GENETIC AND METHODOLOGICAL ASPECTS OF STATIN-INDUCED LIPID RESPONSE

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PART I

General aspects

CHAPTER 1

General introduction and outline of thesis

Cardiovascular disease (CVD) remains a leading cause of morbidity and mortality for all regions of the world. In 2015, there were an estimated 422.7 million prevalent cases of CVD (95% CI: 415.5 to 427.9) and 17.9 million CVD deaths (95% CI: 17.6 to 18.3) (1). Despite this substantial health loss, we should recognize that identification of causal risk factors for atherosclerosis throughout the past century has enabled us to make great strides in tackling CVD occurrence through preventive strategies.

The significance and role of cholesterol in the formation of atherosclerosis was first described in 1913 by Anichkov, who observed that feeding rabbits cholesterol induced the development of aortic atheromatous plaques (2). Shortly thereafter, Bacmeister and Henes demonstrated that elevated cholesterol concentrations associated with atherosclerosis, diabetes, and kidney disease in humans (3). Since then, observational studies have repeatedly shown that particularly low-density lipoprotein (LDL) cholesterol concentrations positively associate with CVD risk. For example, among 44,234 individuals without initial vascular disease, the hazard ratio for coronary heart disease (adjusted for several conventional factors) was observed to be 1.38 (95% CI 1.09-1.73) per standard deviation (0.85 mmol/L) increase in directly measured LDL cholesterol (4).

A multitude of interventional studies of LDL cholesterol lowering strategies across the spectrum of baseline cardiovascular risk have provided compelling evidence that LDL cholesterol has a causal effect on CVD risk. Amongst the pharmacological interventions, HMG-CoA reductase inhibitors, more commonly known as statins, are perhaps the most well-established and well-tolerated class of LDL cholesterol lowering drugs, having shown their worth both in primary and secondary prevention settings (5,6). As a result, statins were the drug class of choice for over 95% of the 2.1 million individuals who used cholesterol level-lowering drugs in the Netherlands in 2016 (7). It is likely that the introduction and proliferation of statin treatment can be counted among the key drivers of the serum total cholesterol-decreasing trend found in Australasia, North America, and western European countries between the 1980s and 2000s (8), notably coinciding with decreasing age-specific rates of CVD occurrence.

However, there exists a large inter-individual variability in response to statin therapy. For example, 13% of the subjects allocated to statin treatment within the PROspective Study of Pravastatin in the Elderly at Risk (PROSPER) did not reach >10% LDL cholesterol lowering after 36 months of pravastatin treatment (9). Similarly, a cross-sectional study of 57,885 statin-treated outpatients demonstrated that only 21.7% of the patients classified at very high risk for

cardiovascular disease attained their LDL cholesterol goals (10). Possible drawbacks regarding statin therapy have also been reported, including a modestly increased risk of new-onset type 2 diabetes (T2D) (11). Finally, it was recently reported that intra-individual variability in LDL cholesterol may associate with risk for both coronary and cerebrovascular events, and that adherence to statin treatment may play a key role (12, 13).

Genetic epidemiology may contribute to our understanding of these issues. For example, it is increasingly recognized that genetic factors also contribute to (non-)response to statin therapy, in addition to (in)adequate dosing and (non-) adherence to treatment (14-16). Pharmacogenomics is a promising research field aimed at discovering genetic variation related to intended and/or unintended drug effects. The large international Genomic Investigation of Statin Therapy (GIST) consortium aims to accomplish this for both lipid and non-lipid response to statin treatment. This collaboration between investigators from different statin trials and prospective population-based cohorts has already led to the discovery of multiple genetic loci of importance to statin-induced LDL cholesterol lowering response (17). Furthermore, methods have been developed to utilize summary level data from genome-wide association studies (GWAS) to estimate causal effects through Mendelian randomization studies, in which germline genetic variants are proposed as proxies ('instruments') for typically modifiable exposures or disease risk factors. This approach, which aims to avoid issues difficult to fully take into account in conventional observational epidemiology (most notably residual confounding and reverse causality), might also be applied to predict unintended drug effects (18).

OUTLINE OF THIS THESIS

In this thesis, we delve both into genetic and methodological aspects of research on statin-induced lipid response, taking a closer look at some of the issues raised above. In **Chapter 2** in **Part I** of this thesis, we provide an introduction to the principles behind Mendelian randomization studies. In **Part II** of this thesis we look at statin pharmacogenomics and how Mendelian randomization studies might complement this field of research. In **Chapter 3** we first describe considerations and assumptions of different response phenotypes and study designs popular in pharmacogenetic research. In **Chapter 4** we present the results of the largest pharmacogenetic meta-analysis of GWAS of high-density lipoprotein (HDL) cholesterol response to statins to date within the GIST consortium. In **Chapter 5** we combine data from the GIST consortium's

previously published pharmacogenetic meta-analysis on statin-induced LDL cholesterol response with publicly available data from the Global Lipids Genetics Consortium (GLGC) in a two-sample Mendelian randomization study, to examine whether overall genetic predisposition to LDL cholesterol has a causal effect on LDL cholesterol response to statin therapy. In **Chapter 6** we subsequently combine summary-level data from the GIST- and DIAbetes Genetics Replication And Meta-analysis (DIAGRAM) consortia to perform a bidirectional two-sample Mendelian randomization study, examining whether the presence of T2D has a causal effect on LDL cholesterol response to statin therapy, and vice versa.

