjects even in the presence of a concurrent strong risk factor in the form of smoking. That is – younger subjects are at such comparably low risk of CAD that the genetic effects would be preserved even if they smoke. Contrary, in older subjects who by means of their age (and the risk factors that come with age) are at much higher risk of CAD, the

addition of yet another strong risk such as smoking may diminish the relative influence of genetic factors on risk of CAD to larger extent.

Potential implications for CVD prevention

The results from Study IV and the reasoning in the former section may indicate that genetic screening for CVD could in fact be valuable in younger subjects and in other lower-risk groups where conventional CVD risk factors are not as prominent. Looking at the results from a more pessimistic point of view, one may also state that genetic screening for the CVD risk variants on chromosome 9p21 is unlikely to be of value in the presence of other strong conventional risk factors.

The hypothesis of genetic screening being more valuable in younger and / or lower-risk subjects is supported by studies showing no or little value over conventional risk factors for the chromosome 9p21 and other CVD risk SNPs in predicting CVD in the general population (239,308,310-312) and in high risk subjects (309), whereas modestly improved CVD risk prediction for genetic risk variants has been reported in low intermediate risk groups (239,344). Also, the previously discussed studies of genetic CAD prediction (240,241), suggesting a "lower number-needed-to-screen" in a younger population, would support this hypothesis.

Even if the design and objectives of Study IV were different from those of Study II, the results from both studies converge on a central question in the context of using genetics in prevention of CVD. This question is, as previously discussed, whether genetic screening would be valuable in young subjects, potentially for allocating anti-atherosclerotic therapy at an early stage, before conventional risk factors and risk markers could be detected (318,319). Such studies would require large cohorts, long follow-up times and would pose a number of ethical issues. Also, even if the risk effect by genetic factors such as the polymorphisms on chromosome 9p21 are larger in relative terms in low-risk subjects compared to conventional high risk subjects, the question of an absolute benefit of genetic screening in low risk subjects would be hard to appreciate, not least if we take a life-time rather than the conventional 10-year perspective.

In conclusion, despite the existence of a number of obstacles concerning study design that need to be overcome if we are to be able to answer the above question, further prospective studies investigating the value of the chromosome 9p21 risk alleles (and other common CVD risk variants) as predictors of CVD risk in groups with few, or successfully treated, conventional risk factors are warranted.

Conclusions

The general aim of this thesis was to explore how common genetic polymorphisms that associate with CVD and CVD risk factors in the population could possibly be implemented in a number of aspects concerning prevention and treatment of CVD.

On the basis of the results of the four studies in the thesis it can be concluded that:

- A lipid genetic risk score, based on variation in nine common LDL- and HDL-associated genetic polymorphisms, is of importance for the magnitude of fluvastatin induced HDL increase in women with asymptomatic carotid atherosclerosis. This genetic risk score may predict a potentially beneficial lipid response to statin treatment in women.
- A CAD and MI genetic risk score, that strongly predicts incident CAD, is associated with increased carotid bulb-IMT and with occurrence of carotid plaque in a middle-aged generally healthy population. These results provide evidence for a genetic association with markers of subclinical atherosclerosis in the population, and suggest that the score may identify individuals at risk of developing atherosclerosis before markers of atherosclerosis can be detected by conventional methods. Further studies should test if genetic screening is valuable for early treatment decisions in subjects that are genetically susceptible to CAD and MI.
- Eight SNPs that are strongly associated with BP levels in the population are unlikely to have any clinically important impact on antihypertensive therapy with beta-blockers, diuretics and diltiazem. Whether or not more recently discovered BP-associated SNPs influence BP treatment response, individually or aggregated into therapy-specific genetic scores, should be addressed in future studies.
- Smoking modifies the associated risk of CAD and CVD-mortality conferred by the strong CVD risk locus on chromosome 9p21. Whether the observed attenuation of the genetic risk reflects a pathophysiological mechanism or is a result of smoking being such a strong risk-factor that it may eliminate the associated genetic effect, should be further investigated. The results raise hypotheses of using genetics in CVD risk prediction in the population, suggesting that common genetic factors may have a relatively larger influence on CVD risk in conventionally assessed low-risk groups compared to high risk groups.

Perspectives

Eventually, all medical research aims at improving health. Improved prevention of CVD is likely to be among the most important areas for improving future health – both in the general population and in the individual patient, accounting for the often severe consequences of CVD manifestations such as MI and stroke. The research underlying this thesis has been focused on genetic aspects of CVD prevention, which is one (but certainly not the only one) of many areas that could lead to major improvements in CVD prevention and thus reduce disease burden. Ever since the dawn of modern genetics, there has been a hope for rapid translations of genetic findings from research into clinical, personal (and commercial) utility. It may be true that we have been a little bit too eager on this point (345), with many subsequent clinical utility studies of CVD genetics suggesting very modest, if any, clinical value (239,308-312). With these lessons learned, it seems sound to considerer new genetic findings, such as those in this thesis, as additional pieces that are needed to finally solve the complicated CVD genetics puzzle, rather than trying to promote an instant "from-research-to-the-clinic-translation" of the results.

The numerous common genetic CAD risk variants discovered thus far are estimated to explain only about 10 % of the total genetic component of CAD (223). However, as discussed by Kessler and colleagues in a recent review article of CAD genetics (346) estimating the actual contribution of such common risk variants to CAD may be complicated, accounting for the fact that that nearly every subject in the population carries a substantial number of risk variants. Thus, if we compare hypothetical subjects with zero risk alleles (or subjects where all risk alleles have been functionally eliminated) to subjects at the high end of the normal distribution, the common genetic risk variants theoretically have a large impact on the overall susceptibility to CAD. Nevertheless, most subjects are still in the middle of the normal distribution and accordingly, the predictive utility of these common genetic risk variants in the general population will be small overall (346).

In terms of prediction, it has been estimated that 100 common genetic risk variants with risk allele frequencies between 0.1 – 0.5 and individual odds ratios of 1.1-1.2 per variant would explain from 1.0 % to 9.1 % of the total variance of CAD and that a model including such genetic variants would yield C-statistics of 0.75 at best, which is similar to that in conventional risk prediction models (347). Thus, from the view of the "common variant – common disease hypothesis" the number

of risk variants would need to increase substantially in order to provide the long awaited clinical utility. With the emerging technique for cheaper exome and whole genome sequencing comes the challenge of interpreting if and how rare heterogeneous CVD risk variants could be incorporated into CVD prediction models. Also the complexity of potential models including gene-gene, gene-protein and gene-environment interaction should be accounted for.

In the context of prediction, as emphasized in the discussion in relation to the results from Study II, a relevant question is that of what time perspective of prediction should be taken when evaluating genetic models. Since genes are present from birth – should we take a life-time perspective rather than a conventional 10-year perspective (318,319)? Logical reasoning would suggest that the former perspective might well be more relevant, and that we need to extent the time-horizon of prediction models. Furthermore, the results of Study IV as well as the recent results of genetic prediction models for CVD (240,241) would support the assessment of CVD genetics primarily in low to intermediate risk subjects (which is what most subjects are early in life), rather than in high risk subjects in whom conventional risk factors should likely first be dealt with.

One can naturally question whether the use of family history alone would be a sufficient assessment in the context of CVD risk estimation, rather than the use of a detailed and much more complicated genetic assessment. However, although family history is an undisputed risk factor for CVD (19,20), the positive predictive value of a self-reported family history of premature CVD has been found to be only between 28 % and 43 % in the Framingham Offspring Study (348). This highlights the doubtful validity of self-reported CVD disease status in the family. Furthermore, the fact that many genetic CVD-associations hold true even after accounting for a family history (238,239) supports that specific genetic assessment for CVD risk would add a value to prediction beyond family history.

Besides enhanced prediction, personalized medicine with tailored prescription of medications has since long been a desirable goal within the field of genetics. This gene-treatment response perspective within CVD was also the focus of Studies I and III in the current thesis. Even though there are some examples of specific drugs where the potential clinical utility of genotyping for treatment outcome is relatively strong (such as for warfarin, clopidogrel and assessing the risk of myopathy with use of statins (251)) the broader clinical utility of using genetics in CVD preventive treatments is still unclear. Future research within this field faces a number of major challenges – including finding study populations of sufficient size where such gene-treatment interactions could be assessed and translating the potential findings into feasible clinical uses (245). Additionally, it should not be forgotten that whereas the genetic architecture is likely to be of importance, the most powerful predictor for the response of a drug is still likely to be whether or not the patient takes the drug. In this context, it could be questioned whether genetics could also be used for improving compliance. An interesting ongoing

study addresses this question and will examine whether patient knowledge of the genetic CVD risk would improve the outcome of CVD preventive treatments (349). However, it should also be considered that knowledge of genetic risk may also lead to worse compliance in cases where the patient is regarded as low genetic risk of CVD.

Whereas the current thesis has focused on epidemiological aspects of CVD prevention it should not be forgotten that an important additional area of CVD genetics is that of molecular studies and how CVD genetics can give important insights into pathophysiology and biologically relevant pathways for disease. The importance of such findings could be exemplified by the enzyme proprotein convertase subtilisin kexin type 9 (PCSK9) (350). PCSK9 promotes degradation of hepatic LDL-receptors and was identified as an important enzyme in LDL-metabolism when an association between mutations in the gene for PCSK9 and rare forms of hypercholesterolemia was found. Subsequent genetic studies revealed mutations that instead caused both lower LDL and decreased CVD risk. Further molecular studies have clarified that these beneficial mutations (causing lower LDL subsequent reduction in CVD risk) are loss-of-function mutations – causing a decreased activity of PCSK9 and thus more LDL-receptors available for clearing LDL from the circulation. Today, there are ongoing phase III studies of PCSK9-inhibition, providing a potential new way of decreasing LDL levels beyond the use of statins (350). Whereas the full clinical role of PCSK9 inhibition remains to be fully evaluated, the story of PCSK9 elegantly shows how genetic findings can be successfully translated into potential clinical utility. Further exome and whole genome sequencing have the potential for revealing similar novel pathways.

The first study included in this thesis was published in 2010 and was based on nine at the time well-validated lipid-associated polymorphisms. Four years later, as this thesis is being printed, the number of common polymorphisms associating with CVD and CVD-traits in the population has increased several-fold, with new techniques as well as novel research designs continuously being implemented and evaluated in genetic research. With the rapid evolvement of CVD genetics in mind, we do not know whether the results of the current thesis will be of relevance in yet another four years. Nevertheless, since research is about continuously building upon earlier knowledge, reconfirming or disregarding previous findings, the author of this thesis still thinks and hopes that these results will constitute additional pieces of the genetic puzzle that we need to solve if CVD genetics is going to be successfully utilized in CVD prevention in the general population.

Populärvetenskaplig sammanfattning

Hjärtkärlsjukdom är den vanligaste dödsorsaken i både Sverige och globalt, och är i Europa tillsammans med cancer den vanligaste underliggande orsaken till förtida död. Hjärtkärlsjukdom utgörs framförallt av kranskärlssjukdom – ett begrepp som bland annat innefattar hjärtinfarkt och dess komplikationer – samt stroke. Både hjärtinfarkt och stroke har i den absoluta majroiteten av fallen sin grund i åderförkalkning i hjärtats respektive hjärnans blodkärl.

Det finns en mängd faktorer – så kallade riskfaktorer – som ökar risken att drabbas av åderförkalkning och därmed också i slutändan hjärtinfarkt och stroke. Sådana riskfaktorer utgörs både av faktorer som man själv kan påverka och behandla - exempelvis höga blodfetter, högt blodtryck eller rökning – men också av faktorer som i sig själva är förutbestämda – så som ålder och kön. Till den senare kategorin hör också ärftlighet. Man vet sedan länge att risken att insjukna i hjärtinfarkt eller stroke ökar om sjukdomarna finns i familjen, speciellt om någon insjuknat i ung ålder. På senare år har man lyckats identifiera en stor mängd vanliga genetiska varianter – så vanliga att i princip alla bär på ett antal av dem – som ökar risken för olika typer av hjärtkärlsjukdom. Det handlar dels om genetiska varianter som hänger ihop med de kända riskfaktorerna för hjärtkärlsjukdom så som ofördelaktiga blodfetter och högt blodtryck, dels om genetiska varianter som ökar risken för hjärtkärlsjukdom utan att man vet på vilket sätt.

Behandlingen av hjärtkärlsjukdom, i synnerhet hjärtinfarkt, har förbättrats påtagligt under de senaste decennierna, både vad gäller effektiva förebyggande åtgärder och vad gäller behandlingen för dem som insjuknar. Fortfarande drabbas dock cirka 30 000 personer av hjärtinfarkt respektive stroke i Sverige varje år, och hos en del av dessa finner man ingen underliggande förklaring. Om, och i så fall hur, vi skulle kunna använda sig av den nya genetiska kunskapen för att ytterligare minska insjuknande och dödlighet i hjärtkärlsjukdom är det idag ingen som vet.

Syftet med denna avhandling, som består av fyra olika delstudier, var att försöka börja utröna svaret på ovanstående fråga, och att vidare undersöka hur vanliga genetiska variationer som ökar risken för hjärtkärlsjukdom skulle kunna användas i syfte att bättre förebygga och behandla hjärtkärlsjukdom i befolkningen.

De fyra delstudierna utfördes i delar av två stora sedan tidigare insamlade studiematerial. I Malmö-Kost-Cancer-studien ingår cirka 30 000 malmöbor som rekryterades under 90-talet. Dessa personer lämnade då bland annat blodprov med sparat DNA för senare genetisk analys och man har sedan haft möjlighet att följa deltagarna för att se vilka som insjuknat i hjärtkärlsjukdom och vilka som förblivit friska. En mindre del (cirka 6 000 personer) fick också under 90-talet genomgå en ultraljudsundersökning av det högra halskärlet, för att på så sätt bedöma om de hade tecken på åderförkalkning. Utav dessa fick ytterligare en mindre del (knappt 800 personer) vara med i en studie där man testade om man med hjälp av medicin kunde bromsa utvecklingen av åderförkalkning i halskärlen.

I det andra studiematerialet, den så kallade NORDIL-studien, ingick knappt 11 000 norska och svenska personer med högt blodtryck. Syftet med NORDIL var att undersöka om en blodtryckssänkande medicin (diltiazem) är bättre på att sänka blodtrycket och förebygga insjuknande i hjärtkärlsjukdom än två andra vanliga blodtryckssänkande läkemedel (beta-blockerare och tiazider). Under studien, som genomfördes under åren 1992-1999 fann man ingen skillnad mellan läkemedlen. Senare har man hos svenska deltagare sparat DNA för genetisk analys, vilket nyttjats i denna avhandling (totalt finns DNA lagrat på drygt 5000 personer från NORDIL-studien).

En av de viktigaste behandlingarna för att förebygga framförallt insjuknande i hjärtinfarkt hos personer med hög risk är användandet av blodfettsänkande behandling med så kallade statiner. I den första studien i avhandlingen testade vi därför om vanliga genetiska varianter som i sig ökar risken för ofördelaktiga blodfetter också påverkar utfallet av sådan blodfettsänkande behandling med statiner. Studien genomfördes hos cirka 400 personer med tecken på åderförkalkning i halskärlen och som därför är personer där sådan blodfettsänkande behandling kan vara motiverad. Vi fann att kvinnor, men inte män, som har genetiska varianter som sammanhänger med ofördelaktiga blodfetter, också uppvisar ett mer fördelaktigt svar på statinbehandling, genom att öka HDL-kolesterolet (ofta kallat ”det goda kolesterolet”) i högre grad än andra. Kvinnor som har dessa genetiska varianter kan alltså tänkas ha speciellt stor nytta av att behandlas med statiner.

I den andra studien testade vi om genetiska varianter som ökar risken för hjärtinfarkt också sammanhänger med tecken på åderförkalkning i halskärlen i befolkningen. Hos cirka 4000 personer i Malmö-Kost-Cancer-studien, varav de allra flesta utan några symtom på hjärtkärlsjukdom, fann vi att de vanliga genetiska variationerna för hjärtinfarkt också ger sig till känna genom tecken på åderförkalkning i halskärlen, form av en ökad tjocklek i kärlväggen och förekomst av åderförkalknings-plack. Resultaten från denna studie visar att personer som har en genetisk benägenhet för hjärtinfarkt redan långt före ett eventuellt insjuknande uppvisar tecken på åderförkalkning. Detta belyser i sin tur möjligheten att med hjälp av genetik mycket tidigt kunna sätta in förebyggande behandling hos

personer som har en genetiskt förhöjd risk att drabbas av hjärtinfarkt. Fler och större studier behövs dock för att vidare utröna detta.

Förutom blodfettsänkande behandling är behandling av högt blodtryck viktigt för att förebygga insjuknande i hjärtkärlsjukdom, framförallt stroke. I den tredje studien testade vi därför om åtta vanliga genetiska varianter som ökar risken för högt blodtryck också påverkar behandlingen med tre olika blodtryckssänkande medel (beta-blockerare, tiazider och diltiazem) hos svenska personer som deltog i NORDIL-studien. Efter sex månaders behandling av de knappt 4 000 personer som ingick i studien kunde vi dock inte finna att någon av de åtta genetiska varianterna säkert påverkande blodtryckssänkningen av de olika behandlingarna.

Även om vi alla föds med våra gener, har miljön stor betydelse för hur genetiska variationer uttrycks och därmed vilken påverkan de har. I den fjärde och sista studien studerade vi därför hur den idag mest kända genetiska variationen som ökar risken för både hjärtinfarkt och stroke – en genetisk variant på kromosom 9 – samverkar med livsstilsfaktorer som i sin tur sammanhänger med hjärtkärlsjukdom: rökning, utbildningsnivå och fysisk aktivitet. Hos 25 000 malmöbor från Malmö-Kost-Cancer-studien som i snitt följdes under cirka 15 år fann vi att den genetiska risken för insjuknande i kranskärlsjukdom (så som hjärtinfarkt) och död i hjärtkärlsjukdom helt försvann hos personer som rökte då studien påbörjades. Detta betyder inte att personer som har en genetisk ökad risk för hjärtkärlsjukdom bör röka för att minska sin risk (rökning ökar risken för hjärtkärlsjukdom oavsett gener), utan indikerar istället att personer som röker i sig har så stor risk att drabbas av hjärtkärlsjukdom att den genetiska risken tycks vara av underordnad betydelse. Studien talar därför för att man hos rökare, och kanske också andra personer med hög risk för hjärtkärlsjukdom, i första hand bör lägga fokus på att eliminera sådana klassiska riskfaktorer snarare än att bestämma genetiska riskvarianter.

Sammanfattningsvis belyser denna avhandling att vanliga genetiska riskvarianter för hjärtkärlsjukdom skulle kunna ha en viktig roll i att tidigt identifiera och behandla personer som har ökad risk att utveckla åderförkalkning, och därmed också ökad risk för hjärtinfarkt och stroke. Omvänt är sannolikt vanliga genetiska riskvarianter av underordnad betydelse hos personer som på grund av andra starka riskfaktorer, så som rökning, redan har en hög risk att drabbas av hjärtkärlsjukdom. Hos dessa personer bör man sannolikt istället i första hand fokusera på att behandla sådana riskfaktorer för att minska risken för hjärtinfarkt och stroke. Vad gäller specifik förebyggande behandling av hjärtkärlsjukdom kan genetiska varianter som ökar risken för ofördelaktiga blodfetter kanske användas för att identifiera kvinnor som har särskilt stor nytta av behandling med blodfettsänkande statiner. Hur man skulle kunna använda vanliga genetiska variationer för bättre behandling av högt blodtryck är oklart.

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Paper I

A gene score of nine LDL and HDL regulating genes is associated with fluvastatin-induced cholesterol changes in women[S]

Viktor Hamrefors,* Marju Orho-Melander,* Ronald M. Krauss,† Bo Hedblad,* Peter Almgren,* Göran Berglund,* and Olle Melander[1,*]

Department of Clinical Sciences,* Lund University, Malmö, Sweden; and Children's Hospital,† Oakland Research Institute, Oakland, CA

Abstract While conventional pharmacogenetic studies have considered single gene effects, we tested if a genetic score of nine LDL- and HDL-associated single nucleotide polymorphisms, previously shown to predict cardiovascular disease, is related to fluvastatin-induced lipid change. In patients with asymptomatic plaque in the right carotid artery, thus candidates for statin therapy, we related score LDL [APOB(rs693), APOE(rs4420638), HMGCR(rs12654264), LDLR(rs1529729), and PCSK9(rs11591147)] and score HDL [ABCA1(rs3890182), CETP(rs1800775), LIPC(rs1800588), and LPL(rs328)] as well as the combined score LDL+HDL to fluvastatin-induced LDL reduction (± metoprolol) (n = 395) and HDL increase (n = 187) following 1 year of fluvastatin treatment. In women, an increasing number of unfavorable alleles (i.e., alleles conferring higher LDL and lower HDL) of score LDL+HDL ($P = 0.037$) and of score LDL ($P = 0.023$) was associated with less pronounced fluvastatin-induced LDL reduction. Furthermore, in women, both score LDL+HDL ($P = 0.001$) and score HDL ($P = 0.022$) were directly correlated with more pronounced fluvastatin-induced HDL increase, explaining 5.9–11.6% of the variance in treatment response in women. There were no such associations in men.[JLR] This suggests that a gene score based on variation in nine different LDL- and HDL-associated genes is of importance for the magnitude of fluvastatin HDL increase in women with asymptomatic plaque in the carotid artery.— Hamrefors, V., M. Orho-Melander, R. M. Krauss, B. Hedblad, P. Almgren, G. Berglund, and O. Melander. **A gene score of nine LDL and HDL regulating genes is associated with fluvastatin-induced cholesterol changes in women. *J. Lipid Res.* 2010.** 51: **625–634.**

Supplementary key words genetic score • statins • pharmacogenetics • atherosclerosis • carotid plaque • lipid levels • treatment response

Cardiovascular disease is a major cause of mortality and morbidity in high- as well as low-income countries (1). Total and LDL-cholesterol is one of the main risk factors for ischemic heart disease in middle aged and older subjects (2) and has been shown to be a predictor of coronary heart disease mortality in a number of different ethnicities (3, 4). Additionally, a high level of HDL-cholesterol has been shown to be protective against ischemic heart disease (2) and to inhibit or even regress atherosclerosis in animal models (5).

Although lifestyle modifications could achieve a more favorable lipid profile with lower LDL and higher HDL (6), drug therapy with statins has been shown to lower LDL and raise HDL in a more prominent way (7, 8) as well as to reduce the risk of cardiovascular morbidity and mortality in both primary (8–10) and secondary (8, 11–13) prevention. Therefore, statins are first line therapy when lifestyle modifications fail to lower LDL to target levels (14). However, only one-third of treated patients do reach their treatment goals (15), and treatment goals for high risk patients are increasingly more strictly set (16). It is possible that one reason for not meeting the desirable lipid levels is the individual genetic differences affecting lipid and/or statin metabolism.

Earlier research suggests a number of genetic polymorphisms influencing blood levels of LDL and HDL (17–22).

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Abbreviations: APO, apolipoprotein; BCAPS, β-blocker Cholesterol-Lowering Asymptomatic Plaque Study; BMI, body mass index; CVD, cardiovascular disease; hmgcr, HMG-CoA reductase; HRT, hormone replacement therapy; IMT, intima-media thickness; LDLR, low density lipoprotein receptor; MDC-CC, Malmö Diet and Cancer-Cardiovascular Cohort; SNP, single nucleotide polymorphism.

[1] To whom correspondence should be addressed.
e-mail: olle.melander@med.lu.se

[S] The online version of this article (available at http://www.jlr.org) contains supplementary data in the form of two tables.

A recent study conducted in 20,000 individuals suggested common variants at 30 loci contributing to polygenic dyslipidemia (23). Furthermore, pharmacogenetic aspects of statin therapy have been evaluated and discussed (24). Several studies have suggested that genetic polymorphisms of individual genes, known to be involved in lipid metabolism (25–30), general drug metabolism (31–34), and other genes whose relation to lipid metabolism is less clear (35–37), could influence the outcome of statin therapy.

As single gene polymorphisms usually explain only a small proportion of population variance in LDL and HDL (38), each of them is expected to affect cardiovascular outcome only modestly. On the other hand, a combination of gene variants adversely affecting LDL and HDL would be expected to have greater clinical importance. In a middle-aged population based cohort, the Malmö Diet and Cancer-Cardiovascular Cohort (MDC-CC) (39), we recently showed that a genetic score based on a combination of nine common single nucleotide polymorphisms (SNPs), with each individual SNP having prior evidence of association with either LDL [rs693 in apolipoprotein B (APOB), rs4420638 in APOE, rs12654264 in HMG-CoA reductase (HMGCR), rs1529729 in LDL receptor (LDLR), and rs11591147 in PCSK9] or HDL (rs3890182 in ABCA1, rs1800775 in cholesteryl ester transfer protein, rs1800588 in hepatic lipase, and rs328 in LPL) is strongly linearly associated with both increasing LDL and decreasing HDL. Furthermore, we found that the same score is independently related to incident cardiovascular events and that it improves individual cardiovascular risk classification as assessed by using both the Net Reclassification Index and the Integrated Discrimination Index (40).

Given its strong relationship with both LDL and HDL levels as well as with increased cardiovascular risk (40), we hypothesized that this gene score would be related to the magnitude of LDL reduction and HDL increase during statin treatment. We tested this hypothesis in a subset of the MDC-CC population who participated in the β-blocker Cholesterol-Lowering Asymptomatic Plaque Study (BCAPS) (41) taking potentially gender-specific response into account.

METHODS

Study population

The MDC study (39) is an epidemiological cohort study of 28,449 subjects recruited between the years of 1991 and 1996. From this cohort, 6,103 subjects were randomly selected to study the epidemiology of carotid artery disease, including measurement of intima media thickness, occurrence of plaques, and a broad range of cardiovascular risk factors, in the MDC-CC. Using B-mode ultrasound, the right carotid artery bifurcation was scanned within a predefined window comprising 3 cm of the distal common carotid artery, the bifurcation, and 1 cm of the internal and external carotid artery. Intima-media thickness (IMT) was measured in the far wall according to the leading edge principle, using a specially designed computer-assisted image analyzing system. Presence of plaque was defined as focal IMT > 1.2mm. Subjects with asymptomatic plaques in the carotid artery (i.e., subjects with plaques meeting the definition but with no symptoms of carotid artery disease) in the MDC-CC and at the enrollment examination (n = 793) were included in BCAPS. This is a randomized, double blind, placebo-controlled study that tested whether treatment with low dose metoprolol (25 mg) and/or fluvastatin (40 mg) could reduce carotid intima media thickening in comparison with placebo. Exclusion criteria in the BCAPS study were a history of myocardial infarction, angina pectoris, or stroke within the preceding 3 months of the study; history of surgical intervention in the right carotid artery; β-blocker or statin use; blood pressure >160 systolic or >95 diastolic or hyperglycemia suspected to require insulin treatment (41). Since a triglyceride level exceeding 4.5 mmol/l invalidates the use of the Friedewald formula, we additionally excluded one subject with a triglyceride level exceeding this level.

To test our hypothesis, we included all subjects in the BCAPS study who were randomized to receive either 40 mg fluvastatin daily (F group, n = 198) or 40 mg fluvastatin plus 25 metoprolol daily (FM group, n = 197; total n = 395). Clinical characteristics of the study population are shown in Table 1.

