PHARMACOGENETIC EPIDEMIOLOGY OF STATINS IN AN AGEING POPULATION

CATHERINE ELISABETH DE KEYSER

The Rotterdam Study is supported by the Erasmus Medical Center and Erasmus University Rotterdam; the Netherlands Organization for Scientific Research (NWO); the Netherlands Organization for Health Research and Development (ZonMW); the Research Institute for Diseases in the Elderly (RIDE); the Netherlands Genomics Initiative (NGI); the Ministry of Education, Culture and Science; the Ministry of Health, Welfare and Sport; the European Commission (DG XII); and the Municipality of Rotterdam.

The contributions of the study participants, general practitioners and pharmacists of the Ommoord district to the Rotterdam Study are gratefully acknowledged.

The publication of this thesis was financially supported by the Health Care Inspectorate.

Cover: 'Het Blauwe Hart' (1998), gemaakt door kunstenaar Marcel Smink in opdracht van de gemeente Delft, en gelokaliseerd op de keerlus voor bussen op de Oude Langendijk in de binnenstad van Delft. De foto voor het ontwerp van de omslag van dit proefschrift is gemaakt door Marijke de Vries.

Printed by Optima Grafische Communicatie, Rotterdam, the Netherlands
ISBN: 978-94-6169-668-7

Pharmacogenetic Epidemiology of Statins in an Ageing Population

Farmacogenetische Epidemiologie van Statines in een Ouder wordende Populatie

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de rector magnificus
Prof.dr. H.A.P Pols
en volgens besluit van het College voor Promoties

De openbare verdediging zal plaatsvinden op
vrijdag 5 juni 2015 om 13.30 uur

door

Catherine Elisabeth de Keyser

geboren te Delft

Erasmus University Rotterdam

PROMOTIECOMMISSIE

Promotoren:	Prof.dr. B.H.Ch. Stricker
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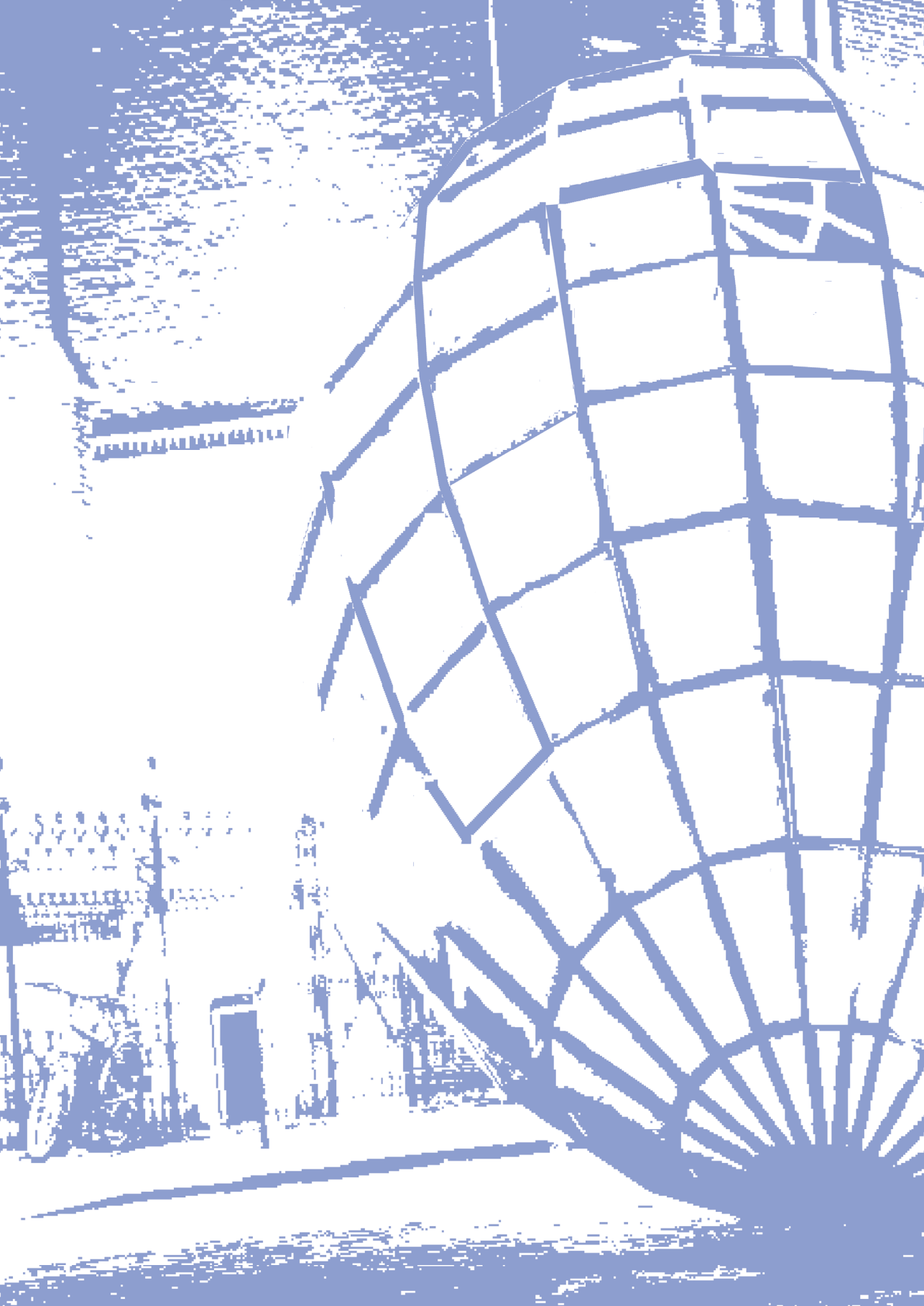
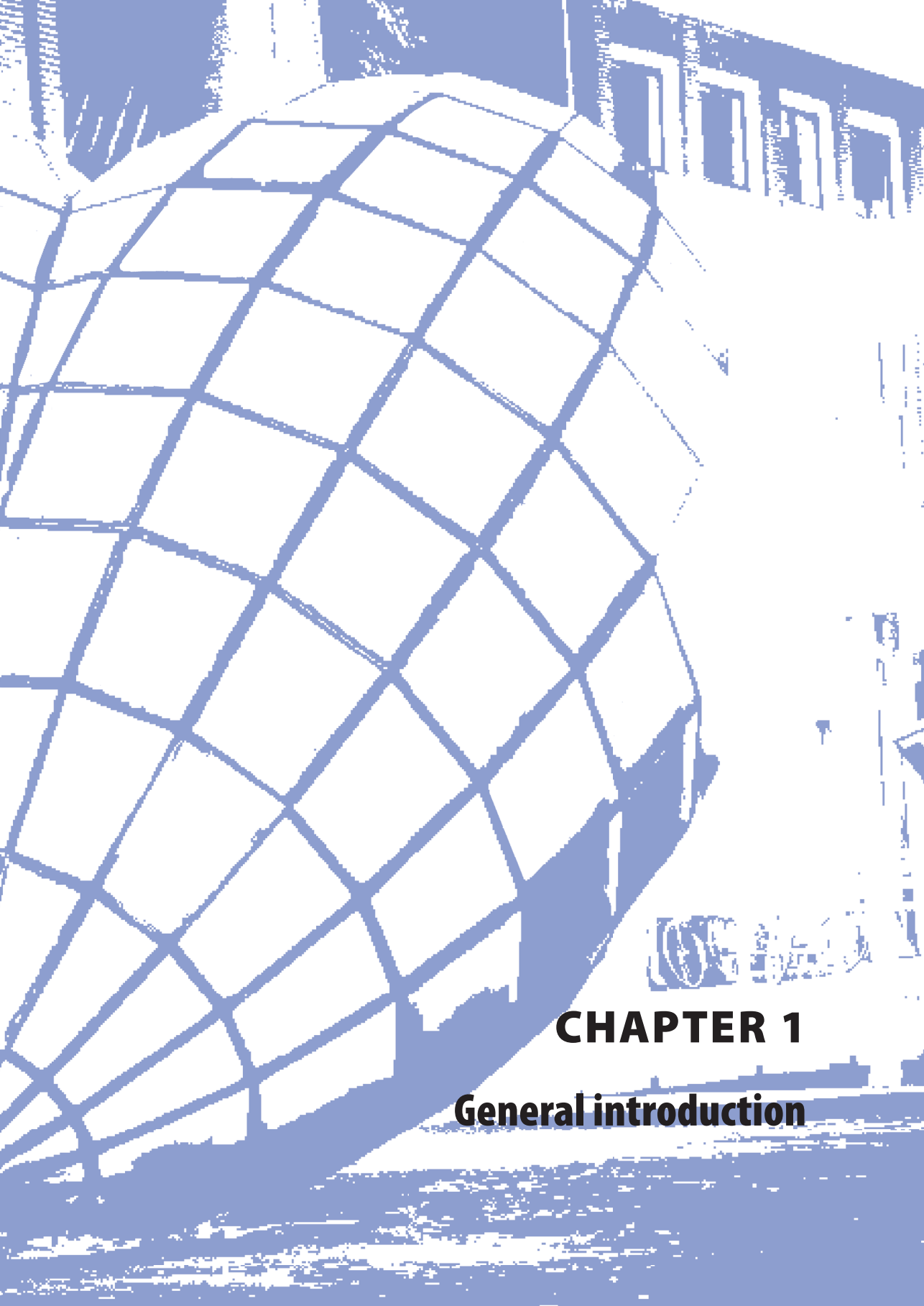




CHAPTER 1

General introduction

Cardiovascular disease (CVD) is an important cause of morbidity and mortality worldwide. Due to factors such as the aging population and an increased number of people with overweight, the incidence and prevalence of CVD are increasing. This constitutes a considerable disease burden and major health challenge for prevention and treatment.[1,2] CVD frequently co-exists with other diseases such as type 2 mellitus (T2DM) and non-alcoholic fatty liver disease (NAFLD). These diseases are strongly related to the metabolic syndrome, a disease entity which consists of the components dyslipidemia, insulin resistance, hypertension, and abdominal obesity.[3-5] The metabolic syndrome is a strong risk factor for the occurrence of CVD, and CVD is an important cause of death in NAFLD and T2DM patients.[3,6]

A broad spectrum of cardiovascular drugs is available for the prevention and treatment of CVD, and often, a patient receives a combination of drugs. The intention for using these drugs is to treat risk factors for CVD, such as hypertension and hypercholesterolemia, or the symptoms of CVD, such as heart failure and rhythm disorders. At the beginning of this thesis, we wrote an inventory on the current state of knowledge on the pharmacogenetics of response to cardiovascular drug therapy.[7] Thereafter, we decided to focus this thesis on one particular drug group: the frequently prescribed cholesterol lowering statins, or 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors. Statins have proven to be beneficial in the primary and secondary prevention of CVD, and act primarily by lowering serum low-density lipoprotein (LDL)-cholesterol levels through inhibition of HMG-CoA reductase, the rate-limiting step in the cholesterol biosynthesis pathway.[8-13] Independent of this cholesterol lowering effect, statins have pleiotropic effects, including anti-inflammatory, immunomodulatory, and anti-oxidants effects, and improvement of endothelium viability.[14]

With the pharmaco-epidemiological studies described in this thesis, we aimed to gain more insight into the use of statins in an ageing population. We focused on statins because several topics with regard to these drugs are important and interesting to consider, and there were many more interesting research hypotheses than we could investigate during this PhD research period. Therefore, we had to restrict our choices to the following ones. First, current statin therapy is not optimal for all patients, and some individuals do not respond adequately to statins. This ranges from lack of therapeutic effect to the occurrence of adverse drugs reactions (ADRs).[15,16] This difference in response between individuals can partly be explained by genetic variation, and insight into underlying genetic variation that predicts drug response could be useful in tailoring treatment for individual subjects or certain patient subgroups, so-called 'personalized medicine'.[14] Second, in 2013, the American guidelines on primary prevention of CVD lowered the threshold for the indication for statin treatment, and thereby widened the target population for these already frequently prescribed drugs.[17] This might have implications for current clinical practice, certainly since one may not ignore the potential risk

of overtreatment or unintended effects that may go with this increased use. Moreover, in observational studies it is a challenge to investigate the association between statin exposure and outcome measures, since these studies are often subject to bias and confounding.

The first part of this introduction is the inventory on the current state of knowledge on the pharmacogenetics of response to cardiovascular drug therapy, which was published in 2012.[7] The second part describes the aim and outline of this thesis.

1.1 PHARMACOGENETICS OF RESPONSE TO CARDIOVASCULAR DRUG THERAPY: WHAT IS THE CURRENT STATE OF KNOWLEDGE?

Catherine E de Keyser, Mark Eijgelsheim, André G Uitterlinden, Bruno H Stricker. *Dialogues in Cardiovascular Medicine* 2012; 17(4): 281-292

CVD is a major cause of morbidity and mortality in developed countries. Besides well-known environmental factors such as smoking and overconsumption of saturated fat, genetic factors contribute to the risk of developing CVD. In addition, genetic factors may modify both the pharmacokinetics and pharmacodynamics of cardiovascular drugs (pharmacogenetics). The most important genetic polymorphisms that influence response to cardiovascular treatment are highlighted, with regard to effectiveness and risk of adverse reactions. Insight into individual genetic risk factors for disease and treatment response could lead to 'personalized medicine' in the future.

CVD is an important cause of morbidity and mortality in developed countries, and insight into the underlying risk factors for the occurrence of CVD and response to treatment are major topics for the understanding and improvement of current clinical practice. CVD consists of a range of predominantly cardiac syndromes that are often caused by atherosclerosis of the vascular system, but may also be of other origin (e.g., idiopathic cardiomyopathy, congenital long QT syndrome).

A broad spectrum of cardiovascular drugs are available for the treatment of CVD. They are all intended to treat risk factors for CVD, such as hypertension and hypercholesterolemia, or the symptoms of CVD, such as heart failure and rhythm disorders. Often, a patient receives a combination of drugs, acting on different pathways or transporters, or acting synergistically on one pathway. According to the World Health Organization (WHO)[18], in the Anatomical Therapeutical Chemical (ATC) classification system, cardiovascular drugs are divided into 9 different subclasses of drugs, namely, cardiac therapy, antihypertensives, diuretics, peripheral vasodilators, vasoprotectors, β-blocking agents, calcium channel blockers, agents acting on the renin-angiotensin system, and lipid-

modifying agents. Every drug subclass contains several different chemical groups, each of which in turn contains a large number of different drugs, each with its own ATC code. Although this underscores the large number of different cardiovascular drugs, it should be emphasized that the number of unique pharmacological entities is rather limited and that many drugs on the market are merely slightly changed copies of each other. Fortunately, research is going on to develop new drugs, acting on other targets/biological systems. Here, pharmacogenomics (i.e., the whole-genome application of pharmacogenetics) plays a pivotal role in the discovery of new drug targets.

There is a large interindividual variability in response to cardiovascular drugs, which is not simply explained by differences in daily doses. Variability in response despite similar dosage can be explained by individual differences in pharmacokinetics, for instance, by comorbidities such as a decrease in hepatic or renal function. However, it may also result from concomitant medication interacting with the cardiovascular drug, e.g., if two drugs are both metabolized by the same cytochrome P450 (CYP450) isoenzyme or if another drug is an inducer or inhibitor of the enzyme by which the cardiovascular drug is metabolized. Interindividual differences in pharmacodynamics response may occur via a difference in the molecular structure of a drug receptor or a smaller number of receptors. Although it is assumed that aging is associated with a decrease in numbers of receptors, determinants for differences in pharmacodynamics are less well documented.

Probably the most important factor that can explain interindividual variability in pharmacokinetics and pharmacodynamic response to drug therapy is genetic variation.[19,20] Pharmacogenetics focuses on genetic variants and polymorphisms that influence response to drug therapy. A single nucleotide polymorphism (SNP) is a DNA sequence variation, in which one single nucleotide differs between individuals. If this variation occurs in 1% or greater of the population, it is called a genetic polymorphism or variant.[21] A SNP or a combination of SNPs (haplotype) can help predict susceptibility to environmental factors and the risk of developing a particular disease, but also the pharmacokinetic and pharmacodynamics response of individuals to certain drugs. Other reasons for genetic variation may be the presence ("insertion") or absence ("deletion") of a series of DNA bases or the presence of "copy number variations" (CNV). In case of genetic variation that modifies pharmacokinetics, the SNP is located in a gene involved in the absorption, distribution, metabolism, or excretion of a drug. For example, genetic variation in one of the important CYP450 isoenzymes, such as CYP2C9 or CYP2D6, or genetic variation in a liver transporter that is involved in the active uptake or excretion of a particular drug, modifies the pharmacokinetics of a drug. As a consequence of this variation, the plasma concentration of the drug changes, which alters efficacy or toxicity risk, because less or more drug, respectively, is available at the receptor site. Regulatory variation in the gene encoding the drug uptake receptor at its target organ can influence the active uptake of the drug to its main organ where it is acting. In this case, the

plasma concentration of the drug has not changed, but the concentration in the primary organ of acting is diminished or increased, depending on whether the SNP increases or decreases the number of uptake transporters, respectively.[22-24] A pharmacodynamic example of a potential genetic variation is the encoding for the structure of cardiovascular and respiratory β-adrenoceptors, which is supposed to lead to differences in response to drugs.

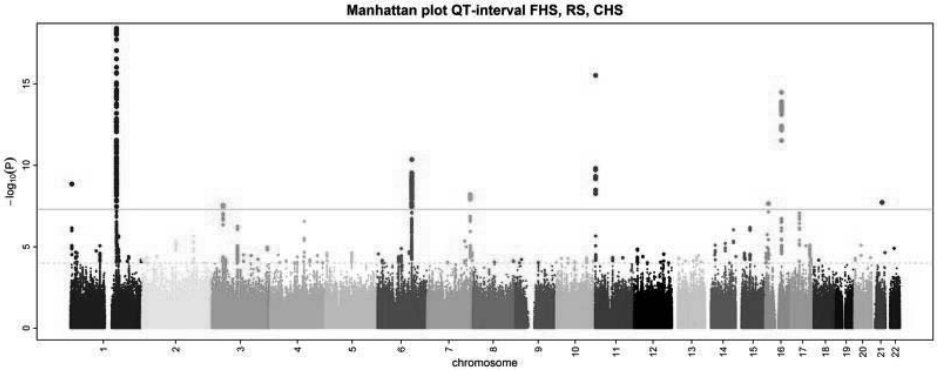
Over the past decades, many studies have focused on identifying genetic determinants that influence response to therapy, using different techniques for analysis. Candidate gene studies investigating SNPs in "biologically plausible" genes, e.g., SNPs in genes involved in the biological pathway of a disease, and genome-wide association studies (GWAS) without an a priori hypothesis of the underlying genetic variation involved in treatment response, are two often used methods.[25] Furthermore, new analyzing techniques, "next-generation sequencing" like exome sequencing and whole-genome sequencing, are currently upcoming and promising techniques to unravel rare coding variants involved in the genetics of complex traits.[26] In future perspective, markers that are predictive of drug efficacy or the occurrence of adverse drug reactions could be useful in tailoring treatment for individual subjects or certain patient subgroups, so-called 'personalized medicine'.

In the following paragraphs, we discuss the most important pharmacogenetic associations that were discovered in the past years for the different cardiovascular drugs, regarding treatment response. This includes both the efficacy of drugs, and the risk of developing adverse reactions. Although there are many drugs with cardiovascular adverse effects, the discussion will be restricted to drugs with a cardiovascular indication, notably antiarrhythmics, diuretics, angiotensin-converting enzyme (ACE) inhibitors, and angiotensin blockers, β-blockers, lipid-lowering drugs, and anticoagulants. Importantly, we restricted ourselves to those associations that were confirmed in other studies because many incidental findings in the pharmacogenetic literature have not been confirmed by others.

Antiarrhytmics: prolonged QT interval duration, calcium antagonists, and digoxin

Several drugs are associated with prolongation of the electrocardiographic QT interval duration, but only some of them have cardiovascular indications. The QT interval is a measure of myocardial repolarization time, and prolongation of the QT interval duration is associated with a higher risk of drug-induced arrhythmias and sudden cardiac death (SCD). In particular, in individuals with the congenital long QT syndrome (a term encompassing more than 10 different mutations, for instance, in the genes *KCNQ1*, *KCNH2*, and *SCN5A*), it is associated with an increased risk of torsades de pointes (TdP), a specific type of ventricular arrhythmia. Although the risk of TdP might also be increased in cases of drug-induced QT prolongation, this seems to be less well documented.

Figure 1 Manhattan plot for the QT interval association analysis, a meta-analysis of three GWAS in 13,685 individuals from three independent cohorts



QT interval association results for 2,543,686 imputed SNPs in 13,685 individuals from 3 cohorts. Results are shown on the $-\log_{10}(P)$ scale and are truncated at $-\log_{10}(P) = 18$ for display purposes. The solid bar corresponds to the genome-wide significance threshold of 5×10^{-8}.
Abbreviations: CHS, Cardiovascular Health Study; FHS, Framingham Heart Study; GWAS, genome-wide association study; RS, Rotterdam Study; SNP, single nucleotide polymorphism.
After reference 27: Newton-Cheh et al. Nat Genet. 2009; 41(4): 399-406.

Moreover, there are rare and more common genetic loci for QT prolongation. In 2009, a meta-analysis of three GWAS in 13 685 individuals of European ancestry discovered 14 independent variants at 10 loci, together explaining 5.4% to 6.5% of the variation in QT interval.[27] The results of this meta-analysis are represented in *Figure 1*[27], the Manhattan plot for the QT interval association results for the three cohorts combined. Other studies also detected several genetic variants in multiple genes associated with prolongation of the normal QT interval duration, and with that provide candidate genes that might predispose to modifying the QT-prolonging effect of drugs.[28,29] A study showed that the minor alleles of two genetic variants (rs10494366 T>G and rs10918594 C>G) in the nitric oxide synthase 1 activating protein (*NOS1AP*) gene potentiate the QTc prolonging effect of the calcium antagonist verapamil.[30] Another study showed that the minor allele of the *NOS1AP* rs10494366 polymorphism was associated with increased all-cause and cardiovascular mortality in users of dihydropyridine calcium channel blockers.[31] Possibly, genetic variation modifies cardiac contractility and repolarization, by influencing calcium and potassium ionic transport in the cardiomyocyte. Although these findings are interesting, studies revealing the underlying biological mechanism are needed.

Not only studies on QT interval duration, but also GWAS in different opulations showed genetic variation that was associated with other electrocardiographic measures such as PR-interval and QRS interval.[32-34] Also, genetic variants associated with the risk of atrial fibrillation were discovered in GWAS.[34,35] Testing these polymorphisms on interac-

tion with drugs used in atrial fibrillation, such as calcium channel blockers, would be an interesting topic for future research.

Digoxin is most frequently used in chronic heart failure with atrial fibrillation. It is a known substrate for the ATP-binding cassette B1 (ABCB1) transporter (P-gp, P-glycoprotein), encoded by the *ABCB1* gene, formerly known as multidrug resistance 1 (*MDR1*) gene. A study showed that three common variants in the *ABCB1* gene—1235C>T, 2677G>T, and 3435C>T—and the associated *TTT* haplotype were associated with increased digoxin serum concentrations.[36] Other studies also showed that *TTT* haplotype and the 3435TT genotype were associated with higher digoxin serum concentrations.[37,38] Thereby, the effect of haplotype analysis seemed superior to single SNP analysis in the prediction of digoxin pharmacokinetics.

Diuretics

Thiazide diuretics are the most commonly used diuretics in the treatment of hypertension. However, large differences in blood pressure lowering response between individuals exist.[39] In 2008, a GWAS discovered a region of chromosome 12q15 that was significantly associated with blood pressure-lowering response in black individuals using hydrochlorothiazide (HCTZ).[40] After fine mapping of the three genes in the region (*FRS2*, *YEATS4*, *LYZ*), the variation in *YEATS4* appeared to be most strongly associated with blood pressure response. This gene is involved in the regulation of the initiation of transcription, and a priori this gene was not expected to be involved in thiazide response.

The study was replicated in an individual population, and variation in the *YEATS4* rs7297610 polymorphism contributed most to the variation in response to HCTZ. In expression analyses, HCTZ-treated African-Americans showed different *YEATS4* expression patterns post-treatment between the rs7297610 genotypes, which could explain the HCTZ response variability.[41]

Other candidate gene studies on the blood pressure–lowering response to thiazides revealed several polymorphisms at different loci. Two polymorphisms in the sodium channel γ-subunit promoter gene (*SCNN1G*, rs5729, and rs5723), and a polymorphism in the endothelial nitric oxide synthase gene (*eNOS*, rs1799983) were significantly associated with blood pressure response to HCTZ.[42] A combination of genetic variation in the alpha adducin (*ADD1*) gene (Gly460Trp) and *NEDD4L* gene (rs4149601, G>A), both genes regulating renal sodium absorption, was associated with a modified antihypertensive response to thiazides.[43] This effect has also been demonstrated for the two polymorphisms separately.[44-46]

Regarding pharmacogenetics of response to loop diuretics, there are several candidate genes possibly relevant for interindividual variability in drug pharmacokinetics and pharmacodynamics, such as genetic variation in the organic anion transporters OAT1 (*SLC22A6* gene) and OAT3 (*SLC22A8* gene), and the primary target of loop diuret-

ics, the Na+-K+-2Cl– cotransporter (*NKCC2, SLC12A1* gene).[47] Some associations were described[48]; however, there is little evidence and more research is needed.

Drugs involved in the renin-angiotensin-aldosterone system (RAAS): ACE-inhibitors and angiotensin receptor blockers

The most frequently investigated polymorphism in the treatment response to ACE inhibitors is the ACE gene insertion/deletion (I/D) polymorphism (rs1799752). This polymorphism is strongly associated with serum ACE levels, and accounts for almost 50% of the phenotypic variance of serum ACE levels. This suggests that the polymorphism might be a good candidate for modifying the response to ACE-inhibitor therapy. However, studies on this topic show conflicting results on blood pressure lowering effect, cardiovascular events, and mortality risk, and currently no final conclusions can be drawn on an association with therapeutic response.[49-52]

A recent review on the effect of genetic variants in the RAAS system on the blood pressure lowering response to RAAS-blocking drugs (ACE inhibitors, angiotensin receptor blockers) also failed to show an association between the *ACE* I/D polymorphism and antihypertensive effects from RAAS blockade.[53]

Another frequently investigated variant in the RAAS system is the Met235Thr polymorphism in the angiotensinogen (*AGT*) gene, which is associated with elevated serum levels of angiotensinogen. But after review, again no association with antihypertensive effects of RAAS blockade could be demonstrated.[53] Furthermore, in the Rotterdam Study, both the *ACE* I/D polymorphism and the *AGT* Met235Thr polymorphism did not significantly modify the risk of atherosclerosis.[54]

Several other genetic variation involved in the RAAS system (*AT1* A1166C and haplotype, *AT2* variants, *AGT* rs7079, *REN* and *ACE2* variants) might be involved in the response to ACE inhibitors and angiotensin receptor blockers, but as confirmative studies are lacking or conflicting, further evaluation within larger populations is needed to confirm associations before definite conclusions can be drawn.

β-blocking agents

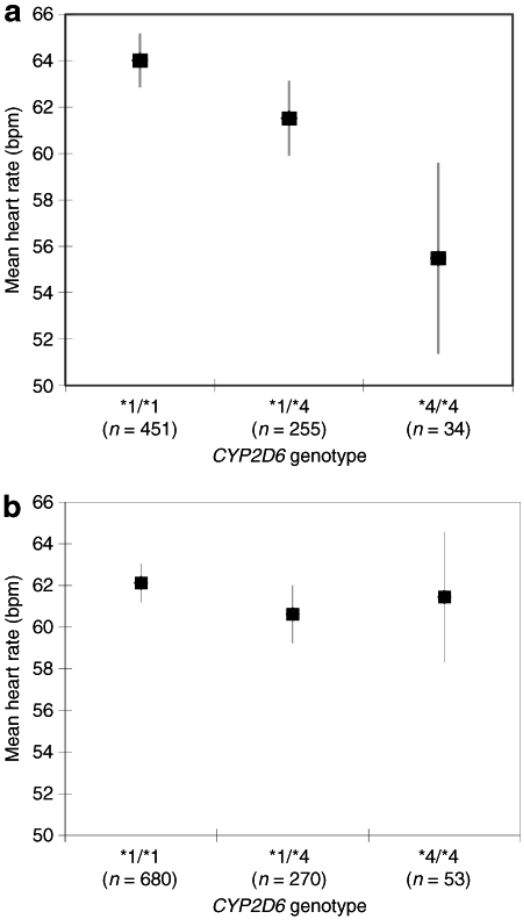
β-Adrenoreceptor antagonists or β-blockers have an important role in the treatment of cardiovascular diseases. Major indications are heart failure, hypertension, angina pectoris, and myocardial infarction (MI). β-Blockers can selectively act on the β1-adregenic receptor (ADRB1) or on the β2-adregenic receptor (ADRB2), or on both receptors.

Regarding the efficacy of β-blocker treatment, *ADRB1* and *ADRB2* are potentially interesting candidate genes for investigation. Within the *ADRB1* gene, the linked polymorphisms rs1801252 (Ser49Gly) and rs1801253 (Arg389Gly) are clinically relevant, and for the *ADRB2* gene three clinically relevant polymorphisms were described: rs1042713 (Arg16Gly), rs1042714 (Gln27Glu), and rs1800888 (Thr164Ile). Many studies have inves-

tigated these polymorphisms and the results are extensive and diverse. For instance, *ADRB1* polymorphisms have been associated with blood pressure reduction[55,56], with overall the largest blood pressure–lowering response for users with the homozygous Arg389 genotype or Ser49/Arg389 haplotype. Furthermore, they have been associated with mortality in heart failure patients on the β-blocker carvedilol[57], and the homozygous Arg389 genotype is associated with significantly better improvement of left ventricular ejection fraction (LVEF) during β-blocker therapy within heart failure patients.[58-62] However, other studies did not find an association, and further investigation in larger populations or studying the combination of different alleles is needed. Regarding the *ADRB2* polymorphisms, these are described in relationship with survival, and the Glu27 allele of the rs1042714 polymorphism seems associated with improved LVEF in response to β-blocker therapy in heart failure patients, compared with the Gln27 allele.[63] Patients heterozygous for the Ile164 rs1800888 genotype may have impaired heart failure survival during β-blocker treatment.[64] No association with blood pressure response is found.[65,66] Also, for these *ADRB2* polymorphisms, results of studies are inconsistent and more research should be performed.

Many β-blockers are metabolized by the polymorphic isoenzyme cytochrome P450 2D6 (CYP2D6), encoded by the *CYP2D6* gene. To date, more than 70 genetic variants within this gene have been described. Several of these variants lead to diminished or absent function of the enzyme. Patients with two inactive alleles are associated with the so-called "poor metabolizer" phenotype, which includes 5% to 10% of the white population. Poor metabolization results in higher plasma concentrations of β-blockers, with a higher risk of toxicity, but also a potentially increased efficacy of the drug. Studies showed that poor metabolizers have a lower heart rate than extensive metabolizers in response to β-blockers that are metabolized by CYP2D6 (e.g. metoprolol), but not in non-metabolized β-blockers such as atenolol.[67] Also, the blood pressure reduction was larger in individuals with the poor metabolizing phenotypes, reflecting a better efficacy of the drug.[67-69] An example of a study in which poor metabolizers showed a stronger lowering of the heart rate than extensive metabolizers is given in *Figure 2*. A stronger heart rate lowering response increases the risk of adverse reactions: a study showed an almost fourfold increased risk of bradycardia with metoprolol in poor metabolizers compared with extensive metabolizers.[67] Another study demonstrated that CYP2D6 poor metabolizers had a fivefold increased risk for the development of adverse reactions during metoprolol treatment in comparison with patients who were not poor metabolizers.[70] The consequences of genetic variation in the *CYP2D6* gene and their clinical effect were mainly demonstrated for metoprolol, which could be explained from the fact that this β-blocker is most extensively metabolized by CYP2D6.[71] As the *CYP2D6* gene may demonstrate copy number variations with ultraextensive metabolism, high doses of metoprolol might be required for a clinical effect in some individuals. Since there is

Figure 2 The *CYP2D6* genotype has an influence on heart rate in metoprolol users, but not in atenolol users



Association between CYP2D6 genotype and adjusted heart rate in users of (a) metoprolol and (b) atenolol. The data are adjusted for age, sex, β-blocker dose, and use of other antihypertensives. The adjusted heart rate in metoprolol users was 8.5 bpm lower in PMs than in EMs (P<0.0001). In *4 heterozygotes, the heart rate was 2.5 bpm lower (P = 0.013).

Abbreviations: *1/*1 – *1/*4 – *4/*4, genotypes; bpm, beats per minute; EMs, extensive metabolizers; PMs, poor metabolizers.

After reference 67: Bijl MJ et al. Clin Pharmacol Ther 2009; 85(1): 45-50.

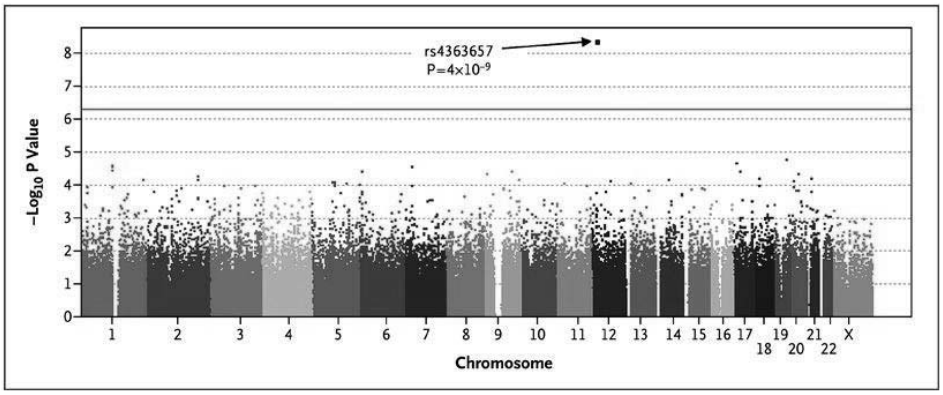
a large difference between β-blockers in their receptor specificity, and in their affinity for metabolizing enzymes and transporters, it is difficult to draw definitive conclusions about the pharmacogenetics of β-blocker response. Currently, genetic testing for the CYP2D6 poor metabolizing genotype is performed only occasionally. In many patients, the dosage will be titrated downward if needed on clinical grounds. According to some, dose adjustments should be considered in certain patients, depending on the particular drug or underlying disease.[72]

Lipid-lowering therapy with statins: cholesterol-lowering effect and muscle toxicity

The HMG-CoA reductase inhibitors, or statins, have a beneficial effect on the primary and secondary prevention of cardiovascular morbidity and mortality, primarily by lowering the concentration of circulating LDL.[73] Statins exert their effect by inhibition of HMG-CoA reductase (HMGCR), the rate-limiting enzyme in the cholesterol biosynthesis pathway. Therefore, the *HMGCR* gene is a good candidate for studies on genetic variation influencing the cholesterol lowering effect of statin therapy. Studies have showed that polymorphisms in the *HMGCR* gene are associated with a lower reduction in levels of total and LDL-cholesterol, within different populations and settings.[74-76]Another important candidate gene is the LDL receptor (*LDLR*) gene, since statins increase LDLR expression. Studies on genetic variation in this gene showed a decreased response to statin therapy.[77,78] Furthermore, cytochrome P450 3A4 (CYP3A4) metabolizes simvastatin, atorvastatin, and lovastatin, and in a recent study the CYP3A4 intron 6 C>T SNP was associated with an increased total and LDL-cholesterol lowering response to simvastatin therapy.[79] Other studies investigating *CYP3A4* polymorphisms also showed an improved LDL-cholesterol lowering response to atorvastatin therapy.[80,81] On the other hand, within the *CYP3A4* gene, polymorphisms showing a diminished lipid-lowering response to statin therapy were also described.[80-82] Regarding the apolipoprotein E (*APOE*) ε2/ε3/ε4 variants (a combination of genetic polymorphisms rs429358 and rs7412) and response to statin therapy, in several studies the ε2 variant seemed to be associated with better cholesterol lowering response to statin therapy and a reduction in cardiovascular outcomes, while in other studies this was not found.[83] The first GWAS on genetic variation and statin response showed an association for variation in *APOE*[84], but in a meta-analysis this was not confirmed.[85] Therefore, the question remains whether *APOE* polymorphisms are associated with a modified statin response. In recent GWAS, 95 new loci that influence lipid concentrations were identified.[86,87] These loci are interesting candidates for pharmacogenetic associations with statin treatment response.

In general, statins are well-tolerated and safe drugs, although adverse reactions do occur. A relatively common adverse reaction is myopathy, which in its severe form may evolve into rhabdomyolysis with muscle necrosis and release of myoglobin. This serious condition can lead to renal failure and death.[88] In 2008, the SEARCH Collaborative Group (Study of the Effectiveness of Additional Reductions in Cholesterol and Homocysteine) published a GWAS on the development of myopathy in simvastatin users and identified a strong significant association for the rs4363657 variant in the solute carrier organic anion transporter family 1B1 (*SLCO1B1*) gene, as shown in *Figure 3*.[89] This polymorphism was in almost complete linkage disequilibrium with the rs4149056 c.521T>C polymorphism, which had already been described in the literature in relation to statin metabolism. Patients homozygous for the minor allele had a 17 times higher risk of myopathy than patients homozygous for the major allele. It is established that the homozygous minor

Figure 3 GWAS on myopathy cases and matched controls, using 80 mg simvastatin daily, showed a genome-wide significant association for a SNP in the *SLCO1B1* gene



Results of tests for a trend in the association between myopathy and each SNP (single nucleotide polymorphism) measured in the genome-wide association study (GWAS). P values are shown for each SNP measured among 85 participants with myopathy and 90 matched controls who were taking 80 mg of simvastatin daily. Analyses are based on 316,184 of the 318,237 SNPs (99.4%) on the Sentrix HumanHap300-Duo BeadChip (Illumina). A result above the horizontal red line indicates strong evidence of an association.
After reference 89: SEARCH Collaborative Group. N Engl J Med. 2008; 359(8): 789-799.

allele genotype of this polymorphism is associated with a higher risk of simvastatin-induced adverse reactions, since other studies also find an association.[90,91] However, the question remains whether there is a class effect, since currently no association could be found between the S*LCO1B1* rs4149056 polymorphism and adverse reactions during use of other statins such as atorvastatin.[90-92]

Anticoagulant therapy with coumarin derivatives and platelet-inhibiting therapy with clopidogrel

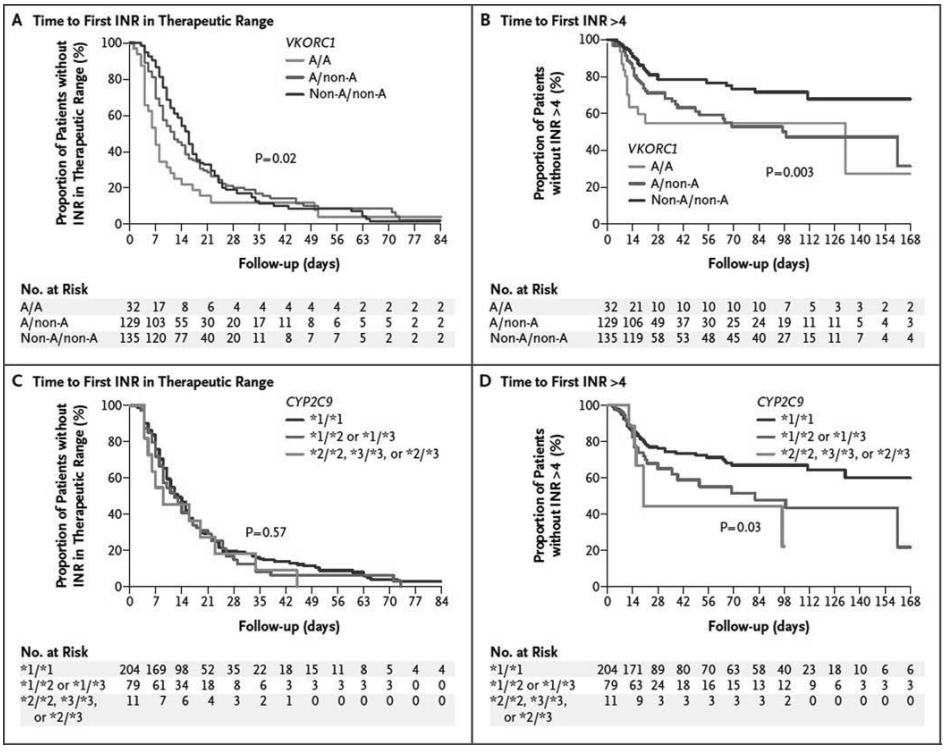
Although in a strict sense one might question whether anticoagulants and platelet inhibitors can be considered as cardiovascular drugs, they are often used in combination with such drugs. In this topic, we mention the most important polymorphisms in the response to oral anticoagulants and platelet inhibitors.

Coumarin derivatives (vitamin K antagonists) are widely used in the prevention and treatment of venous thromboembolism. In models for dose prediction of the coumarin derivative warfarin, polymorphisms in the cytochrome P450 2C9 (CYP2C9) and vitamin K epoxide reductase complex subunit 1 (*VKORC1*) gene substantially contribute to the prediction of stable warfarin dose. Prediction models with *CYP2C9* polymorphisms, *VKORC1* polymorphisms, and clinical factors together included, explained approximately 50% to 60% of the interindividual variability in response to warfarin therapy.[93-95] A study demonstrated that genetic variation in the *VKORC1* gene has a stronger influence on international normalized ratio (INR) response to warfarin therapy at a particular dos-

age, than genetic variation in the *CYP2C9* gene.[96] These results are also represented in *Figure 4*. The variant alleles of the *VKORC1* polymorphisms – 1639A>G (rs9923231) and 1173T>C (rs9934438) – result in patients with this genetic trait being less sensitive to warfarin therapy.[96] Vitamin K epoxide reductase complex subunit 1, encoded by *VKORC1*, is responsible for converting inactive vitamin K back to its active form, and coumarins are in competition with vitamin K for receptors that activate vitamin K-dependent clotting factors. Therefore, genetic variation in the *VKORC1* gene that decreases the function of the VKORC1 enzyme requires higher doses of warfarin to elicit the same effect as in patients with the major genotype.

Cytochrome P450 2C9 (CYP2C9) is the most important enzyme for the elimination of warfarin. The *CYP2C9**2 (rs1799853) and *CYP2C9**3 (rs1057910) alleles are associated with diminished effect of the enzyme, and therefore patients with one or two of these al-

Figure 4 Genetic variation in the *VKORC1* gene has a stronger effect on variability in response to warfarin therapy, than genetic variation in the *CYP2C9* gene



Association between specific genetic variants and study outcomes. The graphs show the association between the time to the first international normalized ratio (INR) within the therapeutic range and the time to the first INR of more than 4 for patients carrying genetic variants for vitamin K epoxide reductase (*VKORC1*) (Panels A and B) and for cytochrome P450 2C9 (*CYP2C9*) (Panels C and D).
After reference 96: Schwarz et al. N Engl J Med. 2008; 358(10): 999-1008.

leles require lower warfarin doses for a particular target INR according to an allele-effect relationship.[97,98] Also for acenocoumarol therapy, a coumarin derivative which is mostly used in several European countries, it has been demonstrated that genetic variation within the *VKORC1* and *CYP2C9* genes play an important role in the response to therapy. In a GWAS within 1451 whites, besides other significant polymorphisms in other genes, polymorphisms within *VKORC1* (most significant polymorphism: rs10871454, chromosome 16, $P=2.0\times10^{-123}$) and *CYP2C9* (most significant polymorphism: rs4086116, chromosome 10, $P=3.3\times10^{-24}$) contributed to the variance in acenocoumarol dosage. It was established that besides age, sex, body mass index, and target INR, one polymorphism within each of the *VKORC1*, *CYP2C9*, *CYP4F2*, and *CYP2C18* genes could explain 48.8% of the variation in acenocoumarol dosage.[99]

For platelet-inhibiting agents, most genetic studies have focused on clopidogrel. Clopidogrel is an inactive prodrug and the cytochrome P450 2C19 (CYP2C19) enzyme is involved in the conversion of clopidogrel into its active metabolite. The *CYP2C19**2 polymorphism (rs4244285) results in a decreased activity of the CYP2C19 enzyme, leading to diminished plasma concentrations of the active metabolite of clopidogrel. This decreases the therapeutic effect of clopidogrel and higher doses are needed to reach their optimal effect.[100-104] Whether these lower plasma levels also lead to increased risk of cardiovascular events due to inefficiency of the drug is questionable: large studies or reviews find an increased risk of cardiovascular events in carriers of a reduced-function allele[101,102], but other studies could not demonstrate an association with cardiovascular events.[103,104]

Overview and future challenges

In this paper, we have summarized current knowledge concerning the most important genetic associations in the response to cardiovascular drug therapy. There is also much literature about pharmacogenetic determinants of cardiovascular effects by drugs with other indications, such as by tricyclic antidepressants, which may prolong the QT interval duration, but these associations were not covered in this paper.[105] A couple of conclusions can be made.

First, it is clear that despite abundant literature we know relatively little about pharmacogenetic determinants of drug response. Much pharmacogenetic literature is contradictory and gives a scattered picture of the topic. Probably, many apparently contradictory results come from lack of power in candidate gene studies, lack of standardization, and lack of collaboration between studies within a large consortium. Also, the likelihood that for complex phenotypes the contribution of pharmacogenetic determinants may be difficult to disentangle from other risk factors may have contributed to the relative lack of consistency between studies. Consequently, most clinically relevant knowledge pertains to pharmacogenetics of drug metabolism by the cytochrome

P450 system, where a relative wealth of clinical pharmacological literature facilitates the performance of candidate gene studies with blood levels as an outcome. Second, despite the fact that during the past decades much effort has been put into pharmacogenetic research and many studies have been published, the pharmacogenetics of cardiovascular drug therapy has not had substantial clinical consequences. Apart from the abovementioned argument of contradictory literature, this is explained by the fact that most drugs can be titrated on clinical symptoms (e.g., digoxin) or biomarkers such as the INR (e.g., warfarin). Although health authorities such as the US Food and Drug Administration give dose recommendations based on genetic polymorphisms for some drugs (e.g., warfarin), there is no indication that this has led to substantial implementation in clinical practice. Although this might seem to be a negative appraisal, it does not mean that pharmacogenetic research was a waste of time, effort, and resources. Pharmacogenetic research provided us with important scientific insights into drug metabolism and actions. For instance, pharmaceutical companies will be reluctant to develop a drug that is metabolized by CYP2D6 because of the high prevalence of "poor metabolizers." Moreover, pharmacogenetic research is only at an early stage because progressive cost reduction of DNA analyses, and increasing international cooperation in study consortia such as CHARGE (Cohorts for Heart and Aging Research in Genomic Epidemiology),[106] are leading to discovery of important polymorphisms in candidate genes studies as well as in GWAS. Moreover, new genetic approaches, the "next-generation sequencing," will undoubtedly have an important role in revealing new and also rare loci of genetic variation. Especially, pharmaceutical companies should play a more active role than they currently do by focusing on variations of known pharmacological entities. New genetic associations, and their synergism with patient characteristics, should be investigated more proactively to determine specific groups of patients for whom genetic testing is relevant. Hopefully, future findings will make it possible that, based on a pharmacogenetics profile, drug therapy can be individualized. This would improve efficacy of therapy, reduce the risk of ADRs, and minimize costs. However, before we reach that point, if ever, there is still a long way to go.

1.2 AIM AND OUTLINE OF THIS THESIS

The aim of this thesis was to gain more insight into the pharmaco(genetic) epidemiology of statins in an ageing population. The main purposes were 1) to identify genetic determinants that modify the response to statin therapy, 2) to investigate unintended effects of the use of statin therapy in clinical practice, and 3) to investigate different methodological techniques to estimate the effect of time-dependent statin use in observational studies. All studies in this thesis are embedded in the Rotterdam Study, a

prospective population-based cohort study among 14,926 inhabitants of Ommoord, a suburb of Rotterdam, aged 45 years and older. The objectives and design of the Rotterdam Study were described in detail previously.[107,108] Since the start of the study in 1990, follow-up examinations were conducted periodically. Blood samples were obtained from which genomic DNA was extracted, medication dispensing data were available on a daily basis through linkage with pharmacies in the Ommoord suburb, and the cohort is continuously monitored for major morbidity and mortality through linkage with general practitioner's records.

In chapter 2, we describe genetic factors that modify the efficacy, effectiveness, and risk of ADRs of statins in clinical practice. We used the GWAS approach to discover new genetic markers without *a priori* hypothesis of the underlying genetic variation, and the candidate gene approach to replicate genetic variation that has previously been associated with a modified statin response or occurred in a pathway that relates to statin pharmacokinetics. Chapter 3 covers general (non-genetic) epidemiological studies on unintended effects of the use of statins in clinical practice. Use of statins is studied in relation with NAFLD, a disease which is frequently associated with dyslipidemia, and in which statins are frequently prescribed. Moreover, the influence of statins on the levels of total and non sex hormone-binding globulin-bound testosterone is investigated. Cholesterol is a precursor in the formation of testosterone, and since statins lower cholesterol, these drugs may also influence testosterone levels. In chapter 4, we investigate whether marginal structural modeling (MSM) produces different effect estimates from more traditional Cox-proportional hazards models in estimating the effect of time-dependent drug use in observational studies. Statin use in the primary prevention of CVD is investigated as an example. Studies can be biased when a time-dependent covariable is simultaneously 1) a reason for prescribing or dose-changing (often termed 'confounding by indication'), 2) influenced by the drug under study, and 3) a potential cause of the outcome of interest. This can be adjusted for by MSM. Chapter 5 investigates whether serum SHBG can serve as a biomarker for the risk of NAFLD. Previous cross-sectional studies have demonstrated an inverse association between serum SHBG levels and NAFLD prevalence, but no statements on causality could be made. Finally, chapter 6 will provide a general discussion, in which we discuss the main findings of this thesis, discuss the consequences of widespread statin use, evaluate several methodological issues, and discuss the implementation of personalized medicine in future clinical practice.

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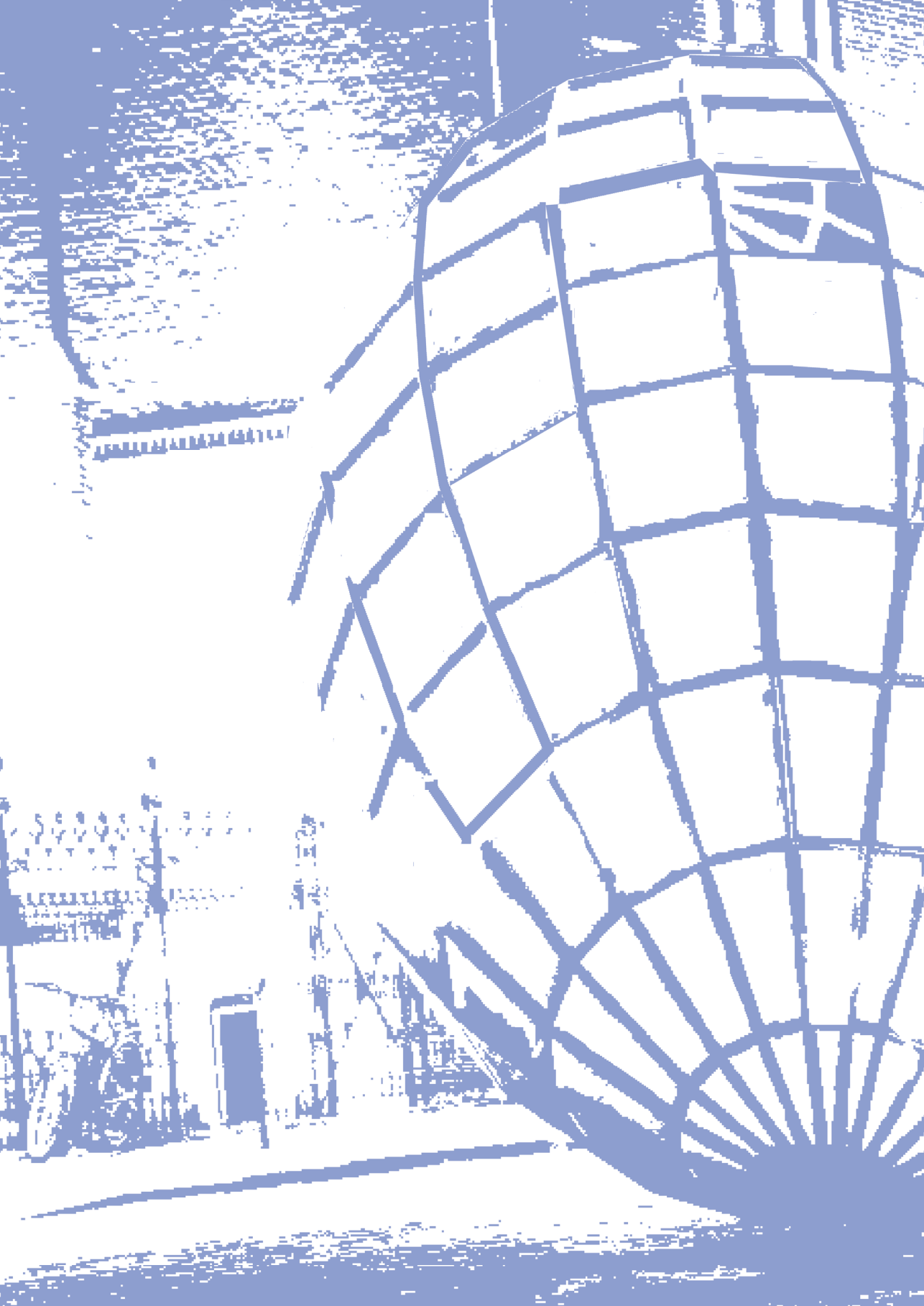

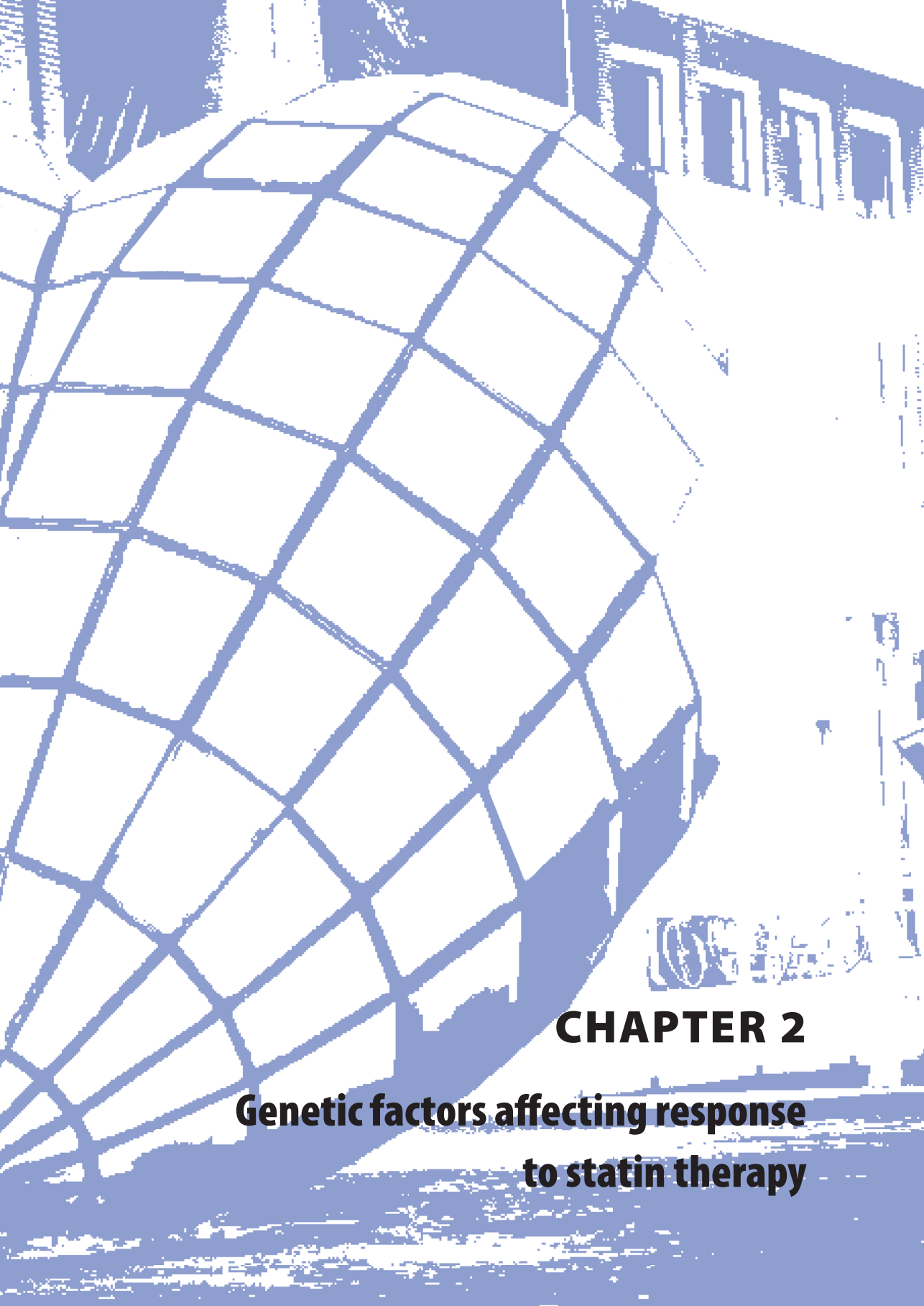

CHAPTER 2

Genetic factors affecting response to statin therapy

2.1

Single nucleotide polymorphisms in genes that are associated with a modified response to statin therapy: the Rotterdam Study

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The Pharmacogenomics Journal 2011; 11(1): 72-80

ABSTRACT

Introduction: The objective of this study was to investigate whether common variation in genes involved in lipid metabolism modify the effect of statins on serum total cholesterol concentration.

Methods: Statin users were identified in the Rotterdam Study, a prospective population-based cohort study of subjects >55 years of age. We studied the association between single nucleotide polymorphisms (SNPs) in genes involved in lipid metabolism and total cholesterol response to statin therapy, using linear regression analysis and adjusting for potential confounders. Replication was performed in an independent extended cohort of the Rotterdam Study.

Results: Genotype data and total cholesterol concentrations after start of statin therapy were available for 554 newly started statin users. Two SNPs were associated with a significantly higher cholesterol concentration under statin therapy: SNP rs1532624 in the CETP gene (β 0.141 mmol/L, P 0.004 per additional allele) and SNP rs533556 in the APOA1 gene (β 0.138 mmol/L, P 0.005 per additional allele). In the replication sample, only the CETP rs1532624 SNP again showed a significant association. The SNPs were not related to baseline total cholesterol in non-statin users.

Conclusion: In conclusion, we found that the CETP rs1532624 polymorphism is associated with cholesterol response to statin therapy in a cohort of elderly subjects in the general population.

INTRODUCTION

Cardiovascular disease (CVD) is a major health problem and is one of the leading causes of death in industrialized countries.[1,2] It is a multifactorial complex disease, composed of several vascular disorders and many factors contribute to the risk of CVD. One of the major risk factors for CVD is high serum cholesterol, especially increased serum low-density lipoprotein (LDL).[3] Pharmacological interventions, principally lipid-lowering therapy, are an essential component of the clinical management of CVD.[3-5] The 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, or statins, have shown to be beneficial for primary and secondary prevention of CVD, due to their cholesterol lowering activity.[6-11] Statins competitively inhibit HMG-CoA reductase (HMGCR), the rate-limiting enzyme in the cholesterol biosynthesis pathway. This leads to the upregulation of LDL-receptor activity and reduced secretion of apolipoprotein B (apoB)-containing lipoproteins from the liver, both of which contribute to lowering of the LDL-cholesterol concentration in plasma.[12,13] By this mechanism, statins have the potential to protect against cardiovascular morbidity and mortality.

Nevertheless, there is a considerable variability between individuals in response to statins, in terms of both cholesterol lowering and clinical outcomes, of which the origins are poorly understood. A part of this variation may be explained by genetic factors.[14,15] Pharmacogenetics focuses on genetic polymorphisms that influence response to drug therapy. Over the past decade, many studies have focused on identifying potential genetic determinants regarding response to statin therapy. Markers that are predictive of statin efficacy or the occurrence of statin-related adverse drug reactions could be useful in tailoring treatment, based on individual subjects or certain subgroups.

Because statins have an influence on serum cholesterol concentration, single nucleotide polymorphisms (SNPs) in genes involved in the lipid metabolism may be involved in the interindividual variability in cholesterol response to statin therapy. In this study, we investigated whether common variation in genes involved in lipid metabolism modify the effect of statins on total serum cholesterol concentration.

METHODS

Setting

The Rotterdam Study is a prospective population-based cohort study of chronic diseases in the elderly population. From 1990 to 1993, all inhabitants of Ommoord, a district of the city of Rotterdam in the Netherlands, aged 55 years or over, were invited to participate (n=10 278) in the Rotterdam Study I (RS-I). Of them, 78% (n=7983) gave the written informed consent, including permission for retrieval of medical records, use of blood

and DNA for research purposes, and publication of obtained results. The medical ethics committee of the Erasmus Medical Center, Rotterdam, the Netherlands, approved the study. Baseline examinations took place from March 1990 through July 1993. Follow-up examinations were conducted periodically, every 4–5 years. The cohort was continuously being monitored for major morbidity and mortality through linkage of the Rotterdam Study database with general practitioner and municipality records. Furthermore, exposure to medication was continuously monitored since 1 January 1991, through fully computerized pharmacy records from the seven linked pharmacies in the Ommoord district. Information on all dispensed drugs was available in computerized format on a day-to-day basis. The data consisted of information on the date of prescribing, the total amount of drug units per prescription, the prescribed daily number of units, the product name of the drugs and the Anatomical Therapeutic Chemical code. DNA for genotyping was available for 6571 (82%) participants from the baseline visit.