Although often assumed to give a valid causal estimate, it has been argued that Mendelian randomization studies can give biased results when performed in selected subgroups. For example, older populations necessarily consist of the non-random subset of individuals who have survived until study inclusion. Therefore, in **Chapter 7** we explore the problem of survival bias in Mendelian randomization studies through simulations of simple causal structures. In **Part III** of this thesis, we turn our attention to the interaction between statins and visit-to-visit lipid variability. In **Chapter 8**, using data from the PROSPER trial, we perform cross-sectional analyses to examine whether intra-individual variability in LDL cholesterol concentrations associates with cognitive test performance and magnetic resonance imaging outcomes in an older population at high risk for vascular disease. Given the possible influence of treatment adherence, we performed these analyses stratified by statin use. In **Chapter 9** we provide an overview of the current evidence linking visit-to-visit lipid variability to (sub)clinical outcomes, discuss its interplay with lipid-lowering treatment, and describe the existing literature into possible genetic factors of interest. We supplement this discussion with an explorative GWAS on visit-to-visit variability of LDL- and HDL cholesterol. Finally, in **Chapter 10** in **Part IV** the main findings are presented and discussed, and we offer some future perspectives for the field.

REFERENCES

1 Roth GA, Johnson C, Abajobir A, et al. Global, Regional, and National Burden of Cardiovascular Diseases for 10 Causes, 1990 to 2015. J Am Coll Cardiol. 2017;70:1-25.

2 Anitschkow NN. Über die veränderungen der kaninchenaorta bei experimenteller Cholesterinsteatose. Beitr Pathol Anat Allgem Pathol. 1913;56:379-404.

3 Bacmeister A, Henes E. Untersuchungen über den Cholesteringehalt des menschlichen Blutes bei verschiedenen inneren Erkrankungen. Deuts Med Wochenschr 1913;39:544-546.

4 Di Angelantonio E, Sarwar N, Perry P, et al. Major lipids, apolipoproteins, and risk of vascular disease. JAMA. 2009;302:1993-2000.

5 Taylor F, Huffman MD, Macedo AF, et al. Statins for the primary prevention of cardiovascular disease. Cochrane Database Syst Rev. 2013;1:CD004816.

6 Cholesterol Treatment Trialist' (CTT) Collaborators. Efficacy and safety of LDL-lowering therapy among men and women: meta-analysis of individual data from 174 000 participants in 27 randomised trials. Lancet. 2015;385:1397-1405.

7 https://www.sfk.nl/publicaties/data-en-feiten/data-en-feiten-2017.

8 Farzadfar F, Finucane MM, Danaei G, et al. National, regional, and global trends in serum total cholesterol since 1980: systematic analysis of health examination surveys and epidemiological studies with 321 country-years and 3·0 million participants. Lancet. 2011;377:578-586.

9 Shepherd J, Blauw GJ, Murphy MB, et al. Pravastatin in elderly individuals at risk of vascular disease (PROSPER): a randomised controlled trial. Lancet. 2002;360:1623-1630.

10 Gitt AK, Lautsch D, Ferrieres J, et al. Low-density lipoprotein cholesterol in a global cohort of 57,885 statin-treated patients. Atherosclerosis. 2016;255:200-209.

11 Sattar N, Preiss D, Murray HM, et al. Statins and risk of incident diabetes: a collaborative meta-analysis of randomised statin trials. Lancet. 2010;375:735-742.

12 Bangalore S, Breazna A, DeMicco DA, et al. Visit-to-visit low-density lipoprotein cholesterol variability and risk of cardiovascular outcomes: insights from the TNT trial. J Am Coll Cardiol. 2015;65:1539-1548.

13 Baber U, Halperin JL. Variability in low-density lipoprotein cholesterol and cardiovascular risk: should consistency be a new target? J Am Coll Cardiol. 2015;65:1549-1551.

14 Heintjes EM, Penning-van Beest FJ, Plat AW, et al. Cholesterol level goal attainment with statins: clinical management guideline recommendations versus management in actual clinical practice. Pharmacotherapy. 2012;32:631-641.

15 Rodondi N, Peng T, Karter AJ, et al. Therapy modifications in response to poorly controlled hypertension, dyslipidemia, and diabetes mellitus. Ann Intern Med. 2006;144:475-484.

16 Watanabe JH, Bounthavong M, Chen T. Revisiting the medication possession ratio threshold for adherence in lipid management. Curr Med Res Opin. 2013;29:175-180.

17 Postmus I, Trompet S, Deshmukh HA, et al. Pharmacogenetic meta-analysis of genome-wide association studies of LDL cholesterol response to statins. Nat Commun. 2014;5:5068.

18 Walker VM, Davey Smith G, Davies NM, Martin RM. Mendelian randomization: a novel approach for the prediction of adverse drug events and drug repurposing opportunities. Int J Epidemiol. 2017;46:2078-2089.