The BCAPS and MDCS-CC study protocols were approved by the ethics committee at Lund University, and all subjects gave their written informed consent.

Genotyping and SNP selection

Our study population is a subset of the MDC-CC, and all subjects have thus been genotyped for the nine SNPs included in the gene score that we have previously shown to be related to blood levels of LDL and HDL as well as to risk of cardiovascular events (rs693 in APOB, rs4420638 in APOE, rs12654264 in HMGCR, rs1529729 in LDLR, and rs11591147 in PCSK9 for LDL and rs3890182 in ABCA1, rs1800775 in cholesteryl ester transfer protein, rs1800588 in hepatic lipase, and rs328 in LPL for HDL) (40). Fifteen percent of the samples were run in duplicate without any inconsistencies. All genotypes were called by two different investigators. None of the SNPs included significantly deviates from the Hardy-Weinberg equilibrium in the study population.

Genotype score construction

The concept of genotype score was implemented in our earlier study (40). The genotype score was constructed on the basis of the number of unfavorable alleles of the nine common SNPs

TABLE 1. Baseline characteristics of the subjects (n = 395)

	Men (n = 180)	Women (n = 215)
Mean age, years	62.3 ± 5.3	61.9 ± 5.1
Cholesterol, mmol/l		
Total	6.03 ± 0.86	6.22 ± 1.01[a]
LDL	4.12 ± 0.80	4.20 ± 0.89
HDL	1.27 ± 0.31	1.47 ± 0.35[c]
Mean IMT_{CCA} thickness, mm	0.90 ± 0.16	0.89 ± 0.21
Cholesterol > 5.0 mmol/l, n (%)	164 (91.1)	193 (89.8)
Triglycerides, mmol/l[d]	1.18 (0.86)	1.05 (0.71)
BMI, kg/m^2	25.8 ± 3.4	25.3 ± 3.5
Systolic blood pressure, mm Hg	139.3 ± 12.8	139.4 ± 14.9
Diastolic blood pressure, mm Hg	86.0 ± 6.2	84.0 ± 7.1[b]
Blood glucose, mmol/l	5.29 ± 0.63	4.98 ± 0.73[c]
History of diabetes, n (%)	4 (2.2)	4 (1.7)
Smokers, n (%)	60 (33.3)	64 (29.8)
History of CVD, n (%)	16 (8.9)	5 (2.3)[e]

Data are shown as mean ± SD if not otherwise specified.
[a–c] For Student's *t*-test: [a] $P < 0.05$, [b] $P < 0.01$, and [c] $P < 0.001$.
[d] Data shown as median (interquartile range).
[e] $P\ \chi^2$ (Pearson) = 0.004.

TABLE 2. Association between score and LDL decrease (all subjects)

Score Name (Possible Points)	Min-Max		Unadjusted Data β-Coefficient *mmol/l per point (% per point)*	Unadjusted Data Variance Explained %	Unadjusted Data *P*	Adjusted Data[a] β-Coefficient	Adjusted Data[a] Variance Explained %	Adjusted Data[a] *P*
Score LDL + HDL	4-15	Absolute	−0.0200	0.185	0.444	−0.026	0.336	0.306
(0–18)		Percentage	−0.500	0.250	0.371	−0.676	0.490	0.219
Score LDL	2-8	Absolute	−0.0540	0.884	0.094	−0.043	0.593	0.176
(0–10)		Percentage	−1.18	0.884	0.093	−0.968	0.640	0.158
Score HDL	0-8	Absolute	0.0380	0.292	0.318	0.004	0.0036	0.913
(0–8)		Percentage	0.721	0.221	0.383	−0.059	0.0016	0.941

[a] For residuals adjusted for age and blood glucose at randomization and percentage of BMI change during the study period.

(alleles associated with higher LDL or lower HDL levels; score LDL + HDL, range 0–18) (40).

In addition to score LDL + HDL, we evaluated a score based on number of unfavorable alleles of the five SNPs associated specifically with LDL levels (score LDL; range 0–10) and a score based on number of unfavorable alleles of the four SNPs associated specifically with HDL levels (score HDL; range 0–8).

Outcomes

Blood levels of LDL and HDL in the BCAPS study were obtained at randomization and after 12, 24, and 36 months of fluvastatin treatment, as described previously (41). LDL was calculated from the formula of Friedewald. Since the effect of statins on blood cholesterol is usually rapid, and because dropout rate increases and compliance may decrease with time, we used the change of LDL and HDL from baseline to12 months of fluvastatin treatment as the outcome variable.

From the two measurements (baseline and 12 months) we calculated an absolute difference and a percentage (absolute difference divided by baseline value * 100) change of LDL and HDL. As mean LDL levels are expected to decrease and mean HDL levels are expected to increase during statin treatment, we defined mean LDL change as (baseline LDL – 12 months LDL) and mean HDL change as (12 months HDL – baseline HDL).

Statistical analysis

All statistical analyses, except from Power, were conducted using SPSS 16.0. Power was calculated by PS 3.0 from Dupont. Data are given as means ± SD unless otherwise specified. The significance of changes in LDL and HDL during fluvastatin treatment was tested using one-sample *t*-test. Group-wise differences in clinical characteristics were tested using independent samples *t*-test for continuous and χ^2 test for dichotomous variables. The LDL and HDL change during fluvastatin treatment related to genotype scores was assessed using linear regression analysis in unadjusted and adjusted models. In the adjusted models, residuals of LDL and HDL change, adjusted for age, percentage of body mass index (BMI) reduction during the study period, and baseline blood glucose, were entered as dependent variable and genotype score as the independent variable. We tested for interaction between genotype score and age (genotype score × age, genotype score and age as independent variables) and between genotype score and sex (genotype score × sex, genotype score and sex as independent variables) on the outcome of LDL and HDL change during fluvastatin treatment. Since hormone replacement therapy (HRT) was not an exclusion criteria, we also tested for interaction between genotype score and HRT (genotype score × HRT, genotype score and HRT as independent variables) on the outcome of LDL and HDL in women.

As a secondary analysis, associations between single SNPs and LDL and HDL change during fluvastatin treatment were tested using linear regression, assuming an additive model of inheritance.

A *P* value of <0.05 was considered significant. The *t*-tests were two-tailed unless otherwise indicated in the text.

RESULTS

Population characteristics

The baseline characteristics of the 395 subjects treated with fluvastatin are shown in **Table 1**. Age and BMI were similar between sexes. The mean IMT thickness in the common carotid artery did not differ significantly between sexes. Women had higher total cholesterol and HDL than men, and a history of cardiovascular disease (CVD) was more common in men than in women. There were no significant differences in baseline variables between the F and the FM group (data not shown).

TABLE 3. Association between score and LDL decrease (men)

Score Name (Possible Points)	Min-Max		Unadjusted Data β-Coefficient *mmol/l per point (% per point)*	Unadjusted Data Variance Explained %	Unadjusted Data *P*	Adjusted Data[a] β-Coefficient	Adjusted Data[a] Variance Explained %	Adjusted Data[a] *P*
Score LDL + HDL	7-15	Absolute	0.0500	1.44	0.154	0.026	0.410	0.449
(0–18)		Percentage	0.774	0.656	0.337	0.206	0.0529	0.790
Score LDL	3-8	Absolute	0.0140	0.0576	0.776	0.002	0.0016	0.966
(0–10)		Percentage	0.111	0.00810	0.919	−0.160	0.0169	0.879
Score HDL	2-7	Absolute	0.100	2.34	0.058	0.061	1.00	0.226
(0–8)		Percentage	1.75	1.37	0.150	0.832	0.348	0.473

[a] For residuals adjusted for age and blood glucose at randomization and percentage of BMI change during the study period.

Complete genotype data to construct score LDL + HDL, score LDL, and score HDL were available for 342, 344, and 363 subjects, respectively. Measures of LDL and HDL after 12 months were available for 371 and 375 subjects, respectively. Consequently, gene score analyses on LDL change could be conducted in a total of 319, 321, and 339 subjects for score LDL + HDL, score LDL, and score HDL, respectively. Similarly, gene score analyses for HDL change could be conducted in 323, 325, and 343 subjects for score LDL + HDL, score LDL, and score HDL, respectively.

Change of LDL

At 12 months of treatment, LDL was significantly reduced by an average of 0.909 ± 0.76 mmol/l and 21.11 ± 16.8% (one-tailed *t*-test, $P < 0.001$ for both). There was no significant difference in LDL reduction between sexes (data not shown). The magnitude of LDL reduction did not significantly differ between the F (n = 185) and the FM (n = 186) groups (0.905 ± 0.77 mmol/l versus 0.913 ± 0.75; $P = 0.92$ and 20.90 ± 16.8% versus 21.33 ± 16.8%; $P = 0.80$). The extent of LDL decrease was positively correlated to age ($P < 0.001$ for absolute and percentage of reduction) and percentage of BMI reduction during the study period ($P = 0.005$ for absolute and $P = 0.003$ for percentage of LDL reduction), with higher age and more BMI reduction resulting in a greater LDL reduction. There was no significant association between baseline blood glucose and the magnitude of LDL reduction (data not shown).

Change of LDL and score models

There was no significant association between genotype scores and LDL change in the group including

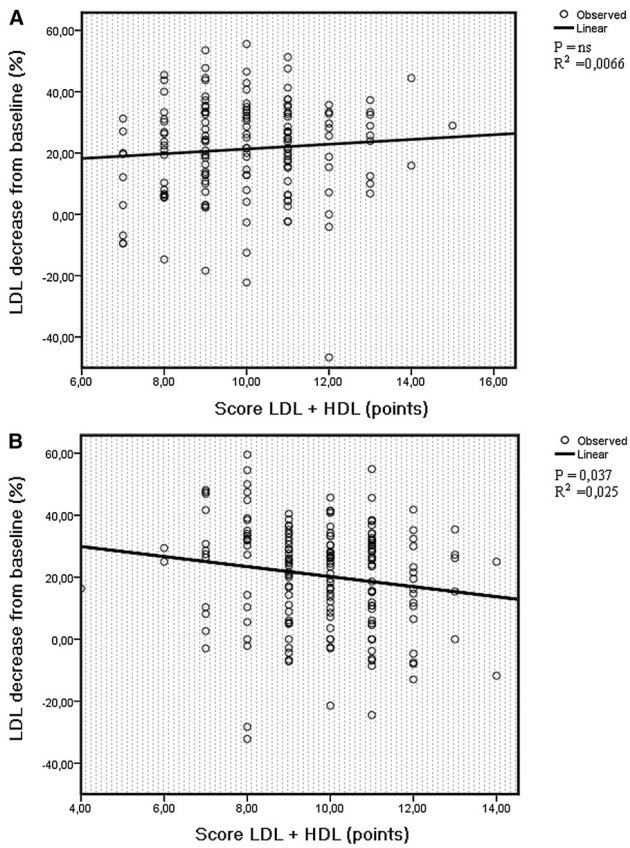

Fig. 1. The relationship between score LDL + HDL and percentage of LDL decrease among men (A) and women (B) after 12 months of fluvastatin therapy. A higher score corresponds to a higher number of unfavorable alleles in lipid-regulating genes (resulting in higher baseline LDL or lower HDL levels). In women, a higher score LDL + HDL confers a less prominent response to statin treatment.

TABLE 4. Association between dcore and LDL decrease (women)

Score Name (Possible Points)	Min-Max		Unadjusted Data β-Coefficient *mmol/l per point (% per point)*	Unadjusted Data Variance Explained %	Unadjusted Data *P*	Adjusted Data[a] β-Coefficient	Adjusted Data[a] Variance Explained %	Adjusted Data[a] *P*
Score LDL + HDL	4-14	Absolute	−0.0800	2.66	**0.031**	−0.073	2.37	**0.046**
(0–18)		Percentage	−1.62	2.50	**0.037**	−1.55	2.43	**0.043**
Score LDL	2-8	Absolute	−0.101	2.92	**0.023**	−0.074	1.69	0.089
(0–10)		Percentage	−2.11	2.92	**0.023**	−1.61	1.82	0.078
Score HDL	0-8	Absolute	−0.014	0.0400	0.789	−0.049	0.49	0.349
(0–8)		Percentage	−0.132	0.00810	0.908	−0.909	0.38	0.411

[a] For residuals adjusted for age and blood glucose at randomization and percentage of BMI-change during the study period.
Bold indicates significant *P*-value as described in the Method section.

both sexes (**Table 2**); however, interaction analyses revealed a significant interaction between sex and score LDL + HDL on fluvastatin-induced LDL change (P = 0.012 for absolute and P = 0.033 for percentage). In line with this finding, there was no significant association between score LDL + HDL and LDL change or between score LDL and LDL change among men (**Table 3**, **Fig. 1A**), whereas among women, score LDL + HDL and score LDL were significantly associated with absolute as well as percentage of LDL change, with a higher score resulting in a smaller LDL reduction (**Table 4**, Fig. 1B). The significance remained after adjustment for age, percentage of BMI reduction, and baseline blood glucose for score LDL + HDL, whereas for score LDL, the significance was slightly attenuated. Score HDL was not associated with fluvastatin-induced change of LDL. We found no evidence of interaction between age and genotype scores or between HRT and genotype scores on the outcome of LDL.

Change of LDL and single SNPs

None of the individual SNPs were associated with LDL change in the entire study population (see supplementary Table IA). Among men, there was an association between absolute LDL change and the APOB polymorphism (rs693) and absolute and percentage of LDL change and the HMGCR polymorphism (rs12654264) (see supplementary Table IB). The association for the APOB polymorphism was positively correlated; thus, the more unfavorable alleles, the greater LDL reduction. The HMGCR polymorphism showed an inverse relationship, with more unfavorable alleles resulting in a smaller magnitude of LDL reduction (see supplementary Table IB). Among women, there was no significant association between any single SNP and LDL change (see supplementary Table IC).

Change of HDL

In the F group (n = 187), there was a significant percentage of increase of HDL of 2.58 ± 13.8% (one-tailed *t*-test, P = 0.006) and a significant absolute increase of 0.0258 ± 0.20 mmol/l (one-tailed *t*-test, P = 0,037) after 12 months fluvastatin treatment. By contrast, in the FM group (n = 188), there was a nonsignificant percentage of HDL decrease by an average of 0.909 ± 11.9% (one-tailed *t*-test, P = 0.149) and a significant absolute decrease of 0.0262 ± 0.18 mmol/l (one-tailed *t*-test, P = 0.021) after 12 months. There was a significant difference in statin-induced HDL change between subjects with and without simultaneous metoprolol treatment (P = 0.007 for absolute and P = 0.009 for percentage of change). Thus, in order to exclude the confounding effect of metoprolol on HDL levels, which is well known from previous trials of β-blockers (42), all the analyses of HDL changes were performed exclusively on the F group (complete data for score LDL + HDL, score LDL, and score HDL for 160, 162, and 169 subjects, respectively).

There was no difference of HDL change between sexes, and age, baseline blood glucose, and percentage of change of BMI during the study period did not affect HDL change (data not shown).

TABLE 5. Association between score and HDL increase (all subjects)

Score Name (Possible Points)	Min-Max		Unadjusted Data β-Coefficient *mmol/l per point (% per point)*	Unadjusted Data Variance Explained %	Unadjusted Data *P*	Adjusted Data[a] β-Coefficient	Adjusted Data[a] Variance Explained %	Adjusted Data[a] *P*
Score LDL + HDL	4-15	Absolute	0.0250	4.80	**0.005**	0.027	5.76	**0.003**
(0–18)		Percentage	1.54	4.00	**0.011**	1.61	4.37	**0.009**
Score LDL	2-8	Absolute	0.0170	1.25	0.155	0.020	1.90	0.085
(0–10)		Percentage	1.12	1.23	0.161	1.24	1.49	0.127
Score HDL	0-7	Absolute	0.0310	3.53	**0.014**	0.032	3.61	**0.014**
(0–8)		Percentage	1.85	2.66	**0.035**	1.90	2.76	**0.033**

[a] For residuals adjusted for age and blood glucose at randomization and percentage of BMI change during the study period.
Bold indicates significant *P*-value as described in the Method section.

TABLE 6. Association between score and HDL increase (men)

Score Name (Possible Points)	Min-Max		Unadjusted Data β-Coefficient *mmol/l per point (% per point)*	Unadjusted Data Variance Explained %	Unadjusted Data *P*	Adjusted Data[a] β-Coefficient	Adjusted Data[a] Variance Explained %	Adjusted Data[a] *P*
Score LDL + HDL	7-15	Absolute	0.00200	0.0361	0.868	0.003	0.116	0.774
(0–18)		Percentage	0.271	0.123	0.763	0.260	0.123	0.769
Score LDL	3-8	Absolute	−0.006	0.212	0.692	−0.003	0.0441	0.860
(0–10)		Percentage	−0.187	0.0289	0.883	−0.147	0.0196	0.907
Score HDL	2-7	Absolute	0.012	0.656	0.473	0.011	0.593	0.499
(0–8)		Percentage	0.841	0.548	0.517	0.818	0.533	0.521

[a] For residuals adjusted for age and blood glucose at randomization and percentage of BMI change during the study period.

Change of HDL and score models

In the F group, there was a significant association between absolute and percentage of HDL increase and score LDL + HDL and score HDL (**Table 5**). The relationship remained significant after adjustment for age, percentage of BMI reduction, and baseline blood glucose. A higher score generated a more prominent HDL increase after fluvastatin treatment (Table 5). As was the case for fluvastatin-induced LDL change, there was a significant interaction between sex and score LDL + HDL on HDL change during fluvastatin treatment ($P = 0.015$ for absolute $P = 0.047$ for percent). In men, there was no significant relationship between score LDL + HDL or score HDL and HDL change (**Table 6**, **Fig. 2A**), while in women, there was a strong direct relationship between fluvastatin-induced HDL increase and score LDL + HDL and between fluvastatin-induced HDL increase and score HDL (**Table 7**, Fig. 2B), implying that a higher score was associated with a more prominent HDL increase following 12 months of fluvastatin treatment. The significance remained after adjustments (Table 7). Score LDL was not significantly associated with fluvastatin-induced HDL change (Tables 5–7). There was no evidence of interaction between genotype scores and age, or between genotype scores and HRT on the outcome of HDL levels.

Change of HDL and single SNPs

In the F group, the APOB polymorphism (rs693) showed an association with percentage of HDL increase, with more unfavorable alleles resulting in a more prominent HDL increase (see supplementary Table IIA). Furthermore, there was a significant association between the LPL polymorphism (rs328) and absolute HDL increase, with more unfavorable alleles resulting in a larger HDL increase response to treatment. No single SNP showed any association among men (see supplementary Table IIB). Among women, LPL (rs328) showed an association to absolute and percentage of HDL increase, with more unfavorable alleles resulting in a higher HDL level after statin treatment (see supplementary Table IIC).

Power calculations

As we detected a significant association between HDL change and LDL + HDL score in women, we tested whether we were powered enough to detect similar effects in the smaller group of males. At α 0.05, we had 89% power to detect such an effect in males. As we also detected a significant association between LDL change and score LDL + HDL in women, we performed the same test here. At α 0.05, we had 48% power to detect such an effect in males.

DISCUSSION

The key findings of our study are that a genotype score, recently shown to influence blood levels of LDL, HDL, and CVD risk at the population level (40), is associated with variation in fluvastatin treatment response in women with asymptomatic carotid plaques. The associations between the genotype score and fluvastatin-induced LDL and HDL changes were dependent on gender, as demonstrated by significant interactions between genotype score and gender. Consequently, there was no association between genotype score and fluvastatin response in males.

The strongest association of genotype scores was observed with fluvastatin-induced HDL response, where score LDL + HDL explained up to 12% of the variance of fluvastatin-induced HDL change in women. The proportion of variance of LDL response explained by score LDL + HDL was ~2% but marginally significant in women only. Although statin treatment primarily affects LDL, the proportion of variance of HDL change explained by score LDL + HDL in women was large. Thus, the genetic association with both HDL and LDL change may be of some clinical importance.

As we hypothesized, introducing the gene score concept in this pharmacogenetic setting seems to be more informative as compared with the study of single gene effects. Although the unfavorable allele of all individual HDL and LDL SNPs had positive point estimates of the β-coefficient in relation to the statin-induced percentage of HDL increase in women, only one of the individual HDL SNPs was significant (LPL rs328 for HDL response). Similarly, all individual LDL SNPs were negatively but nonsignificantly related to percentage of statin-induced LDL decrease among women. Thus, score LDL + HDL seems to be more informative than its individual SNP components regarding the effect of statin treatment, at least in women. However, considering the skew distribution of many of the individual SNPs in this cohort, we were not adequately powered to detect significant associations in many of these

analyses. Thus, our results involving single SNPs should be interpreted with great caution.

The association between score LDL + HDL and LDL response in women was clearly driven by the combined effect of the five LDL SNPs (i.e., score LDL), and the directionality of the association showed that the LDL lowering effect of fluvastatin was gradually attenuated with increasing number of LDL elevating alleles, suggesting resistance to fluvastatin treatment in subjects with a high score LDL and score LDL + HDL. However, the *P* values were modest and did not remain significant after Bonferroni corrections for multiple testing. This weakens the conclusions that can be drawn from the LDL + HDL score on LDL change in women, although the trend is an interesting finding.

The association between score LDL + HDL and HDL response in women seemed to be driven by both the five LDL SNPs and the four HDL SNPs, although more strongly by the four HDL SNPs (score HDL). Here, an increasing number of unfavorable score LDL + HDL and score HDL alleles was associated with a more pronounced statin-induced HDL elevation. The significance remained after Bonferroni corrections for multiple testing were made. The elevation of HDL with more unfavorable alleles (a high score LDL+HDL) could be contrasted to the trend of attenuation of LDL lowering in those subjects. Thus, from a strict clinical point of view, it can be questioned whether the fluvastatin resistance in LDL response or the more beneficial effect of fluvastatin on HDL response, in subjects with a high score LDL + HDL, is the more important

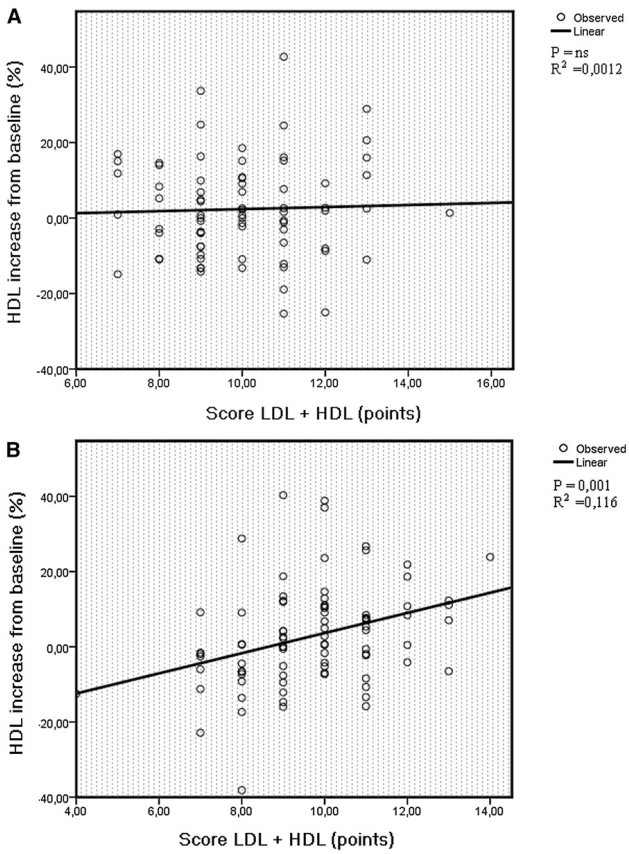

Fig. 2. The relationship between score LDL + HDL and percentage of HDL increase among men (A) and women (B) after 12 months of fluvastatin therapy. A higher score corresponds to a higher number of unfavorable alleles in lipid regulating genes (resulting in higher baseline LDL or lower HDL levels). As a group, the treatment response in HDL was small; however, the figure shows high interindividual variation in HDL treatment response depending on score LDL + HDL in women.

TABLE 7. Association between score and HDL increase (women)

Score Name (Possible Points)	Min-Max		Unadjusted Data β-Coefficient *mmol/l per point (% per point)*	Unadjusted Data Variance Explained %	Unadjusted Data *P*	Adjusted Data[a] β-Coefficient	Adjusted Data[a] Variance Explained %	Adjusted Data[a] *P*
Score LDL + HDL (0–18)	4-14	Absolute	0.045	12.9	**0.001**	0.045	12.6	**0.001**
		Percentage	2.68	11.6	**0.001**	2.64	11.2	**0.002**
Score LDL (0–10)	2-8	Absolute	0.032	4.20	0.056	0.032	4.28	0.059
		Percentage	1.98	4.20	0.057	1.96	4.04	0.067
Score HDL (0–8)	0-7	Absolute	0.048	7.08	**0.012**	0.050	7.13	**0.013**
		Percentage	2.75	5.90	**0.022**	2.84	6.05	**0.022**

[a] For residuals adjusted for age and blood glucose at randomization and percentage of BMI change during the study period.
Bold indicates significant *P*-value as described in the Method section.

and whether the net effect on cardiovascular outcome would be similar as in the population as a whole. As significance after Bonferroni correction for multiple testing remained for the results of HDL only, our study mainly highlights the genetic susceptibility for HDL-elevating properties of fluvastatin in women and suggest this might be the most important finding. However, from a mechanistic point of view, we also find it informative that LDL response is primarily influenced by score LDL, whereas HDL response is mostly influenced by score HDL.

Recently, after score LDL + HDL was originally defined (40), the knowledge of LDL and HDL genetics have greatly advanced with several novel gene discoveries (38). Studies incorporating such novel cholesterol regulating SNPs into extended score models, in order to test if a greater proportion of the variance in statin-induced LDL and HDL response can be explained, are warranted.

There were significant interactions between score LDL + HDL and gender on both LDL response and HDL response, and significant associations between genetic scores and LDL and HDL responses were found in women only. Earlier studies of gender differences in statin-induced changes of lipoproteins are not very abundant. Sakabe et al. (43) found that 3 months atorvastatin treatment lowered small dense LDL more in women than in men. Nakajima (44) noted a greater LDL reduction in women than in men with hypercholesterolemia after 12 months of simvastatin treatment. Fluvastatin pharmacokinetics has not been shown to be different among sexes (45). However, Leitersdorf (46) found a significantly greater HDL increase in women compared with men treated with fluvastatin because of familial hypercholesterolemia.

In our study, there was no significant sex difference in total LDL or HDL hange during the study period; however, the association with score LDL + HDL was clearly dependent on gender. Previous studies on single gene pharmacogenetic associations have suggested that women, but not men, respond with greater HDL increase after statin therapy depending on polymorphisms in genes coding for Estrogen Receptor Alpha (ESR1) and APOA-1 (35), which is in line with our findings that women are more genetically sensitive to HDL response during statin treatment. Pedro-Botet et al. (47), on the other hand, reported a greater magnitude of LDL reduction in men, but not women, depending on the epsilon2 allele of APOE. The lack of evidence regarding effect on clinical outcome following primary prevention with statins among women (10, 48, 49) and the fact that some studies show greater HDL elevating and LDL lowering effects in women compared with men suggests that pharmacogenetic gender differences may be important to take into account in outcome studies of statin therapy involving both sexes. However, we were not adequately powered to detect an association between the magnitude of LDL change and score in men. Thus, these results have to be interpreted with caution. Importantly, the clinical role of the gender-specific response to statins deserves further evaluation. However, although the HDL elevating effect of statins is marginal on the average, statins may be beneficial for improvement of HDL in a subset of women with high score LDL + HDL. Such an effect could be important, considering that a higher HDL level after 3 months of statin treatment has been shown be associated with protection from major cardiovascular events (50) and since novel therapies developed to increase HDL did not lead to an overall benefit on endpoints (51–54).