Furthermore, in 2000, an extended cohort was enrolled, the Rotterdam Study II (RS-II). All inhabitants of Ommoord, aged 55 years or older and not enrolled in the RS-I, were invited to participate in this extended cohort (n=5404). Of them, 3011 (67%) entered the study and took part in the baseline examination. The second visit for the RS-II took place between 2004 and 2005.

Detailed information on design, objectives and methods of the Rotterdam Study has been given elsewhere.[16,17]

Study population

The source population consisted of all participants of the RS-I, who were successfully genotyped as part of a large population-based project on genetics of complex traits and diseases, financed by the Dutch government through the Netherlands Scientific Organization—Large Investments (NWO Groot 175.010.2005.011) (n=5974). The study population consisted of all participants of the RS-I, who received a prescription for statin therapy in the study period between 1 April 1991 and 1 January 2008, and who had at least one serum cholesterol measurement available at any time during the study period after prescription of statin therapy (n=554).

Outcome definition

In this study, the outcome of interest was cholesterol response to statin therapy. For every statin user in the population, the first serum total cholesterol measurement after start of prescription of statin therapy was defined as the outcome variable, with the date of cholesterol measurement at least 1 week after the prescription date of statin therapy. This first cholesterol measurement was considered as a measure of cholesterol response to statin therapy. In the Rotterdam Study, serum cholesterol levels are assessed at baseline examination and subsequently during follow-up examinations. As of April

1997, also fasting total cholesterol levels assessed between follow-up examinations as part of patient care were gathered by linkage to the general practitioners' laboratory 'Stichting Artsenlaboratorium Rotterdam en Omstreken' (Starlab).

Genotyping and SNP selection

At the baseline examination of the Rotterdam Study, blood was taken from which genomic DNA was extracted, using the salting-out method.[18] Microarray genotyping was performed in the whole original Rotterdam Study cohort using the Infinium II HumanHap550K Genotyping Bead-Chip version 3 (Illumina Inc., San Diego, CA, USA). Genotyping procedures were followed according to the manufacturer's protocols. Microarray genotyping procedures in the Rotterdam Study have been previously described.[19]

In this study the selection of SNPs was performed, using a candidate gene approach. On the basis of literature[13,20,21], we selected 18 genes involved in lipid metabolism. The selected genes were *HMGCR*, squalene synthase (*FDFT1*), cholesterol 7a hydroxylase (*CYP7A1*), LDL-receptor (*LDLR*), APOB, apolipoprotein E (*APOE*), apolipoprotein A-I (*APOA1*), cholesteryl ester transfer protein (*CETP*), ATP-binding cassette transporter A1 (*ABCA1*), ATP-binding cassette transporter G8 (*ABCG8*), paraoxonase 1 (*PON1*), sterol regulatory element-binding protein 1 (*SREBF1*), peroxisome proliferator-activated receptor-d (*PPARD*), peroxisome proliferator-activated receptor-g (*PPARG*), lipoprotein lipase (*LPL*), hepatic lipase (*LIPC*), microsomal triglyceride transfer protein (*MTP*) and leptin receptor (*LEPR*). Markers were excluded if they deviated significantly from Hardy-Weinberg equilibrium ($P<1\times10^{-4}$), if they had a low minor allele frequency (MAF<0.05) or if they had an SNP call rate <95% within the samples. All SNPs present within the gene area of each individual gene ±50 kb were extracted from the data set. This resulted in a data set of 667 SNPs in total for the 18 candidate genes together.

Covariables

The following covariates were considered as potential determinants for affecting the association between genotype and serum total cholesterol concentration in a population only consisting of statin users: age, gender, baseline total cholesterol, high-density lipoprotein (HDL)-cholesterol at baseline, average defined daily dose of statin therapy and duration of statin use. During the baseline visit at the research center, nonfasting blood samples were obtained, and serum lipid parameters of total cholesterol and HDL-cholesterol were determined by an enzymatic procedure.[22] The prescribed dose of statin therapy is given as the average defined daily dose, calculated as the total defined daily dose of statin therapy over the total follow-up time divided by the total number of days of exposure over the same time period. Duration of statin use is defined as the time between the prescription date of statin therapy and the date of first serum total cholesterol measurement after at least 1 week of statin therapy.

Analysis

Differences in baseline characteristics between statin users and the source population were tested using a X^2-test for binary variables and a t-test for continuous variables. Hardy-Weinberg equilibrium was tested using a X^2-test.

We performed two statistical analyses in a stepwise approach. First, we investigated which of all selected SNPs (n=667) influenced the cholesterol response to statin therapy, by using linear regression analysis with Plink version 1.01 (Purcell et al., Boston, MA, USA).[23] All selected SNPs were tested on total cholesterol concentration after start of statin therapy under an additive model, adjusted for age, gender, baseline cholesterol, HDL-cholesterol at baseline, statin dose and duration of statin use. To take into account the problem of multiple testing, per individual gene, we applied a Bonferroni correction for the number of SNPs within the gene region. The Bonferroni P-value was calculated by multiplying the observed P-value by the number of SNPs tested for association within the gene region. Because a priori candidate genes in the lipid metabolism were selected based on literature, Bonferroni correction was performed per individual gene. Selected on this Bonferroni P-value, the promising SNPs were also studied in a replication cohort, to reduce the chance of reporting a false-positive association. Because our aim was to investigate polymorphisms that influence cholesterol concentration in response to statin therapy, and not polymorphisms that influence cholesterol concentration in general, we also performed a linear regression analysis on baseline cholesterol in non-statin users to investigate the relationship between each SNP and baseline cholesterol. This linear regression was performed on all subjects who had not received any statin at baseline and who had a baseline cholesterol measurement available (n=5749).

The SNPs that showed a $P<0.1$ after Bonferroni correction, and were not significantly related to baseline cholesterol, were further analyzed. We tested the remaining SNPs in a genotypic model on cholesterol concentration after start of statin therapy, using SPSS for Windows software, version 15.0 (SPSS Inc., Chicago, IL, USA). We compared cholesterol concentration after start of statin therapy between the different genotype categories, whereby we adjusted for age, gender, baseline cholesterol, HDL-cholesterol at baseline, statin dose and duration of statin use.

Replication

To avoid the reporting of a false-positive association, we repeated the analysis for those SNPs that showed a significant effect on cholesterol concentration in response to statin therapy in the discovery cohort (RS-I). This replication study was performed in an independently collected cohort, the RS-II. The source population for replication consisted of all participants in the RS-II, who were successfully genotyped (n=1895). The study population consisted of those subjects in the source population who received a prescription for statin therapy in the study period between baseline date and 1 January

2008, and who had at least one serum cholesterol measurement available at any time during the study period at least 1 week after prescription of statin therapy (n=243). The outcome variable was cholesterol concentration after start of statin therapy, as described earlier. We performed a linear regression analysis, in which both SNPs were tested in an additive and a genotypic model. We compared cholesterol concentration after start of statin therapy between the different genotype categories, whereby we adjusted for age, gender, baseline cholesterol, HDL-cholesterol at baseline, statin dose and duration of statin use. Finally, we performed a fixed effect inverse variance meta-analysis to combine the results of the original analysis (RS-I) and the results of the replication analysis (RS-II).

RESULTS

Population characteristics

The baseline characteristics of the source and study population (RS-I) are shown in Table 1. Overall, the source population consisted of all subjects, of whom genotype data were available (n=5974). The mean age in the population was approximately 69.4 years and 59.4% of the total population were women. For the analysis on cholesterol response to statin therapy the study population consisted of all subjects who received statin therapy between 1 April 1991 and 1 January 2008 (n=554). For all these 554 statin users, a serum cholesterol measurement at least 1 week after start of statin therapy was available. The median time between the prescription date of statin therapy and the date of first serum total cholesterol measurement was 80 days (interquartile range: 40–174 days), with a minimum of 8 days and a maximum of 1068 days. The average defined daily dose of statin therapy was 1.12 (SD: ±0.71). Of the different kind of statins, simvastatin was prescribed most frequently (n=337, 60.8%), followed by atorvastatin (n=108, 19.5%) and pravastatin (n=66, 11.9%).

Cholesterol response

Of all 667 SNPs, only 2 showed evidence for association with cholesterol response to statin therapy under an additive model, after Bonferroni correction at a liberal P-value of <0.1. The two SNPs that remained under this P-value threshold were the SNP rs1532624 in the CETP gene (β 0.153, original P-value 0.002, Bonferroni P-value 0.084) and the SNP rs533556 in the APOA1 gene (β 0.140, original P-value 0.004, Bonferroni P-value 0.039). Both SNPs were not related to baseline total cholesterol in non-statin users in the discovery cohort (RS-I).

The results of the linear regression analysis on cholesterol concentration after start of statin therapy under a genotypic model are shown in Table 2. Subjects with a minor

Table 1 Baseline characteristics

Characteristic	Source population (n = 5974)	Study population (RS-I) (n = 554)	Replication population (RS-II) (n = 243)
Age, years (mean±SD)	69.4 ± 9.1	64.5 ± 5.5	64.5 ± 7.1
Gender, n (%)			
– men	2427 (40.6)	232 (41.9)	110 (54.3)
– women	3547 (59.4)	322 (58.1)	133 (54.7)
Total cholesterol, mmol/L (mean±SD)	6.6 ± 1.2	7.6 ± 1.3	5.5 ± 1.1
HDL-cholesterol, mmol/L (mean±SD)	1.3 ± 0.4	1.3 ± 0.4	1.3 ± 0.3
Hypertension, n (%)	1997 (34.3)	219 (39.8)	–
Smoking, n (%)			
– current	1339 (23.0)	135 (24.7)	56 (23.0)
– former	2425 (41.7)	258 (47.2)	124 (51.0)
– never	2046 (35.2)	154 (28.2)	63 (25.9)
Body mass index, kg/m^2 (mean±SD)	26.3 ± 3.7	26.7 ± 3.6	27.9 ± 3.9
Diabetes mellitus, n (%)	631 (10.6)	54 (9.7)	–
Heart failure, n (%)	194 (3.2)	11 (2.0)	7 (2.9)
Statin therapy, n (%)	–		
– Simvastatin		337 (60.8)	133 (54.7)
– Pravastatin		66 (11.9)	35 (14.4)
– Fluvastatin		38 (6.9)	21 (8.6)
– Atorvastatin		108 (19.5)	52 (21.4)
– Cerivastatin		5 (0.9)	2 (0.8)
Statin dose, AVDDD (mean±SD)	–	1.12 ± 0.71	1.24 ± 0.86

Abbreviations: AVDDD, average defined daily dose; HDL, high-density lipoprotein; n, number; SD, standard deviation.

allele of the SNP rs1532624 in the CETP gene showed a higher cholesterol concentration after start of statin therapy than subjects homozygous for the major allele, the reference category. This effect of genotype on cholesterol concentration was borderline non-significant for the heterozygous genotype compared with the homozygous major allele genotype (β 0.144 mmol/L, P 0.054), and significant for the variant genotype compared with the homozygous major allele genotype (β 0.281 mmol/L, P 0.006). In our population, the MAF of the rs1532624 SNP was 40.6% (A allele). Subjects with a minor allele of SNP rs533556 in the APOA1 gene showed a higher cholesterol concentration after start of statin therapy than subjects homozygous for the major allele, the reference

Table 2 Relationship between genotype and cholesterol response among statin users in RS-I

SNP (gene)	Genotype	Number (%)	Effect genotype on total cholesterol in mmol/L (95% CI)	P
rs1532624 (*CETP*)				
Additive model		546 (100)	**0.141 (0.045; 0.237)**	**0.004**
Genotypic model	CC	191 (35.0)	(ref)	–
	CA	267 (48.9)	0.144 (–0.002; 0.291)	0.054
	AA	88 (16.1)	**0.281 (0.081; 0.481)**	**0.006**
rs533556 (*APOA1*)				
Additive model		546 (100)	**0.138 (0.041; 0.235)**	**0.005**
Genotypic model	CC	230 (42.1)	(ref)	–
	CA	245 (44.9)	**0.178 (0.036; 0.320)**	**0.014**
	AA	71 (13.0)	**0.246 (0.037; 0.456)**	**0.021**

Abbreviations: *APOA1*, apolipoprotein A-I; *CETP*, cholesteryl ester transfer protein; CI, confidence interval; SNP, single nucleotide polymorphism.
Effect of CETP and APOA1 genotype on cholesterol concentration after start of statin therapy, using an additive and genotypic model. Mean differences in total cholesterol concentration in mmol/L after at least 1 week of statin use are shown. For both SNPs, genotype CC is homozygous for the major allele, genotype CA is heterozygous and genotype AA is homozygous for the variant allele. P-values are adjusted for age, gender, baseline cholesterol, HDL-cholesterol at baseline, statin dose and duration of statin use. **Bold** value indicates statistically significant association.

category. This effect of genotype on cholesterol concentration was significantly different for both the heterozygous genotype and the homozygous variant allele genotype, compared with the homozygous major allele genotype (β 0.178 mmol/L, P 0.014 and β 0.246 mmol/L, P 0.021, respectively). In our study population, the MAF of the rs533556 SNP was 35.5% (A allele). Genotype distributions for the CETP genotype and the APOA1 genotype are given in Table 3.

Replication

The baseline characteristics of the replication sample (RS-II) are shown in Table 1. The statins used were comparable between the discovery cohort and the study population for replication. Also, the distribution of statin use was comparable between both cohorts, with in the replication cohort a median time of 78.5 days (interquartile range: 40–148 days) between the prescription date of statin therapy and the date of first serum total cholesterol measurement, with a minimum of 8 days and a maximum of 1260 days. A serum cholesterol measurement after start of statin therapy was available for 385 statin users. Of these statin users, 239 subjects had genotype data available for the CETP polymorphism, and 243 had genotype data available for the APOA1 polymorphism.

Genotype distributions for the CETP and the APOA1 genotypes are given in Table 3. In the replication population, the MAF of the CETP rs1532624 SNP was 40.2% (A allele) and

Table 3 Genotype frequencies of *CETP* and *APOA1* SNPs

SNP (gene)	Genotype	Genotype frequency in source population (RS-I) N (%)	Genotype frequency in study sample (RS-I) N (%)	Genotype frequency in source population (RS-II) N (%)	Genotype frequency in replication sample (RS-II) N (%)
rs1532624 (*CETP*)					
	CC	1890 (31.7)	194 (35.0)	591 (31.5)	83 (34.7)
	CA	2935 (49.2)	272 (49.1)	912 (48.6)	120 (50.2)
	AA	1142 (19.1)	88 (15.9)	373 (19.9)	36 (15.1)
rs533556 (*APOA1*)					
	CC	2589 (43.5)	235 (42.4)	821 (43.3)	92 (37.9)
	CA	2677 (44.9)	247 (44.6)	859 (45.3)	119 (49.0)
	AA	692 (11.6)	72 (13.0)	214 (11.3)	32 (13.2)

Abbreviations: *APOA1*, apolipoprotein A-I; *CETP*, cholesteryl ester transfer protein; SNP, single nucleotide polymorphism.

Genotype distributions for the *CETP* and *APOA1* SNPs in the source population and study population, both for the discovery cohort and for the replication cohort. For both SNPs, genotype CC is homozygous for the major allele, genotype CA is heterozygous, and genotype AA is homozygous for the variant allele.

Table 4 Relationship between genotype and cholesterol response among statin users in RS-II (replication)

SNP (gene)	Genotype	N (%)	Effect genotype on total cholesterol in mmol/L (95% CI)	P
rs1532624 (*CETP*)				
Additive model		239 (100)	**0.203 (0.048; 0.359)**	**0.011**
Genotypic model	CC	83 (34.7)	(ref)	–
	CA	120 (50.2)	0.108 (–0.123; 0.339)	0.358
	AA	36 (15.1)	**0.460 (0.134; 0.785)**	**0.006**
rs533556 (*APOA1*)				
Additive model		243 (100)	–0.003 (–0.161; 0.156)	0.975
Genotypic model	CC	92 (37.9)	(ref)	–
	CA	119 (49.0)	–0.012 (–0.241; 0.216)	0.915
	AA	32 (13.2)	–0.002 (–0.339; 0.344)	0.989

Abbreviations: SNP, single nucleotide polymorphism; *CETP*, cholesteryl ester transfer protein; *APOA1*, apolipoprotein A-I; CI, confidence interval.

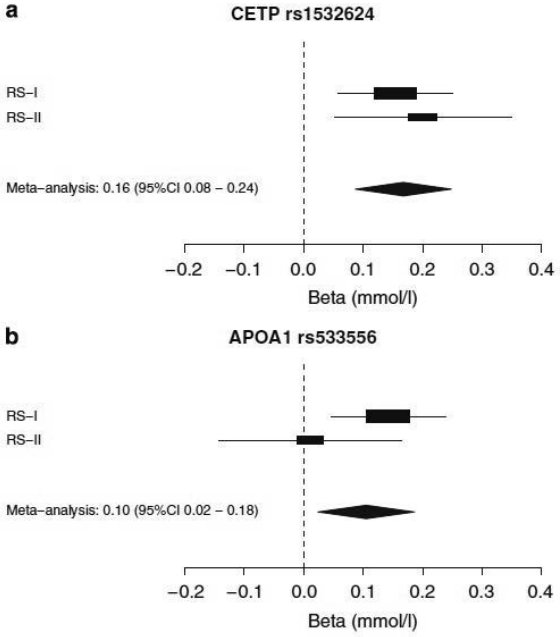
Effect of *CETP* and *APOA1* genotype on cholesterol concentration after start of statin therapy, using an additive model and a genotypic model. Mean differences in total cholesterol concentration in mmol/L after at least 1 week of statin use are shown. For both SNPs, genotype CC is homozygous for the major allele, genotype CA is heterozygous and genotype AA is homozygous for the variant allele. P-values are adjusted for age, gender, baseline cholesterol, HDL-cholesterol at baseline, statin dose and duration of statin use. **Bold** value indicates statistically significant association.

the MAF of the APOA1 rs533556 SNP was 37.7% (A allele). Both genotype distributions were in Hardy–Weinberg equilibrium.

Results of the linear regression analysis on cholesterol concentration after start of statin therapy under a genotypic model are shown in Table 4. Subjects with a minor allele of the rs1532624 SNP in the CETP gene showed a higher cholesterol concentration after start of statin therapy than subjects homozygous for the major allele, the reference category. This effect of genotype on cholesterol concentration was significant for the variant genotype compared with the homozygous major allele genotype (effect: 0.460 mmol/L, P 0.006), but not significant for the heterozygous genotype compared with the homozygous major allele genotype (effect: 0.108 mmol/L, P 0.358). For the rs533556 SNP in the APOA1 gene, no significant difference in cholesterol concentration after start of statin therapy between the different genotype categories was observed.

The results of the fixed effect inverse variance meta-analysis are shown in Figure 1. For the rs1532624 SNP in the CETP gene the β was 0.16 mmol/L (95% CI for β 0.08–0.24 mmol/L), with a P-value of 1.4×10^{-4}. For the rs533556 SNP in the APOA1 gene the β was 0.10 mmol/L (95% CI for β 0.02–0.18 mmol/L, with a P-value of 0.02.

Figure 1 Results of the meta-analysis of the *CETP* and *APOA1* single nucleotide polymorphisms (SNPs)



Fixed effect inverse variance meta-analysis of the effect of the rs1532624 SNP in the *CETP* gene and the rs533556 SNP in the *APOA1* gene on cholesterol concentration after start of statin therapy, in the Rotterdam Study (RS-I) and an extended cohort of the Rotterdam Study (RS-II). For each study, the β with corresponding 95% CI is reported. (a) rs1532624 in the *CETP* gene. (b) rs533556 in the *APOA1* gene.
Abbreviations: *CETP*, cholesteryl ester transfer protein; *APOA1*, apolipoprotein A-I; CI, confidence interval.

DISCUSSION

In this study, we investigated whether SNPs in genes involved in the lipid metabolism modify the relationship between statin therapy and cholesterol response. We showed a statistically significant association between the rs1532624 polymorphism, located in intron 7 of the CETP gene, and cholesterol response to statin therapy. Subjects with a variant allele of the polymorphism showed a significantly smaller cholesterol reduction after start of statin therapy. This association was replicated in an independent cohort, reducing the chance of a false-positive association. There appears to be a dose–allele effect: the cholesterol concentration was on average 0.16 mmol/L higher per additional allele. We also demonstrated that subjects with at least one variant allele of the rs533556 in the APOA1 polymorphism showed a significantly smaller cholesterol reduction after start of statin therapy in the discovery sample, however this association failed to replicate.

To the best of our knowledge, the SNP (rs1532624) identified in this study has not been described before in the literature in association with cholesterol response to statin therapy. Previous pharmacogenetic studies investigated the relationship between CETP gene polymorphisms and lipid concentration.[24-35] The polymorphism in the CETP gene that is most frequently described is the noncoding mutation TaqIB SNP in intron 1. Several studies investigated the relationship between the TaqIB polymorphism and response to statin therapy.[25,30,31,33-35] However, these studies used different study populations and examined different outcomes, for example, serum cholesterol concentrations, atherosclerosis progression and CVD outcomes, and the results are not conclusive. None of these studies showed a difference between the TaqIB genotype categories in total cholesterol concentration after start of statin therapy, the outcome variable we used in our study. Fiegenbaum et al.[33] showed a greater HDL cholesterol increase for the CETP B2B2 homozygotes than for B1B2 and B1B1 subjects (14.1 vs 1.7% and 1.3%, $P<0.05$) in response to simvastatin treatment, but no effect on total cholesterol was seen. De Grooth et al.[34] investigated the TaqIB polymorphism and change in total cholesterol, LDL-cholesterol and HDL-cholesterol, and reduction in nonfatal myocardial infarction and coronary heart disease mortality, in response to pravastatin treatment, but no difference between the genotype categories was shown. We have investigated whether the CETP rs1532624 SNP we found in this study was in linkage disequilibrium with the CETP TaqIB polymorphism. In our study, the TaqIB polymorphism was not available at the Illumina chip, but it was genotyped separately. The R^2 between the CETP TaqIB SNP and the CETP rs1532624 SNP was 0.88.

The main difference between our study and the previous pharmacogenetic studies is the way the SNPs were selected for analysis. Most studies comprised only a few SNPs, selected from known candidate genes in the cholesterol or statin metabolism, and

known to be related to cholesterol. Although our study also used a candidate gene approach, we selected 18 genes all involved in the lipid metabolism from the literature. We aimed to identify those SNPs that define cholesterol concentration in response to statin therapy, and not SNPs that may define cholesterol concentration in general.

In this study, the assumption was made that a response to statin therapy was observed after at least 1 week of statin therapy. It might be argued that this period is too short for statins to exert an effect. In clinical trials, often at least a 4-week period of statin therapy is taken, before the outcome is measured. In our study, 15.5% of all statin users had a first serum total cholesterol measurement between 1 and 4 weeks after the prescription date of statin therapy. However, it is unlikely that this results in a bias, as the time between the prescription date of statin therapy and the date of first serum total cholesterol measurement is likely to be independent of genotype. Inclusion of the time between the prescription of statin therapy and the first serum total cholesterol measurement in the linear regression analysis did not affect the results. Furthermore, when comparing subjects with a short duration of statin therapy use with subjects with a long duration of statin therapy use, the mean cholesterol response measurement was similar for both the groups.

Regarding the genotype distributions of the CETP polymorphism, given in Table 3, both in the discovery cohort and in the replication cohort a difference in genotype frequency between the source population and the study population was observed. In the study population, a lower frequency of subjects homozygous for the variant genotype was observed, and a higher frequency of subjects homozygous for the major allele, compared with the source population. An explanation for this difference might be that CETP genotype is related to the indication for statin therapy. In our population, the CETP genotype was related to HDL-cholesterol: subjects with a variant allele had a significantly higher baseline HDL-cholesterol, whereas the baseline total cholesterol concentration was equivalent to subjects homozygous for the major allele. Therefore, subjects with a variant allele have a better ratio between HDL- and LDL-cholesterol, and may be less likely candidates for statin therapy. This may explain the fact that in the study population of only statin users we observed fewer subjects homozygous for the variant genotype. However, this does not influence the validity of our findings, as our study population only consisted of statin users.

Potential limitations of our study should be considered. Selection bias is unlikely as missing of blood samples and difficulties with genotyping are not related to CETP and APOA1 genotype. Information bias is not likely, as genotype data, data on cholesterol measurements, and prescription data of statin therapy were collected prospectively without prior knowledge of the aim of the study. We controlled for potential confounding factors such as age, gender, baseline total cholesterol, baseline HDL-cholesterol and statin dose in the linear regression analysis, in which we investigated potential effect

modification by genotype of the relationship between statin therapy and cholesterol response. Furthermore, because statins primarily have their effect on serum LDL-cholesterol concentration, we preferred to consider serum LDL-cholesterol concentrations in response to statin therapy as our outcome variable of interest, instead of serum total cholesterol concentrations. Because LDL-cholesterol was determined less frequently in the Rotterdam Study, we unfortunately could not take this measurement as the outcome variable, as we had not enough power because of small sample size. Equally, we had an insufficient number of subjects to take Δ cholesterol, the difference between serum cholesterol concentration before and after start of statin therapy, as our outcome variable of interest. If we could have taken the Δ cholesterol as our outcome variable, this would allow us to include SNPs that were related to both baseline total cholesterol concentration and serum total cholesterol concentration under statin therapy. For example, we had included a polymorphism in the APOE gene, which is one of the most replicated statin pharmacogenetic interactions.[36-39] In this study, the polymorphism in the APOE gene had an effect on serum total cholesterol concentration under statin therapy, but was excluded because it was also related to baseline total cholesterol concentration. Finally, we could not exclude that we did not find a significant association for some of the remaining SNPs, due to a lack of power.

Our study has several strengths. An advantage of the Rotterdam Study is the prospective ascertainment of risk factors and outcome variables over a relatively long period of follow-up, through which extensive information is available for use in the analysis. Also, the Rotterdam Study has a population-based character, which reduces the risk of selection bias. Another strength of our study is the replication in an independent cohort to minimize the chance of reporting a false-positive association, however, the fact that our replication sample is collected from the same general population means that the replication does not add information on the generalizability to other populations.

In conclusion, we found that the CETP rs1532624 polymorphism was associated with total cholesterol concentration in response to statin therapy in a cohort of elderly European statin users and an independent replication sample in the general population. The findings in this study may explain a small part of the interindividual variability in cholesterol response to statin therapy, although further research is recommended to define the genetic profile that predicts response to statin therapy.

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2.2

Genetic variation in the *PPARA* gene is associated with simvastatin-mediated cholesterol reduction in the Rotterdam Study

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Pharmacogenomics 2013; 14(11): 1295-1304

ABSTRACT

Introduction: Recently, minor alleles of two strongly linked polymorphisms in the *PPARA* gene, rs4253728 G>A and rs4823613 A>G, were related to decreased CYP3A4 expression and activity. We studied whether they were associated with the cholesterol lowering effect of simvastatin.

Methods: We identified 123 incident users with cholesterol measurements before and after starting statin therapy in a prospective population-based cohort study. Associations between *PPARA* polymorphisms and change in total and low-density lipoprotein (LDL)-cholesterol levels were analyzed using linear regression.

Results: The minor G allele of the rs4823613 A>G polymorphism was associated with a 0.258 mmol/l (95% CI −0.470; −0.046) and a 0.294 mmol/l (95% CI −0.495; −0.093) larger reduction in total and LDL-cholesterol, respectively, after starting simvastatin therapy. Results were similar for the rs4253728 G>A polymorphism.

Conclusion: The minor alleles of the *PPARA* rs4253728 and rs4823613 polymorphisms are associated with a better total and LDL-cholesterol lowering response to simvastatin, possibly through influence on CYP3A4.

INTRODUCTION

The 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors or statins are cholesterol lowering drugs, widely prescribed for the primary and secondary prevention of cardiovascular disease.[1-4] Statins primarily act by lowering the low-density lipoprotein (LDL)-cholesterol concentration. In practice, the treatment goal of statin therapy is to reduce the LDL-cholesterol levels below a certain threshold, depending on the cardiovascular risk profile of a patient.[5,6] Physicians may achieve this goal by monitoring serum cholesterol levels and adapting the daily dosage accordingly. Nevertheless, despite this dose titration, there is still a substantial proportion of patients who do not achieve their recommended goal.[7,8] This may have reasons such as noncompliance or co-medications interacting with the drug. Variation in response can also be partly explained by genetic factors. SNPs in genes involved in the pharmacokinetics and pharmacodynamics of statins can interfere with the cholesterol lowering effect.[9,10] In previous years, several SNPs that were associated with differences in cholesterol lowering response to statins were discovered in either candidate gene studies or genome-wide association studies.[9-13]

The CYP3A4 enzyme is the main enzyme responsible for the metabolism of simvastatin, whereas there is also a minor contribution of CYP3A5.[14-17] In the past few years, functional polymorphisms in the *CYP3A4* and *CYP3A5* gene have been reported that influence metabolizing activity. The minor allele of the recently discovered intron 6 *CYP3A4*22* polymorphism has been associated with decreased CYP3A4 expression and function, and with a stronger cholesterol lowering response to simvastatin therapy.[18,19] The more commonly described *CYP3A4*1B* polymorphism has been associated with a decreased reduction in LDL-cholesterol levels in atorvastatin users[20], and with a lower risk of a dose decrease or a switch to another cholesterol lowering therapy – as proxies of an adverse drug reaction – in simvastatin and atorvastatin users.[21] *CYP3A5*3*, the most frequent and functionally relevant CYP3A5 polymorphism, has been associated with a decreased CYP3A5 expression and a stronger cholesterol lowering response to statin therapy.[22,23] The *CYP3A4*1B* allele is in strong linkage disequilibrium with the *CYP3A5*1* functional allele, and the *CYP3A5*3* allele is in strong linkage disequilibrium with the *CYP3A4*1A* allele.[24-26]

Recently, genetic variation in the peroxisome proliferator-activated receptor alpha (*PPARA*) gene, coding the nuclear receptor PPARα, was discovered as a novel genetic determinant influencing CYP3A4 activity.[27] The minor alleles of the strongly linked *PPARA* rs4253728 G>A and rs4823613 A>G polymorphisms were associated with significantly decreased CYP3A4 expression and activity. These polymorphisms might therefore influence the pharmacokinetics of drugs that are primarily metabolized by the CYP3A4 enzyme, such as simvastatin. We hypothesized that the minor alleles of the rs4253728

G>A and rs4823613 A>G polymorphisms are associated with an increased cholesterol lowering response of simvastatin.

In our study, the objective was to investigate whether the minor alleles of the rs4253728 G>A and rs4823613 A>G polymorphisms in the *PPARA* gene were associated with response to simvastatin therapy, as assessed by increased serum total cholesterol and LDL-cholesterol level reductions after start of therapy in a large population-based cohort study. We also investigated the association in users of other statins to exclude the possibility of a pharmacodynamic group effect. Analyses were adjusted for the effect of the *CYP3A4* and *CYP3A5* genotypes.

METHODS

Setting

The Rotterdam Study is a prospective population-based cohort study of chronic diseases in the elderly population. From 1990 to 1993, 7983 inhabitants of the suburb Ommoord in Rotterdam, The Netherlands, aged 55 years or older, entered the Rotterdam Study (RS-I) and gave written informed consent. Ethical approval was obtained from the medical ethical committee of the Erasmus Medical Center, Rotterdam, The Netherlands. Participants were invited between 1990 and 1993 and have been continuously followed since then. Medication prescription data were obtained from all seven fully computerized pharmacies in the Ommoord suburb. These pharmacies dispense the prescriptions of more than 99% of all participants. Information on all filled prescriptions from 1 January 1991 until 1 June 2008 was available and included information on the product name of the drug, the Anatomical Therapeutical Chemical code, the amount dispensed, the prescribed dosage regimen and the date of dispensing.[28] Furthermore, in 2000, an extended cohort was enrolled, the Rotterdam Study II (RS-II). A total of 3011 inhabitants entered the study and have been continuously followed since then. Detailed information on design, objectives and methods of this study have been described before.[29,30]

For this study, we used the total cholesterol and LDL-cholesterol assessments from the 'Star-Medisch Diagnostisch Centrum' (Star-MDC), which performs all outpatient laboratory assessments for general practitioners in the Rijnmond area of Rotterdam with a potential source population of more than 1 million inhabitants. All outpatient total cholesterol and LDL-cholesterol assessments from the participants of the Rotterdam Study between 1 April 1997, the time at which a new computer system was introduced at Star-MDC, and 1 June 2008 were obtained.

Study sample

The study sample consisted of all participants in the Rotterdam Study who were incident statin users in the period between 1 April 1997 and 1 June 2008; who had a measurement of total and/or LDL-cholesterol level in the period of 180 days before the first prescription and in the period between 7 and 180 days following the first prescription; and for whom DNA was available. Incident statin use was defined as a first dispensed prescription for a particular statin in the database, without prior prescriptions for other statins in the period between 1 January 1991 and 1 April 1997. Patients who discontinued statin therapy before the first measurement after the start of the study were excluded. Patients who were coprescribed fibrates, bile acid sequestrants, nicotinic acids or ezetimibe at the time of one of the measurements were also excluded.

Outcome

In this study, the outcome of interest was total and LDL-cholesterol response to statin therapy. We analyzed the association between the *PPARA* rs4253728 G>A and rs4823613 A>G polymorphisms and reductions in total and LDL-cholesterol level, measured as the difference in mmol/l between the last measurement before start and the first measurement after start of statin therapy.

Covariables

Age, sex, the level at the last total cholesterol/LDL-cholesterol measurement before start of statin therapy, the daily prescribed dose at the time of the first measurement after the start of statin therapy, *CYP3A4*22* (intron 6, rs35599367 C>T) genotype and *CYP3A5*3* (rs776746 A>G) genotype were considered as potential confounders or effect modifiers for affecting the association between the *PPARA* polymorphisms and the change in total and LDL-cholesterol level. The daily prescribed dose was given in defined daily dose, to facilitate direct dose comparisons between drugs from the same pharmacotherapeutic group. Adjustment for *CYP3A4*22* and *CYP3A5*3* genotype was done by adjusting for the number of minor alleles (additive model) per genotype.

Genotyping & SNP selection

At baseline examination of the Rotterdam Study, blood was taken from which genomic DNA was extracted, using the salting-out method.[31] Microarray genotyping was performed in both Rotterdam Study cohorts, using the Infinium II HumanHap550K Genotyping BeadChip version 3 (Illumina Inc., CA, USA). Genotyping procedures were followed according to the manufacturer's protocols. Microarray genotyping procedures in the Rotterdam Study have been previously described.[32] The *PPARA* polymorphisms rs4253728 G>A and rs4823613 A>G were both genotyped as a tagging SNP on the Illumina BeadChip. The two polymorphisms were in almost complete linkage disequilibrium ($R^2 = 0.96$).

Statistical analysis

We investigated the association between genetic variants and serum total and LDL-cholesterol in all incident simvastatin users, because we expected an association on the basis of the pharmacokinetic mechanism via CYP3A4 expression. The association was also investigated in incident atorvastatin users, since CYP3A4 is also a contributor to the metabolism of atorvastatin, albeit to a lesser extent. Furthermore, we investigated this association in all incident statin users combined to exclude a possible pharmacodynamic effect. Potential deviations from Hardy–Weinberg equilibrium were tested using a X^2 test. A multivariable linear regression model was used to analyze differences between the *PPARA* rs4253728 G>A and rs4823613 A>G genotype groups in change in total cholesterol and LDL-cholesterol. This was investigated for all statin users combined, and for starters with simvastatin and atorvastatin separately. We analyzed both additive models, investigating the change in total and LDL-cholesterol level per additive minor allele, and categorical models, investigating the change in total and LDL-cholesterol for the heterozygous genotype category and the homozygous minor allele genotype category compared with the homozygous major allele genotype category. Multivariable linear regression models were also used to analyze differences between the *PPARA* rs4253728 G>A and rs4823613 A>G genotypes and baseline total and LDL-cholesterol levels, and the differences in time from start of simvastatin therapy and the first total cholesterol measurement after the start of therapy. The analyses were performed using SPSS software (SPSS Inc., version 20.0, IL, USA).

RESULTS

We identified 123 incident statin users in the Rotterdam Study (RS-I and RS-II), who had a serum cholesterol measurement in the period of 180 days before and in the period between 7 and 180 days after start of statin therapy, and who had genotype data available for the *PPARA* polymorphisms. The study population consisted of 77 incident simvastatin users, 29 incident atorvastatin users, and 17 incident users of other statins (pravastatin: eight; fluvastatin: four; cerivastatin: four; and rosuvastatin: one). Baseline characteristics of the study population are shown in Table 1.

In the analysis of all incident statin users combined, no significant associations were found between the *PPARA* rs4253728 G>A and rs4823613 A>G polymorphisms and change in total and LDL-cholesterol level (Table 2).

We subsequently analyzed incident simvastatin users separately. In the subgroup of 77 incident simvastatin users, the minor allele frequency of the rs4253728 G>A polymorphism was 30.5% (A allele), and for the rs4823613 A>G polymorphism the minor allele frequency was 32.5% (G allele). The genotype distributions of both polymorphisms were

Table 1 Baseline characteristics of the study population

Characteristic	All incident statin users (n = 123)		Incident simvastatin users (n = 77)		Incident atorvastatin users (n = 29)	
	rs4823613 (AA)[a]	rs4823613 (AG + GG)	rs4823613 (AA)	rs4823613 (AG + GG)	rs4823613 (AA)	rs4823613 (AG + GG)
N	54	69	33	44	15	14
Sex (N male, %)	21 (38.9)	35 (36.2)	16 (45.7)	18 (42.9)	3 (20.0)	2 (14.30
Age (years, mean ± SD)	71.1 ± 4.6	71.6 ± 4.9	71.5 ±4.9	71.6 ±5.1	70.7 ± 4.1	70.1 ± 3.8
TOTc before start (mmol/L, mean ± SD)	7.09 ±1.01	6.77 ± 1.05	6.92 ± 0.88	6.80 ± 1.11	7.54 ± 1.30	6.77 ± 1.11
LDLc before start (mmol/L, mean ± SD)	4.70 ± 1.00	4.51 ± 0.93	4.73 ± 0.89	4.59 ±1.00	4.84 ± 1.29	4.35 ± 0.98
ΔTOTc (mmol/L, mean ± SD)	−2.27 ± 0.79	−2.14 ± 0.72	−2.04 ± 0.63	−2.19 ±0.66	−2.94 ± 0.73	−2.51 ± 0.65
ΔLDLc (mmol/L, mean ± SD)	−2.04 ± 0.77	−1.97 ± 0.71	−1.91 ± 0.67	−204 ± 0.61	−2.48 ±0.86	−2.21 ± 0.72
Statin dose (mean ± SD)						
– at first TOTc measurement after start	0.55 DDD[b] ± 0.25	0.62 DDD ± 0.32	15.1 mg ±6.7	15.0 mg ± 6.3	11.3 mg ± 3.5	15.0 ± 5.2
– at first LDLc measurement after start	0.55 DDD ± 0.25	0.65 DDD ±0.33	14.7 mg ± 7.2	14.0, m ±7.3	11.3 mg ± 3.5	15.4 ± 5.2

Abbreviations: DDD, defined daily dosage; TOTc, total cholesterol; LDLc, low-density lipoprotein cholesterol; SD, standard deviation. [a] Baseline characteristics are only shown for the rs4823613 A>G polymorphism and not for the rs4253728 G>A polymorphism, because they are almost in complete linkage disequilibrium ($R^2 = 0.96$); the AA genotype is the homozygous major allele genotype (reference category). [b] DDD, facilitates direct dose comparisons between drugs from the same therapeutic group. ΔLDLc: Change in low-density lipoprotein cholesterol from start of statin therapy; ΔTOTc: Change in total cholesterol from start of statin therapy.

in Hardy–Weinberg equilibrium (P 0.24 for the rs4253728 G>A polymorphism, P 0.27 for the rs4823613 A>G polymorphism; Table 1). No significant differences among genotype groups were found when considering the time between the last cholesterol measurement and start of simvastatin therapy or the time between start of simvastatin therapy and the first cholesterol measurement. Neither were significant differences observed in baseline LDL-cholesterol or baseline total cholesterol measurements. The average time between start of simvastatin therapy until the first cholesterol measurement was 51 days for the total cholesterol measurement (standard deviation [SD] 33 days), and 53 days for the LDL-cholesterol measurement (SD 34 days). The average decrease in total and in LDL-cholesterol levels after start of simvastatin therapy were 2.1 mmol/l (SD 0.6 mmol/l) and 2.0 mmol/l (SD 0.7 mmol/l), respectively.

The results of the multivariable linear regression analyses on the association between the *PPARA* rs4253728 G>A and rs4823613 A>G polymorphisms and the change in total and LDL-cholesterol levels during simvastatin therapy are shown in Table 2. For the

Table 2 Association between the *PPARA* rs4253728 G>A and rs4823613 A>G polymorphisms and total and low-density lipoprotein cholesterol reduction during statin therapy – all incident statin users versus incident simvastatin users versus incident atorvastatin users

	All incident statin users (n = 123)		Incident simvastatin users (n = 77)		Incident atorvastatin users (n = 29)	
Genotype	**Full model[a]: Δ cholesterol[b] (95% CI)**	**P**	**Full model: Δ cholesterol (95% CI)**	**P**	**Full model: Δ cholesterol (95% CI)**	**P**
rs4253728 G>A: total cholesterol						
Additive model[c]	−0.028 (−0.213; 0.157)	0.761	**−0.247 (−0.458; −0.035)**	**0.023**	0.224 (−0.111; 0.560)	0.177
Categorical model[d]						
– GG	(ref)	–	(ref)	–	(ref)	–
– GA	0.070 (−0.145; 0.285)	0.522	−0.189 (−0.437; 0.059)	0.132	0.224 (−0.111; 0.560)	0.177
– AA	−0.379 (−0.901; 0.142)	0.152	**−0.603 (−1.130; −0.076)**	**0.026**	–[e]	–
rs4253728 G>A: LDL-cholesterol						
Additive model	0.067 (−0.157; 0.291)	0.557	**−0.253 (−0.458; −0.049)**	**0.016**	0.099 (−0.180; 0.379)	0.461
Categorical model						
– GG	(ref)	–	(ref)	–	(ref)	–
– GA	0.096 (−0.181; 0.373)	0.495	−0.165 (−0.410; 0.079)	0.181	0.099 (−0.180; 0.379)	0.461
– AA	0.071 (−0.497; 0.639)	0.804	**−0.641 (−1.140; −0.142)**	**0.013**	–[e]	–
rs4823613 A>G: total cholesterol						
Additive model	−0.011 (−0.189; 0.167)	0.904	**−0.258 (−0.470; −0.046)**	**0.018**	0.198 (−0.093; 0.488)	0.170
Categorical model						
– AA	(ref)	–	(ref)	–	(ref)	–
– AG	0.054 (−0.166; 0.274)	0.629	−0.207 (−0.454; 0.040)	0.099	0.215 (−0.136; 0.566)	0.213
– GG	−0.163 (−0.618; 0.292)	0.479	**−0.614 (−1.140; −0.088)**	**0.023**	0.339[f] (−0.500; 1.178)	0.406
rs4253728 G>A: LDL-cholesterol						
Additive model	0.034 (−0.186; 0.253)	0.761	**−0.294 (−0.495; −0.093)**	**0.005**	0.019 (−0.222; 0.260)	0.869
Categorical model						
– AA	(ref)	–	(ref)	–	(ref)	–
– AG	0.074 (−0.204; 0.352)	0.599	−0.224 (−0.465; 0.016)	0.066	0.150 (−0.126; 0.427)	0.265
– GG	−0.007 (−0.547; 0.534)	0.981	**−0.672 (−1.154; −0.180)**	**0.008**	−0.337[f] (−0.977; 0.303)	0.279

Abbreviations: CI, confidence interval; LDL, low density lipoprotein.

[a] Full model: adjusted for age, sex, statin dose, cholesterol level before start, *CYP3A4*22* genotype and *CYP3A5*3* genotype. [b] Average change in total cholesterol and LDL-cholesterol level between the last measurement before start and the first measurement after start of statin therapy. [c] Number of copies of the minor allele. [d] Categorical model with the homozygous major allele genotype as the reference category. [e] No atorvastatin users with the rs4253728 minor allele AA genotype. [f] One atorvastatin user with the rs4823613 minor allele GG genotype. **Bold** value indicates statistically significant association.

PPARA rs4823613 A>G polymorphism, the minor G allele was associated with a trend towards a stronger cholesterol response to simvastatin after the start of therapy, as reflected by the larger reduction in total and LDL-cholesterol for carriers of the minor G allele compared with the homozygous major allele AA genotype. In the full model with the homozygous major allele AA genotype as the reference category, the increased reduction in total cholesterol was −0.207 mmol/l (95% CI −0.454; 0.040, P 0.099) for the heterozygous AG genotype, and −0.614 mmol/l (95% CI −1.140; −0.088, P 0.023) for the homozygous minor allele GG genotype. For LDL-cholesterol, the increased reduction was −0.224 mmol/l (95% CI −0.465; 0.016, P 0.066) for the heterozygous AG genotype, and −0.672 mmol/l (95% CI −1.164; −0.180, P 0.008) for the homozygous minor allele GG genotype.

The results of the analyses for the *PPARA* rs4253728 G>A polymorphism showed similar significant results as for the rs4823613 A>G polymorphism (Table 2).

We did not find an interaction between the rs4823613 A>G polymorphism and the *CYP3A4*22* (P-value for interaction term 0.748) or the *CYP3A5*3* polymorphism (P-value for interaction term 0.624) in the analysis on change in total cholesterol. Also, we did not find an interaction between the rs4823613 A>G polymorphism and the *CYP3A4*22* (P-value for interaction term: 0.748) or the *CYP3A5*3* polymorphism (P-value for interaction term: 0.624) in the analysis on change in LDL-cholesterol. These results were similar for the rs4253728 G>A polymorphism. In the full model, both CYP polymorphisms showed a non-significant association towards a stronger cholesterol lowering response to simvastatin, with a mean β of −0.254 mmol/l for the *CYP3A4*22* polymorphism and a mean β of −0.319 mmol/l for the *CYP3A5*3* polymorphism.

An analysis in the 29 incident atorvastatin users separately showed no significant association (Table 2). For atorvastatin, we could not investigate the association in the different genotype categories, since there was only one atorvastatin user with two minor alleles of the rs4823613 A>G polymorphism, and no atorvastatin user with two minor alleles of the rs4253728 G>A polymorphism.

DISCUSSION

In this population-based cohort study, the rs4253728 G>A and rs4823613 A>G polymorphisms in the *PPARA* gene were associated with the total and LDL-cholesterol lowering effect of simvastatin therapy. For the *PPARA* rs4253728 G>A polymorphism, the minor A allele was associated with a 0.247 mmol/l larger reduction in total cholesterol and a 0.253 mmol/l larger reduction in LDL-cholesterol. For the *PPARA* rs4823613 A>G polymorphism, the minor G allele was associated with a 0.258 mmol/l larger reduction in

total cholesterol and a 0.294 mmol/l larger reduction in LDL-cholesterol. The association disappeared when we considered all incident statin users combined.

This is the first study that demonstrates an association between the strongly linked *PPARA* rs4253728 G>A and rs4823613 A>G polymorphisms and the cholesterol lowering effect of simvastatin. The *PPARA* gene encodes PPARα, which is part of a family of nuclear receptors – PPARα, PPARδ and PPARγ –, each encoded by a different gene, and with a specific tissue expression in which they regulate the transcription of multiple genes. PPARα is highly expressed in the liver and is involved in the regulation of lipid metabolism, inflammation and vascular function, and hence interferes with the process of atherogenesis. The effects of PPARα on gene regulation result in an increased high-density lipoprotein production by the liver, a decrease in atherogenic LDL-cholesterol levels, a decrease in triglyceride levels, and a decreased production as well as an increased clearance of very-low-density lipoprotein. These effects tend to decrease the atherosclerotic risk.[33-35] Genetic polymorphisms in the *PPARA* gene have been described and studies conducted in rodents and humans have suggested a link between genetic variation in the *PPARA* gene and serum lipid levels and cardiovascular disease.[36-42] However, only a few studies thus far investigated the effect of the rs4253728 G>A and rs4823613 A>G polymorphisms. A recent study in Chinese patients with hypercholesterolemia could not demonstrate an association between the *PPARA* rs4823613 A>G polymorphism and the change in LDL-cholesterol level after 6 weeks of treatment with simvastatin 40 mg.[43] Although this study also investigated simvastatin response by determining the difference in LDL-cholesterol levels before and after start of simvastatin treatment, in contrast with our study the population consists of Asian people with a relatively lower age (55.6 years). Also, the Rotterdam Study has a population-based setting in which medication prescription data are collected through linkage with pharmacies, in contrast with the Chinese study in which all patients were treated with 40 mg of simvastatin for 6 weeks. Furthermore, genetic structure differs between races and the rs4253728 polymorphism is absent in Japanese and Chinese populations. Another study demonstrated an association between the minor allele of the rs4253728 G>A polymorphism and increased triglycerides and apolipoprotein CIII levels in African-Americans but not in Caucasians. However, the effect of statin therapy was not investigated.[36] No association with total cholesterol, LDL-cholesterol or high-density lipoprotein cholesterol levels was found. Another study did not find an association between the rs4253728 G>A polymorphism and myocardial infarction.[37] In our study of only Caucasians, both *PPARA* polymorphisms were not associated with baseline total and LDL-cholesterol levels but only with the response to simvastatin.

Based on the results in the study of Klein et al.[27], as mentioned in the introduction, and in the findings in the current study, a pharmacokinetic mechanism seems to be the most plausible explanation for the effect of the *PPARA* polymorphisms on the cholesterol low-

ering effect of simvastatin therapy. The CYP3A4 enzyme is involved in the metabolism of several statins – simvastatin, atorvastatin, lovastatin and cerivastatin –, with the largest effect of CYP3A4 on simvastatin metabolism. A recent study established that the nuclear receptor PPARa directly influenced the expression of CYP3A4.[44] In the study of Klein et al., the minor alleles of the *PPARA* rs4253728 G>A and rs4823613 A>G polymorphisms were associated with significantly lower CYP3A4 protein expression and enzyme activity *in vitro*.[27] Further testing of the rs4253728 G>A polymorphism in atorvastatin-treated volunteers confirmed a decrease in atorvastatin metabolism in carriers with two minor alleles compared with heterozygous or homozygous major allele carriers. Our results are in line with this. The minor alleles of the *PPARA* rs4253728 G>A and rs48236313 A>G polymorphisms significantly decrease the CYP3A4 enzyme activity, leading to a decreased simvastatin metabolism and therefore an increased plasma simvastatin concentration. Thus, the minor alleles of the *PPARA* polymorphisms are likely to be associated with a better cholesterol lowering response to simvastatin therapy. In the study of Klein et al.[27], the effect of the rs4253728 G>A polymorphism was demonstrated in participants using atorvastatin, while in our study we did not find an association for incident atorvastatin users, neither for all statin users combined. For atorvastatin, the CYP3A4 enzyme is also a contributor to its metabolism. In the study of Klein *et al.* the decrease in atorvastatin metabolism was tested by measuring atorvastatin-2-hydroxylation, the major metabolite of atorvastatin, after a single-dose of atorvastatin. In our population-based study the outcome measure was cholesterol response, derived from clinically driven cholesterol measurements from outpatient laboratory assessments. Simvastatin undergoes more extensive metabolism by CYP3A4 than atorvastatin, thus inhibition of CYP3A4 by co-medication or genetic factors such as the *PPARA* polymorphisms produces a more increased serum simvastatin concentration than serum atorvastatin concentration.[45,46] This may explain the fact that in our study, we did find an association in simvastatin users, but could not demonstrate an association in atorvastatin users. Atorvastatin users with a minor allele had a significantly higher dose than atorvastatin users homozygous for the major allele. This would have led to an overestimation of the results in carriers of a minor allele, but no association in atorvastatin users was found at all. However, our study had too low a power to investigate the association in atorvastatin users, since we only had 29 incident atorvastatin users available. Only one user had two minor alleles of the rs4823613 A>G polymorphism, and there was no user with two minor alleles of the rs4253728 polymorphism. Therefore, an association in atorvastatin users could not be excluded and investigating the association in a larger population of atorvastatin users is necessary before definite conclusions can be drawn regarding this association.

Potential biases and limitations in our study should be considered. The *PPARA* polymorphisms were not associated with baseline cholesterol level before start of simvastatin therapy. Therefore, it is unlikely that differences in cholesterol levels were already pres-

ent before the start of simvastatin therapy. Genetic variation in the *PPARA* gene was also not associated with plasma lipid levels in the meta-analyses by Teslovich et al.[47], a large study that revealed 95 loci significantly associated with plasma lipid levels. This agrees with our finding that the *PPARA* polymorphisms really influence cholesterol response. The effect of statins is already measurable after 1 week of treatment, with the maximum effect reached after 4–6 weeks. We included patients with a cholesterol measurement 7 days after start of statin therapy to prevent potential biases. In response to a cholesterol measurement in the first 1–4 weeks after start of therapy, adjustments in therapy could have occurred such as a change in dose or switching to another statin. The fact that the cholesterol lowering effect during that period has not reached its maximum would have led to an underestimation of the results, and the actual effect of the *PPARA* polymorphisms on the cholesterol lowering effects of statins is possibly even larger. We had a low sample size of only 77 incident simvastatin users. Despite this limited number we found a statistically significant association, indicating that the effect of the *PPARA* polymorphisms on CYP3A4 activity and thus the cholesterol lowering effect of simvastatin might be large. The association was adjusted for the effect of the *CYP3A4*22* and *CYP3A5*3* polymorphisms. Both CYP polymorphisms showed a non-significant association towards a stronger cholesterol lowering response, as was expected on the basis of previous literature. Unfortunately, we had insufficient numbers of users of other statins other than simvastatin to analyze these statins individually. It would be interesting to investigate whether in our population the *PPARA* polymorphisms influence CYP3A4 expression and activity *in vitro*. Unfortunately, we have no *in vitro* expression data available for this study.

In conclusion, in the Rotterdam Study the strongly linked rs4253728 G>A and rs4823613 A>G polymorphisms in the *PPARA* gene were associated with a stronger total and LDL-cholesterol lowering response to simvastatin therapy. This is the first study that demonstrates an effect of these *PPARA* polymorphisms on the cholesterol lowering effect of simvastatin. With this study, we provide further evidence for the hypothesis that the influence of these *PPARA* polymorphisms on the cholesterol lowering effect of simvastatin acts through influence of these polymorphisms on CYP3A4 enzyme activity. Although these findings are interesting, one might question whether this will lead to tailored pharmacotherapy. After all, even if patients with a minor allele have a stronger intended cholesterol lowering effect, the majority of patients with two major alleles will still keep their indication for statin therapy. However, they might possibly respond better with a higher dose, in which case pharmacogenetics might have clinical consequences.

FUTURE PERSPECTIVE

We observed that genetic variation in the *PPARA* gene is associated with a cholesterol lowering response to simvastatin. We hypothesize that this effect appears through influence of genetic variation in the *PPARA* gene on CYP3A4 enzyme activity. This might be considered as a significant step towards a better management of treatment with drugs that are predominantly metabolized by the CYP3A4 enzyme. Since CYP3A4 is one of the most important enzymes in drug metabolism, this could be clinically relevant. Future pharmacogenetic studies should focus on the impact of *PPARA* polymorphisms on the response to drugs metabolized by CYP3A4, and should further evaluate whether this impact should lead to adjustment of the current treatment guidelines in place for optimization of treatment efficacy and avoidance of drug toxicity. Namely, statin efficacy (cholesterol lowering) and the occurrence of adverse drug reactions are both related to plasma statin concentrations. According to our hypothesis, one would expect that carriers of the minor alleles of the *PPARA* polymorphisms would have a higher risk of statin-induced adverse drug reactions. This would be an interesting topic for further research.

In general, the field of statin pharmacogenomics will evolve during the next few years and new genetic variation involved in statin response will be discovered through the implementation of new technologies such as exome sequencing and whole-genome sequencing.

EXECUTIVE SUMMARY

Background

- The CYP3A4 enzyme is the main enzyme responsible for the metabolism of simvastatin.
- Recently, it was discovered that the minor alleles of the two strongly linked polymorphisms in the *PPARA* gene, rs4253728 G>A and rs4823613A>G, decrease CYP3A4 expression and activity.
- Decreased CYP3A4 activity may result in increased simvastatin plasma levels and stronger cholesterol lowering response.

Methods

- We analyzed whether these *PPARA* polymorphisms were associated with a reduction in total and low-density lipoprotein (LDL)-cholesterol levels after start of simvastatin therapy.
- We adjusted for *CYP3A4*22* and *CYP3A5*3* genotype, since both polymorphisms have been associated with response to statin therapy.

Results: main findings

- The minor G allele of the rs4823613 A>G polymorphism was associated with a 0.258 mmol/l (95%CI −0.470; −0.046, P 0.018) larger reduction in total cholesterol and a 0.294 mmol (95% CI −0.495; −0.093, P 0.005) larger reduction in LDL-cholesterol after start of simvastatin therapy.
- Categorical analyses demonstrated a −0.614 mmol/l (95% CI −1.140; −0.088; P 0.023) larger reduction in total cholesterol and a −0.672 mmol/l (95% CI −1.164; −0.180, P 0.008) larger reduction in LDL-cholesterol, for the homozygous minor allele GG genotype compared with the reference homozygous major allele AA genotype.
- The results were similar for the strongly linked rs4253728 G>A polymorphism.
- No association was found with all incident statin users combined, to exclude the possibility of a pharmacodynamic group effect.

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The rs13064411 polymorphism in the *WDR52* gene, associated with PCSK9 levels, modifies statin-induced changes in serum total and LDL-cholesterol levels

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Pharmacogenetics and Genomics 2015; 25(3): 134-142

ABSTRACT

Introduction: Recently, the minor allele of the rs13064411A>G polymorphism in the *WD repeat domain 52* (*WDR52*) gene was associated with increased statin-induced proprotein convertase subtilisin/kexin type 9 (PCSK9) levels, and with low-density lipoprotein (LDL)-cholesterol response to statins. PCSK9 promotes LDL-receptor degradation, leading to increased serum LDL-cholesterol. We investigated whether the polymorphism was associated with cholesterol response to statins.

Methods: We identified 1105 current, 322 past, and 4831 never statin users during follow-up in the prospective population-based Rotterdam Study. Mean delta total, LDL- and high density lipoprotein (HDL)-cholesterol levels between current and no current statin users with the same number of minor alleles were analyzed using random effect repeated measurements. We adjusted for age, sex, number of cholesterol measurements, and follow-up time.

Results: Compared to no users with the same genotype, current statin users carrying a minor allele showed a statistically significantly lower delta total and LDL-cholesterol compared to reference homozygous major allele carriers (total: Δ-0.551 mmol/L [AG+GG] vs. Δ-0.732 mmol/L [AA], $P_{interaction}$ 5.2×10^{-7}; LDL: Δ-0.566 mmol/L [AG+GG] vs Δ-0.720 mmol/L[AA], $P_{interaction}$ 1.8×10^{-5}). The effect was stronger in women ($P_{interaction}$: 2.0×10^{-5} for LDL-cholesterol, 8.0×10^{-6} for total cholesterol) and in high dose users (defined daily doses >1.00) ($P_{interaction}$ 7.0×10^{-5} for LDL-cholesterol, $P_{interaction}$ 0.081 for total cholesterol). The polymorphism was not associated with HDL-cholesterol in current statin users, nor with total, LDL- and HDL-cholesterol in never statin users.

Conclusion: The minor G allele of the rs13064411 polymorphism, associated with statin-induced PCSK9-levels, was associated with a decreased LDL- and total cholesterol-lowering response to statins.

INTRODUCTION

The 3-hydroxy-3-methylglutary coenzyme A (HMG-CoA) reductase inhibitors or statins are cholesterol lowering drugs, widely prescribed for the primary and secondary prevention of cardiovascular disease. Statins primarily act on serum low density lipoprotein (LDL)-cholesterol by interfering with the cholesterol metabolism through inhibition of HMG-CoA reductase, the rate-limiting step in the cholesterol metabolism pathway. This leads to upregulation of LDL-receptors in the hepatocyte, resulting in an increased uptake of LDL-cholesterol from the serum and a decreased serum cholesterol concentration.[1-4] In clinical practice, there is a substantial difference between individuals in cholesterol lowering response to statin therapy. A part of this variability in response can be explained by genetic factors. Several single nucleotide polymorphisms have already been discovered that were associated with a modified cholesterol lowering response to statin therapy.[5-7] For example, the apolipoprotein (*APOE*) E2 variant has frequently been associated with a better LDL-cholesterol lowering response in both candidate and genome-wide association studies.[8-14]

Proprotein convertase subtilisin/kexin type 9, or PCSK9, is involved in the cholesterol homeostasis and promotes LDL-receptor degradation. In the liver, PCSK9 binds to the LDL-receptor and is internalized together with the LDL-receptor. Subsequently, this binding of PCSK9 to the LDL-receptor induces modification of LDL-receptor conformation, avoiding normal cycling of the LDL-receptor to the plasma membrane and enhancing lysosomal degradation. As a result, the number of LDL-receptors at the cell surface of hepatocytes is decreased and less LDL-cholesterol is taken up from the serum, with consequently a higher serum LDL-cholesterol level.[15-17] Serum PCSK9 levels have been shown to be positively correlated with both LDL-cholesterol and total cholesterol levels.[18-22] Also, it was demonstrated that statin therapy increases serum PCSK9 concentrations.[19,20,22-26] The influence of PCSK9 on cholesterol metabolism and the interaction with statin therapy, is of such interest that currently PCSK9-inhibitors are developed as a potential effective LDL-cholesterol lowering treatment alone or in combination with statin therapy.[27-30]

A recent study discovered a polymorphism that was genome-wide significantly ($P = 8.2 \times 10^{-8}$) associated with statin-induced changes in plasma PCSK9 levels, and modified the LDL-cholesterol lowering response to statins.[31] The minor allele of the rs13064411 polymorphism in the *WD repeat domain 52* (*WDR52*) gene was associated with increased simvastatin-induced PCSK9 concentrations compared to the homozygous major allele genotype. This study was the first one that showed the influence of genetic variation on statin-induced changes in serum PCSK9 levels. We aimed to investigate whether this polymorphism showed interaction with the effect of statin therapy on serum cholesterol measurements. Due to the increased statin-induced PCSK9-levels, statin users carrying a minor allele are expected to have an increased LDL-receptor degradation with con-

sequently less uptake of LDL-cholesterol from the serum into the hepatocytes, thus a decreased cholesterol lowering response to statin therapy.