CHAPTER 2

Using genetic variation for establishing causality of cardiovascular risk factors: overcoming confounding and reverse causality

Roelof AJ Smit, Stella Trompet, Anton JM de Craen, J Wouter Jukema

Netherlands Heart Journal. 2014;22(4):186-9.

ABSTRACT

Cardiovascular disease (CVD) remains the leading cause of death in developed countries, despite the decline of CVD mortality over the last two decades. From observational, predictive research, efforts have been made to find causal risk factors for CVD. However, in recent years some of these findings have been shown to be mistaken. Possible explanations for the discrepant findings are confounding and reverse causation. Genetic epidemiology has tried to address these problems through the use of Mendelian randomization. In this paper we discuss the promise and limitations of using genetic variation for establishing causality of cardiovascular risk factors.

Cardiovascular disease (CVD) remains the leading cause of death in developed countries. This is unlikely to change within the near future, despite the decline of CVD mortality over the last two decades (1). One of the pivotal studies that broadened our understanding of cardiovascular risk is the Framingham Heart Study. Since its inception in 1948, this study has identified various major risk factors contributing to CVD, including hypertension and elevated lipid concentrations (2). Moreover, the Framingham Study has generated one of the first multivariate cardiovascular risk prediction scores (3). From observational, predictive research, efforts have been made to also assess likely causal relationships. However, in recent years some of these findings have been called into question and ultimately proven wrong.

One of the most profound examples of such high-profile misidentification is the risk-lowering effect of hormone-replacement therapy on coronary heart disease found in observational studies, leading to widespread prescription of hormones for post-menopausal women. Subsequent randomized controlled trials (RCTs) showed that hormone therapy not only fails to lower cardiovascular risk, it may even increase mortality risk and lead to other adverse clinical outcomes (4, 5). Similar over-turnings were seen for vitamins E and C after RCTs disproved any cardioprotective effects (6). It has been argued that the most likely explanations for these discrepancies have been confounding by environmental and behavioral factors, baseline health status and prescription policies, combined with reverse causation and selection bias (7). This shows that observational studies have certain weaknesses. Similar limitations might be present for RCTs, which are still viewed as the gold standard in estimation of causality. Firstly, it is sometimes unethical or impractical to allocate participants to exposures of interest (e.g. elevated blood pressure or physical inactivity). Additionally, participants are often relatively healthy with few co-morbidities which limits the applicability of the study findings to the general population, worsened by the possibility of consent bias. Lastly, trials may need significant follow-up time to produce meaningful results, which means RCTs are relatively resource-intensive and expensive.

Genetic epidemiology has tried to address these concerns through the use of Mendelian randomization studies. While this term was introduced by Gray and Wheatley in 1991 (8), the underlying principles have long been recognized and applied in the field of econometrics, taking the form of instrumental variable analysis. An instrumental variable (or instrument) is a variable associated with the exposure, but not with the outcome of interest except through its association with the exposure (9). The application of Mendelian randomization in biomedical research, credited to Katan (10), is based on the concept that inheritance

of germline genetic variants is subject to the random allocation of alleles at conception, more commonly known as Mendel's second law or the law of independent assortment (11). As the associations between genotype and clinical outcome are generally unrelated to environmental or behavioral exposures, use of single nucleotide polymorphisms (SNPs) known to be associated with modifiable risk factors makes it possible to avoid possible confounding or reverse causality (**Fig. 1**). In other words, causality of these risk factors can accurately be estimated using observational data in a research design resembling an RCT (**Fig. 2**) (12).

A clear example where Mendelian randomization was successfully used to prove the causality of a possible risk factor is the secretory phospholipase A2 (sPLA2) story. Higher circulating levels of sPLA2-IIA mass or sPLA2 enzyme activity have been associated with increased risk of cardiovascular events in observational studies (13). However, a recent RCT with Varespladib, a sPLA2 inhibitor, was stopped because of lack of efficacy. Subjects randomized to Varespladib had an increased risk for cardiovascular events compared with subjects receiving placebo (14). A Mendelian randomization study was conducted to investigate the causality of sPLA2 in cardiovascular disease. The polymorphism rs11573156, which was associated with significantly lower sPLA2 levels, was not associated with coronary events (OR 1.02 (95% CI 0.98-1.06)). The conclusion from this Mendelian randomization study was that sPLA2-lowering therapy would not be a useful therapeutic tool to prevent cardiovascular disease (15).

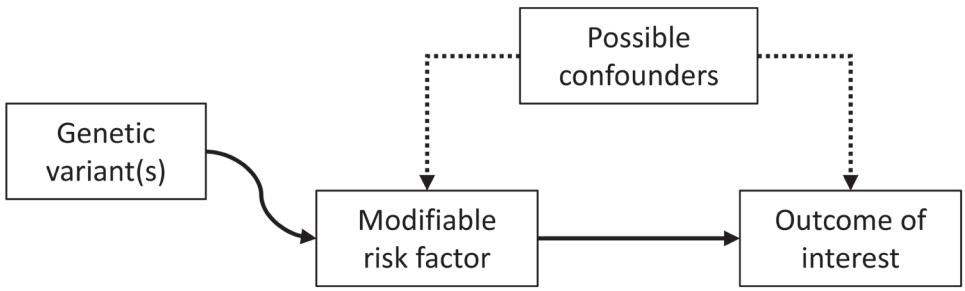

Fig. 1 Causal relationships which satisfy the core assumptions of Mendelian randomization: (1) genotype is associated with phenotype, (2) genotype is independent of confounding factors, (3) genotype is associated with outcome, but only through phenotype