Our study population consisted of middle-aged and older subjects with asymptomatic carotid plaques. Thus, the population has significant atherosclerosis and is thus relevant to study in this respect as such patients would commonly be subject to statin therapy in clinical practice. Furthermore, considering the generally high prevalence of carotid plaque at these ages (55), the results could be generalized to a large proportion of the population aged over 50 years.

We do acknowledge that our study included a small number of subjects and that our results need to be replicated in a larger cohort before any clinical conclusions can be drawn. Furthermore, from a clinical point of view, the debate on how much the protective effect of statins could be attributed to factors other than their cholesterol-modifying effects (56–60) makes pharmacogenetic studies with hard endpoints as the outcome warranted. In our study, we did not have power to study whether the association between gene score and LDL and/or HDL response is of any relevance for outcome, such as differences in IMT progression or cardiovascular endpoints; however, our data encourage such studies to be performed.

In conclusion, a genotype score based on nine common lipid-linked SNPs was associated with the magni-

tude of HDL increase achieved after 12 months of fluvastatin treatment in middle-aged women with asymptomatic carotid plaque. The score was also associated with the magnitude of LDL decrease among the same subgroup, but with a marginal significance that did not withstand multiple testing corrections. No associations were seen in men.

This suggests gender differences in genetic susceptibility to statin therapy and implies the possibility of genotyping being used for predicting subjects having the greatest benefit of statin therapy, mainly considering HDL increase. Further studies involving larger cohorts are warranted in order to investigate whether this could have a role in clinical practice.

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Supplementary tables

Supplementary Table 1A **Assocation between LDL-decrease and single SNPs (All subjects)** [a]

SNP	Gene		Non-Risk Allele Homozygote		Heterozygote		Risk Allele Homozygote		βcoefficient [b]	Variance explained	P [c]
			N	LDL decrease *mmol/L (%)*	N	LDL decrease *mmol/L (%)*	N	LDL decrease *mmol/L (%)*		*%*	
LDL-associated											
rs693	APOB		76		181		95				
		Absolute		0.858 ± 0.82		0.940 ± 0.72		0.890 ± 0.80	0.013	0.0121	0.831
		Percent		19.7 ± 18		22.0 ± 16		19.8 ± 17	-0.105	0.00160	0.935
rs12654264	HMGCR		145		170		41				
		Absolute		0.981 ± 0.76		0.874 ± 0.76		0.851 ± 0.78	- 0.079	0.476	0.197
		Percent		22.9 ± 17		19.9 ± 16		19.9 ± 17	-1.94	0.593	0.148
rs1529729	LDLR		107		164		86				
		Absolute		0.944 ± 0.76		0.878 ± 0.81		0.947± 0.65	-0.02	0.0400	0.970
		Percent		21.6 ± 17		19.9 ± 18		22.7 ± 15	0.421	0.0361	0.727
rs11591147	PCSK9		0		5		343				
		Absolute		-		1.26 ± 0.78		0.908 ± 0.76	-0.352	0.303	0.302
		Percent		-		29.7 ± 18		20.9 ± 17	-8.82	0.397	0.238
rs4420638	APOE Cluster		211		131		19				
		Absolute		0.967 ± 0.75		0.845 ± 0.75		0.937 ± 0.68	-0.0740	0.348	0.267
		Percent		22.5 ± 16		19.4 ± 17		22.1 ± 15	-1.81	0.436	0.212

HDL-associated									
rs3890182	ABCA1		267	86	2				
		Absolute	0.912 ± 0.76	0.871 ± 0.78	1.30 ± 0.14	-0.0180	0.0121	0.843
		Percent	21.0 ± 16	20.2 ± 18	33.6 ± 11	-0.109	0.0009	0.956
rs1800775	CETP		80	178	91			
		Absolute	0.864 ± 0.78	0.909 ± 0.74	0.963 ± 0.77	0.0500	0.2116	0.393
		Percent	20.2 ± 18	21.0 ± 16	21.8 ± 17	0.800	0.116	0.529
rs1800588	LIPC		17	100	238			
		Absolute	0.671 ± 0.77	0.874 ± 0.78	0.948 ± 0.74	0.104	0.6241	0.136
		Percent	15.4 ± 17	20.5 ± 17	21.7 ± 16	2.07	0.518	0.174
rs328	LPL		5	69	278			
		Absolute	0.880 ± 0.76	0.986 ± 0.73	0.895 ± 0.76	-0.066	0.1521	0.460
		Percent	19.9 ± 16	22.6 ± 16	20.7 ± 17	-1.37	0.137	0.484

[a] Results are displayed as mean ± SD for absolute and percent LDL-decrease
[b] For linear model
[c] For linear regression

Supplementary Table 1B **Assocation between LDL-decrease and single SNPs (Men)** [a]

SNP	**Gene**		**Non-Risk Allele Homozygote**		**Heterozygote**		**Risk Allele Homozygote**		**βcoefficient** [b]	**Variance explained**	**P** [c]
			N	LDL decrease *mmol/L (%)*	N	LDL decrease *mmol/L (%)*	N	LDL decrease *mmol/L (%)*		*%*	
LDL-associated											
rs693	APOB		33		83		45				
		Absolute		0.621 ± 0.65		0.922 ± 0.68		0.998 ± 0.82	0.179	2.96	**0.029**
		Percent		14.9 ± 16		22.2 ± 16		22.1 ± 18	3.33	1.96	0.077
rs12654264	HMGCR		70		73		14				
		Absolute		1.04 ± 0.75		0.770 ± 0.67		0.742 ± 0.79	-0.201	3.17	**0.026**
		Percent		24.5 ± 17		18.2 ± 16		18.1 ± 16	-4.41	2.86	**0.035**
rs1529729	LDLR		46		69		43				
		Absolute		0.804 ± 0.70		0.884 ± 0.80		0.986 ± 0.62	0.0910	0.884	0.238
		Percent		18.9 ± 16		21.2 ± 19		22.9 ± 13	2.02	0.828	0.255
rs11591147	PCSK9		0		1		154				
		Absolute				2.00		0.896 ± 0.71	-	-	0.126
		Percent				46.5		21.0 ± 16	-	-	0.121
rs4420638	APOE Cluster		88		66		9				
		Absolute		0.917 ± 0.71		0.897 ± 0.71		0.956 ± 0.69	-0.00200	0.0004	0.981
		Percent		21.9 ± 16		21.1 ± 17		21.6 ± 13	-0.494	0.0361	0.815

HDL-associated									
rs3890182	ABCA1		115	46	0				
		Absolute	0.889 ± 0.71	0.863 ± 0.77	-	-0.0260	0.0256	0.840	
		Percent	20.9 ± 16	20.1± 18		-0.831	0.0529	0.774	
rs1800775	CETP		40	73	43				
		Absolute	0.795 ± 0.77	0.859 ± 0.66	1.07± 0.74	0.136	1.9321	0.083	
		Percent	18.9± 18	20.3± 16	24.4/- 16	2.77	1.376	0.124	
rs1800588	LIPC		5	52	102				
		Absolute	0.700 ± 0.81	0.808 ± 0.72	0.949 ± 0.71	0.136	1.1025	0.189	
		Percent	17.0 ± 19	19.7 ± 17	21.8± 16	2.175	0.533	0.358	
rs328	LPL		3	33	122				
		Absolute	1.10 ± 0.95	0.870 ± 0.60	0.901 ± 0.74	-0.008	0.0025	0.949	
		Percent	24.2 ± 22	21.0 ± 15	20.9 ± 17	-0.541	0.0256	0.845	

[a] Results are displayed as mean ± SD for absolute and percent LDL-decrease

[b] For linear model

[c] For linear regression

Supplementary Table 1C **Assocation between LDL-decrease and single SNPs (Women)** [a]

SNP	Gene		Non-Risk Allele Homozygote		Heterozygote		Risk Allele Homozygote		βcoefficient [b]	Variance explained	P [c]
			N	LDL decrease *mmol/L (%)*	N	LDL decrease *mmol/L (%)*	N	LDL decrease *mmol/L (%)*		*%*	
LDL-associated											
rs693	APOB		43		98		50				
		Absolute		1.04 ± 0.89		0.956 ± 0.76		0.794 ± 0.78	-0.124	1.19	0.133
		Percent		23.4 ± 19		21.9 ± 16		17.6 ± 16	-2.95	1.49	0.093
rs12654264	HMGCR		75		97		27				
		Absolute		0.923 ± 0.77		0.952 ± 0.81		0.907 ± 0.78	0.00200	0.000400	0.983
		Percent		21.4 ± 17		21.2 ± 16		20.9 ± 18	-0.241	0.0100	0.892
rs1529729	LDLR		61		95		43				
		Absolute		1.05 ± 0.80		0.874 ± 0.83		0.907 ± 0.69	-0.0800	0.533	0.307
		Percent		23.7 ± 16		19.0 ± 17		22.5 ± 16	-0.948	0.168	0.568
rs11591147	PCSK9		0		4		189				
		Absolute				1.08 ± 0.76		0.918 ± 0.79	-0.158	0.0784	0.694
		Percent				25.5 ± 18		20.8 ± 17	-4.68	0.16	0.581
rs4420638	APOE Cluster		123		65		10				
		Absolute		1.00 ± 0.78		0.792 ± 0.79		0.920 ± 0.71	-0.133	1.00	0.160
		Percent		23.0 ± 17		17.7 ± 16		22.5 ± 18	-2.99	1.12	0.137

HDL-associated									
rs3890182	ABCA1		152	40	2				
		Absolute	0.930 ± 0.80	0.880 ± 0.80	1.300 ± 0.14	-0.00600	0.000900	0.964	
		Percent	21.0 ± 17	20.3 ± 18	33.6 ± 11	0.560	0.0225	0.838	
rs1800775	CETP		40	105	48				
		Absolute	0.933 ± 0.80	0.944 ± 0.79	0.871 ± 0.79	-0.0330	0.0784	0.697	
		Percent	21.5 ± 18	21.5 ± 16	19.5 ± 17	-1.06	0.185	0.554	
rs1800588	LIPC		12	48	136				
		Absolute	0.658 ± 0.79	0.946 ± 0.84	0.946 ± 0.77	0.0810	0.384	0.391	
		Percent	14.8 ± 17	21.4 ± 18	21.6 ± 16	2.00	0.518	0.319	
rs328	LPL		2	36	156				
		Absolute	0.550 ± 0.35	1.092 ± 0.83	0.890 ± 0.78	-0.127	0.476	0.337	
		Percent	13.3 ± 4.2	24.1 ± 17	20.4 ± 17	-2.20	0.325	0.432	

[a] Results are displayed as mean ± SD for absolute and percent LDL-decrease

[b] For linear model

[c] For linear regression

Supplementary Table 2A **Association between HDL-increase and single SNPs (All subjects)** [a]

SNP	Gene		Non-Risk Allele Homozygote		Heterozygote		Risk Allele Homozygote		βcoefficient [b]	Variance explained	P [c]
			N	HDL increase *mmol/L (%)*	N	HDL increase *mmol/L (%)*	N	HDL increase *mmol/L (%)*		*%*	
LDL-associated											
rs693	APOB		47		88		44				
		Absolute		- 0.0121± 0.18		0.0199 ± 0.22		0.0677 ± 0.16	0.040	2.13	0.051
		Percent		- 0.325 ± 13		2.14 ± 14		5.47 ± 13	2.89	2.37	**0.039**
rs12654264	HMGCR		71		88		19				
		Absolute		0.00890 ± 0.20		0.0268 ± 0.17		0.0495 ± 0.24	0.019	0.449	0.377
		Percent		1.94 ± 14		2.42 ± 12		3.35 ± 14	0.629	0.0961	0.681
rs1529729	LDLR		50		85		43				
		Absolute		0.0184 ± 0.19		0.0266 ± 0.19		0.0174 ± 0.19	0.000	0.000100	0.993
		Percent		1.74 ± 13		2.66 ± 13		2.36 ± 14	0.333	0.0324	0.809
rs11591147	PCSK9										
		Absolute	0		2	- 0.0600 ± 0.042	172	0.0220 ± 0.19	0.082	0.2025	0.553
		Percent				- 4.77 ± 3.1		2.38 ± 14	7.15	0.3249	0.458
rs4420638	APOE Cluster		114		58		9				
		Absolute		0.0254 ± 0.20		0.0047 ± 0.17		0.0678 ± 0.12	- 0.0020	0.0025	0.949
		Percent		2.41 ± 13		1.28 ± 14		6.21 ± 11	0.259	0.0121	0.879

HDL-associated									
rs3890182	ABCA1		142	36	1				
		Absolute	0.0181 ± 0.20	0.0303 ± 0.18	0.5000	0.038	0.672	0.275
		Percent	1.90 ± 13	2.86 ± 14	40.3	3.00	0.903	0.207
rs1800775	CETP		38	87	49			
		Absolute	0.0174 ± 0.24	0.0122 ± 0.18	0.0435 ± 0.19	0.014	0.270	0.497
		Percent	2.10 ± 15	1.42 ± 12	4.14 ± 14	1.13	0.348	0.437
rs1800588	LIPC		7	48	122			
		Absolute	- 0.0643 ± 0.14	- 0.0056 ± 0.19	0.0370 ± 0.19	0.046	1.77	0.078
		Percent	- 2.17 ± 7.5	0.678 ± 14	3.20 ± 13	2.59	1.17	0.154
rs328	LPL		4	34	137			
		Absolute	- 0.0475 ± 0.20	- 0.0474 ± 0.21	0.0412 ± 0.19	0.073	3.28	**0.017**
		Percent	- 1.10 ± 10	- 1.30 ± 14	3.35 ± 13	3.79	1.82	0.075

[a] Results are displayed as mean ± SD for absolute and percent HDL-increase

[b] For linear model

[c] For linear regression

Supplementary Table 2B **Association between HDL-increase and single SNPs (Men)** [a]

SNP	Gene		Non-Risk Allele Homozygote		Heterozygote		Risk Allele Homozygote		βcoefficient [b]	Variance explained	P [c]
			N	HDL increase *mmol/L (%)*	N	HDL increase *mmol/L (%)*	N	HDL increase *mmol/L (%)*		*%*	
LDL-associated											
rs693	APOB		23		42		19				
		Absolute		0.00910 ± 0.20		0.0162 ± 0.18		0.0600 ± 0.17	0.025	0.941	0.380
		Percent		0.961 ± 15		1.51 ± 13		5.57 ± 15	2.220	1.28	0.305
rs12654264	HMGCR		33		43		4				
		Absolute		0.0133 ± 0.18		0.0258 ± 0.15		0.0375 ± 0.25	0.012	0.185	0.703
		Percent		2.72 ± 15		1.59 ± 11		2.57 ± 16	- 0.692	0.0961	0.782
rs1529729	LDLR		21		36		23				
		Absolute		0.0629 ± 0.18		0.0197 ± 0.14		- 0.0143 ±0.18	- 0.039	3.03	0.122
		Percent		4.35 ± 13		2.11 ± 12		0.0400 ± 14	- 2.151	1.59	0.266
rs11591147	PCSK9		0		0		81				
		Absolute						0.0175 ± 0.17	-	-	-
		Percent						1.84 ± 13			
rs4420638	APOE Cluster		52		27		5				
		Absolute		0.0321 ± 0.16		- 0.0289 ± 0.18		0.0880 ± 0.13	- 0.016	0.3364	0.603
		Percent		2.61 ± 12		- 1.36 ± 14		8.95 ± 13	- 0.344	0.0256	0.885

HDL-associated									
rs3890182	ABCA1		63	21	0				
		Absolute	0.0144 ± 0.17	0.0533 ± 0.20	-	0.039	0.8836	0.394
		Percent	1.51 ± 13	4.59 ± 16		3.08	0.9216	0.383
rs1800775	CETP		16	43	22			
		Absolute	0.0313 ± 0.20	0.00120 ± 0.17	0.0395 ± 0.14	0.007	0.0841	0.800
		Percent	2.88± 15	0.314 ± 13	4.08 ± 13	0.867	0.2025	0.688
rs1800588	LIPC		2	21	59			
		Absolute	- 0.0400 ± 0.071	0.0171 ± 0.19	0.0197 ± 0.16	0.011	0.1156	0.760
		Percent	- 1.96 ± 4.1	2.49 ± 16	1.73 ± 12	0.069	0.00090	0.981
rs328	LPL		2	17	63			
		Absolute	0.0950 ± 0.12	- 0.0076 ± 0.15	0.0219 ± 0.17	0.006	0.0324	0.874
		Percent	6.27 ± 7.88	0.370 ± 11	2.09 ± 14	0.369	0.0196	0.901

[a] Results are displayed as mean ± SD for absolute and percent HDL-increase

[b] For linear model

[c] For linear regression

Suppementary Table 2C **Association between HDL-increase and single SNPs (Women)**[a]

SNP	Gene		Non-Risk Allele Homozygote		Heterozygote		Risk Allele Homozygote		βcoefficient[b]	Variance explained	P[c]
			N	HDL increase *mmol/L (%)*	N	HDL increase *mmol/L (%)*	N	HDL increase *mmol/L (%)*		*%*	
LDL-associated											
rs693	APOB		24		46		25				
		Absolute		- 0.0325 ± 0.17		0.0233 ± 0.25		0.0736 ± 0.15	0.0530	3.39	0.075
		Percent		- 1.56± 11		2.71 ± 15		5.40± 10	3.47	3.69	0.062
rs12654264	HMGCR										
		Absolute	38	0.00500 ± 0.22	45	0.0278 ± 0.18	15	0.0527 ± 0.25	0.0240	0.6241	0.440
		Percent		1.26 ± 14		3.21± 13		3.55 ± 14	1.327	0.4761	0.503
rs1529729	LDLR		29		49		20				
		Absolute		- 0.0138 ± 0.19		0.0316 ± 0.23		0.0540 ± 0.19	0.035	1.39	0.248
		Percent		- 0.153 ± 13		3.06 ± 14		5.02 ± 14	2.646	1.88	0.177
rs11591147	PCSK9		0		2		91				
		Absolute				- 0.0600 ± 0.042		0.0260 ± 0.22	0.0860	0.3481	0.577
		Percent				- 4.77 ± 3.1		2.85 ± 14	7.62	0.6400	0.445
rs4420638	APOE Cluster		62		31		4				
		Absolute		0.0198 ± 0.24		0.0339 ± 0.16		0.0425 ± 0.13	0.013	0.1225	0.733
		Percent		2.25 ± 14		3.59 ± 13		2.79 ± 9.2	0.905	0.1444	0.712

HDL-associated										
rs3890182	ABCA1		79		15		1			
		Absolute	0.0210 ± 0.21		- 0.00200 ± 0.15		0.500	0.037	0.533	0.483
		Percent	2.22 ± 13		0.440 ± 9.8		40.3	2.98	0.884	0.364
rs1800775	CETP		22		44		27			
		Absolute	0.00730 ± 0.27		0.0230 ± 0.18		0.0467 ± 0.22	0.0200	0.4624	0.519
		Percent	1.52 ± 16		2.51 ± 12		4.19 ± 15	1.35	0.504	0.498
rs1800588	LIPC		5		27		63			
		Absolute	- 0.0740 ± 0.16		- 0.0233 ± 0.20		0.0532 ± 0.22	0.070	3.80	0.058
		Percent	- 2.25 ± 9.0		- 0.734 ± 12		4.57 ± 15	4.37	3.53	0.069
rs328	LPL		2		17		74			
		Absolute	- 0.190 ± 0.17		- 0.0871 ± 0.25		0.0576 ± 0.20	0.137	9.06	**0.003**
		Percent	- 8.48 ± 5.7		- 2.96 ± 17		4.41 ± 13	7.05	5.71	**0.021**

[a] Results are displayed as mean ± SD for absolute and percent HDL-increase

[b] For linear model

[c] For linear regression

Paper II

Original Article

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A myocardial infarction genetic risk score is associated with markers of carotid atherosclerosis

V. Hamrefors, B. Hedblad, G. Engström, P. Almgren, M. Sjögren & O. Melander

Department of Clinical Sciences, Lund University, Malmö, Sweden

Abstract. Hamrefors V, Hedblad B, Engström G, Almgren P, Sjögren M, Melander O (Lund University, Malmö, Sweden). A myocardial infarction genetic risk score is associated with markers of carotid atherosclerosis. *J Intern Med* 2012;**271**: 271–281.

Objective. To assess whether or not a genetic risk score that was previously shown to be associated with myocardial infarction (MI) and coronary artery disease (CAD) is also associated with markers of carotid atherosclerosis.

Design. A total of 4022 middle-aged subjects from the general Swedish population were genotyped and individually assigned a genetic risk score based on 13 single-nucleotide polymorphisms (SNPs), previously associated with MI and CAD. The genetic score (Score-MI) was then related to carotid bulb intima–media thickness (IMT), common carotid artery (CCA) IMT and to the occurrence of carotid plaques in the study population.

Results. Score-MI was associated with IMT of the bulb ($P < 0.001$) and the CCA ($P < 0.001$) in unadjusted analyses, and with IMT of the bulb after adjustment for cardiovascular risk factors ($P = 0.003$). The effect size of Score-MI on IMT of the bulb was similar to that of LDL cholesterol. After adjustment for cardiovascular risk factors, Score-MI was also associated with the occurrence of carotid plaques (odds ratio per quintile of Score-MI = 1.11; 95% confidence interval 1.04–1.18; $P = 0.001$). In addition to SNPs with known effects on LDL levels, Score-MI showed nominal associations with increasing systolic blood pressure and decreasing C-reactive protein levels.

Conclusions. This genetic risk score was independently associated with carotid bulb IMT and carotid plaques, providing evidence of an association with early markers of atherosclerosis. This might imply that the genetic MI risk conferred by the score is related to early atherosclerosis and that the risk score may identify at an early stage candidates at risk of developing intermediate phenotypes of atherosclerosis. Further studies should test whether or not assessing the genetic score could be valuable for early treatment decisions in these subjects.

Keywords: carotid atherosclerosis, carotid IMT, chromosome 9p21, genetic score, myocardial infarction genetics, single-nucleotide polymorphisms.

Introduction

Family history is an important and independent risk factor for myocardial infarction (MI), particularly for premature-onset disease [1]. However, many mechanisms of this heredity are not well understood.

Most genetic–epidemiological studies in recent years have focused on identifying the most common genetic variants in the population – single-nucleotide polymorphisms (SNPs) – and relating them to disease in genome-wide association studies (GWASs).

For MI, GWASs have shown associations between particular SNPs and risk factors such as dyslipidaemia [2] and blood pressure [3], as well as risk of ischaemic heart disease independently of known risk factors [4].

Recent GWASs have suggested a strong association between common SNPs in a Linkage Disequilibrium block on chromosome 9p21 and the risk of early MI, independently of traditional risk factors [5–7]. These SNPs seem to influence vascular cell proliferation [8] and some studies have suggested that there are associations between the SNPs on chromosome 9p21 and coronary [9, 10], carotid [11] and peripheral [12] atherosclerosis. However, other studies have shown either no or uncertain associations between these SNPs and the extent of coronary atherosclerosis

[13, 14] or early markers of carotid atherosclerosis [15–19]. Thus, whether or not there is an association between SNPs on chromosome 9p21 and generally increased atherosclerosis remains unclear.

In addition to the locus on chromosome 9p21, there are a number of other loci with well validated associations with MI and coronary artery disease (CAD) [20–23], and new SNPs with associations with these conditions are continually being discovered [24]. Some of these SNPs are associated with LDL cholesterol or other known risk factors for MI (Table S1), whereas the relevant functions of others are unknown. To accurately direct preventive therapy in people with a genetic predisposition for MI, it is logical that understanding the mechanisms underlying the increased risk associated with these SNPs should be of great importance.

Increased carotid intima–media thickness (IMT) is considered a marker of early atherosclerosis [25] and is correlated with angiography-verified atherosclerosis in coronary and other arteries [26–28]. Increased carotid IMT is also a strong risk factor for incident clinical atherosclerotic manifestations such as MI and stroke [29]. The occurrence of carotid plaques, representing overt atherosclerotic lesions, is an even more direct marker of atherosclerotic burden [25].

In the current study we assessed whether or not a genetic risk score, based on 13 well-validated MI-/CAD-related SNPs [20–23] and identical to a genetic score recently shown to strongly predict CAD [30], is also associated with carotid IMT and carotid plaques in the Malmö Diet and Cancer (MDC) study-cardiovascular (CV) cohort, a middle-aged population in which carotid IMT and plaques are strongly associated with incident MI and stroke [31, 32]. In addition, we also assessed the relationships between the genetic risk score and traditional MI risk factors and C-reactive protein (CRP).

Methods

Subjects and clinical data

The MDC study is a prospective population-based cohort study including 28 449 subjects recruited during 1991–1996 [33]. Subjects aged 45–69 years, living in the city of Malmö, Sweden were eligible for participation. Between November 1991 and February 1994, every other enrolled subject was also invited to take part in a substudy of the epidemiology of carotid artery disease [31, 32]. This MDC-CV cohort consists of 6103 subjects (60% women), 5540 of whom also agreed to have blood collected under standardized fasting conditions. Using B-mode ultrasound, the right carotid artery was scanned within a pre-defined window of 3 cm of the distal common carotid artery (CCA), the bifurcation and 1 cm of the internal and external carotid artery. IMT was measured 'off-line' in the far wall according to the leading edge principle, using a specially designed computer-assisted image analysing system. In the bulb the maximum IMT was measured, whereas in the CCA the mean IMT of a 1 cm distance just proximal to the bulb was recorded. The occurrence of plaque was defined as focal IMT >1.2 mm.

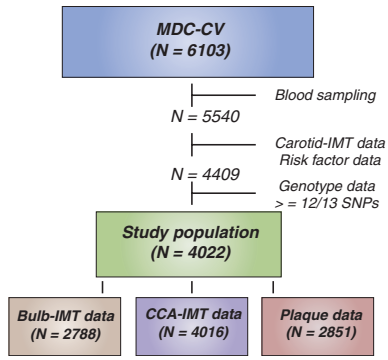

Fig. 1 *Selection of study subjects.*

The current study population included subjects (n = 4022) from the MDC-CV cohort who had IMT measurements of either the bulb or the CCA, cardiovascular risk factor data (age, gender, smoking, systolic and diastolic blood pressure, antihypertensive medication, diabetes mellitus, LDL, HDL, CRP and waist circumference) and genetic data for at least 12 of 13 MI-/CAD-associated SNPs selected for the study (Fig. 1).

The MDC-CV study protocol complies with the Declaration of Helsinki and was approved by the ethics committee at Lund University. All subjects gave their written informed consent to participate.

SNPs and genotyping

We based our study on a genetic risk score previously shown to strongly predict incident CAD [30], includ-

ing a total of 13 SNPs that are all robustly associated with MI and CAD: chr9p21-rs4977574, SORT1-rs646776, MIA3-rs17465637, CXCL12-rs1746048, KCNE2-rs9982601, PHACTR1-rs9349379, WDR12-rs6725887, LDLR-rs1122608, PCSK9-rs11206510 [20]; MRAS-rs9818870, HNF1A-rs2259816 [21]; SH2B3-rs3184504 [22] and LPA-rs3798220 [23]. A brief description of each SNP as well as their MI/CAD effect sizes are shown in Table S1.