In our study, the objective was to investigate whether the minor allele of the rs13064411 polymorphism in the WDR52 gene showed interaction with statin therapy, as assessed by a modified effect of the minor allele on serum total, LDL- and high density lipoprotein (HDL)-cholesterol levels in statin users compared with non-statin users in a large population-based cohort study.

METHODS

Setting

The current study was performed within the Rotterdam Study, a prospective population-based cohort study that aims to study the frequency and determinants of diseases in the middle-aged and elderly people. The rationale and design of the Rotterdam Study have been described in detail previously.[32,33] In short, all 10,275 persons aged 55 years and over in the Ommoord district of Rotterdam, the Netherlands, were invited to participate. Of them, 7,983 (78%) were enrolled between 1990 and 1993 (RS-I). At baseline, all participants were interviewed at home and underwent extensive clinical examination at the research center. Additional re-examinations took place in 1993-1995, 1997-1999, 2002-2004, and 2009-2012. In 2000, an extended cohort was enrolled[when 3011 inhabitants entered the study and have been continuously followed since then (RS-II). Furthermore, in 2006 a third cohort started (RS-III) including 3932 inhabitants aged 45 years and over. The Rotterdam Study has been approved by the medical ethics committee according to the Wet Bevolkingsonderzoek: ERGO (Population Study Act: Rotterdam Study), executed by the Ministry of Health, Welfare and Sports of the Netherlands. All participants gave informed consent to participate in the study and to obtain information from treating physicians and pharmacies, separately.

Medication prescription data are continuously obtained from all seven fully computerized pharmacies in the Ommoord district. These pharmacies dispense the prescriptions of more than 95% of all participants. Information on all filled prescriptions from 1 January 1991 until 31 January 2012 was available and included information on the product name of the drug, the Anatomical Therapeutical Chemical code (ATC-code), the amount dispensed, the prescribed dosage regimen and the date of dispensing.[34]

For this study, we used the total cholesterol, LDL- and HDL-cholesterol assessments from the 'Star-Medisch Diagnostisch Centrum' (Star-MDC), which performs all outpatient laboratory assessments for general practitioners in the Rijnmond area of Rotterdam with a potential source population of more than 1 million inhabitants. All outpatient cholesterol assessments from the participants of the Rotterdam study between 1 April

1997, the time at which a new computer system was introduced at Star-MDC, and 18 August 2011 were obtained.

Study population

The study sample consisted of all participants in the three cohorts of the Rotterdam Study who had at least one cholesterol measurement (total or LDL- of HDL-cholesterol) available in the period between 1 January 1997 and 18 August 2011, and for whom DNA was available. Participants were excluded if they were prescribed fibrates, bile acid sequestrants, nicotinic acids or ezetimibe at the time of one of the measurements. Per participant, follow-up started at the date of the first serum cholesterol measurement (baseline for the current study).

Exposure to statins

We investigated whether the rs13064411 polymorphism modified the effect of current statin use on serum cholesterol levels, by assessing the delta cholesterol between current and no current statin users per genotype. Use of statin therapy was obtained from dispensing records of fully computerized pharmacies, as described above. Repeated prescriptions which were filled within 7 days after ending the previous filled prescription were considered as continuous use. If a cholesterol measurement occurred within a prescription period, this contributed to current statin use, and if a cholesterol measurement occurred outside a prescription period, this contributed to no current statin use.

Outcome

The outcome of interest was serum cholesterol. We investigated whether the polymorphism showed significant interaction with response to statin therapy on both serum total cholesterol, serum LDL-cholesterol, as well as serum HDL-cholesterol measurements. Cholesterol measurements were derived from the Star-MDC, as described above, and were measured in mmol/L.

Covariables

Age, sex, and daily prescribed dose of statin therapy were considered as potential confounders or effect modifiers for affecting the association between the rs13064411 polymorphism and serum cholesterol levels. To compare doses of the different statins, the daily dose of statin therapy was expressed in standardized defined daily doses (DDD), according to the WHO.[34]

Genotyping

At the baseline examination of the Rotterdam Study, blood was taken from which genomic DNA was extracted, using the salting-out method.[35] Microarray genotyping was

performed in all three Rotterdam Study cohorts, using the Infinium II HumanHap 50K Genotyping BeadChip version 3 (Illumina Inc., San Diego, California, USA). Genotyping procedures were followed according to the manufacturer's protocol. During genetic quality control, participants with a non-Caucasian background were excluded from the genetic dataset. Microarray genotyping procedures in the Rotterdam Study have been described previously.[36]

Statistical analysis

Deviation from Hardy-Weinberg equilibrium was tested using a X^2-test. Differences in baseline characteristics between the genotype categories were tested for significance with a t-test for continuous variables and a X^2-test for binary variables.

We used random effect repeated measurements to investigate whether there was significant statistical interaction between the rs13064411 polymorphism and current use of statin therapy on serum cholesterol measurements.[37] Past users were included in the group of non-users. Separate analyses were performed for the outcomes total, LDL- and HDL-cholesterol. We investigated the mean difference in cholesterol levels in current statin users compared to non-statin users with the same number of minor alleles. Analyses were adjusted for age, sex, number of cholesterol measurements per participant, and follow-up time. We investigated both additive models, investigating the effect on serum cholesterol levels per additive minor allele, and categorical models, investigating the effect on serum cholesterol levels for the heterozygous genotype category and the homozygous minor allele genotype category compared with the homozygous major allele category. In additional analyses, we stratified the population by sex, statin dose, and individual statin to see whether the association was different in these categories. In sensitivity analyses, we investigated whether there was significant statistical interaction between the rs13064411 polymorphism and the daily dose of current statin therapy, instead of current use of statin therapy yes/no. Furthermore, in a sensitivity analysis we excluded past users of statin therapy.

To investigate whether the polymorphism only interacts with statin therapy on serum cholesterol levels, and not influences cholesterol levels in general, we investigated whether the polymorphism influences serum cholesterol levels in participants who had never used any statin during follow-up. Since the aim of the study was to investigate whether the rs13064411 polymorphism showed significant interaction with the cholesterol lowering effect of statin therapy, it is important to exclude an association between the polymorphism and cholesterol levels in general in a population not using statins.

All P-values were two-sided with level of significance of $P<0.05$. The analyses were performed using SPSS software (SPSS Inc., version 21.0, Chicago, Illinois, USA).

RESULTS

From the three cohorts in the Rotterdam Study, we identified 1,105 participants who were current statin users during follow-up, 322 participants who were past statin users during follow-up, and 4,831 participants who never used statin therapy, who had serum cholesterol measurements available, and who had genotype data available for the rs13064411 polymorphism. Simvastatin was the most frequently used statin. The median number of cholesterol measurements per participant was 4.1 (SD 3.8). The minor allele frequency (MAF) of the rs13064411 polymorphism was 14.9%, and the genotype distributions of the polymorphism were in Hardy-Weinberg Equilibrium in both statin and no statin users (P 0.06 and P 0.18 respectively). Baseline characteristics of the study population are shown in table 1.

In the analyses, we compared the effect of the rs13064411 polymorphism on serum cholesterol levels in current statin users compared with no current statin users (table 2).

Table 1 Characteristics of the study population

Characteristic	Current statin users (n = 1105)	Past statin users (n = 322)	Never statin users (n = 4831)
Age, yrs (SD)	68.4 (7.4)	72.0 (8.1)[a]	63.8 (11.8)[b]
Sex, N males (%)	491 (44.4%)	139 (43.3%)	2017 (41.8%)[b]
Baseline total cholesterol, mmol/L, mean (SD)	5.8 (1.1)	5.9 (1.4)	6.0 (1.3)[b]
Baseline LDL cholesterol, mmol/L, mean (SD)	3.7 (1.2)	3.6 (1.3)	3.6 (1.0)[b]
Baseline HDL cholesterol, mmol/L, mean (SD)	1.5 (0.4)	1.4 (0.4)[a]	1.5 (0.4)
Follow-up time, yrs, mean (SD)	7.9 (4.3)	5.4 (4.6)[a]	4.7 (4.5)[b]
rs13064411 genotype, n (%)			
– homozygous major allele (AA)	778 (70.4%)	233 (72.4%)	3544 (73.4%)
– heterozygous (AG)	288 (26.1%)	79 (24.5%)	1174 (24.3%)
– homozygous minor allele (GG)	39 (3.5%)	10 (3.1%)	113 (2.3%)
Starting dose, DDD, mean (SD)	1.27 (0.8)	NA	NA
Starting ATC-code		NA	NA
– simvastatin	612 (55.4%)		
– atorvastatin	238 (21.5%)		
– pravastatin	138 (12.5%)		
– fluvastatin	78 (7.1%)		
– rosuvastatin	39 (3.5%)		

Abbreviations: yrs, years; SD, standard deviation; LDL, low-density lipoprotein; HDL, high-density lipoprotein; DDD, defined daily doses; ATC, Anatomical Therapeutical Chemical; NA, not applicable.
[a] current statin users differ significantly from never statin users (P<0.05); [b] current statin users differ significantly from past statin users (P<0.05).

Table 2 Delta cholesterol in mmol/L: difference in mean cholesterol levels between current statin users and no users with the same number of minor alleles of the rs13064411 polymorphism – statistical interaction by genotype on the effect of statins on cholesterol levels

No use		Current use		Δ Cholesterol per genotype category[a] (mmol/L)	P_{int}[b]	N[c]
Model	**Mean cholesterol in mmol/L (SE)**	**Model**	**Mean cholesterol in mmol/L (SE)**			
LDL-cholesterol		**LDL-cholesterol**				
Categorical model[d]		*Categorical modeld*				
no use – AA	2.66 (0.114)	current use – AA	1.94 (0.115)	−0.720	(ref)	4555
no use – AG	2.64 (0.115)	current use – AG	2.08 (0.119)	**−0.566**	**4.0×10^{-5}**	1541
no use – GG	2.60 (0.133)	current use – GG	2.04 (0.152)	**−0.565**	**0.094**	162
Dominant model[e]		*Dominant modele*				
no use – AA	2.66 (0.114)	current use – AA	1.94 (0.115)	−0.620	(ref)	4555
no use – AG + GG	2.64 (0.115)	current use – AG + GG	2.07 (0.119)	**−0.566**	**1.8×10^{-5}**	1703
Total cholesterol		**Total cholesterol**				
Categorical model		*Categorical model*				
no use – AA	4.73 (0.107)	current use – AA	4.00 (0.109)	−0.732	(ref)	4296
no use – AG	4.72 (0.109)	current use – AG	4.16 (0.113)	**−0.561**	**6.0×10^{-6}**	1468
no use – GG	4.78 (0.131)	current use – GG	4.31 (0.150)	**−0.471**	**0.005**	154
Dominant model		*Dominant model*				
no use – AA	4.73 (0.107)	current use – AA	4.00 (0.109)	−0.732	(ref)	4296
no use – AG + GG	4.73 (0.109)	current use – AG + GG	4.18 (0.112)	**−0.551**	**5.2×10^{-7}**	1622
HDL-cholesterol		**HDL-cholesterol**				
Categorical model		*Categorical model*				
no use – AA	1.47 (0.029)	current use – AA	1.51 (0.031)	0.033	(ref)	3237
no use – AG	1.47 (0.031)	current use – AG	1.53 (0.034)	0.056	0.182	1090
no use – GG	1.48 (0.045)	current use – GG	1.51 (0.057)	0.028	0.910	121
Dominant model		*Dominant model*				
no use – AA	1.47 (0.029)	current use – AA	1.51 (0.031)	0.033	(ref)	3237
no use – AG + GG	1.48 (0.031)	current use – AG + GG	1.53 (0.033)	0.052	0.231	1211

Abbreviations: SE, standard error; CI, Confidence Interval; N, number; LDL, low density lipoprotein; HDL, high density lipoprotein.

[a] Delta cholesterol: difference in mean cholesterol levels between current statin users compared to no current statin users with the same number of minor G alleles; adjusted for age, sex, number of cholesterol measurements per participant, follow-up time. [b] P-values for the interaction between genotype and current statin use on delta cholesterol, with the AA genotype as the reference. [c] N contains the total number of participants: current users + no current users (past and never use). [d] Categorical model with the homozygous major allele AA genotype as the reference category. [e] Dominant model: effect in participants carrying at least one minor G allele compared to the homozygous major allele AA genotype as the reference category. The degrees of freedom (df) in the categorical model were 11 for the covariates and 48 for the repeated measurements; the df in the dominant model were 9 for the covariates and 48 for the repeated measurements. **Bold** value indicates statistically significant association.

Current statin users carrying a minor G allele showed a decreased cholesterol lowering response compared to the reference homozygous major allele AA carriers, as reflected by a smaller delta cholesterol for minor allele carriers. In current users compared to no current users with the same number of minor alleles, the mean LDL-cholesterol level was 0.720 mmol/L lower in homozygous major allele carriers, 0.566 mmol/L lower in heterozygous carriers, and 0.565 mmol/L lower in homozygous minor allele carriers. The interaction between the number of minor alleles and current statin use was statistically significant for heterozygous carriers (P_{int} 4.0×10^{-5}) but not for homozygous minor allele carriers (P_{int} 0.094), however, it was statistically significant when we combined minor allele carriers in the dominant model (P_{int} 1.8×10^{-5}). In current users compared to no current users with the same number of minor alleles, the mean total cholesterol levels was 0.732 mmol/L lower in homozygous major allele carriers, 0.561 mmol/L lower in heterozygous carriers, and 0.471 mmol/L lower in homozygous minor allele carriers. The interaction between the number of minor alleles and current statin user was statistically significant for both heterozygous carriers (P_{int} 6.0×10^{-6}), homozygous minor allele carriers (P_{int} 0.005), as well as for the dominant model (P_{int} 5.2×10^{-7}). For the serum HDL-cholesterol measurements, no statistically significant interaction was found between the polymorphism and current statin use.

In additional analyses we investigated whether the association for LDL- and total cholesterol was different if we stratified the current statin users in the population on mean dose, compared to all non-current users (figure 1A and 1B), and if we stratified the population on sex (figure 1C and 1D). For both LDL- and total cholesterol, the interaction between the rs13064411 polymorphism and current statin use was stronger in the higher dose category (DDD>1.00) and in women. The difference in effect between men and women showed a P_{int} of 2.0×10^{-5} for LDL-cholesterol and 8.0×10^{-6} for total cholesterol. The difference in effect between low dose and high dose users showed a P_{int} of 7.0×10^{-5} for LDL-cholesterol, the P_{int} for total cholesterol almost reached significance (P 0.081).

In analyses in which we stratified on individual statin, the interaction between the polymorphism and current statin use remained significant on the LDL- and total cholesterol measurements for every statin (simvastatin, atorvastatin, pravastatin, fluvastatin, rosuvastatin), except for the analyses within fluvastatin users on total cholesterol, possibly due to low numbers (results not shown).

Table 3 showed the analyses on the association between the rs13064411 polymorphism and serum cholesterol levels in participants who never used a statin. In both additive and categorical models, the number of minor alleles was not statistically significantly associated with both LDL-, total and HDL-cholesterol levels.

In sensitivity analyses, we investigated whether there was statistically significant interaction between the polymorphism and the DDD of current statin use. In both the

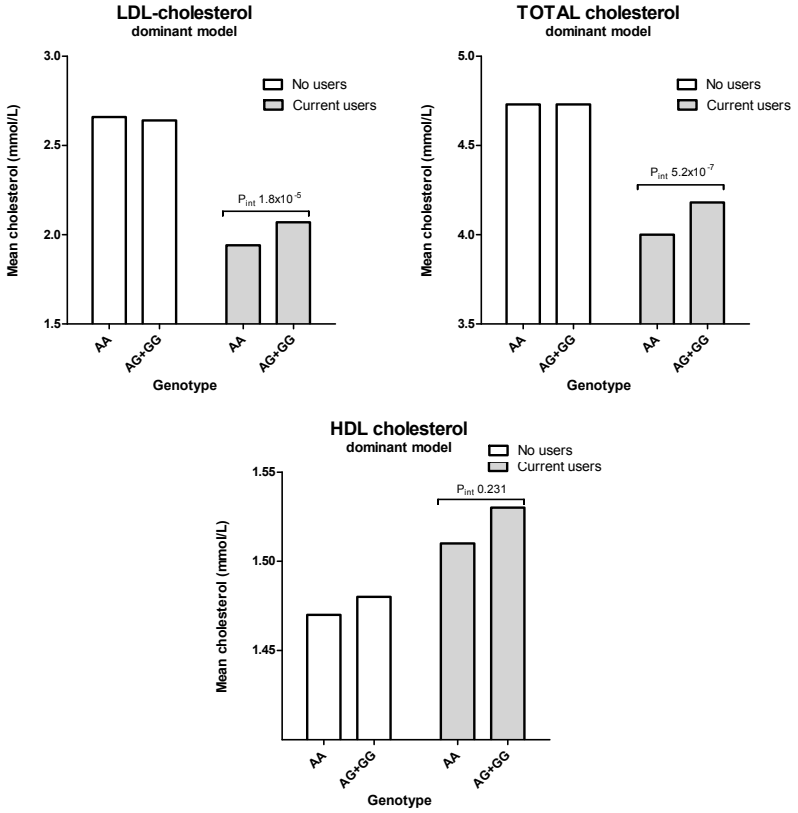

Figure 1 Mean delta in cholesterol levels (in mmol/L) between current statin users and no current statin users with the same number of minor alleles of the rs13064411 polymorphism

Abbreviations: LDL, low-density lipoprotein; P_{int}, P-value for interaction between genotype and statin use in the dominant model, with the AA genotype as reference.

Dominant model: effect in participants carrying at least one minor G allele compared to the homozygous major allele AA genotype as the reference category. Adjusted for age, sex, number of cholesterol measurements per participant, and follow-up time.

additive and dominant model, the interaction between the polymorphism and statin DDD remained statistically significant for LDL- and total cholesterol, with carriers of a minor allele having a smaller delta cholesterol in response to statin therapy than homozygous major allele carriers. For HDL-cholesterol, again no association was found. In a second sensitivity analysis, in which we excluded past users of statin therapy, the association remained statistically significant for LDL- and total cholesterol in the additive and dominant model. The mean delta in cholesterol levels for current statin users compared to non-statin users with the same number of minor alleles was similar to the main analysis presented in table 2, albeit non-significantly for the categorical model probably due to low power.

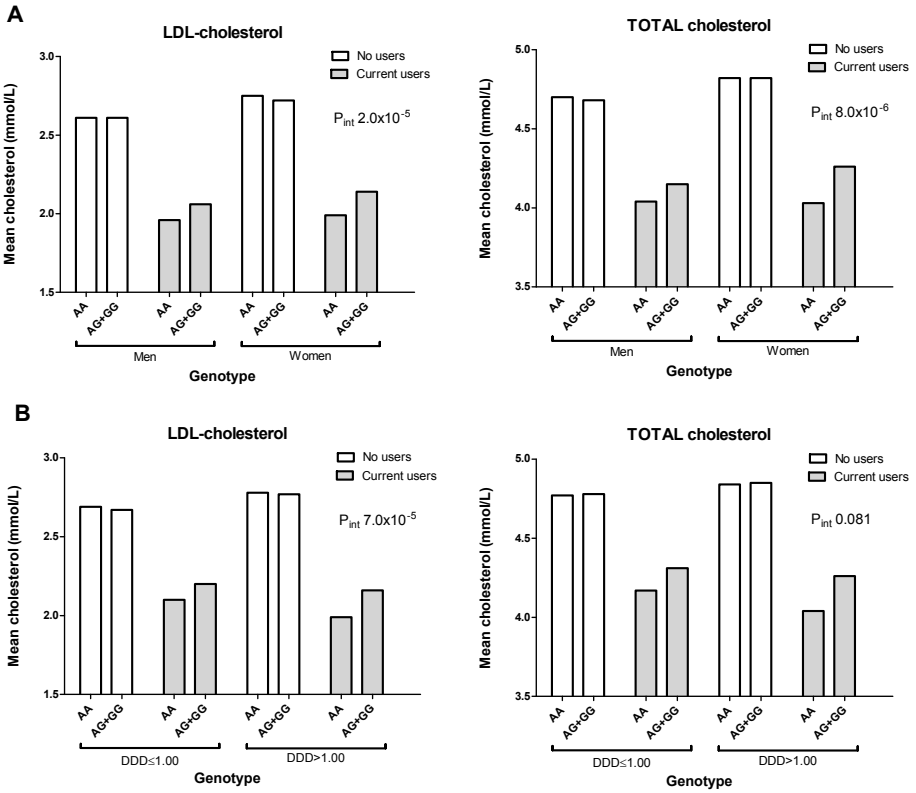

Figure 2 Mean delta cholesterol levels (in mmol/L) between current statin users and no current statin users with the same number of minor allele of the rs13064411 polymorphism – stratified by sex and statin dose
A: stratified by sex; B: stratified by statin dose.
Abbreviations: DDD, defined daily doses; LDL, low-density lipoprotein; P_{int}, P-value for the difference between men/women and low dose/high dose users for the interaction between genotype and statin use on cholesterol levels.
Dominant model investigating the effect in participants carrying at least one minor G allele compared to the homozygous major allele AA genotype as the reference category; the heterozygous AG genotype and homozygous minor allele GG genotype categories were combined due to low number in the GG category. Adjusted for age, sex (B), number of cholesterol measurements per participant, and follow-up time.

DISCUSSION

In this population-based cohort study, the rs13064411 polymorphism showed a statistically significant interaction with the effect of statin therapy on serum total and LDL-cholesterol concentrations. Compared to past and never statin users with the same number of minor alleles, current statin users carrying a minor allele showed a mean 0.154 mmol/L smaller delta LDL-cholesterol and a mean 0.181 mmol/L smaller delta total cholesterol, compared to homozygous major allele carriers. The polymorphism

Table 3 Association between the rs13064411 polymorphism and serum cholesterol levels (in mmol/L) in never statin users

	Mean cholesterol in mmol/L per genotype (SE)[a]	Beta (95% CI)[b]	P	N[c]
LDL-cholesterol				
Additive modeld	–	−0.00045 (−0.048; 0.047)	0.985	4831
Categorical modele				
AA	2.71 (0.071)	(ref)	–	3544
AG	2.73 (0.074)	0.020 (−0.036; 0.076)	0.487	1174
GG	2.62 (0.106)	−0.089 (−0.249; 0.071)	0.274	114
Total cholesterol				
Additive model	–	0.034 (−0.019; 0.087)	0.212	4486
Categorical model				
AA	4.94 (0.153)	(ref)	–	3283
AG	4.97 (0.126)	0.035 (−0.027; 0.098)	0.266	1099
GG	5.00 (0.153)	0.061 (−0.117; 0.240)	0.502	104
HDL-cholesterol				
Additive modeld	–	−0.077 (−0.018; 0.033)	0.557	3347
Categorical modele				
AA	1.50 (0.034)	(ref)	–	2456
AG	1.51 (0.036)	0.014 (−0.016; 0.045)	0.356	811
GG	1.48 (0.054)	−0.012 (−0.098; 0.073)	0.776	80

Abbreviations: SE, standard error; CI, Confidence Interval; N, number; LDL, low density lipoprotein; HDL, high density lipoprotein.

[a] Mean cholesterol levels in mmol/L. [b] Change in cholesterol levels in minor G allele carriers compared to the homozygous major allele AA reference category. [c] N contains the total number of participants. [d] Additive model: change in cholesterol level per additional minor G allele. [e] Categorical model: change in cholesterol level in never statin users heterozygous and homozygous for the minor allele compared to never statin users homozygous for the major allele, the reference category. Analyses are adjusted for age, sex, number of cholesterol measurements per participant, and follow-up time.

was not associated with cholesterol levels in never statin users, which indicates that this polymorphism only modifies response to statin therapy.

Theusch et al. showed that the rs13064411 polymorphism was associated with statin-induced changes in plasma PCSK9 levels and a modified LDL-cholesterol response to statin therapy.[31] Our study is a first replication in an independent population of this new finding by Theusch et al. that the rs13064411 polymorphism was associated with inter-individual variation in cholesterol response to statins. In the Rotterdam Study, we analyzed whether the polymorphism was associated with a modified response of cholesterol levels to statin therapy. The minor G allele is associated with increased statin-induced levels of PCSK9, leading to increased LDL-receptor degradation, higher

LDL-cholesterol levels and thus a decreased cholesterol lowering response. Therefore, we hypothesized that the rs13064411 polymorphism was associated with increased serum LDL and total cholesterol levels. However, in the study by the Theusch et al.[31], the minor allele of the polymorphism was weakly associated with greater statin-induced decreases in plasma LDL-cholesterol levels. Other studies reported that statin-induced increase in serum PCSK9 levels led to a stronger LDL-cholesterol reduction during statin therapy.[19,23-25]. This is in contrast with the findings in our study. Other studies are in line with our findings and showed that statin-induced increase in PCSK9 levels reduced statin efficacy.[38,39] Statins lower intracellular cholesterol levels in hepatocytes, with as a result upregulation of the activity of transcription factor sterol regulatory element binding protein-2 (SREBP-2). This leads to gene expression of SREBP-2 target genes, including LDL-receptor (*LDLR*) and *PCSK9*.[16,40] Thus statins induce both LDL-receptor configuration, as well as they counteract their therapeutic effect through promoting LDL-receptor degradation via PCSK9. The balance between configuration and degradation determines the net effect on the number of LDL-receptors and LDL-concentration, but also depends on other factors such as the statin dose or the normal fluctuation in PCSK9 against little fluctuation in LDL-cholesterol during the day.[41]

The rs13064411 polymorphism is located in the *WDR52* gene coding for the WDR52 protein, of whom the function is unknown. Research on other WD-repeat proteins indicated that they contain a beta-propeller domain structure and play a role in the coordination of several protein interactions.[42,43] The WDR52 protein possibly influences the effect of PCSK9 on LDL-receptor degradation. The LDL-receptor contains a beta-propeller domain, which is essential for successful directing the LDL-receptor for lysosmal degradation by PCSK9.[44] An alternative explanation for the genetic variation in response is that the polymorphism influences other genes that have their effect on PCSK9 levels. Further research is needed to elucidate the mechanism by which the rs13064411 influences the change in serum PCSK9 levels and response to statin therapy.

We had a large sample size of 5936 participants, of whom 1105 statin users, and in total 29,103 LDL- and 30,630 total cholesterol measurements available. In our study, we only demonstrated an association on LDL- and total cholesterol levels, and not on serum HDL-cholesterol levels. This is in line with previous studies that could not demonstrate an association between PCSK9 levels and serum HDL-cholesterol and triglycerides.[18,19,21] Our results showed a stronger effect of the polymorphism on total and LDL-cholesterol levels in women than in men (P_{int} 8.0×10^{-6} for total cholesterol, P_{int} 2.0×10^{-5} for LDL-cholesterol). The study by Theusch et al.[31] showed that women had higher serum PCSK9 levels than men at baseline and during statin treatment, but no significant sex-difference in statin-induced PCSK9 levels was present. Other studies demonstrated that baseline PCSK9 levels were higher in women than in men.[20,21,24] A sex-difference in the interaction of the rs13064411 polymorphism with the effect of

statin therapy on serum cholesterol concentrations, as demonstrated in our study, has not been investigated before and needs further replication. Hormone levels might play a role, e.g. it is known that estrogen increases LDL receptor levels, while androgens diminish this effect.[45,46] This sex-difference in genetic effect might also be explained by underlying higher baseline serum PCSK9 levels in women. However, since in the Rotterdam Study no serum PCSK9 levels are available, we could not investigate this. Furthermore, in our study the effect of the polymorphism on LDL- and total cholesterol levels was higher in statin users with a DDD>1.00. This makes it more plausible that the influence of the rs13064411 polymorphism on statin-induced changes in serum cholesterol levels is based on a true-positive finding. Also, the effect on total and LDL-cholesterol is consistently present in both men and women, in both the low and high DDD statin users, and in the different type of statins separately, which further supports this finding.

Potential biases and limitations of our study should be considered. The Rotterdam Study is a population-based cohort study, in which data is collected prospectively without prior knowledge of the research hypothesis of the current analyses. We used the cholesterol measurements requested by general practitioners for healthcare purposes and the reasons for the measurements may differ between statin users and non-statin users, inducing potential information bias. However, as prescribing physicians were not aware of the genotype status, this will not have influenced results. The genetic variant was in Hardy-Weinberg equilibrium, thus Mendelian randomization occurred and therefore, selection bias was unlikely. We adjusted for potential confounding factors such as age, sex and the number of cholesterol measurements per individual. In additional and sensitivity analyses, the association remained present, indicating that the association is true and not based on a false-positive finding. In the Rotterdam Study, no expression data was available for the PCSK9 enzyme. Therefore, we could not investigate whether the polymorphism was associated with statin-induced PCSK9 levels, such as in the study by Theusch et al. Although non-compliance with statin therapy might have occurred[47], this would lead to random misclassification and an underestimation of the true effect.

In conclusion, in the Rotterdam Study the rs13064411 polymorphism in the *WDR52* gene was associated with a modified effect of statin therapy on serum LDL- and total cholesterol levels. The minor G allele of the polymorphism, with a MAF of 14.9%, was associated with a decreased cholesterol lowering response to statins, and this effect was stronger in women and in high dose users (DDD>1.00). Currently, PCSK9-inhibitors are developed as a potentially effective LDL-cholesterol lowering treatment alone or in combination with statin therapy. Considering the potential inter-individual variation in response to statin therapy, it may be worthwhile to investigate whether patients carrying a minor allele who respond less to statin therapy might benefit from a PCSK9-inhibitor additional to statin therapy.

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2.4

The *SLCO1B1* c.521T>C polymorphism is associated with dose decrease or switching during statin therapy in the Rotterdam Study

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Pharmacogenetics and Genomics 2014; 24(1): 43-51

ABSTRACT

Introduction: The *SLCO1B1* c.521T >C polymorphism is associated with statin plasma levels and simvastatin-induced adverse drug reactions. We studied whether the c.521T >C polymorphism is associated with dose decreases or switches to other cholesterol lowering drugs during simvastatin and atorvastatin therapy, because these events are indicators of adverse drug reactions.

Methods: We identified 1939 incident simvastatin and atorvastatin users in the Rotterdam Study, a population-based cohort study. Associations were studied using Cox proportional hazards analysis. Meta-analysis was performed with data from the Utrecht Cardiovascular Pharmacogenetics study.

Results: Simvastatin users with the c.521 CC genotype had a significantly higher risk of a dose decrease or switch than users with the TT genotype [hazard ratio (HR) 1.74, 95% confidence interval (CI) 1.05; 2.88]. Female sex, age below 70 years, and low starting dose were risk factors. In atorvastatin users with starting dose of more than 20 mg, the risk of a dose decrease or switch was higher in users carrying a C allele than in users with the TT genotype (HR 3.26, 95% CI 1.47; 7.25). In the meta-analysis the association in simvastatin users remained, with a significantly higher risk of a dose decrease or switch in simvastatin users with two minor alleles (HR 1.69, 95% CI 1.05; 2.73). For atorvastatin users no significant association was found.

Conclusion: In simvastatin users in the Rotterdam Study, we demonstrated an association between the c.521T >C polymorphism and dose decrease or switching, as indicators of adverse drug reactions, and provided risk factors for this association. For atorvastatin, an association was found in users with a starting dose of more than 20 mg.

INTRODUCTION

The 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors or statins are cholesterol lowering drugs, prescribed for the primary and secondary prevention of cardiovascular diseases.[1-4] Statins have a protective effect on cardiovascular morbidity and mortality primarily by lowering the low-density lipoprotein cholesterol concentration.[5,6] In general, statins are well-tolerated and safe drugs, although adverse drug reactions do occur.[7] A common adverse drug reaction is myopathy, which can vary from myalgia to life-threatening rhabdomyolysis.[8] There is considerable variation between individuals in the risk of developing adverse drug reactions during statin therapy. This variation is partly explained by genetic factors. Single-nucleotide polymorphisms in genes involved in the pharmacokinetics of statins can change the uptake, metabolism, or clearance of a particular statin and therefore increase the serum statin concentration and the risk of developing adverse drug reactions.[9,10] Furthermore, there is a dose–response relationship in the risk of developing statin-induced adverse reactions, and patient characteristics may also contribute to the risk. For example, women are at higher risk of statin-induced musculoskeletal adverse reactions.[11]

The solute carrier organic anion transporter family member 1B1 (*SLCO1B1*) gene encodes the organic anion transporting polypeptide 1B1 (OATP1B1). This influx transporter is mainly expressed on the membranes of hepatocytes and regulates the influx of both endogenous and exogenous compounds.[12] It is involved in the transport of statins, including simvastatin and atorvastatin, into hepatocytes. The rs4149056 c.521T>C minor allele in the *SCLO1B1* gene alters the OATP1B1 transporter function and statin uptake, which increases the serum statin concentration and increases the risk of developing adverse drug reactions.[12-18]

In 2008, the SEARCH Collaborative Group published a genome-wide association (GWA) study about the risk of developing myopathy during simvastatin therapy.[19] They identified common genetic variation in the *SLCO1B1* gene that was strongly associated with an increased risk of simvastatin-induced myopathy. The strongest association was found for the *SLCO1B1* rs4363657 polymorphism, which was significantly associated genome wide with myopathy ($P=4\times10^{-9}$), and was in almost complete linkage disequilibrium with the c.521T>C polymorphism, which has already been described in the literature in relation to statin pharmacokinetics.[13,14,17,18] Patients homozygous for the minor allele had a 16.9 times higher risk of myopathy than patients with the wild-type genotype.

This GWA study demonstrated an association between the c.521T>C polymorphism and myopathy among simvastatin users, but the question is whether this effect is only present for simvastatin or also for the other statins. In 2010, Niemi published a paper about the role of transporter pharmacogenetics in statin therapy.[20] All statins are substrate for the OATP1B1 transporter, but the effect of the c.521T>C polymorphism on the

pharmacokinetics of statins differs. This polymorphism exerts its greatest effect on simvastatin, and the effect diminishes according to the sequence pitavastatin, atorvastatin, pravastatin, rosuvastatin and fluvastatin, the last one with only a minor effect.

In our study, the objective was to investigate whether the c.521T>C polymorphism in the *SLCO1B1* gene modifies the risk of adverse drug reactions during simvastatin and atorvastatin therapy in the Rotterdam Study and in the Utrecht Cardiovascular Pharmacogenetics (UCP) study. We assessed whether this polymorphism is associated with the occurrence of a dose decrease or a switch to another cholesterol lowering drug, as indicators of adverse drug reactions. Our aim was to confirm the previously described association between the c.521T>C polymorphism and simvastatin-associated adverse drug reactions, and to investigate whether the effect was also present among atorvastatin users. We stratified the analyses for sex, age, and dose categories to determine whether these are risk factors for the occurrence of adverse drug reactions during statin therapy.

METHODS

Setting

The Rotterdam Study is a prospective population-based cohort study of chronic diseases in the elderly population. From 1990 to 1993, 7983 inhabitants (98% White) of the suburb of Ommoord in Rotterdam (the Netherlands) aged 55 years or older, participated in the Rotterdam Study I (RS-I) and gave written informed consent. Ethical approval was obtained from the Medical Ethics Committee of the Erasmus Medical Center (Rotterdam, the Netherlands). Baseline examinations took place from March 1990 through July 1993. Follow-up examinations were conducted periodically, every 4–5 years. Medication prescription data were obtained from the fully computerized pharmacies in the Ommoord suburb. These pharmacies dispense the prescriptions of more than 99% of all participants. Information on all filled prescriptions from 1 January 1991 until 1 June 2011 was available and included the product name of the drug, the Anatomical Therapeutical Chemical code, the amount dispensed, the prescribed dosage regimen, and the date of dispensing.[21] Furthermore, in 2000, an extended cohort was enrolled, RS-II. A total of 3011 inhabitants entered the study and have been continuously followed since then. DNA for genotyping was available for 6571 (82%) participants from RS-I and 2157 (71.6%) participants from RS-II. Detailed information on design, objectives, and methods of the Rotterdam Study have been described previously.[22,23]

Study sample

The study population consisted of all participants with at least one filled prescription for simvastatin or atorvastatin between 1 January 1991 and 1 June 2011, and for whom DNA was available. Follow-up started at the date of the first prescription for simvastatin or atorvastatin. Participants were followed until the occurrence of an event as described below, the end of the last prescription for simvastatin or atorvastatin, or until the end of 3 years of follow-up, whichever came first. In addition, participants were censored when they had a gap of at least 180 days between a previous statin prescription and the start of a next prescription. A maximum follow-up period of 3 years was chosen because the risk of an adverse drug reaction is greater during the first years of therapy, and decreases afterwards. To ensure that all participants were incident users and did not have a prescription for simvastatin or atorvastatin before 1 January 1991, for which we did not have the prescription data, we excluded all simvastatin and atorvastatin users with a first prescription before 1 July 1991.

Outcome

In this study, the outcome of interest is adverse drug reactions during statin therapy. In our database, adverse drug reactions were not registered as such. Therefore, we analyzed the occurrence of either a dose decrease or a switch to another cholesterol lowering drug as an indicator of an adverse drug reaction or a too strong reduction in cholesterol level. The first time during follow-up after start of simvastatin or atorvastatin therapy that a patient had a dose decrease or switched to another cholesterol lowering drug (statins, fibrates, bile acid sequestrants, nicotinic acid, acipimox, or ezetimibe) was considered as an event. If a physician notices an adverse drug reaction, he can proceed in two ways. First, the physician can lower the dose of the particular drug, if he considers that there is a dose-effect relationship between the drug dose and the adverse drug reaction. Second, switching to another cholesterol lowering drug may be considered. Therefore, we used both dose decrease and switching as outcome measures, as an indicator of a potential adverse drug reaction.

In a previous study by Becker et al.[24], the reason for the change in medication was retrieved by checking medical patient records of 32 cases, available as of 1 January 1997. For this study, we could additionally retrieve the change in medication for 31 cases. Of the 63 cases that could be checked in the patient records, 43 cases (68%) had a complaint of an adverse drug reaction as reason for the decrease or switch to another cholesterol lowering drug, 17 cases (27%) had a too strong reduction in cholesterol level, and three cases (5%) switched to another cholesterol lowering drug after a cholesterol measurement; this was most likely due to ineffective drug therapy.

Covariables

Age, sex, and the prescribed dose of the first prescription for simvastatin or atorvastatin were considered as potential confounders or effect modifiers for the association between the SLCO1B1 c.521T>C polymorphism and the occurrence of an event. To compare the doses of atorvastatin and simvastatin, the daily dose of the first prescription of statin therapy was expressed in standardized defined daily doses (DDD), according to the WHO. The DDD for simvastatin is 30 mg and the DDD for atorvastatin is 20 mg.[21]

Genotyping and SNP selection

At baseline examination of the Rotterdam Study, blood was taken, from which genomic DNA was extracted, using the salting-out method.[25] Microarray genotyping was performed in both Rotterdam Study cohorts using the Infinium II HumanHap550K Genotyping BeadChip version 3 (Illumina Inc., San Diego, California, USA). Genotyping procedures were followed according to the manufacturer's protocol. During genetic quality control, participants with a non-White background were excluded from the genetic dataset. Microarray genotyping procedures in the Rotterdam Study have been described previously.[26]

Since the *SLCO1B1* c.521T>C polymorphism was not genotyped as a tagging SNP on the Illumina

HumanHap550K Genotyping Beadchip, we selected the genotyped rs1871395 polymorphism in the *SLCO1B1* gene for the analysis. This polymorphism is available on the Illumina Beadchip and is in complete linkage disequilibrium with the c.521T>C polymorphism (R^2=1.0).[27]

Statistical analysis

Deviation from Hardy–Weinberg equilibrium was tested using a X^2-test. Differences in baseline characteristics between the genotype categories were tested for significance with a t-test for continuous variables and a X^2-test for binary variables.

We used Cox proportional hazards models to investigate differences in the incidence of the outcome variable between the *SLCO1B1* c.521T>C genotypes. We used categorical models in which we analyzed the occurrence of events per different genotype categories, since from the results in the GWA study from the SEARCH Collaborative group the greatest effect can be found in the homozygous minor allele genotype. In additional analyses, we stratified for sex, age, and the prescribed dose of the first prescription of statin therapy, to investigate whether they are risk factors for the outcome. The stratification by age and starting dose was based on the mean value of the covariables in the study population, respectively, a mean age of 70.6 years for simvastatin users and 70.2 years for atorvastatin users, and a mean starting dose of 20 mg (0.67 DDD) for simvastatin users and 17.8 mg (0.89 DDD) for atorvastatin users. On the basis of the

calculated values in the study population, we used a cut-off point of 20 mg or less (0.67 DDD) for simvastatin users and 20 mg or less (1.00 DDD) for atorvastatin users. Finally, we tested whether we had enough power to test our research hypotheses. The analyses were performed using SPSS software (version 20.0; SPSS Inc., Chicago, Illinois, USA).

Meta-analysis

We repeated the analyses in an independent cohort, the UCP study. This is a myocardial infarction case-control study in patients with hypercholesterolemia and/or hypertension. Participants from this study were enrolled from the population-based Pharmaco-Morbidity Record Linkage System (PHARMO, http://www.pharmo.nl), which links drug dispensing histories from a representative sample of Dutch community pharmacies to the national registration of hospital discharges [Dutch National Medical Registry (LMR)]. In PHARMO, complete pharmacy records were available as of 1991, including the day of delivery, daily dose, and durations of therapy. Details of this study can be found in earlier publications.[28,29] The *SLCO1B1* rs4149056 c.521T>C polymorphism was genotyped using TaqMan allelic discrimination. For this cohort, the same outcome variables, covariates, and analysis methods were used as in the Rotterdam Study cohort. The study population consists of 98% White participants.

Finally, we performed a fixed effect inverse variance meta-analysis to combine the results of the original analysis (Rotterdam Study) and the results of the UCP study analysis.

RESULTS

We identified 2080 participants in RS-I and RS-II, who were prescribed simvastatin or atorvastatin during follow-up and for whom the *SLCO1B1* genotype was available. Of these statin users, 141 had a first prescription before 1 July 1991 and were excluded from the analysis, finally leading to 1939 statin users eligible for analysis (Table 1). The genotype distribution of c.521T>C polymorphism was in Hardy-Weinberg equilibrium (P 0.055). The minor allele frequency for the c.521T>C polymorphism was 15.5%.

The results of the Cox proportional hazards regression analyses for simvastatin and atorvastatin users are shown in Table 2. Among simvastatin users in the Rotterdam Study, the c.521T>C polymorphism was significantly associated with the outcome. Patients homozygous for the minor allele (CC genotype) had a 1.7 times higher risk of a dose decrease or switch to another cholesterol lowering drug than patients with the TT genotype [hazard ratio (HR) 1.74, 95% confidence interval (CI) 1.05; 2.88, n=319 events]. Among atorvastatin users, we did not find a significant association with the study outcome (HR 1.49, 95% CI 0.54; 4.10, n=110 events).

Table 1 Baseline characteristics of 1939 incident statin users in the Rotterdam Study and 637 incident statin users in the Utrecht Cardiovascular Pharmacogenetics study

	Rotterdam Study population (original analysis)		UCP study population (included in meta-analysis)	
	Simvastatin users (n = 1462)	**Atorvastatin users (n = 477)**	**Simvastatin users (n = 393)**	**Atorvastatin users (n = 244)**
Age [years, mean (SD)]	70.6 (8.2)	70.2 (7.8)	62.3 (9.4)	62.3 (10.3)
Sex [N male (%)]	631 (43.2)	249 (52.2)	296 (75.3)	194 (79.5)
Starting dose [mg, mean (SD)]	20.0 (11.4)	17.8 (13.2)	41.7 (18.9)	45.0 (34.8)
Follow-up [years, mean (SD)]	2.3 (1.1)	2.4 (1.0)	2.3 (1.0)	2.0 (1.0)
Event [n (%)]				
total	319	110	88	42
dose decrease	191 (59.9)	80 (72.7)	63 (71.6)	26 (61.9)
switching	128 (40.1)	30 (27.3)	25 (28.4)	16 (38.1)
SLCO1B1 genotype [n (%)]				
TT	1058 (72.4)	336 (70.4)	286 (72.8)	179 (73.4)
TC	361 (24.7)	126 (26.4)	99 (25.2)	60 (24.6)
CC	43 (2.9)	15 (3.1)	8 (2.0)	5 (2.0)

Abbreviations: UCP, Utrecht Cardiovascular Pharmacogenetics; SD, standard deviation; *SLCO1B1*, solute carrier organic anion transporter family 1B1.

Table 2 The association between the *SLCO1B1* c.521T>C polymorphism and dose decrease or switching to another cholesterol-lowering drug in simvastatin and atorvastatin users in the Rotterdam Study

	Unadjusted HR	**Adjusted HR[a]**	**95% CI**	**P**
Simvastatin users (n=1462)[b]				
TT	1.00 (ref)	1.00 (ref)	–	–
TC	0.74	0.77	0.58; 1.02	0.065
CC	1.69	**1.74**	**1.05; 2.88**	**0.033**
Atorvastatin users (n = 477)[b]				
TT	1.00 (ref)	1.00 (ref)	–	–
TC	1.11	1.17	0.77; 1.79	0.454
CC	1.25	1.49	0.54; 4.10	0.445

Abbreviations: HR, hazard ratio; CI, confidence interval; ref, reference.
[a] Adjusted for age, sex, and starting dose. [b] Categorical model with the TT genotype as reference. **Bold** value indicates statistically significant association.

The analyses were stratified for sex, age, and starting dose (Table 3). For simvastatin users, female sex, low starting dose of statin therapy (>20 mg), and age below 70 years were associated with a higher risk of a dose decrease or a switch to another cholesterol lowering drug in patients with the c.521 CC genotype. Women homozygous for the minor allele had a more than two-fold higher risk for the outcome than women with

Table 3 The association between the *SLCO1B1* c.521T>C polymorphism and dose decrease or switching to another cholesterol-lowering drug in simvastatin and atorvastatin users in the Rotterdam Study – stratified for sex, starting dose, and age

		Unadjusted HR	Adjusted HR[a]	95% CI	P
Stratified by sex[b]					
Men					
Simvastatin (n = 631)	CC vs TT	1.15	1.10	0.41; 2.99	0.851
Atorvastatin (n = 249)	CC vs TT	1.25	1.39	0.43; 4.52	0.580
Women					
Simvastatin (n = 831)	CC vs TT	**2.00**	**2.18**	**1.20; 3.96**	**0.010**
Atorvastatin (n = 228)	CC vs TT	1.11	1.59	0.21; 11.83	0.653
Stratified by starting dose[b]					
Lower starting dose ≤ 20 mg					
Simvastatin (n = 1142)	CC vs TT	**1.87**	**1.83**	**1.06; 3.16**	**0.029**
Atorvastatin (n = 406)	CC vs TT	1.12	1.21	0.38; 3.90	0.746
Higher starting dose > 20 mg					
Simvastatin (n = 320)	CC vs TT	1.02	1.12	0.27; 4.65	0.877
Atorvastatin (n = 71)	TC + CC vs TT[c]	**3.67**	**3.26**	**1.47; 7.25**	**0.004**
Stratified by age[b]					
Age < 70 years					
Simvastatin (n = 722)	CC vs TT	**2.14**	**2.14**	**1.18; 3.88**	**0.012**
Atorvastatin (n = 252)	CC vs TT	0.84	0.81	0.11; 5.91	0.835
Age ≥ 70 years					
Simvastatin (n = 690)	CC vs TT	0.98	0.98	0.36; 2.66	0.971
Atorvastatin (n = 225)	CC vs TT	1.50	1.73	0.53; 5.69	0.364

Abbreviations: HR, hazard ratio; CI, confidence interval.

[a] Adjusted for age and starting dose for the analysis stratified by sex; adjusted for age and sex for the analysis stratified by starting dose; adjusted for sex and starting dose for the analysis stratified by age. [b] Categorical model comparing the homozygous minor allele genotype (CC genotype) with the reference category (TT genotype. [c] The TC and CC genotype categories are combined since the number of cases with two minor alleles (CC genotype) was too small (two cases, one event). **Bold** value indicates statistically significant association.

the reference genotype (HR 2.18, 95% CI 1.20; 3.96), whereas in men no significant association was found (HR 1.10, 95% CI 0.41; 2.99). In the lower age category (<70 years) a similar effect was found, with a more than two-fold higher risk in the minor allele genotype category (HR 2.14, 95% CI 1.18; 3.89).

For atorvastatin users, we found an association between the c.521T>C polymorphism and dose decrease or switching to another cholesterol lowering drug in the highest-dose category (>20 mg). We combined the heterozygous and homozygous minor allele genotype categories for this analysis, since the number of events in patients with minor variant alleles was too small (two events). Patients in this category with at least one

minor allele (TC+CC genotypes) showed a more than three-fold higher risk for a dose decrease or switch compared with the reference TT genotype category (HR 3.26, 95% CI 1.47; 7.35).

In simvastatin users, the power was 100.0% to find an HR of 2.0 and the power was 87.8% to find an HR of 1.5, whereas for atorvastatin users the power was 90.0% to find an HR of 2.0 and 51.7% to find an HR of 1.5.

Meta-analysis

The baseline characteristics of the UCP study sample are shown in Table 1. Within the UCP study, we identified 393 incident simvastatin users and 244 incident atorvastatin users eligible for the analysis. The genotype distribution of the c.521T>C polymorphism was in Hardy–Weinberg equilibrium (P 0.89), and the minor allele frequency was 14.5%.

The results of the Cox proportional hazards regression on dose decrease or switching in the UCP study are shown in Table 4. Within the UCP study, the c.521T>C polymorphism was not associated with dose decrease or switching.

Table 4 The results of Utrecht Cardiovascular Pharmacogenetics study and meta-analysis

UCP study	Unadjusted HR	Adjusted HR[c]	95% CI	P
Simvastatin users (n = 393)[a]				
TT	1.00 (ref)	1.00 (ref)	–	–
TC	0.78	0.74	0.45; 1.24	0.259
CC	1.26	1.38	0.34; 5.71	0.653
Atorvastatin users (n = 244)[a]				
TT	1.00 (ref)	1.00 (ref)	–	–
TC or CC[b]	0.96	0.97	0.68; 1.40	0.871
Meta-analysis (Rotterdam Study and UCP study combined)	**β**	**Adjusted HR[c]**	**95% CI**	**SE**
Simvastatin and atorvastatin users (n = 3576)				
CC (n = 71) vs TT (n=1859)	**0.454**	**1.57**	**1.02; 2.42**	**0.220**
Simvastatin users (n = 1855)				
CC (n = 51) vs TT (n = 1344)	**0.526**	**1.69**	**1.05; 2.73**	**0.244**
Atorvastatin users (n = 721)				
TC + CC (n = 206) vs TT (n = 515)	0.066	1.07	0.82; 1.40	0.137

Abbreviations: UCP, Utrecht Cardiovascular Pharmacogenetics; HR, hazard ratio; CI, confidence interval; ref, reference; SE, standard error.

[a] Categorical model with the TT genotype as reference. [b] The TC and CC genotype categories are combined since the number of cases with two minor alleles (CC genotype) was too small. [c] Adjusted for age, sex, and starting dose. **Bold** value indicates statistically significant association.

In simvastatin users the power was 83.3% to find an HR of 2.0 and the power was 44.5% to find an HR of 1.5, while for atorvastatin users the power was 56.0% to find an HR of 2.0 and 29.0% to find an HR of 1.5.

The results of the fixed effect inverse variance meta-analysis are shown in Table 4. For simvastatin users, compared with the reference homozygous major allele genotype, the heterozygous genotype was associated with a significantly lower risk of a dose decrease or switching to another cholesterol lowering drug (HR 0.76, β – 0.270, 95% CI 0.60; 0.98), and the homozygous minor allele genotype was significantly associated with a higher risk of the outcome (HR 1.69, β 0.526, 95% CI 1.05; 2.73).

For atorvastatin users in the UCP study the number of patients with two minor alleles was too low, and therefore we combined the heterozygous and homozygous minor allele genotype in the meta-analysis of atorvastatin users. For atorvastatin users, no significant association was found. When we combined simvastatin users and atorvastatin users, the heterozygous genotype showed no significant association with the outcome (HR 0.86, β – 0.156, 95% CI 0.70; 1.05), whereas patients homozygous for the minor allele showed a significantly higher risk of the outcome (HR 1.57, β 0.454, 95% CI 1.02; 2.42).

DISCUSSION

The c.521T>C polymorphism in the *SLCO1B1* gene is associated with a higher risk of a dose decrease or switch to another cholesterol lowering drug during simvastatin therapy. These changes in medication are indicators of adverse drug reactions or too strong reductions in plasma cholesterol concentration. In simvastatin users, the determinants female sex, low starting dose (≤20 mg), and age under 70 years were associated with a higher risk of these events. In atorvastatin users, we found a significant association among patients with a high dose (>20 mg) at first prescription. In a meta-analysis in which we combined the original Rotterdam Study results and the results from an independent cohort, the UCP study, the association in simvastatin users remained.

In this study, we demonstrated that the *SLCO1B1* c.521 CC genotype is associated with a higher risk of a dose decrease or switch, as indicators of adverse drug reactions, associated with simvastatin therapy, but in the group of all atorvastatin users there was no association. The question remains whether this association is mainly present for simvastatin therapy, or whether the polymorphism also significantly influences the risk of adverse drug reactions associated with other statins, in particular atorvastatin. For simvastatin therapy, it is established that the variant genotype of the polymorphism is associated with a higher risk of simvastatin-induced adverse drug reactions.[11,19,30,31] Although it has been demonstrated that the c.521T>C minor allele is associated with higher serum statin plasma concentrations for all statins except for fluvastatin[20], previous studies did not

show an effect of this polymorphism on atorvastatin associated adverse drug reactions. Voora et al.[11] demonstrated a significant association of the polymorphism with adverse drug reactions during simvastatin therapy, and patients showed the same trend during atorvastatin therapy, but the association did not reach statistical significance. Santos et al.[32] could not find a significant association of the c.521T>C polymorphism with myalgia or abnormal creatinine kinase values among atorvastatin users. In the study by Brunham et al.[30], the *SLCO1B1* c.521T>C polymorphism was significantly associated with myopathy in simvastatin users, whereas for atorvastatin users no significant association was found. In our study, we demonstrated a significant association between the *SLCO1B1* polymorphism and the risk of the outcome in atorvastatin users with a starting dose of more than 20 mg and with at least one minor allele. These results suggest that there may be an association with the *SLCO1B1* polymorphism in high-dose atorvastatin users, although other studies did not find an association. However, all studies found an odds ratio above one for the association with adverse drug reactions among atorvastatin users, but the number of cases was lower than in our study. As shown in our results above, we had sufficient power to test our main hypotheses. Another explanation for our finding might be that the high-dose atorvastatin group contains mainly too strong reductions in cholesterol levels instead of adverse drug reactions, which were treated by decreasing the dose subsequently. For the majority of cases, we could not distinguish between the underlying reasons for the medication change. However, the majority of the events were due to adverse drug reactions.

Whether the association between the c.521T>C polymorphism and adverse drug reactions is present for all statins remains to be determined. The effect of the c.521T>C polymorphism on serum statin concentration is greater for simvastatin than for atorvastatin[20], but despite this difference, our study was able to detect a pharmacogenetic interaction for atorvastatin with the outcome. Since the serum statin concentration is potentially related to the risk of adverse drug reactions, one would expect the effect of the c.521T>C polymorphism on the risk of developing adverse drug reactions to be greater in high-dose atorvastatin users as compared with low-dose users. This finding is also corroborated by results from the SEARCH study.[19] They found a greater risk of simvastatin in the 80 mg daily SEARCH study, compared with the 40 mg daily Heart Protection Study replication. Furthermore, simvastatin and atorvastatin are not only transported by the OATP1B1 transporter; other OATP transporters also contribute to their uptake. Atorvastatin is also a substrate for the OATP2B1 transporter whereas simvastatin is not.[33] This may indicate that atorvastatin is a less important substrate for the OATP1B1 transporter than simvastatin, and therefore the c.521T>C polymorphism also has a more important effect in simvastatin users than in atorvastatin users, and an effect for atorvastatin can only be seen in high-dose users. Furthermore, in our study the power was lower for atorvastatin compared with simvastatin. However, in the UCP study

we did not have enough power to investigate this association, and replication of the finding in atorvastatin users in an independent population is necessary.

In simvastatin users, the heterozygous genotype showed a non-significant decreased association with dose decrease and switching, with an HR of 0.77. We could not explain this association, and it is in contrast with previous findings from the literature and probably due to chance. In the analysis in the UCP study cohort, in which the same methods were used as in the original analysis, we could not demonstrate an association for simvastatin and atorvastatin users. However, the number of statin users in the UCP study was only one-third of those from the Rotterdam Study, and consequently we had substantially less power to demonstrate an association. Furthermore, among atorvastatin users it was not possible to investigate the association in the homozygous minor allele genotype category separately because of too low numbers, while based on the results in the Rotterdam Study and the results of the SEARCH collaborative group the greatest effect can be found in that category. Nevertheless, in the meta-analysis of the two studies combined, the association in the homozygous minor allele genotype category was still significant for simvastatin, and for simvastatin and atorvastatin combined.

In the Rotterdam Study, among simvastatin users, women showed an approximately two-fold higher risk for a dose decrease or switch to another cholesterol lowering drug. No significant association was found in men. The biological mechanism for this difference is unclear, but our findings are consistent with previous studies that showed an effect of sex. Voora et al.[11] investigated the association between the c.521T>C polymorphism and statin-induced adverse drug reactions and showed that female sex was associated with a higher risk of statin-induced adverse drug reactions, in simvastatin, atorvastatin, and pravastatin users. Niemi et al.[34] demonstrated that women carrying the variant allele of the c.521T>C polymorphism had a higher serum pravastatin concentration than men carrying the variant allele. In the participants of the SEARCH trial, the relative risk of myopathy was higher in women.[19] In a recent study, women homozygous for the minor allele of the c.521T>C polymorphism showed a significantly greater decrease in total cholesterol compared with men.[35] It is possible that the OATP1B1 transporter is more strongly expressed in women than in men, resulting in the effect of the c.521T>C polymorphism being stronger in women. Furthermore, statins are not only dependent on the OATP1B1 transporter for their uptake; other transporters also play a role. It is possible that another uptake transporter has a lower activity in women, through which the OATP1B1 transporter plays a more important role and therefore the effect of the c.521T>C polymorphism is more pronounced. Differences between men and women in transporter expression have been described previously. The ATP-binding cassette B1 (ABCB1) transporter, for example, has a lower expression in women than in men.[36]

We found an age difference in the risk of the events for simvastatin, with a significant effect in the younger age category (<70 years) and no significant association in the

older age category (≥70 years). Possibly, the older age group is a more selective group of simvastatin users who are relatively healthier. This group reached the older age category, whereas the more diseased patients had already died. Furthermore, in the older population other factors, such as a decrease in liver and renal function and a change in body composition, may be of more importance in the risk of developing adverse drug reactions, making the effect of pharmacogenetics lower.[37-39] Another explanation might be that the OATP1B1 transporter expression is influenced by age. For example, this has been described for the P-glycoprotein transporter expression.[40]

We found a significant association between the c.521T> C polymorphism and the outcome in simvastatin users in the lower-dose category (≤20 mg), and in atorvastatin users in the high-dose category (>20 mg). There is a dose–response relationship in the risk of developing statin-induced adverse drug reactions. A possible explanation is that physicians are more careful in prescribing statins to patients with risk factors for myopathy (high-risk patients), and therefore start with a lower dose in these patients. For example, a physician starts with a lower dose in a female patient with impaired liver function.

Potential biases in our study should be considered. Omission of blood samples and difficulties with genotyping were completely random and not related to the c.521T>C genotype. The results of our study are consistent with previous studies on this topic. In the medical patient records, we could not retrieve the reasons for all dose decreases or switches and assumed that these events were associated with increased serum statin levels resulting in adverse drug reactions. The majority of the checked events were due to adverse drug reactions. Only in a minority of the cases was the cause of the event too strong cholesterol lowering or ineffective drug therapy. Furthermore, we missed events caused by adverse drug reactions, since we did not consider, for example, patients who stopped statin therapy due to adverse drug reactions. This may have resulted in less power of the study but will not change the effect size. We explicitly did not choose discontinuation of treatment as an indicator of an adverse drug reaction, since discontinuation of statins could be related to many reasons, such as noncompliance, the disappearance of the indication over time, the patient reaches a certain age, and the preventive effect becomes too small. Noncompliance frequently occurs in the case of statin therapy; in a previous study it was demonstrated that 53% of new users of statin therapy discontinued therapy within 2 years.[41] The risk of information or selection bias is unlikely since the Rotterdam Study is a population-based cohort study, in which data are collected prospectively without prior knowledge of the aim of this study. In the analysis, we controlled for potential confounding factors such as age, sex, and starting dose of the prescribed statin therapy. Although we had 1939 statin users available and we had the option of stratifying the analysis by age, sex, and starting dose, in some categories we lost power because of small sample size.