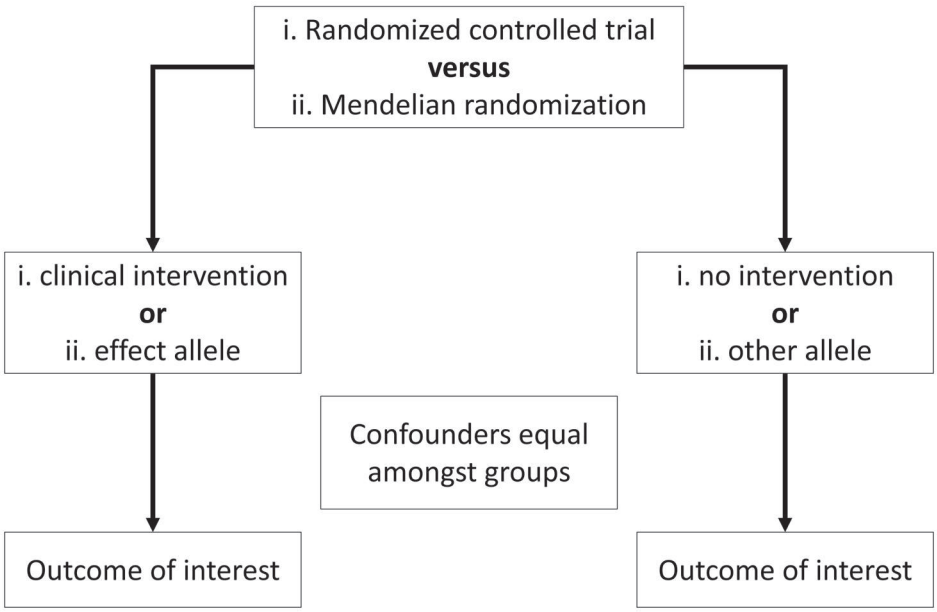

Fig. 2 Comparison of randomized controlled trial and Mendelian randomization study designs

To test whether elevated levels of C-reactive protein (CRP) are causally associated with ischemic vascular disease, Zacho et al. conducted genotyping for four CRP polymorphisms. They found that the risk of ischemic heart disease and ischemic cerebrovascular disease was increased by a factor of 1.6 and 1.2, respectively, in persons who had CRP levels above 3 mg/l, as compared with persons who had CRP levels below 1 mg mg/l. Polymorphisms in the CRP gene were associated with considerable increases in CRP levels and thus with a theoretically predicted increase in the risk of ischemic vascular disease. However, these polymorphisms were not associated with an increased risk of ischemic vascular disease, thereby demonstrating that a causal relationship of CRP levels with adverse cardiovascular outcome is unlikely (16).

Another example of a Mendelian randomization, originating from Katan's original hypothesis, used apolipoprotein E (ApoE) genotype to infer causality between cholesterol and cancer (10, 17). The background for this was the uncertainty whether the associations found between low plasma cholesterol levels and increased risk of cancer might actually reflect a hypocholesterolemic

effect of cancer in preclinical stages (10). Trompet et al. reported that subjects within the lowest third of plasma cholesterol levels had increased risk of cancer incidence (HR 1.9 (95% CI 1.34-2.70)) and cancer mortality (HR 2.03 (95% CI 1.23-3.34)), when compared with those within the highest third of plasma cholesterol levels. However, they also found that carriers of the ApoE2 genotype, who had 9% lower plasma cholesterol than carriers of the ApoE4 genotype, did not have increased risk of cancer incidence (HR 0.86 (95% CI 0.50-1.47)) or cancer mortality (HR 0.70 (95% CI 0.30-1.60) compared with ApoE4 carriers. These findings suggested that low cholesterol levels are not causally related to increased cancer risk (17).

An important limitation of Mendelian randomization is that genetic variants generally explain a modest amount of the variation in exposure levels, which means large sample sizes are needed to obtain valid results. It has been suggested that combining multiple SNPs into an allele score increases power and facilitates avoidance of weak instrument bias (18, 19). Genome-wide association studies (GWAS), which scan large numbers of genetic markers in genomes of different individuals to find genetic variations associated with a particular disease or trait, have made construction of these genetic risk scores feasible. Teslovich et al. found 95 loci associated with plasma lipids in more than 100,000 individuals, explaining 9.6-12.4% of total variance of lipid levels in the Framingham Heart Study and corresponding to ~25-30% of the genetic variance for each trait (20). Other large-scale GWAS have examined traits of blood pressure (21), body mass index (22) and CRP (23), providing more insight into the genetics and biology of these possible risk factors.