The SNPs were genotyped using IPLEX on a MassARRAY platform (Sequenom, San Diego, CA, USA) according to the manufacturer's standard protocols. Fifteen per cent of the samples were run in duplicate without any inconsistencies. All genotypes were called by two different investigators. No SNP failed the Hardy-Weinberg equilibrium at a *P*-value of 0.001.

Genotyping of at least 12 of the 13 SNPs was successful in 4022 subjects (n = 3544 with 13 SNPs, and n = 478 with 12 SNPs) from the MDC-CV cohort and this was, as already stated, a criterion for inclusion in the current study population (Fig. 1). In subjects with missing data for one of the 13 SNPs, cohort-specific averages were imputed for the missing SNP.

Genetic score construction

The primary genotype score (Score-MI) for each subject was constructed on the basis of the number of unfavourable alleles (maximum 26) of the 13 SNPs (i.e. alleles associated with a higher risk of MI/CAD). To take into account the magnitude of risk for each included SNP (Table S1), every unfavourable SNP allele was weighted based on the effect sizes for MI/CAD for that allele, as previously described by Ripatti *et al.* who first used this score [30] (Table S1). Each of the weighted unfavourable alleles was added to the sum that constituted Score-MI. As already stated, cohort-specific averages were imputed in cases in which subjects had missing data for a single SNP.

In addition to Score-MI, two weighted subscores were constructed: Score-MI-LDL including the five SNPs that were formerly shown to be associated with LDL (LDLR-rs1122608, SORT1-rs646776, PCSK9-rs11206510, HNF1A-rs2259816 and LPA-rs3798220) and Score-MI-non-LDL including the remaining eight SNPs.

Statistical analyses

General statistics. All statistical analyses were performed using spss Statistics 19.0 (SPSS Inc., Chicago, IL, USA). Applying conservative Bonferroni correction for nine tests [three genotype scores (Score-MI, Score-MI-LDL and Score-MI-non-LDL) all tested against three outcome variables (IMT of the bulb, IMT of the CCA and occurrence of carotid plaques)], a *P*-value of 0.05/9 = 0.0056 was considered significant in the main analyses.

Association between genotype scores and IMT. Because IMT values were positively skewed, they were log-transformed in all analyses. The relationships between bulb IMT, CCA IMT and the genetic scores were assessed using linear regression in a model in which the genetic scores were entered as independent variables and IMT as the dependent variable. In addition, separate variables denoting the second to fifth quintiles of the genetic scores were entered as independent variables in the regression models; that is, the second to fifth quintiles of the genetic scores were separately tested for association with IMT, relative to the first (reference) quintile.

Unadjusted and adjusted models were used. In the adjusted models, residuals of IMT of the bulb and CCA, adjusted for age, gender, smoking, diabetes mellitus, systolic blood pressure, antihypertensive treatment, LDL, HDL, CRP and waist circumference, were entered as dependent variables and genotype score as the independent variable, as described in the paragraph above.

To compare the effect size of the genetic scores with that of LDL and systolic blood pressure levels on IMT, the two variables LDL and systolic blood pressure were additionally tested as independent variables in the regression analyses for IMT. Finally, for assessing their relative contribution in the genetic score, associations between specific SNPs and IMT were tested in unadjusted analyses, using linear regression, assuming an additive model.

Association between genotype scores and carotid plaques. To additionally assess the association between the genotype scores and manifest carotid atherosclerotic lesions, the genotype scores were related to the occurrence of significant (≥10 mm^2) carotid plaques. Using binary logistic regression analysis, the genotype scores were related to a dichotomous plaque variable denoting occurrence of at least one carotid plaque ≥10 mm^2, representing moderate to severe carotid atherosclerosis. Assessment and grading of carotid plaques in the MDC-CC cohort was performed 'on-line' as previously described [31].

Association between genotype scores and cardiovascular risk factors. In a secondary analysis, the main genotype score (Score-MI) was related to risk factors for MI, using linear regression for association with LDL, HDL, systolic and diastolic blood pressure, log-transformed CRP and waist circumference and using binary logistic regression for association with prevalent diabetes mellitus (n = 4022).

Results

Population characteristics

Intima–media thickness measurements of the bulb and CCA were available for 2788 and 4016, respectively, of the 4022 study subjects. The dichotomous plaque variable denoting moderate to severe carotid atherosclerosis could be assessed for 2851 partici pants. Subjects in the subpopulation without bulb IMT data had slightly higher waist circumference, slightly lower HDL and were more frequent users of antihypertensive medications but less frequent smokers compared to the entire study cohort (Table 1).

Subjects from the MDC-CV cohort excluded from the current study because of missing data for carotid IMT or genetic or cardiovascular risk factors (n = 2081) had slightly higher blood pressure, waist circumference and CRP and slightly lower HDL than

Table 1 *Clinical characteristics*

	Study population		
	All included subjects	No IMT bulb data	Excluded from study[a]
Total subjects, n	4022	1234	2081
Male (%)	1634 (40.6)	476 (38.6)	938 (45.1)
Female (%)	2388 (59.4)	758 (61.4)	1143 (54.9)
Age, years	57.5 ± 5.9	57.1 ± 5.8	57.4 ± 5.9
SBP, mmHg	140.9 ± 18.8	140.9 ± 18.9	142.2 ± 19.7
DBP, mmHg	86.8 ± 9.4	87.6 ± 9.4	87.4 ± 9.6
Antihypertensive treatment, n (%)	668 (16.6)	251 (20.3)	342 (16.4)
Waist circumference, cm	83.5 ± 13	85.9 ± 13	85.6 ± 13
Total cholesterol, mmol L^{-1}	6.15 ± 1.1	6.12 ± 1.1	6.20 ± 1.2
HDL, mmol L^{-1}	1.39 ± 0.37	1.35 ± 0.35	1.34 ± 0.38
LDL, mmol L^{-1}	4.17 ± 0.98	4.15 ± 0.99	4.15 ± 1.0
CRP, mg L^{-1}[b]	1.3 (0.1–60.2)	1.5 (0.1–51.4)	1.7 (0.1–51.3)
Diabetes, n (%)	303 (7.5)	103 (8.4)	183 (12.3)
Smoking, n (%)	1033 (25.7)	266 (21.6)	582 (33.5)
IMT of bulb, mm (range)[b]	1.23 (0.47–4.94)	NA	1.24 (0.46–5.08)
IMT of CCA, mm (range)[b]	0.714 (0.33–2.03)	0.694 (0.33–1.73)	0.714 (0.41–2.63)
Carotid plaque data, n	2851	420	1391
≥10 mm^2, n (%)	849 (29.8)	65 (15.5)	442 (31.8)
Score-MI	1.825 ± 0.33	1.814 ± 0.32	NA

Values displayed as mean ± SD unless otherwise specified.
CCA, common carotid artery; CRP, C-reactive protein; IMT, intima–media thickness; MI, myocardial infarction.
[a]Subjects in the Malmö Diet and Cancer Cardiovascular cohort (total n = 6103) excluded from the current study because of missing IMT, genetic and/or risk factor data. Numbers displayed are based on available data in this group: gender: n = 2081; age: n = 2080; SBP/DBP: n = 2080; antihypertensive treatment: n = 2072; waist circumference: n = 2072; cholesterol: n = 1589; HDL: n = 1431; LDL: n = 1349; CRP: n = 713; diabetes: n = 1487; smoking: n = 1739; bulb IMT: n = 1358; CCA IMT: n = 2040; plaque data: n = 1391.
[b]Displayed as median (range).

those included in the present study. Excluded subjects were also more likely to be men, to smoke and to have diabetes. IMT and occurrence of plaques did not differ between included and excluded subjects (Table 1).

Associations between Score-MI, carotid IMT and occurrence of plaques

There was a significant association between Score-MI and bulb IMT ($P < 0.001$), which remained significant after adjustments for cardiovascular risk factors and Bonferroni correction ($P = 0.003$). For CCA IMT, the association with Score-MI was significant after adjustments for cardiovascular risk factors, but not after accounting for additional Bonferroni correction ($P = 0.008$) (Table 2).

After adjustments, the strength of the relationship between Score-MI and bulb IMT ($P = 0.003$) was weaker than the strength of the relationships between LDL levels and bulb IMT ($P < 0.001$) and between systolic blood pressure levels and bulb IMT ($P < 0.001$). However for the effect estimate on bulb IMT, the upper quintiles of Score-MI were comparable to the upper quintiles of LDL levels ($\beta = 0.12$ SDs of IMT for the upper two quintiles of Score-MI versus $\beta = 0.09$–0.20 SDs of IMT for the upper two quintiles of LDL levels; Fig. 2). By contrast, Score-MI had a smaller effect estimate on bulb IMT compared to the effect estimate for systolic blood pressure levels on bulb IMT ($\beta = 0.12$ SDs of IMT for the upper two quintiles of Score-MI versus $\beta = 0.32$–0.34 SDs of IMT for the upper two quintiles of systolic blood pressure levels; Fig. 2).

The adjusted strength of the relationship between Score-MI and CCA IMT ($P = 0.008$) was also weaker than the adjusted strength of the relationships between LDL levels and CCA IMT ($P < 0.001$) and between systolic blood pressure levels and CCA IMT ($P < 0.001$). As in the case of bulb IMT, the upper quintiles of Score-MI were, however, comparable to the upper quintiles of LDL levels for the effect estimate on CCA IMT ($\beta = 0.11$–0.12 SDs of IMT for the upper two quintiles of Score-MI versus $\beta = 0.10$–0.16 SDs of IMT for the upper two quintiles of LDL levels; Fig. 3). Also similar to the case of bulb IMT, effect estimates on CCA IMT for Score-MI were small compared to the effect estimates on CCA IMT for systolic blood pressure levels ($\beta = 0.11$–0.12 SDs of IMT for the upper two quintiles of Score-MI versus $\beta = 0.31$–0.47 SDs of IMT for the upper two quintiles of systolic blood pressure levels; Fig. 3).

Score-MI was significantly associated with the occurrence of moderate to severe carotid atherosclerosis, defined as the presence of at least one carotid plaque ≥10 mm^2. Association remained significant after adjustment for cardiovascular risk factors and Bonferroni correction (odds ratio per quintile of Score-MI = 1.11; 95% confidence interval 1.04–1.18; $P = 0.001$) (Table 2).

Associations between genotype subscores, carotid IMT and the presence of plaque

There were no significant associations between Score-MI-LDL and IMT of the bulb or the CCA after accounting for Bonferroni corrections. There was also

Table 2 *Association between Score-MI and carotid parameters*

	Unadjusted model			Adjusted model[a]		
	β[b]	*P*-quintiles[c]	*P*-continuous[d]	β[b]	*P*-quintiles[c]	*P*-continuous[d]
IMT of carotid-bulb	0.043	0.001	<0.001	0.038	0.005	0.003
IMT of CCA	0.033	0.003	<0.001	0.028	0.011	0.008
	OR (95% CI)	*P* in model		OR (95% CI)	*P* in model	
Carotid atherosclerosis[e]	1.12 (1.06–1.18)	<0.001		1.11 (1.04–1.18)	0.001	

Applying Bonferroni correction, a *P*-value of 0.05/9 = 0.0056 was considered significant.
Additional data is shown in Table S2.
CCA, common carotid artery; IMT, intima–media thickness; MI, myocardial infarction.
[a]Adjusted for age, gender, smoking, diabetes mellitus, systolic blood pressure, antihypertensive treatment, LDL, HDL, C-reactive protein and waist circumference.
[b]β-coefficients relating to number of standard deviations from mean of log IMT per quintile of Score-MI.
[c]*P*-value for linear regression with quintiles of Score-MI as independent variable.
[d]*P*-value for linear regression with continuous Score-MI as independent variable.
[e]Defined as occurrence of at least one carotid plaque ≥10 mm^2. Odds ratios (ORs) are average per quintile of Score-MI in binary logistic regression model.

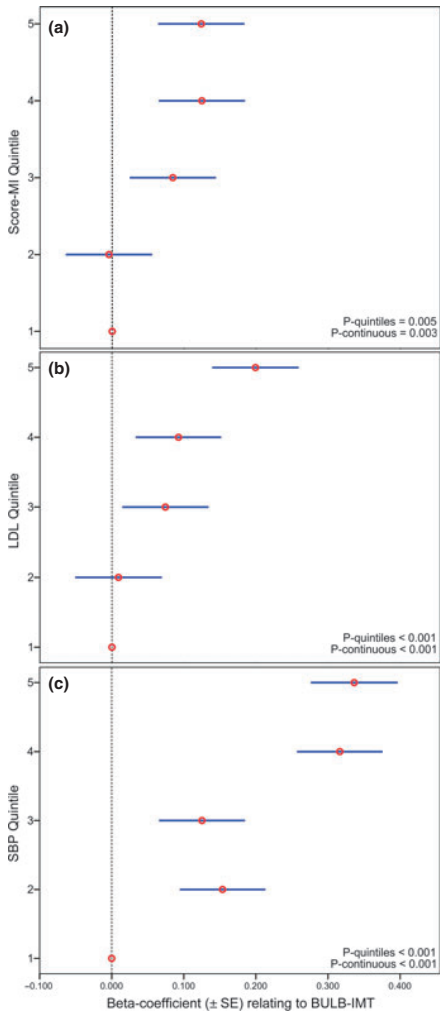

Fig. 2 *Impact of quintiles of Score-MI, LDL levels and systolic blood pressure levels on intima–media thickness (IMT) of the bulb. β-coefficients (red circles) ± SE (blue lines) relating to standard deviations of log-transformed IMT of the bulb for quintiles of (a) Score-MI, (b) LDL and (c) systolic blood pressure (SBP). Models were adjusted for age, gender, diabetes, smoking, antihypertensive medication, C-reactive protein and HDL (all), as well as systolic blood pressure (only in a and b), LDL (only in a and c) and Score-MI (only in b and c). MI, myocardial infarction.*

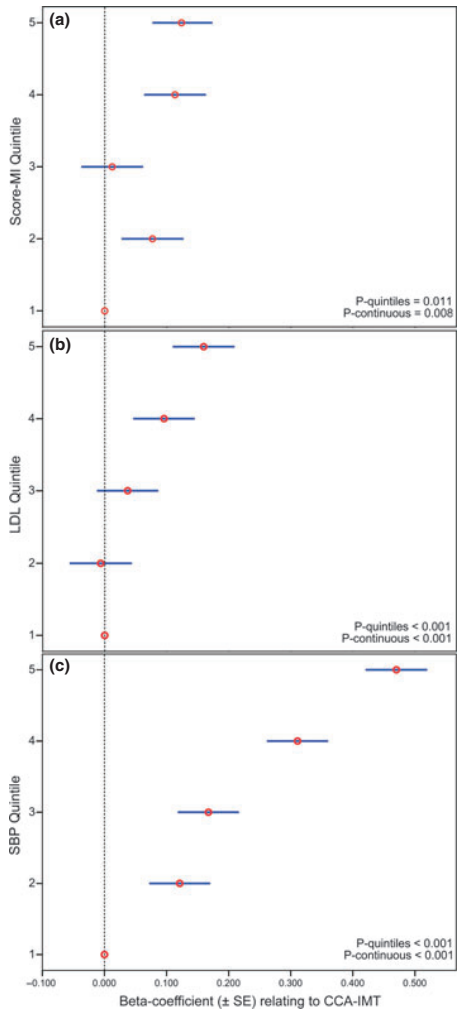

Fig. 3 *Impact of quintiles of Score-MI, LDL levels and systolic blood pressure levels on intima–media thickness (IMT) of the common carotid artery (CCA). β-coefficients (red circles) ± SE (blue lines) relating to standard deviations of log-transformed IMT of the CCA for quintiles of (a) Score-MI, (b) LDL and (c) systolic blood pressure (SBP). Models were adjusted for age, gender, diabetes, smoking, antihypertensive medication, C-reactive protein and HDL (all), as well as systolic blood pressure (only in a and b), LDL (only in a and c) and Score-MI (only in b and c). MI, myocardial infarction.*

no significant association between Score-MI-LDL and the occurrence of moderate to severe carotid atherosclerosis (Table 3).

Score-MI-non-LDL showed a significant association with IMT of the bulb ($P = 0.001$) and the CCA ($P = 0.004$). Significance for bulb and CCA IMT remained after adjustments for cardiovascular risk factors but not after accounting for Bonferroni corrections ($P = 0.011$ and $P = 0.038$, respectively) (Table 4). Score-MI-non-LDL was however associated with the occurrence of moderate to severe carotid atherosclerosis after cardiovascular risk factor adjustment and Bonferroni correction (odds ratio per quintile of Score-MI-non-LDL = 1.09; 95% confidence interval 1.03–1.16; $P = 0.005$).

Association between genotype scores and risk factors for MI

Score-MI was associated with LDL levels in the study cohort ($P < 0.001$; $\beta = 0.041$ mmol L^{-1} per quintile of Score-MI). As expected, the association was explained by Score-MI-LDL which was significantly associated with LDL levels ($P < 0.001$) whereas there was no significant association for Score-MI-non-LDL ($P = 0.307$). Score-MI was nominally associated with

Table 3 *Association between Score-MI-*LDL *and carotid parameters*

	Unadjusted model			Adjusted model[a]		
	β[b]	*P*-quintiles[c]	*P*-continuous[d]	β[b]	*P*-quintiles[c]	*P*-continuous[d]
IMT of carotid-bulb	0.018	0.175	0.126	0.020	0.141	0.157
IMT of CCA	0.024	0.033	0.065	0.024	0.029	0.112
	OR (95% CI)	*P* in model		OR (95% CI)	*P* in model	
Carotid atherosclerosis[e]	1.06 (1.00–1.12)	0.056		1.06 (1.00–1.12)	0.066	

Applying Bonferroni correction, a *P*-value of 0.05/9 = 0.0056 was considered significant.
Additional data is shown in Table S3.
CCA, common carotid artery; IMT, intima–media thickness; MI, myocardial infarction.
[a]Adjusted for age, gender, smoking, diabetes mellitus, systolic blood pressure, antihypertensive treatment, LDL, HDL, C-reactive protein and waist circumference.
[b]β-coefficients relating to number of standard deviations from mean of log IMT per quintile of Score-MI-LDL.
[c]*P*-value for linear regression with quintiles of Score-MI-LDL as independent variable.
[d]*P*-value for linear regression with continuous Score-MI-LDL as independent variable.
[e]Defined as occurrence of at least one carotid plaque ≥10 mm^2. Odds ratios (ORs) are average per quintile of Score-MI-LDL in binary logistic regression model.

Table 4 *Association between Score-MI-non-LDL and carotid parameters*

	Unadjusted model			Adjusted model[a]		
	β[b]	*P*-quintiles[c]	*P*-continuous[d]	β[b]	*P*-quintiles[c]	*P*-continuous[d]
IMT of carotid-bulb	0.045	0.001	0.001	0.037	0.006	0.011
IMT of CCA	0.031	0.005	0.004	0.022	0.049	0.038
	OR (95% CI)	*P* in model		OR (95% CI)	*P* in model	
Carotid atherosclerosis[e]	1.10 (1.04–1.17)	0.001		1.09 (1.03–1.16)	0.005	

Applying Bonferroni correction, a *P*-value of 0.05/9 = 0.0056 was considered significant.
Additional data is shown in Table S4.
CCA, common carotid artery; IMT, intima–media thickness; MI, myocardial infarction.
[a]Adjusted for age, gender, smoking, diabetes mellitus, systolic blood pressure, antihypertensive treatment, LDL, HDL, C-reactive protein and waist circumference.
[b]β-coefficients relating to number of standard deviations from mean of log IMT per quintile of Score-MI-non-LDL.
[c]*P*-value for linear regression with quintiles of Score-MI-non-LDL as independent variable.
[d]*P*-value for linear regression with continuous Score-MI-non-LDL as independent variable.
[e]Defined as occurrence of at least one carotid plaque ≥10 mm^2. Odds ratios (ORs) are average per quintile of Score-MI-non-LDL in binary logistic regression model.

increasing systolic blood pressure levels ($P = 0.037$) and decreasing CRP levels ($P = 0.040$). There were no associations between Score-MI and diastolic blood pressure, HDL, diabetes mellitus or waist circumference (Table S5.)

Association between specific SNPs and IMT

Chr9p21-rs4977574 was associated with bulb ($P = 0.001$) and CCA IMT ($P = 0.004$); SORT1-rs646776 was also associated with both IMT measurements ($P = 0.013$ and $P = 0.038$, respectively). HNF1a-rs2259816 was negatively associated with CCA IMT ($P = 0.015$) and LDLR-rs1122608 was positively associated with CCA IMT ($P = 0.041$). None of the remaining nine SNPs showed statistically significant associations with IMT. (Table S6).

Discussion

Main findings

In this population-based study we found a significant association between a genetic score of 13 well-validated MI-/CAD-associated SNPs, carotid bulb IMT and plaques. By contrast, association between the genetic score and CCA IMT did not reach Bonferroni-corrected significance after adjustment for cardiovascular risk factors.

Associations between carotid IMT and SNPs adjacent to genes involved in haemostasis, the renin–angiotensin system, inflammation and the extracellular matrix have previously been suggested [19, 34]. SNPs on chromosome 9p21 have been associated with carotid atherosclerotic lesions [11] but not with carotid IMT [11, 15–18], in contrast to our results. For the combined effects, a genetic score of SNPs associated with total cholesterol has been shown to be associated with carotid IMT [18, 35]. Whereas the genetic risk score used in our study has been shown previously to strongly predict CAD [30], our study is, as far as we are aware, the first to investigate the effect of this genetic score – and thus the combined effects of only specifically well-validated MI-/CAD-associated SNPs – on carotid IMT and plaques.

MI genetic risk score and increased atherosclerosis: is there an association?

Increased carotid IMT is considered an early marker of atherosclerosis [25] and is strongly related to manifest atherosclerosis in carotid and other arteries [26–28]. Increased IMT is also associated with other factors (i.e. increased expression of mediators of coagulation, vasoconstriction and inflammation) that have been implicated in atherogenesis [25]. Carotid plaques represent focal atherosclerotic lesions that are directly linked with atherosclerosis [25].

Our results showed associations between the main genetic risk score (Score-MI) and maximum bulb IMT with an effect size comparable to that of LDL, a well-known risk factor for carotid disease in our cohort [31]. In addition, the genetic score was associated with the occurrence of carotid plaques representing moderate to severe carotid atherosclerosis. Considering these consistent results of correlations with bulb IMT and plaque, this genetic risk score might be associated with increased atherosclerosis, providing a plausible explanation for its association with MI. However, it must be stressed that we have not shown a direct link with atherosclerosis for the score, but rather an association with intermediate phenotypes for atherosclerosis. Therefore, a possible direct association between atherosclerosis and this genetic risk score requires further investigation before any definite conclusions can be drawn.

We suggest two possible explanations for the fact that the genetic score was significantly associated with carotid bulb IMT and plaques, but did not show a Bonferroni-corrected significant association with mean IMT in the CCA in our population. First, CCA IMT is strongly correlated with risk factors for stroke, especially blood pressure [36], and therefore there is reason to believe that such factors could have greater impact on CCA IMT than a genetic score that is primarily associated with MI/CAD and not stroke. This was also the case in the present study (Fig. 3). Second, the association with maximal carotid bulb IMT might in fact represent an association with carotid plaques, as these are most often found at the bulb in our cohort. Increased mean CCA IMT on the other hand often represents more diffuse changes of smooth muscle proliferation and ground substance accumulation [37].

Impact of genotype subscores

It is noteworthy that the associations between the genetic score, bulb IMT and plaques could be substantially attributed to SNPs unrelated to LDL cholesterol (Score-MI-non-LDL). However, Score-MI-LDL included only five SNPs, possibly reducing the effect size of this score.

Association with biochemical MI risk factors

It has been shown that the SNPs on chromosome 9p21 are not associated with biochemical risk factors for MI [5, 16, 17] As expected, the main genetic risk score in our study (Score-MI) was strongly associated with LDL levels. Although we also found associations between Score-MI and increasing levels of systolic blood pressure as well as decreasing CRP levels, these associations were weak, in particular compared to the association between Score-MI and LDL levels. SH2B3-rs3184504 has been previously shown to be associated with population blood pressure [3], possibly explaining the modest association found between Score-MI and blood pressure. Whether or not this weak association as well that between Score-MI and decreasing CRP levels indeed represent true relationships remains unclear.

Possible clinical implications

The association between this MI genetic risk score and intermediate phenotypes of atherosclerosis could in turn suggest that the main mechanism through which the genetic score confers increased risk of MI is accelerated atherosclerosis. Although not directly proven by our study, this suggestion is in line with the results from a recent GWAS showing associations between the individual SNPs of the score and CAD, but not MI on top of CAD [38]. Thus it is possible that clinical use of known anti-atherosclerotic therapy is warranted to reduce the speed of development of the intermediate phenotypes of atherosclerosis in the form of increased IMT and carotid plaques, and in turn ultimately to minimize the development of atherosclerosis and the increased risk of MI in subjects with a high genetic risk score. Also taking into consideration the fact that atherosclerosis is a disease that develops over decades and as such should logically be most effectively treated before intermediate markers can be detected, assessing this genetic score might prove valuable for decision of early initiation of known anti-atherosclerotic treatment in young individuals with a family history of MI (and in whom these intermediate atherosclerotic phenotypes in the form of increased IMT and plaques are not yet detectable by conventional methods). However, it must be stressed that in the current study we did not specifically investigate whether assessing the MI genetic score with regard to early anti-atherosclerotic treatment decision-making is valuable for prevention of intermediate atherosclerotic phenotypes as well as for prevention of fully developed atherosclerosis and MI. Nevertheless, our results of an association between intermediate phenotypes of atherosclerosis and the genetic score provide support for such studies to be performed to test this hypothesis.

Limitations

Our study has several limitations. First, although in terms of validity we emphasize the strength of using a genetic score with strong association with CAD [30], new SNPs associated with MI and CAD are continually being discovered. Considering this, the genetic risk score used in the current study could be criticized for not being entirely up to date and, accordingly, adding newly discovered MI-/CAD-related SNPs [24] to the genetic score would be valuable.

Secondly, as already stated, we have not shown a direct link with atherosclerosis for the genetic score, but only an association with an intermediate marker of disease. It also remains unclear whether or not other mechanisms, such as plaque instability, haemostasis and factors that influence angiogenesis as well as the sensitivity of heart muscle to ischaemia, could also explain the pathophysiological background for the MI risk conferred by this genetic score.

Thirdly, as Lorentz *et al.* concluded from their meta-analysis [29], IMT studies show substantial methodological heterogeneity of measurement of IMT segments. This could limit the value of comparing different IMT segments and also complicate the comparison of different studies.

Finally, although methods of quality control in the MDC-CV cohort have been shown to be satisfactory [39], ultrasound is a method with some inter- and intra-observer variability.

Conclusions

In conclusion, we have shown associations between a genetic score of 13 MI-/CAD-associated SNPs and both IMT of the bulb and the occurrence of carotid plaques in middle-aged subjects. The results provide evidence of an association between the genetic risk score and intermediate phenotypes of atherosclerosis, further suggesting that accelerated atherosclerosis could be a mechanism through which this genetic score confers its increased risk of MI. The risk score may prove helpful in identifying subjects at risk of intermediate phenotypes of atherosclerosis before these phenotypes can be detected by conventional methods. Whether or not the genetic risk score could be valuable for early treatment decisions in young

individuals with a family history of premature-onset MI should be further tested.