In conclusion, our study provides further evidence for the role of the *SLCO1B1* c.521T>C polymorphism in simvastatin-associated adverse drug reactions. We demonstrated an association between the c.521T>C polymorphism and dose decrease or switching, as indicators of adverse drug reactions, among simvastatin users, and describe risk factors that increase the risk for these events. In addition, in atorvastatin users we found an association between the c.521T>C polymorphism and dose decrease or switching among users with a starting dose of more than 20 mg. For simvastatin users with the homozygous minor allele genotype, female sex, age under 70 years, and a low starting dose (<20 mg) were risk factors for the outcome. Our findings suggest that in patients carrying two minor alleles of the c.521T>C polymorphism, an alternative statin instead of simvastatin, or a cholesterol lowering drug from a different drug group, may be a reasonable first choice in the treatment of hypercholesterolemia.

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2.5

No association between *CYP3A4*22* and statin effectiveness in reducing the risk for myocardial infarction

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Pharmacogenomics 2014; 15(11): 1471-1477

ABSTRACT

Introduction: Genetic variation has been shown to influence statin response in terms of lowering LDL-cholesterol. The recently discovered *CYP3A4*22* allele (defined as rs35599367) has been shown to affect statin-induced LDL-cholesterol lowering. Our objective was to investigate whether this polymorphism modifies the risk reduction for myocardial infarction (MI) by statins.

Methods: We analyzed the interaction between the *22 minor allele and statin use in the independent Utrecht Cardiovascular Pharmacogenetics study and Rotterdam Study, using logistic and Cox regression models.

Results: In total, 771 MI cases and 6131 controls were included in the analyses. There was no effect of the CYP3A4*22 allelic status in the studies separately, nor when the estimates from both studies were combined (interaction odds ratio: 1.27, 95% CI 0.73; 2.21; P 0.40 for carriers of the minor T-allele).

Conclusion: We found no association of the CYP3A4*22 minor allele (rs35599367) with the effectiveness of statins in reducing MI risk.

INTRODUCTION

Statins are widely prescribed to reduce plasma LDL-cholesterol (LDL-C), and by this action reduce the risk for cardiovascular disease (CVD). Statins act by inhibiting HMG-CoA reductase, the rate-controlling enzyme in the cholesterol biosynthesis pathway.[1] The response to statin therapy shows a degree of interindividual variability influenced by genetic variation and environmental factors. Based on a variety of selection criteria, a whole range of candidate genes and SNPs have been associated with the lipid-lowering response to statins.[2] Due to several factors (e.g., small samples, differences in analysis methods and preference to publish new results), results have rarely been replicated.

One way to reduce false-positive findings in addition to using stringent criteria for claiming associations is to investigate well-characterized SNPs with effects on gene expression or protein function. This method is especially relevant in pharmacogenetics, where the biologic pathway involved (e.g., enzymes metabolizing the drug and the proteins that the drug targets) is known, at least to a degree.

Lovastatin, simvastatin and atorvastatin are all extensively metabolized on the first pass through the liver, primarily by cytochrome P450 isoform CYP3A4, which leads to a systemic availability of 5-10%.[3] A change in CYP3A4 activity is expected to be associated with a change in the metabolism of the drug, which may in turn affect systemic statin levels. Several genetic variants in the *CYP3A4* gene have been described, but none of these have explained a large proportion of the variability in CYP3A4 enzyme activity. Recently, a SNP (rs35599367, *CYP3A4*22*) in the *CYP3A4* gene was reported to be associated with reduced CYP3A4 enzyme expression and activity and increased response to statin therapy.[4] In separate studies, this SNP was associated with reduced CYP3A4 activity, resulting in an increased lipid-lowering response to simvastatin[5], tacrolimus pharmacokinetics[6], and an increased risk of delayed graft function.[7]

While the association of rs35599367 with CYP3A4 activity and a lipid-lowering response to statins has been replicated, it is unknown whether this SNP also affects the relative risk reduction for cardiovascular events.[8] Here, we aimed to investigate whether this SNP in intron 6 of *CYP3A4* modifies the risk-lowering effect of statins on myocardial infarction (MI) in two large population-based studies.

METHODS

We assessed the association of rs35599367 with statin effectiveness in reducing MI risk in two separate data sets, the Utrecht Cardiovascular Pharmacogenetics (UCP) study and the Rotterdam Study. The study setup has been described for both the UCP study[9] and the Rotterdam Study[10,11], and will be described briefly here (for further information, see

Supplementary Methods). The UCP study was approved by the Medical Ethics Committee of the University Medical Center Utrecht, The Netherlands, and participants gave written informed consent to the researcher allowing them to collect, store and analyze their DNA material. The Rotterdam Study was approved by the Medical Ethics Committee according to the Wet Bevolkingsonderzoek: ERGO (Population Study Act: Rotterdam Study), executed by the Ministry of Health, Welfare and Sports of The Netherlands. At baseline of the Rotterdam Study, participants gave written informed consent for the researchers to use their DNA for research purposes.

We included 1483 individuals from the UCP study and 5484 individuals from the Rotterdam Study. From this total of 6967 individuals (Table 1), 771 subjects were MI cases. This gave us a power of 80% to detect a SNP-statin interaction odds ratio (OR) of 0.5, and a power of 8% to detect an interaction OR of 0.92, both at a P-value of 0.05.

The UCP study

The UCP study participants were enrolled from the population-based Pharmaco-Morbidity Record Linkage System (PHARMO) System, which links drug-dispensing histories from a representative sample of Dutch community pharmacies to the national registration of hospital discharges. Patients who received a prescription for an antihypertensive drug and/or had hypercholesterolemia (prescription for a cholesterol lowering drug or total cholesterol >5.0mmol/l) were selected from the PHARMO database for pharmacogenetic studies on antihypertensive drugs and statins. From this cohort, patients hospitalized for MI (International Classification of Diseases [ICD]-9 code 410) were included as cases if they were registered in the PHARMO database for at least 1 year. Controls were selected using risk set sampling and if they met the same eligibility criteria as the cases but had not developed a MI. Coded pharmacy records were used to ascertain exposure to statins and other drugs. Self-reported data on covariates (e.g. smoking, hypertension and diet, among others) were assessed using questionnaires. We used conditional logistic regression for analysis, with cases matched to controls based on age and sex. Analyses were corrected for BMI, use of antihypertensive medication, family history of CVD and smoking. Analyses were performed using R (version 3.0.2[12]).

The Rotterdam study

The Rotterdam Study is a prospective, population-based cohort study of chronic diseases in the general population. At baseline between 1990 and 1993, all persons aged 55 years and over in the Ommoord district of Rotterdam, the Netherlands, were invited to participate. Participants have been continuously followed since then during follow-up rounds (1993–1995, 1997–1999, 2002–2004 and 2009–2012). Medication-dispensing data were obtained from all seven fully computerized pharmacies in the Ommoord suburb. Information on the presence and occurrence of MI is available through collaboration

Table 1 Baseline characteristics by case-control status

	UCP study			Rotterdam Study		
	Cases (n = 275)	**Controls (n = 1121)**	**P[a]**	**Cases (n = 474)**	**Controls (n = 5010)**	**P[a]**
Males	216 (78.5%)	879 (78.4%)	1[b]	264 (55.2%)	1852 (37.0%)	<0.0001
Age	62.25 (9.68)	62.19 (9.45)	0.9219[b]	68.54 (7.58)	69.33 (9.17)	0.033
BMI	27.65 (9.36)	26.98 (9.45)	0.2343	26.42 (3.44)	26.27 (3.75)	0.408
Familial history of CVD	164 (59.6%)	595 (53.1%)	0.0382	282 (60.8%)	2588 (53.2%)	0.002
Current smoker	176 (64.0%)	452 (40.3%)	<0.0001	130 (27.2%)	1103 (22.0%)	<0.0001
Use of statins	126 (45.8%)	595 (53.0%)	0.0364	69 (14.7%)	899 (17.9%)	0.562
Use of antihypertensives	161 (58.5%)	621 (55.3%)	0.3817	149 (31.2%)	1273 (25.4%)	0.010
Total cholesterol (mmol/L)	NA	NA	NA	6.84 (1.18)	6.61 (1.23)	<0.0001
HDL-cholesterol (mmol/L)	NA	NA	NA	1.25 (0.33)	1.37 (0.37)	<0.0001
Hypertension	NA	NA	NA	214 (44.8%)	1599 (32.7%)	0.066
Diabetes patients	NA	NA	NA	67 (14.1%)	715 (14.3%)	0.237
Genotypes						
CC (n, %)	247 (89.9%)	1006 (89.7%)	NA	413 (87.1%)	4412 (88.1%)	NA
CT (n, %)	25 (9.09%)	107 (9.54%)	NA	60 (12.6%)	586 (11.7%)	NA
TT (n, %)	3 (1.09%	8 (0.71%)	NA	1 (0.21%)	12 (0.24%)	NA
Statin type	**n (%)**	**Dose[c]**		**n (%)**	**Dose[c]**	
Simvastatin (C10AA01)	421 (49.0%)	1.48 (0.80)	NA	627 (64.8%)	1.18 (0.73)	NA
Pravastatin (C10AA03)	99 (11.5%)	1.50 (0.59)	NA	106 (11.0%)	0.94 (0.58)	NA
Fluvastatin (C10AA04)	23 (2.7%)	0.93 (0.55)	NA	47 (4.9%)	0.90 (0.46)	NA
Atorvastatin (C10AA05)	271 (31.5%)	2.10 (1.47)	NA	162 (16.7%)	1.48 (0.91)	NA
Cerivastatin (C10AA06)	5 (0.6%)	1.30 (0.45)	NA	11 (1.1%)	1.16 (0.41)	NA
Rosuvastatin (C10AA07)	41 (4.8%)	1.28 (0.61)	NA	15 (1.5%)	1.12 (0.71)	NA

Abbreviations: BMI, body mass index; CVD, cardiovascular disease; HDL, high-density lipoprotein; ATC, Anatomical Therapeutical Chemical Code.
Categorical data are presented as n (%), while continuous data are presented as mean (standard deviation).
[a] P-value from t-test (continuous variables) or X^2-tests (categorical variables). [b] Analyses were matched by sex and age; cases and controls are therefore very similar for these variables. [c] Defined daily doses given as mean dose (standard deviation).

with the general practitioners in the study area[13]. In order to control for confounding, analyses were corrected for age, sex, family history of CVD, hypertension, current and past smoking status, BMI, use of antihypertensives, serum total and HDL-cholesterol levels and the presence of type 2 diabetes mellitus. Cox proportional hazard regression analyses were used, and all analyses were performed using SPSS software (SPSS, Inc., version 20.0, IL, USA).

Statin use & case definition

For this study, participants with more than 180 cumulative defined daily dosages (DDDs) of total use of statins were considered to be users, while participants with less than 180 cumulative DDDs were considered to be nonusers. An exposure of 180 DDDs was chosen as the cumulative statin exposure necessary to exert a protective effect on MI. Participants that developed a MI (ICD-9 code 410) during follow-up were defined as a case, whereas controls did not develop a MI during follow-up.

Genotyping

For both studies, genotyping was carried out using TaqMan® (Applied Biosystems, CA, USA) genotyping assays (catalog identifier: C_59013445_10) on the ABI PRISM® 7500 Fast Real-Time PCR Systems (Applied Biosystems), according to manufacturer instructions.

Statistical analysis

Analyses were performed separately for both data sets, consisting of logistic regression analysis for the UCP study and Cox proportional hazards regression analysis for the Rotterdam Study. A SNP–statin interaction in the regression model was used to assess the influences of the SNP on statin efficacy. The SNP–statin interaction analyses were investigated in all statins combined, and second, only in statins that are metabolized by CYP3A4, which include simvastatin, atorvastatin, lovastatin and cerivastatin. Estimates from both studies were combined using inverse-variance meta-analysis. Due to the low minor allele frequency (6%), a dominant genetic model was used. QUANTO was used for the power analyses.[14] A Hardy–Weinberg equilibrium p-value of <0.01, calculated with the exact test[15] due to low genotype counts, was considered to be an indication of problems with the genotyping. We performed two sensitivity analyses: one in which only patients using statins at the date of MI (or corresponding date for matched controls) were considered as being exposed, and one in which start and end dosages were stratified by genotype in order to investigate whether the genotype group with a theoretically stronger statin effect was titrated towards lower dosages.

RESULTS

A total of 771 MI cases and 6131 controls were eligible for the analysis (Table 1). Cases in the UCP study (n = 275) more frequently had a familial history of CVD and smoked more frequently than controls in this study. Cases in the Rotterdam Study (n = 474) also had a familial history of CVD more frequently than controls, and had increased rates of hypertension and use of blood pressure-lowering medication. Compared with the par-

ticipants in the Rotterdam Study, participants in the UCP study were younger and more frequently males. Genotype distributions were similar in both studies, and rs35599367 was in Hardy–Weinberg equilibrium in both studies.

Statins were protective for MI in the UCP study (OR 0.66, 95% CI 0.49; 0.89, Supplementary Table 1) and in the Rotterdam Study (OR 0.95, 95% CI 0.71; 1.27, Supplementary Table 2). Statins were more protective in the Rotterdam Study, but not in the UCP study, when only current users were considered (sensitivity analysis; Supplementary Tables 3 & 4). The CYP3A4*22 polymorphism had no main effect on the risk for MI (i.e., no SNP effect without considering an interaction with statins), with the OR in the UCP study being 0.98 (95% CI 0.63; 1.53, P 0.93) and the OR in the Rotterdam Study being 1.15 (95% CI 0.87; 1.52, P 0.32).

SNP-statin interaction

In both the UCP study and the Rotterdam study, there was no evidence of a SNP-statin interaction (Table 2). In the UCP study, the adjusted interaction OR of 0.94 (95% CI 0.37;2.40) indicated a slightly higher statin benefit in carriers of the minor T-allele. However, in the Rotterdam Study, the interaction effect estimator was above 1 (adjusted interaction OR 1.48, 95% CI 0.75; 2.94), indicating less benefit. When the estimates from

Table 2 Interactions between statin exposure and rs35599367 and the risk of myocardial infarction

Genotype	Statin exposure	Cases (n)	Controls (n)	OR (95% CI)[a]	Interaction-OR[b] (95% CI)[a]
UCP study					
CC	**No statin exposure**	135	476	Reference value	
	Statin exposure	112	530	0.75 (0.56; 1.01)	
CT/TT	**No statin exposure**	14	50	Reference value	
	Statin exposure	14	65	0.70 (0.17; 2.99)	0.94 (0.37; 2.40)
Rotterdam Study					
CC	**No statin exposure**	356	3618	Reference value	
	Statin exposure	57	794	0.86 (0.63; 1.19)	
CT/TT	**No statin exposure**	49	493	Reference value	
	Statin exposure	12	105	1.19 (0.55; 2.54)	1.48 (0.75; 2.94)
Meta-analysis					
CC	**No statin exposure**	491	4094	Reference value	
	Statin exposure	169	1324	0.80 (0.65; 0.99)	
CT/TT	**No statin exposure**	63	543	Reference value	
	Statin exposure	26	170	1.06 (0.54; 2.09)	1.27 (0.73; 2.21)

Abbreviations: OR, odds ratio; CI, confidence interval.

[a] Values are from unadjusted conditional logistic regression for the UCP study (adjusted models cannot be calculated in all strata). [b] Adjusted values for both data sets.

both studies are combined, the interaction OR was 1.27 (95% CI 0.73; 2.21), with a P-value of 0.40. SNP-statin interaction analyses in which we only considered the statins that are metabolized by CYP3A4 (i.e., simvastatin, atorvastatin, cerivastatin and lovastatin) showed results similar to the analysis with all statin users combined (Supplementary Table 5); the overall interaction OR was 1.21 (95% CI 0.66; 2.22, P 0.54) in this analysis. An additional sensitivity analysis showed that there was no effect of genotype on end dosages of statins in the UCP study or in the Rotterdam Study (Supplementary Tables 6 & 7).

DISCUSSION

Here, we examined the functional SNP rs35599367 in *CYP3A4*, coding for the **22* isoform, and its relationship with the effectiveness of statins in terms of reducing the risk for MI. Although this study presents a 'negative' result in that we did not find an association, the result is still of interest. We showed that the rs35599367 polymorphism in the metabolizing CYP3A4 enzyme, with a known and replicated association with either necessary dose[4] or LDL-C reduction with a standard dose[5], is not associated with the clinical outcome MI.

The SNP under investigation was linked to a 0.34 mmol/l stronger lipid-lowering response for rs35599367 minor T-allele carriers when compared with non-carriers. Because stronger reductions in LDL-C correspond to further reductions in the incidence of cardiovascular events (relative risk of 0.78 per mmol/l of LDL-C reduction[16]), we hypothesized that this SNP is associated with better statin effectiveness. When considering the effect the T-allele has on the lipid-lowering response, we expected an interaction OR of 0.92 for the clinical outcome. When assuming a 22% reduction of cardiovascular risk through normal statin-induced LDL-C lowering, this translates to a 28% risk reduction for carriers of one or more minor T-alleles. The size of the difference made it reasonable to search for the clinical effect of this polymorphism in observational studies.

There may be several reasons for not finding an association of rs35599367 with statin effectiveness. The most probable explanation seems to be that the effect on LDL-C response is too small to affect clinical outcome. While our study had a statistical power of 80% for detecting a SNP–statin interaction OR of 0.5, it had much less power for detecting a smaller effect (i.e., an interaction OR of 0.92). The power to find an interaction of this size was 8%, emphasizing the difficulty of finding small effects on clinical benefit, even in large studies. However, if the association can only be found in an extremely large dataset, the clinical relevance of the interaction is uncertain. Secondly, we have analyzed the data from two separate studies, each with their own study design. For example, the studies demonstrated different values regarding the effectiveness of statins. However, by adjusting for specific covariates in both studies, we showed a protective effect of

statins, and it is unlikely that the SNP–statin interaction was influenced by confounding, because the prescribing physicians had no knowledge of the genetic information. Another possible reason for not finding an effect is that the drug dosage is titrated for a particular reduction of LDL-C, thus negating any potential clinical effect. However, we did not see any difference between the genotype groups regarding dosages of statins. Lastly, it cannot be excluded that in a different population (e.g., of Asian ancestry), this SNP might have a larger effect. This is not uncommon in (pharmaco)genetics, but we were unable to study this with the current Caucasian data sets.

The strengths of this study are that we used two separate studies with an accurate measure of drug use and a well-defined outcome, being that of MI. Both studies separately did not show an interaction, nor was there an effect when the data from both studies were combined. The current sample size was larger than that of the previous studies[4,5], which investigated 235 and 80 statin users, respectively, in terms of LDL-C outcome. Here, with 771 cases and 4713 controls, we could not show an effect on risk reduction for MI.

In two population-based studies, we could not demonstrate any effect of modification of the *CYP3A4* rs35599367 polymorphism on the effectiveness of statins in reducing the risk for MI. Although this polymorphism has been shown to influence other clinical outcomes, this study suggests a limited value of genotyping patients for this specific polymorphism in order to improve the clinical effectiveness of statins on MI.

FUTURE PERSPECTIVE

To date, several polymorphisms have been found to affect responses to statin therapy based on achieving reduced LDL-C. A further increase in the number of loci associated with LDL-C response could probably be achieved with larger studies using genome-wide association study methodologies, although large numbers of statin users would be required in order to effectively conduct such studies. The main problem with statin pharmacogenetics is that using these findings to improve clinical effectiveness has not been very successful. When more loci are identified, a dosing algorithm based on the whole collection of SNPs influencing statin response might improve effectiveness. Given the existing findings in this field, it seems unlikely that genotyping an incident statin user for any one SNP and using this information to adjust statin dose will reduce the risk for cardiovascular events.

EXECUTIVE SUMMARY

Background

- The response to statin therapy shows a degree of interindividual variability, which is influenced by genetic variation and environmental factors.
- Statins are extensively metabolized on the first pass through the liver, primarily by cytochrome P450 isoform CYP3A4.
- The *CYP3A4*22* isoform (defined as rs35599367) was associated with lower CYP3A4 expression and activity and increased response to statin therapy, based on LDL-cholesterol lowering. It is unclear whether this polymorphism has effects on the reduction of cardiovascular events by statin use.

Patients & methods

- We analyzed the association of rs35599367 with clinical effectiveness of statins in two separate data sets, the Utrecht Cardiovascular Pharmacogenetics (UCP) study and the Rotterdam Study.
- A total of 771 myocardial infarction cases and 6131 controls were included in the analyses.

Results & conclusion

- There was no effect of *CYP3A4*22* in either of the studies separately, nor when the estimates from both studies were combined (interaction odds ratio 1.27, 95% CI 0.73 – 2.21, P 0.40 for carriers of the minor T-allele).
- Although this polymorphism has been shown to influence other clinical outcomes, this study suggests a limited value of genotyping patients for this specific polymorphism in order to improve the clinical effectiveness of statins in prevention of myocardial infarction.

All supplementary files can be found via:
http:// www.futuremedicine.com/doi/suppl/10.2217/pgs.14.90

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2.6

Pharmacogenetic meta-analysis of genome-wide association studies of LDL cholesterol response to statins

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Nature Communications 2014

ABSTRACT

Statins effectively lower LDL-cholesterol levels in large studies and the observed inter-individual response variability may be partially explained by genetic variation. Here we perform a pharmacogenetic meta-analysis of genome-wide association studies (GWAS) in studies addressing the LDL-cholesterol response to statins, including up to 18,596 statin-treated subjects. We validate the most promising signals in a further 22,318 statin recipients and identify two loci, *SORT1/CELSR2/PSRC1* and *SLCO1B1*, not previously identified in GWAS. Moreover, we confirm the previously described associations with *APOE* and *LPA*. Our findings advance the understanding of the pharmacogenetic architecture of statin response.

INTRODUCTION

The 3-hydroxymethyl-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, also known as statins, are widely prescribed and are highly effective in the management and prevention of cardiovascular disease. Statin therapy results in a lowering of low-density lipoprotein cholesterol (LDL-C) levels by up to 55%[1] and a 20–30% reduction of cardiovascular events.[2] Despite the clinical efficacy of statins in a wide range of patients[2], interindividual variability exists with regard to LDL-C-lowering response as well as efficacy in reducing major cardiovascular events.[3] The suggestion that some of this variability may be due, in part, to common pharmacogenetic variation is supported by previous studies that have identified genetic variants associated with differential LDL-C response to statin therapy.[4-6]

A small number of genome-wide association studies (GWAS) have previously identified loci associated with statin response on a genome-wide level. A GWAS in the JUPITER trial identified three genetic loci, *ABCG2* (rs2199936), *LPA* (rs10455872) and *APOE* (rs7412), that were associated with percentage LDL-C reduction following rosuvastatin therapy.[7] In the CARDS and ASCOT studies, single nucleotide polymorphisms (SNPs) at *LPA* (rs10455872) and *APOE* (rs445925 and rs4420638) were associated with LDL-C response to atorvastatin treatment.[8] A combined GWAS in three statin trials identified a SNP within *CLMN* (rs8014194) that is associated with the magnitude of statin-induced reduction in plasma cholesterol.[9] However, two other GWAS identified no genetic determinants of LDL-C response to statin therapy at a genome-wide significant level.[6,10] On the basis of these studies, as well as previous candidate gene studies[4,6], the only genetic variants that have been consistently identified to be associated with variation in LDL-C response to statin therapy, irrespective of statin formulation, are located at or nearby *APOE* and *LPA*. To determine whether additional loci may influence LDL-C response to statins, we formed the Genomic Investigation of Statin Therapy (GIST) consortium and conducted a pharmacogenetic meta-analysis using GWAS data sets from randomized controlled trials (RCTs) and observational studies. We identify two loci not previously identified in GWAS, *SORT1/CELSR2/PSRC1* and *SLCO1B1*. In addition, we confirm the associations within the *APOE* and *LPA* genes. These findings will extend the knowledge of the pharmacogenetic architecture of statin response.

METHODS

Study populations

The meta-analysis was conducted in the GIST consortium, which includes data from 8 randomized controlled statin trials (RCTs) and 11 prospective, population-based studies.

The initial analysis (first stage) was performed in 8,421 statin-treated subjects from 6 RCTs (ASCOT, CARDS, CAP, PRINCE, PROSPER and TNT) and 10,175 statin-treated subjects from 10 observational studies (AGES, ARIC, BioVU, CHS, FHS, GoDARTS I, GoDARTS II, Health ABC, HVH and MESA). Further investigation (second stage) was performed in 21,975 statin-treated subjects from two randomized trials (HPS and JUPITER) and one observational study (Rotterdam Study). Six SNPs were additionally genotyped in the Scandinavian participants of the ASCOT study. The details of the first- and second-stage studies can be found in the Supplementary Tables 1 and 2 and Supplementary Notes 1 and 2.

Subjects

Response to statin treatment was studied in statin-treated subjects only and not in those treated with placebo. Subjects included in the observational studies' analysis should be treated with statins and have LDL-C measurements before and after start of statin treatment. Subjects of reported or suspected non-European ancestry were excluded. All participants gave written informed consent and the study was approved by all institutional ethics committees.

Outcome measurements

The response to statin treatment was defined as the difference between the natural log-transformed on- and off-treatment LDL-C levels. The beta of the corresponding regression thus reflects the fraction of differential LDL lowering in carriers versus non-carriers of the SNP. For observational studies, the on-treatment LDL-C levels were taken into account for all kinds of prescribed statins, at any dosage, for any indication and for at least 4 weeks before measurement. Characteristics of on- and off-treatment LDL-C levels and statins used in each study are shown in Supplementary Table 2. For each individual, at least one off-treatment LDL-C measurement and at least one on-treatment LDL-C measurement were required. When multiple on- or off-treatment measurements were available, the mean of the cholesterol measurements was used. Subjects with missing on- or off-treatment measurements were excluded, with the exception of the GoDARTS cohorts for which missing off-treatment LDL-C levels were estimated using imputation methods (Supplementary Note 2). In the HPS, proportional LDL-C response was defined by the changes in natural log lipid levels from the screening visit before starting statin therapy to the randomization visit.[6]

Genotyping and imputation

Genotyping, quality control, data cleaning and imputation were performed independently in each study using different genetic platforms and software as outlined in Supplementary Table 4. In all studies, genotyping was performed using Illumina, Af-

fymetrix or Perlegen genotyping arrays, and MACH, Impute or BIMBAM software was used for imputation.

GWAS analysis

Each study independently performed the GWAS on the difference between natural log-transformed on- and off-treatment LDL-C levels. To control for possible associations with off-treatment LDL-C levels, analyses were adjusted for the natural log-transformed off-treatment LDL-C level. An additive genetic model was assumed and tested using a linear regression model. For imputed SNPs, regression analysis was performed onto expected allele dosage. Analyses were additionally adjusted for age-, sex- and study-specific covariates (for example, ancestry principal components or country). Analyses in the observational studies were, if available, additionally adjusted for the statin dose by the natural logarithm of the dose equivalent as defined in Supplementary Table 3. This table shows the dose equivalent per statin type; dividing the statin dosage of an individual by the dose equivalent shown in Supplementary Table 3 will give the adjusted statin dosage.

Quality control and meta-analysis

Centrally, within each study, SNPs with MAF <1% or imputation quality <0.3 were excluded from the analysis. QQ-plots were assessed for each study to identify between-study differences (Supplementary Fig. 1). The software package METAL was used for performing the meta-analysis (http://www.sph.umich.edu/csg/abecasis/Metal/index.html). A fixed effects, inverse variance weighted approach was used. Using an inverse variance weighted meta-analysis will give smaller weights to studies with large SE. To correct for possible population stratification, genomic control was performed by adjusting the within-study findings and the meta-analysis results for the genomic inflation factor.

Second stage

SNPs with P values $<5\times10^{-4}$ in the first-stage meta-analysis were selected for further investigation in a second stage. A maximum of two SNPs per locus were selected, based on statistical significance, except for the *APOE* locus, for which all genome-wide significant associated SNPs were selected for validation. A total of 246 SNPs, within 158 independent loci, were selected for the second stage, which was performed in the JUPITER trial, HPS study and the Rotterdam Study, which all had GWAS data and response to statin treatment available. For 2 of the 246 SNPs, a proxy was used in the JUPITER trial, and 31 SNPs were not available, nor was a proxy SNP. HPS provided data on 151 directly genotyped SNPs from GWAS and IPLEX experiments, including 48 of the requested SNPs and 103 proxy SNPs (R^2> 0.8). Analysis in HPS was not adjusted for ln baseline LDL-C

levels. In addition, the number of subjects with data varied from SNP-to-SNP and ranges from ~4,000 for variants with GWAS data to ~18,000 for some candidate genes. Results of the first and second stage were combined using fixed effects, inverse variance weighted meta-analysis and analyzed by METAL. As a third stage, six SNPs with P values $5 \times 10^{-8} < P < 5 \times 10^{-7}$ in the combined meta-analysis were selected for additional genotyping in the Scandinavian participants of the ASCOT study. Kaspar assays were designed for four of the SNPs using the KBioscience Primerpicker software, and oligos were provided by Intergrated DNA technologies (http://eu.idtdna.com/site). Full Kaspar methodology is available from LGC SNP genotyping (http://www.lgcgenomics.com/genotyping/kasp-genotypingreagents/). Two SNPs (rs981844 and rs13166647) were genotyped using Taqman assays supplied by Life Technologies (http://www.lifetechnologies.com/uk/en/home.html) using the standard Taqman protocol. Results of the additional genotyping were combined with results from the first and second stages using a fixed effects, inverse variance weighted meta-analysis and analyzed by METAL.

Determination of changes in LDL subfractions

LDL subclasses were analyzed as described previously[29] using non-denaturing gradient gel electrophoresis of fasting plasma samples taken at baseline and after 6 weeks of simvastatin 40 mg per day (CAP study, n = 579) or 12 weeks of pravastatin 40 mg per day (PRINCE study, n = 1,284). Aliquots of 3.0ml of whole plasma were mixed 1:1 with a sampling buffer of 20% sucrose and 0.25% bromophenol blue. Electrophoresis of samples and size calibration standards was performed using 2–14% polyacrylamide gradients at 150V for 3 h following a 15-min pre-run at 75 V. Gels were stained with 0.07% Sudan black for 1h and stored in a 0.81% acetic acid, 4% methanol solution until they were scanned by computer-assisted densitometry for determination of areas of LDL IVb (22.0–23.2 nm), LDL IVa (23.3–24.1nm), LDL IIIb (24.2–24.6 nm), LDL IIIa (24.7–25.5nm), LDL IIb (25.6–26.4 nm), LDL IIa (26.5–27.1nm) and LDL I (27.2–28.5 nm). The cholesterol concentrations of the subfractions (mg/dL plasma) were determined by multiplying percent of the total stained LDL area for each subfraction by the LDL-C for that sample. For genetic association analyses, subfractions were grouped into large LDL (LDL IIa), medium LDL (LDL IIb), small LDL (LDL IIIa) and very small LDL (LDL IIIb+Iva+IVb) as described previously.[18] A generalized estimating equation method was used to test the association of log change with the interaction of the four SNPs by LDL subfraction.

Effect of off-treatment LDL-C

Effects of genetic variation on treatment response as measured by on-treatment LDL-C could be mediated through effects on the off-treatment LDL-C. To evaluate whether genetic on-treatment LDL-C likely reflects residual effect on off-treatment LDL-C, it is necessary to adjust for the off-treatment LDL-C levels and to correct the maximum likeli-

hood estimate of the adjusted effect of genotype on on-treatment value for the noise in off-treatment values (the noise is both random measurement error and intra-individual variation in usual LDL-C). This analysis was only carried out in CARDS in which multiple baseline measurements were available. From the rules of path analysis, we calculated the direct effect γ of genotype on an on-treatment trait value as $\beta - \alpha\delta (1 - \rho)/ \rho$, where β is the coefficient of regression for on-treatment trait value on genotype adjusted for measured off-treatment value, α is the coefficient of regression of baseline LDL on genotype, ρ is the intraclass correlation between replicate measurements of off-treatment values and δ is the coefficient of regression for on-treatment value on observed off-treatment value8. For these calculations, we used $\rho = 0.8$ as a plausible value for the intraclass correlation based on the within-person correlation in LDLC values taken over two off-treatment visits in CARDS. The interaction of candidate SNPs with statin versus placebo allocation was assessed in the JUPITER trial, since this study was not involved in the first-stage meta-analysis. Regression models were applied to the combined population of statin- and placebo-treated subjects by including extra terms encoding placebo allocation and the product of placebo allocation with SNP minor allele dose.[7]

GWCA using Genome-Complex Trait Analysis

There may be multiple causal variants in a gene and the total variation that could be explained at a locus may be underestimated if only the most significant SNP in the region is selected. To identify independent SNPs, we ideally can perform a conditional analysis, starting with the top associated SNP, across the whole genome followed by a stepwise procedure of selecting additional SNPs, one by one, according to their conditional P values. Such a strategy would allow the discovery of more than two associated SNPs at a locus. To identify independent SNPs across the genome-wide data, we used an approximate conditional and joint analysis approach implemented in Genome-Complex Trait Analysis (GCTA) software (http://www.complextraitgenomics.com/software/gcta/). We used summary-level statistics from the first- and second-stage-combined meta-analysis and LD corrections between SNPs estimated from CARDS GWAS data. SNPs on different chromosomes or more than 10Mb distant are assumed to be in linkage equilibrium. The model selection process in GCTA starts with the most significant SNP in the single-SNP meta-analysis across the whole genome with P-value $<5 \times 10^{-7}$. In the next step, it calculates the P-values of all the remaining SNPs conditional on the top SNP that have already been selected in the model. To avoid problems due to colinearity, if the squared multiple correlations between a SNP to be tested and the selected SNP(s) is larger than a cut-off value, such as 0.9, the conditional P-value for that SNP will be set to 1. Select the SNPs with minimum conditional P-value that is lower than the cut-off P-value. Fit all the selected SNPs jointly in a model and drop the SNPs with the P value that is greater than the cut-off P value. This process is repeated until no SNPs can be added or removed from the model.

Pathway analysis and construction of a statin response network

Genes showing evidence of association (based on direct association or LD (HapMap CEU R^2 >0.8)) were reviewed for evidence of involvement in statin response at a pathway level using GeneGo Metacore (Thomson Reuters (portal.genego.com)). A statin response network was constructed in two stages. First, all genes with a literature-reported involvement in statin response (based on Medical Subject Headings (MeSH)) were identified using GeneGo MetaCore (Supplementary Data 3). Second, these genes were combined with all genes in associated loci (including genes in LD) and a network was constructed based on direct interactions only. By including direct interactions only, we created a conservative network of direct gene interactions that have been consistently linked to statin response in the literature.

eQTL analysis

LDL-C-associated index SNPs (246 SNPs) were used to identify 1,443 LD proxy SNPs displaying complete LD ($R^2 = 1$) across four HapMap builds in European ancestry samples (CEU) using the SNAP tool (http://www.broadinstitute.org/mpg/snap/). The primary index SNPs and LD proxies were searched against a collected database of expression SNP (eSNP) results, including the following tissues: fresh lymphocytes[30], fresh leukocytes[31], leukocyte samples in individuals with Celiac disease[32], whole-blood samples[33-36], lymphoblastoid cell lines (LCL) derived from asthmatic children[37,38], HapMap LCL from three populations[39], a separate study on HapMap CEU LCL[40], additional LCL population samples[41-43] (Mangravite et al., unpublished), CD19+ B cells[44], primary phytohaemagglutinin-stimulated T cells[41], CD4+ T cells[45], peripheral blood monocytes[44,46,47], CD11+ dendritic cells before and after Mycobacterium tuberculosis infection[48], omental and subcutaneous adipose[33,43,49], stomach[49], endometrial carcinomas[50], ER+ and ER– breast cancer tumour cells[51], brain cortex[46,52,53], prefrontal cortex[54,55], frontal cortex[56], temporal cortex[53,56], pons[56], cerebellum[53,56], three additional large studies of brain regions including prefrontal cortex, visual cortex and cerebellum, respectively[57], liver[49,58,59], osteoblasts[60], ileum[49,61], lung[62], skin[43,63] and primary fibroblasts.[41] Micro-RNA QTLs were also queried for LCL[64] and gluteal and abdominal adipose.[65] The collected eSNP results met the criteria for association with gene expression levels as defined in the original papers. In each case where a LDL-C-associated SNP or proxy was associated with a transcript, we further examined the strongest eSNP for that transcript within that data set (best eSNP), and the LD between the best eSNP and GIST-selected eSNPs to estimate the concordance of the LDL-C and expression signals.

Statin response connectivity map analysis

The Connectivity Map (Cmap) data set is available at the Broad Institute (www.broadinstitute.org/cmap) and contains more than 7,000 expression profiles representing 1,309

compounds used on five different cultured human cancer cell lines (MCF7, ssMCF7, HL60, PC3 and SKMEL5). We selected (prostate tumour-derived) PC3 cells as they showed the most responsiveness to statins at a genome-wide level. Four statins were included in our analysis, including pravastatin, atorvastatin, simvastatin and rosuvastatin. PC3 Instance reference files for each statin treatment were extracted (as defined by Lamb et al.[12]), that is, a treatment associated to its control pair. Transcripts were considered to show evidence of differential expression with a fold change >2. A fold change >1.5 was considered to be suggestive of differential expression only.

Exploration of functional impact among directly and indirectly associated variants

Genes and variants across all LDL-C-associated loci were investigated for evidence of functional perturbation using a range of bioinformatics tools and databases. Variants showing LD (CEU R^2 >0.8) with associated variants were explored for impact on coding gene function using Annovar[66] and regulatory function using a combination of HaploReg[67] and Regulomedb[68], which both draw on comprehensive data from the Encyclopedia of DNA Elements (ENCODE)[69] and the NIH Roadmap Epigenomics consortium[70]. Building on the functional annotation, we also identified variants that were shown to mediate eQTLs. Genes in associated loci were also used to query the NIH connectivity map for evidence of differential expression in PC3 cell lines treated with pravastatin, simvastatin and rosuvastatin. By combining a wide range of functional data and pathway support, we were able to build up a view of genes with the highest level of support in statin response.

RESULTS

First-stage meta-analysis

The GIST consortium includes 6 RCTs (n = 8,421 statin recipients) and 10 observational studies (n = 10,175 statin recipients) that participated in the first stage (see Methods; Supplementary Tables 1 and 2; Supplementary Notes 1 and 2). To search for genetic variants associated with differential LDL-C response to statin therapy, each study independently performed a GWAS among statin users, using the difference between the natural log-transformed LDL-C levels on- and off-treatment as the response variable (see Methods).

The first-stage meta-analysis identified three loci, including 13 SNPs, that attained genome-wide significance ($P<5\times10^{-8}$) for association with LDL-C response to statin treatment (Fig. 1; Table 1). The most significant association was for a SNP on chromosome 19, at *APOE* (rs445925, minor allele frequency (MAF) 0.098, β −0.043, SE 0.005, P 1.58×10^{-18}; Fig. 2a), indicating that carriers of the rs445925 SNP respond to statins with an addi-

Table 1 Genome-wide significant associations in stage 1, stage 2, and combined meta-analysis

Chr	Position	Lead SNP	Gene	Coding allele	Noncoding allele	Phase	N	Frequency-coding allele	Beta[a]	SE	% Extra reduction[b]	P
1	109620053	rs646776	*SORT1/CELSR2/PSRC1*	C	T	Stage 1	16,697	0.230	−0.015	0.003	1.5	6.70×10^{-7}
						Stage 2	21,902	0.216	−0.010	0.003	1.0	2.43×10^{-4}
						Combined	38,599		−0.013	0.002	1.3	1.05×10^{-9}
6	160930108	rs10455872	*LPA*	G	A	Stage 1	12,981	0.069	0.041	0.006	−4.1	1.95×10^{-11}
						Stage 2	18,075	0.087	0.059	0.005	−5.9	7.14×10^{-35}
						Combined	31,056		0.052	0.004	−5.2	7.41×10^{-44}
12	21260064	rs2900478	*SLCO1B1*	A	T	Stage 1	16,749	0.165	0.016	0.003	−1.6	2.26×10^{-6}
						Stage 2	7,504	0.164	0.017	0.006	−1.7	3.54×10^{-3}
						Combined	24,253		0.016	0.003	−1.6	1.22×10^{-9}
19	50107480	rs445925	*APOE*	A	G	Stage 1	13,909	0.098	−0.043	0.005	4.3	1.58×10^{-18}
						Stage 2	3,613	0.157	−0.088	0.011	8.8	1.41×10^{-15}
						Combined	17,522		−0.051	0.005	5.1	8.52×10^{-29}

Abbreviations: Chr, chromosome; SNP, single nucleotide polymorphism; S.E., standard error.

[a] Beta for difference between the natural log-transformed on- and off-treatment low-density lipoprotein cholesterol (LDL-C) levels adjusted for natural log-transformed off-treatment LDL-C-, age-, sex- and study-specific covariates. The beta reflects the fraction of differential LDL-C lowering in carriers versus non-carriers of the SNP; a negative beta indicates a better statin response (stronger LDL-C reduction), a positive beta a worse statin response. Betas and P values were generated using linear regression analysis. [b] This percentage reflects the % extra LDL-C lowering in carriers versus non-carriers of the SNP.

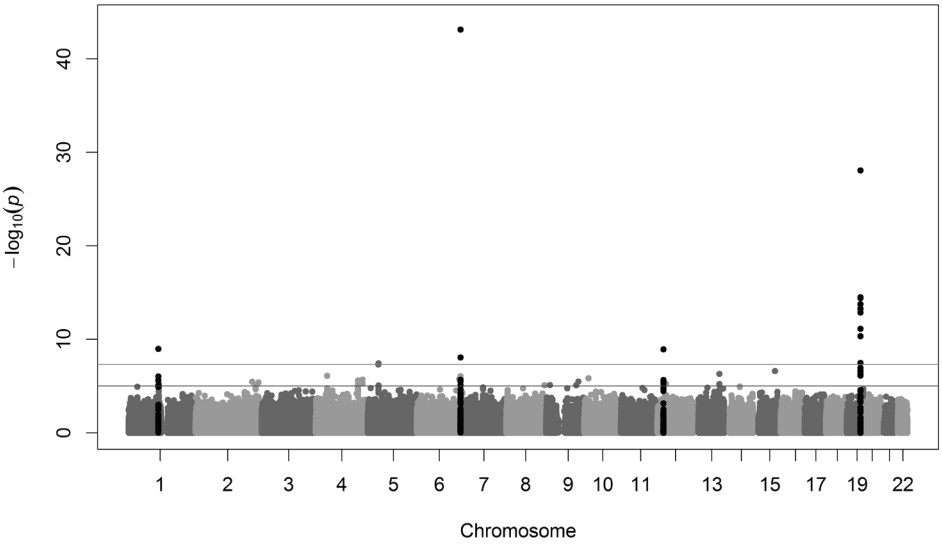


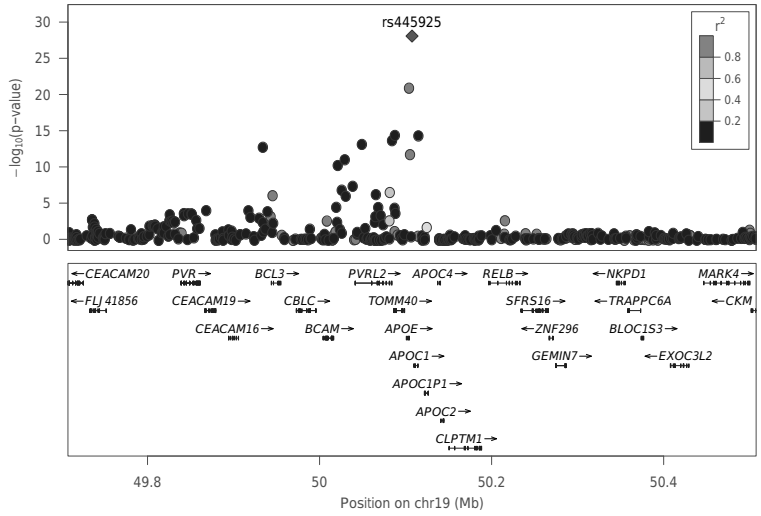
Figure 1 Results of the GWAS meta-analysis
Manhattan plot presenting the $-\log_{10}$ P-values from the combined meta-analysis (n = 40,914) on LDL-C response after statin treatment. P-values were generated using linear regression analysis.

tional 4.3% increase per allele in LDL-C lowering effect compared with non-carriers. The second strongest association was with a SNP at *LPA* on chromosome 6 (rs10455872, MAF 0.069, β 0.041, SE 0.006, P 1.95×10^{-11}; Fig. 2b), indicating a 5.9% smaller LDL-C lowering per minor allele for carriers of the SNP compared with non-carriers. Associations at both loci have previously been described.[7,8] A third genome-wide significant association was found with a SNP at *RICTOR* on chromosome 5 (rs13166647, MAF 0.230, β −0.253, SE 0.046, P 4.50×10^{-8}), although genotypes for this SNP were only available in two studies within the first stage (n = 2,144).

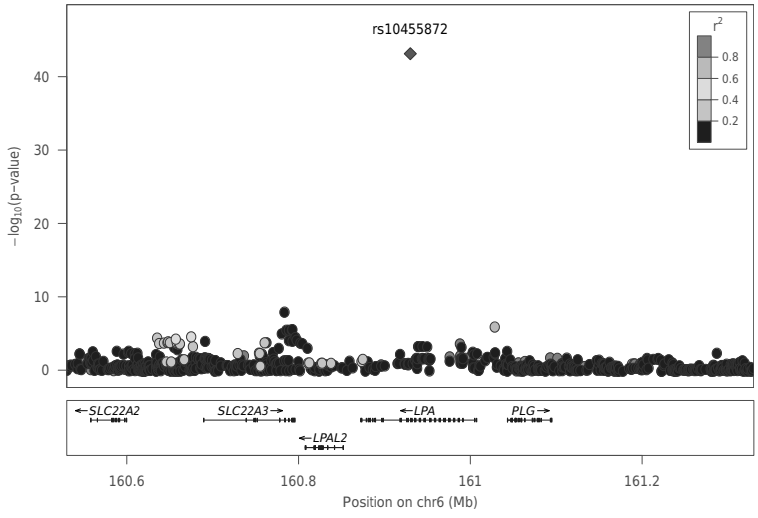
Second-stage meta-analysis

We selected 246 SNPs with P $<5 \times 10^{-4}$ from 158 loci for further investigation in three additional studies comprising up to 22,318 statin-treated subjects (see Methods; Supplementary Tables 1 and 5; Supplementary Note 3). This second stage confirmed the genome-wide significant associations between variations within the *APOE* and *LPA* loci and LDL-C response, as observed in the first stage (Table 1; Supplementary Fig. 2; Supplementary Table 5). In addition, SNPs at two new loci with P values between 6.70×10^{-7} and 2.26×10^{-6} in the first phase were shown to be significantly associated with statin-induced LDL-C lowering after statin treatment in the total combined meta-analysis at a genome-wide level: *SORT1/CELSR2/PSRC1* (rs646776, β −0.013, SE 0.002, P 1.05×10^{-9} and rs12740374, β −0.013, SE 0.002, P 1.05×10^{-9}; Fig 2c) and *SLCO1B1* (rs2900478, β

APOE locus



LPA locus



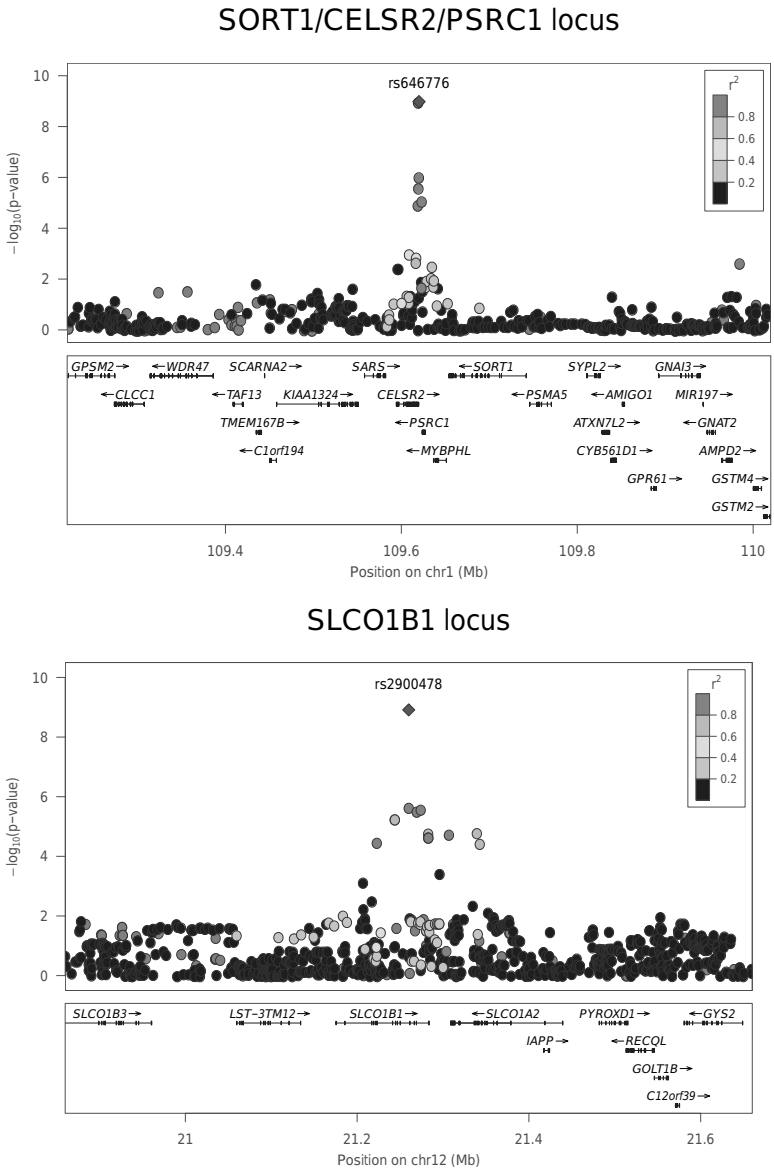


Figure 2 Regional association plots of the genome-wide significant associations with LDL-C response after statin treatment

The plots show the genome-wide significant associated loci in the combined meta-analysis (n = 40,914), the *APOE* locus (a), the *LPA* locus (b), the *SORT1/CELSR2/PSRC1* locus (c) and the *SLCO1B1* locus (d) (generated using LocusZoom (http://genome.sph.umich.edu/wiki/LocusZoom)). The RefSeq genes in the region are shown in the lower panel. P-values were generated using linear regression analysis.

0.016, SE 0.003, P 1.22×10^{-9}; Fig 2d), indicating an additional 1.5% increase per allele in LDL-C lowering effect for carriers of the *SORT1/CELSR2/PSRC1* SNP and a 1.6% smaller LDL-C lowering per minor allele for carriers of the *SLCO1B1* SNP.

The six next-ranked SNPs with P values just below 5×10^{-8} in the combined meta-analysis, including the two SNPs at *RICTOR* (rs13166647 and rs13172966), were selected for additional genotyping in the Scandinavian ASCOT participants (see Methods). None of these six SNPs reached genome-wide significance after this additional genotyping (Supplementary Table 6). Therefore, our overall genome-wide significant findings were the SNPs at *APOE*, *LPA*, *SORT1/CELSR2/PSRC1* and *SLCO1B1*.

Subfraction analyses

To extend our results for the novel GWAS finding *SORT1/CELSR2/PSRC1*, we performed additional association analyses, using measurements of cholesterol levels in four LDL subfractions (large, medium, small and very small) from two of the trials in GIST, CAP and PRINCE (Table 2; see Methods). The minor allele of *SORT1* rs646776 was associated with greater statin-induced reductions in levels of all LDL subfractions, and there was a non-significant trend for larger effect sizes and greater statistical significance for lowering of small and very small LDL (Table 2). In contrast, the *APOE* SNP associated with greater LDL-C response to statins (rs445925) showed a small and nonsignificant association with change in very small LDL (Table 2). For the minor allele of rs2900478 (*SLCO1B1*), the borderline significant association with smaller magnitude of LDL-C reduction showed a trend for preferential association with larger versus smaller LDL subfractions. The lack of association of rs10455872 (*LPA*) with changes in LDL subfractions is consistent with evidence discussed below that this locus affects levels of lipoprotein(a) (Lp(a)) and not

Table 2 Associations of the minor alleles of rs646776, rs445925, rs2900478 and rs10455872 with changes in LDL-C and LDL subfractions in response to statin in the combined CAP and PRINCE studies

Change[a]	*SORT1/CELSR2/PSRC1* rs646776 (MAF 0.2)			*APOE* rs445925 (MAF 0.086)			*SLCO1B1* rs2900478 (MAF 0.16)			*LPA* rs10455872 (MAF 0.056)		
	Beta	**SE**	**P**	**Beta**	**SE**	**P**	**Beta**	**SE**	**P**	**Beta**	**SE**	**P**
LDL-C total	−0.023	0.008	0.003	−0.046	0.018	0.008	0.010	0.005	0.04	0.032	0.019	0.09
Large LDL-C	−0.028	0.014	0.042	−0.075	0.029	0.009	0.020	0.008	0.01	0.036	0.031	0.23
Medium LDL-C	−0.027	0.015	0.075	−0.079	0.032	0.012	0.016	0.009	0.07	0.010	0.034	0.77
Small LDL-C	−0.047	0.018	0.009	−0.071	0.037	0.050	0.002	0.010	0.83	−0.024	0.039	0.54
Very small LDL-C	−0.034	0.009	0.00006	−0.022	0.017	0.202	0.001	0.005	0.90	0.008	0.019	0.67

Abbreviations: S.E., standard error; LDL-C, low-density lipoprotein cholesterol; MAF, minor allele frequency.
[a] Change: ln (on treatment) – ln (baseline) models adjusted for log (baseline variable), age, sex, body mass index, smoking(y/n) and study (CAP versus PRINCE). Betas and P values were assessed using a generalized estimating equation method.

LDL particles. Using generalized estimating equations, we tested the association of log change in each of the LDL subfractions with interactions of the four SNPs. For very small LDL, the association with the rs646776 minor allele was significantly different from that of the other minor alleles (P 0.03 after adjustment for multiple testing).

Effects of off-treatment LDL-C

To demonstrate that our findings for LDL-C response to statin treatment are unlikely to be explained through associations with baseline LDL-C levels, we performed a number of additional analyses (see Methods). First, Supplementary Table 7 shows regression coefficients for baseline-adjusted and measurement noise-corrected estimates of the direct effect of genotype on on-treatment LDL-C at the strongest SNPs in the GIST meta-analysis ($P < 1 \times 10^{-8}$), which were available in the CARDS data set. Correcting our effect size estimate further and modeling measurement noise at baseline reduced the apparent effect only slightly for all the markers, suggesting that there is little effect of measurement noise. Next, within the JUPITER trial, additional analyses were performed to determine whether there was an interaction between LDL-C change and statin or placebo allocation. Supplementary Table 8 shows significant P values for interaction (all $< 5 \times 10^{-2}$) for SNPs at the four genome-wide significant loci in the GIST meta-analysis, also suggesting that genetic effects on baseline LDL-C as manifested in the placebo group contribute at most only in part to genetic effects on LDL-C response in the statin group.

Genome-Wide Conditional Analysis

To investigate whether there were multiple SNPs within any gene and multiple loci associated with differential LDL-C lowering to statin therapy, we performed a conditional analysis across the genome using the summary statistics of the combined meta-analysis. The results of the Genome-Wide Conditional Analysis (GWCA; see Methods; Supplementary Table 9) showed 14 SNPs independently associated with statin response and these explained ~5% of the variation in LDL-C response to statin treatment. Of the 14 independent SNPs, 6 were genome-wide significant in the combined GWAS meta-analysis (Supplementary Table 5).

Previous findings

In Supplementary Table 10, we performed a look-up in our GWAS meta-analysis for SNPs previously described in the literature (NHGRI Catalogue[11] of Published GWAS and Candidate gene studies) to be associated with statin response, besides the loci associated at a genome-wide level in the current study. None of these SNPs was associated with statin response in our GWAS after correcting for multiple testing.

Functional analyses

Functional characterization of the 246 SNPs selected for the second stage was performed using a range of bioinformatics tools (see Methods). A total of 420 expression quantitative trait loci (eQTL) associations were identified across a wide range of tissues (Supplementary Data 1), which comprised 67 independent gene eQTL associations. Eleven genes, including *APOE*, *SORT1*, *CELSR2* and *PSRC1*, showed eQTLs in liver, which considering its primary role in mediating statin-induced LDL reduction may be particularly relevant to statin response. Putative gene eQTLs were combined with genes annotated to variants in linkage disequilibrium (LD) with LDL-C response-associated variants, resulting in a list of 185 candidate gene loci, defined by 2,681 SNPs (Supplementary Data 2 and 3). To identify statin responsive genes among the candidate loci, gene expression data measured in response to statin treatment in a range of cell lines was retrieved from the Connectivity Map resource[12] (see Methods). Five genes (*APOE*, *BRCA1*, *GRPEL1*, *ADRB2* and *ETV1*) showed convincing evidence of statin responsiveness on the basis of greater than twofold differential expression in response to statin treatment. Eight genes showed suggestive evidence (1.5- to 2-fold change; *TOMM40*, *SREBP1*, *PSRC1*, *BCL3*, *BCAM*, *ANK3*, *SIVA1* and *RANBP9*; Supplementary Data 3).

Finally, involvement in statin response was investigated at a pathway level using GeneGo Metacore (Thomson Reuters[13]). Briefly, 87 literature-reported genes linked to statin response were combined with the 185 candidate gene loci reported here (Supplementary Data 3). A conservative network of direct interactions was constructed between query genes (Supplementary Data 4). The network included 24 genes located in the LDL-C-associated loci (Supplementary Fig. 4). Collectively, our functional and pathway analysis confirms a strong biological and functional role in statin response for several strongly associated gene loci, including *APOE*/*TOMM40*/*PVRL2* and *SORT1*/*CELSR2*/*PSRC2*.

DISCUSSION

We have performed a meta-analysis of GWAS including more than 40,000 subjects, investigating genetic variants associated with variation in LDL-C lowering on statin treatment independent from associations with baseline LDL-C. We identified four loci at genome-wide significance, including the previously identified *APOE* and *LPA*, and the novel GWAS loci *SORT1*/*CELSR2*/*PSRC1* and *SLCO1B1*.

Nine SNPs in the *APOE* gene region reached genome-wide significance for LDL-C response. The minor allele of the lead SNP rs445925, which is a proxy for the apoE ε2 protein variant defining SNP rs7412[14], was associated with a larger LDL-C-lowering response to statins compared with carriers of the major allele. The magnitude and direc-

tion of the effect size was similar to previously reported findings for the rs445925 variant in the GWAS study performed in CARDS and ASCOT[8] and of the SNP rs7412 in JUPITER.[7] Since the apoE ε2 protein results in increased hepatic cholesterol synthesis, it may also predispose to stronger inhibition of cholesterol synthesis by statin treatment.[8,10]

Three independent SNPs at *LPA* were significantly associated with LDL-C response to statins. The minor G allele of the lead SNP rs10455872 was associated with smaller LDL-C reduction than the major allele. This result was similar to the previous GWAS findings for this SNP in the JUPITER trial and the combined ASCOT and CARDS study.[7,8] The rs10455872 SNP was strongly associated with the KIV-2 copy number variant in Lp(a), which encodes variability in apo(a) size and is responsible for ~30% of variance in Lp(a) levels.[8,15] Furthermore, rs10455872 was shown to be strongly associated with plasma Lp(a) levels.[16] Standard assays of LDL-C, as well as the Friedewald formula, include cholesterol that resides in Lp(a).[6,8] Carriers of this *LPA* variant are characterized by higher Lp(a) levels and a larger proportion of their measured LDL-C resides in Lp(a) particles.[8,10] Since statin therapy does not reduce the number of Lp(a) particles[17], their presence attenuates the measured LDL-C response to statins.

Two SNPs at *SORT1/CELSR2/PSRC1* (rs646776 and rs12740374) on chromosome 1p were associated with an enhanced statin LDL-C response. A similar association was previously observed in a large candidate gene study in HPS[6]; however, we demonstrate this finding now first at a genome-wide significance level. The minor allele of rs12740374 has been shown to generate a binding site for the transcription factor C/EBPa.[18] Transcription results in upregulation of hepatic expression of three genes at this locus, *SORT1*, *CELSR2* and *PSRC1*[18], which we also showed in our eQTL analysis (Supplementary Data 1). Of these, *SORT1* is most notable, in that it encodes the multifunctional intracellular trafficking protein sortilin, which has been shown to bind tightly to apoB.[19] Sortilin-induced lowering of plasma LDL-C results from two mechanisms: reduced secretion of apoB-containing precursors, and, perhaps of greater importance, increased hepatic LDL uptake via binding to sortilin at the cell surface, with subsequent internalization and lysosomal degradation.[19] Notably, the minor allele of rs646776 is preferentially associated with lower levels of small and very small LDL (Table 2), suggesting that sortilin is of particular importance for regulating levels of these particles.[18] Smaller LDL subfractions have been shown to be relatively enriched in particles with reduced LDL receptor binding affinity and cellular uptake[20], a property that may contribute to their associations with increased risk for cardiovascular disease.[21] This property may also underlie the diminished efficacy of statins for reduction of these particles (Supplementary Fig. 3)[22], since statins act to reduce LDL-C levels to a large extent by increasing LDL receptor expression as a result of upregulation of the transcription factor SREBP2, whereas SORT1 is not regulated by this mechanism. Hence, the greater statin-mediated reduction of LDL-C among carriers of the rs646776 minor allele could be attributed to relative depletion of LDL particles

dependent on sortilin for clearance and hence a residually greater proportion of those LDL particles whose uptake is more dependent on the LDL receptor than on sortilin.