Various studies have applied GWAS findings to examine causality of cardiovascular risk factors. For example, Voight et al. constructed a genetic risk score comprising 14 SNPs known to be associated with HDL cholesterol but not with other lipid traits. While observational epidemiology showed that an increase of 1 SD in HDL cholesterol was associated with decreased occurrence of myocardial infarction (OR 0.62 per SD (95% CI 0.58-0.66)), genetically raised HDL was not associated with risk of myocardial infarction (OR 0.93 per SD (95% CI 0.68-1.26)), thereby challenging the concept that raising plasma HDL cholesterol leads to reductions in risk of myocardial infarction. In contrast, the estimate from observational epidemiology for LDL cholesterol (OR 1.54 per SD (95% CI 1.45-1.63)) was concordant with that from genetically raised LDL (OR 2.13 per SD (95% CI 1.69-2.69)) (24). In another recent study, a total of 30 SNPs were combined by Lieb et al. to evaluate whether hypertension truly acts as a causative factor for coronary artery disease, finding that those individuals

carrying most systolic and diastolic blood pressure raising risk alleles had the highest odds of having coronary artery disease (25).

Most research has been performed using data from Caucasian populations only, which illustrates one of the limitations to the application of genetic risk scores in clinical practice. It is unlikely that Mendelian randomization findings will uniformly translate into treatment effects as clinical interventions may have additional biological and biochemical pathways through which they affect clinical outcome, though the findings will generally be informative for the direction of effect and may further the design of an intervention study. In general, Mendelian randomization studies must examine the possibility of potential confounders to genotype. This includes confounding through multiple functions of a genotype (pleiotropy), the non-random association of alleles at two or more loci (linkage disequilibrium), population stratification and canalization, which describes a fetal developmental change in response to a potentially harmful genetic variant (12).

Despite its current challenges, genetic epidemiology has great potential for extending the knowledge base of cardiovascular risk assessment. With increasing sample sizes and next-generation sequencing, GWAS will be able to detect increasing numbers of trait- and disease-associated genetic variants. Recently, the Global Lipids Genetics Consortium identified 157 loci associated with lipid levels, including 62 loci not previously associated with lipid levels in humans, thereby extending the findings of Teslovich et al. and opening up new possibilities for construction of genetic risk scores (26). Moreover, in coming years academic cooperation through international research consortia (e.g. CHARGE, GIANT, IDEAL) will present unprecedented possibilities for translational and (pre)clinical research.

REFERENCES

1 Global Atlas on Cardiovascular Disease Prevention and Control. Mendis S, Puska P, Norrving B editors. World Health Organization, Geneva 2011.

2 Kannel WB, Dawber TR, Kagan A, et al. Factors of risk in the development of coronary heart disease--six year follow-up experience. The Framingham Study. Ann Intern Med. 1961;55:33-50.

3 Wilson PW, D'Agostino RB, Levy D, et al. Prediction of coronary heart disease using risk factor categories. Circulation. 1998;97:1837-47.

4 Rossouw JE, Anderson GL, Prentice RL, et al. Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results From the Women's Health Initiative randomized controlled trial. JAMA. 2002;288:321-33.

5 Gabriel SR, Carmona L, Roque M, et al. Hormone replacement therapy for preventing cardiovascular disease in post-menopausal women. Cochrane Database Syst Rev. 2005;2:CD002229.

6 Lawlor DA, Davey Smith G, Kundu D, et al. Those confounded vitamins: what can we learn from the differences between observational versus randomized trial evidence? Lancet. 2004;363:1724-7.

7 Lawlor DA, Harbord RM, Sterne JA, et al. Mendelian randomization: using genes as instruments for making causal inferences in epidemiology. Stat Med. 2008;27:1133-63.

8 Gray R, Wheatley K. How to avoid bias when comparing bone marrow transplantation with chemotherapy. Bone Marrow Transplant. 1991;7 Suppl 3:9-12.

9 Greenland S. An introduction to instrumental variables for epidemiologists. International journal of epidemiology. 2000;29:722-9.

10 Katan MB. Apolipoprotein E isoforms, serum cholesterol, and cancer. Lancet. 1986;1:507-8.

11 Mendel G. Experiments in plant hybridization. 1865. Available from: www.mendelweb.org/archive/Mendel.Experiments.txt

12 Davey Smith G, Ebrahim S. What can mendelian randomization tell us about modifiable behavioural and environmental exposures? BMJ. 2005;330:1076-9.

13 Koenig W, Khuseyinova N. Lipoprotein-associated and secretory phospholipase A2 in cardiovascular disease: the epidemiological evidence. Cardiovasc Drugs Ther. 2009;23:85-92.

14 Nicholls SJ, Kastelein JJ, Schwartz GG, et al. Varespladib and cardiovascular events in patients with an acute coronary syndrome: the VISTA-16 randomized clinical trial. JAMA. 2014;311:252-62.

15 Holmes MV, Simon T, Exeter HJ, et al. Secretory phospholipase A(2)-IIA and cardiovascular disease: a mendelian randomization study. J Am Coll Cardiol. 2013;62:1966-76.

16 Zacho J, Tybjaerg-Hansen A, Jensen JS, et al. Genetically elevated C-reactive protein and ischemic vascular disease. N Engl J Med. 2008;359:1897-908.

17 Trompet S, Jukema JW, Katan MB, et al. Apolipoprotein e genotype, plasma cholesterol, and cancer: a Mendelian randomization study. Am J Epidemiol. 2009;170:1415-21.