Conflict of interest statement

No conflicts of interest to declare.

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Correspondence: Viktor Hamrefors and Olle Melander, Clinical Research Centre, Lund University, Entrance 72, Building 91, Floor 12, Skåne University Hospital Malmö, SE-205 02 Malmö, Sweden.
(fax: +46-40-391222; e-mails: viktor.hamrefors@med.lu.se; olle.melander@med.lu.se).

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Description of included SNPs.

Table S2. (A) Association between Score-MI and IMT of the bulb and CCA. (B) Association between Score-MI and occurrence of moderate to severe carotid atherosclerosis.

Table S3. (A) Association between Score-MI-LDL and IMT of the bulb and CCA. (B) Association between Score-MI-LDL and occurrence of moderate to severe carotid atherosclerosis.

Table S4. (A) Association between Score-MI-non-LDL and IMT of the bulb and CCA. (B) Association between Score-MI-non-LDL and occurrence of moderate to severe carotid atherosclerosis.

Table S5. Association between Score-MI and cardiovascular risk factors.

Table S6. Distribution and association between SNPs and carotid-IMT of bulb and CCA.

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Paper III

Original Article

Pharmacogenetic implications for eight common blood pressure-associated single-nucleotide polymorphisms

Viktor Hamrefors[a], Marketa Sjögren[a], Peter Almgren[a], Björn Wahlstrand[b], Sverre Kjeldsen[c], Thomas Hedner[b], and Olle Melander[a]

Objective: We aimed to test whether eight common recently identified single-nucleotide polymorphisms (SNPs), strongly associated with blood pressure (BP) in the population, also have impact on the degree of BP reduction by antihypertensive agents with different mechanisms.

Methods: In 3863 Swedish hypertensive patients, we related number of unfavorable alleles of each SNP (i.e. alleles associated with higher baseline BP) to the magnitude of BP reduction during 6 months of monotherapy with either a beta-blocker, a thiazide diuretic or diltiazem.

Results: For six SNPs (*rs16998073*, *rs1378942*, *rs3184504*, *rs1530440*, *rs16948048*, *rs17367504*) no pharmacogenetic interactions were suggested, whereas two SNPs showed nominal evidence of association with treatment response: *PLCD3-rs12946454* associated with more SBP (beta = 1.53 mmHg per unfavorable allele; $P = 0.010$) and DBP (beta = 0.73 mmHg per unfavorable allele; $P = 0.014$) reduction in patients treated with diltiazem, in contrast to those treated with beta-blockers or diuretics wherein no treatment response association was found. *CYP17A1-rs11191548* associated with less DBP reduction (beta = −1.26 mmHg per unfavorable allele; $P = 0.018$) in patients treated with beta-blockers or diuretics, whereas there was no treatment response association in diltiazem-treated patients. However, if accounting for multiple testing, the significant associations for *rs12946454* and *rs11191548* were attenuated.

Conclusion: For a majority of these, eight recently identified BP-associated SNPs, there are probably no important pharmacogenetic interactions for BP reduction with use of beta-blockers, diuretics or diltiazem. Whether the nominally significant associations for *rs12946454* and *rs11191548* are true signals and could be of possible clinical relevance for deciding treatment of polygenic essential hypertension should be further tested.

Keywords: adrenergic, beta-blockers, calcium channel blockers, diuretics, hypertension treatment, pharmacogenetics, polymorphism, single nucleotide

Abbreviations: ACE, angiotensin-converting enzyme; GWAS, genome wide association study; SNPs, single nucleotide polymorphisms

INTRODUCTION

Hypertension is a major factor implicated in global morbidity and mortality [1], and is affecting more than one-quarter of the world's adult population [2]. Unfortunately, despite the extensive use of various antihypertensive therapies worldwide, control of blood pressure among hypertensive patients is generally poor, with only approximately one-third of treated patients estimated to reach target blood pressure levels [3]. Although lack of compliance is a well known problem in treatment of hypertension, the very high proportion of patients that do not reach the treatment goals also emphasizes the need of new approaches for finding optimal and better individualized antihypertensive therapies.

It has been known for a long time that blood pressure levels are considerably influenced by hereditable factors [4]. The currently most used genetic epidemiological approach of performing genome wide association studies (GWASs) in order to identify the most common genetic variants in the population – single-nucleotide polymorphisms (SNPs) – that associate with blood pressure has proved challenging. Lately, however, large GWASs have succeeded in identifying a number of common SNPs that are strongly associated with SBP and/or DBP in the population [5–7].

Many of the newly discovered blood pressure-associated SNPs are located near genes encoding possible targets for various antihypertensive agents [5–7], suggesting that they might possibly also affect the blood pressure treatment response of various antihypertensive medications. Although previous pharmacogenetic studies of blood pressure treatment have examined various formerly discovered genetic variants affecting the renin–angiotensin–aldosterone system (RAAS) system [8], other forms of renal sodium retention [9–11], beta-adrenergic receptors [12–14] and

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[a]Department of Clinical Sciences, Lund University, Malmö, [b]Department of Clinical Pharmacology, Sahlgrenska University Hospital, Gothenburg, Sweden and [c]Ullevaal University Hospital, University of Oslo, Oslo, Norway

Correspondence to Viktor Hamrefors, MD, Clinical Research Centre, Lund University, Entrance 72, Building 91, Floor 12, Skåne University Hospital Malmö, SE-205 02 Malmö, Sweden. Tel: +46 703996529; fax: +46 40391222; e-mail: Viktor.Hamrefors@med.lu.se

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atrial natriuretic peptides [15], little is known about the pharmacogenetic aspects of the common blood pressure-associated SNPs that were discovered in the recent GWASs.

The vast majority of antihypertensive treatment studies in recent years involve multiple simultaneous antihypertensive agents from an early stage, limiting the potential of detecting gene–single drug treatment interactions. The Nordic Diltiazem (NORDIL) antihypertensive study [16] involved mainly monotherapy during the first 6 months. In the NORDIL study, with an ethnically homogenous cohort of Swedish and Norwegian middle-aged hypertensive individuals, diltiazem-based antihypertensive therapy was compared with beta-blockers and/or diuretics therapy, showing no difference in incident cardiovascular events over a period of 4.5 years [16]. Considering the use of monotherapy during the first 6 months for the majority of study participants, as well as the ethnically homogenous population, the NORDIL material offers a unique possibility for pharmacogenetic studies of blood pressure therapy.

Our objective of the current study was to test whether eight widely spread blood pressure-associated SNPs, that were identified in a large GWASs in 2009 [5], also have impact on the degree of blood pressure reduction achieved by antihypertensive agents with substantially different mechanisms. By genotyping and using blood pressure treatment data of Swedish participants in the NORDIL cohort, we related each of the eight blood pressure-associated SNPs to blood pressure reduction after 6 months of beta-blocker/diuretic or diltiazem-based antihypertensive monotherapy.

METHODS

Patients and clinical data

The NORDIL antihypertensive study [16] is a prospective, randomized, open, blinded endpoint study conducted in the years of 1992–1999. Ten thousand, eight hundred and eighty-one middle-aged Swedish and Norwegian patients, who had DBP of 100 mmHg or more on two occasions, were included. Participants were randomized to treatment with either the nonselective calcium channel blocker diltiazem or to therapy with beta-blockers and/or diuretics. Blood pressure was measured in recumbent position every 6 months as previously described [11], and patients with DBP still over 90 mmHg on follow-ups received additional therapy in steps. For the diltiazem group, therapy for the first 6 months of the study period was 180–360 mg of diltiazem daily. Step 2 was addition of an angiotensin-converting enzyme (ACE) inhibitor. In step 3, a diuretic or an alpha-blocker was added to the ACE inhibitor. If still not meeting criteria of DBP below 90 mmHg, any antihypertensive agent compound could be added. In the beta-blocker and diuretics group, patients were initially treated with a beta-blocker or thiazide diuretic. In step 2, the two were combined if needed for adequate blood pressure reduction. In step 3, an ACE inhibitor or an alpha-blocker was added. If patients were still hypertensive, any other antihypertensive compound except a calcium antagonist could be added. Patients were followed for a mean time of 4.5 years, with no differences of the primary endpoint (fatal and nonfatal stroke and myocardial infarction, death from cardiovascular causes) between diltiazem and beta-blocker/diuretics groups.

As formerly described, DNA was extracted from 5152 Swedish patients, constituting 72.4% of the Swedish NORDIL cohort [11]. From these, patients on monotherapy with either a beta-blocker, a thiazide diuretic or diltiazem during the first 6 months of the NORDIL study were selected for the current study ($N = 4052$). However, only patients with complete data of baseline (inclusion and randomization in NORDIL) and 6 months SBP and DBP levels, combined with baseline data of covariates including age, sex, diabetes, smoking, serum creatinine, BMI and previous cardiovascular disease, were included in the final study population ($N = 3863$). Of these, 1323 (34.2%) were treated with beta-blockers, 521 (13.5%) with thiazide diuretics and 2019 (52.3%) with diltiazem in monotherapy during the first 6 months of the NORDIL study period (Table 1).

The study protocol was formally approved by the ethics committee at Lund University and Gothenburg University. All patients had formerly given their informed consent. The procedures followed were in accordance with institutional guidelines.

Single-nucleotide polymorphisms and genotyping

The current study included eight SNPs associated with SBP and/or DBP at the population level, published in a major GWASs in 2009: *rs12946454*, *rs11191548*, *rs16998073*, *rs1378942*, *rs3184504*, *rs1530440*, *rs16948048* and *rs17367504* [5]. Short descriptions of the included SNPs are shown in Supplementary Table 1, Supplemental Digital Content 1, http://links.lww.com/HJH/A169. On the basis of the distribution in the study population, Hardy–Weinberg statistics were calculated for all SNPs [17], with no included SNP significantly deviating from the Hardy–Weinberg equilibrium at a P value of 0.05 (Tables 2–9).

The SNPs were genotyped by IPLEX on a MassARRAY platform (Sequenom, San Diego, California, USA) according to standard protocols from the manufacturer. Fifteen percent of the samples were run in duplicate without any inconsistencies. All genotypes were called by two different investigators.

Design and statistical analyses

General statistics

Statistical analyses were performed using SPSS Statistics 17.0/19.0 from SPSS Inc. (Chicago, Illinois, USA), except for power calculations that were done by PS 3.0 by WD Dupont and WD Plummer Jr from the Department of Biostatistics, Vanderbuilt University (Nashville, Tennessee, USA). A P value of 0.05 was considered statistically significant.

Relation of single-nucleotide polymorphisms with blood pressure reduction during treatment

It follows from the design of the NORDIL study that during the first 6 months of the study period, a majority of patients received antihypertensive monotherapy with a

TABLE 1. Population characteristics

	All patients	BB/diuretics	Diltiazem
Total patients (*n*)	3863	1844	2019
Men [*n* (%)]	1883 (48.7)	872 (47.3)	1011 (50.1)
Women [*n* (%)]	1980 (51.3)	972 (52.7)	1008 (49.9)
Age (years)	60.3 (6.6)	60.3 (6.7)	60.4 (6.6)
Baseline SBP	172.7 (16.2)	171.9 (15.9)*	173.3 (16.5)*
−ΔSBP (mmHg)	17.1 (16.1)	18.1 (16.5)†	16.1 (15.7)†
−ΔSBP (%)	9.57 (8.9)	10.3 (9.1)†	8.9 (8.6)†
Baseline DBP	103.9 (5.0)	103.6 (5.0)*	104.1 (5.0)*
−ΔDBP (mmHg)	14.4 (8.1)	14.2 (8.3)	14.5 (8.0)
−ΔDBP (%)	13.7 (7.6)	13.6 (7.8)	13.8 (7.4)
Smokers [*n* (%)]	780 (20.2)	371 (20.1)	409 (20.3)
Diabetes [*n* (%)]	324 (8.4)	142 (7.7)	182 (9.0)
BMI (kg/m^2)	28.0 (4.3)	27.9 (4.4)	28.0 (4.3)
eGFR (ml/min per m^2)‡			
Men	81.14 (16.7)	81.34 (17.4)	80.97 (16.2)
Women	86.42 (18.3)	86.73 (18.3)	86.13 (18.4)
Previous CVD [n (%)]	146 (3.8)	69 (3.7)	77 (3.8)

Continuous variables are all displayed as mean (SD). CVD, cardiovascular disease.
*$P < 0.01$ between beta-blocker (BB)/diuretic and diltiazem groups (Student's *t*-test).
†$P < 0.001$ between BB/diuretic and diltiazem groups (Student's *t*-test).
‡Glomerular filtration rate (GFR) calculated by using Cockcroft–Gault formula and divided by body surface area.

beta-blocker, a thiazide diuretic or diltiazem. Considering the favorable pharmacogenetic study aspects of patients on only one antihypertensive therapy at a time, the 6-month period was chosen as the defined treatment period for studying blood pressure reduction in the current study. Thus, the individual absolute and percentage SBP and DBP reduction achieved after 6 months of monotherapy (mean of blood pressure levels at inclusion and randomization – blood pressure level at 6 months) on either diltiazem, beta-blockers or diuretics was calculated for each included participant.

Each of the eight SNPs were related to absolute and percentage reduction in SBP and DBP during 6 months of antihypertensive monotherapy, using linear regression, with number of unfavorable SNP alleles (i.e. alleles formerly shown to be associated with higher base line blood pressure) as independent and blood pressure reduction (positive direction) as dependent variable. We assumed an additive model (0–1–2 alleles) for all included SNPs, as such a model has proven to be the most appropriate in the relation with population blood pressure. As we primarily aimed to discover significant pharmacogenetic effects involving either mechanisms of sodium retention in the kidney (an important mechanism for genes near some of the included SNPs) or effects on the vascular smooth muscle (a mechanism for genes near other of the included SNPs) (see Supplementary Table1, Supplemental Digital Content 1, http://links.lww.com/HJH/A169), analyses were performed separately in two groups: in group 1 were participants treated with either beta-blockers or diuretics

TABLE 2. Association of rs16998073 with blood pressure reduction

				$Trend_{unadjusted}$		$Trend_{adjusted}$[a]		
	AA	AT	TT	Beta[b]	*P*	Beta[b]	*P*	Power[c]
Beta-blockers/diuretics								
Number of patients	694	863	266					
Baseline SBP (mmHg)	171.2 (15.6)	172.3 (16.0)	172.2 (15.6)					
−ΔSBP (mmHg)	16.9 (16.2)	19.2 (16.6)	17.6 (16.4)	0.819	0.145	0.863	0.124	94.6%
−ΔSBP (%)	9.6 (9.0)	10.9 (9.2)	9.9 (9.3)	0.448	0.151	0.464	0.136	
Baseline DBP (mmHg)	103.6 (5.0)	103.7 (4.9)	103.6 (5.0)					
−ΔDBP (mmHg)	13.7 (8.2)	14.7 (8.4)	13.7 (8.1)	0.245	0.388	0.262	0.355	94.1%
−ΔDBP (%)	13.1 (7.7)	14.1 (7.9)	13.1 (7.6)	0.237	0.372	0.251	0.344	
Diltiazem								
Number of patients	811	931	254					
Baseline SBP (mmHg)	173.0 (16.5)	173.2 (16.0)	174.0 (17.6)					
−ΔSBP (mmHg)	16.3 (16.2)	16.1 (15.2)	15.1 (15.6)	−0.466	0.370	−0.405	0.433	97.1%
−ΔSBP (%)	9.0 (8.8)	9.0 (8.3)	8.4 (8.5)	−0.245	0.386	−0.218	0.440	
Baseline DBP (mmHg)	104.0 (5.1)	104.0 (5.0)	104.5 (5.0)					
−ΔDBP (mmHg)	14.7 (8.0)	14.3 (8.0)	14.3 (7.9)	−0.277	0.295	−0.246	0.349	96.6%
−ΔDBP (%)	14.0 (7.5)	13.7 (7.4)	13.6 (7.5)	−0.266	0.280	−0.235	0.336	

Genes: *PRDM8*, *FGF5*, *c4orf22*. Main phenotype: DBP. Risk allele: T[d]. *P* value of χ^2 distribution for Hardy–Weinberg equilibrium: 0.689. Reduction (positive direction) of blood pressure after 6 months of diuretic/beta-blocker or diltiazem antihypertensive therapy. Displayed as mean (SD).
[a]Age, sex, smoking, s-creatinine, BMI, diabetes mellitus and previous cardiovascular disease entered as covariates in linear model.
[b]For linear additive model, with genotype as independent and reduction of blood pressure as dependent variable.
[c]Calculated for absolute blood pressure reduction in unadjusted analyses, detecting beta = 2 mmHg per allele for SBP and beta = 1 mmHg per allele for DBP.
[d]Allele associated with higher baseline blood pressure in discovery studies.

TABLE 3. Association of rs1378942 with blood pressure reduction

				$Trend_{unadjusted}$		$Trend_{adjusted}$[a]		
	AA	AC	CC	Beta[b]	*P*	Beta[b]	*P*	Power[c]
Beta-blockers/diuretics								
Number of patients	799	830	204					
Baseline SBP (mmHg)	171.8 (15.9)	172.1 (16.0)	172.0 (15.5)					
−ΔSBP (mmHg)	18.6 (16.0)	17.6 (17.1)	18.6 (15.8)	−0.300	0.604	−0.306	0.598	93.2%
−ΔSBP (%)	10.5 (8.9)	9.9 (9.5)	10.6 (8.6)	−0.182	0.570	−0.203	0.526	
Baseline DBP (mmHg)	103.6 (5.0)	103.7 (5.0)	103.4 (4.9)					
−ΔDBP (mmHg)	14.6 (8.3)	13.9 (8.4)	13.8 (8.3)	−0.480	0.101	−0.446	0.129	92.7%
−ΔDBP (%)	13.9 (7.7)	13.3 (7.8)	13.2 (7.9)	−0.445	0.104	−0.408	0.136	
Diltiazem								
Number of patients	875	904	226					
Baseline SBP (mmHg)	172.8 (16.5)	173.6 (16.2)	174.1 (17.1)					
−ΔSBP (mmHg)	15.8 (15.7)	16.6 (15.5)	15.5 (16.2)	0.176	0.737	0.237	0.651	96.8%
−ΔSBP (%)	8.8 (8.6)	9.2 (8.4)	8.5 (8.8)	0.062	0.828	0.084	0.769	
Baseline DBP (mmHg)	104.0 (5.1)	104.0 (5.0)	104.7 (5.3)					
−ΔDBP (mmHg)	14.5 (8.0)	14.6 (7.8)	14.1 (8.6)	−0.122	0.647	−0.071	0.791	96.3%
−ΔDBP (%)	13.9 (7.5)	13.9 (7.2)	13.4 (8.0)	−0.159	0.523	−0.110	0.655	

Genes: *CYP1A1*, *CYP1A2*, *CSK*, *LMAN1L*, *CPLX3*, *ARID3B*. Main phenotype: DBP. Risk allele: C[d]. *P* value of χ^2 distribution for Hardy–Weinberg equilibrium: 0.549. Reduction (positive direction) of blood pressure after 6 months of diuretic/beta-blocker or diltiazem antihypertensive therapy. Displayed as mean (SD).
[a]Age, sex, smoking, s-creatinine, BMI, diabetes mellitus and previous cardiovascular disease entered as covariates in linear model.
[b]For linear additive model, with genotype as independent and reduction of blood pressure as dependent variable.
[c]Calculated for absolute blood pressure reduction in unadjusted analyses, detecting beta = 2 mmHg per allele for SBP and beta = 1 mmHg per allele for DBP.
[d]Allele associated with higher baseline blood pressure in discovery studies.

(BB/diuretics group; $N = 1844$), whereas group 2 consisted of participants treated with diltiazem (diltiazem group; $N = 2019$). The rationale for grouping together participants on beta-blockers and diuretics was based on the fact that both diuretics (direct effects) and beta-blockers (by blocking renin release) inhibit sodium reabsorption in the nephron, thus substantially differing from the mechanisms of the calcium antagonist diltiazem that have primarily vasodilatatory effects and have no obvious effect on sodium retention in the nephron.

All analyses were performed unadjusted and adjusted. In adjusted analyses, age, sex, smoking (current and former), serum creatinine, diabetes mellitus, BMI and previous cardiovascular disease were entered as covariates in the linear regression model.

In order to test the possible cumulative effects of the eight SNPs, we also constructed a genetic risk score (Score-BP) for participants in study cohort who had data of at least seven of the eight SNPs ($N = 3647$). Score-BP for each participant was constructed on the basis of the number of unfavorable alleles (maximum 16) of the eight SNPs. Cohort-specific averages for alleles in this group were used in case of missing data of a single SNP. In order to take into account the effect sizes for the individual SNPs (Supplementary Table 1, Supplemental Digital Content 1, http://links.lww.com/HJH/A169), every unfavorable SNP allele

TABLE 4. Association of rs3184504 with blood pressure reduction

				$Trend_{unadjusted}$		$Trend_{adjusted}$[a]		
	CC	CT	TT	Beta[b]	*P*	Beta[b]	*P*	Power[c]
Beta-blockers/diuretics								
Number of patients	498	837	373					
Baseline SBP (mmHg)	172.8 (16.4)	172.0 (15.9)	171.5 (14.9)					
−ΔSBP (mmHg)	18.6 (16.8)	18.6 (16.3)	17.7 (16.2)	−0.395	0.481	−0.391	0.486	94.7%
−ΔSBP (%)	10.4 (9.1)	10.5 (9.1)	10.1 (9.1)	−0.158	0.609	−0.171	0.581	
Baseline DBP (mmHg)	103.7 (5.0)	103.6 (4.9)	103.5 (5.0)					
−ΔDBP (mmHg)	14.2 (9.0)	14.3 (8.0)	14.1 (8.2)	−0.024	0.933	0.010	0.973	93.9%
−ΔDBP (%)	13.6 (8.4)	13.7 (7.5)	13.5 (7.6)	0.001	0.966	0.038	0.866	
Diltiazem								
Number of patients	552	922	402					
Baseline SBP (mmHg)	173.8 (16.0)	173.5 (16.5)	172.9 (16.6)					
−ΔSBP (mmHg)	15.9 (15.1)	16.3 (15.7)	16.5 (16.0)	0.328	0.519	0.371	0.464	97.6%
−ΔSBP (%)	8.8 (8.2)	9.0 (8.6)	9.1 (8.7)	0.175	0.527	0.186	0.500	
Baseline DBP (mmHg)	104.0 (5.2)	104.1 (4.8)	104.1 (5.4)					
−ΔDBP (mmHg)	14.2 (7.8)	14.9 (7.9)	14.4 (8.1)	0.162	0.531	0.199	0.438	97.3%
−ΔDBP (%)	13.5 (7.3)	14.2 (7.3)	13.7 (7.5)	0.132	0.582	0.168	0.483	

Genes: *SH2B3*, *ATXN2*. Main phenotype: DBP. Risk allele: T[d]. *P* value of χ^2 distribution for Hardy–Weinberg equilibrium: 0.451. Reduction (positive direction) of blood pressure after 6 months of diuretic/beta-blocker or diltiazem antihypertensive therapy. Displayed as mean (SD).
[a]Age, sex, smoking, s-creatinine, BMI, diabetes mellitus and previous cardiovascular disease entered as covariates in linear model.
[b]For linear additive model, with genotype as independent and reduction of blood pressure as dependent variable.
[c]Calculated for absolute blood pressure reduction in unadjusted analyses, detecting beta = 2 mmHg per allele for SBP and beta = 1 mmHg per allele for DBP.
[d]Allele associated with higher baseline blood pressure in discovery studies.

TABLE 5. Association of rs1530440 with blood pressure reduction

				$Trend_{unadjusted}$		$Trend_{adjusted}$[a]		
	TT	TC	CC	Beta[b]	*P*	Beta[b]	*P*	Power[c]
Beta-blockers/diuretics								
Number of patients	49	535	1225					
Baseline SBP (mmHg)	170.8 (13.4)	172.0 (15.7)	172.0 (16.1)					
−ΔSBP (mmHg)	20.1 (14.9)	17.9 (17.0)	18.2 (16.4)	−0.080	0.913	−0.071	0.923	77.9%
−ΔSBP (%)	11.5 (8.4)	10.1 (9.4)	10.3 (9.1)	−0.093	0.818	−0.092	0.820	
Baseline DBP (mmHg)	104.1 (3.9)	103.7 (5.1)	103.6 (4.9)					
−ΔDBP (mmHg)	14.6 (7.6)	14.1 (8.7)	14.2 (8.2)	−0.061	0.868	−0.065	0.861	77.4%
−ΔDBP (%)	14.0 (7.2)	13.5 (8.1)	13.6 (7.7)	−0.050	0.884	−0.056	0.871	
Diltiazem								
Number of patients	63	597	1307					
Baseline SBP (mmHg)	173.8 (17.9)	174.5 (17.3)	172.7 (16.0)					
−ΔSBP (mmHg)	16.2 (13.9)	16.7 (15.8)	15.9 (15.8)	−0.595	0.360	−0.518	0.424	86.8%
−ΔSBP (%)	9.0 (7.7)	9.2 (8.5)	8.8 (8.7)	−0.279	0.432	−0.246	0.486	
Baseline DBP (mmHg)	104.5 (5.3)	104.2 (5.1)	104.0 (5.1)					
−ΔDBP (mmHg)	14.9 (6.8)	14.6 (7.9)	14.4 (8.0)	−0.218	0.509	−0.179	0.586	85.9%
−ΔDBP (%)	14.2 (6.3)	13.9 (7.3)	13.7 (7.5)	−0.194	0.527	−0.151	0.620	

Genes: *c10orf107*, *TMEM26*, *RTKN2*, *RHOBTB1*, *ARID5B*. Main phenotype: DBP. Risk allele: C[d]. *P* value of χ^2 distribution for Hardy–Weinberg equilibrium: 0.282. Reduction (positive direction) of blood pressure after 6 months of diuretic/beta-blocker or diltiazem antihypertensive therapy. Displayed as mean (SD).
[a]Age, sex, smoking, s-creatinine, BMI, diabetes mellitus and previous cardiovascular disease entered as covariates in linear model.
[b]For linear additive model, with genotype as independent and reduction of blood pressure as dependent variable.
[c]Calculated for absolute blood pressure reduction in unadjusted analyses, detecting beta = 2 mmHg per allele for SBP and beta = 1 mm Hg per allele for DBP.
[d]Allele associated with higher baseline blood pressure in discovery studies.

was weighted based on the beta-coefficient for association with blood pressure for that allele. Each of the weighted unfavorable alleles was added to the sum that constituted Score-BP. Score-BP was related to the 6-month absolute and percentage reduction in SBP and DBP, using linear regression with quintiles of Score-BP as independent and blood pressure reduction as dependent variable.