Notably, the strong association of rs646776 with statin-induced reductions in small and very small LDL particles contrasts to the weaker associations of changes in these particles with rs445925, likely the result of differing mechanisms underlying the effects of these SNPs on statin response. As noted above, rs445925 is a proxy for the SNP defining the apoE ε2 protein variant that is thought to predispose to heightened statin response as a result of greater statin inhibition of cholesterol synthesis and hence upregulation of SREBP and LDL receptor activity.

The *SLCO1B1* rs2900478 minor allele was associated with a smaller LDL-C reduction in response to statin treatment. *SLCO1B1* encodes the organic anion-transporting polypeptide OATP1B1 and facilitates the hepatic uptake of statins.[23] SNP rs2900478 is in strong LD ($R^2 = 0.89$) with rs4149056, which represents the Val174Ala substitution resulting in complete loss of function. In the HPS trial, which used simvastatin, this candidate gene SNP was associated with a 1% lower LDL-C reduction per allele.[6] Single-dose studies have shown that the observed area under the curve of plasma level of active simvastatin after a dose of 40 mg was 221% higher in rs4149056 CC homozygotes compared with rs4149056 TT homozygotes, as compared with atorvastatin 20 mg (144% higher for CC versus TT) and rosuvastatin 40 mg (117% higher for CC versus TT).[24] This finding results from the slower hepatic uptake of statins caused by the genetic variant, which would also be expected to result in a reduction in the cholesterol lowering effect.[25] In a GWAS of the genetic risk factors for simvastatin-induced myopathy, *SLCO1B1* showed the strongest association.[25] Homozygous carriers of the *SLCO1B1* variant had a 16.9 times higher risk for myopathy compared with non-carriers. This might have led to a decrease in study medication adherence, and consequently a decreased effect on LDL-C in carriers of this SNP. In addition, previous analysis in the GoDARTS study showed that the effect of the *SLCO1B1* gene on statin efficacy was abolished after removal of individuals who showed signs of intolerance.[26]

GWCA identified three independent loci in the *APOE* gene region and two loci in the *LPA* gene region (Supplementary Table 9). GWCA also showed several other loci with $P < 5 \times 10^{-8}$ that were not GWAS significant on single-SNP analysis (*HGD*, *RNF175*, *ISCA1L-HTR1A*, *GLIS3-SLC1A1*, *LOC100128657*, *NKX2-3-SLC25A28* and *PELI2*). These findings will require replication in independent, larger data sets. The significant SNPs in the GWCA analysis explained ~5% of the variation in LDL-C response to statin treatment. Whether this 5% is clinically relevant should be investigated by other studies. For example, it would be of interest to investigate whether this differential LDL-C lowering is also associated with differential event reduction by statin treatment.

In the current study, we combined the results of 6 randomized clinical trials and 10 observational studies in the first stage. This approach resulted also in combining

several types of statins, since different statins were studied in the trials and within the observational studies (Supplementary Table 2). This, and the variation in statin dosage during follow-up for an individual, is a limitation of the current study, since, for example, the impact of the *SLCO1B1* variant on statin pharmacogenetics is known to be highly dependent on statin type and dose.[24,27] To overcome this limitation, the individual study analyses were adjusted for statin dose. Dividing the actual statin dose given by the statin-specific dose equivalent (Supplementary Table 3) gives the statin-adjusted equivalent based on the daily dosages required to achieve a mean 30% LDL-C reduction. Using this table, we made the different statin dosages and types comparable within the studies. To correct for between-study variance, we used a fixed effect meta-analysis with inverse variance weighting. Since we observed that the *SLCO1B1* gene was genome-wide significantly associated with LDL lowering, this highlights the thoroughness of our analytical approach, in which the analyses were correctly adjusted for the type and dose of statins used (Supplementary Table 3). Moreover, a comparison of the estimates of the SNPs between the RCTs (where there are no intra-individual differences in dosages) with the estimates of the SNPs in the observational studies showed large homogeneity between the estimates in the various study designs (Supplementary Fig. 2), indicating that our adjustment for dosage seems to be sufficient within this study.

Another possible limitation of the current study is the influence of the identified genetic variants on baseline LDL-C levels. In pharmacogenetic studies investigating the LDL-C-lowering response to statins, it is important to eliminate the effect of association between the genetic variant and baseline LDL-C levels, since those findings may confound the response to treatment associations. Previous large GWAS studies have shown strong associations between baseline LDL-C levels and genetic variants in *SORT1*/*CELSR2*/*PSRC1*, *APOE* and *LPA*.[28] To eliminate those possible confounding effects, our response to treatment analyses were adjusted for baseline LDL-C levels. In addition, additional analysis in CARDS and JUPITER suggests no or little influence of genetic associations with baseline LDL-C on the genetic effects on LDL-C-lowering response.

In conclusion, this study is the largest meta-analysis of GWAS for LDL-C response to statin therapy conducted to date. Our results demonstrate that apart from the previously identified *APOE* and *LPA* loci, two new loci, *SORT1*/*CELSR2*/*PSRC1* and *SLCO1B1*, also have a modest but genome-wide significant effect on LDL-C response. The minor alleles of the *APOE* rs445925 and *SORT1*/*CELSR2*/*PSRC1* rs646776 SNPs were associated with a larger statin response, whereas the minor alleles of the *LPA* rs10455872 and *SLCO1B1* rs2900478 SNPs were associated with a smaller statin response. Our findings advance the understanding of the pharmacogenetic architecture of statin response.

All supplementary files and figures can be found via:
http://www.nature.com/ncomms/2014/141028/ncomms6068/full/ncomms6068.html

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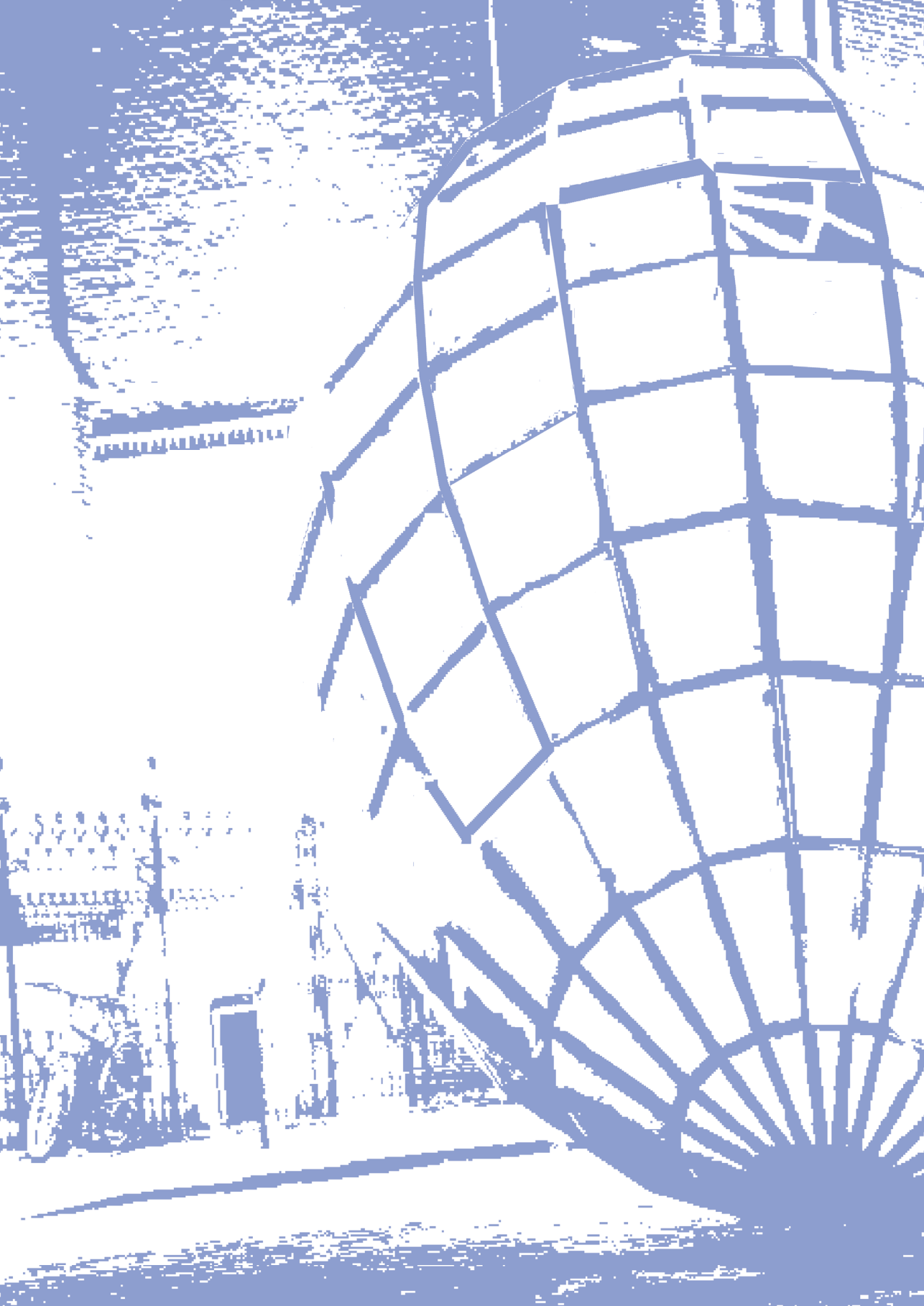

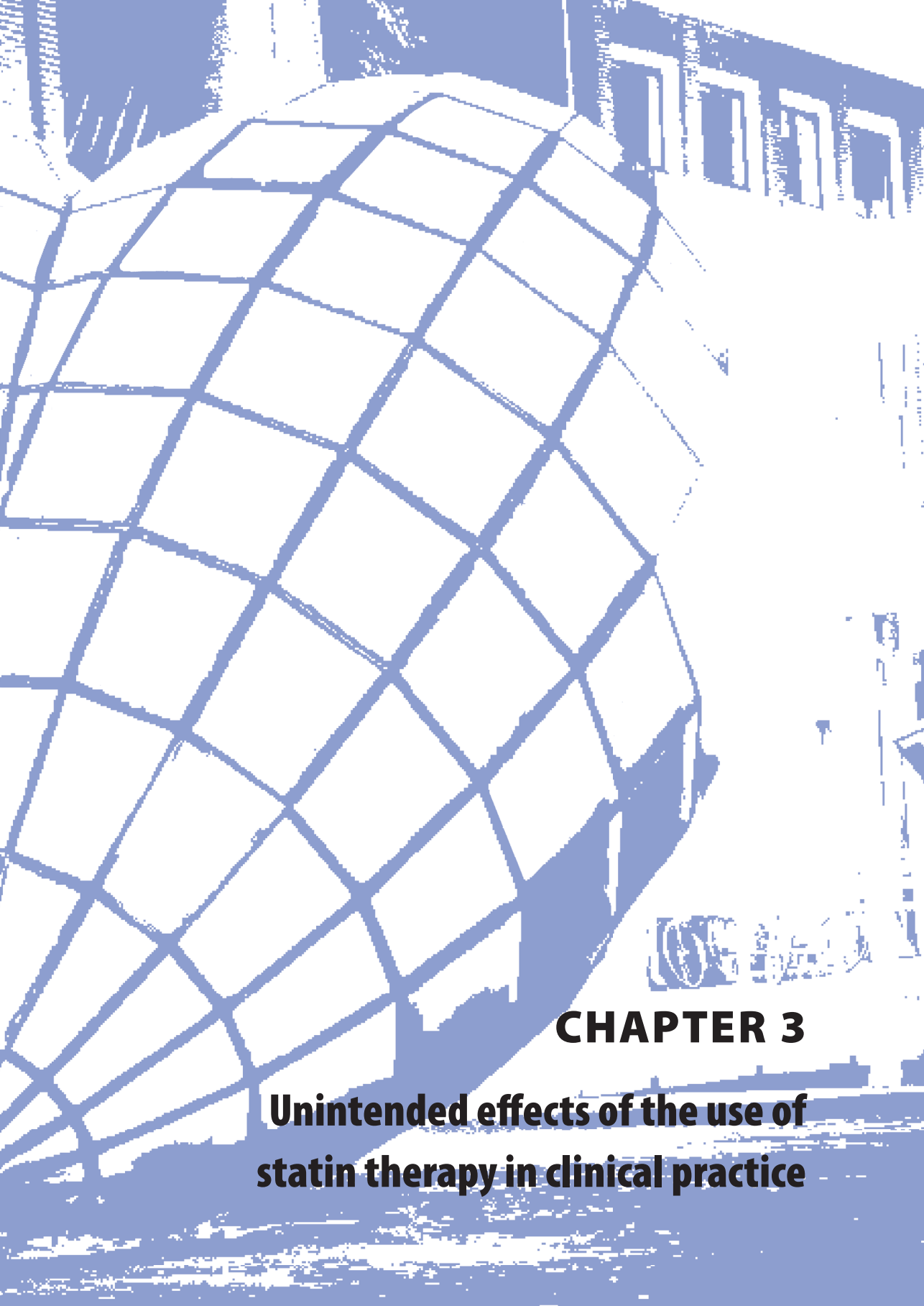

CHAPTER 3

Unintended effects of the use of statin therapy in clinical practice

3.1

Statin therapy is associated with a reduced risk of non-alcoholic fatty liver in overweight individuals

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Digestive and Liver Disease 2014; 46(8): 720-725

ABSTRACT

Introduction: Non-alcoholic fatty liver or hepatic steatosis is considered the hepatic manifestation of the metabolic syndrome. Statins are often used by patients with metabolic syndrome, but their effect in steatosis is not well established. We aimed to study the association between statins and the presence of steatosis.

Methods: In the population-based Rotterdam Study, 2578 subjects underwent liver ultrasonography and had prescription data available. In a cross-sectional design, we investigated the effect of current, past, and duration of statin use. Logistic regression analyses were adjusted for age, sex, and other known risk factors.

Results: The prevalence of steatosis was 35.3%. We identified 631 current and 359 past statin users. In multivariable analyses, current statin use >2 years was associated with a significantly lower steatosis prevalence (OR 0.43, 95% CI 0.19; 0.96). Stratification by mean body mass index showed that this association was stronger in patients with body mass index ≥27.5 (OR 0.30, 95% CI 0.11; 0.81 for current use >2 years), while in patients with body mass index <27.5 the association was non-significant.

Conclusion: Within the Rotterdam study, in patients with body mass index ≥27.5 current use of statins for >2 years was associated with a lower prevalence of steatosis.

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is the most common cause of serum alanine aminotransferase (ALT) elevation and of chronic liver disease in Western countries. The term NAFLD encompasses a spectrum of disease activity, ranging from simple hepatic steatosis or non-alcoholic fatty liver (NAFL), to non-alcoholic steatohepatitis (NASH) and NASH cirrhosis, which may lead to a decreased liver function, hepatocellular carcinoma, and liver failure.[1-3] NAFLD is considered as the hepatic manifestation of the metabolic syndrome. It is frequently associated with dyslipidemia, with elevated serum triglycerides and low-density lipoprotein (LDL)-cholesterol, and a decrease in serum high-density lipoprotein (HDL)-cholesterol.[1-5] Furthermore, NAFLD has been associated with the risk of incident cardiovascular disease (CVD), independently of the components of the metabolic syndrome, and the major cause of death in NAFLD is CVD.[2,5-10]

Statins interfere with cholesterol metabolism in the liver by inhibiting HMG-CoA reductase, the rate-limiting enzyme of the cholesterol synthesis pathway. This leads to upregulation of LDL receptors in the liver, increased uptake of circulating LDL-cholesterol, and subsequently to a decrease in LDL-cholesterol concentration. Besides this reduction in LDL-cholesterol, statins are effective in lowering of the triglyceride concentration and modestly effective in raising the HDL-cholesterol concentration.[11-16] Statins are beneficial in the prevention of CVD with an approximately 20% relative risk reduction on mortality and major cardiovascular events in persons free of CVD.[17,18] In general, statins are well-tolerated and safe drugs.[19]

Statins are frequently used for several indications such as dyslipidemia, type 2 diabetes mellitus, and in patients at high risk of CVD.[20] There is some discussion as to whether statins are safe and effective in NAFLD, and whether they worsen hepatic steatosis, despite improvement of serum lipid concentration.[21-25] Clarification of this topic is important, since due to the co-existence of dyslipidemia and NAFLD, and a higher risk of CVD mortality in NAFLD patients, these patients will often be treated with statins for the primary and secondary prevention of CVD.

In the present study, the objective was to investigate whether statin therapy is associated with the presence of NAFL, considering both current and past use of statin therapy, and duration of statin therapy, in a large prospective cohort study in community-dwelling elderly. In an extended analysis, we investigated whether the association between the use of statin therapy and NAFL was modified by body mass index (BMI) because obesity is a strong and independent risk factor for NAFL.

METHODS

Setting

The Rotterdam Study is a prospective population-based cohort study of chronic diseases in the elderly population. From 1990 to 1993, 7983 inhabitants of the suburb Ommoord in Rotterdam, the Netherlands, aged 55 years or older, participated in the Rotterdam Study (RS-I) and gave written informed consent. Ethical approval was obtained from the Medical Ethical Committee of the Erasmus Medical Center, Rotterdam, the Netherlands. Baseline examinations took place from March 1990 through July 1993. Follow-up examinations were conducted periodically, every 4–5 years. In 2000, an extended cohort was enrolled, the Rotterdam Study II (RS-II). Three-thousand-eleven inhabitants entered the study and have been continuously followed since then. Furthermore, in 2006, a younger cohort was enrolled, the Rotterdam Study III (RS-III), containing 3932 inhabitants aged 45 years or older. Abdominal ultrasonography was added to the core protocol at the fifth survey of the Rotterdam Study (February 2009–February 2012), which constitutes the baseline survey for the present study.

Medication dispensing data were obtained from the fully computerized pharmacies in the Ommoord suburb. Information on all filled prescriptions from 1 January 1991 until 1 December 2011 was available and included information on the product name of the drug, the Anatomical Therapeutical Chemical code, the amount dispensed, the prescribed dosage regimen and the date of dispensing.[26]

Detailed information on design, objectives and methods of the Rotterdam Study has been described before.[27,28]

Study population

The study population consisted of all participants with complete data on the extensive interview and clinical examination at the fifth survey of the Rotterdam Study (February 2009–February 2012). The clinical examination included a fasting blood sample, abdominal ultrasonography, and anthropometric assessment. Medication prescription data on the use of statin therapy was avail-able until 1 December 2011. Therefore, all participants with an interview and clinical examination date after 1 December 2011 were excluded.

Exposure to statins

For every prescription of a statin, the duration was calculated by dividing the number of dispensed tablets by the prescribed daily number. Repeated prescriptions which were filled within seven days after ending a previous one, were considered as one single episode of continuous use. At the date of ultrasonography, every cohort participant was classified into the following mutually exclusive categories: 'current use' if the ultrasonog-

raphy was performed within a prescription episode; 'past use' if the patient had been treated with statins in the past but did not use statins on the day of ultrasonography; 'non-use' meant that the participant had not used statins at all during the study period. The prescribed daily dose of statin therapy was expressed in standardized defined daily doses (DDD), according to the World Health Organization.[26]

Outcome

The outcome of interest was the presence of NAFL, assessed by abdominal ultrasonography in all study participants. Abdominal ultrasonography was performed by certified and experienced technicians on a Hitachi HI VISION 900. Images were stored digitally and re-evaluated by a hepatologist with more than ten years experience in ultrasonography. The diagnosis and grading of fatty liver was determined according to the protocol by Hamaguchi et al.[29] Severity of fatty liver was classified as 'no fatty liver' (score 0–1), 'mild fatty liver' (score 2–3), or 'moderate to severe fatty liver' (score 4–6). Individuals with any of the following possible secondary causes of fatty liver were excluded from the analyses: (1) current excessive alcohol consumption or a history of excessive alcohol consumption, (2) positive HBsAg or anti-HCV, and (3) use of pharmacological agents historically associated with fatty liver (i.e. amiodarone, corticosteroids, methotrexate, and tamoxifen).

Covariables

To control for confounding, we adjusted the analyses for age, sex, prescribed dose of statin therapy, serum total cholesterol level, number of ethanol consumptions weekly, presence of type2 diabetes mellitus, the individual components of the metabolic syndrome, presence of CVD in history, and use of fibrates or other cholesterol lowering medication. CVD in history was defined as a myocardial infarction (MI), percutaneous transluminal coronary angioplasty (PTCA), coronary artery bypass grafting (CABG), heart failure, carotid desobstruction, cerebrovascular accident (CVA), or a transient ischemic attack (TIA) in the history.[30-32] Information on covariables was obtained by an interview at home, laboratory measurements, and anthropometric assessments at the research center. The interview was designed to obtain data concerning demographics, medical history, co-morbid conditions, smoking behaviour, physical activity, and alcohol consumption.

Fasting blood samples were collected on the morning of ultrasound examination. Blood lipids, serum glucose, ALT, aspartate aminotransferase (AST), gamma-glutamyltransferase (GGT), alkaline phosphatase (ALP), and total bilirubin were measured using automatic enzymatic procedures (Roche Diagnostics GmbH, Mannheim, DE). HbsAg and anti-HCV antibodies were measured by automatic immunoassay (Roche Diagnostics GmbH, Mannheim, DE).

Anthropometric measurements were performed by well trained nurses. Waist and hip circumference were measured in centimeters. BMI was calculated as the weight (in kg) divided by height (in m^2). The average of two blood pressure measurements, obtained at a single visit in sitting position after a minimum of 5 min rest, was used for analysis. Presence of type 2 diabetes mellitus was defined as the use of glucose-lowering drugs, a non-fasting glucose level of more than 11.0 mmol/L, or a fasting glucose level of more than 6.9 mmol/L. The metabolic syndrome was defined according to the following criteria: (1) abdominal obesity, defined as a waist circumference in men >102 cm (40 in.) and in women >88 cm(35 in.), (2) serum triglycerides ≥150 mg/dL (1.7 mmol/L), (3) serum HDL-cholesterol <40 mg/dL (1.0 mmol/L) in men and <50 mg/dL (1.3 mmol/L) in women, (4) blood pressure ≥130/85 mmHg or drug treatment for elevated blood pressure, (5) elevated fasting glucose ≥100 mg/dL (5.6 mmol/L) or drug treatment for hyperglycaemia. Although this is according to Adult Treatment Panel III criteria, use of cholesterol lowering drugs was not used as a criteria because of the research objective.[33,34]

Statistical analysis

Differences in the distributions of characteristics between statin users and non-statin users were tested for significance with a t-test (means) for continuous variables and a Wilcoxon rank sum test (medians) for categorical variables. We used logistic regression analysis to investigate the association between statin therapy and the presence of NAFL. We considered both current, past, and never use of statin therapy, as well as the duration of current and past use. Duration of use was distinguished a priori, based on the median duration of past and current use in the population: a cut-off point of 2 years for past use and a cut-off point of a half year for current use. To investigate the effect of longer duration of use, also current use for >2 years was investigated. In an extended analysis, we stratified the population by the mean BMI. In this analysis, we investigated the association between statin therapy and the presence of NAFL in participants with BMI <27.5 and in participants with BMI ≥27.5. We performed a sensitivity analysis in which we excluded all NAFL patients with 'mild fatty liver' at ultrasonography. Furthermore, we performed a sensitivity analysis on the outcome steatosis defined by the noninvasive Fatty Liver Index (FLI) according to the definition by Bedogni et al.[35], including BMI, waist circumference, GGT, and triglycerides. According to Bedogni et al. a FLI ≥60 can be used to rule in hepatic steatosis.

Statistical analyses were performed using SPSS software (SPSSInc., version 20.0, Chicago, IL, USA).

RESULTS

Baseline characteristics

In total, 3205 participants underwent abdominal ultrasonography. Three hundred ninety-four participants were excluded because of the presence of potential secondary causes of fatty liver (excessive alcohol consumption (n = 255), positive HBsAg (n = 3) or anti-HCV (n = 24), use of pharmacological agents historically associated with fatty liver (n = 121)). Of these 2811 remaining participants, 2578 had information on statin dispensing data available and were eligible for the analysis. Differences in characteristics between statin users and non-statin users are shown in Table 1. Of the 2578 study participants, 1588 (61.6%) had never used any statin, 631 (24.5%) were current users of statin therapy and 359(13.9%) were past users of statin therapy. The prevalence of NAFL was 35.3%; 134 participants (5.2%) had 'mild fatty liver' and 776 participants (30.1%) had 'moderate to severe fatty liver'.

Table 1 Characteristics of 2578 study participants

Characteristic	Statin users (n = 990)	Non statin users (n = 1588)	p
Age (mean, years)	76.8 ± 5.5	76.2 ± 6.2	0.014
Gender, male (n, %)	423 (42.7%)	604 (38.0%)	0.018
Serum total cholesterol (mean±SD, mmol/L)	4.9 ± 1.1	5.7 ± 0.9	<0.0001
Serum HDL-cholesterol (mean±SD, mmol/L)	1.3 ± 0.3	1.5 ± 0.4	<0.0001
Serum LDL-cholesterol (mean±SD, mmol/L)	2.6 ± 1.0	3.5 ± 0.8	<0.0001
Serum triglycerides (mean±SD, mmol/L)	1.6 ± 0.8	1.3 ± 0.6	<0.0001
Serum ALT (mean±SD, mmol/L)	21.6 ± 11.3	19.5 ± 10.2	<0.0001
Serum AST (mean±SD, mmol/L)	26.8 ± 10.4	25.7 ± 7.0	0.004
NAFL (n, %)	404 (40.8%)	506 (31.9%)	<0.0001
Ethanol use (drinks/week)	3.6 ± 3.7	3.8 ± 3.8	0.30
Body mass index (mean±SD, kg/m^2)	27.9 ± 4.3	27.1 ± 4.2	<0.0001
Hypertension (n, %)	960 (97.0%)	1440 (90.7%)	<0.0001
Waist circumference (mean±SD, cm)	94.5 ± 12.3	91.2 ± 11.8	<0.0001
Insulin resistance (HOMA-IR >3) (n, %)	520 (52.5%)	537 (33.8%)	<0.0001
Type 2 diabetes mellitus (n, %)	251 (26.0%)	125 (8.1%)	<0.0001
Metabolic syndrome (n, %)	519 (52.4%)	515 (32.4%)	<0.0001
Cardiovascular disease in history (n, %)	428 (43.2%)	164 (10.3%)	<0.0001

Abbreviations: HDL, high-density lipoprotein; LDL, low-density lipoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; NAFL, non-alcoholic fatty liver; HOMA-IR, Homeostasis Model of Assessment-Insulin Resistance.

Statin therapy and the prevalence of NAFLD

The results of the logistic regression analysis on the association between the use of statin therapy and NAFL are shown in Table 2. In this analysis, current and past use of statin therapy were compared with never use as the reference category, and duration of use was considered by dividing current and past use into categories with different durations of use, based on the median duration of use.

In the multivariable analysis, adjusted for age and sex, ever use of statin therapy (including current and past use) was associated with a higher prevalence of NAFL (OR 1.51, 95% CI 1.28; 1.78, P < 0.001) compared with never use, whereas this association disappeared when the analysis was adjusted for all co-variables (full model: OR 1.06, 95% CI 0.82; 1.73, P 0.648).

When we analyzed ever use as a categorical variable, dividing past use into two categories (>2 year past use and ≤2year past use), and current use into three categories (≤half year current use, >half year – ≤2 year current use, >2 year current use), and adjusted for all covariables, past use of statin therapy was not significantly associated with NAFL (OR

Table 2 Association between the use of statins and the presence of non-alcoholic fatty liver

Current + past vs. never use[a]	OR	Beta	95% CI	P	N
Crude analyses[b]					
Additive model	**1.51**	**0.411**	**1.28; 1.78**	**<0.0001**	2578
Categorical model					
never use	1.00	(ref)	–	–	1588
>2 years past use	**1.69**	**0.523**	**1.24; 2.30**	**0.001**	189
1 days to ≤2 years past use	**1.75**	**0.559**	**1.27; 2.41**	**0.001**	170
>1 day to ≤half year current use	**1.41**	**0.340**	**1.10; 1.80**	**0.007**	326
>half year to ≤2 years current use	**1.53**	**0.425**	**1.17; 2.01**	**0.002**	256
>2 years current use	**0.81**	**–0.209**	**0.43; 1.55**	**0.526**	49
Adjusted analyses[c]					
Additive model	1.06	0.060	0.82; 1.73	0.648	2578
Categorical model					
never use	1.00	(ref)	–	–	1588
>2 years past use	1.34	0.292	0.92; 1.94	0.125	189
1 days to ≤2 years past use	1.14	0.131	0.76; 1.70	0.520	170
>1 day to ≤half year current use	0.77	–0.267	0.49; 1.20	0.239	326
>half year to ≤2 years current use	0.80	–0.227	0.51; 1.26	0.328	256
>2 years current use	**0.43**	**–0.843**	**0.19; 0.96**	**0.040**	49

Abbreviations: OR, odds ratio; CI, confidence interval; N, number of participants.

[a] In all analyses, never use is the reference category. [b] Adjusted for age and sex. [c] Adjusted for age and sex, statin dose, total cholesterol level, number of ethanol containing drinks per week, type 2 diabetes mellitus, individual components of the metabolic syndrome, cardiovascular disease in history, and use of fibrates or other cholesterol-lowering drugs. **Bold** value indicates statistically significant association.

1.34, 95% CI 0.92; 1.94, P 0.125 for >2 year past use; OR 1.14, 95% CI 0.76; 1.70, P 0.520 for ≤2 year past use). Only current use for more than 2 years was significantly associated with a lower prevalence of NAFL (OR 0.77, 95% CI 0.49; 1.20, P 0.239 for ≤half year current use; OR 0.80, 95% CI 0.51; 1.26, P 0.328 for >half year – ≤2 year current use; OR 0.43, 95% CI 0.19; 0.96, P 0.040 for >2 year current use).

Fig. 1 shows the results of the logistic regression analysis on the association between the use of statin therapy and NAFL stratified by BMI. Stratification by BMI was based on the mean BMI in the population (27.44 kg/m2) and the population was stratified by BMI <27.5 and BMI ≥27.5. In participants with BMI ≥27.5, current use of statin therapy for more than two years was significantly associated with a lower prevalence of NAFL and this association was stronger than for the non-stratified analyses (Table 2). There was a trend towards a lower prevalence of NAFL the longer statin therapy was used (OR 0.66, 95% CI 0.38; 1.17, P 0.153 for ≤half year current use; OR 0.58, 95% CI 0.32; 1.05, P 0.071 for >half year– ≤2 year current use; OR 0.30, 95% CI 0.11; 0.81, P 0.017 for >2 year current use). Past use of statin therapy was not significantly associated with the prevalence of NAFL in participants with BMI ≥27.5. In participants with BMI <27.5, the association between statin therapy and a lower prevalence of NAFL was not present.

We performed sensitivity analyses in which we excluded all NAFL participants with 'mild fatty liver' at ultrasonography(n = 134). In these analyses on the outcome measure

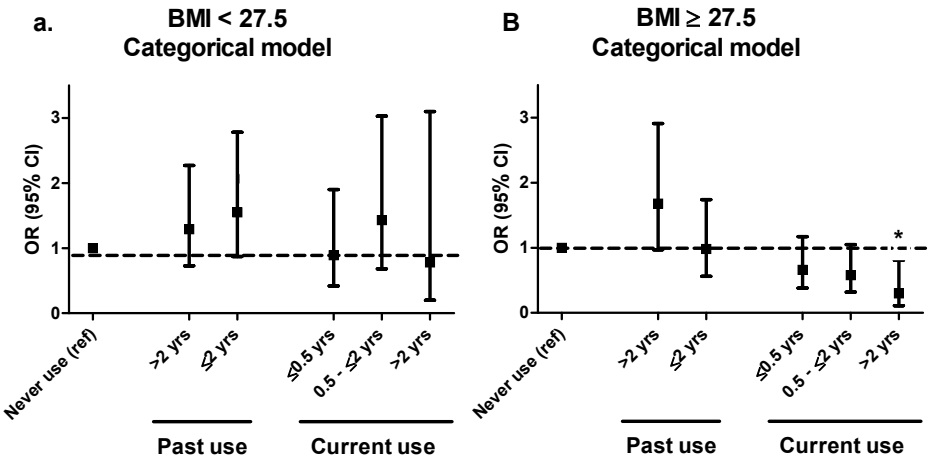

Figure 1 Association between the use of statins and the presence of non-alcoholic fatty liver in participants with body mass index <27.5 (A) and in participants with body mass index ≥27.5 (B)
Abbreviations: BMI, body mass index; OR, odds ratio; CI, confidence interval; yrs, years.
In all analyses, never use is the reference category. Analyses are adjusted for age and sex, statin dose, total cholesterol level, number of ethanol containing drinks per week, type 2 diabetes mellitus, individual components of the metabolic syndrome, cardiovascular disease in history, and use of fibrates or other cholesterol lowering drugs.

'moderate to severe fatty liver', the association between current use statin therapy >2 years and the lower prevalence of NAFL remained (OR 0.42, β −.862, 95% CI 0.18; 0.98, P 0.045). In a second additional sensitivity analysis the outcome measure was FLI ≥60 (steatosis) vs. FLI <60, based on the noninvasive calculation of the FLI. In this analysis, there was a trend for an association between current use of statin therapy and a lower prevalence of NAFL, albeit non-significantly. However, the number of participants in the categories were smaller than in the original analysis (n = 24 in statin users >2 years with BMI≥27.5).

Discussion

In this cross-sectional analysis in a large population-based prospective study, current use of statin therapy for more than 2 years was significantly associated with an approximately two times lower prevalence of NAFL. This association was only found in patients with a BMI ≥27.5, who showed an approximately three times lower prevalence of NAFL with more than 2 years current use of statin therapy.

It has been hypothesized that statins may exacerbate or worsen NAFLD despite an improvement in serum lipid levels. Statins may increase de novo cholesterol and fatty acid synthesis through induction of transcription factor sterol response regulatory element-binding protein-2 (SREBP-2) that activates genes involved in the synthesis of cholesterol, fatty acids, triglycerides and the LDL receptor.[36,37] Furthermore, by inhibition of HMG-CoA reductase, the number of hepatic LDL receptors increases, leading to an enhanced uptake of LDL-cholesterol.[13] Both effects might increase hepatic fatty infiltration and thereby exacerbate or worsen NAFLD. Conversely, the effect of statins might be beneficial as well, both by their most widely known lipid-lowering function, as well as through their pleiotropic effects acting on other mechanisms than the HMG-CoA reductase pathway, independent of their cholesterol lowering effect. Anti-inflammatory and immunomodulatory effects, anti-oxidant effects, and an improvement of the endothelial function are examples of pleitropic effects of statins.[38] Although the exact pathogenesis of NAFLD is currently not clarified and probably many factors contribute, insulin resistance, lipid abnormalities and chronic inflammation are considered to be the central pathway for the development of diseases related to obesity such as NAFLD and CVD.[1,39,40]

Similar to our results, no association between ever use of statins and NAFLD was demonstrated in a cross-sectional analysis in the Dallas Heart Study.[7] That such a crude exposure measure can lead to non-differential misclassification and bias towards the null hypothesis, is known.[41] However, we demonstrated a protective effect of >2 years current statin use and the prevalence of NAFL. We were able to distinguish between current and past users, and adjust for statin dose, in contrast with other studies which mostly obtained medication data by questionnaire. Our findings are supported by stud-

ies on the effect of statin therapy on hepatic histology by liver biopsy, showing that statin therapy was associated with improvement in liver steatosis.[5,42,43] Although studies have suggested that statins might induce hepatic injury, statin therapy was infrequently associated with acute or chronic liver failure, and significant injury from statins is rare.[21-25] In February 2012, the Food and Drug Administration (FDA) approved important safety label changes for statins to remove the need for routine periodic monitoring of liver enzymes in patients taking statins.[44] Furthermore, recent reviews from literature support our findings and clearly showed the safety of statins in NAFLD, and demonstrated that statins may lead to a reduction in the extent of hepatic steatosis.[45-47]

It seems plausible that statins may protect against NAFLD by their favourable effect on blood lipids. In our study, in patients with a BMI ≥27.5 who were current statin users for more than 2 years, statins showed a strong protective association with NAFL, while inpatients with a BMI <27.5 no protection could be demonstrated. The metabolic syndrome is the most important risk factor for NAFLD, and the individual components of the metabolic syndrome are all related to obesity.[1] Furthermore, overweight people are more likely to have a disturbed lipid profile. The accumulation of lipids in the hepatocytes, mainly triglycerides, is essential for the development of NAFLD.[1] Statins also exert their effect on serum triglycerides, with a mean reduction of 10–30% in serum triglycerides concentration.[13-15] Furthermore, obesity is associated with inflammation[48], and anti-inflammatory effects of statins might play a protective role.

This is the first observational study on this topic with continuous information on medication data, whereby past, current and duration of use was investigated. Another strength of the study was that we were able to adjust our analyses for a history of CVD, whereas no other studies did control for this. Adjustment for CVD is important to minimize confounding by indication. Since statin therapy is mainly prescribed for the primary and secondary prevention of CVD, and previous studies suggest a strong link between NAFLD and CVD, independently from the metabolic syndrome[8,9], the prevalence of NAFLD may also be higher in patients who use statins for CVD. When no adjustment is made for a history of CVD in the analyses, one may not detect or underestimate a potential favourable effect of statin therapy on NAFLD.

However, also some potential limitations and biases in our study should be considered. The risk of information or selection bias is unlikely, since the Rotterdam Study is a population-based cohort study, in which data are collected prospectively without prior knowledge of the aim of this study. A 'healthy user' effect seems unlikely because in that case ever use would also have been associated with a lower prevalence of NAFL. We investigated whether participants in the statin user categories differ from non-users in change in BMI or fasting serum glucose over time, thus whether life-style modifications might have played a role in the demonstrated association. No statistically significant differences between the groups were present. In the analysis, we controlled for potential

confounding factors such as dose of the prescribed statin therapy and a history of cardiovascular disease. In the present study, the diagnosis and severity of hepatic steatosis was assessed by ultrasonography. Ultrasonography may be less sensitive than more advanced imaging techniques such as CT/MRI, since ultrasonography is not appropriate for the detection of less than 30 percent steatosis. However, Hernaez et al.[49] showed that ultrasonography is comparable with other imaging modalities in the detection of NAFLD with an acceptable sensitivity of 80–100%. Ultrasonography is especially insensitive in the detection of mild steatosis. However, in a sensitivity analysis in patients with moderate to severe fatty liver (mild fatty liver patients excluded), the investigated association remained. Unfortunately, no histology was available in this population-based study, and therefore we could not investigate the effect of statin therapy on hepatic histology by liver biopsy. Finally, we had only a very low number of users of cholesterol lowering medication other than statins, such as ezetimibe, to investigate the effect of these drugs in NAFL patients.

In conclusion, we did not demonstrate an overall association between current and past use of statin therapy and the presence of NAFL in this large population-based cohort study. However, current use of statin therapy for more than two years was significantly associated with a lower prevalence of NAFL, and this association was even stronger in patients with a BMI ≥27.5. We think that this protective association warrants further investigation through replication in an independent cohort. In the meantime, and given the association between NAFLD and CVD, lipid lowering treatment with statins may be considered in the treatment of NAFLD patients.

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3.2

Use of statins is associated with lower serum total and non-SHBG-bound testosterone levels in male participants of the Rotterdam Study

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Eur J Endocrinology, accepted

ABSTRACT

Introduction: Statins or HMG-CoA reductase inhibitors decrease cholesterol production. Because cholesterol is a precursor of the testosterone biosynthesis pathway, there is some concern that statins might lower serum testosterone levels. Our objective was to investigate the association between use of statins and serum testosterone levels in men.
Methods: We used a cross-sectional design within the prospective population-based Rotterdam Study. We included 4166 men with total testosterone, non-SHBG-bound testosterone, and medication dispensing data available. Multivariable linear regression analysis was used to compare differences in serum testosterone levels (nmol/L) between current, past, and never statin users. We considered dose and duration of use. Analyses were adjusted for age, body mass index, cardiovascular disease history, diabetes mellitus, hypertension, and estradiol levels.
Results: We identified 577 current (mean age 64.1 years), 148 past (mean age 64.6 years), and 3441 never (mean age 64.6 years) statin users. Adjusted for all covariables, current statin use 1–≤6 months and >6 months was significantly associated with lower total testosterone levels compared to non-users (β −1.24, 95%CI −2.17; −0.31 and β −1.14, 95%CI −2.07; −0.20 respectively). Current use 1–≤6 months was also associated with significantly lower non-SHBG-bound testosterone levels (β −0.42, 95%CI −0.82; −0.02). There was a trend towards lower testosterone levels at higher statin doses, both for total (P_{trend} 2.9×10^{-5}) and non-SHBG-bound testosterone (P_{trend} 2.0×10^{-4}). No association between past statin use and testosterone levels was found.
Conclusion: We showed that current use of statins was associated with significantly lower serum total and non-SHBG-bound testosterone levels. Clinical relevance of the association should be further investigated.

INTRODUCTION

Statins or HMG-CoA reductase inhibitors are widely used cholesterol lowering drugs which are effective in the primary and secondary prevention of cardiovascular disease (CVD).[1-3] They competitively inhibit HMG-CoA reductase, mainly in the liver, which is the rate-limiting enzyme in the cholesterol biosynthesis pathway. By inhibiting this cholesterol biosynthesis, the number of low-density lipoprotein (LDL) receptors in the hepatic membrane is increased. This leads to increased serum uptake of LDL-cholesterol, and thus a decrease in serum cholesterol concentration.[4]

Testosterone is the main circulating androgenic hormone in men with important effects on libido, bone mass, fat distribution, muscle mass, strength and production of blood cells and sperm.[5,6] It is synthesized in the testes, and this process requires a continuous supply of cholesterol, which can be derived from plasma, mostly originating from the liver, or from *de novo* production within the gland.[7] In contrast, women have in general 8 to 9 times lower levels of serum total testosterone; the hormone here also plays a role in sexual function and libido.[8] Circulating testosterone in men is for 40-65% bound to sex hormone-binding globulin (SHBG), which regulates the serum concentration of testosterone and its transport to target tissues. SHBG has a high binding affinity for testosterone and the serum concentrations of total testosterone and SHBG are strongly correlated. In contrast, the non-SHBG-bound fraction of testosterone, which is considered to be bioactive, is barely associated with the serum SHBG concentration.[9-11] This indicates that non-SHBG-bound testosterone, rather than total testosterone plays an important role in maintaining equilibrium in the negative feedback of the hypothalamo-pituitary-testicular axis and in other androgenic effects.

Since statins decrease cholesterol biosynthesis, and cholesterol is the precursor of testosterone, there is some concern whether statins might impair testosterone production. Statins decrease serum availability of the substrate cholesterol, in vitro studies showed that statins decrease cholesterol production in testicular Leydig cells[12], or inhibit enzymes within the testosterone biosynthesis pathway (e.g. 17β-hydroxysteroid dehydrogenase).[13] A lower testosterone level due to statins may be undesired in men with already low testosterone levels, since it may lead to symptoms such as a decrease in mood, libido, muscle strength, or bone mineral density. The 2013 American guidelines for cardiovascular disease prevention lowered the threshold for treatment with statins and widened the target population.[14] Furthermore, the prevalence of diseases such as type 2 diabetes mellitus (T2DM) and CVD is increasing.[15,16] Therefore, the already substantial use of statins in clinical practice may further increase, and this might come along with an increase in non-beneficial effects of statins.[17] Consequently, it is important to elucidate whether statins decrease serum testosterone levels, and more specifically non-SHBG bound testosterone, as potential undesired effect of their use.

In this large population-based cohort study, our objective was to investigate whether the use of statins was associated with decreased serum levels of total and non-SHBG-bound testosterone in male persons aged 45 years or older.

METHODS

Setting and Study Population

This research was conducted within the Rotterdam Study, a prospective population-based cohort study, which aims to examine the frequency and determinants of diseases in middle-aged and elderly people. The rationale and design of the Rotterdam Study have been described previously.[18,19]

In short, at start, all 10,275 persons aged ≥55 years in the Ommoord district of Rotterdam, the Netherlands, were invited to participate. Of them, 7,983 (78%) were enrolled between 1990 and 1993 (RS-I). At baseline, participants underwent extensive clinical examination at the research center. Participants have been followed during up to four follow-up rounds (1993-1995, 1997-1999, 2002-2004, 2009-2012). In 2000, an extended cohort was enrolled (RS-II), in which 3,011 inhabitants aged ≥55 years entered the study and are continuously followed since then. Furthermore, in 2006, a third cohort started (RS-III) including 3,932 inhabitants aged ≥45-54 years at enrollment.

The Rotterdam Study has been approved by the medical ethics committee according to the Wet Bevolkingsonderzoek: ERGO (Population Study Act: Rotterdam Study), executed by the Ministry of Health, Welfare and Sports of the Netherlands. All participants gave informed consent to participate in the study and to obtain information from treating physicians and pharmacies, separately.

All three cohorts of the Rotterdam study were considered in the current research, including a study population aged 45 years or over. The study population consisted of all male participants of the Rotterdam Study (n=4,255), for whom a serum total testosterone measurement was available, for whom a serum non-SHBG-bound testosterone concentration could be calculated, and for whom medication dispensing data were available. As most of the women in the Rotterdam Study are postmenopausal and have very low testosterone levels, we expected that any potential effect of statins could probably only be demonstrated in males or would not be clinically relevant in females. Therefore, we restricted our study population to only male participants.

Outcome assessment

Our outcomes of interest were the serum levels of total testosterone and non-SHBG -bound testosterone. Serum hormone data were assessed from laboratory measurements during the third visit of the first cohort (RS-I-3, 1997-1999), first visit of the second

cohort (RS-II-1, 2000-2001), and the first visit of the third cohort (RS-III-1, 2006-2008). Serum levels of non-SHBG-bound testosterone were used as a measure of bioactive testosterone.

Exposure assessment

The exposure of interest was the use of statin therapy. Medication dispensing data were obtained from all seven fully computerized linked pharmacies in the Ommoord district. Information on all filled prescriptions from January 1st 1991 until February 1st 2012 was available and included information on the product name of the drug, the Anatomical Therapeutical Chemical Code (ATC-code), the amount dispensed, the prescribed dose regimen and the date of dispensing.[20] For every dispensing of a statin, the duration of use (prescription episode) was calculated by dividing the number of dispensed tablets by the prescribed daily number. Repeated prescriptions, which were filled within 7 days after ending the previous filled prescription, were considered as continuous use.

At the date of the testosterone measurement, every participant was classified into mutually exclusive categories: 'Current use' if the measurement occurred within a prescription episode, 'Past use' if the participant previously stopped using statins, or 'Never use' if the participant had not used statins during the study period. Current and past statin users were further stratified into 5 categories according to the duration of exposure to statins: current use ≤1 month, current use >1 to ≤6 months, and current use >6 months; past use >6 months, and past use ≤6 months since the end of the last prescription episode. The 6-month cut-off was applied based on the median duration of current and past use in the population. To facilitate direct dose comparisons between drugs from the same therapeutic drug group, the daily dose of statin therapy was expressed in 'Defined Daily Doses' (DDD).[20]

Analytical determinations

All steroids and SHBG were estimated in the same serum sample obtained from blood taken in the morning in the fasting state. Testosterone, dehydroepiandrosterone (DHEA) and DHEA sulphate (DHEAS) were measured simultaneously with a LC-MS/MS method using the CHS™ MSMS Steroids Kit (Perkin Elmer, Turku, Finland). The Steroids Kit uses a combined solvent extraction and protein precipitation method with acetonitrile containing the deuterated internal standards $^{2}H_5$-testosterone, $^{2}H_6$-DHEA and $^{2}H_6$-DHEAS. The internal standard underwent processing identical to the analytes. The chromatographic separation was performed on a Waters® (Milford, MA, USA) Acquity™ UPLC HSS T3 1.8 µm column (diameter 1 mm, length 10 cm) and in-line filter frit 0.2 µm with an acetonitrile/MeOH gradient. A Waters XEVO-TQ-S system equipped with an ESI source operating in the electrospray positive mode was used for quantitation. The lower limits of quantitation for testosterone, DHEA and DHEAS were 0.07, 2.2 and 24.7 nmol/L, respectively.

Non-SHBG-bound testosterone was calculated according to the method of Södergard et al.[21], using previously described equations[9] assuming a fixed albumin level of 40 g/L. SHBG, estradiol and insulin were measured using a Cobas 8000 Modular Analyzer (Roche Diagnostics GmbH, Mannheim, Germany). Fasting total cholesterol was measured on a cobas c702 system (Roche Diagnostics GmbH,Mannheim, DE).

Covariables

Variables were considered as potential confounders or effect modifiers influencing the association between the use of statins and serum testosterone levels, based on their clinical relevance or confounding effect in the analysis (a variable that changed the estimate >10% was considered as a confounder).[22-24] Our selected confounders were: age, body mass index (BMI), a history of CVD, T2DM, hypertension, and serum estradiol levels. Testosterone is known to decrease with increasing age and BMI. CVD, hypertension and T2DM are conditions associated with lower testosterone levels. Estradiol changed the estimate >10% and is also correlated with testosterone and SHBG. Furthermore, analyses were adjusted for the prescribed DDD of statin therapy.

In addition, we investigated whether additional adjustment for insulin, total cholesterol, DHEA, and DHEAS influenced the association between SHBG and testosterone, and investigated whether these variables were effect modifiers or intermediates. Insulin influences SHBG levels that are strongly correlated with total testosterone, total cholesterol and DHEA are part of the pathway from cholesterol to testosterone, and DHEAS is a metabolite of DHEA.

BMI was calculated as the weight (in kg) divided by height-squared (in m^2). CVD in history was defined as the occurrence of a myocardial infarction (MI), percutaneous transluminal coronary angioplasty (PTCA), coronary artery bypass grafting (CABG), heart failure, carotid desobstruction, cerebrovascular accident (CVA), or transient ischemic attack (TIA) before the date of testosterone measurement.[25-27] T2DM was defined as a current prescription for an oral glucose-lowering drug or insulin (ATC-code A10). Hypertension was defined as blood pressure higher than 140/90 mmHg or a current prescription for an antihypertensive agent (ATC-code C02, C03, C07, C08 or C09).[20]

Statistical analysis

Kolmogorov-Smirnov tests were used to test the normality of the distribution of the parameters. Correlations between variables were tested using Pearson's correlation method for normally distributed data, and Spearman's correlation method for non-normally distributed data. Differences in characteristics between current, past and never statin users were tested using unpaired two-sided Student's T-test for normally distributed parameters and Mann-Whitney U-test for non-normally distributed parameters.

In a cross-sectional design, we investigated the association between use of statins and serum total and non-SHBG-bound testosterone levels, using multivariable linear regression analysis to adjust for confounders. Non-normally distributed data were log transformed in these analyses. First, we compared current and past use of statin therapy on serum testosterone levels, with never use as the reference category. In a second analysis, we considered duration of statin use (current use ≤1 month, >1-≤6 months and >6 months; past use ≤6 and >6 months), and investigated the effect of duration of use on serum testosterone levels, with never use as the reference category. Furthermore, we investigated whether additional adjustment for insulin, total cholesterol, DHEA, and DHEAS influenced this association, and investigated whether these variables were effect modifiers or intermediates.

In an additional analysis, we investigated whether the association between statins and testosterone levels was dose-dependent. We stratified the DDD of statin therapy into tertiles, and compared current and past use of statins with never use in each stratum.

A sensitivity analysis was performed in participants without CVD, to investigate whether associations are constant in men without CVD. Another sensitivity analysis was performed re-allocating most recent past users (within 14 days) from ≤6 months past use into the current use category. This was done because we believed that patients recently exposed to statins might still be affected by the drug (i.e. carry-over effect).

Statistical analyses were performed using SPSS software (SPSS Inc., version 21.0, Chicago, Illinois, USA). All p-values are two-sided and were considered statistically significant if $p<0.05$.

RESULTS

In total, 4,255 male participants with a serum total testosterone measurement, a calculated serum non-SHBG-bound testosterone measurement, and medication dispensing data were available. Since we analyzed complete case sets, 89 (2%) of 4,255 participants were excluded due to incomplete data on covariables. Of all 4,166 eligible male participants in the study population, 577 (14%) were current statin users and 148 (3.5%) were past statin users. Characteristics of current, past and never users are shown in Table 1. All hormone levels were non-normally distributed in the population and were therefore log transformed in the analyses. Compared to never users, current and past users had a significantly higher BMI and a higher prevalence of CVD, T2DM and hypertension. Furthermore, current statin users had significantly higher serum insulin and estradiol levels, and significantly lower levels of SHBG, DHEA, DHEAS and cholesterol. Both current and past statin users had significantly lower mean total (current users 13% ↓, past users 9% ↓) and non-SHBG-bound (current users 8% ↓, past users 7% ↓) testosterone

Table 1 Characteristics of the study population of 4166 male participants

Characteristic	Current users (n = 577)	Past users (n = 148)	Never users (n = 3441)
Age (mean±SD, years)	64.1 ± 8.1	64.6 ± 7.9	64.6 ± 9.7
BMI (mean±SD, kg/m^2)	28.1 ± 3.9[a]	28.0 ± 4.1[b]	26.8 ± 3.5
CVD in history (n, %)	179 (31.0%)[a]	39 (26.4%)[b]	115 (3.3%)
T2DM (n, %)	100 (17.3%)[a]	24 (16.2%)[b]	18 (5.4%)
Hypertension (n, %)	298 (51.9%)[a]	72 (49.0%)[b]	896 (26.0%)
Total T (median, IQR, nmol/L)	14.8 (11.5 – 18.8)[a]	15.5 (12.0 – 19.9)[b]	17.0 (13.3 – 21.4)
Non-SHBG-bound T (median, IQR, nmol/L)	8.1 (6.6 – 9.9)[a]	8.2 (6.8 – 9.7)[b]	8.8 (7.2 – 10.6)
Insulin (median, IQR, pmol/L)	90 (63 – 136)[a]	90 (62 – 137)[b]	71 (49 – 101)
Estradiol (median, IQR, pmol/L)	105 (79 – 132)[a]	97 (75 – 122)[b]	99 (77 – 126)
SHBG (median, IQR, nmol/L)	40 (31 – 52)[a]	42 (33-57)[b]	46 (35 – 58)
DHEA (median, IQR, nmol/L)	8 (6 – 14)[a]	8 (6 – 14)[b]	10 (6 – 15)
DHEAS (median, IQR, µmol/L)	2.44 (1.43 – 3.90)[a]	2.48 (1.49 – 3.87)[b]	2.81 (1.73 – 4.31)
Total cholesterol (median, IQR, mmol/L)	4.69 (4.14 – 5.32)[a]	5.47 (4.70 – 6.31)[b]	5.59 (4.96 – 6.20)
Duration of current use (mean±SD, days)	190.8 ± 185.3	–	–
Statin DDD (mean±SD)	1.6 ± 1.2	–	–
Type of statin		–	–
– Simvastatin (C10AA01)	345 (5.8%)		
– Pravastatin (C10AA03)	68 (11.8%)		
– Fluvastatin (C10AA04)	34 (5.9%)		
– Atorvastatin (C10AA05)	112 (19.4%)		
– Rosuvastatin (C10AA07)	18 (3.1%)		

Abbreviations: SD, standard deviation; T, testosterone; IQR, interquartile range; BMI, body mass index; CVD, cardiovascular disease; T2DM, type 2 diabetes mellitus; SHBG, sex hormone-binding globulin; DHEA, dehydroepiandrosterone; DHEAS, dehydroepiandrosterone sulphate; DDD, defined daily doses.
[a] Current users/[b] Past users are statistically significantly different from never users (P<0.05).

levels compared to never users. The majority of current statin users used simvastatin (n = 345, 59.8%), followed by atorvastatin (n = 112, 19.4%), pravastatin (n = 68, 11.8%), fluvastatin (n = 34, 5.9%), and rosuvastatin (n = 18, 3.1%).

In multivariable linear regression analysis, after adjustment for all covariables, current use of statins was associated with statistically significantly lower total testosterone levels compared to never use, with a beta of −1.18 nmol/L (95%CI −1.96; −0.40, P 0.003). Past statin use was not significantly associated with lower total testosterone levels compared to never use (β −0.79, 95% CI −1.70; 0.12, P 0.089) (Table 2). When duration of use was considered, >1 to ≤6 months current use and >6 months current use were associated with statistically significantly lower total testosterone levels, compared to never use

Table 2 Multivariable linear regression on the association between use of statin therapy and serum total and non-SHBG-bound testosterone levels

	Beta (SE)[a]	95% CI	P	N
Total testosterone				
Current – Past – Never use[b]				
never use	(ref)	–	–	3441
current use	**−1.18 (0.40)**	**−1.96; −0.40**	**0.003**	577
past use	−0.79 (0.47)	−1.70; 0.12	0.089	148
Current and past use categories[c]				
never use	(ref)	–	–	3441
≤1 month current use	−1.13 (0.71)	−2.52; 0.26	0.111	78
>1 – ≤6 months current use	**−1.24 (0.47)**	**−2.17; −0.31**	**0.009**	288
>6 months current use	**−1.14 (0.48)**	**−2.07; −0.20**	**0.017**	226
≤6 months past use	−1.17 (0.62)	−2.39; 0.05	0.061	81
>6 months past use	−0.35 (0.67)	−1.67; 0.97	0.600	67
Non-SHBG-bound testosterone				
Current – Past – Never use				
never use	(ref)	–	–	3441
current use	**−0.35 (0.17)**	**−0.68; −0.01**	**0.042**	577
past use	−0.26 (0.20)	−0.65; 0.13	0.191	148
Current and past use categories				
never use	(ref)	–	–	3441
≤1 month current use	−0.29 (0.30)	−0.89; 0.31	.341	78
>1 – ≤6 months current use	**−0.42 (0.20)**	**−0.82; −0.02**	**.039**	288
>6 months current use	−0.29 (0.21)	−0.70; 0.11	.153	226
≤6 months past use	−0.34 (0.27)	−0.87; 0.18	.200	81
>6 months past use	−0.17 (0.29)	−0.73; 0.40	.563	67

Abbreviations: β, beta; SE, standard error; CI, confidence interval; SHBG, sex hormone-binding globulin.
[a] Analyses are adjusted for age, body mass index, history of cardiovascular disease, diabetes mellitus, hypertension, estradiol level, and statin dose. [b] Current and past use of statin therapy compared to never use as the reference category. [c] Current and past use categories: the 1-month current use cut-off was applied to investigate whether statins exert an effect already after 1 month of therapy; the 6-month current and past use cut-off was applied based on the median duration of current and past use in the population. **Bold** value indicates a statistically significant association.

(β −1.24, 95%CI −2.17; −0.31, P 0.009 for >1 to ≤6 months current use; β −1.14, 95%CI −2.07; −0.20, P 0.017 for >6 months current use). For the past use categories and ≤1 month current use, no significant association with serum total testosterone levels was found (Table 2).

In the multivariable analyses on non-SHBG-bound testosterone levels, current statin use was associated with lower non-SHBG-bound testosterone levels compared to never

use (β −0.35, 95%CI −0.68; −0.01, P 0.042). Past statin use was not significantly associated with lower non-SHBG-bound testosterone levels compared to never use (β −0.26, 95% CI −0.65; 0.13, P 0.191) (Table 2). When duration of use was considered, >1 to ≤6 months current use was associated with statistically significantly lower non-SHBG-bound testosterone levels with a beta of −0.42 (95%CI −0.82; −0.02, P 0.039). For the other past and current use categories, no significant association was found (Table 2).

We investigated whether other hormones and total cholesterol were intermediates in the association between statins and testosterone levels. Additional adjustment for insulin and DHEAS did not change the results importantly. Additional adjustment for total cholesterol and DHEA showed the largest effects on the associations, and reduced the magnitude of the effect. Additional adjustment for total cholesterol showed a beta of −0.97 (95%CI −1.75; −0.18, P 0.015) for total testosterone and a beta of −0.25 (95%CI −0.58; 0.90, P 0.151) for non-SHBG-bound testosterone for current use, instead of the original betas of −1.18 and −0.35 respectively. Additional adjustment for DHEA showed similar results as for total cholesterol adjustment. (*Results not shown*).

Moreover, we stratified statin DDD in tertiles to investigate whether a higher statin dose was associated with a stronger testosterone lowering effect. As shown in Figure 1, there was a trend towards a stronger testosterone lowering effect at a higher statin dose, both for total testosterone (P for trend 2.9×10^{-5}) and non-SHBG-bound testosterone (P for trend 2.0×10^{-4}). Current statin use in the mid and high dosage tertiles was associ-

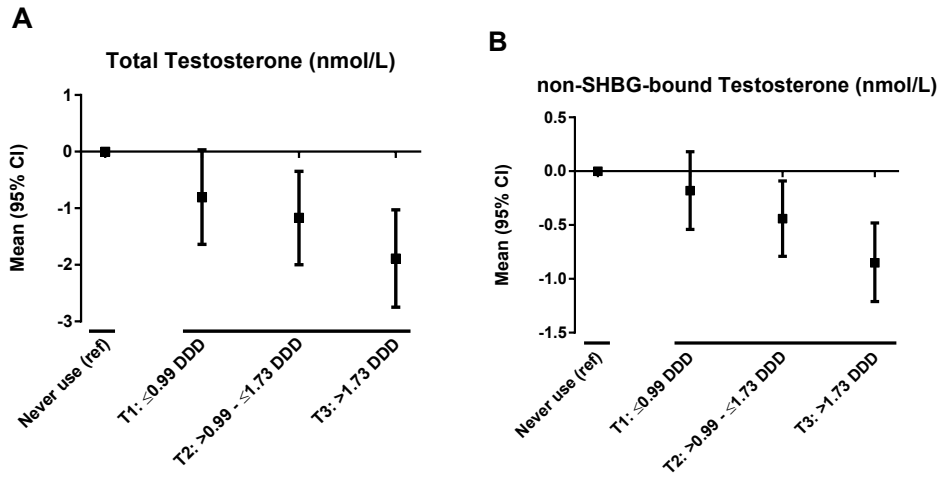

Figure 1 Multivariable linear regression of the association between tertiles of dosage of statin therapy in all current users and serum total and non-SHBG-bound testosterone levels

Abbreviations: SHBG, sex hormone-binding globulin; CI, confidence interval; DDD, defined daily doses.