18 Pierce BL, Ahsan H, Vanderweele TJ. Power and instrument strength requirements for Mendelian randomization studies using multiple genetic variants. Int J Epidemiol. 2011;40:740-52.

19 Burgess S, Thompson SG. Use of allele scores as instrumental variables for Mendelian randomization. Int J Epidemiol. 2013;42:1134-44.

20 Teslovich TM, Musunuru K, Smith AV, et al. Biological, clinical and population relevance of 95 loci for blood lipids. Nature. 2010;466:707-13.

21 Ehret GB, Munroe PB, Rice KM, et al. Genetic variants in novel pathways influence blood pressure and cardiovascular disease risk. Nature. 2011;478:103-9.

22 Speliotes EK, Willer CJ, Berndt SI, et al. Association analyses of 249,796 individuals reveal 18 new loci associated with body mass index. Nat Genet. 2010 ;42:937-48.

23 Dehghan A, Dupuis J, Barbalic M, et al. Meta-analysis of genome-wide association studies in >80 000 subjects identifies multiple loci for C-reactive protein levels. Circulation. 2011;123:731-8.

24 Voight BF, Peloso GM, Orho-Melander M, et al. Plasma HDL cholesterol and risk of myocardial infarction: a mendelian randomization study. Lancet. 2012;380:572-80.

25 Lieb W, Jansen H, Loley C, et al. Genetic predisposition to higher blood pressure increases coronary artery disease risk. Hypertension. 2013;61:995-1001.

26 Willer CJ, Schmidt EM, Sengupta S, et al. Discovery and refinement of loci associated with lipid levels. Nat Genet. 2013;45:1274-83.

PART II

Statin pharmacogenomics and Mendelian randomization

CHAPTER 3

A critical appraisal of pharmacogenetic inference

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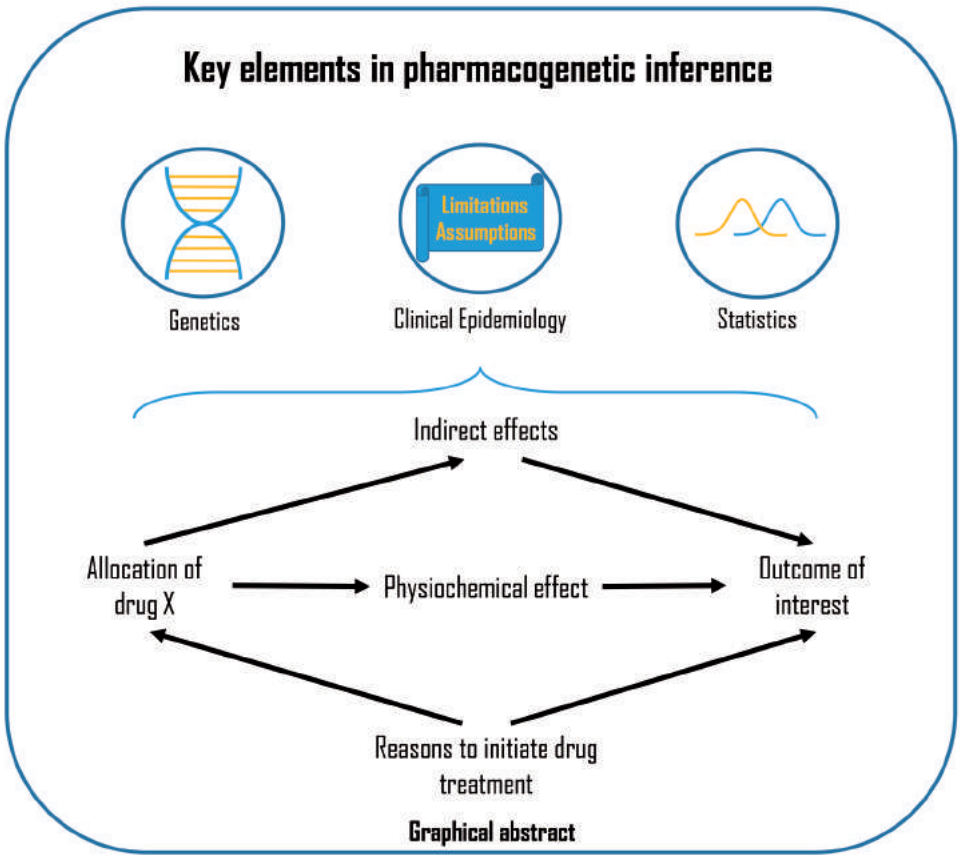
ABSTRACT

In essence, pharmacogenetic research is aimed at discovering variants of importance to gene-treatment interaction. However, epidemiological studies are rarely set up with this goal in mind. It is therefore of great importance that researchers clearly communicate which assumptions they have had to make, and which inherent limitations apply to the interpretation of their results. This review discusses considerations of, and the underlying assumptions for, utilizing different response phenotypes and study designs popular in pharmacogenetic research to infer gene-treatment interaction effects, with a special focus on those dealing with of clinical effects of drug treatment.

Graphical abstract



INTRODUCTION

Pharmacogenetics can be thought of as a classic example of gene-environment interaction. Namely, in the search for genetic variation which can explain inter-individual drug response variability, researchers typically aim to answer the question whether a treatment effect differs between subjects with different genotypes. In other words, whether an inherited genetic variant acts as an effect measure modifier for a certain (drug) treatment.