Power calculations

As there are no previously reported data on standard deviations of linear regression errors of blood pressure reduction relating to number of alleles of the blood pressure-associated SNPs, we performed a power calculation based on the current study data. Thus, at an alpha of 0.05, number of participants in each analysis, standard deviations of the independent variable (i.e. allele frequency for each SNP respectively) and standard deviations of linear regression errors of the dependent variable (i.e. blood pressure reduction) were implemented in the power calculations. Detectable difference in linear regression slope (i.e. beta-coefficient) for SBP/DBP reduction was set to 2/1 mmHg per allele, as these are blood pressure

TABLE 6. Association of rs16948048 with blood pressure reduction

				$Trend_{unadjusted}$		$Trend_{adjusted}$[a]		
	AA	AG	GG	Beta[b]	*P*	Beta[b]	*P*	Power[c]
Beta-blockers/diuretics								
Number of patients	626	868	272					
Baseline SBP (mmHg)	171.3 (16.5)	172.3 (15.7)	171.7 (15.5)					
−ΔSBP (mmHg)	17.9 (17.2)	18.5 (16.5)	17.9 (15.7)	0.127	0.827	0.240	0.679	93.3%
−ΔSBP (%)	10.1 (9.5)	10.5 (9.1)	10.1 (8.7)	0.080	0.804	0.134	0.676	
Baseline DBP (mmHg)	103.4 (4.7)	103.8 (5.1)	103.7 (5.1)					
−ΔDBP (mmHg)	14.3 (8.4)	14.3 (8.4)	13.9 (8.0)	−0.163	0.575	−0.079	0.787	93.0%
−ΔDBP (%)	13.7 (7.9)	13.6 (7.9)	13.3 (7.5)	−0.182	0.503	−0.096	0.725	
Diltiazem								
Number of patients	720	915	296					
Baseline SBP (mmHg)	173.5 (16.1)	173.6 (16.9)	171.9 (16.5)					
−ΔSBP (mmHg)	16.0 (15.2)	16.5 (16.1)	15.6 (15.6)	−0.080	0.877	0.009	0.985	97.2%
−ΔSBP (%)	8.9 (8.3)	9.1 (8.7)	8.7 (8.6)	−0.083	0.767	−0.036	0.899	
Baseline DBP (mmHg)	104.1 (5.0)	104.3 (5.1)	103.8 (5.4)					
−ΔDBP (mmHg)	14.6 (8.0)	14.6 (8.2)	14.2 (7.3)	−0.128	0.625	−0.096	0.713	96.8%
−ΔDBP (%)	13.9 (7.4)	13.9 (7.6)	13.5 (6.8)	−0.126	0.604	−0.087	0.719	

Genes: *ZNF652*, *PHB*. Main phenotype: DBP. Risk allele: G[d]. *P* value of χ^2 distribution for Hardy–Weinberg equilibrium: 0.573. Reduction (positive direction) of blood pressure after 6 months of diuretic/beta-blocker or diltiazem antihypertensive therapy. Displayed as mean (SD).
[a]Age, sex, smoking, s-creatinine, BMI, diabetes mellitus and previous cardiovascular disease entered as covariates in linear model.
[b]For linear additive model, with genotype as independent and reduction of blood pressure as dependent variable.
[c]Calculated for absolute blood pressure reduction in unadjusted analyses, detecting beta = 2 mmHg per allele for SBP and beta = 1 mmHg per allele for DBP.
[d]Allele associated with higher baseline blood pressure in discovery studies.

TABLE 7. Association of r17367504 with blood pressure reduction

				$Trend_{unadjusted}$		$Trend_{adjusted}$[a]		
	GG	GA	AA	Beta[b]	*P*	Beta[b]	*P*	Power[c]
Beta-blockers/diuretics								
Number of patients	36	360	1201					
Baseline SBP (mmHg)	172.7 (18.0)	173.3 (16.2)	171.3 (15.5)					
−ΔSBP (mmHg)	18.5 (19.9)	18.8 (16.2)	17.9 (16.6)	−0.750	0.374	−0.777	0.357	66.0%
−ΔSBP (%)	10.3 (10.7)	10.6 (8.8)	10.1 (9.3)	−0.344	0.462	−0.383	0.413	
Baseline DBP (mmHg)	104.7 (4.7)	103.5 (5.2)	103.5 (4.9)					
−ΔDBP (mmHg)	13.4 (9.4)	14.7 (8.6)	14.0 (8.4)	−0.326	0.447	−0.287	0.503	64.5%
−ΔDBP (%)	12.8 (9.0)	14.1 (8.0)	13.4 (7.8)	−0.319	0.426	−0.275	0.492	
Diltiazem								
Number of patients	28	408	1317					
Baseline SBP (mmHg)	175.3 (18.6)	173.2 (16.6)	173.4 (16.3)					
−ΔSBP (mmHg)	16.3 (12.5)	16.7 (15.9)	15.7 (15.9)	−0.780	0.327	−0.758	0.339	71.2%
−ΔSBP (%)	9.0 (6.8)	9.2 (8.7)	8.7 (8.7)	−0.443	0.308	−0.416	0.338	
Baseline DBP (mmHg)	105.9 (5.2)	103.7 (5.4)	104.2 (4.8)					
−ΔDBP (mmHg)	15.0 (7.8)	14.7 (7.9)	14.3 (8.1)	−0.408	0.311	−0.421	0.293	70.0%
−ΔDBP (%)	14.2 (7.3)	14.0 (7.3)	13.6 (7.6)	−0.383	0.308	−0.393	0.292	

Genes: *MTHFR, CLCN6, NPPA, NPPB, AGTRAP*. Main phenotype: SBP. Risk allele: A[d]. *P* value of χ^2 distribution for Hardy–Weinberg equilibrium: 0.542. Reduction (positive direction) of blood pressure after 6 months of diuretic/beta-blocker or diltiazem antihypertensive therapy. Displayed as mean (SD).
[a]Age, sex, smoking, s-creatinine, BMI, diabetes mellitus and previous cardiovascular disease entered as covariates in linear model.
[b]For linear additive model, with genotype as independent and reduction of blood pressure as dependent variable.
[c]Calculated for absolute blood pressure reduction in unadjusted analyses, detecting beta = 2 mmHg per allele for SBP and beta = 1 mmHg per allele for DBP.
[d]Allele associated with higher baseline blood pressure in discovery studies.

reductions that have formerly been suggested to translate into applicable reductions in cardiovascular disease, implying clinical relevance [18]. Implementing the calculated values for each analysis, a calculated power of 80% or more was considered adequate to correctly reject a false null hypothesis stating that there are no differences in blood pressure reduction depending on number of SNP alleles.

Power calculations for the genetic score analyses were performed in a similar way, aiming to detect a difference in linear regression slope (i.e. beta-coefficient) for SBP/DBP reduction of 1/0.5 mmHg per quintile of Score-BP. As for single SNP, a calculated power of 80% or more was considered adequate to correctly reject a false null hypothesis stating that there are no differences in blood pressure reduction depending on quintiles of Score-BP.

Power for the respective analyses is shown in Tables 2–9 and Supplementary Table 2, Supplementary Digital Content 1, http://links.lww.com/HJH/A169.

RESULTS

Baseline characteristics

Baseline characteristics for the 3863 participants included in the study are shown in Table 1. Participants in the BB/diuretics group overall showed lower baseline blood pressure levels as well as a more pronounced SBP reduction

TABLE 8. Association of rs12946454 with blood pressure reduction

				$Trend_{unadjusted}$		$Trend_{adjusted}$[a]		
	AA	AT	TT	Beta[b]	*P*	Beta[b]	*P*	Power[c]
Beta-blockers/diuretics								
Number of patients	1000	702	115					
Baseline SBP (mmHg)	171.4 (15.7)	172.9 (15.8)	169.9 (17.4)					
−ΔSBP (mmHg)	17.6 (16.4)	18.9 (16.4)	18.1 (18.2)	0.761	0.228	0.779	0.218	88.6%
−ΔSBP (%)	10.0 (9.1)	10.7 (9.0)	10.2 (10.1)	0.409	0.242	0.417	0.233	
Baseline DBP (mmHg)	103.5 (4.9)	103.7 (5.1)	103.6 (5.0)					
−ΔDBP (mmHg)	14.2 (8.2)	14.1 (8.3)	14.8 (9.6)	0.053	0.869	0.054	0.866	88.0%
−ΔDBP (%)	13.6 (7.7)	13.4 (7.7)	14.1 (8.9)	0.020	0.947	0.019	0.949	
Diltiazem								
Number of patients	1126	757	107					
Baseline SBP (mmHg)	172.6 (16.3)	174.5 (16.8)	174.0 (16.4)					
−ΔSBP (mmHg)	15.3 (15.4)	17.2 (16.3)	17.6 (15.5)	1.529	0.010	1.557	0.008	92.4%
−ΔSBP (%)	8.5 (8.5)	9.4 (8.7)	9.7 (8.3)	0.767	0.017	0.773	0.016	
Baseline DBP (mmHg)	104.1 (5.0)	104.1 (5.2)	104.4 (5.2)					
−ΔDBP (mmHg)	14.2 (7.7)	14.9 (8.3)	15.7 (8.0)	0.734	0.014	0.751	0.011	91.8%
−ΔDBP (%)	13.5 (7.2)	14.2 (7.7)	14.8 (7.3)	0.661	0.018	0.678	0.014	

Genes: *PLCD3, ACBD4, HEXIM1, HEXIM2*. Main phenotype: SBP. Risk allele: T[d]. *P* value of χ^2 distribution for Hardy–Weinberg equilibrium: 0.172. Reduction (positive direction) of blood pressure after 6 months of diuretic/beta-blocker or diltiazem antihypertensive therapy. Displayed as mean (SD).
[a]Age, sex, smoking, s-creatinine, BMI, diabetes mellitus and previous cardiovascular disease entered as covariates in linear model.
[b]For linear additive model, with genotype as independent and reduction of blood pressure as dependent variable.
[c]Calculated for absolute blood pressure reduction in unadjusted analyses, detecting beta = 2 mmHg per allele for SBP and beta = 1 mmHg per allele for DBP.
[d]Allele associated with higher baseline blood pressure in discovery studies.

TABLE 9. Association of rs11191548 with blood pressure reduction

				$Trend_{unadjusted}$		$Trend_{adjusted}$[a]		
	CC	CT	TT	Beta[b]	*P*	Beta[b]	*P*	Power[c]
Beta-blockers/diuretics								
Number of patients	10	244	1496					
Baseline SBP (mmHg)	176.1 (18.8)	171.5 (15.1)	172.0 (15.9)					
−ΔSBP (mmHg)	16.6 (11.3)	18.8 (18.0)	17.9 (16.3)	−0.665	0.529	−0.719	0.496	47.4%
−ΔSBP (%)	9.3 (6.0)	10.6 (10.1)	10.1 (9.0)	−0.366	0.531	−0.418	0.475	
Baseline DBP (mmHg)	106.0 (3.4)	104.1 (4.6)	103.6 (5.0)					
−ΔDBP (mmHg)	14.0 (6.4)	15.4 (8.4)	13.9 (8.4)	−1.263	0.018	−1.272	0.017	46.5%
−ΔDBP (%)	13.2 (6.0)	14.7 (7.8)	13.3 (7.8)	−1.161	0.020	−1.172	0.019	
Diltiazem								
Number of patients	16	282	1617					
Baseline SBP (mmHg)	175.8 (18.3)	172.9 (16.7)	173.4 (16.5)					
−ΔSBP (mmHg)	16.8 (14.8)	15.9 (14.8)	16.2 (16.0)	0.126	0.891	0.128	0.889	58.5%
−ΔSBP (%)	9.2 (7.4)	8.9 (8.1)	9.0 (8.7)	0.074	0.882	0.063	0.900	
Baseline DBP (mmHg)	104.3 (4.2)	103.8 (4.9)	104.1 (5.1)					
−ΔDBP (mmHg)	14.7 (11.4)	13.8 (8.4)	14.5 (7.9)	0.556	0.235	0.561	0.229	56.9%
−ΔDBP (%)	14.0 (10.6)	13.2 (7.9)	13.9 (7.4)	0.523	0.231	0.531	0.220	

Genes: *CYP17A1, AS3MT, CNNM2, NT5C2*. Main phenotype: SBP. Risk allele: T[d]. *P* value of χ^2 distribution for Hardy–Weinberg equilibrium: 0.465. Reduction (positive direction) of blood pressure after 6 months of diuretic/beta-blocker or diltiazem antihypertensive therapy. Displayed as mean (SD).
[a]Age, sex, smoking, s-creatinine, BMI, diabetes mellitus and previous cardiovascular disease entered as covariates in linear model.
[b]For linear additive model, with genotype as independent and reduction of blood pressure as dependent variable.
[c]Calculated for absolute blood pressure reduction in unadjusted analyses, detecting beta = 2 mmHg per allele for SBP and beta = 1 mmHg per allele for DBP.
[d]Allele associated with higher baseline blood pressure in discovery studies.

than participants in the diltiazem group. There were no other statistical significant differences between the BB/diuretics group and the diltiazem group (Table 1). Baseline blood pressure (mean of blood pressure levels at inclusion and randomization) according to genotype is shown in Tables 2–9.

Associations of single-nucleotide polymorphisms with blood pressure reduction

For six out of the eight SNPs included in the study (*rs16998073*, *rs1378942*, *rs3184504*, *rs1530440*, *rs16948048* and *rs17367504*), there were no significant associations with SBP or DBP reduction in neither the BB/diuretics nor the diltiazem group (Tables 2–7).

Two of the eight SNPs (*rs12946454* and *rs11191548*) showed nominal evidence of association with blood pressure reduction during the study period.

rs12946454, in relation to genes *PLCD3/ACBD4/HEXIM1/HEXIM2*, was associated with a more pronounced absolute and percentage SBP and DBP reduction in the diltiazem group, that is more unfavorable alleles resulted in a more pronounced mean SBP/DBP reduction of 1.53/0.73 mmHg per allele (*P* for trend = 0.010/0.014; Table 8). Significance remained after adjustments for covariates (*P* for trend = 0.008/0.011; Table 8). However, if multiple testing was taken into account, significance was attenuated. No significant associations were seen for *rs12946454* in the BB/diuretics group (Table 8).

rs11191548, in relation to genes *CYP17A1/AS3MT/CNNM2/NT5C2* and the strongest blood pressure-associated SNP identified thus far, was associated with a less pronounced DBP reduction in the BB/diuretics group, that is more unfavorable alleles were additionally associated with a less mean DBP reduction of 1.26 mmHg per allele in this group (*P* for trend = 0.018; Table 9). Results were consistent after covariates adjustment (*P* for trend = 0.017; Table 9). However, as for *rs12946454*, the significance was attenuated if multiple testing was taken into account. No effect on blood pressure reduction could be detected for *rs11191548* in the diltiazem group. Analyses for *rs11191548* were somewhat underpowered (Table 9).

In summary, six out of eight SNPs showed consistently negative results for association with blood pressure reduction. Two SNPs (*rs12946454* and *rs11191548*) showed nominally significant associations with blood pressure reduction; however, if multiple testing was taken into account, the significance for these two SNPs was attenuated as well (Tables 2–9).

Associations of the genetic risk score with blood pressure reduction

The genetic risk score (Score-BP) did not show any significant associations with the magnitude of SBP or DBP reduction in neither the BB/diuretics group nor the diltiazem group (Supplementary Table 2, Supplemental Digital Content 1, http://links.lww.com/HJH/A169).

DISCUSSION

In this pharmacogenetic study, we examined the impact of eight common blood pressure-associated SNPs on the degree of blood pressure reduction achieved with beta-blockers/diuretics-based or diltiazem-based antihypertensive therapy, respectively. Although the examined SNPs each explain only 0.04–0.09% of the proportion of variance in population blood pressure levels [5], their widespread distribution in the population and their relation to genes encoding possible targets for antihypertensive therapy make them particularly interesting from a pharmacogenetic point of view.

Overall, our results for pharmacogenetic interactions for the examined SNPs were negative, with six of eight SNPs showing no significant associations with blood pressure reduction in neither the beta-blockers/diuretics

nor the diltiazem group. With exception of *rs17367504*, these analyses were well powered, substantially reducing the risk of type II error (Tables 2–7). *rs17367504* is a pharmocogenetically interesting SNP, considering its adjacency to the NPPA/NPPB genes and the concurrent possible association with diuretics treatment in particular, and this SNP should be focus of further studies with better power.

Two of the examined SNPs in our study showed nominally significant associations for impact on the achieved mean blood pressure reduction in the diltiazem (*rs12946454* for SBP and DBP) and beta-blockers/diuretics (*rs11191548* for DBP) groups, respectively. However, if multiple testing was taken into account, significances for these two SNPs were attenuated and the results for these SNPs should be interpreted with great caution. *rs12946454* is located in an intron of the gene *PLCD3* which encodes one of the *Phospholipase C* enzymes. Since *Phospholipase C* is essential for calcium release in smooth muscle, thus affecting vascular tonus [19], it would seem mechanistically logical if polymorphisms in the *PLCD3* gene also affect the response of the calcium antagonist and vasodilator diltiazem. The second SNP, *rs11191548*, is located in a locus involving the gene *CYP17A1* which encodes the cytochrome P450 enzyme, *CYP17A1* (also known as p450c17). Mediating steroid 17α-hydroxylase and 17,20-lyase activity, the *CYP17A1* enzyme regulates an essential step in the production of mineralocorticoids and glucocorticoids [5]. This locus is interesting from a pharmocogenetic point of view, concerning use of both beta-blockers and diuretics. Thus, even if our results for *rs12946454* and *rs11191548* should be considered negative if multiple testing is taken into account, we do think that the unadjusted signals found combined with the possible involvement of these SNPs in genes with mechanisms that could be relevant for the use of calcium antagonists, beta-blockers and diuretics, respectively, qualifies them for further pharmacogenetic investigations.

Using a genetic risk score cumulating the individually small effects of the SNPs is a common strategy for increasing the possible outcome effect sizes in genetic studies. This is also a way of testing the clinically more relevant scenario for polygenic disease, in which individuals are affected not by genes one by one but by a combined genetic susceptibility. Our results for the genetic risk score (Score-BP) was consistently negative and we had a power of more than 90% in all these analyses (Supplementary Table 2, Supplemental Digital Content 1, http://links.lww.com/HJH/A169). A likely explanation for the absence of association with treatment response might be that the eight SNPs of Score-BP involve many different potential mechanisms, possibly resulting in opposite directions on treatment response for a given antihypertensive. Thus, the heterogeneity of SNPs involved might well complicate the task of finding specific treatment associations for a combined genetic score like Score-BP.

The International Consortium for Blood Pressure Genome-Wide Association Studies (ICBP-GWAS) recently published a large meta-analysis of GWAS data that identified 29 independent genetic variants (16 of which were novel discoveries) associated with SBP and/or DBP [7]. Noteworthy, the ICBP-GWAS did not support the association with blood pressure for the previously strongly associated SNP *rs12946454*. Nevertheless, this SNP might still be an interesting target for further pharmacogenetic studies, as discussed above.

Accounting for the fact that many loci from the ICBP-GWAS study involves potential biological pathways, such as natriuretic peptides and their metabolism, nitric oxide signaling and other potentially vasodilatatory pathways and adrenomedullin [7], an obvious follow-up to our current study would be to test whether any of these variants could influence blood pressure treatment response. Furthermore, a recent GWAS that looked specifically at gene regions encoding antihypertensive drug targets found associations with population blood pressure for SNPs within two loci for beta-adrenergic receptors and the RAAS system, respectively [20]. Although these and nearby loci have already been subject to some previous pharmacogenetic studies (*rs1801253* in ADRB1 has been found to affect response to beta-blocker therapy [8,13]), these loci should naturally be considered in future pharmacogenetic studies, as well.

Strengths and limitations

Our current study has several strengths. First, this study provides novelty in that it is, as far as we know, the first study to investigate pharmacogenetic aspects of these eight widely spread blood pressure-associated SNPs. Second, our study cohort, involving the Swedish part of the NORDIL cohort, provides a unique material for pharmacogenetic studies; the use of monotherapy for 6 months for a majority of patients enhances the chance of finding specific single-drug pharmacogenetic interactions and the ethnically homogenous cohort involved decreases the risk of misinterpretating ethnicity-related differences in treatment response. Third, as discussed, our analyses, with a few exceptions, were generally well powered.

Our study naturally shows a number of limitations, as well. Six months is a relatively short follow-up time, especially if considering hard endpoints (such as stroke and myocardial infarction). Furthermore, we did not have data on specific drug doses and were thus unable to account for differences in doses between individuals with various genotypes. As at least one of the included SNPs (*rs3184504* in perfect correlation with *rs653178*) has been shown to additionally associate with myocardial infarction [21], hard endpoint pharmacogenetic analyses for the SNPs would definitely add valuable insights to this study. However, accounting for the fact that there were only approximately a total of 100 cardiovascular events during the first 6 months of the NORDIL study [16], power for these analyses are very limited. Extending the study period to more than 6 months would mean that we would have to include a large proportion of patients treated with multiple simultaneous antihypertensive medications, something that would considerably lower the possibility of detecting specific gene treatment interactions.

A second limit of our study could be attributed to the fact that we assumed an additive model for all of the included SNPs. Although this seems to be the case regarding their relation to population blood pressure levels, we naturally

cannot include that some of the SNPs have either a dominant or recessive model for impact on blood pressure treatment response.

Third, although beta-blockers, diuretics and calcium channel blockers are antihypertensive medications widely used today, pharmacogenetic analyses concerning the use of ACE inhibitors and angiotensin II receptor blockers were not included in our study. At the time of the NORDIL study, these drugs were not as extensively used as today, and this group of antihypertensive medication was thus not included from an early stage. As ACE inhibitors or angiotensin II receptor blockers today are first choice for many hypertensive patients, pharmacogenetic analyses of the eight SNPs relating to use of these drugs would naturally have added valuable information to the current study.

Finally, lack of compliance is a well known problem in antihypertensive treatment, which obviously could reduce power to detect pharmacogenetic effects. Although we cannot exclude a certain degree of lack of compliance also in our study, the problem is likely to be less pronounced in a clinical trial than in clinical practice.

In conclusion, this is, as far as we are aware, the first study to investigate the impact on pharmacological blood pressure treatment for these eight common blood pressure-associated SNPs. The major conclusion that can be drawn from the study is that for a majority of the eight SNPs, there are probably no important pharmacogenetic interactions of blood pressure reduction for beta-blockers, diuretics and diltiazem. Whether or not the nominally significant associations seen for *rs12946454* and *rs11191548* in this study are true remains to be addressed in further studies. However, even if these two loci turn out to be true signals, the effect size on blood pressure treatment response is likely to be small. Despite this, we believe it will be important to attempt replication of these SNPs as well as to test the new blood pressure-associated variants from the most recent GWAS [7,20] as even small differences in treatment response are likely to affect cardiovascular prognosis [18]. Ultimately, the desirable goal would be the possibility of applying novel pharmacogenetic approaches in order to individualize and thus further improve treatment of essential hypertension in the population.

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Conflicts of interest

There are no conflicts of interest.

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Reviewers' Summary Evaluations

Reviewer 1

This study investigates whether SNPs recently identified in GWAS on blood pressure levels might have an effect on BP reduction by antihypertensive agents. The study was conducted in 3863 Swedish subjects participating in the NORDIL antihypertensive cohort.

The study is well-designed and appropriately powered to detect modest BP reduction. The results are mainly negative if multiple testing is taken into account. Another limitation is the relatively short follow-up duration (6 months).

Reviewer 2

The current pharmacogenetic study had enough power to give reasonable results also for small blood pressure reductions, depending on the patients' allele carrier status. With respect to the effect sizes, (I) drug dose-related associations and (II) compound "unfavourable" allele carriage (at least two, depending on individual allele frequencies) would have been interesting to study, but certainly at the expense of the study's power due to smaller subgroups. The overall negative result of the study can be interpreted as such: it is unlikely that the studied SNPs, retrieved from genome-wide association study results, have a clinically important pharmacogenetic effect.

Paper IV

Smoking modifies the associated increased risk of future cardiovascular disease by genetic variation on chromosome 9p21

Authors: Viktor Hamrefors, MD * †; Bo Hedblad, MD, PhD *; George Hindy, MD *; J Gustav Smith, MD, PhD * ‡ § ‖ ; Peter Almgren, MSc *; Gunnar Engström MD, PhD *; Marketa Sjögren, PhD *; Klas Gränsbo, MD * ¶; Marju Orho-Melander, PhD * and Olle Melander, MD, PhD * ¶

Affiliations: * Department of Clinical Sciences, Faculty of Medicine, Lund University, Malmö, Sweden
† Clinical Physiology and Nuclear Medicine Unit, Skåne University Hospital, Malmö, Sweden
‡ Department of Cardiology, Faculty of Medicine, Lund University, Lund, Sweden
§ Department of Cardiology, Skåne University Hospital, Lund, Sweden
‖ Program in Medical and Population Genetics, Broad Institute of Harvard and MIT, Cambridge, Massachusetts, USA
¶ Department of internal medicine, Skåne University Hospital, Malmö, Sweden

Address for correspondence:
Viktor Hamrefors
Clinical Research Centre, Lund University
Jan Waldenströms Gata 35, Building 91, Floor 12
Skåne University Hospital Malmö
SE-205 02 Malmö, Sweden
E-mail: Viktor.Hamrefors@med.lu.se / Phone: +46 703996529 / Fax: +46 40391222

ABSTRACT

Aims

Genetic predisposition for cardiovascular disease (CVD) is likely to be modified by environmental exposures. We tested if the associated risk of CVD and CVD-mortality by the single nucleotide polymorphism rs4977574 on chromosome 9p21 is modified by life-style factors.

Methods and results

A total of 24944 middle-aged subjects (62% females) from the population-based Malmö-Diet-and-Cancer-Cohort were genotyped. Smoking, education and physical activity-levels were recorded. Subjects were followed for 15 years for incidence of coronary artery disease (CAD; N=2309), ischemic stroke (N=1253) and CVD-mortality (N=1156). Multiplicative interactions between rs4977574 and life-style factors on endpoints were tested in Cox-regression-models.

We observed an interaction between rs4977574 and smoking on incident CAD (P=0.035) and CVD-mortality (P=0.012). The hazard ratios (HR) per risk allele of rs4977574 were highest in never smokers (N=9642) for CAD (HR=1.26; 95% CI 1.13-1.40; P<0.001) and for CVD-mortality (HR=1.40; 95 % CI 1.20-1.63; P<0.001), whereas the risk increase by rs4977574 was attenuated in current smokers (N=7000) for both CAD (HR=1.05; 95%CI 0.95-1.16; P=0.326) and CVD-mortality (HR=1.08; 95%CI 0.94-1.23; P=0.270). A meta-analysis supported the finding that the associated increased risk of CAD by the risk-allele was attenuated in smokers. Neither education nor physical activity-levels modified the associated risk of CAD, ischemic stroke and CVD mortality conferred by rs4977574.

Conclusion

Smoking may modify the associated risk of CAD and CVD-mortality conferred by genetic variation on chromosome 9p21. Whether the observed attenuation of the genetic risk reflects a pathophysiological mechanism or is a result of smoking being such a strong risk-factor that it may eliminate the associated genetic effect, requires further investigation.

INTRODUCTION

Family history is a well recognized important risk factor for cardiovascular disease (CVD) [1]. Similar to most other common diseases, the inheritance of CVD is multifactorial, with genetic and environmental factors and interactions between them affecting the risk [2].