A: Total Testosterone; B: Non-SHBG-bound Testosterone.

Tertiles of statin drug dosage compared to never use as the reference category. Analyses are adjusted for age, body mass index, history of cardiovascular disease, diabetes mellitus, hypertension, and estradiol level.

Table 3 Sensitivity analysis: multivariable linear regression on the association between use of statin therapy and serum total testosterone levels – recent past users reallocated

	Beta (SE)[a]	95% CI	P	N
Total testosterone				
Current – Past – Never use[b]				
never use	(ref)	–	–	3441
current use	**−1.31 (0.38)**	**−2.06; −0.56**	**0.001**	600
past use	−0.49 (0.50)	−1.47; 0.49	0.331	125
Non-SHBG-bound testosterone				
Current – Past – Never use				
never use	(ref)	–	–	3441
current use	**−0.42 (0.16)**	**−0.74; −0.10**	**0.011**	600
past use	−0.12 (0.21)	−0.54; 0.30	0.569	125

Abbreviations: β, beta; SE, standard error; CI, confidence interval; SHBG, sex hormone-binding globulin.
[a] Analyses are adjusted for age, body mass index, history of cardiovascular disease, diabetes mellitus, hypertension, estradiol level, and statin dose. [b] Current and past use of statin therapy compared to never use as the reference category. **Bold** value indicates a statistically significant association.

ated with betas of −1.17 (95%CI −2.00; −0.35, P 0.005) and −1.89 (95%CI −2.75; −1.03, P 1.7×10^{-5}) nmol/L of lower serum total testosterone, respectively, when compared to never users. For non-SHBG-bound testosterone, current statin use in the mid and high dosage tertiles was associated with betas of −0.44 (95%CI −0.79; −0.09, P 0.015) and −0.85 (95%CI −1.21; −0.48, P 6.0×10^{-6}) nmol/L, respectively, when compared to never users.

In a sensitivity analysis, we excluded all participants with a history of CVD (8% of the population). Current users again showed significantly lower total testosterone levels (β −1.12, SE 0.43, P 0.048). The effect estimate for current users on lower non-SHBG-bound testosterone levels was similar to the original analysis, although non-significant (β −0.29, SE 0.27, P 0.125). For past statin use, again no association was found.

In an additional sensitivity analysis, we re-allocated 23 participants from ≤6 months past use to current use. These patients were the most recent past users (they had stopped statins ≤14 days before testosterone assessment). Results showed no significant association of past use with lower total and non-SHBG-bound testosterone levels. Current users again showed a stronger and more significant association with total testosterone levels (β −1.31, 95%CI −2.06; −0.56, P 0.001) and non-SHBG-bound testosterone levels (β −0.42, 95%CI −0.74; −0.10, P 0.011), than never users (Table 3). When past users within 30 days were re-allocated to current use (40 participants re-allocated), the results were similar to the 14 days re-allocation (*Results not shown*).

DISCUSSION

In this cross-sectional population-based study, we showed that current use of statins was associated with statistically significantly lower levels of serum total and non-SHBG-bound testosterone in males. We demonstrated that the magnitude of the decrease in testosterone was directly proportional to the dosage of statin therapy. Considering duration of statin use, the association between current statin use and lower testosterone levels was present after at least 1 month current use. For past use, no association was found with testosterone levels.

As far as we know, there are five cross-sectional studies in the literature that studied the association between statins and testosterone levels. Three studies are in line with our findings on total testosterone levels and showed that statins were associated with lower total testosterone levels[28-30], while two showed no association at all[31,32]. The studies which showed lower total testosterone levels demonstrated a difference of 1.5, 1.6 and 3.0 nmol/L, respectively, while in our study mean total testosterone levels were 2.2 nmol/L lower in current users compared to never users. Three of these five studies also investigated free testosterone, of which two did not find an association[29,31] and one showed significantly lower free and bioavailable testosterone levels[30]. Bioavailable testosterone levels were 1.0 nmol lower in statin users than in non-users[30], while in our study mean non-SHBG-bound testosterone levels were 0.7 nmol/L lower in current users than in never users. Advantages of our study compared with these studies are that one did not adjust for potential confounders[29], another had only 25 statin users[32], only one study had statin dosage[30], while in none of these studies duration of therapy was analyzed. Moreover, one study was conducted only in patients consulting for erectile dysfunction[30], while in another exclusively T2DM patients were selected[29].

Similarly, placebo controlled randomized trials studied total testosterone levels before and after statin therapy. A recent meta-analysis including 5 such trials concluded that current statin therapy (4 weeks – 3 months) induced a decrease in total testosterone of –0.66 nmol/L in men.[33]

Theoretically, there are several mechanisms by which statins may inhibit testosterone production. The main one is by inhibiting HMG-CoA reductase in the testis.[12] By operating in the testis, statins consequently suppress *de novo* cholesterol production, impairing the substrate source for the testosterone biosynthesis pathway. The second mechanism is by decreasing serum cholesterol concentrations, and therefore cholesterol uptake by the testis, which could, again, limit its availability and impact on testosterone production. A third mechanism is by directly inhibiting other enzymes in the testosterone biosynthesis pathway.[13] An alternative theory for the decrease in testosterone levels is that statin users have increased insulin levels, because these patients often have metabolic syndrome, and statins themselves may increase insulin levels.[34] Insulin suppresses SHBG

production in the liver[35,36], with consequently lower total testosterone levels, because free testosterone levels are kept constant in response to the decrease in SHBG levels.[9]

The impact of statins as testosterone lowering drugs has been criticized[37] and several arguments were used. First, a decrease of −0.66 nmol/L, as shown in the recent meta-analysis[33], is a small mean decrease in total testosterone level. Second, in literature there is a poor correlation between the use of statins and symptoms such as decreased libido or muscle strength, which questions the clinical relevance of this decrease. However, there is a variability in response to statins and some patients might be more vulnerable to statins and will have a stronger decrease.[38] A modest average decrease in a population might hide a substantial decrease in a handful of individuals and in those with an already low testosterone, it might be clinically meaningful. Moreover, even modest effects on a population-based scale may gain more relevance now that statin therapy is increasingly used. For instance, application of the adapted American guidelines on male participants of the Rotterdam Study would imply that nearly all elderly men (i.e. 96.4%) should be prescribed statins.[39] This corroborates to the idea that adverse reactions to statins, e.g. a testosterone lowering effect, deserve attention. In our study, additional adjustment for total cholesterol or DHEA attenuated the association between statins and lower testosterone levels, while DHEAS and insulin showed no important effect. Both cholesterol and DHEA are substrates for testosterone formation, and attenuation of the association through additional adjustment supports the hypothesis that statins lower testosterone through reducing cholesterol. Also, DHEA is a reflection of adrenal steroid production, and suppression of DHEA may suggest an effect on adrenal steroidogenesis.[40] Moreover, when the decrease in testosterone levels is clinically relevant, statin users can become eligible for testosterone replacement therapy (TRT). However, evidence as to whether TRT is associated with an increase in cardiovascular events is controversial, since a first meta-analysis[41] on RCTs supported the association, while a reviewing second meta-analysis[42] did not.

Our study is cross-sectional, because regular assessment of serum testosterone levels is very unusual in a population-based setting. Nevertheless, we were able to study duration of use to establish a temporal relationship. Current use for >1 to ≤6 months and >6 months showed a significantly lower total testosterone level. The first month of therapy was investigated separately to gain insight into how testosterone levels behave during the beginning of statin use. During the first month, the testosterone lowering effect was non-significant and less strong than for the other current use categories, suggesting that more time is needed to induce a complete effect on testosterone levels. For >1 to ≤6 months of statin use, non-SHBG-bound testosterone levels decreased in parallel with total testosterone. However, for the > 6 months statin users, non-SHBG-bound testosterone levels also showed a negative beta which was, however, not significant. As described in the introduction, non-SHBG-bound testosterone is driving the negative feedback

mechanism of the hypothalamo-pituitary-gonadal axis to keep its concentration constant. Possibly, in long-term statin users, the setpoint has adapted to the effect of statins on testosterone lowering, as a compensatory effect to prevent the levels of bioactive non-SHBG-bound testosterone to fall. This might explain why we could not demonstrate an effect of long-term statin use on the levels of bioactive non-SHBG-bound testosterone.

The magnitude of the association between current statin use and lower serum total and non-SHBG-bound testosterone was stronger at a higher statin doses. This is in line with what is expected, and strengthens our finding. Some misclassification of exposure may have occurred in very recent past users who may actually be current users who took a drug holiday or be subject to a carry-over effect after stopping the drug. In sensitivity analyses, the association was strengthened for current users, which supports the possibility of some misclassification of exposure.

Strengths and potential limitations should be considered. The Rotterdam Study is a large prospective population-based cohort study with extensive data collection. For instance, we were able to consider statin dose and were the first study that considered the effect of duration of statin use. Furthermore, we accounted for potential confounding variables (e.g. T2DM, hypertension and CVD). Compared to other cross-sectional studies, we managed not only to overcome potential flaws, but also to further extend and strengthen the analysis. We adjusted for confounding by indication which potentially plays a role since diseases such as CVD and T2DM are independently associated with lower testosterone levels.[43]

A strength is that we measured total testosterone, DHEA and DHEAS by LC-MS/MS. Although we measured estradiol by electrochemiluminescence immunoassay, the method used has been standardized via ID-GC/MS. In the Rotterdam Study no direct measurement of free testosterone by equilibrium dialysis was performed. Instead, we calculated non-SHBG-bound testosterone using the formula by Sodergard et al.[20] Results of both types of estimations of free testosterone yielded highly correlated results.[44] Ideally, the study should be performed longitudinally with several testosterone assessments over time. However, this is very unusual in a population-based setting. Unfortunately, we had too low numbers of current statin users to investigate the association in users of the different types of statins separately. However, we expected a class effect of statins on testosterone lowering. This study supports the hypothesis that statins lower testosterone through cholesterol lowering, and thus all statins will show this effect. Analyses of the association between current statin use and testosterone levels showed a similar direction of the effect (negative beta) for all statins separately. However, the association only remained significant for the association of total testosterone in simvastatin users, due to decreased power because of sample size.

In conclusion, our study showed that current use of statin therapy is associated with significantly lower serum total and non-SHBG-bound testosterone levels after adjust-

ment for important confounders. Given the large number of statin-treated males and important biological role of testosterone, the clinical relevance of this association should be further investigated.

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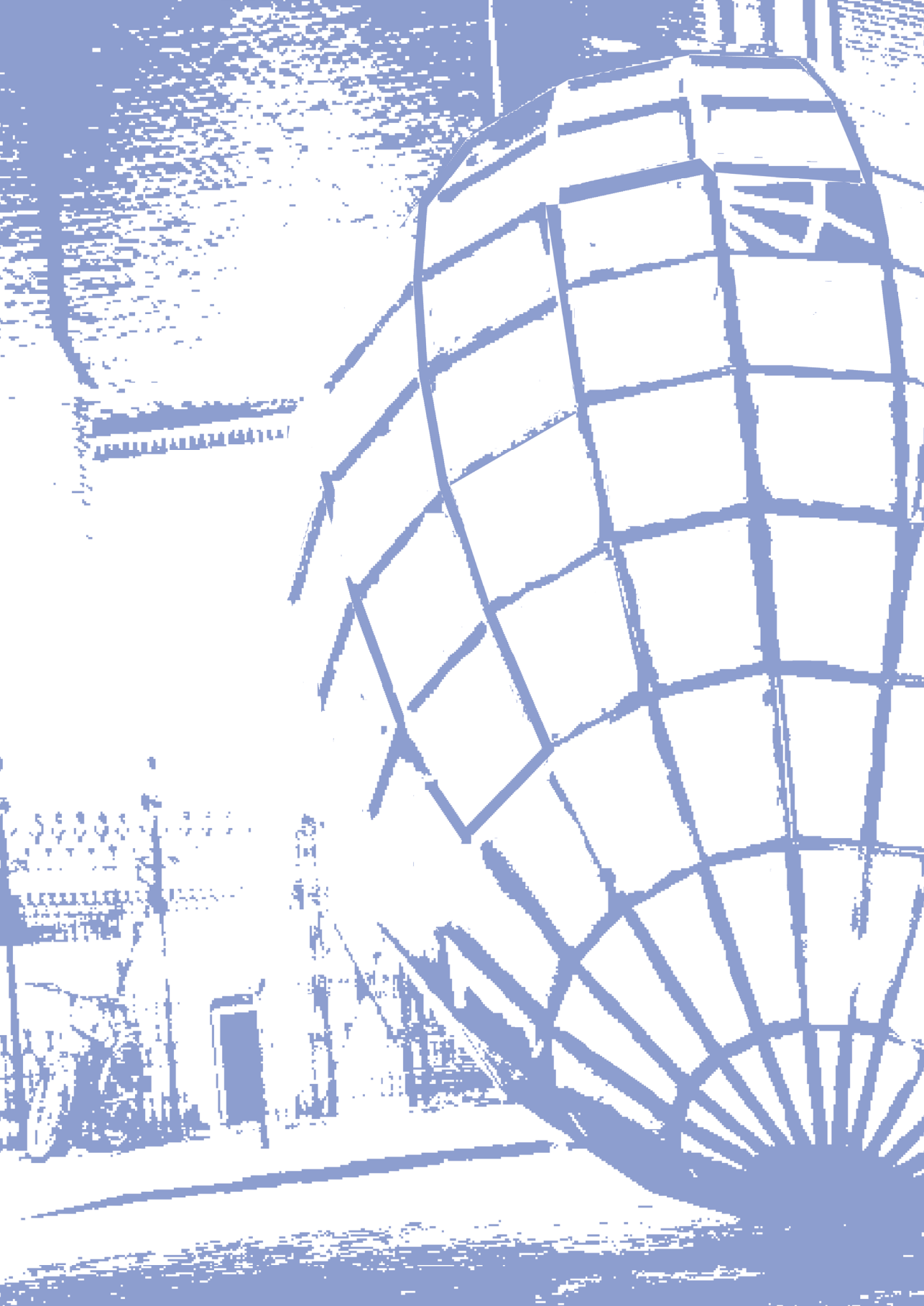

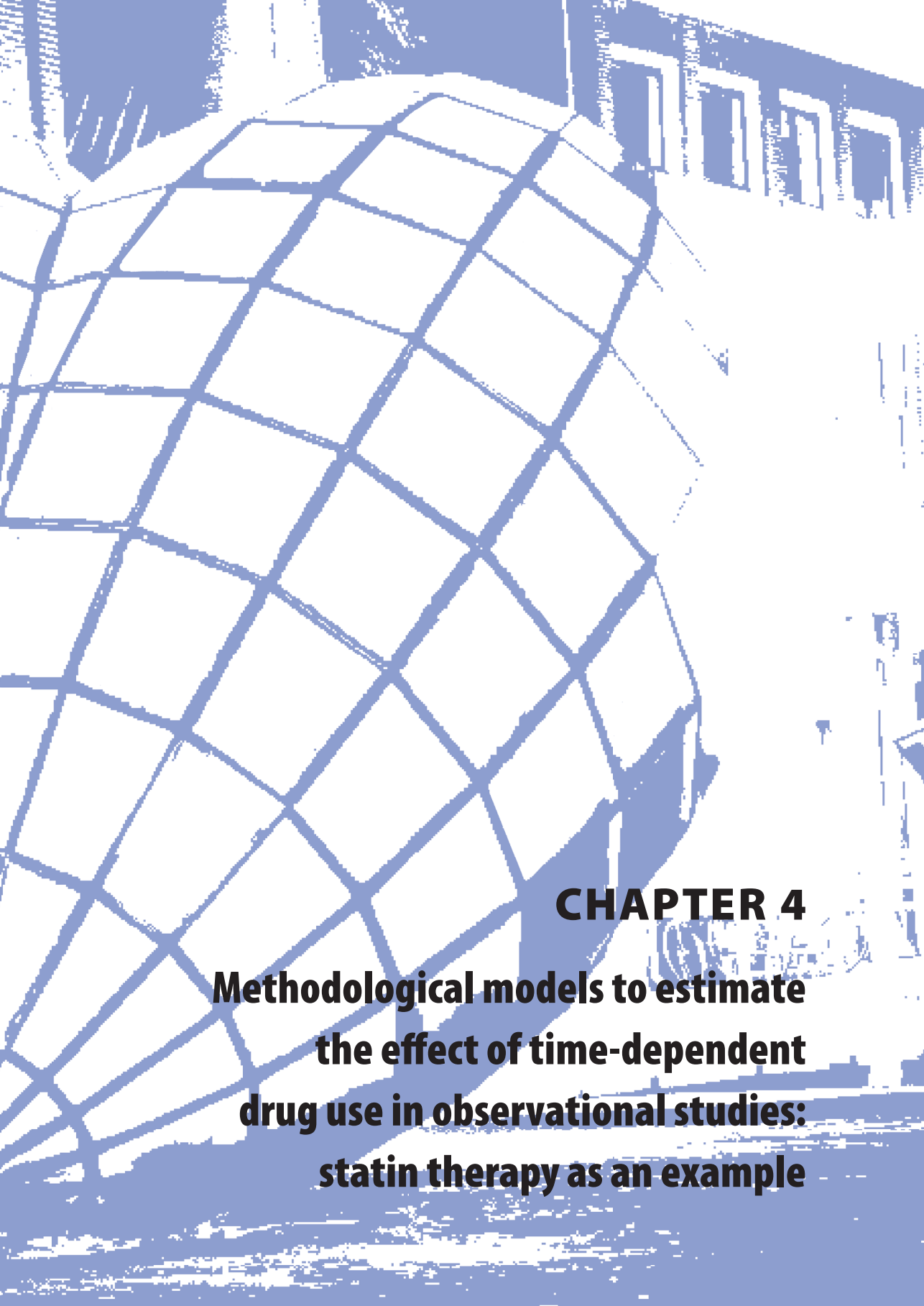

CHAPTER 4

Methodological models to estimate the effect of time-dependent drug use in observational studies: statin therapy as an example

4.1

Comparing a marginal structural model with a Cox proportional hazard model to estimate the effect of time-dependent drug use in observational studies: statin use for primary prevention of cardiovascular disease as an example from the Rotterdam Study

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Eur J Epidemiol 2014; 29(11): 841-850

ABSTRACT

Introduction: When studying the causal effect of drug use in observational data, marginal structural modeling (MSM) can be used to adjust for time-dependent confounders that are affected by previous treatment. The objective of this study was to compare traditional Cox proportional hazard models (with and without time-dependent covariates) with MSM to study causal effects of time-dependent drug use.

Methods: The example of primary prevention of cardiovascular disease (CVD) with statins was examined using up to 17.7 years of follow-up from 4,654 participants of the observational prospective population-based Rotterdam Study. In the MSM model, the weight was based on measurements of established cardiovascular risk factors and co-morbidity.

Results: In general, we could not demonstrate important differences in results from the Cox models and MSM. Results from analysis on duration of statin use suggested that substantial residual confounding by indication was not accounted for during the period shortly after statin initiation.

Conclusion: In conclusion, although on theoretical grounds MSM is an elegant technique, lack of data on the precise time-dependent confounders, such as indication of treatment or other considerations of the prescribing physician jeopardizes the calculation of valid weights. Confounding remains a hurdle in observational effectiveness research on preventive drugs with a multitude of prescription determinants.

INTRODUCTION

Increasingly, comparative effectiveness research is performed using data from large health care databases on drug utilization and clinical outcomes. Such databases have traditionally been utilized to study rare adverse drug reactions and prescription patterns in unselected populations. It is imperative that these health care databases contain accurate and complete information on drug use, clinical outcomes, and relevant covariates. However, because the consequences of invalid findings can be substantial, also proper methods for statistical analysis need to be employed to estimate causal effects.[1]

Observational studies with information on drug use at baseline only (e.g. based on interviews at enrollment) may have substantial misclassification of exposure during follow-up. Drug use is in most circumstances a time-varying determinant since discontinuation, change in dosage, or switching to another drug is common. To circumvent this, we previously proposed a method based on a Cox model for the analysis of drug use as a time-dependent determinant.[2] Nonetheless, effect estimates may be biased because some risk factors which change during follow-up may have been influenced by preceding drug use, and in the presence of a time-dependent risk factor for the event of interest which also predicts subsequent drug use. Both conditions will hold when a time-dependent covariate is simultaneously (1) a reason for prescribing (often termed 'confounding by indication'[3]); (2) influenced by the drug treatment under study; and (3) a potential cause of the outcome of interest. This can be adjusted for by marginal structural modeling (MSM), in which drug-outcome associations can be modeled with the drug as a time-dependent determinant, along with adjustment for time-dependent confounding through the application of weights. In short, all observations are assigned a weight based on the inverse of the conditional probability of receiving observed treatment.[4,5] However, there is limited experience with the application of such MSMs in observational studies with complete data on drug use during follow-up and a multitude of treatment determinants. This is unfortunate as the method may be valuable for the rapidly increasing number of observational studies on the effectiveness of drug use in health care databases which serve as a basis for decision making or initiation of clinical trials.

The objective of this study was to investigate whether MSM produces different risk estimates from more traditional Cox models with and without time-dependent covariates in observational (i.e. nonrandomized) data with detailed information on medication use, clinical outcomes, and relevant covariates. We used data from the prospective population-based Rotterdam Study to examine the example of primary prevention of cardiovascular disease (CVD) with statins.

METHODS

Setting

The current study was performed within the Rotterdam Study, a prospective population-based cohort study in the general population. The rationale and design of the Rotterdam Study have been described in detail elsewhere.[6,7] In short, all 10,275 persons aged 55 years and over in the Ommoord district of Rotterdam, the Netherlands, were invited to participate. Of them, 7,983 (78 %) were enrolled between 1990 and 1993. At baseline, all participants were interviewed at home and 7,085 underwent extensive clinical examination at the research center. We additionally used data from re-examinations that took place in 1993–1995, 1997–1999, and 2002–2004.

The Rotterdam Study has been approved by the medical ethics committee according to the Wet Bevolkingsonderzoek: ERGO (Population Screening Act: Rotterdam Study), executed by the Ministry of Health, Welfare and Sports of the Netherlands.

Study population

We excluded all participants who did not visit the research center at baseline, were 80 years or older, and those with established CVD, diabetes mellitus, or statin use at baseline. Prevalent CVD was defined as a history of myocardial infarction (MI), heart failure, stroke, transient ischemic attack (TIA), or coronary-, carotid-, or abdominal aortic revascularization. Diabetes mellitus at baseline was defined as filling a prescription according to the pharmacy dispensing records for oral glucose lowering drugs or insulin before the baseline examination. Statin use at baseline was defined as filling a prescription according to pharmacy records for statins or if the participant reported statin use during the interview.

Drug exposure

Medication dispensing data were obtained from all seven fully computerized pharmacies in the Ommoord district. Information on all filled prescriptions from 1 January 1991 until 1 February 2012 were available and included information on the product name of the drug, the WHO Anatomical Therapeutical Chemical (ATC) code[8], the amount dispensed, the prescribed dosage regimen, and the date of dispensing.[6,7] We excluded participants who filled a first prescription of statins prior to 1 April 1991 in order to ensure that all participants were incident users and did not use statins before. We used the ATC codes C10AA and C10B for statins; C02 (antihypertensives), C03 (diuretics), C07 (b-blockers), C08 (calcium channel blockers) or C09 (renin-angiotensin system modifying agents) for blood pressure lowering medication; and A10 for oral glucose-lowering medication and insulin.[8]

For every dispensing of a statin, the duration of use was calculated by dividing the number of dispensed tablets by the prescribed daily number. Repeated prescriptions which were filled within 7 days after ending the previous filled prescription were considered as continuous use. At each month during follow-up, every participant was classified into mutually exclusive categories: 'ever use' if the participant had filled a prescription for statins during this month or at any point during follow-up prior to this month; 'never-use' if the participant had not used statins during the study period leading up to that month. We did not distinguish between current use and past use of statins since we did not have good determinants for discontinuation available and thereby assumed that any participant starting statins during follow-up remained exposed thereafter ('intention to treat'). We considered duration of statin use by dividing the total number of days of statin use into mutually exclusive categories of cumulative use: first month of use, cumulative use equals 31 days to 365 days, cumulative use for more than 365 days.

Cardiovascular outcomes

Information on the presence and occurrence of CVD is available through collaboration with the general practitioners in the study area. Methods of data collection and definitions of cardiovascular outcomes in the Rotterdam Study have been described in detail previously.[9-11]

For the present analysis, we used two different combined clinical endpoints as outcome of interest. First, we considered 'hard' atherosclerotic cardiovascular endpoints, defined as the first occurrence of any of the following events: fatal or nonfatal MI, non-hemorrhagic stroke, or atherosclerotic CVD death. Second, we extended this definition by including all strokes and arterial revascularization procedures (defined as a surgical or percutaneous coronary, carotid revascularization, or abdominal aortic surgery). Physicians prescribe statins to those whom are expected to benefit most from statins during their remaining lifespan[12] which carry similar considerations as the patient selection for arterial revascularization procedures. Heart failure and TIA were not considered as endpoints, but if one of these events occurred during follow-up, the participant was censored since statin use after the occurrence of such an event could not be considered as primary prevention of CVD.

Covariables

The variables age, sex, current smoking status, systolic blood pressure, and serum level of total cholesterol were considered in the analysis. This was based on the variables used in the risk charts used for cardiovascular risk management in clinical practice: the European Society of Cardiology guidelines on CVD prevention.[13] Besides these established cardiovascular risk factors, we additionally considered body mass index (BMI) in kg/m2, family history of premature MI (first degree relative aged <65 years), level of highest

education attained as a measure of socioeconomic status[14], incident non-skin cancer (n = 1,327) and chronic obstructive pulmonary disease (COPD) at baseline as measures of co-morbidity, and use of blood pressure lowering medication.

Statistical analysis

Throughout the analysis we used pooled logistic regression models using generalized estimating equations with each person-month of follow-up as a separate observation. This was done to emulate time-dependent Cox proportional hazards models with the ability to incorporate time-dependent weighing of the observations.[15,16]

We compared three different models. First, we used a Cox proportional hazards model with statin use during follow-up as a time-dependent exposure (including categories based on the cumulative duration of use) (model I). This analysis was adjusted for the baseline values of age, sex, smoking status, systolic blood pressure, total cholesterol, BMI, level of education, incident non-skin cancer, COPD, and the use of blood pressure lowering medication.

In the second time-dependent Cox proportional hazards model (model II), we included statin use during follow-up as a time-dependent exposure, as well as updated variables for the potential confounding variables. At every monthly interval, the values for the covariates smoking status, systolic blood pressure, total cholesterol level, and BMI were updated to the most recent visit of the Rotterdam Study research center. Use of blood pressure lowering medication, based on pharmacy records, and incident non-skin cancer were also included as a time-dependent covariate. Furthermore, model II was adjusted for age, sex, level of education, and COPD at baseline.

Last, we used a marginal structural Cox proportional hazards model (model III) to adjust for time-dependent confounding by indication.[17] To adjust for time-dependent confounding by indication, all observations are weighted by the inverse of the conditional probability of the observed status of statin use, based on variables that are considered to influence the initiation of statins: age, sex, smoking status, systolic blood pressure, total cholesterol, BMI, level of education, incident non-skin cancer, COPD, and use of blood pressure lowering medication. We used stabilized weights as described by Hernán et al.[4,17] The range and distribution of the stabilized weights used in model III are visualized in Fig. 1 (mean 1.012; median 0.996). At every monthly interval, the time-dependent measures of the covariates used to compute the weights were updated to the values from the most recent visit to the Rotterdam Study research center. The regression model was adjusted for the baseline values of the covariates in model I. Statin use was included as a categorical time-dependent exposure based on the cumulative duration of use. Adjustment for follow-up time was done by including dummy variables for the tertiles of follow-up time (<43 months, ≥43-<98 months, and ≥98 months, respectively). Model III was based on a previously published SAS macro (statistical code available in the online supplement).[17]

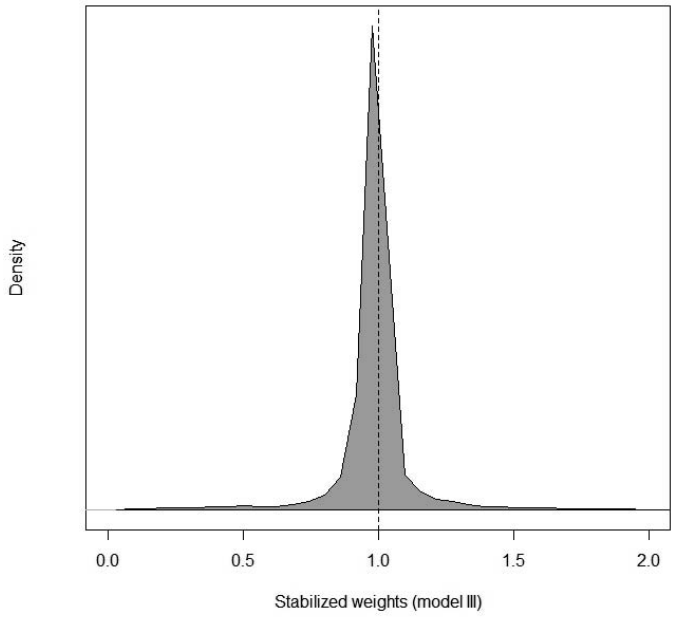

Figure 1 Distribution of stabilized weights for the marginal structural model (model III)

In a sensitivity analysis we excluded observations with extreme stabilized weights (<0.01th and >99.99th percentile in line with Cook et al.[18]; <0.044 and >17.756, respectively,) in order to assess the impact of these observations on the results.

For the present study, follow-up started at 1 April 1991 or the date of clinical examination when enrolled later. The end of the follow-up was 1 January 2009. Since we aimed to study primary prevention of CVD in accordance with the European and American guidelines[13,19], participants were followed until the date of any cardiovascular event, the date of first dispensing of oral blood glucose lowering drugs or insulin, the 80th birthday, or the last date of data collection, whichever came first.

Missing values for one or more of the covariates at baseline were present in 105 (2.3 %) participants. These were handled by single imputation using an expectation-maximization algorithm.[20] Throughout follow-up we used last observation carried forward for all confounders. All measures of association are presented with 95% confidence intervals (95% CIs). We used the level of significance of $P<0.05$. The analyses were performed using SAS version 9.3 (SAS Institute Inc., Cary, NC, U.S.).

RESULTS

The baseline characteristics of the study population are presented in Table 1. A total of 4,654 participants were eligible for the analysis. During up to 17.7 years of follow-up, 583 participants initiated statin therapy. At the end of follow-up, 378 (64.8 %) participants that initiated statin therapy were still using statins. Out of the 3,795 person-months of follow-up after initial statin dispensing, 2,697 person-months were covered by statin dispensing, corresponding to a 71.1 % coverage of person-time exposed to 'ever statin use'. The mean duration of cumulative use was 4.6 years. A total of 510 'hard' atherosclerotic CVD and 655 'extended' atherosclerotic CVD endpoints occurred. The follow-up and reasons for ending follow-up for the present study are presented in Table 2.

In the first Cox regression model with time-dependent statin use (model I), ever use of statins was significantly associated with a lower risk of the hard CVD endpoint (HR 0.68, 95% CI 0.47; 1.00), but it was not significantly associated with the extended CVD endpoint (HR 1.19, 95% CI 0.89; 1.61). These results are shown in Table 3 and Table 4 respectively. When we divided use of statins into categories based on the cumulative duration of use, in the analysis on the hard CVD endpoint, use of statins for more than 1 year was significantly associated with a lower risk of hard CVD with a HR of 0.50 (95% CI 0.31; 0.82) (Table 3). This association could not be demonstrated in the analysis on the extended endpoint. In this analysis however, cumulative statin use for 1–30 days

Table 1 Characteristics of the study population at baseline (1990-1993)

Characteristic	Study population (n = 4,654)
Age, years (mean, SD)	66.3 (6.7)
Men (n, %)	1,835 (39.4%)
BMI, kg/m^2 (mean, SD)	26.3 (3.6)
Current smoking (n, %)	1,135 (24.4%)
Systolic blood pressure, mmHg (mean, SD)	138 (22)
Hypertension (n, %)	2,387 (51.3)
Total cholesterol, mmol/L (mean, SD)	6.7 (1.2)
HDL-cholesterol, mmol/L (mean, SD)	1.3 (0.4)
COPD (n,%)	180 (3.9%)
Use of blood pressure lowering drugs (n, %)	1006 (21.6%)
– Diuretics (n, %)	389 (8.4%)
– β-blockers (n, %)	524 (11.3%)
– Calcium channel blockers (n, %)	172 (3.7%)
– ACE-inhibitors (n, %)	194 (4.2%)
– Other blood pressure lowering drugs (n, %)	44 (0.9%)

Abbreviations: ACE, angiotensin-converting enzyme; BMI, body mass index; HDL, high-density lipoprotein; COPD, chronic obstructive pulmonary disease.

and cumulative statin use for 31–365 days were associated with a significantly increased risk of CVD with HRs of 3.73 (95% CI 1.51; 9.21) and 2.82 (95% CI 1.73; 4.61), respectively (Table 4).

The analyses of the time-dependent Cox regression model (model II) with both statin therapy and covariates updated during follow-up showed similar results compared to model I on the association between use of statins and both CVD endpoints (Tables 3; 4).

In the Cox proportional hazards MSM analyses (model III), ever use of statins was associated with a lower hard CVD risk. However, this association was not statistically significant (HR 0.89, 95% CI 0.5; 1.43). When distinguishing categories of the cumulative duration of use, use of statins for more than 1 year was significantly associated with a lower risk of hard CVD (HR 0.57, 95% CI 0.33; 0.99) (Table 3). For the extended CVD endpoint, ever use of statins was not significantly associated with CVD (HR 1.05, 95%

Table 2 Details regarding the indications for end of follow-up and amount of follow-up (1991–2009)

CVD events	
Total CVD (n, %)	655 (14.1%)
– Coronary heart disease (n, %)	353 (7.6%)
– Myocardial infarction (n, %)	199 (4.3%)
– Coronary artery bypass grafting (n, %)	43 (0.9%)
– Percutaneous coronary intervention (n, %)	50 (1.1%)
– Coronary death (n, %)	61 (1.3%)
– Cerebrovascular disease (n, %)	268 (5.8%)
– Non-hemorrhagic stroke (n, %)	231 (5.0%)
– Hemorrhagic stroke (n, %)	36 (0.8%)
– Carotid revascularization (n, %)	1 (0.0%)
– Abdominal aortic surgery (n, %)	28 (0.6%)
– Other atherosclerotic CVD mortality (n, %)	6 (0.1%)
Indications for censoring follow-up	
Last date of data collection (n, %)	1,170 (25.1%)
Non-atherosclerotic death (n, %)	421 (9.0%)
80th birthday (n, %)	1,695 (36.4%)
First dispensing of glucose-lowering medication or insulin (n, %)	314 (6,7%)
Diagnosis of heart failure (n, %)	245 (5.3%)
Diagnosis of cerebrovascular transient ischemic attack (n, %)	154 (3.3%)
Follow-up	
Total, pys	44,065
Median (interquartile range), years	9.8 (4.8–15.0)
Incidence	
Crude incidence rate (95% CI), per 1,000 pys	14.9 (13.7–16.0)

Abbreviations: CVD, cardiovascular disease; pys, person-years; CI, confidence interval.

Table 3 Cox regression and MSM analyses for the association between statin use and the occurrence of hard atherosclerotic CVD

	N_{cases}	pys	I. First time-dependent Cox regression model[a] HR (95% CI; P-value)	II. Second time-dependent Cox regression model[b] HR (95% CI; P-value)	III. MSM[c] model HR (95% CI; P-value)
Ever use versus never use[d]					
Never use	476	40,326	1.00 (ref)	1.00 (ref)	1.00 (ref)
Ever use	34	3,739	**0.68 (0.47; 1.00, 0.048)**	**0.69 (0.48; 1.00, 0.050)**	0.89 (0.55; 1.43, 0.627)
Duration of use[e]					
Never use	476	40,326	1.00 (ref)	1.00 (ref)	1.00 (ref)
1-30 days use	4	201	1.77 (0.44; 7.16, 0.425)	1.63 (0.40; 6.69, 0.495)	4.50 (0.86; 23.5, 0.074)
31-365 days use	11	825	1.15 (0.51; 2.62, 0.736)	1.09 (0.48; 2.48, 0.831)	2.00 (0.77; 5.18, 0.154)
≥366 days use	19	2,714	**0.50 (0.31; 0.82, 0.006)**	**0.51 (0.31; 0.83, 0.007)**	**0.57 (0.33; 0.99, 0.045)**

We used pooled logistic regression models using generalized estimating equations with each person-month of follow-up as a separate observation. This was done to emulate time-dependent Cox proportional hazards models with the ability to incorporate time-dependent weighting of the observations.[15,16] 'Hard' atherosclerotic CVD is a composite endpoint of fatal and nonfatal myocardial infarction, stroke, and atherosclerotic CVD death.

Abbreviations: MSM, marginal structural model; CVD, cardiovascular disease; N_{cases}, number of CVD cases; pys, person-years of observed follow-up; HR, hazard ratio; CI, confidence interval; ref, reference; BMI, body mass index; COPD, chronic obstructive pulmonary disease.

[a] First time-dependent Cox regression model: statin use during follow-up as time-dependent variable; adjusted for the baseline values age, sex, BMI, smoking status, systolic blood pressure, total cholesterol, use of blood pressure lowering drugs, presence of COPD, incident non-skin cancer, and level of education.

[b] Second time-dependent Cox regression model: statin use during follow-up as time-dependent variable; adjusted for the updated values of BMI, smoking status, systolic blood pressure, total cholesterol level (updated at every monthly interval to the most recent visit of the Rotterdam Study) and adjusted for age, sex, use of blood pressure lowering drugs at baseline, presence of COPD, incident non-skin cancer, and level of education.

[c] MSM: exposure and adjustment is the same as for the first time-dependent Cox regression model. All observations are weighted by the inverse of the conditional probability of the observed status of statin use, based on variables that are considered to influence the initiation of statins: age, sex, smoking status, systolic blood pressure, total cholesterol, BMI, level of education, incident non-skin cancer, COPD, and use of blood pressure lowering medication.

[d] Ever use of statins versus never use of statins at the occurrence of a cardiovascular event.

[e] Use of statins divided in categories based on the duration of cumulative use: first month of use of statins, cumulative use of statins equals more than 31 days to 1 year, cumulative use of statins for more than 1 year.

Bold value indicates statistically significant association.

CI 0.67; 1.64). When we divided statin use into categories, use of statins for more than 1 year was significantly associated with a lower risk of CVD (HR 0.70, 95% CI 0.15; 0.99). Use of statins in the first month after initiation and between 31 days and 1 year after start was significantly associated with a higher risk of CVD, with HRs of 4.36 (95% CI 1.17; 16.28) and 2.47 (95% CI 1.25; 4.87), respectively (Table 4). After exclusion of observations with extreme weights, results from the MSM analyses did not markedly change, albeit

Table 4 Cox regression and MSM analyses for the association between statin use and the occurrence of atherosclerotic CVD (extended endpoint including arterial revascularization procedures)

	N_{cases}	pys	I. First time-dependent Cox regression model[a] HR (95% CI; P-value)	II. Second time-dependent Cox regression model[b] HR (95% CI; P-value)	III. MSM[c] model HR (95% CI; P-value)
Ever use versus never use[d]					
Never use	586	40,326	1.00 (ref)	1.00 (ref)	1.00 (ref)
Ever use	69	3,739	1.19 (0.89; 1.61, 0.241)	1.10 (0.84; 1.45, 0.499)	1.05 (0.67; 1.64, 0.836)
Duration of use[e]					
Never use	586	40,326	1.00 (ref)	1.00 (ref)	1.00 (ref)
1-30 days use	7	201	**3.73 (1.51; 9.21, 0.004)**	**3.11 (1.24; 7.76, 0.015)**	**4.36 (1.17; 16.28, 0.028)**
31-365 days use	26	825	**2.82 (1.73; 4.61, <0.0001)**	**2.47 (1.52; 4.01, <0.001)**	**2.47 (1.25; 4.87, 0.009)**
≥366 days use	36	2,714	0.86 (0.59; 1.27, 0.452)	0.79 (0.55; 1.14, 0.205)	**0.70 (0.15; 0.99, 0.047)**

We used pooled logistic regression models using generalized estimating equations with each person-month of follow-up as a separate observation. This was done to emulate time-dependent Cox proportional hazards models with the ability to incorporate time-dependent weighting of the observations.[15,16]
'Extended' atherosclerotic CVD is a composite endpoint of fatal and nonfatal myocardial infarction, stroke, atherosclerotic CVD death, and arterial revascularization procedures defined as surgical or percutaneous coronary revascularization, abdominal aortic surgery, or carotid revascularization.
Abbreviations: MSM, marginal structural model; CVD, cardiovascular disease; N_{cases}, number of CVD cases; pys, person-years of observed follow-up; HR, hazard ratio; CI, confidence interval; ref, reference; BMI, body mass index; COPD, chronic obstructive pulmonary disease.
[a] First time-dependent Cox regression model: statin use during follow-up as time-dependent variable; adjusted for the baseline values age, sex, BMI, smoking status, systolic blood pressure, total cholesterol, use of blood pressure lowering drugs, presence of COPD, incident non-skin cancer, and level of education.
[b] Second time-dependent Cox regression model: statin use during follow-up as time-dependent variable; adjusted for the updated values of BMI, smoking status, systolic blood pressure, total cholesterol level (updated at every monthly interval to the most recent visit of the Rotterdam Study) and adjusted for age, sex, use of blood pressure lowering drugs at baseline, presence of COPD, incident non-skin cancer, and level of education.
[c] MSM: exposure and adjustment is the same as for the first time-dependent Cox regression model. All observations are weighted by the inverse of the conditional probability of the observed status of statin use, based on variables that are considered to influence the initiation of statins: age, sex, smoking status, systolic blood pressure, total cholesterol, BMI, level of education, incident non-skin cancer, COPD, and use of blood pressure lowering medication.
[d] Ever use of statins versus never use of statins at the occurrence of a cardiovascular event.
[e] Use of statins divided in categories based on the duration of cumulative use: first month of use of statins, cumulative use of statins equals more than 31 days to 1 year, cumulative use of statins for more than 1 year.
Bold value indicates statistically significant association.

the HRs for the extended endpoint in the 1–30 days and 31–365 days of use categories were no longer statistically significant (data not shown).

DISCUSSION

In this study, we compared MSM and traditional Cox regression models using empirical data on time-varying statin use in the primary prevention of CVD as an example. We investigated whether the use of MSM to estimate the causal effect of time-dependent drug use in the presence of time-dependent confounders, produces different relative risk estimates from traditional Cox proportional hazards models with and without time-dependent covariates. In general, we could not demonstrate important differences between these different analyses.

Marginal structural modeling of time-dependent drug use

Efficacy of drugs is tested in double-blind randomized clinical trials. However, the homogeneous characteristics of patients in clinical trials differ substantially from those of patients in a real-life setting.[21-24] Therefore, effectiveness in a real-life setting requires observational studies, ideally in unselected populations. The chance of confounding by (contra) indication in observational studies is well-recognized and generally dealt with by adjustment for the (contra) indication.[25] Hernán et al.[17] pointed out that such adjustment may be flawed in the presence of time-varying confounders that are influenced by the drug under study. As a potential remedy, they proposed a model to adjust for time-dependent confounding in observational studies by weighting of the observations with the inverse conditional probability of receiving their observed treatment.[4,17]

Marginal structural modeling resembles the use of propensity scores in which the baseline probability of being treated is used to deal with potential confounding by indication by matching, stratification, or adjustment on score at baseline.[26] On theoretical grounds, MSM is a more valid approach than the use of baseline propensity scores, since MSM allows for taking into account time-dependent confounders that are affected by prior treatment.

However, propensity scores are increasingly used in observational (comparative) effectiveness research[27], whereas MSM is still less frequently encountered in pharmacoepidemiological literature.[28,29]

In 2000, Hernán et al.[17] published a seminal study on the use of MSM in studying causal effects of drugs in observational data. They investigated the effect of zidovudine on survival 0f human immunodeficiency virus (HIV)-positive men using data from an observational cohort study. They demonstrated an important difference between the unadjusted model and the weighted MSM with adjustment for time-dependent confounders. In the unadjusted model, zidovudine was spuriously associated with an increased risk of death, while the weighted MSM showed a significantly reduced mortality rate ratio for zidovudine. In our study, a 32% CVD risk reduction with the Cox model with time-dependent statin use decreased to 11% with MSM. The study by Hernán et al.

differs from our study on several aspects. First, they investigated treatment by specialists for a disease entity with a very strictly defined indication for initiation of zidovudine, namely $CD4^+$ lymphocyte count in the case of HIV positivity[30], while in our study the indication to start statins for primary prevention of CVD is far less stringent and prescribing of statins is mostly initiated by general practitioners on the basis of the global cardiovascular risk profile and expected merits of treatment.[12,13] Second, once a patient with HIV starts on zidovudine, this will generally be continued indefinitely since continued treatment is necessary for survival. Opposite, statins can be used intermittently and are far less crucial for survival, and may therefore be more frequently discontinued because of non-adherence to therapy or perceived adverse reactions such as myalgia. Discontinuation frequently occurs in the case of statin therapy, a previous study demonstrated that 53 % of new users of statins discontinued within 2 years.[31]

In line with our results, previous MSM studies on statin effectiveness in observational data reported small differences between the unweighted risk estimates and the weighted risk estimates.[32,33] A recent literature review of application of MSMs in pharmacoepidemiology summarized discrepancies between HRs from conventional regression analysis and MSM.[28] Of the 14 studies that directly compared the HRs from different regression techniques, only six studies concluded that the MSM results differed from the conventional analysis. Another recent literature review on the use of MSM estimated that in only 40 % of the studies the effect estimates from MSMs materially differed from results from conventional models.[29] In all, this indicates that there is often insufficient information on the time-dependent confounding of treatment, or that the influence of time-varying confounding is small.[4,17]

Confounding by indication of statin treatment

Current guidelines on prevention of CVD recommend clinicians to start statins in persons without established CVD, but with a high global absolute CVD risk.[13,19] These recommendations are based on large randomized clinical trials that demonstrate a 25% risk reduction on the occurrence of major cardiovascular endpoints and 14% risk reduction in all-cause mortality in persons free of CVD.[34] There is no substitute for randomized clinical trials since proper randomization minimizes the risk of confounding in such studies.[35] This was recently confirmed by Danaei et al.[32] who emulated a hypothetical randomized trial using observational data. They demonstrated that there was substantial confounding by indication for statins in primary prevention of CVD. In line with our results, the HRs during the initial months after statin initiation suggest a non-beneficial effect of statin use on CVD endpoints. This seems to indicate that there was still substantial unmeasured confounding by indication which was not accounted for, such as due to symptoms of angina.

Limitations

Potential limitations of our study should be considered. First, we did not observe a large difference in effect estimates between the results from the Cox model without time-dependent covariates (model I) and the Cox model with time-dependent covariates (model II). A possible explanation for this could be that the changes in cardiovascular risk factors and other potential confounders we evaluated do not materially relate to the probability of initiating statin treatment, i.e. for our particular research question no or only a limited degree of time-dependent confounding by indication was present. The observed increased risk in the category 1–30 days of statin use also argues that other factors may be of greater importance for statin prescription in everyday clinical practice, besides measurements of established cardiovascular risk factors. Therefore, other time-dependent predictors, such as more detailed measures of the burden of co-morbidity and frailty or symptoms like angina or intermittent claudication, may be more appropriate indicators of both statin initiation and subsequent prognosis. Also, information on the confounders was updated at 4–5 year time intervals during follow-up visits of the Rotterdam Study. As a consequence, some participants had only a small number of updates on the time-dependent covariates. A density plot of the weights used in model III showed a steep curve, with a 5th and 95th percentile of 0.824 and 1.194, respectively (Fig. 1), indicating that the weights carried a limited amount of information. Next, in the MSM analysis we made an attempt to model weights for statin initiation. Since use of statins is frequently discontinued it would be useful to also compute weights for discontinuation of treatment to more adequately deal with potential confounding by contra-indication. Furthermore, we conducted an 'intention to treat' analysis because information on determinants for statin discontinuation, such as adverse drug reactions or patient preferences are not available in the Rotterdam Study. This may have affected our estimates given that one third of the participants in our study who started statins discontinued treatment during follow-up (71.1 % coverage of exposed person-time). Last, we did not take into account the differences in equivalent statin dose used or changes in dosage over time.[36]

Conclusions

Although, on theoretical grounds, MSM is an elegant statistical technique to adjust for time-dependent confounding by indication, the absence of knowledge about detailed confounder status on a daily basis may be a hurdle to the use of MSM in real-life population-based cohort studies. Even if drug use is registered on a daily basis, the absence of data on time-dependent confounders, such as the precise prescription indication or other treatment considerations jeopardizes the calculation of the actual valid weights. Confounding by indication remains a hurdle in observational effectiveness research on preventive drugs with a multitude of prescription determinants.

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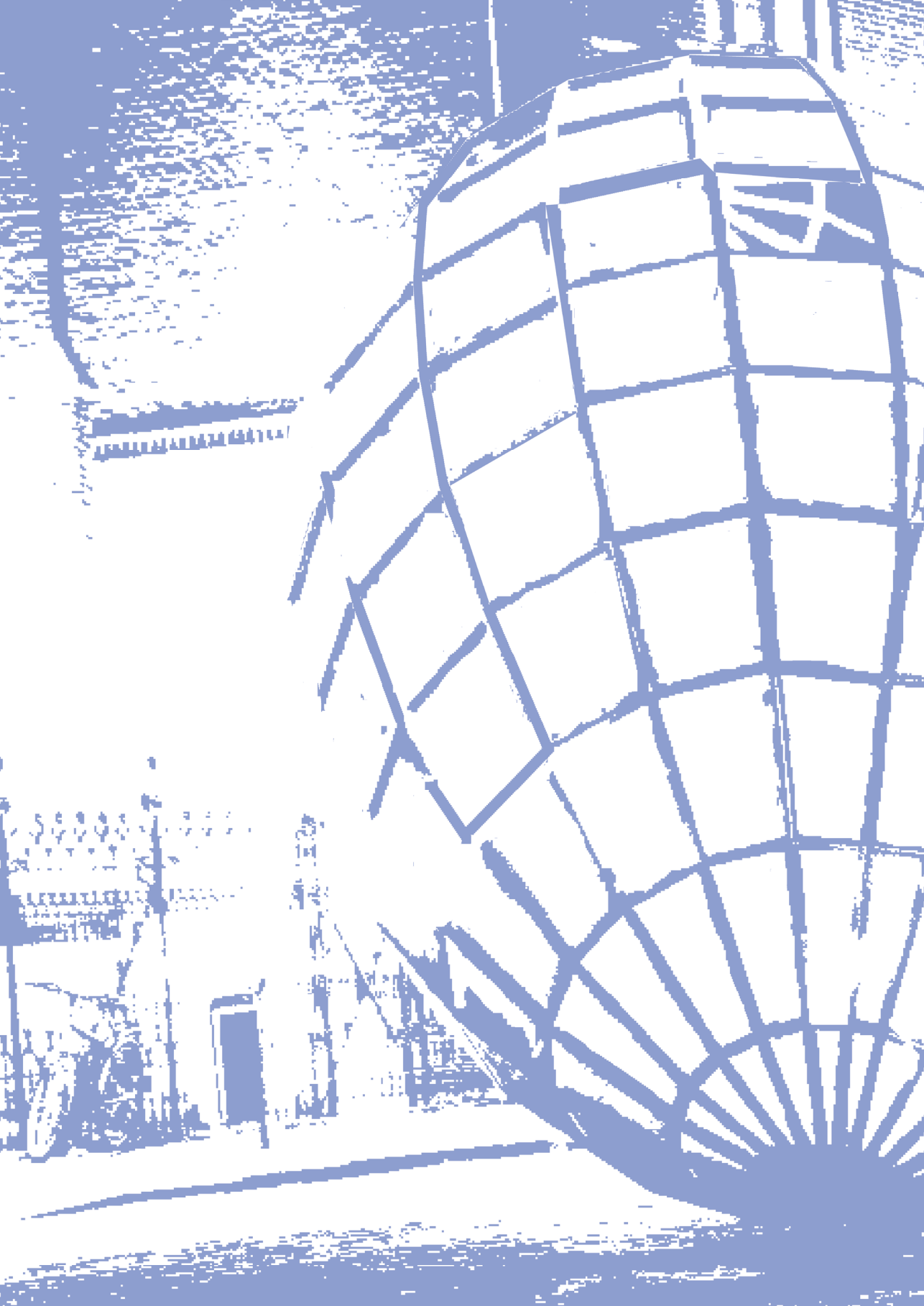

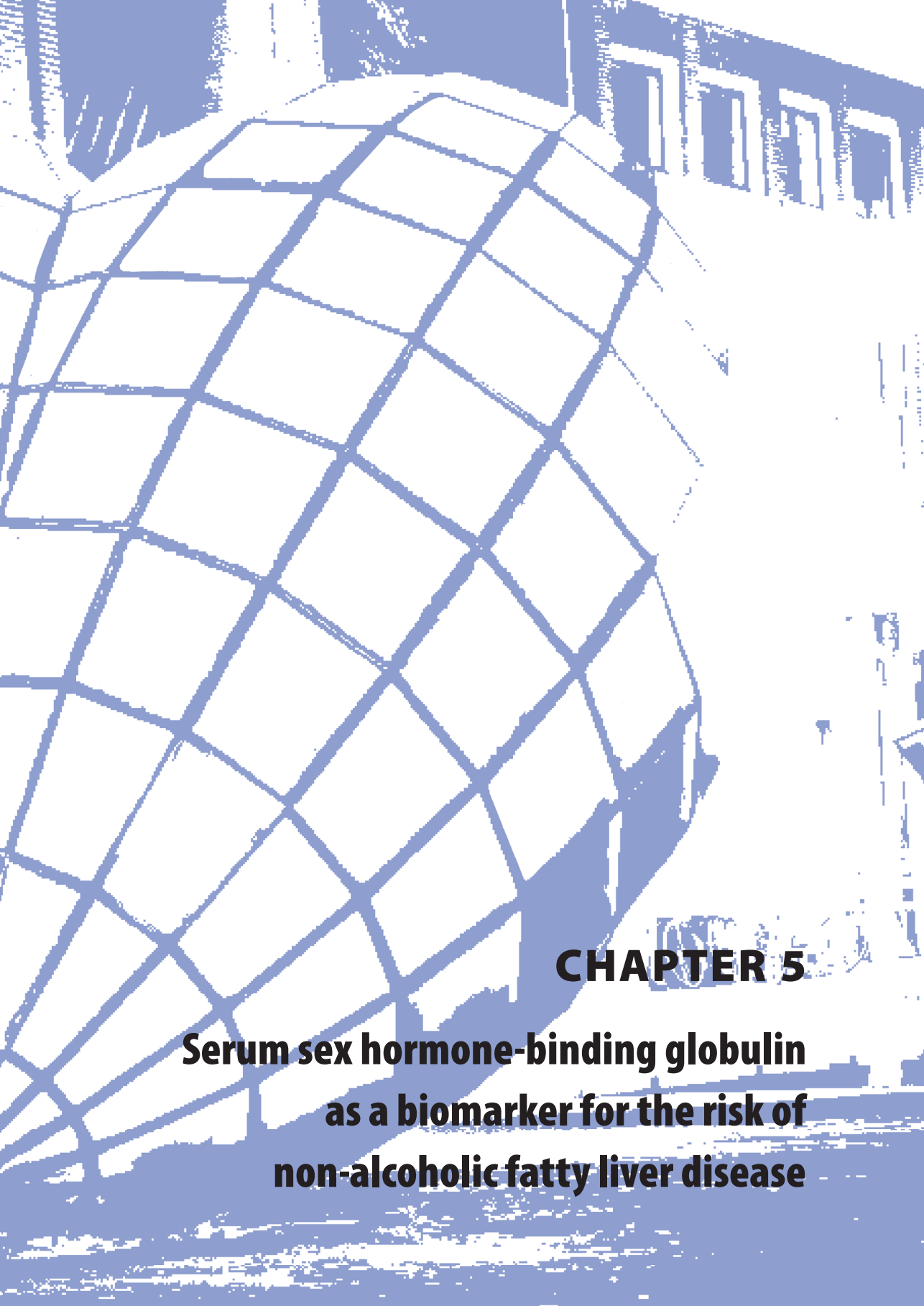

CHAPTER 5

Serum sex hormone-binding globulin as a biomarker for the risk of non-alcoholic fatty liver disease

5.1

Higher serum SHBG levels at baseline are associated with a lower incidence of NALFD during follow-up: the Rotterdam Study

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Submitted

ABSTRACT

Introduction: Non-alcoholic fatty liver disease (NAFLD), or hepatic steatosis, is considered as the hepatic manifestation of the metabolic syndrome. Previous cross-sectional studies suggested an association between higher serum sex hormone-binding globulin (SHBG) levels and a lower prevalence of NAFLD. Our aim was to investigate whether serum SHBG levels, measured in a population with a very low probability of steatosis at baseline, were associated with the development of NAFLD during follow-up.

Methods: In the population-based Rotterdam Study, 632 men and 1132 women had a fatty liver index (FLI) below 60 (very low probability of steatosis) and a serum SHBG measurement available at baseline, and underwent liver ultrasonography after a mean follow-up period of 11 years. In multivariable logistic regression analysis, we investigated the association between SHBG levels in nmol/L and NAFLD incidence. In an additional analysis, we selected participants with a FLI<30 (no steatosis).

Results: In men and women with FLI<60, higher baseline SHBG levels were significantly associated with a lower incidence of NAFLD during follow-up, after adjustment for total testosterone level and other co-variables. The odds ratio per SD increase in SHBG was 0.64 (95%CI 0.46; 0.89) in men and 0.78 (95%CI 0.64; 0.94) in women. Analyses in participants with FLI<30 showed similar effect estimates, although non-significant.

Conclusion: Higher SHBG levels were independently associated with a lower risk of developing NAFLD after around 11 years follow-up. This study showed evidence that SHBG might be an early biomarker for the development of NAFLD.

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) or hepatic steatosis is a condition in which fat is accumulated in hepatocytes. It encompasses a spectrum of disease activity, ranging from simple hepatic steatosis to non-alcoholic steatohepatitis (NASH) and NASH cirrhosis, which may finally lead to a decreased liver function, liver failure, and hepatocellular carcinoma.[1-3] NAFLD is considered the hepatic manifestation of the metabolic syndrome and its prevalence increases, especially in developed countries.[1,4] NAFLD is frequently associated with dyslipidemia, obesity, and type 2 diabetes mellitus (T2DM), and has been associated with an increased risk of incident cardiovascular disease (CVD), independently of the components of the metabolic syndrome.[1-3,5,6] The pathogenesis of NAFLD is not completely clear, but insulin resistance, hyperlipidemia and inflammation are considered to play a major role in NAFLD development.[1,7,8]

Sex hormone-binding globulin (SHBG) is a glycoprotein that is mostly produced in the liver.[9] Serum SHBG binds to sex steroid hormones, regulates the serum concentration of these circulating hormones and their transport to target tissues. SHBG has a high binding affinity for testosterone and the serum concentrations of total testosterone and SHBG are strongly correlated.[10-12] Previous studies have shown that low serum SHBG levels were associated with the metabolic syndrome, insulin resistance and T2DM, and the risk of incident CVD.[13-16] Furthermore, low serum SHBG levels were associated with an increased prevalence of NAFLD in previous cross-sectional studies.[17-20]

Serum testosterone levels are also linked to obesity, insulin resistance, and the metabolic syndrome[15,16,21], and previous studies also suggested an association between serum testosterone levels and NAFLD.[19,20,22-24] Whether serum SHBG levels contribute to the development of NAFLD, and whether this effect is independent of testosterone, is unclear. Studies on this association were relatively small, were performed cross-sectionally, and further longitudinal research is needed before conclusions on the role of SHBG in the development of NAFLD can be drawn.

In this large prospective population-based cohort study, the aim was to investigate whether higher serum SHBG levels at baseline were associated with a lower risk of developing NAFLD during follow-up, independent of testosterone levels. Thereby, we aimed to investigated whether SHBG levels could serve as a biomarker for the development of NAFLD.

METHODS

Setting

The current study was performed within the Rotterdam Study, a prospective population-based cohort study that aims to study the frequency and determinants of diseases in the middle-aged and elderly people. The rationale and design of the Rotterdam Study have been described in detail previously.[25,26] In short, all 10,275 persons aged 55 years and over in the Ommoord district of Rotterdam, the Netherlands, were invited to participate. Of them, 7,983 (response rate 78%) were enrolled between 1990 and 1993 (RS-1). At baseline, all participants were interviewed at home and underwent extensive clinical examination at the research center. Additional re-examinations took place in 1993-1995, 1997-1999, 2002-2004, and 2009-2012. In 2000, an extended cohort was enrolled, when 3011 inhabitants (response rate 67%) aged 55 years and over entered the study (RS-II). The Rotterdam Study has been approved by the medical ethics committee according to the Wet Bevolkingsonderzoek: ERGO (Population Screening Act: Rotterdam Study), executed by the Ministry of Health, Welfare and Sports of the Netherlands. All participants gave informed consent to participate in the study and to obtain information from treating physicians and pharmacy records, separately.