Although the term pharmacogenetics was coined halfway through the 20th century by Fredrich Vogel (1), widespread interest into the field truly emerged with the completion of the Human Genome Project (2) (**Figure 1**). There now exist large publicly available web resources and pharmacogenetic databases, made possible by methodological advances in sequencing technology and the emergence of genome-wide testing strategies (3, 4). Regrettably, contemporary pharmacogenetic research often depends on the type of study data readily available, as most epidemiological studies are not developed with pre-specified pharmacogenetic research questions in mind. Therefore, a heterogeneous body of literature exists. Collective interpretation can be difficult, as limitations and assumptions inherent to different epidemiological study designs must be recognized. Unfortunately, there also exist notable examples in the literature where authors overextend the scope and significance of their findings.

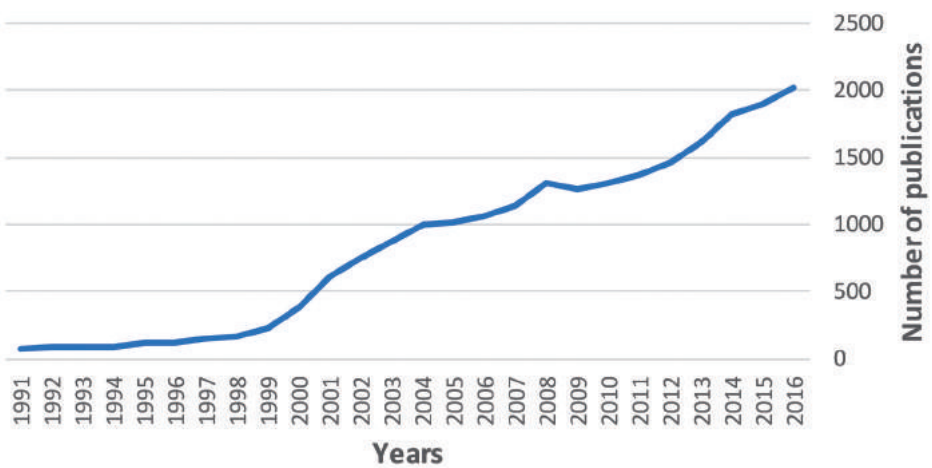


Figure 1. Appearance of the terms pharmacogenetic(s) or pharmacogenomic(s) in PubMed-indexed publications across the past 25 years. The Human Genome Project was completed in 2003.

Here, we discuss considerations relating to different response phenotypes and study designs typically found throughout the pharmacogenetic literature. Though many of the considerations and pitfalls described in this paper will also apply to other types of pharmacogenetic investigations (e.g. those focusing on ADME properties), we will especially focus on studies dealing with clinical effects of drug treatment, an area where we feel invalid inference is more prevalent or at least more visible. We will clarify which conclusions may be drawn and which limitations naturally follow from which methodological approach. Where applicable we provide illustrative examples from the field of statin pharmacogenetics, in which a diverse range of phenotypes and study designs have been combined and investigated (5). Here, we will focus specifically on investigations into the intended effects of cholesterol reduction, on the prevention of vascular events, or on the unintended occurrence of myopathy-related complaints after starting statin therapy.

RESPONSE PHENOTYPES

Except for sharply defined clinical outcomes such as mortality, effects of treatment can often be visualized as lying on a possible spectrum of outcomes. For example, the clinical spectrum of statin-induced myopathy ranges from commonly occurring myalgia to very rare incidents of life threatening rhabdomyolysis (6). The narrow approach of dichotomization will thus lead to a loss of information and possibly reduced statistical power (7). This may particularly be the case for drug efficacy or toxicity phenotypes related to drug dosage. Furthermore, dichotomizing outcomes may induce unnecessary phenotypic heterogeneity between studies (complicating systemic reviews and meta-analyses), and might conceal possible non-linearity in the associations under investigation. Therefore, continuously distributed outcome-traits are often preferable when available. However, these outcomes come with their own challenges (e.g. non-normal distributions), and may hinder translating the results to clinically meaningful findings. For example, prior knowledge of clear clinical bimodality (e.g. disease remission) may guide researchers in choosing a response phenotype which most closely aligns with the biology of interest. In addition, dichotomous outcomes more often allow for simple visual presentation of results and categorization may mitigate the effects of including significant outliers in your analysis.

Most pharmacogenetic investigations of interest are inherently longitudinal in nature, as one wishes to measure a phenotype just before and then after a drug treatment has started. This goal corresponds to a criterion essential to causal inference, namely temporality: that exposure preceded the outcome (i.e. onset

of disease or change over time in a trait) (8). Even for binary outcomes (e.g. clinical or adverse events) it will be essential to compare incidence between drug exposure categories, including the absence of drug exposure. Whenever possible, incorporating both on- and off-treatment observations into the data analysis is therefore considered superior to solely basing conclusions on data from one or more observations made on-treatment. There exist additional reasons why utilizing repeated measurements is often preferable for quantitative traits. Firstly, a single measurement is merely a snapshot of the underlying response-curve, not representative of the true response characteristics over the whole treatment phase, which is likely to differ per individual (9). Secondly, methods that do involve baseline values can eliminate much of the between-subject variability from the treatment comparison, and are therefore typically more powerful. Thirdly, limiting the analysis to a single on-treatment value ignores possible baseline imbalances between the groups, which are likely to occur in non-randomized studies. Taking these into account may help to control for confounding by (contra)indication and in distinguishing genetic effects on the response phenotype from those on off-treatment levels. Finally, having both on- and off-treatment measurements allows for the calculation of change over time, which is easy to communicate to a broad non-statistical audience.