Genome wide association studies (GWAS) have been successful in identifying common genetic factors that associate with multifactorial diseases including CVD [3, 4]. Single nucleotide polymorphisms (SNPs) on chromosome 9p21 have been found to strongly associate with coronary artery disease (CAD) and myocardial infarction (MI) in the population, with risk allele frequencies of around 50 % in populations of European ancestry and odds ratios for CAD and MI of ~ 1.30 per allele [5-7]. The association of these SNPs with CAD and MI has been confirmed in numerous populations of European ancestry [4, 8, 9] and in other ethnicities [10-11]. Beyond CAD and MI, the same SNPs on Chromosome 9p21 associate with other CVD manifestations, including ischemic stroke [12]. Importantly, the chromosome 9p21 SNPs have been found not to associate with any of the traditional cardiovascular risk factors [5-7].

It is likely that the associated effect of genetic factors on CVD is modified by different environmental exposures [13]. Today, a number of modifiable environmental and life-style related risk factors show consistent evidence as risk factors for CVD. These include tobacco smoking, a low socioeconomic status, often measured as a low educational level, and physical inactivity [14]. Accounting for the complex nature of CVD, knowledge of how such life-style related risk factors may interact with genetic susceptibility variants on CVD risk is important for CVD risk prediction and prevention [15, 16]. However, very little is known about such putative gene-environment interactions.

In this study we tested whether the associated increased risk of future CVD and CVD-mortality by the common CVD risk SNP on chromosome 9p21 (rs4977574) is modified by life-style risk factors including smoking, educational level and physical activity level. We tested this hypothesis in 24944 middle aged Swedish subjects from the Malmö Diet and Cancer Cohort Study (MDCS), with around 15 years follow-up.

METHODS

Study population

MDCS is a prospective population-based cohort study that initially recruited a total of 30447 subjects during the years 1991-1996. Subjects born between 1923 and 1950 living in the city of Malmö in Sweden were eligible for participation [17]. At baseline, participants underwent measurement of anthropometric variables and blood pressure, and provided blood samples. Subjects were also asked to complete a self-administered questionnaire of health and life-style related factors, including current and previous disease, medication, tobacco smoking, education and physical activity.

DNA was extracted and successfully genotyped for the rs4977574 in 27885 subjects in MDCS. After excluding subjects with previous CVD at baseline (i.e. a history of MI, coronary-artery-by-pass graft surgery (CABG), percutaneous coronary intervention (PCI), or stroke) a total of 26855 subjects remained. Of these, we selected subjects that had complete baseline data for all variables and covariates of interest including smoking status, educational level, physical activity, systolic blood pressure, use of antihypertensive medication and body mass index (BMI), leaving us with a total of 24944 subjects for the current study (Figure 1).

The study was approved by the Regional Ethical Review Board in Lund at Lund University, Sweden. Written informed consent was obtained from all participants.

Assessment of end-points

The three primary endpoints of our study were CAD, ischemic stroke and CVD-mortality (defined below). The endpoints were identified through linkage of the 10-digit personal identification number of each Swedish citizen with four registers: the Swedish Hospital Discharge Register, Swedish Coronary Angiography and Angioplasty Registry (SCAAR), the Stroke Register of Malmö and the Swedish Cause of Death Register. The registers have been previously described and validated for classification of outcomes [18-21]. Follow-up for the study extended to June 30, 2009.

CAD was defined as fatal or non-fatal MI, death from ischemic heart disease, CABG or PCI. MI was defined on the basis of International Classification of Diseases 9th and 10th Revisions

(ICD9 and ICD10) codes 410 and I21, respectively. Death due to ischemic heart disease was defined on the basis of codes 412 and 414 (ICD9) or I22–I23 and I25 (ICD10). CABG was identified from national Swedish classification systems of surgical procedures, the KKÅ system from 1963 until 1989 and the Op6 system since then. CABG was defined as a procedure code of 3065, 3066, 3068, 3080, 3092, 3105, 3127, 3158 (Op6) or FN (KKÅ97). PCI was defined based on the operation codes FNG05 and FNG02. Fatal or nonfatal stroke was assessed using codes 430, 431, 434 and 436 (ICD9) and I60, I61, I63, and I64 (ICD10). Hemorrhagic strokes were however censored in the analyses, meaning that only cerebral infarctions (code 434 for ICD9 / I63 for ICD 10) were included in the endpoint definition. CVD-mortality was defined as underlying cause of death classified as ICD-9 diagnoses 390-459 and ICD-10 diagnoses I00-199.

Genotyping and definition of the independent variables at baseline

In MDCS, DNA was extracted from frozen granulocyte or buffy coat samples from blood from the baseline examination using QIAamp 96 spin blood kits (QIAGEN, VWR, Gaithersburg, MD, USA). The rs4977574 SNP (A/G) on chromosome 9p21 was genotyped using "Assay by design" TaqMan probes with a real time polymerase chain reaction assay on an ABI-7900HT equipment (Applied Biosystems, Foster City, CA) according to the manufacturer's standard protocols. 20% of the samples were run in duplicate as part of the quality control process and the concordance was > 99.9%. The number of rs4977574 risk alleles (G) for each subject was coded as a linear variable assuming an additive effect.

The status of smoking was self-reported and coded as 0 = never, 1= former or 2 = current (i.e. any smoking within the past year) in a categorical variable. Passive smoking was defined as exposure to smoking either at home ("Do the persons you live with smoke indoors, or have they done so previously?") or at work ("Do you regularly stay in places of work [apart from your home] where people smoke, or have you previously been staying in such places regularly?") and was coded as a dichotomous variable. Education was defined as the self-reported highest level of education and coded as a six-graded categorical variable (0 = did not complete elementary school, 1 = elementary school (6-8 yrs), 2 = junior secondary school (9-10 yrs), 3 = education at advanced level (12 yrs); 4 = at least one additional year, 5 = university degree). For physical activity the information reported by the study participants for leisure-time physical activity level during the preceding year was used. A summary score of all physical activities was obtained by using intensity factors for each activity combined with

information on the time spent on the activity. This physical activity (PA)-score has previously been described in detail and it has been validated with an accelerometer monitor in a random sample of 369 subjects in MDCS [22].

Statistics

Main effects as well as interactions were all tested in multivariable proportional-hazards models using Cox regression analysis to test associations between the independent variables and time to the first event of each end-point. The proportional-hazards assumption was confirmed by visual inspection of survival curves.

Evidence of multiplicative interaction between the number of rs4977574 risk alleles and smoking, educational level and physical activity on the end-points was tested by constructing Cox regression models that included the respective multiplicative interaction terms (rs4977574 x smoking status; rs4977574 x educational level; rs4977574 x quintiles of physical activity score) in addition to the main effect terms. The likelihood ratio (LR) tests were performed comparing model fit with and without the interaction terms in order to test for evidence of significant interaction. We compared the fit of simple models adjusted for age and sex only, as well as models including additional covariates BMI, systolic blood pressure and use of antihypertensive medication in addition to all the three main effect terms. A P-value of less than 0.05 was considered significant.

For incident CAD, we also performed a meta-analysis including additional data from a recent report of interaction analyses between chromosome 9p21 variation and various environmental factors in 9877 subjects from the Atherosclerosis Risk in Communities (ARIC) Study [23]. The meta-analysis was performed on the study level, by pooling the effect estimates for the associated risk of incident CAD by the chromosome 9p21 risk locus in smokers and non-smokers respectively.

Statistical analyses were performed using SPSS Statistics 19.0-21.0 (SPSS Inc., Chicago, IL, USA) and Stata 11.0 (StataCorp LP, College Station, Texas, USA).

RESULTS

Study population and incidence of CVD during follow-up

Characteristics of the study population according to genotype are shown in Table 1. For the incident end-points of CAD (n=2309), ischemic stroke (n=1253) and CVD-mortality (n=1156) the subjects were followed for a median time of 14.5, 14.6 and 14.7 years, respectively.

Subjects in MDCS excluded from the current study (N = 5503) because of incomplete genotype data, covariate data and/or previous CVD at baseline (Figure 1) generally had a higher burden of cardiovascular risk factors (more likely to be males, less likely to be never-smokers, slightly higher systolic blood pressure and BMI) compared to included subjects. In accordance with these observations, there was also a higher incidence of end points in excluded subjects (Table S1A in File S1). Most of the higher CVD risk burden in excluded subjects could be attributed to subjects with previous CVD at baseline. Compared to included subjects and to excluded subjects without previous CVD, these subjects were much more likely to be men and former smokers; they were older, had higher blood pressure despite an extensive use of antihypertensive medication and they had higher BMI. As expected, the incidence of CAD, ischemic stroke and CVD death was considerably higher in subjects with previous CVD. (Table S1B in File S1).

Main effects of the independent variables on CVD incidence

In Cox regression models adjusted for age and sex the rs4977574 associated with all three end-points with hazard ratios of 1.12-1.16 per risk allele. Current smoking showed a strong association with all three end-points, whereas former smoking was associated with incident CAD and CVD-mortality, but not with incident ischemic stroke. Level of education was associated with incident ischemic stroke, and a significant trend for association also with incident CAD and CVD-mortality could be observed across education categories. Level of physical activity showed non-linear associations with all end-points (Table 2). Results for main effects were similar in the multivariate adjusted models (Table S2 in File S1).

Interaction between Chromosome 9p21 risk alleles and smoking status

We observed a significant interaction between the number of rs4977574 risk alleles and smoking status on incidence of CAD ($P_{interaction}$ = 0.035) and CVD-mortality ($P_{interaction}$ = 0.012). These interactions remained significant in the fully adjusted models for both incident CAD ($P_{interaction}$ = 0.035) and CVD-mortality ($P_{interaction}$ = 0.029). No interaction was observed between the rs4977574 risk allele and smoking status on incident ischemic stroke ($P_{interaction}$ = 0.702).

As we found significant interactions between rs4977574 and smoking status on incident CAD and CVD-mortality, we tested the associated effect of rs4977574 on these two endpoints according to smoking status (Figures 2-3). For incident CAD, the associated effect of rs4977574 was found to be highly significant in never-smokers (HR 1.26 per risk allele; 95% CI 1.13-1.40; P<0.001) and former smokers (HR = 1.20 per risk allele; 95% CI 1.08-1.32; P<0.001), whereas this associated effect was attenuated and not significant in current smokers (HR = 1.05 per risk allele; 95% CI 0.95-1.16; P=0.326) (Table 3, Figure 2). Since we had additional data also on the exposure to passive smoking for 22049 subjects we performed stratification within the groups of never and former smokers according to this variable. In never smokers, the significant associated effect of rs4977574 risk alleles on incident CAD was attenuated among subjects that reported passive exposure to smoking (HR 1.14 per risk allele; 95% CI 0.99-1.32; P=0.068), contrasting to subjects that were not exposed to passive smoking, in whom rs4977574 showed a high hazard ratio per allele (HR 1.56 per risk allele; 95% CI 1.29-1.88; P<0.001). The results were similar in the adjusted models and were similar in both sexes (Tables S3A-B in File S1). In current smokers we had information also on baseline "pack-years" (number of cigarette packs per day x years of smoking; N = 6256) and cigarettes smoked per day (N = 6311). Thus, within the group of current smokers we additionally stratified for pack-years and number of daily cigarettes in order to test if there was a suggestive dose-relationship for the modification of the genetic effect by smoking. There was however no such evident pattern for pack-years or number of daily cigarettes further modifying the chromosome 9p21 genetic association for incident CAD. (Table 3)

For CVD-mortality (Figure 3), the associated effect of rs4977574 was found to be highly significant only in the group of never smokers (HR 1.40 per risk allele; 95 % CI 1.20-1.63;P<0.001), whereas the associated effect was attenuated and not significant among both current (HR 1.08 per risk allele; 95 % CI 0.94-1.23; P=0.270) and former smokers (HR 1.05

per risk allele; 95 % CI 0.91-1.21; P=0.525) (Table 4, Figure 3). The highest HRs were observed in never smokers that were not exposed to passive smoking. Including covariates in the models did not change the results which were similar in both sexes (Tables S4A-B in File S1). In contrast to the results for CAD, there was a suggestive pattern for a dose-response association modifying the genetic effect within current smokers, as the genetic effect seemed to be attenuated to a larger extent in subjects with more extensive smoking habits. (Table 4).

The fact that the associated risk of CAD and CVD-mortality by rs4977574 was attenuated in current smokers provided the rationale for studying also if the risk of incident events associated with smoking would be less in risk allele carriers. As expected, smoking was observed to be a strong risk factor regardless of genotype. However, we did observe a pattern of smoking having a less effect on risk of incident CAD and CVD-mortality in rs4977574 risk allele carriers compared to non-risk allele carriers (Tables 5-6).

Meta-analysis: Risk of incident CAD by Chromosome 9p21 stratified by smoking status

In the meta-analysis of MDCS and ARIC, the associated risk of incident CAD by the chromosome 9p21 locus was found to be attenuated in smokers (Overall HR per allele = 1.07; 95% CI 0.99-1.15). Contrary, when pooling the results from MDCS and ARIC in non-smokers (i.e. never and former smokers) there was an increased risk of incident CAD by chromosome 9p21 (Overall HR per allele = 1.23; 95% CI 1.16-1.30) (Figure 4).

Interaction between Chromosome 9p21 risk alleles, education and physical activity

No interactions were observed between rs4977574 and educational level or physical activity on incident CAD (P = 0.082 and P = 0.457, respectively), incident ischemic Stroke (P = 0.876 and P = 0.251, respectively) or CVD-mortality (P = 0.681 and P = 0.286, respectively). Results were similar also after adjusting the models for putative confounders (Table S5 in File S1).

DISCUSSION

In this population-based prospective study we evaluated gene-environment interactions for one of the strongest reported cardiovascular risk SNPs rs4977574 on chromosome 9p21. We report a significant interaction with smoking for incidence of CAD and CVD-mortality, with similar results in both men and women.

During the approximately 15 years of follow-up time we observed a strong association between rs4977574 and risk of incident CAD in non-smokers, whereas the significance of rs4977574 was fully attenuated in current smokers (Figure 2). These findings were similar in both sexes (Tables S3A-B in file S1). Interestingly, when stratifying additionally for passive smoking within the group of non-smokers we could observe that rs4977574 had the highest hazard ratio for incident CAD in never smokers that were never exposed to passive smoking (Table 3). There was however no evidence for a dose-response relationship for the genetic effect modification by smoking within the group of current smokers (Table 3). The finding of an attenuation of the associated increased risk of CAD by the risk locus on chromosome 9p21 in smokers was further supported by a meta-analysis including recent results from ARIC [23] (Figure 4).

For CVD-mortality the results were similar to the result for CAD, showing that in relative terms the risk influence of rs4977574 was consistently highest in never-smokers who had no previous exposure to passive smoking at baseline. However, for CVD-mortality, attenuation of the genetic effect was observed also in former smokers. Furthermore there was a suggestive pattern of a dose-response genetic effect modification, as the genetic effect for chromosome 9p21 seemed to be more markedly attenuated in subjects who smoked more. (Table 4). Naturally, a similar dose-response-test would have been very interesting to perform within the group of former smokers, however unfortunately, baseline pack-year-data and daily cigarettes consumption was available only in current smokers.

The interactions between smoking and the chromosome 9p21 CVD risk locus on CAD and CVD-mortality were evident also from a reverse point of view. That is, although smoking was a strong risk factor regardless of genotype, we observed a pattern of the risk conferred by

smoking being lower in the risk allele carriers (Tables 5-6), with similar results in both sexes (Tables S6-S7 in File S1).

The reported effect size for the chromosome 9p21 risk locus on CAD and MI is larger for early-onset than for later onset disease [5]. A question that arose from the current study results for CAD was thus if the observed modification of the genetic effect on CAD by smoking could be further influenced by age. In order to address this question we stratified the group of non-smokers / smokers according to age at baseline. These analyses did reveal a pattern of smoking seeming to be a more evident modifier of the genetic effect of rs4977574 in older subjects, compared to younger subjects (Table 7). A possible explanation is that the chromosome 9p21 CVD risk locus might confer a substantial relative risk for CAD in the low-risk group of younger subjects even in the presence of a concurrent strong risk factor in the form of smoking. That is – younger subjects are at such comparably low risk for CAD that the weaker genetic effects would be preserved even if they smoke. Contrary, in older subjects who by means of their age (and the risk factors that come with age) are already at much higher risk for CAD, the addition of yet another strong risk such as smoking may diminish the relative influence of genetic factors on risk of CAD to larger extent.

Even though the meta analysis including the results from ARIC further supports the finding that the increased risk of incident CAD by the chromsome 9p21 CVD risk locus was attenuated in smokers, the fact that the ARIC-based study in itself did not reveal a significant interaction between chromosome 9p21 and smoking ($P_{interaction} = 0.14$) is worth discussing. The ARIC-study examined the SNP rs10757274 which is in strong LD with rs4977574 ($r^2 = 0.94$ in data from 1000 genomes pilot 1; value obtained from SNP Annotation and proxy search (SNAP) from Broad Institute; http://www.broadinstitute.org/mpg/snap/) and the endpoints in both studies were similar. We suggest two possible main explanations for the difference of the results. First, the ARIC-study included less subjects (approximately 10 000 versus 25 000 subjects) and even though the mean follow-up time in the ARIC-study was longer than in our study (17 vs 14 years) there were less CHD/CAD events in the ARIC-study (1653 versus 2309 events). The absence of significance for the interaction term in the ARIC-study ($P = 0.14$) might thus well be a matter of power. Second, subjects from the ARIC-study were generally younger at baseline than subjects from MDCS included in our study. Based on the above reasoning of smoking being a more evident modifier of the genetic effect by

chromosome 9p21 in older subjects, this may be an additional possible explanation for the lack of significant interaction in the ARIC-study.

The causes behind the observed interaction between chromosome 9p21 and smoking on risk of CVD from our results could be looked at from both a pathophysiological and genetic-epidemiological point of view. From a pathophysiological view the chromosome 9p21 CVD risk locus has been consistently found not to associate with conventional cardiovascular risk factors. Molecular studies have found that the locus involves a specific non-coding RNA, termed antisense non-coding RNA in the INK4 locus (ANRIL), which has been suggested to be an epigenetic regulator of other genes potentially involved in CVD pathophysiology [24]. To the best of our knowledge, there are no reported associations between ANRIL and smoking or the known pathophysiological pathways of smoking. However, considering that smoking is likely to affect the risk of CVD via multiple pathways, one could speculate that smoking and the chromosome 9p21 risk locus (and ANRIL) might act via at least partially same (yet unknown) pathway(s) on CVD, and that smoking in itself would be sufficient cause for disease.

The results could also be looked from a more genetic-epidemiological view. As outlined in the previous discussion of the influence of age on genetic effect modification, smoking is in itself a very strong risk factor for CVD - and smokers constitute a high risk group for all cardiovascular events. It is thus tempting to speculate that the relative influence of genetic factors on CVD risk could be attenuated in such a high risk group. Among individuals with low conventional risk of CVD, the relative effect of genetic factors might instead be accentuated. This argument is supported by the higher effect estimates of the chromosome 9p21 CVD risk locus for early onset cases of MI [5], in which conventional cardiovascular risk factors are usually less prominent than in later onset-cases of MI and CAD. Also, our age-stratification analysis revealing a suggestive pattern of a more evident genetic effect attenuation by smoking in older (higher risk) subjects compared to younger (lower risk) subjects support this hypothesis, as already discussed.

Practically, our results and the reasoning above may indicate that genetic screening for CVD could in fact be valuable in younger subjects and in other low-risk groups where conventional cardiovascular risk factors are not as prominent. This hypothesis is supported by studies showing no or little value over conventional risk factors for the chromosome 9p21 and other

CVD risk SNPs in predicting CVD in the general population [25, 26], whereas modestly improved CVD risk prediction has been reported in low to intermediate risk groups [25, 27]. Further prospective studies investigating the value of chromosome 9p21 risk alleles as predictors of CVD risk in groups with few, or successfully treated conventional risk factors, are warranted.

We are aware of that our study has a number of limitations that deserve to be mentioned. First, the MDCS cohort consists of subjects mainly of European ancestry, thus limiting the generalization of the results to populations of non-European ancestry. Also, community based cohorts involving self reported data might suffer from an uncertainty concerning the validity of the data, although this problem should be less in prospective studies than in case-control studies. Furthermore, we were not able to evaluate how a change of exposure to the environmental risk factors during the follow-up period may have affected the results, for example how the risk conferred by rs4977574 was changed in subjects that quitted smoking during the follow-up. Finally, from a statistical point of view, if strict Bonferroni-correction is applied in order to correct for multiple interaction tests, the significance of the interaction between rs4977574 and smoking on incident CAD ($P = 0.035$) would be attenuated. However, we do think that the conclusive results in the subsequent stratification as well as the results from the meta-analysis still provide adequate evidence for considering an interaction between and chromosome 9p21 and smoking on risk of CAD to be likely.

Conclusion

In this large prospective gene-environment interaction study we observed that smoking attenuated the associated increased risk of incident CAD and CVD-mortality by rs4977574 on chromosome 9p21. A meta-analysis of 35 000 subjects further supported the finding that the associated increased risk of CAD by the risk locus on chromosome 9p21 was attenuated in smokers. Whether a specific pathophysiological mechanism can explain these findings remains to be explored. The results raise hypotheses regarding strategies for genetic cardiovascular risk prediction in the population, suggesting that genetic factors may have a relatively larger influence on CVD risk in conventionally assessed low-risk groups compared to high risk groups.

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TABLES

TABLE 1: Population characteristics

	Chromosome 9p21 rs4977574 genotype		
	0 risk alleles A/A	*1 risk allele A/G*	*2 risk alleles G/G*
Total subjects, n (%)	7609	12311	5024
Men	2881 (37.9)	4682 (38.0)	1892 (37.7)
Women	4728 (62.1)	7629 (62.0)	3132 (62.3)
Age, years	58.0 (7.7)	58.0 (7.6)	57.9 (7.7)
Smoking status, n (%)			
Never smokers	2896 (38.1)	4737 (38.5)	2010 (40.0)
Former smokers	2537 (33.3)	4105 (33.3)	1658 (33.0)
Current smokers	2176 (28.6)	3469 (28.2)	1356 (27.0)
Highest level of education, n (%)			
No elementary school	57 (0.7)	95 (0.8)	51 (1.0)
Elementary school (6-8 yrs)	3084 (40.5)	5024 (40.8)	2015 (40.1)
Junior Sec. School (9-10 yrs)	2015 (26.5)	3283 (26.7)	1260 (25.1)
Advanced level (12 yrs)	656 (8.6)	1084 (8.8)	497 (9.9)
At least one additional year	671 (8.8)	1086 (8.8)	457 (9.1)
University degree	1126 (14.8)	1739 (14.1)	744 (14.8)
Low physical activity, n (%) *	1492 (19.6)	2355 (19.1)	1011 (20.1)
Systolic blood pressure, mmHg	141.0 (20.0)	141.0 (20.0)	141.4 (20.2)
Use of AHT, n (%)	1321 (17.4)	2023 (16.4)	821 (16.3)
BMI, kg/m^2	25.8 (4.0)	25.7 (4.0)	25.7 (4.0)
Incidence of events during follow-up			
CAD, events (events/1000 p-ys)	633 (6.0)	1134 (6.7)	542 (7.9)
Ischemic Stroke, events (events/1000 p-ys)	355 (3.3)	609 (3.5)	289 (4.1)
Cardiovascular mortality, events (events/1000 p-ys)	310 (2.8)	586 (3.4)	260 (3.6)

Mean (SD) if not stated other.
p-ys= person-years
* Defined as the lowest quintile of the Physical Activity score in MDCS.

TABLE 2: Main effects

	CAD HR (95 % CI)	Ischemic Stroke HR (95 % CI)	Cardiovascular mortality HR (95 % CI)
rs4977574, per allele	1.16 (1.09-1.23)	1.12 (1.04-1.22)	1.14 (1.05-1.24)
Smoking status*			
Former smoker	1.21 (1.09-1.34)	1.02 (0.89-1.17)	1.27 (1.09-1.48)
Current smoker	2.01 (1.8-2.23)	1.65 (1.44-1.89)	2.67 (2.31-3.09)
Highest education*			
Elementary school (6-8 yrs)	0.97 (0.63-1.50)	0.53 (0.34-0.82)	0.82 (0.46-1.45)
Junior Sec. School (9-10 yrs)	0.81 (0.52-1.25)	0.45 (0.29-0.71)	0.68 (0.38-1.22)
Advanced level (12 yrs)	0.75 (0.48-1.17)	0.38 (0.23-0.62)	0.58 (0.32-1.07)
At least one additional year	0.75 (0.48-1.18)	0.40 (0.24-0.64)	0.54 (0.29-0.99)
University degree	0.63 (0.40-0.98)	0.35 (0.22-0.57)	0.55 (0.30-1.00)
P for trend	*<0.001*	*<0.001*	*<0.001*
Quintiles of PA score*			
Q2	0.68 (0.60-0.78)	0.70 (0.59-0.83)	0.62 (0.52-0.75)
Q3	0.70 (0.62-0.80)	0.70 (0.59-0.83)	0.64 (0.54-0.77)
Q4	0.73 (0.64-0.83)	0.63 (0.53-0.75)	0.63 (0.53-0.75)
Q5	0.75 (0.67-0.85)	0.66 (0.56-0.78)	0.61 (0.51-0.72)

Adjusted for age and sex.
PA score = Physical Activity Score.
* Hazard ratio (HR) in relation to the first category in the categorical variables (never smokers, "did not complete elementary school", and Q1 of PA-score respectively)

TABLE 3: Risk of incident CAD by rs4977574 stratified by smoking status

	Events (total cases)	rs4977574 HR per allele	95 % CI	P-value
Never smokers	**675 (9642)**	**1.26**	**1.13-1.40**	**<0.001**
No passive smoking	220 (3339)	1.56	1.29-1.88	<0.001
Passive smoking	379 (5069)	1.14	0.99-1.32	0.068
Former smokers	**814 (8300)**	**1.20**	**1.08-1.32**	**<0.001**
No passive smoking	177 (2146)	1.30	1.05-1.60	0.015
Passive Smoking	528 (5221)	1.19	1.06-1.35	0.004
Current smokers	**820 (7000)**	**1.05**	**0.95-1.16**	**0.326**
Pack-years < median	298 (3090)	1.05	0.89-1.23	0.572
Pack-years ≥ median	407 (3165)	1.02	0.89-1.18	0.751
Daily Cigs < median	321 (3057)	0.97	0.83-1.13	0.704
Daily Cigs ≥ median	390 (3253)	1.08	0.93-1.24	0.317

Adjusted for age and sex
Daily cigs = number of daily cigarettes

TABLE 4: CVD-mortality by rs4977574 stratified by smoking status

	Events (total cases)	rs4977574 HR per allele	95 % CI	P-value
Never smokers	**327 (9642)**	**1.40**	**1.20-1.63**	**<0.001**
No passive smoking	113 (3339)	1.78	1.37-2.32	<0.001
Passive smoking	174 (5070)	1.27	1.03-1.57	0.025
Former smokers	**383 (8297)**	**1.05**	**0.91-1.21**	**0.525**
No passive smoking	88 (2145)	1.38	1.02-1.85	0.034
Passive smoking	247 (5218)	0.96	0.81-1.15	0.676
Current smokers	**446 (7000)**	**1.08**	**0.94-1.23**	**0.270**
Pack-years < median	154 (3089)	1.20	0.96-1.50	0.11
Pack-years ≥ median	242 (3165)	1.00	0.84-1.21	0.972
Daily cigs < median	179 (3057)	1.14	0.93-1.41	0.214
Daily cigs ≥ median	218 (3252)	1.03	0.85-1.25	0.775

Adjusted for age and sex
Daily cigs = number of daily cigarettes

TABLE 5: Smoking as a risk factor for incident CAD, stratified by number of rs4977574 risk alleles

rs4977574 risk alleles	Events (total cases)	Former smoker HR (95 % CI)	P	Current smoker HR (95% CI)	P
0	633 (7608)	1.24 (1.01-1.52)	0.038	2.21 (1.81-2.70)	<0.001
1	1134 (12309)	1.24 (1.07-1.45)	0.005	2.21 (1.90-2.56)	<0.001
2	542 (5024)	1.12 (0.92-1.38)	0.265	1.48 (1.19-1.84)	<0.001

Adjusted for age and sex

TABLE 6: Smoking as a risk factor for CVD-mortality, stratified by number of rs4977574 risk alleles

rs4977574 risk alleles	Events (total cases)	Former smoker HR (95 % CI)	P	Current smoker HR (95% CI)	P
0	310 (7604)	1.76 (1.30-2.38)	<0.001	3.35 (2.48-4.52)	<0.001
1	586 (12309)	1.20 (0.96-1.48)	0.106	2.77 (2.26-3.39)	<0.001
2	260 (5024)	1.04 (0.77-1.42)	0.781	2.00 (1.48-2.70)	<0.001

Adjusted for age and sex

TABLE 7: Risk of incident CAD by rs4977574 stratified by smoking status and age

	Events (total cases)	rs4977574 HR per allele	95 % CI	P-value
Non-smokers	**1489 (17942)**	**1.22**	**1.14-1.32**	**<0.001**
Age < median	346 (8316)	1.23	1.06-1.43	0.006
Age > median	1143 (9624)	1.21	1.11-1.31	<0.001
Smokers	**820 (7000)**	**1.05**	**0.95-1.16**	**0.326**
Age < median	316 (4154)	1.15	0.99-1.34	0.070
Age > median	504 (2846)	0.98	0.86-1.11	0.693

Adjusted for (age) and sex

FIGURES AND FIGURE LEGENDS

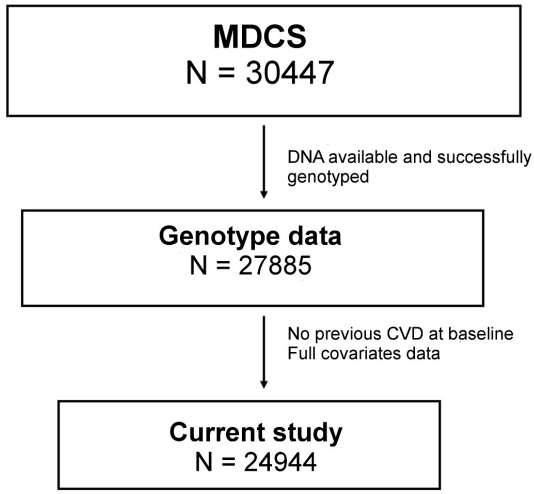

FIGURE 1: Selection of MDCS subjects

The current study included subjects with stored DNA who were successfully genotyped for rs4977574 on chromosome 9p21 and who had no previous history CVD at baseline and complete covariate data (age, sex, smoking status, education, physical activity, systolic blood pressure, antihypertensive medication and BMI). MDCS = Malmö Diet and Cancer Study.