Study sample

We aimed to investigate whether serum SHBG levels could serve as an early biomarker for the development of NAFLD. Therefore, we created a longitudinal component and selected a population with a very low probability of steatosis at baseline (as measured by a Fatty Liver Index [FLI] of <60[27]), with a SHBG measurement available at baseline, and investigated whether these participants developed NAFLD after years of follow-up (*Figure 1*). In the Rotterdam Study data for calculation of the FLI were available from the third visit of the first cohort (RSI-3, March 1997 – December 1999) and first visit of the second cohort (RSII-1, February 2000 – December 2001) onwards. These visits constitute the baseline survey for the current study (*Figure 1*). The FLI is a noninvasive measure to predict hepatic steatosis and was calculated according to the formula by Bedogni et al.[27], including triglycerides, body mass index (BMI), gamma-glutamyltransferase (GGT), and waist circumference. For every participant, a FLI at baseline was calculated. According to Bedogni et al., a FLI≥60 can be used to rule in hepatic steatosis, and a FLI<30 can be used to rule out hepatic steatosis. In our previous study using Rotterdam Study data, we have validated the use of FLI to predict NAFLD as measured by ultrasound with an area under the receiver operating characteristic curve of 0.81, a sensitivity of 92% and 64% and a specificity of 49% and 82% when using a cut-off of FLI >60 and <30, respectively.[28] Hence, participants with a FLI<60 were included in the study population, to create a study population with a very low probability of having hepatic steatosis at

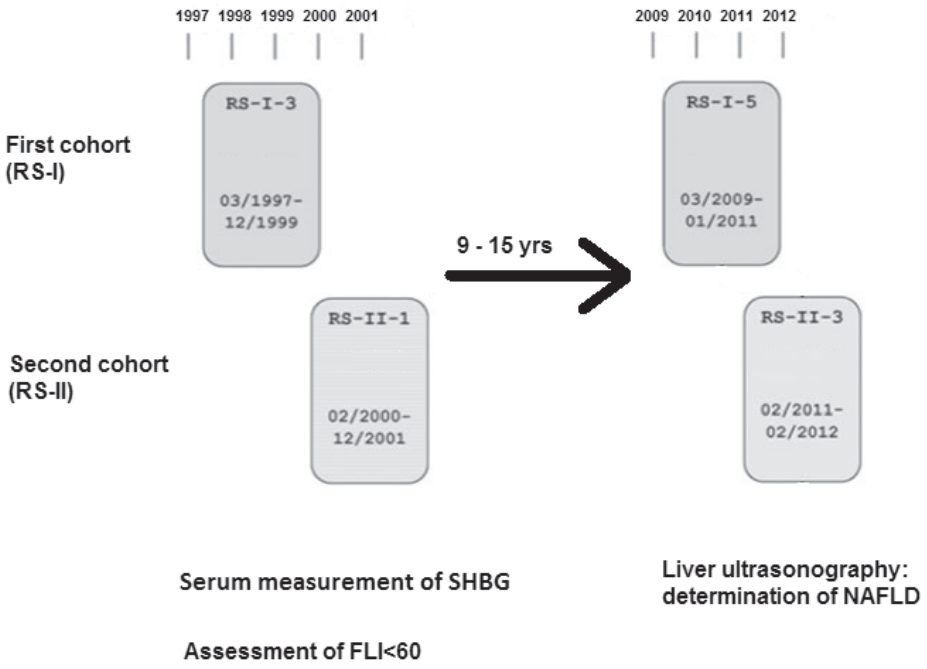

Figure 1 Overview of the assessment of the exposure and the outcome during follow-up of the Rotterdam Study
Abbreviations: RS, Rotterdam Study; SHBG, sex hormone-binding globulin; NAFLD, non-alcoholic fatty liver disease

baseline. In a second analysis, we selected only participants with no steatosis (FLI<30) at baseline.

Exposure assessment

The exposure of interest was serum SHBG level. At baseline of this study during the center visit (RSI-3, RSII-1), fasting blood samples were collected. Serum SHBG levels were measured using double antibody radioimmunoassays (Diagnostic Systems Laboratories, Inc., Webster, TX) and expressed in nmol/L.

Outcome assessment

The outcome of interest was NAFLD, assessed by abdominal ultrasonography in all study participants. Abdominal ultrasonography was added to the core protocol at the fifth survey of the first cohort and the third survey of the second cohort of the Rotterdam Study (RS-I-5, February 2009 – January 2011; RS-II-3, February 2011 – February 2012) (*Figure 1*). Abdominal ultrasonography was performed by certified and experienced ultrasonographists on a Hitachi HI VISION 900. Images were stored digitally and re-

evaluated by a hepatologist with more than ten years experience in liver ultrasonography. The diagnosis and grading of fatty liver was determined according to the protocol by Hamaguchi et al.[29] Severity of fatty liver was classified as 'no fatty liver' (score 0-1), 'mild fatty liver' (score 2-3), or 'moderate to severe fatty liver' (score 4-6). Individuals with any of the following possible secondary causes of fatty liver were excluded from the analyses: 1) current excessive (i.e. more than 14 drinks per week) alcohol consumption or a history of excessive alcohol consumption, 2) positive HBsAg or anti-HCV, and 3) use of pharmacological agents associated with fatty liver (i.e. amiodarone, corticosteroid, methotrexate, and tamoxifen).

Medication prescription data were obtained from all seven fully computerized pharmacies in the Ommoord district. Information of all filled prescriptions from January 1st 1991 until January 31st 2012 was available and included information of the product name of the drug, the Anatomical Therapeutical Chemical Code (ATC-code), the amount dispensed, the prescribed dosage regimen and the date of dispensing.[30]

Covariables

To control for confounding, we adjusted for the following baseline variables: age; sex; BMI; waist circumference; number of alcoholic drinks weekly; hypertension or use of blood pressure lowering drugs; diabetes mellitus; Insulin resistance, as assessed by the Homeostasis Model of Assessment – Insulin Resistance (HOMA-IR); history of CVD; use of statins; serum levels of total testosterone, estradiol, dehydroepiandrosterone sulfate (DHEAS), high-density lipoprotein (HDL)-cholesterol, and triglycerides. Furthermore, we adjusted for follow-up time, i.e. the time between the date of the SHBG measurement and the date of the abdominal ultrasonography.

Information on covariables was obtained by an interview at home, laboratory measurements, and anthropometric assessments at the research center. The interview was designed to obtain data concerning demographics, medical history, co-morbid conditions, smoking behaviour, physical activity, and alcohol consumption. Anthropometric measurements were performed by well-trained research assistants. Waist circumference was measured in centimeters. BMI was calculated as the weight (in kg) divided by height (in m^2). The average of two blood pressure measurements, obtained at a single visit in sitting position after a minimum of 5 minutes rest, was used for analysis. A blood pressure below 130/95 mmHg was defined as hypertension. The HOMA-IR was calculated as: fasting glucose (mmol/L) x fasting insulin (mU/L)/22.5.[31] Blood lipids (HDL, total cholesterol, triglycerides), serum alanine aminotransferase (ALT), and GGT were measured using automatic enzymatic procedures (Roche Diagnostics GmbH, Mannheim, DE). Low-density lipoprotein (LDL)-cholesterol was calculated with the Friedewald formula, using total cholesterol, HDL-cholesterol and triglycerides (<4.5 mmol/L).[32] HbsAg and anti-HCV antibodies were measured by automatic immunoassay (Roche

Diagnostics GmbH, Mannheim, DE). Serum levels of total testosterone, estradiol, and DHEAS were measured using radioimmunoassays (Diagnostic Systems Laboratories, Inc., Webster, TX). Serum blood measurements were measured at the same date of serum SHBG measurement. CVD in history was defined as a myocardial infarction (MI), percutaneous transluminal coronary angioplasty (PTCA), coronary artery bypass grafting (CABG), heart failure, carotid desobstruction, cerebrovascular accident (CVA), or transient ischemic attack (TIA) in the history.[33-35] Diabetes mellitus developed before the date of liver ultrasonography was defined as a dispensing of ATC code A10, oral glucose lowering medication and insulin, at baseline. Use of blood pressure lowering medication was defined as a dispensing of ATC codes C02 (miscellaneous antihypertensives), C03 (diuretics), C07 (β-blockers), C08 (calcium channel blockers) or C09 (rennin-angiotensin system modifying agents) at baseline. Use of statins was defined as a dispensing of ATC code C10AA or C10B at baseline.

Statistical analysis

Baseline characteristics of the study population were stratified by sex, because normal values for serum SHBG differ between men and women.

We used multivariable logistic regression models, with relative risks expressed as odds ratios (ORs), to investigate the association between serum SHBG levels and the risk of developing NAFLD (yes/no), and adjusted for all potential confounding covariables. All analyses were stratified by sex. SHBG levels were expressed in sex-specific standard deviations (SDs). We investigated the association between an increase in serum SHBG levels and the incidence of NAFLD during follow-up. This is a cumulative incidence over a mean follow-up period of 11 years under the assumption that participants have a very low probability of steatosis at baseline. The cumulative incidence was calculated as the number of NAFLD cases that occurred during follow-up divided by the number of participants at risk at baseline. First, participants with a FLI<60 at baseline were investigated on the risk of NAFLD during follow-up. Subsequently, this association was investigated in an additional analysis in participants with a FLI<30 at baseline. In a sensitivity analysis, we excluded participants with mild steatosis on ultrasonography, since ultrasonography is less sensitive for the detection of mild steatosis. We investigated the association between serum SHBG levels and 'moderate to severe fatty liver' vs no fatty liver, using multivariable logistic regression analyses.

All analyses were performed complete case, using SPSS software (SPSS Inc., version 21.0, Chicago, Illinois, USA).

RESULTS

In total, we identified 632 men and 1,132 women with a FLI<60 and a serum SHBG measurement at baseline, who underwent ultrasonography during follow-up. Baseline characteristics of the study population are shown in Table 1. Compared to women, men had a significantly lower BMI but higher waist circumference, more frequently a history of CVD, consumed more alcoholic drinks weekly, and used more frequently statins (P<0.05). Furthermore, men had a significantly lower total and HDL cholesterol levels, and higher serum levels of ALT and GGT, compared to women (P<0.05)

The mean follow-up time between the SHBG measurement at baseline and the NAFLD ultrasonography during follow-up was 11 years (minimum 9 years, maximum 15 years) (*Figure 1*). The incidence of NAFLD was 23.6% for men and 26.1% for women.

Serum SHBG levels at baseline were significantly lower in NAFLD patients than in non-NAFLD patients, both for men (mean 44.0 [SD 15.3] vs mean 52.3 [SD 19.3] respectively,

Table 1 Baseline characteristics of the present study

Characteristic	Men (n = 632)	Women (n = 1132)
Age (mean, years)	65.6 (5.9)	65.2 (6.0)
Body mass index (mean±SD, kg/m^2)	24.9 (2.1)	25.5 (3.0)
Waist circumference (mean±SD, cm)	91.9 (6.5)	84.6 (8.4)
Hypertension (n, %)	596 (94.3%)	1051 (92.8%)
Cardiovascular disease (n, %)	126 (19.9%)	109 (9.6%)
Diabetes Mellitus (n, %)	39 (6.1%	69 (6.0%)
HOMA-IR (mean±SD)	2.8 (2.1)	2.9 (3.0)
Alcohol consumption (drinks/week)	6.2 (6.2)	3.8 (4.6)
Use of statins (n, %)	141 (22.3%)	206 (18.2%)
Serum total cholesterol (mean±SD, mmol/L	5.0 (1.0)	5.7 (1.0)
Serum HDL cholesterol (mean±SD, mmol/L	1.4 (0.4)	1.7 (0.4)
Serum LDL cholesterol (mean±SD, mmol/L)	3.1 (0.9)	3.5 (1.0)
Serum triglycerides (mean±SD, mmol/L)	1.2 (0.5)	1.2 (0.6)
Serum ALT (mean±SD, mmol/L)	20.6 (0.4)	18.9 (10.3)
Serum GGT (mean±, mmol/L)	30.6 (29.0)	25.7 (26.4)
Serum fasting glucose (mean±SD, mmol/L)	5.7 (0.9)	5.6 (1.1)
Serum fasting insulin (mean±SD, pmol/L)	63.8 (54.1)	64.3 (34.9)
Serum total testosterone (mean±SD, nmol/L)	18.8 (5.7)	0.9 (1.1)
Serum estradiol (mean±SD, pmol/L)	105.9 (37.2)	49.5 (63.1)
Serum DHEAS (mean±SD, nmol/L)	3016.7 (1591.3)	1865.7 (1075.4)

Abbreviations: SD, standard deviation; HOMA-IR, Homeostasis Model of Assessment – Insulin Resistance; HDL, high-density lipoprotein; LDL, low-density lipoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma-glutamyltransferase; DHEAS, dehydroepiandrosterone sulfate.

P 1.3×10^{-7}) and women (mean 57.5 [SD 28.4] vs mean 73.5 [SD 31.4] respectively, P 3.0×10^{-15}). Furthermore, in men, NAFLD patients had statistically significantly lower total testosterone levels at baseline than non-NAFLD patients.

Over a mean follow-up period of 11 years, 152 out of 632 men and 299 out of 1,132 women at risk developed NAFLD, corresponding with a cumulative incidence of 24.1% and 26.4%, respectively. The results of the multivariable logistic regression analysis on the association between serum SHBG levels and the incidence of NAFLD are shown in Table 2. For men, the mean SHBG-level was 50.3 nmol/L (with sex-specific SD 18.7); for women, the mean SHBG level was 69.3 nmol/L (with sex-specific SD 31.4). Adjusted for all covariables, in participants with FLI<60 at baseline, higher SHBG levels were associated with a lower incidence of NAFLD during follow-up. In men, per SD increase in serum SHBG levels the OR for NAFLD was 0.64 (95%CI 0.46; 0.89, P.008). In women, per SD increase in serum SHBG levels the OR for NAFLD was 0.78 (95%CI 0.64; 0.94, P.011).

In a second analysis, we selected 229 men and 642 women with a FLI<30 at baseline. In this population, for men the mean SHBG level was 55.5 nmol/L (sex-specific SD 19.7), and for women the mean SHBG level was 76.9 nmol/L (sex-specific SD 31.0). The results of the multivariable logistic regression analysis between SHBG levels and incidence of NAFLD in participants with a FLI<30 at baseline were similar to the results of the analy-

Table 2 The relative risk of NAFLD per SD increase in serum SHBG levels – stratified by sex

Mean SHBG level and sex-specific SD	Mean (nmol/L)	SD	N
Within participants with FLI<60 at baseline			
Men	50.3	18.7	632
Women	69.3	31.4	1132
Within participants with FLI<30 at baseline			
Men	55.5	19.7	227
Women	76.9	31.0	634
Logistic regression analyses	**OR (95% CI)[a]**	**Beta (SE)**	**P**
Within participants with FLI<60 at baseline			
Men	0.64 (0.46; 0.89)	−0.45 (0.17)	0.008
Women	0.78 (0.64; 0.94)	−0.25(0.10)	0.011
Within participants with FLI<30 at baseline			
Men	0.59 (0.33; 1.07)	−0.52 (0.23)	0.084
Women	0.81 (0.60; 1.07)	−0.22 (0.15)	0.139

Abbreviations: NAFLD, non-alcoholic fatty liver disease; SD, standard deviation; SHBG, sex hormone-binding globulin; N, number of men/women; OR, odds ratio; CI, confidence interval; SE, standard error.

[a] Adjusted for: age; body mass index; waist circumference; number of alcoholic drinks weekly; hypertension; diabetes mellitus; HOMA-IR; history of cardiovascular disease; use of statins; serum levels of total testosterone, estradiol, dehydroepiandrosterone sulfate, high-density lipoprotein cholesterol, triglycerides; time between SHBG measurement and steatosis echo.

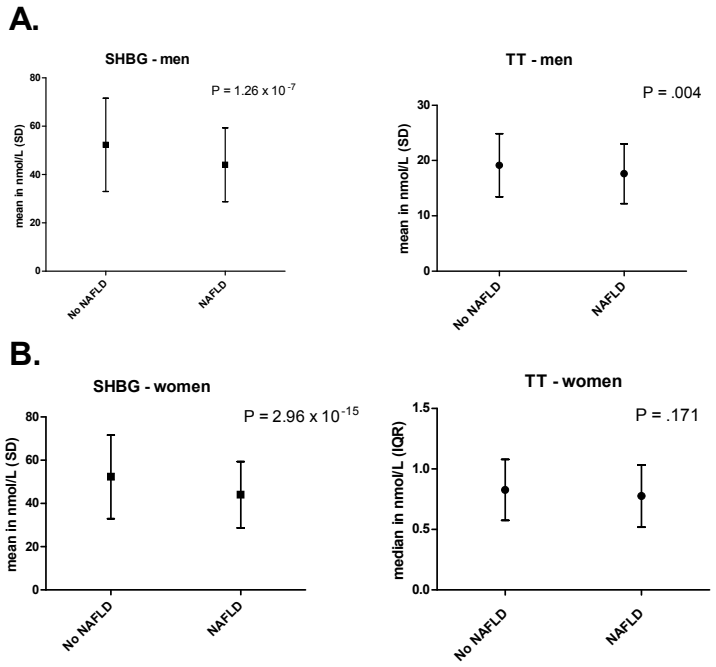

Figure 2 Differences in baseline values of SHBG and total testosterone between patients with and without NAFLD during follow-up

Abbreviations: SD, standard deviation; CI, confidence interval; SHBG, sex hormone-binding globulin; TT, total testosterone.

A: Baseline values in men; B: Baseline values in women.

ses in participants with FLI<60 at baseline, albeit not statistically significant anymore. In men, the OR for NAFLD was 0.59 (95%CI 0.33; 1.07, P.084), and in women the OR for NAFLD was 0.81 (95%CI 0.60; 1.07, P.139) (Table 2).

In a sensitivity analysis, we excluded participants with mild fatty liver at ultrasonography, and investigated the association between serum SHBG levels and incidence of 'moderate to severe fatty liver'. In both men and women, the association between higher SHBG levels and a lower incidence of NAFLD remained present. In men with FLI<60, per SD increase in serum SHBG levels the OR was 0.70 (95%CI 0.50; 0.99, P.043), and in women with FLI<60, per SD increase in serum SHBG levels the OR was 0.72 (95% CI 0.58; 0.90, P.004) (*Results not shown*).

DISCUSSION

In this population-based cohort study, we demonstrated that higher SHBG levels in a population with a very low probability of steatosis at baseline were associated with a

lower risk of developing NAFLD during follow-up. This association was present in both men and women and independent of serum levels of total testosterone.

Previous cross-sectional studies also showed an inverse association between serum SHBG levels and the presence of NAFLD. One study showed that serum SHBG levels were associated with a statistically significantly lower prevalence of biopsy-proven NAFLD in postmenopausal women, after adjustment for age, BMI and waist circumference.[19] Others showed that serum SHBG levels decreased with increasing fatty liver disease severity in type 2 diabetes patients, after adjustment for total testosterone and other confounding factors.[17] Hua and colleagues showed that low serum SHBG levels were associated with NAFLD after adjustment for total testosterone and other covariables in patients with type 2 diabetes mellitus.[18] Other studies also showed an inverse association between serum SHBG levels and hepatic steatosis[20] and amount of liver fat.[36,37]

A role of SHBG in the development of NAFLD has been discussed in literature. However, a convincing explanation has not been revealed yet, and possibly SHBG is not more than a biomarker for the development of NAFLD. The pathogenesis of NAFLD is currently not completely clarified, but insulin resistance, hyperlipidemia, and inflammation play a role. Insulin resistance leads to accumulation of fat in hepatocytes through both lipolysis and hyperinsulinemia.[1,38] Hepatic lipogenesis and the accumulation of lipids in the hepatocytes, especially triglycerides, is essential for the development of NAFLD.[1,7,8,39] A previous study demonstrated that a decrease in liver fat, independent from a change in total body fat and visceral adiposity, was associated with an increase in SHBG levels during a lifestyle intervention[37], and another study found an association between increased intrahepatic fat and decreased SHBG levels.[40] Furthermore, a study showed that monosaccharides (fructose, glucose) induced hepatic lipogenesis, and this subsequently reduced hepatic *SHBG* gene expression and SHBG production.[41] Otherwise, insulin has shown to inhibit SHBG production in the liver in vivo and vitro.[42,43] Insulin resistance with hyperinsulinemia is often present in conditions such as the metabolic syndrome, type 2 diabetes mellitus, and obesity.[1,8,44] Both hepatic lipogenesis and increased insulin levels could thus affect the hepatic SHBG production, and subsequently decrease serum SHBG levels. In line with this, in our study serum SHBG levels were negatively correlated with triglycerides levels, positively correlated with HDL cholesterol, and negatively correlated with fasting insulin and the HOMA-IR.

The association between serum SHBG levels and the presence of NAFLD has only been investigated in cross-sectional studies, but in this study type reverse causation cannot be excluded, and it is difficult to draw firm conclusions about temporal associations.[17-19] In our analyses, we created a longitudinal component by only including those participants who had a FLI<60 at the moment of the baseline serum measurements. Abdominal ultrasonography for detection of NAFLD was performed 9-15 years after the serum measurement (*Figure 1*). This enabled us to study the effect longitudinally, since

there was a mean period of 11 years in which participants with initially a low probability of having steatosis based on the FLI (FLI<60, according to Bedogni et al.[27]) could develop steatosis on abdominal ultrasonography. Analyses in participants with FLI<30 at baseline showed similar estimates as for the association in participants with FLI<60, albeit non-significantly probably due to low numbers. Our results suggest that a low SHBG level can be considered as an early biomarker for the development of NAFLD. Although no steatosis could be established according to the FLI, a measure which has shown to agree with steatosis detection with SteatoTest and abdominal ultrasound[45], the serum levels of SHBG have already decreased significantly. The association between SHBG and NAFLD remained present after adjustment for testosterone, estradiol, and other hormones. This indicates that especially SHBG could serve as a biomarker for later NAFLD development. Furthermore, another advantage of the current study compared to other studies on this topic, is that we included 1764 participants in the analysis, while other studies on this topic were performed in smaller populations (40 to 279 participants).

However, also some potential biases and limitations in our study should be considered. The risk of information bias or selection bias is unlikely, since the Rotterdam Study is a population-based cohort study, in which data are collected prospectively without prior knowledge of the research hypothesis in this study. We adjusted for potential confounding factors which could interfere in the association between SHBG levels and the incidence of NAFLD, such as BMI and serum triglycerides levels. The change in these factors during follow-up was not significantly different for the participants that developed steatosis compared to those that did not develop steatosis. The diagnosis and severity of NAFLD was assessed by abdominal ultrasonography. Ultrasonography may be less sensitive than more advanced imaging techniques such as CT/MRI, since ultrasonography is not appropriate for the detection of less than 30 percent steatosis. However, Hernaez et al.[46] showed that ultrasonography is comparable with other imaging modalities in the detection of NAFLD with an acceptable sensitivity of 80-100%. Furthermore, in a sensitivity analysis in patients with moderate to severe fatty liver, the investigated association remained statistically significant present in both men and women. Unfortunately no pathology was available in this population-based study, and therefore we could not investigate the effect of serum SHBG levels on hepatic histology. Only one study on this topic was performed in 22 biopsy-proven NAFLD patients and demonstrated an inverse association between serum SHBG levels and biopsy-proven NAFLD.[19]

In conclusion, in this large prospective population-based cohort study, higher serum levels of SHBG were independently associated with a lower risk of developing NAFLD after more than ten years follow-up. This study demonstrates evidence that SHBG might be an early biomarker for the development of NAFLD, however, future longitudinal studies are needed to determine whether SHBG could indeed be used in clinical practice as a sensitive predictor of NAFLD.

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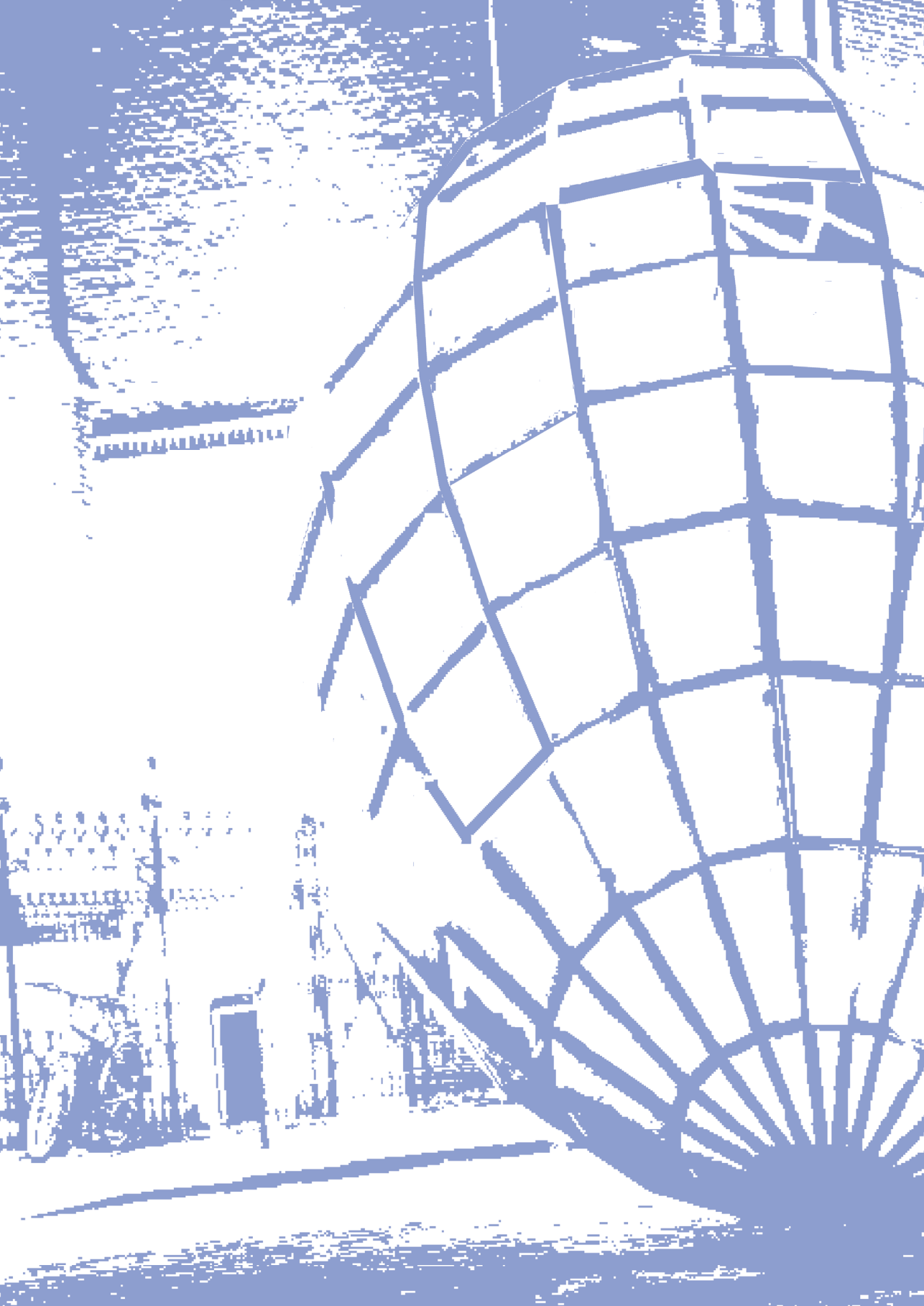

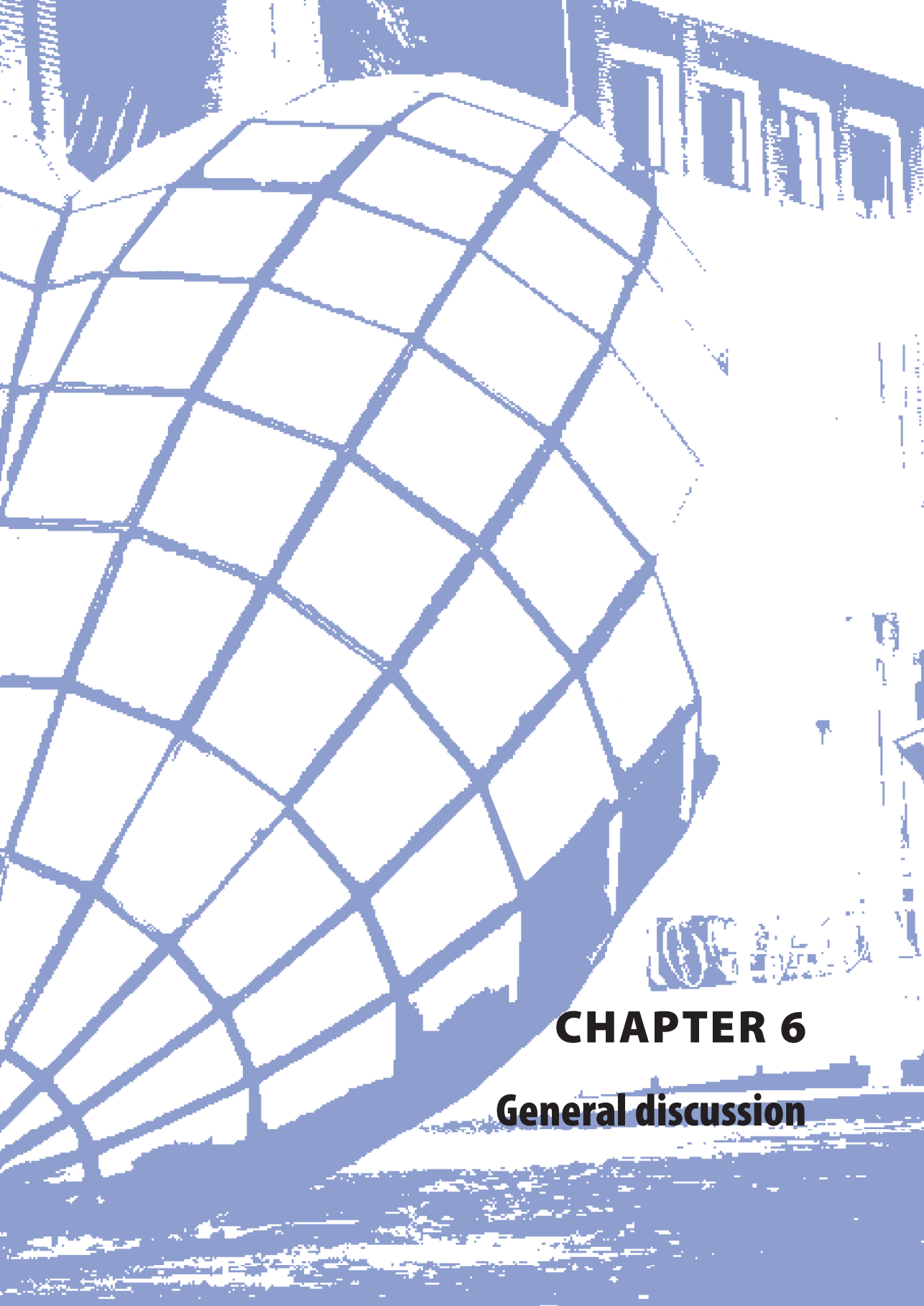

CHAPTER 6

General discussion

INTRODUCTION

Worldwide, cardiovascular disease (CVD) is a public health challenge, and therefore a large focus is on prevention and better treatment of CVD.[1-4] Cholesterol-lowering statins have proven to be beneficial in the primary and secondary prevention of CVD.[5-7] They have a broad spectrum of indications, ranging from hypercholesterolemia and other forms of dyslipidemia, to type 2 diabetes mellitus (T2DM), a history of a previous vascular event, and as a new potential indication inflammatory (rheumatic) diseases.[5-9] Meta-analyses on large clinical trials showed a risk reduction by statins of approximately 25% on the occurrence of major cardiovascular endpoints in persons free from CVD (primary prevention)[5-7], and a risk reduction of 20% on the occurrence of major cardiovascular endpoints in patients with a history of vascular disease.[7] Therefore, statins have definitely entered daily clinical practice, and their use is expected to increase even more with a widening of therapeutic indications.[10] However, current pharmacotherapy may not be optimal for all patients, and individuals may not respond adequately to statins. This ranges from a lack of therapeutic effect to the occurrence of adverse drug reactions (ADRs), and can have several underlying reasons. Insight into why people do not respond adequately to statins might improve clinical practice, can partly avoid these events, and may finally lead to tailored drug therapy.[11,12]

This thesis contains several pharmaco-epidemiological studies investigating the use of statins in an ageing population. We investigated genetic variants that modified response to statins; considered unintended effects of the use statins in clinical practice; and investigated methodological aspects of studying drug effectiveness in observational studies. Last, in a separate study we investigated sex hormone-binding globulin (SHBG) levels as early biomarker for non-alcoholic fatty liver disease (NAFLD).

In this section, the main findings will be discussed and placed in a broader perspective. We will discuss methodological issues, the consequences of widespread use of statins in clinical practice, and the implementation of pharmacogenetics in clinical practice including recommendations for future research.

MAIN FINDINGS

Genetic factors modifying statin response

Individual variation in drug response is an important clinical problem and is influenced by factors such as patient's overall health status and prognosis (e.g. age, renal or hepatic failure), severity of disease for which the drug is given, potential interaction of co-medication with the drug, quality of drug prescribing, patient's compliance with prescribed therapy, and genetic profile.[13]

In clinical practice, a treatment goal of statin therapy is to reduce serum low density lipoprotein (LDL)-cholesterol levels below a certain threshold, depending on the cardiovascular risk profile of a patient.[14,15] Statins lower LDL-cholesterol with a mean reduction of 25-55%, but are also effective in lowering triglycerides (mean reduction 10-30%) and modestly increase high-density lipoprotein (HDL)-cholesterol (mean increase 5-15%).[16-19] Two large meta-analyses of randomized controlled trials (RCTs) showed that per 1 mmol/L decrease in LDL-cholesterol, the average risk reduction in major cardiovascular events is just over 20%.[7,20] In clinical practice, physicians may achieve the LDL treatment goal by monitoring serum cholesterol levels and adapting the daily dosage accordingly. Nevertheless, despite this dose titration, there is still a substantial number of patients who do not achieve their recommended goal, which can be explained by genetic variation.[21,22]

Genetic polymorphisms in protein coding regions of the DNA (exons) may change the amino acid sequence in a protein, resulting in decreased or increased activity of the protein, e.g. metabolizing enzymes, influx or efflux transporters. Genetic polymorphisms in non-coding regions of the DNA (introns) may change gene expression and transcription, resulting in higher or lower protein concentrations. The net result of these effects can be an increased or decreased drug effectiveness.[23] For example, a genetic polymorphism may decrease the activity of an influx transporter that regulates the uptake of a particular drug from the serum into a target organ. The decreased activity of the transporter increases the serum concentration of the drug. Since there is a direct relationship between serum concentration and the risk of developing ADRs, the net effect of the polymorphism is a combination of decreased activity in combination with an increased risk of ADRs.

In chapter 2, we investigated the influence of genetic variation on statin efficacy and effectiveness, i.e. on the cholesterol lowering response to statins, the risk of statin-induced ADRs, and the primary clinical outcome myocardial infarction (MI).

In three candidate gene studies we investigated whether genetic polymorphisms modified the cholesterol lowering response of statins, whereby we aimed to confirm previous plausible biological mechanisms. In chapter 2.1, we investigated genetic variation in genes involved in lipid metabolism. Because statins act on the cholesterol pathway, genetic variation in these genes is likely involved in the variability in cholesterol response to statins. In this candidate-gene approach, we created a hypothesis-free component by testing all polymorphisms in these genes, while most candidate-gene studies comprise only a few polymorphisms which are already described in relation to cholesterol and statin metabolism. Furthermore, the interaction between a polymorphism and cholesterol response to statins should not be explained by an effect of the polymorphism on cholesterol levels itself. We showed that two polymorphisms were associated with a

smaller reduction in total cholesterol after start of statin therapy: rs1532624 in the cholesteryl ester transfer protein (*CETP*) gene and rs533556 in the apolipoprotein A-I (*APOA1*) gene. Only the finding for the *CETP* polymorphism was replicated in an independent population. The *CETP* rs1532624 polymorphism was not described before in association with statin response. However, it was in linkage (R^2 0.88) with the non-coding TaqIB polymorphism in the *CETP* gene, which is frequently described in relation with cholesterol response to statins.[24-29] The non-replication of the finding for the *APOA1* polymorphism could be explained by a false positive association in the discovery cohort, or fewer numbers (decreased power) in the replication cohort of 243 participants. However, in the meta-analysis of both cohorts the association for the *APOA1* polymorphism remained significant. Further investigation of these polymorphisms in an independent population seems worthwhile, particularly on LDL-cholesterol, the primary target of statins, or to investigate delta cholesterol, the difference between serum cholesterol before and after start of statins. In our study, we had insufficient numbers to investigate these outcomes.

The cytochrome P450 (CYP) 3A4 enzyme is the main enzyme responsible for the metabolism of simvastatin, whereas there is also a minor contribution of CYP3A5. The CYP3A4 enzyme contributes to a lesser extent to the metabolism of atorvastatin, lovastatin, and cerivastatin.[30-33] Several functional polymorphisms in the *CYP3A4* and *CYP3A5* enzymes have been described that influence their metabolizing activity, and changed the cholesterol response to statins. The *CYP3A4*22* and *CYP3A5*3* polymorphisms have been associated with a stronger cholesterol lowering response to statin therapy[34-37], while the *CYP3A4*1B* polymorphism has been associated with a decreased cholesterol lowering response to statin therapy.[38-41] A study by Klein and colleagues discovered novel genetic variation in the peroxisome proliferator-activated receptor alpha (*PPARA*) gene that influenced CYP3A4 enzyme expression and activity.[42] The minor alleles of the strongly linked rs4253728 G>A and rs4823613 A>G polymorphisms were associated with significantly decreased CYP3A4 expression and activity in vitro and in vivo, and might therefore influence the pharmacokinetics of simvastatin. In the study described in chapter 2.2, we therefore hypothesized that the minor alleles of both polymorphisms were associated with a stronger cholesterol lowering response to simvastatin, since decreased metabolism of simvastatin would lead to an increased simvastatin concentration, and thus to increased availability to exert its effect. Results indeed showed a stronger total and LDL-cholesterol lowering effect of simvastatin in minor allele carriers of the polymorphisms, and this effect was independent of the *CYP3A4*22* and *CYP3A5*3* polymorphisms. We performed the first study that demonstrated this effect, and confirmed the previous finding by Klein and colleagues on a pharmacokinetic mechanism.[42] Their in vivo study was performed in atorvastatin users, and showed a decreased atorvastatin metabolism for homozygous minor allele carriers compared to major allele carriers. Although in our study we could not demonstrate an association in 29 atorvastatin

users, probably due to low power, an association could not be excluded. Atorvastatin undergoes less extensive metabolism by CYP3A4 than simvastatin, thus inhibition of CYP3A4 enzyme activity and expression by the *PPARA* polymorphisms affects serum atorvastatin concentration to a lesser extent.[43,44]

Inhibition of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase by statins leads to upregulation of LDL receptors in the hepatocytes, consequently an increased uptake of LDL-cholesterol and a decreased serum LDL concentration.[45-47] Proprotein convertase subtilisin/kexin type 9 (PCSK9) binds to the LDL receptor and promotes its degradation, resulting in a decreased uptake of LDL and a higher serum LDL-cholesterol concentration.[48-50] Statins have shown to increase PCSK9 concentrations, and thereby counteract their own mechanism of action.[51-57] A recent genome-wide association study (GWAS) was the first to show genetic variation that modified statin-induced PCSK9 concentrations. The minor allele of the rs13064411 polymorphism was significantly associated with increased simvastatin-induced PCSK9 concentrations.[58] In chapter 2.3, we therefore hypothesized that statin users carrying a minor allele would have increased LDL receptor degradation with consequently less uptake of LDL-cholesterol from the serum into the hepatocytes and a decreased cholesterol lowering response to statins. We confirmed this hypothesis and our study serves as a first replication in an independent population of the new GWAS finding. The rs13064411 polymorphism showed significant effect modification of the total and LDL-cholesterol lowering response by statins, with minor allele carriers having a decreased response. This effect was stronger in women and in users of a high dose of statins. No association was found for HDL-cholesterol, which is in line with previous literature. Also, the polymorphism did not influence cholesterol levels in general in participants who had never used statins, which indicates that this polymorphism indeed modifies response to statin therapy. The dose-response effect, the consistent findings in the different type of statins separately, and confirmation in sensitivity analyses, makes it more plausible that the association is based on a true-positive finding. Nevertheless, replication of our findings is desirable, especially since previous studies show contradictory results on increased PCSK9 levels and increased[51,53,54,56,58] or decreased[59,60] statin efficacy on serum cholesterol levels. The research question would be even more specifically addressed, if we had serum PCSK9 levels available in the Rotterdam Study, to directly link the polymorphism to PCSK9 levels, and subsequently PCSK9 levels to statin response. However, this information was not available in our study.

In general, statins are well-tolerated and safe drugs, although ADRs do occur. The most common ADR is myopathy, which can vary from myalgia to life-threatening rhabdomyolysis.[61] In chapter 2.4, we investigated whether the rs4149056 c.521T>C polymorphism in the solute carrier organic anion transporter family member 1B1 (*SLCO1B1*) gene

modified the risk of developing statin-induced ADRs. In a GWAS, the minor allele of this polymorphism was associated with an increased risk of simvastatin-induced myopathy.[62] Patients carrying two minor alleles had a 16.9 times higher risk of myopathy compared to homozygous major allele carriers. The *SLCO1B1* gene encodes a hepatic influx transporter – organic anion transporting polypeptide 1B1 (OATP1B1) – that transports statins into hepatocytes.[63] The *SLCO1B1* rs4149056 minor allele is associated with an altered transporter function and increased serum statin concentration, with a subsequently increased risk of developing ADRs.[63-68] In the Rotterdam Study, no data on myopathy was available, and we therefore considered the occurrence of either a dose decrease or a switch to another cholesterol lowering drug as an indicator of an ADR or a too strong reduction in cholesterol level. Within the Rotterdam Study, we confirmed the previous finding from the GWAS and thereby provided further evidence for the role of this polymorphism in adverse reactions to simvastatin. The association failed to replicate within the independent Utrecht Cardiovascular Pharmacogenetics (UCP) study, but remained present in a meta-analysis of both studies combined. For atorvastatin, we only found an association in users with a higher starting dose of more than 1.00 standardized defined daily doses. The question remains whether there is a class effect, or that the association is only present for simvastatin. A class effect is most likely, since the rs4149056 minor allele was associated with higher serum statin concentrations for all statins except for fluvastatin.[69] Also, the minor allele was associated with atorvastatin concentration to a lesser extent than simvastatin concentration[69], and other transporters may contribute to atorvastatin and not simvastatin uptake such as the OATP2B1 transporter.[70] This makes atorvastatin a less important substrate for the OATP1B1 transporter, and might explain why we only found an association in high-dose atorvastatin users. Ideally, we had directly used myopathy or alternatively serum creatinine kinase (CK) measurements as outcome measure. However, this was not possible in the Rotterdam Study. Although our outcome measure was less precise, we were able to retrieve the reasons for dose decreases or switches in medical patient records from general practitioners. The majority of the events were due to ADRs or too strong cholesterol lowering, and in a minority (5%) due to ineffective drug therapy. Besides lowering the dose or prescribing another cholesterol lowering drug, the statin may also be stopped once an ADR occurs. We explicitly did not choose discontinuation of treatment as an indicator of ADRs, since this is a heterogeneous outcome measure which could also relate to reasons such as non-adherence (non-compliance) to therapy, or disappearance of the indication over time.

The risk of MI is indirectly influenced by statins via cholesterol lowering, but is also influenced by underlying diseases, such as hypertension and T2DM. The heterogeneity represented by the underlying mechanism of the outcome MI affects the probability of detecting a gene-statin treatment interaction, and requires more power than with an

intermediate endpoint.[71] However, an advantage of considering a hard clinical endpoint as outcome is that it directly allows for evaluation of the effect of a polymorphism on statin effectiveness in reducing the risk, as opposed to a surrogate parameter such as LDL-cholesterol reduction. In chapter 2.5, we could not demonstrate significant effect modification by the *CYP3A4*22* polymorphism on the effect of statins in reducing the risk of MI, neither in the independent UCP study and Rotterdam Study separately, nor in a meta-analysis of the two studies combined. The *CYP3A4*22* polymorphism (rs35599367) has previously been associated with a stronger cholesterol lowering response to statins in two independent studies[34,35], but its association with cardiovascular events was never investigated.[72] Minor allele carriers had a 0.34 mmol/L stronger cholesterol lowering compared to homozygous major allele carriers, and stronger reductions in LDL-cholesterol correspond to further reductions in the incidence of cardiovascular events.[20] A 1 mmol/L decrease in cholesterol leads to a 21-22% risk reduction in major cardiovascular events, and this translates to a 28% risk reduction in carriers of a minor allele.[20] Therefore, this polymorphism seemed an interesting candidate to investigate on the outcome MI. One reason for not finding an association is that the effect on LDL-cholesterol may be too small to affect clinical outcome while we had too low power to detect an association. Another potential reason is heterogeneity of the UCP study and Rotterdam Study. It could not be explained by titration of the statin dose below a certain LDL threshold since there were no differences between the genotypes in statin start and end dosages.

Besides these candidate studies, we also investigated the cholesterol lowering response to statins in a GWAS, a hypothesis-free approach without a priori thought of the underlying genetic variation involved. The Genomic Investigation of Statin Therapy (GIST) consortium is a collaboration in which both RCTs and observational studies participate, and aimed to discover genetic factors that modified the effect of statins on total, LDL- and HDL-cholesterol. The Cohorts for Heart and Aging Research in Genomic Epidemiology (CHARGE) Consortium was formed to facilitate GWAS meta-analyses and replication opportunities among multiple large and well-phenotyped longitudinal population-based cohort studies.[73] For the GIST-consortium analyses, the observational studies came mostly from the CHARGE consortium, one of which is the Rotterdam Study. In a pharmacogenetic meta-analysis of GWAS, described in chapter 2.6, including in total 18,596 statin users (6 RCTs and 10 observational studies), genetic variation modifying the delta LDL-cholesterol lowering response to statins was investigated. The most promising signals were further validated in 22,318 statin users (2 RCTs and 1 observational study, the Rotterdam Study). Two loci at Sortilin 1 (*SORT1*)/Cadherin EGF LAG seven-pass G-type receptor 2 (*CELSR2*)/Proline-Serine-rich coiled-coil 1 (*PSRC1*) and *SLCO1B1* were newly discovered. Furthermore, previously described associations with Apolipoprotein

E (*APOE*) and Lipoprotein, Lp(a) (*LPA*) were confirmed[74,75], the only two loci that have nowadays consistently been identified to be associated with variation in LDL-cholesterol response to statins in both GWAS and candidate-gene studies.[74-77] Genetic variation in *SORT1* and *APOE* was associated with a stronger LDL-lowering response to statins, genetic variation in *LPA* and *SLCO1B1* was associated with a decreased LDL-lowering response to statins, independent of baseline LDL-cholesterol. Additional functional and pathway analyses confirmed a strong biological and functional role in statin response for several strongly associated gene loci, including *APOE/TOMM40/PVRL2* and *SORT1/CELSR2/PSRC2*. In general, with this largest meta-analysis of GWAS on LDL-lowering response to statins conducted to date, we advanced the understanding of the pharmacogenetic architecture of statin response.

A connection between the candidate-gene study in chapter 2.4 and this GWAS in chapter 2.6 can be made. The *SLCO1B1* rs2900478 polymorphism, which resulted from the meta-analysis of GWAS and was associated with a smaller reduction in LDL-cholesterol, is in strong linkage disequilibrium with the rs4149056 polymorphism (R^2 0.89), which is associated with an increased risk of statin-induced ADRs. The minor allele of rs4149056 was previously associated with increased serum statin concentrations[63,64,66], and with a smaller LDL-cholesterol reduction in response to simvastatin in candidate gene studies.[77,78] Thus, besides the conclusion that this polymorphism is associated with an increased risk of statin-induced ADRs due to increased statin serum concentrations[62,78,79], it is also associated with a decreased efficacy. The decreased uptake of statins into hepatocytes by the OATP1B1 uptake transporter is expected to result in decreased cholesterol lowering.[62] An alternative explanation is that the increased number of ADRs in patients carrying a minor allele might lead to decreased statin adherence, and consequently decreased measured efficacy on a population-based scale.

Unintended effects of the use of statins in clinical practice

Statins are frequently used in clinical practice, and with the increasing prevalence of chronic diseases such as CVD and T2DM, the number of patients using statins will increase even more. Although, these drugs are prescribed because of their well-proven beneficial effect, one should not ignore potential ADRs that may occur. In chapter 3, we investigated unintended effects of the use of statins in daily practice. Possibly, statins have beneficial effects on diseases for which currently no therapeutic indication exists, or otherwise, have adverse effects that raise questions as to whether the risk-benefit balance is acceptable to allow the widespread use of these drugs in clinical practice.

In chapter 3.1, we investigated the association between statins and the prevalence of NAFLD. NAFLD, or hepatic steatosis, is considered as the hepatic manifestation of the metabolic syndrome, independently of the components of the metabolic syndrome. Also, CVD is the major cause of death in patients with NAFLD.[80-83] NAFLD patients often

have dyslipidemia, and therefore these patients frequently have an indication for statin therapy. Given the association between NAFLD and CVD, these drugs may be beneficial in these patients. However, there is some concern as to whether statins are safe and effective in NAFLD, and whether they may exacerbate or worsen hepatic steatosis despite improvement in lipid profile.[84-88] In the Rotterdam Study, we provided further evidence for the safe use of statins in NAFLD patients. We did not find an overall association between current and past use of statins and NAFLD prevalence. In patients with a body mass index (BMI) ≥27.5, current use of statins for more than two years was significantly associated with an approximately three times lower NAFLD prevalence. Our study adds further to the field since we performed the first study that distinguished between the effects of current and past use, and adjusted for statin dose. In contrast, other studies mostly obtained medication data by questionnaire, which gives no insight into duration of use and thereby leads to non-differential misclassification. Obesity is a strong and independent risk factor for NAFLD. We could not give a clear explanation for the finding in only patients with a high BMI but it seems plausible that in this high-risk group a beneficial effect is most clearly visible. The exact pathogenesis of NAFLD is currently not clarified. Insulin resistance, lipid abnormalities and chronic inflammation are considered to be central in the development of diseases related to obesity such as NAFLD and CVD.[80,89,90] The accumulation of lipids, especially triglycerides, is essential for the development of NAFLD.[80] Statins might protect against NAFLD through their lipid-lowering effect, but also through their pleiotropic effect such as their anti-inflammatory and immunomodulatory effects.[91] Since these factors are more explicit in obese individuals, a protective effect of statins may be more pronounced.

In chapter 3.2, we demonstrated a non-beneficial effect of statins, since current statin use was significantly associated with lower total and bioactive non-SHBG-bound testosterone levels in males. Testosterone is synthesized in the testes, and this process requires a continuous supply of cholesterol.[92] Since statins decrease cholesterol levels, and cholesterol is a precursor of testosterone, statins may also decrease serum testosterone levels. This hypothesis was further based on theoretical mechanisms that statins lower testosterone levels by direct inhibition of HMG-CoA-reductase in the testis[93], by lowering the serum cholesterol level and thereby decrease cholesterol uptake by the testis, and by direct inhibition of other enzymes in the testosterone pathway.[94] Our finding that statins significantly lowered total and non-SHBG-bound testosterone was further supported by the finding that the magnitude in decrease was directly proportional to the dosage of statin therapy. Overall, in current statin users the mean decrease in total and non-SHBG-bound testosterone was −1.18 and −0.35 nmol/L, respectively. A recent meta-analysis of 5 RCTs showed a mean decrease of −0.66 nmol/L in total testosterone level.[95] One might question the clinical relevance of such a small decrease. Testosterone is biologically important for mood, libido, muscle strength, and protects against osteo-

penia, however, poor correlation exists between testosterone level and symptoms.[96] Nevertheless, a modest average decrease in a population might hide a substantial decrease in a handful of individuals with stronger response, and in those with an already low testosterone level. Therefore, this association might be clinically meaningful on a population-based scale, since statins are increasingly recommended and consequently used in nowadays practice.

Estimating the effect of time-dependent statin use in observational studies

Unlike constant characteristics such as sex and genetic constitution, drug exposure is essentially a time-varying determinant. In observational studies, effect estimates may be biased in the presence of a time-dependent covariable which is simultaneously 1) a reason for prescribing or dose-changing (often termed 'confounding by indication'[97]), 2) influenced by the drug treatment under study, and 3) a potential cause of the outcome of interest.[98,99] In chapter 4, we investigated whether the use of a marginal structural model (MSM) to estimate the causal effect of time-dependent drug use in the presence of time-varying confounders, produces different relative risk estimates from traditional Cox proportional hazard models with and without time-dependent covariables. Currently, there is limited experience with the application of the MSM method in observational studies with complete data on drug use during follow-up and a multitude of treatment determinants. In our study, we used observational data from the Rotterdam study and investigated the use of statins for the primary prevention of CVD. In the Cox models with time-dependent statin use, and with and without time-dependent covariables, we demonstrated a 31% and 32% risk reduction respectively in ever statin users on atherosclerotic CVD (composite endpoint of fatal and nonfatal MI, stroke, and atherosclerotic CVD death). This risk decreased to a 11% risk reduction with MSM, in which all observations were weighted by the inverse of the conditional probability of the observed status of statin use. Three main conclusions could be made on the basis of the results. First, we could not observe a large difference in effect estimates between the results from the Cox model with time-dependent covariables and the Cox model without time-dependent covariables. A possible explanation for this could be that the changes in cardiovascular risk factors and other potential confounders we evaluated do not materially relate to the probability of initiating statin treatment, i.e. for our particular research question no or only a limited degree of time-dependent confounding by indication may have been present. Second, we could not demonstrate important differences in risk estimates from the MSM compared to the traditional Cox proportional hazards models. This is similar to results from other MSM studies on statin effectiveness in observational studies[100,101], and general literature about the application of MSMs.[102,103] Last, we concluded that in our MSM analysis, we were not able to deal with all confounding factors, and there was still substantial unmeasured confounding by indication in the initial period after statin initia-

tion which was not accounted for. Possibly, other factors may be of greater importance for statin prescription in everyday clinical practice, or the design and data of the Rotterdam Study provide insufficient information for an adequate MSM analysis. This topic on estimating drug effectiveness and time-dependent drug use in observational studies will be further discussed in the 'Methodological considerations' part of this discussion.

SHBG level as a biomarker for NAFLD

SHBG is a glycoprotein which is mostly produced in the liver and regulates the serum concentration of sex steroid hormones. SHBG has a high binding affinity for testosterone, and the serum concentrations of total testosterone and SHBG are strongly correlated.[104,105] Previous cross-sectional studies suggested an association between low serum SHBG levels and an increased NAFLD prevalence.[106-109] In these studies, it is difficult to draw firm conclusions about temporal associations and reverse causation cannot be excluded. It is therefore unclear whether SHBG contributes to the development of NAFLD. Also, studies showed an association between serum testosterone levels and NAFLD[107,109-112], and it should be unraveled whether the association between SHBG and NAFLD is independent of testosterone. A potential role of SHBG in the development of NAFLD has been discussed, such as interference with hepatic lipogenesis and the influence of insulin resistance.[113-116] However, a convincing explanation has not been revealed yet. In the Rotterdam Study, we created a longitudinal component by selecting a population with a very low probability of steatosis (defined as fatty liver index (FLI) <60) and a serum SHBG measurement available at baseline, and investigated whether these participants developed NAFLD over a mean follow-up time of 11 years. We demonstrated that higher SHBG levels at baseline were associated with a lower risk of developing NAFLD during follow-up, both in men and women and independent of serum total testosterone levels. Sensitivity analyses in participants with FLI <30 showed similar effect estimates and supported our findings. Our study provided evidence for the assumption that low SHBG level might be considered as an early biomarker for the development of NAFLD. Although steatosis could not be established yet according to the FLI, a measure which has shown to agree with steatosis detection with SteatoTest and abdominal ultrasound[117], serum levels of SHBG have already decreased significantly. Future longitudinal studies should determine whether SHBG could indeed be used in clinical practice as a sensitive predictor of NAFLD.

WIDESPREAD USE OF STATINS IN CLINICAL PRACTICE

Currently, CVD is the leading cause of death worldwide.[1] The aging population and population growth will contribute to a further increase in the number of patients with

coronary heart disease (CHD).[118] Furthermore, the increase in number of people with overweight due to food overconsumption will increase the prevalence of metabolic diseases such as T2DM and NAFLD, both diseases with CVD as important cause of death. Therefore, prevention of CVD is of utmost importance and could be achieved through lifestyle interventions or through pharmacotherapy with different classes of drugs, as described in the introduction of this thesis. Guidelines on primary prevention of CVD recommend clinicians to treat persons without established CVD, but with an increased global absolute risk of future CVD with lipid-lowering therapy.[17,119-121] These recommendations are based on large clinical trials that demonstrated an approximately 25% risk reduction on the occurrence of major cardiovascular endpoints (nonfatal MI, coronary death, stroke, coronary revascularization) and 14% risk reduction in all-cause mortality in persons free of CVD.[5-7] In 2013, the US guidelines were substantially changed by lowering the cutoff for indication for statin treatment for primary prevention. While in the Adult Treatment Panel III (ATP-III) guidelines the threshold for statin therapy starts at a 20% risk of CHD, this recommendation was lowered to a 7.5% risk on hard atherosclerotic CVD in the new American College of Cardiology/American Heart Association (ACC/AHA) guidelines.[119] A recent study showed that application of the new guidelines on participants of the Rotterdam Study would imply that in 96.4% of men and 65.8% of women treatment with statins is recommended, while in an additional 3.3% of men and 14.2% of women treatment should be considered.[10] This would imply that in only a few men (0.3%, n = 6, of 1894 men in this study population) and in the minority of women (20.0%, n = 462, of 2315 women in this study population) no treatment with statins is needed.[10] Strictly following the ACC/AHA guidelines would have a large impact on healthcare expenditure but also raise questions. Should statins be considered as the new wonder drugs, similar to what was initially said about the polypill strategy, a combination of drugs in one pill that should be prescribed to everyone aged 55 years and older to prevent CVD, which was published in 2003?[122] Should statins be used as widespread as our water from the tap? Until we find an attractive new pharmacotherapeutic panacea against the consequences of our increasingly luxurious way of life?

Nowadays, the benefits of statins on cholesterol lowering and CVD prevention are well-established on the basis of large clinical trials with an average 22% risk reduction per 1 mmol/L decrease in LDL-cholesterol.[6,20] Epidemiological studies have shown a log-linear association between cholesterol concentration and CVD risk, with no flattening of the curve at lower serum cholesterol levels. It seems therefore attractive to decrease LDL-cholesterol levels as much as possible. Clinical trials showed also benefit in patients with lower-than-average cholesterol levels, with a risk reduction proportional to the magnitude of the achieved cholesterol reduction, which seemed to be largely independent of the starting cholesterol level.[18,20,123] Consequently, the treatment threshold for

LDL-cholesterol was lowered over the years. Moreover, intensive treatment regimens with higher statin dosages of e.g. 80 mg simvastatin or atorvastatin also showed one fifth reduction in major vascular events[123,124], and these higher dosages did not lead to a significant increase in non-vascular deaths.[123-125] Additionally, a large meta-analysis of 27 RCTs showed that LDL-cholesterol reduction with statins in individuals with a 5-year risk of major vascular disease of less than 10%, significantly and safely reduced the risk of major vascular events, both in those with and without a previous history of vascular disease.[7] The proportional reduction was at least as big in the two lowest risk categories (<5% and ≥5-<10%, with RR 0.62 and 0.69 per 1 mmol/L decrease in LDL respectively) as in the higher risk categories (≥20%-<30% and ≥30%, with RR 0.81 and 0.79 per 1 mmol/L decrease in LDL respectively). From an efficacy point of view, these findings seemed to justify the widespread use of statins in clinical practice for a broader indication. Nevertheless, one should be aware that a 20% risk reduction in people with an already low 5-years risk of CVD, does not add much benefit on the absolute risk of CVD, and in these people the number needed to treat to prevent one CVD event is high.[126] In the meantime, therapy may lead to unintended adverse effects. Also, it is hardly known what individual reassuring thoughts about the benefits of life-long statin treatment will do to the ability of individuals to exert self-discipline regarding their daily fat and carbohydrate intake. A drug which is healthy in individuals can still be toxic to societal tenacity.[127]

Increased use of statins and more intensive treatment regimens raises concerns about the occurrence of ADRs. Although statins are generally recognized as well-tolerated and safe drugs, myopathy may occur, and in its severe form this may lead to life-threatening rhabdomyolysis.[61,128] Intensive treatment regimens have not shown to increase myopathy substantially[129], except for simvastatin 80 mg daily.[62] In this large SEARCH trial, the excessive number of myopathy cases with 80 mg simvastatin was four per 1000 per year in the first year of treatment, and decreased to one per 1000 per year thereafter. The latter is still ten times more common than the relatively low incidence of one per 10,000 patients with 20-40 mg simvastatin daily. About a fifth developed rhabdomyolysis, and this number might even be higher in daily clinical practice since the trial participants were monitored thoroughly, which resulted in earlier detection and timely prevention of rhabdomyolysis.[62] However, the myopathy cases were largely confined to minor allele carriers of the rs4149056 polymorphism in the *SLCO1B1* gene, which was also associated with statin-induced ADRs in our study described in chapter 2.4. More than 60% of the myopathy cases in patients using 80 mg simvastatin could be attributed to the minor C allele, and in theory, this genetic variation could be detected before treatment initiation. These minor allele carriers may be treated with newer, more potent statins (e.g. 80 mg atorvastatin, 20-40 mg rosuvastatin, daily) or a combination of a standard dose

statin with another cholesterol lowering drug, without increasing the risk of myopathy substantially.