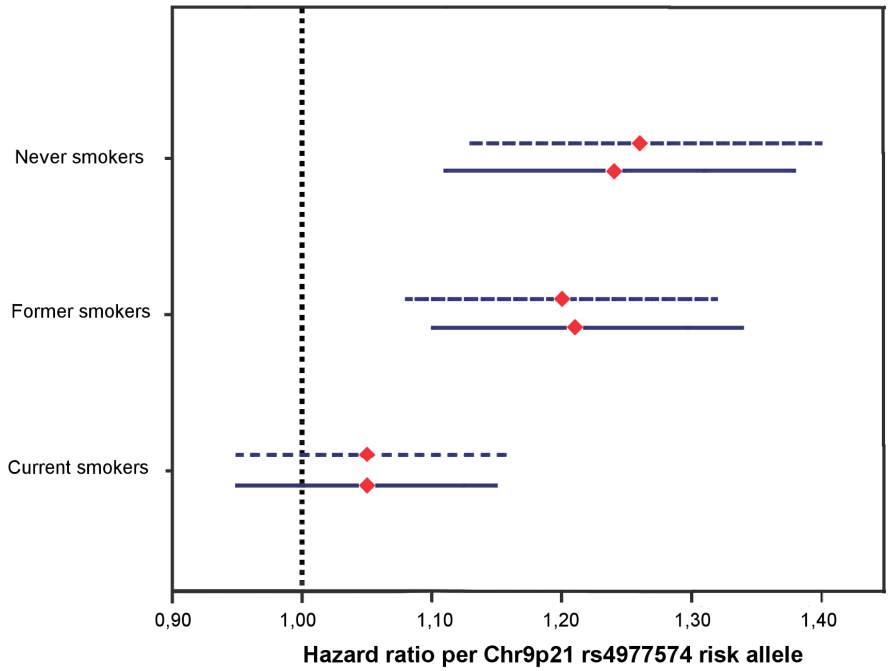

FIGURE 2: Risk of incident CAD by rs4977574 stratified by smoking status

Hazard ratios (HR) with 95 % Confidence Interval per risk allele of rs4977574 in never (N=9642) former (N =8300) and current (N=7000) smokers respectively. Models adjusted for age and sex (dotted upper lines) and adjusted for covariates age, sex, smoking status, education, physical activity, systolic blood pressure, antihypertensive medication and BMI (continuous lower lines).

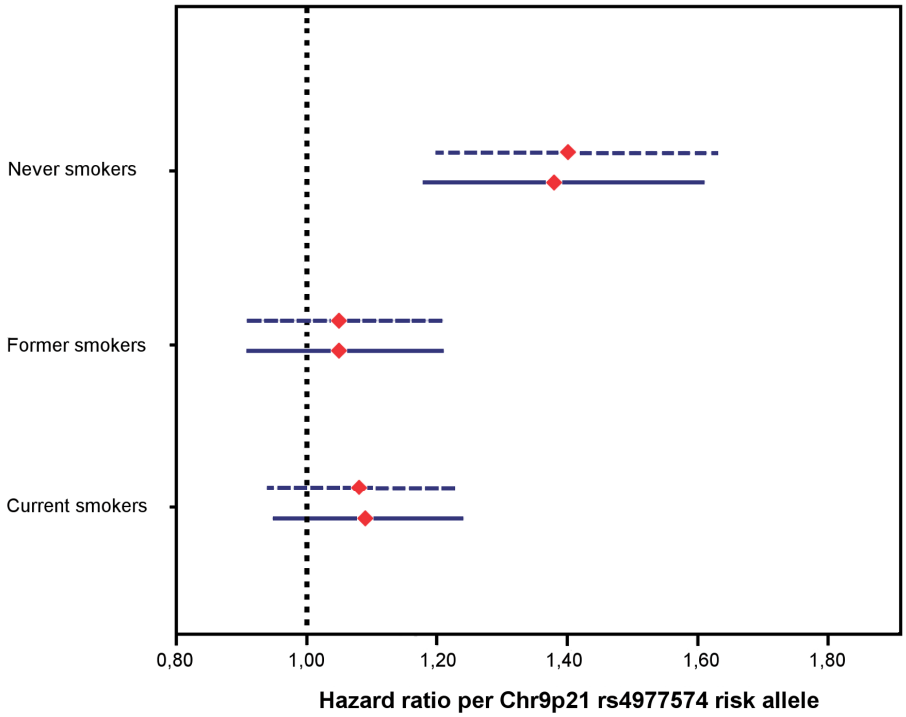

FIGURE 3: CVD-mortality by rs4977574 stratified by smoking status

Hazard ratios (HR) with 95 % Confidence Interval per risk allele of rs4977574 in never (N=9642) former (N = 8297) and current (N=7000) smokers respectively. Models adjusted for age and sex (dotted upper lines) and adjusted for covariates age, sex, smoking status, education, physical activity, systolic blood pressure, antihypertensive medication and BMI (continuous lower lines).

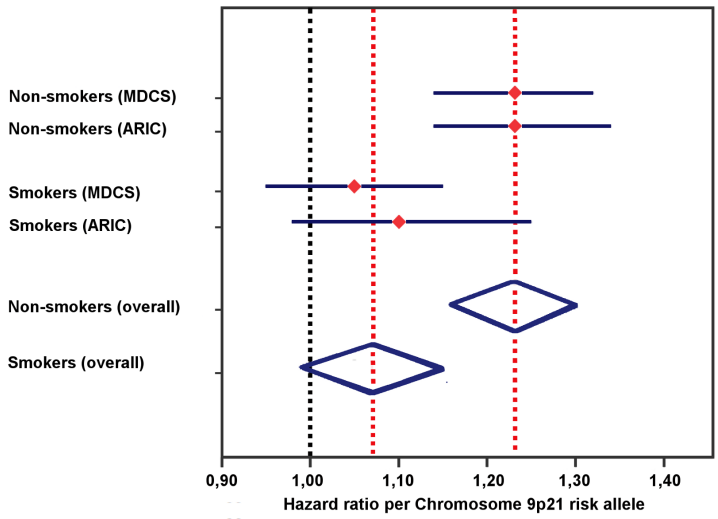

FIGURE 4: Meta analysis: Risk of incident CAD by Chromosome 9p21 stratified by smoking status

Hazard ratios (HR) with 95 % Confidence Interval (CI) per Chromosome 9p21 risk allele in MDCS (rs4977574; N = 24944; 28 % smokers) and ARIC (rs10757274; N = 9877; 25 % smokers). Pooled HR per allele for incident CAD in non-smokers and smokers were 1.23 (95 % CI 1.16-1.30) and 1.07 (95 % CI 0.99-1.15) respectively. MDCS = Malmö diet and cancer study. ARIC = Atherosclerosis Risk in Communities Study.

SUPPORTING INFORMATION “File S1”

Table S1A: Population characteristics

	Current study population (N = 24944)	Subjects in MDCS excluded from current study (N = 5503) [a]
Sex, n (%)		
Men	9455 (37.9)	2666 (48.4)
Women	15489 (62.1)	2837 (51.6)
Age, years	58.0 (7.7)	58.3 (7.2)
Chromosome 9p21, n (%)		
0 risk alleles: A/A	7609 (30.5)	911 (31.0)
1 risk allele: A/G	12311 (49.4)	1373 (46.7)
2 risk alleles: G/G	5024 (20.1)	657 (22.3)
Smoking status, n (%)		
Never smokers	9643 (38.7)	1176 (32.5)
Former smokers	8300 (33.3)	1358 (37.5)
Current smokers	7001 (28.1)	1086 (30.0)
Highest education, n (%)		
No elementary school	203 (0.8)	40 (1.1)
Elementary school (6-8 yrs)	10123 (40.6)	1605 (45.1)
Junior Sec. School (9-10 yrs)	6558 (26.3)	867 (24.4)
Advanced level (12 yrs)	2237 (9.0)	303 (8.5)
At least one additional year	2214 (8.9)	275 (7.7)
University degree	3609 (14.5)	465 (13.1)
Low physical activity, n (%)[b]	4858 (19.5)	765 (22.4)
Systolic blood pressure, mmHg	141.0 (20.0)	141.5 (20.1)
Use of AHT, n (%)	4165 (16.7)	1114 (20.2)
BMI, kg/m^2	25.7 (4.0)	26.3 (4.3)
Incidence of events during follow up		
CAD, total events (events/1000 p-ys)	2309 (6.7)	936 (13.2)
Ischemic Stroke, total events (events/1000 p-ys)	1253 (3.6)	440 (6.1)
Cardiovascular mortality, events (events/1000 p-ys)	1156 (3.2)	590 (7.8)

[a] Subjects from MDCS excluded from the current study because of missing genetic and / or covariates data. Proportion numbers are based on available data in excluded subjects.

[b] Defined as the lowest quintile of the Physical Activity score in MDCS (for definition of Physical Activity Score, (see the methods section)

Table S1B: Population characteristics

	Current study population (N = 24944)	Subjects in MDCS excluded from the current study (N = 5503) [a]	
		No CVD at baseline (N = 4381)	CVD at baseline (N = 1121) [b]
Sex, n (%)			
Men	9455 (37.9)	1854 (42.3)	811 (72.3)
Women	15489 (62.1)	2527 (57.7)	310 (27.7)
Age, years	58.0 (7.7)	57.2 (6.9)	62.9 (6.6)
Chromosome 9p21, n (%)			
0 risk alleles: A/A	7609 (30.5)	613 (32.1)	298 (29.0)
1 risk allele: A/G	12311 (49.4)	893 (46.7)	479 (46.6)
2 risk alleles: G/G	5024 (20.1)	405 (21.2)	252 (24.5)
Smoking status, n (%)			
Never smokers	9643 (38.7)	961 (37.0)	215 (21.0)
Former smokers	8300 (33.3)	814 (31.4)	544 (53.0)
Current smokers	7001 (28.1)	819 (31.6)	267 (26.0)
Highest education, n (%)			
No elementary school	203 (0.8)	27 (1.1)	13 (1.3)
Elementary school (6-8 yrs)	10123 (40.6)	1039 (41.0)	566 (55.6)
Junior Sec. School (9-10 yrs)	6558 (26.3)	671 (26.4)	196 (19.3)
Advanced level (12 yrs)	2237 (9.0)	222 (8.8)	81 (8.0)
At least one additional year	2214 (8.9)	206 (8.1)	69 (6.8)
University degree	3609 (14.5)	372 (14.7)	93 (9.1)
Low physical activity, n (%)[c]	4858 (19.5)	765 (22.2)	232 (22.8)
Systolic blood pressure, mmHg	141.0 (20.0)	140.0 (19.9)	147.2 (19.9)
Use of AHT, n (%)	4165 (16.7)	440 (10.0)	674 (60.1)
BMI, kg/m^2	25.7 (4.0)	26.2 (4.3)	27.0 (4.0)
Incidence of events during follow up			
CAD, events (events/1000 p-ys)	2309 (6.7)	483 (8.1)	453 (39.8)
Ischemic Stroke, events (events/1000 p-ys)	1253 (3.6)	296 (4.9)	144 (11.4)
Cardiovascular mortality, events (events/1000 p-ys)	1156 (3.2)	295 (4.8)	295 (21.5)

Numbers are displayed as mean (SD) if not stated other

p-ys = person-years

[a] Subjects from MDCS excluded from the current study because of missing genetic and / or covariates data. Proportion numbers are based on available data in excluded subjects.

[b] One subject had missing data on prevalent CVD at baseline, and could therefore not be assigned a group.

[c] Defined as the lowest quintile of the Physical Activity score in MDCS (for definition of Physical Activity Score, (see the methods section)

Table S2: Main effects with adjustment for covariates

	CAD HR (95 % CI)	Ischemic Stroke HR (95 % CI)	Cardiovascular mortality HR (95 % CI)
rs4977574, per allele	1.16 (1.10-1.23)	1.13 (1.04-1.22)	1.16 (1.06-1.25)
Smoking status[a]			
Former smoker	1.19 (1.07-1.32)	1.01 (0.88-1.17)	1.26 (1.08-1.46)
Current smoker	2.11 (1.90-2.35)	1.73 (1.51-1.99)	2.85 (2.45-3.30)
Highest education[a]			
Elementary school (6-8 yrs)	0.99 (0.65-1.53)	0.53 (0.34-0.83)	0.83 (0.47-1.47)
Junior Sec. School (9-10 yrs)	0.87 (0.56-1.35)	0.48 (0.30-0.75)	0.73 (0.41-1.30)
Advanced level (12 yrs)	0.84 (0.54-1.33)	0.42 (0.26-0.68)	0.66 (0.36-1.21)
At least one additional year	0.86 (0.55-1.36)	0.45 (0.28-0.73)	0.62 (0.33-1.14)
University degree	0.75 (0.48-1.17)	0.42 (0.26-0.67)	0.70 (0.37-1.22)
P for trend	*<0.001*	*0.001*	*0.002*
Quintile of PA score[a]			
Q2	0.75 (0.66-0.85)	0.76 (0.64-0.90)	0.69 (0.58-0.83)
Q3	0.78 (0.69-0.89)	0.77 (0.65-0.91)	0.73 (0.61-0.87)
Q4	0.83 (0.73-0.94)	0.70 (0.59-0.83)	0.73 (0.62-0.88)
Q5	0.85 (0.75-0.96)	0.73 (0.62-0.86)	0.71 (0.60-0.84)

Models adjusted for main effect variables, age, sex, systolic blood pressure, body mass index and antihypertensive treatment.

PA score = Physical Activity Score

[a] Hazard ratio (HR) in relation to the first category in the categorical variables (never smokers, did not complete elementary school, and Q1 of PA-score respectively)

Table S3: Risk of incident CAD by rs4977574 stratified by smoking status

Table S3A. Model adjusted for age (and sex):

	Events (total cases included)	rs4977574 HR per allele	95 % CI	P
Never smokers	**675 (9642)**	**1.26**	**1.13-1.40**	**<0.001**
Men	340 (2745)	1.27	1.09-1.48	0.002
Women	335 (6895)	1.24	1.07-1.45	0.005
No passive smoking	220 (3339)	1.56	1.29-1.88	<0.001
Passive smoking	379 (5069)	1.14	0.99-1.32	0.068
Former smokers	**814 (8300)**	**1.20**	**1.08-1.32**	**<0.001**
Men	612 (4015)	1.15	1.03-1.29	0.012
Women	202 (4285)	1.31	1.08-1.59	0.006
No passive smoking	177 (2146)	1.30	1.05-1.60	0.015
Passive Smoking	528 (5221)	1.19	1.06-1.35	0.004
Current smokers	**820 (7000)**	**1.05**	**0.95-1.16**	**0.326**
Men	520 (2693)	1.06	0.94-1.20	0.329
Women	300 (4307)	1.03	0.87-1.21	0.743

Table S3B. Model adjusted for age, (sex), SBP, BMI, AHT, Education, Quintiles of PA:

	Events (total cases included)	rs4977574 HR per allele	95 % CI	P
Never smokers	**675 (9642)**	**1.24**	**1.11-1.38**	**<0.001**
Men	340 (2745)	1.26	1.08-1.47	0.004
Women	335 (6895)	1.23	1.06-1.43	0.006
No passive smoking	220 (3339)	1.53	1.26-1.85	<0.001
Passive smoking	379 (5069)	1.12	0.97-1.29	0.116
Former smokers	**814 (8300)**	**1.21**	**1.10-1.34**	**<0.001**
Men	612 (4015)	1.18	1.05-1.32	0.004
Women	202 (4285)	1.33	1.09-1.61	0.004
No passive smoking	177 (2146)	1.31	1.06-1.61	0.012
Passive smoking	528 (5221)	1.22	1.08-1.38	0.001
Current smokers	**820 (7000)**	**1.05**	**0.95-1.15**	**0.368**
Men	520 (2693)	1.06	0.94-1.20	0.362
Women	300 (4307)	1.03	0.88-1.21	0.729

Table S4: CVD Mortality by rs4977574 stratified by smoking status

Table S4A. Model adjusted for age (and sex):

	Events (total cases included)	rs4977574 HR per allele	95 % CI	P
Never smokers	**327 (9642)**	**1.40**	**1.20-1.63**	**<0.001**
Men	137 (2745)	1.35	1.07-1.72	0.013
Women	190 (6895)	1.43	1.17-1.75	<0.001
No passive smoking	113 (3339)	1.78	1.37-2.32	<0.001
Passive smoking	174 (5070)	1.27	1.03-1.57	0.025
Former smokers	**383 (8297)**	**1.05**	**0.91-1.21**	**0.525**
Men	270 (4011)	0.98	0.83-1.16	0.807
Women	113 (4284)	1.22	0.94-1.58	0.136
No passive smoking	88 (2145)	1.38	1.02-1.85	0.034
Passive smoking	247 (5218)	0.96	0.81-1.15	0.676
Current smokers	**446 (7000)**	**1.08**	**0.94-1.23**	**0.270**
Men	265 (2689)	1.08	0.91-1.28	0.362
Women	181 (4307)	1.07	0.86-1.32	0.552

Table S4B. Model adjusted for age, (sex), SBP, BMI, AHT, Education, Quintiles of PA:

	Events (total cases included)	rs4977574 HR per allele	95 % CI	P
Never smokers	**327 (9642)**	**1.38**	**1.18-1.61**	**<0.001**
Men	137 (2745)	1.33	1.04-1.70	0.021
Women	190 (6895)	1.44	1.18-1.76	<0.001
No passive smoking	113 (3339)	1.71	1.31-2.24	<0.001
Passive smoking	174 (5070)	1.28	1.04-1.57	0.023
Former smokers	**383 (8297)**	**1.05**	**0.91-1.21**	**0.489**
Men	270 (4011)	0.99	0.83-1.17	0.870
Women	113 (4284)	1.22	0.94-1.58	0.128
No passive smoking	88 (2145)	1.36	1.01-1.84	0.042
Passive smoking	247 (5218)	0.98	0.82-1.17	0.824
Current smokers	**446 (7000)**	**1.09**	**0.95-1.24**	**0.212**
Men	265 (2689)	1.09	0.92-1.30	0.312
Women	181 (4307)	1.08	0.87-1.33	0.498

Table S5: Interactions between rs4977574 and smoking, education and physical activity on end points

	CAD	Ischemic Stroke	Cardiovascular mortality
	$P_{interaction}$	**$P_{interaction}$**	**$P_{interaction}$**
Crude model [a]			
Smoking status	**0.035**	0.702	**0.012**
Highest Education	0.082	0.876	0.681
Physcial activty	0.457	0.251	0.286
Adjusted model [b]			
Smoking status	**0.035**	0.569	**0.029**
Highest Education	0.080	0.924	0.696
Physcial activty	0.565	0.217	0.372

P-values based on Chi 2 distribution from likelihood ratio tests comparing model fit with and without the interaction terms.

[a] Model adjusted for age and sex

[b] Model adjusted for covariates age, sex, systolic blood pressure, body mass index, antihypertensive treatment and (smoking), (education), (quintiles of physical activity)

Table S6: Smoking as a risk factor for incident CAD, stratified by number of rs4977574 risk alleles

Table S6A. Model adjusted for age (and sex):

rs4977574 risk alleles	Events (total cases included)	Former smoker HR (95 % CI)	P	Current smoker HR (95% CI)	P
0	**633 (7608)**	**1.24 (1.01-1.52)**	**0.038**	**2.21 (1.81-2.70)**	**<0.001**
Men	412 (2881)	1.41 (1.09-1.83)	0.010	2.29 (1.75-2.99)	<0.001
Women	221 (4727)	1.02 (0.72-1.45)	0.908	2.25 (1.66-3.05)	<0.001
1	**1134 (12309)**	**1.24 (1.07-1.45)**	**0.005**	**2.21 (1.90-2.56)**	**<0.001**
Men	717 (4680)	1.18 (0.98-1.43)	0.087	1.97 (1.62-2.39)	<0.001
Women	417 (7629)	1.40 (1.09-1.80)	0.009	2.66 (2.12-3.33)	<0.001
2	**542 (5024)**	**1.12 (0.92-1.38)**	**0.265**	**1.48 (1.19-1.84)**	**<0.001**
Men	343 (1892)	1.18 (0.91-1.54)	0.215	1.57 (1.18-2.09)	0.002
Women	199 (3132)	1.13 (0.80-1.58)	0.496	1.43 (1.01-2.02)	0.043

TableS6B. Model adjusted for age, (sex), SBP, BMI, AHT, Education, Quintiles of PA:

rs4977574 risk alleles	Events (total cases included)	Former smoker HR (95 % CI)	P	Current smoker HR (95% CI)	P
0	**633 (7608)**	**1.17 (0.95-1.43)**	**0.141**	**2.29 (1.87-2.80)**	**<0.001**
Men	412 (2881)	1.29 (0.99-1.68)	0.059	2.32 (1.77-3.04)	<0.001
Women	221 (4727)	1.05 (0.74-1.50)	0.783	2.38 (1.75-3.25)	<0.001
1	**1134 (12309)**	**1.23 (1.06-1.43)**	**0.007**	**2.34 (2.01-2.71)**	**<0.001**
Men	717 (4680)	1.14 (0.95-1.38)	0.170	2.02 (1.66-2.45)	<0.001
Women	417 (7629)	1.44 (1.12-1.85)	0.005	2.99 (2.38-3.76)	<0.001
2	**542 (5024)**	**1.12 (0.91-1.37)**	**0.301**	**1.55 (1.24-1.93)**	**<0.001**
Men	343 (1892)	1.14 (0.87-1.49)	0.338	1.58 (1.18-2.11)	0.002
Women	199 (3132)	1.18 (0.84-1.66)	0.339	1.55 (1.09-2.20)	0.016

Table S7: Smoking as a risk factor for CVD Mortality, stratified by number of rs4977574 risk alleles

TableS7A. Model adjusted for age (and sex):

rs4977574 risk alleles	Events (total cases included)	Former smoker HR (95 % CI)	P	Current smoker HR (95% CI)	P
0	**310 (7604)**	**1.76 (1.30-2.38)**	**<0.001**	**3.35 (2.48-4.52)**	**<0.001**
Men	190 (2877)	1.97 (1.30-2.99)	0.002	3.32 (2.15-5.12)	<0.001
Women	120 (4726)	1.49 (0.93-2.40)	0.099	3.62 (2.38-5.50)	<0.001
1	**586 (12309)**	**1.20 (0.96-1.48)**	**0.106**	**2.77 (2.26-3.39)**	**<0.001**
Men	343 (4676)	1.14 (0.85-1.52)	0.380	2.69 (2.03-3.57)	<0.001
Women	243 (7629)	1.33 (0.95-1.85)	0.098	2.85 (2.13-3.81)	<0.001
2	**260 (5024)**	**1.04 (0.77-1.42)**	**0.781**	**2.00 (1.48-2.70)**	**<0.001**
Men	139 (1892)	1.06 (0.69-1.62)	0.803	2.03 (1.31-3.14)	0.002
Women	121 (3130)	1.09 (0.70-1.71)	0.708	2.02 (1.32-3.10)	0.001

Table S7B. Model adjusted for age, (sex), SBP, BMI, AHT, Education, Quintiles of PA:

rs4977574 risk alleles	Events (total cases included)	Former smoker HR (95 % CI)	P	Current smoker HR (95% CI)	P
0	**310 (7604)**	**1.68 (1.24-2.29)**	**0.001**	**3.43 (2.53-4.65)**	**<0.001**
Men	190 (2877)	1.83 (1.20-2.80)	0.005	3.29 (2.12-5.11)	<0.001
Women	120 (4726)	1.54 (0.95-2.48)	0.080	3.72 (2.43-5.71)	<0.001
1	**586 (12309)**	**1.20 (0.96-1.49)**	**0.108**	**2.95 (2.40-3.63)**	**<0.001**
Men	343 (4676)	1.08 (0.81-1.45)	0.583	2.70 (2.03-3.59)	<0.001
Women	243 (7629)	1.37 (0.98-1.92)	0.064	3.21 (2.38-4.33)	<0.001
2	**260 (5024)**	**1.05 (0.78-1.43)**	**0.738**	**2.15 (1.58-2.94)**	**<0.001**
Men	139 (1892)	1.00 (0.65-1.54)	0.985	2.12 (1.36-3.32)	0.001
Women	121 (3130)	1.17 (0.74-1.84)	0.502	2.21 (1.42-3.43)	<0.001