Moreover, statins have been associated with elevated liver enzymes, and studies suggested that statins might induce hepatic injury and worsen hepatic steatosis. However, statins were rarely associated with acute or chronic liver failure or significant liver injury.[84-88] In February 2012, the Food and Drug Administration approved important safety label changes for statins to remove the need for routine periodic monitoring of liver enzymes in patients taking statins.[130] Concerning hepatic steatosis, our study described in chapter 3.1 showed no overall association between use of statins and NAFLD prevalence, and longer duration of use was even associated with a lower NAFLD prevalence in patients with a high BMI. Studies with pathological data including liver biopsy and recent literature reviews showed a rather beneficial effect by showing a reduction in the extent of hepatic steatosis in statin users.[131-136]

Third, statins have been associated with an increased risk of new onset T2DM in meta-analyses of RCTs[137,138] and observational studies.[139-141] The mechanism is currently not clarified, but a recent study discovered the interesting finding that this increase in risk is partly explained by inhibition of HMG-CoA reductase.[142] As a consequence, this adverse effect of statins, inherent to their primary mechanism, seems to be partly unavoidable. Other mechanisms proposed were a detrimental effect on glucose mechanism via an effect of statins on the glucose-transporter 4[143], an increase in insulin resistance[144-146], or blocking of calcium channels in the β-cells of the pancreas and consequently reduced insulin secretion.[147] Nevertheless, the increase in T2DM risk in statin users corresponded to only a slight increase in T2DM in absolute terms. It did not outweigh the reduction in major cardiovascular events, implying that clinical decision-making should not be changed for patients with an indication for statins for moderate to high CVD risk or existing CVD.[138,148] However, it might question the use of statins at low CVD risk.

Furthermore, as described previously in literature and in our study in chapter 3.2, use of statins was associated with lower levels of both total and non-SHBG bound testosterone. Given the important biological role of testosterone, the increased use of statins, and the fact that this decrease might be substantial in individuals with stronger response or an already low testosterone, this might be clinically relevant.

Last, besides the risk-benefit balance, judgments about the appropriateness of the widespread use of statins, especially in patients at lower risk of major cardiovascular events, depends also on the cost-effectiveness.[149]

METHODOLOGICAL CONSIDERATIONS

Study setting and design

The studies described in this thesis were all embedded in the prospective population-based Rotterdam Study. The Rotterdam Study started in 1990 in the suburb Ommoord in Rotterdam, when 7,983 participants aged 55 years and older were enrolled in the first cohort (RS-I). In 2001, a second cohort started including 3,011 participants aged 55 years and older (RS-II), and in 2006, a third cohort started including 3,932 participants aged 45 years and older. The overall response rate (number of enrolled participants divided by the number of invited eligible inhabitants) over the three cohorts was 72.0%. Detailed medication dispensing data is available on a daily basis through linkage with computerized pharmacies in the Ommoord suburb. Furthermore, the cohort is continuously monitored for major morbidity and mortality through linkage with general practitioner's records. Detailed information on design, objectives and methods of the Rotterdam Study has been described before.[150,151]

The Rotterdam Study has several advantages. Detailed information and follow-up data is available on many covariables, which were measured with standardized methods and over a relatively long period of follow-up. The population-based character of the Rotterdam study reduces the risk of selection bias, and the only inclusion criteria besides residing in the Ommoord suburb is age. The prospective ascertainment of risk factors and outcome variables without prior knowledge of the aim of the research hypotheses of studies, minimizes information bias. The data from general practitioners are of great value since it contains detailed information on disease but, for example, also determinants of prescribing can be figured out. In our study described in chapter 2.4 on the *SLCO1B1* rs4149056 polymorphism and statin-induced ADRs, we used these general practitioners' records to retrieve the reason for a dose decrease of statin therapy or switch to another cholesterol-lowering drug, since these events were considered as indicators of ADRs or too strong reductions in cholesterol level. By checking these records, we were able to validate our outcome measure. Moreover, the detailed pharmacy data on every day of follow-up enabled us to investigate the effects of dose and duration of use, and limited non-differential (random) misclassification of exposure. In addition, differential (non-random) misclassification is restricted through prospective gathering of complete drug dispensing data. In studies that assessed drug information at baseline on the basis of an interview or during repeated rounds of cross-sectional measuring, the risk of information bias is increased because so-called 'recall bias' may lead to differential misclassification and biased risk estimates. People with severe disease tend to have a better recall of drug exposure data than healthy controls.[152] Although non-compliance with statin therapy could have occurred[153], this would have led to random misclassification and underestimation of the true effect.

A potential limitation of the Rotterdam Study is that the cohort includes mostly white (99% Caucasian) individuals aged 45 years and older, which limits external validity. The generalizability of our findings to younger and non-Caucasian populations remains doubtful. With reference to our genetic studies, it has been suggested that at older age the effect of genetics is less important and other factors such as a change in body composition and organ function may contribute more to differences in drug response.[154-156] Also, genetic structure differs between races, e.g. the rs4253728 polymorphism described in chapter 2.2 is not present in Chinese and Japanese populations. Moreover, in the Rotterdam Study, information on ADRs is not collected on a structural basis.

A frequently encountered problem in genetic studies is power, which depends on sample size, magnitude of the effect of the polymorphism on drug response, proportion of cases exposed to the drug of interest, and the minor allele frequency (MAF) of a gene variant. To increase power and limit the risk of both false positive and false negative results, it is important to collaborate with researchers from other studies to increase sample size. The GWAS in chapter 2.6 was performed in 19 independent studies with in total over 40,000 statin users. However, in some of our candidate gene studies samples size may be limited. To prevent non-valid results, findings were validated in an independent population (2.1, 2.4, 2.5), or we confirmed previously well-characterized genetic findings/mechanisms from literature (2.2, 2.3, 2.4). Although, in two studies an association failed to replicate (2.1, 2.4), it remained present in a meta-analysis.

In chapter 2.1, we selected one – most significant – polymorphism per candidate gene. There can be multiple causal variants in a gene and the total variation explained by a locus may be underestimated. Although we found two polymorphisms in two different genes associated with statin response, this study would have been further strengthened if we had used a method described in chapter 2.6. A conditional analysis, starting with the top associated polymorphism across the whole genome, followed by a stepwise procedure in which one by one additional polymorphisms are selected, according to their conditional P-values, would allow the discovery of more polymorphisms at a locus.

Omission of blood samples for genetic data and difficulties with genotyping were completely random and not related to genotype status. Patient and prescribing physician are both unaware of the patient's genetic profile. Also, the modified response to a drug caused by genetic variation in the pharmacokinetic and pharmacodynamic pathway of the drug is only present once a drug is administered to the body. Genetic variation at baseline will therefore be random. This can be referred to as Mendelian randomization, a method that enables to investigate causal effects in observational data in the presence of confounders, as long as first use of a drug is taken into consideration as switching between drugs with a similar indication can lead to confounding (by contra-indication). It assumes that the genotype is assigned randomly and population genotype distribu-

tion is unrelated to confounders. The genotype acts as an instrument for the exposure of interest and only affects the outcome indirectly via its effect on the exposure.[157,158] In all our genetic studies, polymorphisms were in Hardy-Weinberg equilibrium, thus Mendelian randomization occurred and selection bias was therefore unlikely.

The studies described in chapter 2.2 and 2.3 could have been further strengthened with the use of expression data, since in both studies the hypothesis was based on a previous finding in literature between genetic variation and altered expression of an enzyme. Unfortunately, these data were not available in the Rotterdam Study.

Our outcome measure NAFLD in chapter 3.1 and 5.1 was assessed by ultrasonography, while the gold standard for NAFLD detection is liver biopsy. In the population-based Rotterdam Study, it would have been unethical to study participants with this invasive diagnostic technique, and therefore hepatic histology data were not available. Moreover, ultrasonography may be less sensitive to detect NAFLD than more advanced imaging techniques such as CT/MRI, since ultrasonography is not appropriate for detection of less than 30 percent steatosis. However, in a previous study, ultrasonography was similar to other imaging modalities in NAFLD detection with an acceptable sensitivity of 80-100%.[159] Also, in both of our studies, exclusion of patients with mild steatosis did not affect the associations.

In two studies (chapter 2.2 and 2.3), we used laboratory measurements from the 'Star-Medisch Diagnostic Centrum' (Star-MDC), which performs all outpatients laboratory assessments for general practitioners in the Rotterdam Rijnmond area with a potential source population of more than 1 million inhabitants. Reasons for the laboratory measurements requested by general practitioners for healthcare purposes may have differed between statin users and non-statin users, inducing a potential information bias. However, we used these measurements in genetic studies, and as prescribing physicians were not aware of the genotype status, this will not have influenced our results.

Measuring drug effectiveness in observational studies

The efficacy of a drug is defined as how well the drug achieves its intended effect under optimal circumstances, often on a secondary endpoint such as serum cholesterol or blood pressure. The effectiveness of a drug is defined as how well the drug achieves its intended effect in a real-life setting, mostly on a primary endpoint such as stroke or cardiovascular mortality. Often, a discrepancy exists between efficacy and effectiveness, and the drug does not perform as expected in everyday clinical practice. The efficacy of drugs is tested in double-blind RCTs, in which the randomization and blinding minimizes the risk of selection bias and confounding.[160] However, the homogeneous characteristics of patients in these trials differ substantially from those of patients in a real-life setting.[161-164] Moreover, outside the controlled setting of trials, dose adjustments, non-compliance, and discontinuation frequently occur over time. Therefore, effectiveness research requires

observational studies, ideally in unselected populations, to reflect a more real-life setting and to enhance the generalizability of the results. Comparative effectiveness research (CER) is defined by the Institute of Medicine committee as 'the generation and synthesis of evidence that compares the benefits and harms of alternative methods to prevent, diagnose, treat, and monitor a clinical condition, or improve the delivery of care. The purpose of CER is to assist consumers, clinicians, purchasers, and policy makers to make informed decisions that will improve health care at both the individual and population levels'.[165] In short, it compares existing health care interventions with the question which treatment works best, for whom, and under what circumstances.

Increasingly, CER on drug utilization and clinical outcomes is performed in large health care databases, but besides above mentioned value and advantages, these observational studies are subject to bias and confounding. It is assumed that these databases contain accurate and complete information on drug use, clinical outcomes, and relevant covariables. As mentioned previously, drug exposure is essentially a time-varying determinant. Observational studies with drug use available at baseline only, may lead to substantial differential and non-differential misclassification of exposure during follow-up, with as a consequence non-valid effect estimates. To reduce this misclassification, drug exposure should precisely be defined by dividing drug use during follow-up into mutually exclusive episodes of non-use, past use and current use per individual. Previously, a method was proposed based on a Cox model for the analysis of drug use as a time-dependent determinant.[152] Unfortunately, effect estimates may be biased in the presence of time-varying confounding: some risk factors which change during follow-up may have been influenced by preceding drug use, and in the presence of a time-dependent risk factor for the event of interest which also predicts subsequent drug use. Both conditions will always be the case when a time-dependent co-variable is simultaneously 1) a reason for prescribing or dose-changing (often termed 'confounding by indication'[97]); 2) influenced by the drug treatment under study; and 3) a potential risk factor for the outcome of interest.[98,99] For example, serum LDL-cholesterol is a time-varying confounder in the association between statins and MI risk. Confounding by indication arises from the fact that patients who are prescribed a certain drug have a different (mostly poorer) prognosis than people who are not prescribed that drug. The indication for the drug is a risk factor for the outcome under study, and the association between drug exposure and outcome is erroneously attributed to the drug, while in fact the underlying disease (indication for the drug) explains the association.[166] Ways to deal with this in observational studies is to study the association in patients who receive different treatments for the same underlying disease status, or to adjust for the indication. However, such adjustment may remain insufficient in the presence of time-varying confounders that influence the drug under study.

In its routine definition, a confounder is assumed to precede and to be independently associated with exposure, to be a risk factor for the outcome, and not to be a step in the causal pathway between exposure and outcome.[166] In the case of time-varying confounding, the confounder is both associated with past and future exposure.[98,99] Analyses with the presence of time-varying confounders are complex in several ways. First, since the time-varying confounder is affected by past exposure and independently predicts outcome, it acts as an intermediate for the effect of past exposure on the outcome. We would not want to adjust for intermediates when estimating the total effect, but otherwise we have to adjust for the confounder because it may confound future exposure and outcome. Second, the time-varying confounder is affected by past exposure and other covariables that also predict outcome, then adjusting for this confounder may create selection bias[99]. In the example in chapter 4 with use of statins for primary prevention of CVD, several analytical methods can induce biased results: examining the unadjusted effect of baseline statin use; examining the unadjusted effect of time-updated statin use; controlling for baseline covariables; and controlling for time-dependent covariables. The unadjusted estimate of baseline statin use will be biased since statin users more frequently have comorbidities associated with increased CVD risk. Therefore, they differ in baseline risk from non-statin users, and statins will be falsely associated with increased CVD risk. Furthermore, baseline statin use does not take into account changes in dose and duration of therapy over time. This is accounted for in the unadjusted time-updated statin use analysis, but still has the problem of differences in baseline risk. Controlling for baseline co-variables also gives biased effect estimates because it ignores the fact that covariables frequently change over time and may be influenced by statin therapy. Controlling for the time-updated values of co-variables, such as serum cholesterol, will still give biased estimates of the effect of statins, since statins protect against CVD (mostly) by lowering serum cholesterol, and hereby we would control for an intermediate which is in the pathway between statins and CVD. Because the consequences of invalid findings can be substantial, adequate methods for statistical analysis need to be employed to estimate causal effects and overcome this time-varying confounding.[167] Methods to deal with this are G-estimation of structural accelerated failure time modeling, G-estimation of structural cumulative failure time modeling, and inverse probability weighted estimation of MSM.[168] In MSM, which was used in chapter 4, all observations are assigned a weight based on the conditional probability of receiving observed treatment.[169] This resembles the use of propensity scores, but differs in the sense that with propensity scores only the probability of being treated is used. To deal with confounding by indication, matching, stratification, or adjustment on propensity score is performed.[170]

As described in our study chapter 4, and in previous MSM studies, it remains difficult to deal completely with time-varying confounding and still substantial unmeasured confounding by indication remains present. Furthermore, a lack of difference between tradi-

tional Cox proportional hazard models and MSM is described. In general, on theoretical grounds MSM is an elegant statistical technique to adjust for time-varying confounding by indication. However, the absence of knowledge about detailed confounder status on a daily basis may be a hurdle to the use of MSM in real-life population-based cohort studies. Even if drug use is registered on a daily basis, the absence of data on time-dependent confounders, such as the precise prescription indication or other treatment considerations jeopardizes the calculation of the actual valid weights. Confounding by indication remains a hurdle in observational effectiveness research on preventive drugs with a multitude of prescription determinants.

CLINICAL IMPLEMENTATION AND FUTURE DIRECTIONS: PERSONALIZED MEDICINE

Genetic variation influencing statin response, and treatment response in general, has been investigated with different techniques. From the hypothesis-based candidate gene studies, hypothesis-generating GWAS, to more recently 'next-generation sequencing', such as whole-genome sequencing (investigates the complete DNA sequence) and exome sequencing (investigates the protein-coding regions of the genome).[171-174] GWAS have succeeded in discovering common gene variants (MAF>~ 5 %), but with often very small effect sizes, weak correlations with neighbouring variants, and infrequently tracking of the causal variant. Whole-genome analyses are expected to increase the discovery of causal variants and to unravel rare genetic variants with larger impact on drug response.[175] Next-generation sequencing also considers other genetic variation besides single nucleotide polymorphisms, such as insertions, deletions and copy number variations. These are not well captured with a GWAS approach. To completely understand the genetic profile that predicts drug response, the field of pharmacogenetics bridges with the fields of epigenomics (investigates change in gene expression that occurs without a change in DNA sequence, such as DNA methylation), transcriptomics (investigates the expression levels of mRNA), proteomics (investigates the structure and function of the entire range of proteins expressed by a genome), and metabolomics (investigates the metabolites in a cell, tissue, organ, or organism).

Insight into underlying genetic variation that predicts treatment response has two main purposes. First, it generates insight into underlying biological mechanisms that facilitates the discovery and development of new targets for drug therapy. Genetic mutations in PCSK9 were discovered to influence cholesterol metabolism, and nowadays, large clinical trials from pharmaceutical companies investigate the effect of PCSK9 inhibitors as cholesterol lowering drugs for monotherapy or in combination with statins.[176-179] Second, drug response ranges from therapeutic effect to the risk of developing ADRs. Insight into the

genetic profile that predicts drug response may help to identify patients with inadequate response, may optimize drug effectiveness and safety, and may reduce the utilization and costs of daily health care. This may ultimately lead to clinical decision making based on patient's genetic profile: 'tailored pharmacotherapy' or 'personalized medicine'. However, the genetic studies described in this thesis, and pharmacogenetic findings in general, do not have direct clinical implications. Although many polymorphisms are already discovered that modify drug response, the clinical application of pharmacogenetics is lagging behind. Nowadays in the Netherlands, pharmacogenetics-based (dose) recommendations are developed for only a few dozen of drugs.[180] Several hurdles and future steps have to be taken before genotype-guided clinical decision making can be applied. The main reason for limited clinical implementation is lack of evidence that genetic testing leads to improvement in clinical outcomes. Genetic variation may lead to a decreased cholesterol response to statins, but the effect may be too small to be relevant. For example, in chapter 2.2, minor allele carriers of two *PPARA* polymorphisms had a better cholesterol lowering response to statins. Although this is interesting, it will not directly lead to tailored pharmacotherapy. After all, even if patients with a minor allele have a stronger intended cholesterol lowering effect, the majority of patients with two major alleles will still keep their indication for statins. Nevertheless, homozygous major allele carriers might respond better with a higher dose – or with a PCSK9-inhibitor in future practice –, in which case pharmacogenetics might have clinical consequences. Moreover, response to drugs is not likely based on a single polymorphism or gene, but more likely relies on the combination of or interaction between several polymorphisms in different genes.[181] Therefore, future research should focus on combinations of polymorphisms, e.g. by considering haplotypes (a combination of polymorphisms on a single chromatid that is inherited together), by performing pathway analyses, or by using a genetic risk score approach. If more loci are identified, a dosing algorithm based on the whole collection of polymorphisms influencing statin response may predict and improve statin effectiveness. Third, discovered genetic associations failed to replicate or results are contradictory.[182] To avoid false-positive associations and to clarify current contradictions, findings should be replicated in independent populations. To achieve this, large meta-analyses of independent studies in a consortium, such as the CHARGE consortium, are needed. Future research should prioritize the search for and set up of adequate databases for genetic research. Fourth, although decreased over recent years, the costs of genetic testing are still higher than simply routinely monitoring of patient's blood (e.g. cholesterol levels for statin efficacy, CK levels for statin toxicity), and titrate the dose accordingly. Last, other factors influencing drug response, such as a decrease in liver and renal function and change in body composition with increasing age, may be of more importance in drug response and overrule the effect of pharmacogenetics. However, investigating genetic associations and their interaction with patient characteristics might be relevant.

In the end, one might question whether genotyping for tailoring therapy will be broadly implemented in clinical practice. Possibly, it will be confined to a small selection of polymorphisms with large clinical consequences in selected groups of patients. Although this might seem a somewhat negative appraisal, it does not mean that pharmacogenetic research is a waste of time, effort and resources. It provided us with important scientific insights into underlying biological mechanisms involved in drug metabolism and action. Hopefully, future findings will make it possible that each individual receives treatment based on his own genetic profile, and thereby increase treatment efficacy, reduce the risk of drug toxicity, and minimize costs of health care. However, before we reach that point, there is still a long way to go.

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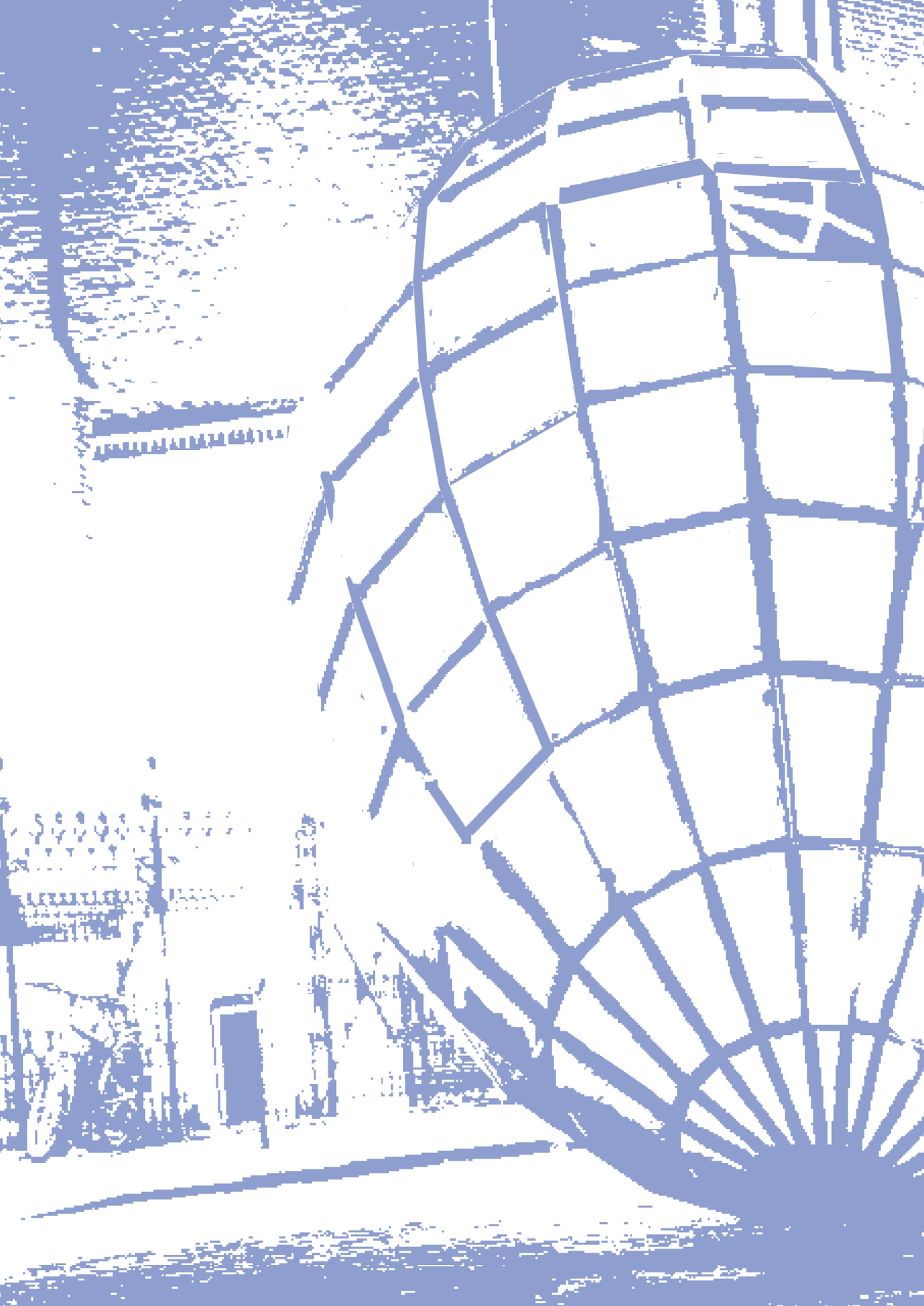

CHAPTER 7

Appendices

Summary

Cardiovascular disease (CVD) is an important cause of morbidity and mortality worldwide. The increasing incidence and prevalence of CVD constitutes a considerable disease burden and major health challenge for prevention and treatment. CVD frequently co-exists with other diseases such as type 2 diabetes mellitus (T2DM) and non-alcoholic fatty liver diseases (NAFLD), which are both strongly related to the metabolic syndrome. The 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase inhibitors or statins are cholesterol-lowering drugs that are beneficial in the primary and secondary prevention of CVD. With an approximately 20-25% reduction of the risk of major cardiovascular endpoints, these drugs have definitely entered daily clinical practice, and their use is expected to increase even more with a widening of therapeutic indications. However, current pharmacotherapy may not be optimal for all patients, and some individuals may not respond adequately to statins. This inadequate response may consist of a lack of therapeutic effect or the occurrence of adverse drug reactions (ADRs), and can have several underlying reasons. Insight into why people do not respond adequately to statins might improve clinical practice, can partly avoid these events, and may finally lead to tailored drug therapy. Moreover, in 2013, the American guidelines on primary prevention of CVD lowered the threshold for the indication for statin treatment, and thereby widened the target population for these already frequently prescribed drugs. This might have implications for current clinical practice, since prescribers should not ignore the potential risk of overtreatment or unintended effects that may go with this increased use.

Chapter 1 describes the current state of knowledge on the pharmacogenetics of response to cardiovascular drug therapy, written some years ago at the beginning of this PhD-project. Subsequently, in this thesis we focused on the pharmacogenetic epidemiology of statins in an ageing population. We describe genetic factors that modify the response to statins, whereby we discovered new genetic variation and validated previous findings from genome-wide research and other relevant literature on the pharmacogenetics of statins. Furthermore, we describe unintended effects of the use of statins in clinical practice, and methodological approaches to estimate statin effectiveness in observational studies.

All studies described in this thesis are embedded in the Rotterdam Study, a prospective population-based cohort study among 14,926 inhabitants of Ommoord, a suburb of Rotterdam, aged 45 years and older.

Genetic factors modifying statin response

In **chapter 2**, we investigated genetic variation that modified the efficacy and effectiveness of statins, and their risk of ADRs in clinical practice. We used the genome-wide association study (GWAS) approach to discover new genetic markers without *a priori* hypothesis of the underlying genetic variation, and the candidate gene approach to replicate genetic variation that has previously been associated with a modified statin response or occurred in a pathway that relates to statin pharmacokinetics. In three candidate gene studies, we investigated the influence of genetic polymorphisms on the cholesterol-lowering response to statins. In **chapter 2.1**, we investigated the role of genetic variation in genes involved in the cholesterol metabolism. We selected polymorphisms in these genes based on a hypothesis-free approach, and subsequently tested the most promising ones in a candidate gene analysis. We showed that two polymorphisms in two different genes, rs1532624 in the cholesteryl ester transfer protein (*CETP*) gene and rs533556 in the apolipoprotein A-I (*APOA1*) gene, were associated with a decreased cholesterol lowering response to statin therapy. The association for the *CETP* polymorphism was subsequently replicated in an independent population. Moreover, in **chapter 2.2**, we were the first study that demonstrated that two strongly linked polymorphisms in the peroxisome proliferator-activated receptor alpha (*PPARA*) gene, rs4253728 and rs4823613, were associated with a stronger cholesterol lowering response to statins. Thereby, we confirmed a pharmacokinetic mechanism which was previously discovered, namely that these two polymorphisms were associated with significantly decreased cytochrome P450 3A4 (CYP3A4) enzyme expression and activity. In **chapter 2.3**, we performed a first replication of a recent finding that the rs13064411 polymorphism was associated with increased statin-induced serum proprotein convertase subtilisin/kexin type 9 (PCSK9) concentrations, and cholesterol response to statins. PCSK9 binds to the low-density lipoprotein (LDL-) receptor, and subsequently promotes the receptor for degradation. We showed that the rs13064411 polymorphism was associated with a decreased cholesterol lowering response to statins, and this effect was stronger in women and in users of a high dose of statins.

In general, statins are safe and well-tolerated drugs, although a common ADR is myopathy, which can vary from myalgia to life-threatening rhabdomyolysis. In **chapter 2.4**, we confirmed the previously described association between the rs4149056 c.521T>C polymorphism in the solute carrier organic anion transporting polypeptide (*SLCO1B1*) gene and an increased risk of developing ADRs to statins. A previous GWAS showed that patients carrying two minor alleles had a 16.9 times higher risk of simvastatin-induced myopathy than patients carrying two major alleles. Within simvastatin users in the Rotterdam Study, we demonstrated that the rs4149056 polymorphism was associated with an increased risk of a dose decrease or switch to another cholesterol lowering drug,

as indicators for ADRs. For atorvastatin users, an association was found in users with a starting dose of more than 1.00 standardized defined daily doses.

The risk of myocardial infarction (MI) is indirectly influenced by statins via cholesterol lowering, but is also influenced by underlying diseases, such as hypertension and T2DM. The heterogeneity in causal risk factors for a hard clinical endpoint such as MI, may affect the probability of detecting one specific gene-statin interaction and may therefore require more power than with an intermediate endpoint. The *CYP3A4*22* polymorphism was previously associated with a stronger cholesterol lowering response to statins. Based on the magnitude of its effect on cholesterol, this polymorphism seemed a good candidate to investigate on the outcome MI. However, in **chapter 2.5**, we could not demonstrate significant effect modification by the *CYP3A4*22* polymorphism on the effect of statins in reducing the risk of MI, neither in the independent UCP study and Rotterdam Study separately, nor in a meta-analysis of the two studies.

Besides candidate gene studies, in **chapter 2.6** we investigated the LDL-cholesterol lowering response to statins in a GWAS, as part of the GIST consortium including more than 40,000 statin users in both randomized controlled trials and observational studies. In this large pharmacogenetic meta-analysis, two loci at Sortilin 1 (*SORT1*) and *SLCO1B1* were newly discovered to be associated with a stronger, and decreased LDL-cholesterol lowering response to statins, respectively. Furthermore, previously described associations with Apolipoprotein E (*APOE*) and Lipoprotein, Lp(a) (*LPA*) were confirmed, that showed a respectively stronger and decreased LDL-cholesterol lowering response.

Unintended effects of the use of statins in clinical practice

Statins are increasingly prescribed in clinical practice and although relatively safe, prescribers should not ignore potential ADRs that may occur. **Chapter 3** investigated unintended effects of the use of statins in daily practice. Possibly, statins have beneficial effects on diseases for which currently no therapeutic indication exists. In **chapter 3.1**, we did not find an overall association between current and past use of statins and NAFLD prevalence. In patients with a body mass index (BMI) ≥27.5, current use of statins for more than two years was significantly associated with an approximately three times lower NAFLD prevalence. With our study we provide further evidence for the safe use of statins in NAFLD patients. Since these patients frequently have dyslipidemia, and the major cause of death in NAFLD patients is CVD, statins may be beneficial. On the other hand, statins might have adverse effects that raise questions as to whether the risk-benefit balance is acceptable to allow the widespread use of these drugs in clinical practice. In **chapter 3.2** we demonstrated a non-beneficial effect of statins. Current use of statins was associated with lower total and (bioactive) non-sex hormone-binding globulin (SHBG)-bound testosterone levels in males. Statins decrease cholesterol production, and cholesterol is a precursor in the testosterone biosynthesis pathway. This

association should be further investigated but might be clinically relevant, given the important biological role of testosterone, the increased use of statins, and the fact that a modest average decrease in a population might hide a substantial decrease in a handful of individuals and in those with an already low testosterone level.

Estimating the effect of time-dependent statin use in observational studies

Drug exposure is essentially a time-varying determinant. In observational studies, effect estimates may be biased in the presence of a time-dependent co-variable which is simultaneously 1) a reason for prescribing or dose-changing (often termed 'confounding by indication'), 2) influenced by the drug under study, and 3) a potential cause of the outcome of interest. A method to deal with this time-varying confounding is marginal structural modeling (MSM), in which all observations are assigned a weight based on the conditional probability of receiving the observed treatment. In **chapter 4**, we compared MSM and traditional Cox proportional hazard models with and without time-dependent covariables using empirical data on time-varying statin use in the primary prevention of CVD. First, we could not observe a large difference in effect estimates between the results from the Cox models with and without time-dependent covariables. Second, we could not demonstrate important differences in risk estimates from MSM compared to the Cox models. Last, in our MSM, there was still substantial unmeasured confounding by indication in the initial period after statin initiation which was not accounted for. In general, although on theoretical grounds MSM is an elegant technique, lack of data on the precise time-dependent confounders, such as indication of treatment or other considerations of the prescribing physician jeopardizes the calculation of valid weights. Confounding remains a hurdle in observational effectiveness research on preventive drugs with a multitude of prescription determinants.

SHBG level as a biomarker for NAFLD

In **chapter 5**, we provided evidence for the assumption that a low serum SHBG level might be considered as an early biomarker for the development of NAFLD. In addition to previous cross-sectional studies, we created a longitudinal component by selecting at baseline a population with a very low probability of steatosis (defined as fatty liver index <60) and a serum SHBG measurement available, and investigated whether these participants developed NAFLD over a mean follow-up time of 11 years. We demonstrated that higher SHBG levels at baseline were associated with a lower risk of developing NAFLD during follow-up.

Conclusions

Finally, in **chapter 6** we discussed the main findings of this thesis and placed them in a broader perspective. We discussed methodological issues, speculated about the con-

sequences of widespread use of statins in clinical practice, and the implementation of pharmacogenetics in daily practice.

Overall, in this thesis we have provided more insight into the use of statins in an ageing population. Statins are expected to be used even more frequently in clinical practice, i.e. through widening of the indication for statins, the aging population and the increasing prevalence of welfare diseases associated with dyslipidemia and an increased CVD risk. Future research should investigate the consequences of this increased use for daily clinical practice. Hopefully, there will be a future role for genotype-based clinical decision making, with statin therapy tailored to an individual's needs on the basis of patient's specific characteristics.

Samenvatting

Hart- en vaartziekten (HVZ) zijn een belangrijke oorzaak van ziekte en sterfte wereldwijd. De toename in de incidentie en prevalentie van HVZ is een groot gezondheidsprobleem en vormt een grote uitdaging voor preventie en behandeling. HVZ gaan vaak samen met andere ziekten zoals type 2 diabetes mellitus (T2DM) en niet-alcoholische leververvetting, twee aandoeningen die beide sterk geassocieerd zijn met het metabool syndroom.

De 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase remmers, oftewel statines, zijn cholesterol-verlagende geneesmiddelen, die werkzaam zijn in de primaire en secundaire preventie van HVZ. Met circa 20 tot 25 procent afname in het risico op belangrijke cardiovasculaire eindpunten zijn deze geneesmiddelen niet meer weg te denken uit de huidige klinische praktijk. Er wordt verwacht dat het gebruik van statines alleen maar verder toe zal nemen, met een uitbreiding van therapeutische indicaties, zoals inflammatoire reumatische ziekten. Echter, in de klinische praktijk reageert niet iedere patiënt optimaal op statines, haalt bijvoorbeeld het beoogde therapiedoel niet of krijgt vaker bijwerkingen op statines. Dit kan verschillende oorzaken hebben. Inzicht in de vraag waarom mensen niet adequaat op statines reageren, kan de klinische praktijk verbeteren en het optreden van verminderde effectiviteit en bijwerkingen deels vermijden. Dit kan uiteindelijk leiden tot het voorschrijven van geneesmiddelen afgestemd op de individuele patiënt of bepaalde patiëntengroepen, zogenaamde 'personalized medicine'. Daarnaast is in de Amerikaanse richtlijnen voor primaire preventie van HVZ in 2013 de drempel voor het instellen van een behandeling met statines verlaagd, en daarbij de populatie vergroot, die in aanmerking komt voor deze reeds frequent voorgeschreven medicijnen. Dit heeft implicaties voor de huidige klinische praktijk. Voorschrijvers mogen niet het potentiële risico op overbehandeling of andere onbedoelde effecten negeren dat kan samengaan met deze toename in gebruik.

Hoofdstuk 1 beschrijft de huidige kennis over de farmacogenetica van verschillende cardiovasculaire geneesmiddelen. Dit is een aantal jaren geleden geschreven aan het begin van het PhD-project. Vervolgens richtte het proefschrift zich op de farmacogenetische epidemiologie van statines in een ouder wordende populatie. We beschrijven genetische factoren die de reactie op statines beïnvloeden, waarbij we nieuwe genetische variatie ontdekten en eerdere bevindingen van genoombreed onderzoek en andere relevante literatuur over de farmacogenetica van statines valideerden. Verder beschrijven we in dit proefschrift onbedoelde effecten van het gebruik van statines in de klinische praktijk, en behandelen we methodologische benaderingen om effectiviteit van statines in observationele studies te onderzoeken.

Alle studies beschreven in dit proefschrift zijn uitgevoerd binnen het Erasmus Rotterdam Gezondheid Onderzoek (Rotterdam Study), een prospectief observationeel bevolkingsonderzoek onder 14,926 inwoners van Ommoord, een deelgemeente van Rotterdam, in de leeftijd van 45 jaar en ouder.

Genetische factoren die de respons op statines beïnvloeden

In **hoofdstuk 2** onderzochten we genetische variatie die de werkzaamheid en doelmatigheid van statines, en het risico op bijwerkingen in de klinische praktijk beïnvloeden. Met gebruik van genoombrede associatiestudies (GWAS) wilden we nieuwe genetische markers ontdekken zonder een a priori hypothese over de onderliggende genetische variatie. Met behulp van de kandidaatgen benadering wilden we genetische variatie repliceren, die eerder geassocieerd was met een veranderde respons op statines of die zich in een 'pathway' bevindt die een rol speelt in de farmacokinetiek van statines. In drie kandidaatgen studies onderzochten we de invloed van genetische polymorfismen op de cholesterolverlagende respons van statines. In **hoofdstuk 2.1** onderzochten we de rol van genetische variatie in genen die betrokken zijn bij het cholesterol metabolisme. We selecteerden polymorfismen in deze genen gebaseerd op een hypothesevrije benadering, en testten vervolgens de meest belovende polymorfismen in een kandidaatgen analyse. We toonden aan dat twee polymorfismen in twee verschillende genen, rs1532624 in het cholesterol ester transfer protein (*CETP*) gen en rs533556 in het apolipoprotein A-I (*APOA1*) gen, waren geassocieerd met een verminderde cholesterolverlagende reactie op statines. We repliceerden de associatie voor het *CETP* polymorfisme vervolgens in een onafhankelijke populatie. Verder geven we in **hoofdstuk 2.2** de eerste studie weer, die aantoonde dat twee sterk gelinkte polymorfismen in het peroxisome proliferator-activated receptor alpha (*PPARA*) gen, rs4253728 en rs4823613, waren geassocieerd met een sterker cholesterolverlagend effect van statines. Daarbij bevestigden we een farmacokinetisch mechanisme dat eerder was ontdekt, namelijk dat deze twee polymorfismen waren geassocieerd met significant verlaagde cytochroom P450 3A4 (CYP3A4) enzym expressie en activiteit. In **hoofdstuk 2.3** repliceerden we als eerste de resultaten uit een recente studie, namelijk dat het rs13064411 polymorfisme was geassocieerd met toegenomen statine-geïnduceerde serum proprotein convertase subtilisin/kexin type 9 (PCSK9) concentraties, en cholesterolrespons op statines. PCSK9 bindt de low-density lipoprotein (LDL) receptor, en bevordert vervolgens de afbraak van de receptor. We toonden aan dat het rs13064411 polymorfisme was geassocieerd met een afgenomen cholesterolrespons op statines, en dit effect was sterker bij vrouwen en bij gebruikers van een hogere dosis statines.

Over het algemeen zijn statines veilige geneesmiddelen die goed worden verdragen, hoewel myopathie een veel voorkomende bijwerking is. Myopathie kan variëren van spierpijn (myalgie) tot rhabdomyolyse, een levensbedreigende overmatige afbraak

van spierweefsel. In **hoofdstuk 2.4** bevestigden we de eerder beschreven associatie tussen het rs4149056 c.521T>C polymorfisme in het solute carrier organic transporting polypeptide (*SLCO1B1*) gen en een toegenomen risico op bijwerkingen van statines. Een eerdere GWAS toonde aan dat patiënten met twee minor allelen (de variant, het minst voorkomende allel in de populatie) een 16.9 maal hoger risico hadden op simvastatine-geïnduceerde myopathie dan patiënten met twee major allelen. Bij gebruikers van simvastatine in de Rotterdam Study toonden we aan dat het rs4149056 polymorfisme was geassocieerd met een toegenomen risico op een dosis verlaging of het switchen naar een ander cholesterolverlagend geneesmiddel, als indicatoren voor een bijwerking. Bij gebruikers van atorvastatine vonden we een toegenomen risico bij gebruikers van een hoge startdosering.

Het risico op een myocard infarct (MI) wordt indirect beïnvloed door statines via het verlagen van cholesterol maar wordt ook beïnvloed door onderliggende ziekten zoals hypertensie en T2DM. De heterogeniteit in causale risicofactoren in het geval van een harde klinische uitkomst zoals MI kan de kans op het detecteren van een specifieke gen-statine interactie beïnvloeden, en kan daardoor meer power vereisen dan in het geval van een tussenliggend eindpunt zoals cholesterol. Het *CYP3A4*22* polymorfisme was eerder geassocieerd met een sterkere cholesterolverlagende reactie op statines. Gezien de grootte van het effect van het polymorfisme op cholesterolconcentraties, leek dit polymorfisme een geschikte kandidaat om te onderzoeken op de uitkomstmaat MI. Echter, in **hoofdstuk 2.5** konden we geen significante effect modificatie door het *CYP3A4*22* polymorfisme aantonen van statines op het risico op MI, noch in de UCP study en Rotterdam Study apart, noch in een meta-analyse van de twee studies samen.

Naast kandidaatgen studies, onderzochten we in **hoofdstuk 2.6** de cholesterolverlagende respons op statines in een GWAS, als onderdeel van het GIST consortium dat meer dan 40,000 statinegebruikers omvat in zowel gerandomiseerde gecontroleerde studies als in observationele studies. In deze grote farmacogenetische meta-analyse werden twee loci op Sortilin 1 (*SORT1*) en *SLCO1B1* ontdekt die waren geassocieerd met een sterkere en verminderde LDL-cholesterolverlagende respons op statines. Verder werden eerder beschreven associaties met Apolipoprotein E (*APOE*) en Lipoprotein, Lp(a) (*LPA*) bevestigd, die een respectievelijk sterkere en verminderde LDL-cholesterolverlagende respons vertoonden.

Onbedoelde effecten van het gebruik van statines in de klinische praktijk

Statines worden steeds meer voorgeschreven in de klinische praktijk en hoewel deze geneesmiddelen relatief veilig zijn, mogen voorschrijvers niet het potentiële risico op bijwerkingen negeren. In **hoofdstuk 3** onderzochten we onbedoelde effecten van het gebruik van statines in de dagelijkse praktijk. Mogelijk hebben statines gunstige effecten op ziektes waarvoor op dit moment geen therapeutische indicatie bestaat.

In **hoofdstuk 3.1** vonden we geen associatie tussen huidig gebruik van statines en de prevalentie van non-alcoholische leververvetting. Ook bleek er geen associatie te zijn tussen gebruik van statines in het verleden en non-alcoholische leververvetting. In patiënten met een body mass index (BMI) ≥27.5 was huidig gebruik van statines voor een periode van meer dan twee jaar geassocieerd met significante, ongeveer drie maal lagere prevalentie van non-alcoholische leververvetting. Met onze studie leveren we verder bewijs voor het veilige gebruik van statines in patiënten met non-alcoholische leververvetting. Aangezien deze patiënten frequent dyslipidemie hebben, en de belangrijkste doodsoorzaak in deze patiënten HVZ is, kan het gebruik van statines gunstig zijn. Aan de andere kant kunnen statines bijwerkingen veroorzaken, hetgeen altijd de vraag moet oproepen of de balans werkzaamheid/schadelijkheid het wijdverspreide gebruik van deze geneesmiddelen in de klinische praktijk rechtvaardigt. In **hoofdstuk 3.2** toonden we een ongunstig effect van statines aan. Huidig gebruik van statines was geassocieerd met lagere totaal en (bioactief) niet-gebonden testosteronconcentraties bij mannen. Statines verlagen de cholesterolproductie, en cholesterol is een voorloper in de testosteron biosynthese 'pathway'. Deze associatie moet verder worden onderzocht maar kan klinisch relevant zijn, gezien de belangrijke biologische rol van testosteron, het toegenomen gebruik van statines, en het feit dat een relatief kleine daling op populatieniveau een substantiële daling kan maskeren bij een handvol individuen en bij diegenen met een al lage testosteronspiegel.

Het schatten van het effect van tijdsafhankelijk statinegebruik in observationele studies

Blootstelling aan geneesmiddelen is in principe een over de tijd variërende determinant. In observationele studies kan er vertekening (bias) zijn van effectschattingen indien er een tijdsafhankelijke covariabele aanwezig is die gelijktijdig 1) een reden is voor voorschrijven of een verandering in dosering (vaak 'confounding by indication' genoemd), 2) wordt beïnvloed door het geneesmiddel dat bestudeerd wordt, 3) een onafhankelijke oorzaak kan zijn van de uitkomst van de studie. Een methode om met deze tijdsafhankelijke confounding om te gaan is 'marginal structural modeling (MSM)', waarbij alle observaties een gewicht toegewezen krijgen gebaseerd op de conditionele kans op het krijgen van de geobserveerde behandeling. In **hoofdstuk 5** vergeleken we MSM en traditionele Cox proportionele hazard modellen met en zonder tijdsafhankelijke covariabelen, met gebruik van empirische data over tijdsafhankelijk statinegebruik voor de primaire preventie van HVZ. Allereerst konden we geen groot verschil aantonen in effectschattingen tussen de resultaten van de Cox modellen met en zonder tijdsafhankelijke covariabelen. Ten tweede konden we geen belangrijke verschillen aantonen in risicoschattingen van MSM vergeleken met de Cox modellen. Als laatste, bij de MSM methode was er nog steeds substantiële niet-gemeten 'confounding by indication' in

de eerste periode na het starten van een statine, waar geen rekening mee kon worden gehouden. In het algemeen kan geconcludeerd worden dat, hoewel op theoretische gronden MSM een elegante techniek is, gebrek aan data over de precieze tijdsafhankelijke confounders, zoals de indicatie voor behandeling of andere overwegingen van de voorschrijvende arts, een obstakel vormt bij het berekenen van valide gewichten. Confounding blijft een lastig probleem bij observationeel doelmatigheidsonderzoek naar preventieve medicijnen met een veelvoud aan determinanten van voorschrijven.

SHBG als een biomarker voor NAFLD

In **hoofdstuk 5** leveren we bewijs voor de hypothese dat een lage serumwaarde van 'Sex Hormone-Binding Globulin' (SHBG) kan worden overwogen als een vroege biomarker voor het ontwikkelen van NAFLD. In aanvulling op eerdere cross-sectionele studies creëerden we een longitudinale component door op baseline een populatie te selecteren met een erg lage kans op steatose (gedefineerd als een 'fatty liver index' <60) die een serum SHBG meting beschikbaar had. We onderzochten of deze deelnemers non-alcoholische leververvetting ontwikkelden over een gemiddelde follow-up periode van 11 jaar. We toonden aan dat hogere SHBG concentraties op baseline waren geassocieerd met een lager risico op het ontwikkelen van non-alcoholische leververvetting gedurende follow-up.

Conclusies

Als laatste bediscussieerden we in **hoofdstuk 6** de belangrijkste bevindingen van dit proefschrift en plaatsten we deze in een breder perspectief. We bediscussieerden methodologische aspecten, speculeerden over de consequenties van het wijdverspreid gebruik van statines in de klinische praktijk, en de implementatie van farmacogenetica in de dagelijkse praktijk.

Concluderend, in dit proefschrift leverden we meer inzicht in het gebruik van statines in een ouder wordende populatie. Er wordt verwacht dat het gebruik van statines in de klinische praktijk alleen maar verder zal toenemen, onder andere door het verbreden van de indicatie voor statines, de vergrijzende populatie en de toename in prevalentie van welvaartsziekten die geassocieerd zijn met dyslipidemie en een toegenomen risico op HVZ. Toekomstig onderzoek moet de gevolgen voor de klinische praktijk van deze toename in gebruik onderzoeken. Mogelijk is er een toekomstige rol voor klinische besliskunde gebaseerd op het genotype met statinetherapie toegespitst op de behoeften van een individu op de basis van patiëntspecifieke karakteristieken.

Dankwoord

Bijna vier jaar geleden startte ik met het promotieonderzoek gebundeld in dit proefschrift. Ik heb nooit spijt gehad van mijn vastberaden keus om te gaan promoveren, en kijk met plezier terug op de jaren in het onderzoek. Naast dat ik met dit promotietraject uiteindelijk de doctorsgraad bereik, was het tevens een weg waarin ik mij binnen de wereld van de wetenschap en als persoon ontwikkeld heb. Tijdens dit traject hebben vele naasten mij terzijde gestaan. Ik wil hierbij met heel mijn (blauwe) hart, iedereen bedanken die voor mij tijdens dit traject belangrijk is geweest.

Allereest wil ik mijn promotoren Prof.dr. B.H.Ch. Stricker en Prof.dr. A.G. Uitterlinden bedanken. Beste Bruno, vanaf dat ik als masterstudent voor het eerst aan wetenschappelijk onderzoek begon, heb je mij met je creativiteit en gedrevenheid enthousiast gemaakt voor het epidemiologisch onderzoek. Bedankt voor de kans en ruimte die je me gaf om me binnen het onderzoek te ontwikkelen, waarbij je me met je ervaring en brede kennis van het vak stimuleerde om veel verschillende projecten te onderzoeken. Maar daarnaast waardeer ik ook zeer je collegialiteit, humor, eigenzinnigheid en vermakelijke doch wijze levenslessen. André, bedankt voor je kennis en bijdrage wat betreft de genetische kant van het onderzoek. Mede door jouw inzet zijn er in Rotterdam goede mogelijkheden voor internationaal genetisch onderzoek, wat voor mij een leerzame ervaring is geweest.

Mijn copromotor Dr. L.E. Visser, beste Loes, bedankt voor je nuchterheid en het geloof dat je in mij toonde. Naast dat je een kritische en snelle reactie op manuscripten en analyses gaf, wist je me soms ook het nodige relativeringsvermogen en vertrouwen in mijn onderzoek te geven.

Dank aan Prof.dr. T. van Gelder, Prof.dr. J.L.C.M. van Saase, en Prof.dr. O.H. Klungel voor het deelnemen in de kleine commissie en de inhoudelijke beoordeling van dit proefschrift. Professor van Gelder, beste Teun, via jou heb ik kennis gemaakt met de klinische farmacologie, een mooi en dynamisch vak dat daarom altijd hoog op de lijst stond als mogelijk aandachtsgebied binnen de interne geneeskunde. Professor van Saase, bedankt voor de mogelijkheid die u mij gaf door te mogen starten met de opleiding tot het mooiste vak: internist. Professor Klungel, de samenwerking met de UCP study in Utrecht hebben mijn promotietraject verbreed, en ik heb deze samenwerking als prettig en leerzaam ervaren.

Dank aan Prof.dr. R.H.N. van Schaik en Prof.dr. E.P. van Puijenbroek voor hun bereidheid om zitting te nemen in de grote commissie. Professor van Schaik, beste Ron, de

farmacogenetische studies waarin een polymorfisme via een verklaarbaar biologisch mechanisme de respons op een geneesmiddel bepaalde, bleven mijn favoriete onderzoeken.

Beste Matthijs, aan het begin van mijn onderzoek initieerde je projecten die geleid hebben tot een aantal mooie publicaties, maar daarnaast kon ik altijd bij je terecht voor vragen bij revisies op papers ondanks je drukke baan. Bedankt voor je betrokkenheid en de fijne samenwerking.

Alle co-auteurs wil ik bedanken voor hun bijdrage aan diverse papers.

Alle collega's van de afdelingen Epidemiologie en Medische Informatica wil ik bedanken voor de leuke jaren, de discussies, de goede sfeer en gezelligheid die er heerste op werk, de ervaringen tijdens congressen, en de mooie samenwerkingen. Beste Prof.dr. A. Hofman, de master Klinische Epidemiologie heeft mijn kennis en mogelijkheden verbreed. Ik ben dankbaar dat ik nu een proefschrift mag afronden waarin ik gebruik heb gemaakt van de gegevens van het ERGO-onderzoek (Rotterdam Study). Daarom ook veel dank aan de deelnemers van het ERGO-onderzoek in Ommoord, de medewerkers van het ERGO-centrum, de huisartsen en apothekers binnen het ERGO-onderzoek, en de medewerkers van het STAR-lab voor hun bijdrage.

Mijn promotietraject was nog leuker door de aanwezigheid van de meisjeskamer, opeens in het leven geroepen door een verhuizing van de ene toren naar de andere toren. Brenda, Kiki, Lies, Marieke en Nikkie: bedankt voor jullie hulp bij lastige methodologie en kromme zinnen, jullie steun als het soms even tegen zat, maar bovenal voor de gezelligheid tijdens werk en daarbuiten. Ik hoop dat er nog vele 'meisjeskamer-uitjes' zullen volgen!. En ze blijven hangen hè, zoek maar een nieuwe collega met net zo'n goede smaak als ik. Mannen, Robert-Jan, Raymond en Marten: gelukkig waren jullie er als tegenhanger, soms zuchtend en schouder-ophalend, maar ook zeer behulpzaam bij onbegrijpelijke analyses.

Maarten, jouw kennis en kritische (cynische) blik waren soms onuitputtend, bedankt voor de mooie samenwerking. Daan, als voorheen goede collega op het onderzoek en bij de inspectie, ben ik blij dat ik je nu weer als collega vol Brabantse gezelligheid terug heb. Myrte en Gwen, ik hoop dat er nog veel culturele avondjes uit zullen volgen! Edith, Mariana, Annemarie, Lisette, Marjolein, Inge, Annelies: bedankt voor de afwisseling van werk met leuke klets/koffie/lunch/congres-momenten. Rikje, bedankt voor de samenwerking op projecten en steun tijdens onderzoek, ik bewonder je gedrevenheid en positieve insteek hierbij. Ik hoop dat er nog vele etentjes en internistendagen zullen volgen!

Mijn promotieonderzoek combineerde ik met een baan bij de Inspectie voor de Gezondheidszorg, binnen het programma Geneesmiddelen en Medische Technologie. Dank

aan mijn collega's bij de IGZ voor het verbreden van mijn kennis binnen het systeem overheid/gezondheidszorg; de gezelligheid tijdens lunch, verbindingsdagen en een cultureel avondje weg: het was een goede afwisseling naast het onderzoek! Janny en Rajka, dank voor de ondersteuning bij alles wat ingevuld en bijgehouden moest worden; en Janny ook voor het heerlijke geklets samen. Jitske, bedankt voor je betrokkenheid. Maris, bedankt voor je collegialiteit, ik ging graag samen op leuke en interessante inspecties. Ik zal ons belachelijke shopgedrag samen met Brenda afgelopen zomer niet snel vergeten.

Sinds 1 januari heb ik er een hele schare aan collega's bij in het Amphia ziekenhuis. Bedankt voor het warme welkom in een leuke groep met enthousiaste collega's. Ik kijk uit naar de samenwerking de komende jaren en heb er alle vertrouwen in dat deze periode erg leerzaam zal zijn, afgewisseld met gezellige (met zachte g) momenten.

En niet te vergeten een groot woord van dank aan de vele vrienden en vriendinnen om mij heen. Jullie aanwezigheid en gezelligheid doen me beseffen hoe belangrijk en mooi het leven naast het drukke werkende leven is. Bedankt voor alle momenten samen, jullie steun en betrokkenheid. Een promotietraject is zo veel leuker als het wordt afgewisseld met fijne en hilarische AH-momenten met Marie-Emilie; bourgondische etentjes met Gaya; glaasjes port met mede-liefhebber Rianne (paplepel toch?); sportieve hardloop-rondjes met Alyt; gezellige avonden met de Eetclub; Amerika-sessies; gezellige momenten met oud-koordames Karlijn, Marjolein, Tirza, Maritgen en Carina, en met mijn vriendinnen uit Delft; geweldige Roparuns met leuke sportieve lui; en op weg naar een mooi doel samen met collega's van het Leontienhuis. Marijke, ik drink graag een goede wijn op de prachtige voorkant! Hild, wat bijzonder om al zo lang vriendinnen te zijn! Eef, je betrokkenheid en steun de afgelopen jaren waren erg belangrijk voor mij, laten we nog vaak gezellige momenten samen plannen! Ellen, alias dokter van de Wouw, bedankt voor je luisterende oor en fijne momenten samen. Het past wel bij ons planmatig karakter dat deze datum uitkomt, bedankt dat je mij terzijde wilt staan als paranimf! Lieve Bas, op naar nog vele malen live long!

En dan natuurlijk ook dank aan al mijn familieleden. Lieve opa de Keyser, wat fijn dat u bij dit moment bent; lieve oma, wat jammer om u te moeten missen. Lieve oma Postma, fijn dat u er ook bent als bijna 92-jarige toch, een deel van het wetenschapsbloed heb ik vast van opa.

Ik prijs me zeer gelukkig met een lieve broer en lieve zussen. Krijn, ik bewonder je om de manier waarop jij je weg gaat in je carrière en daarbuiten, gedreven en met een sociaal hart. Sien, ik had me geen betere schoonzus kunnen bedenken tussen de drie andere zusjes! Ceciel, ik ga ervan uit dat je dansend je weg vindt naar de mooie

toekomst, ik hoop samen nog veel te ondernemen. Mirte, ik waardeer onze gesprekken en dagjes samen erop uit zeer, waarin naast al het geklets over onder andere mode en werk/studie, je luisterende oor voor mij belangrijk was. Fijn dat je mij terzijde wilt staan als paranimf. Puck, vol enthousiasme en energie ga je je eigen weg, met daarin als grote passie de muziek: ik ben fan!

Lieve mama en papa, de grootte van jullie hart is niet in woorden uit te drukken, en had niet op de voorkant van dit proefschrift gepast. Bedankt voor alle mogelijkheden die jullie mij hebben gegeven om mij te ontwikkelen tot wie ik nu ben; jullie alom aanwezige geloof en vertrouwen in mij; en jullie betrokkenheid en interesse. Het is heel fijn om een warm liefdevol thuis te hebben waar ik nog graag naar terug kom.

List of publications

Manuscripts based on this thesis

1.1 **de Keyser CE**, Eijgelsheim M, Uitterlinden AG, Stricker BH. Pharmacogenetics of response to cardiovascular drug therapy: what is the current state of knowledge? *Dialogues in Cardiovascular Medicine* 2012; 17(4): 281-292

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Phd Portfolio

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PhD period:	2011-2014
Supervisors:	Prof.dr. B.H.Ch. Stricker
	Prof.dr. A.G. Uitterlinden

1. PhD training

Research skills and in-depth courses

2006-2009	Master of Health Sciences, specialization Clinical Epidemiology, Netherlands Institute for Health Sciences (NIHES), Rotterdam, the Netherlands.
2008	SNPs and Human Diseases, Molecular Medicine, Rotterdam, the Netherlands.
2013	Pharmacovigilance Inspectors Working Group Training, European Medicines Agency, London, United Kingdom.
2011-2014	Research Seminars, Department of Epidemiology, Erasmus Medical Center, Rotterdam, the Netherlands.

International conferences and presentations

2009	25th International Conference on Pharmacoepidemiology & Therapeutic Risk Management, Providence, USA. ▪ Single nucleotide polymorphisms in genes that are associated witha modified response to statin therapy: the Rotterdam Study. *Oral presentation; Award for the Best Molecular Epidemiology/Pharmacology Abstract submitted by a student for presentation*
2011	CHARGE Investigators Meeting, Los Angeles, USA.
2012	Netherlands Consortium for Healthy Ageing Congress, Amersfoort, the Netherlands.
2012	CHARGE Investigators Meeting, Reykjavik, Iceland.
2012	28th International Conference on Pharmacoepidemiology & Therapeutic Risk Management, Barcelona, Spain. ▪ The *SLCO1B1* c.521T>C polymorphism is associated with dose decrease or switching during statin therapy in the Rotterdam Study. *Oral presentation*

2013 CHARGE Investigators Meeting, Rotterdam, the Netherlands.

2013 29th International Conference on Pharmacoepidemiology & Therapeutic Risk Management, Montréal, Canada.

- Genetic variation in the *PPARA* gene is associated with simvastatin-induced cholesterol reduction in the Rotterdam Study. *Oral presentation*
- Statin therapy is associated with a reduced risk of non-alcoholic fatty liver in overweight individuals. *Oral presentation*

2014 Nederlandse Vereniging voor Klinische Farmacologie en Biofarmacie, Mededelingendag 2014, Leiden, the Netherlands.

- Statin therapy is associated with a reduced risk of non-alcoholic fatty liver in overweight individuals. *Oral presentation*

2. Teaching

Supervising practicals

2009-2012 Pharmacoepidemiology, 4th year medical students, Erasmus Medical Center, Rotterdam, the Netherlands.

2012-2014 Data-analysis in Pharmacoepidemiology practicals, NIHES, Rotterdam, the Netherlands.

Supervising students

2014 Supervision of Filipe Valério de Lima, research project on the association between statins and serum testosterone levels in the Rotterdam Study, Erasmus Medical Center, Rotterdam, the Netherlands.

About the author

Catherine Elisabeth de Keyser was born on February 27th 1986 in Delft, the Netherlands. In 2004, she graduated from secondary school at the Christelijk Lyceum Delft. In the same year, she started the study Medicine at the Erasmus University of Rotterdam. During her second year in medical school, she started with a Master of Science programme in Clinical Epidemiology at the Netherlands Institute for Health Sciences in Rotterdam. She attended the summer school of the Johns Hopkins Bloomberg School of Public Health in Baltimore, USA. For the study Medicine, she did an internship Emergency Medicine at the Academic Hospital in Paramaribo, Suriname. In 2009, she obtained the Master of Science degree in Clinical Epidemiology, and in 2011, she obtained her Medical degree (*cum laude*).

During 2008-2009, as part of the study Medicine and Master of Science in Clinical Epidemiology, she performed a research project at the department of Epidemiology (*head:* Prof.dr. A. Hofman) on the pharmacogenetics of response to statin therapy, under the supervision of Prof.dr. B.H.Ch Stricker. This research project resulted in a PhD project, that started in 2011, and which resulted in the work described in this thesis.

During her study and PhD period, she was an active member of the Erasmus Students choir, participated in the Roparun as a cyclist and as a runner, and since 2014, she is active as a volunteer for the Leontienhuis.

In January 2015, she started her residency in Internal Medicine at the Amphia Hospital in Breda (*head:* Dr C. van Guldener/Dr. J.W. van Esser) as part of her specialist training at the Erasmus Medical Center (*head*: Prof.dr. J.L.C.M. van Saase/Dr. S.C.E. Klein Nagelvoort-Schuit).