A further consideration is the selection of a valid time interval to assess treatment response, which should be based on clinical experience. For example, a steady-state in low-density lipoprotein cholesterol (LDL-C) may be expected 4-6 weeks after start of statin treatment (10). However, when one is interested in onset of myopathy symptoms a longer period should be considered, e.g. the mean duration of statin therapy before onset of symptoms was 6.3 months (range 0.25-48.0) in a retrospective study of 45 patients (11).

For adverse drug reactions, response phenotypes suitable for pharmacogenetic research will generally be those which appear to be strongly tied to the drug exposure. This will often depend on baseline disease incidence, whether relative effect sizes observed in large-scale studies are of apparent clinical importance, but also whether sufficient evidence supports a causal link between the drug exposure and the adverse event. Additional practical considerations such as data availability may guide or limit researchers in their investigations. For example, while it has been reliably shown that new-onset diabetes mellitus may be caused by statin therapy (12), repeated glucose measurements have historically not been assessed within statin trials. This likely explains why statin-induced glucose changes have not been examined in the pharmacogenetic setting to date.

DEFINING TREATMENT EFFECT

The observed average treatment response in a study does not always reflect the benefit of the treatment *per se*, as the context wherein this observation is made is of great importance (**Figure 2**). This is because an individuals' treatment response, defined here as the clinical outcome after starting the treatment, is not just a combination of the drug effect (i.e. the underlying (un)measured physiochemical response) and the natural course of the disease, but may also reflect secondary effects of initiating drug treatment (13, 14). Examples include placebo effects, the possibility that the individual may have been motivated to concurrently alter lifestyle habits of prognostic significance to the outcome of interest, or that the researcher or study participant may (un)knowingly influence the measurement of the endpoint if he/she is aware of the purpose of the study (i.e. observer bias) (14). The latter issue is more likely to occur with subjective outcomes, but may be avoided through blinding both researcher and study participants.

A serious problem in non-randomized studies is the issue of confounding by (contra)indication. In routine healthcare the decision to initiate or refrain from drug treatment is based on the prognosis of the patient. Consequently, the prognoses of treated and untreated individuals in observational studies are typically not comparable. In other words, individuals with more indications for treatment are more likely to be treated, but also more likely to have a worse outcome. If this is not taken into account through study design or statistical adjustment, straightforward inference of treatment benefits may be invalid, as it could seem that treatment actually leads to worse outcomes (15). While no statistical adjustment method can fully resolve confounding by (contra)indication in observational studies if not all confounders are known, its effects should be minimized when possible. Given that genotype is set at conception and remains fixed throughout life, confounding by (contra)indication is unlikely to bias the effect estimate of a genetic variant on the outcome of interest. However, if confounding bias is present for the association between the drug exposure and the outcome of interest, this may in select cases carry over to the assessment of interaction between the genetic variant and this drug exposure (16).

In the next sections we show that the degree to which different study designs are able to avoid or disentangle these considerations is paramount to the interpretation of results and conclusions that can be drawn, also in the field of pharmacogenetics.

STUDY DESIGNS

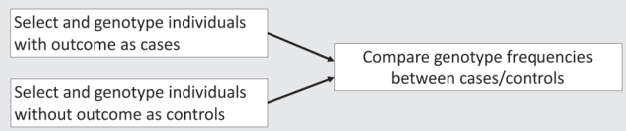
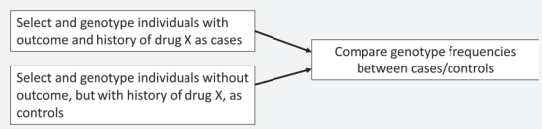
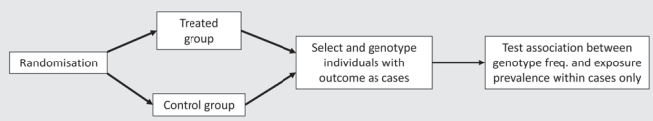
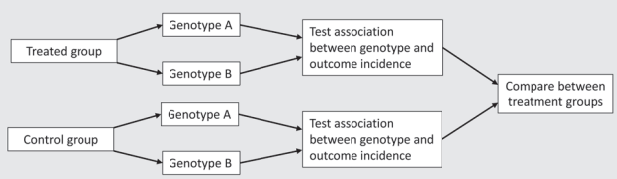
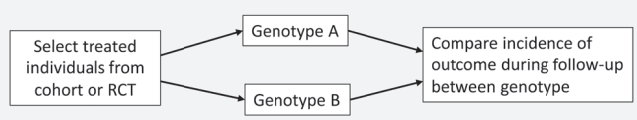
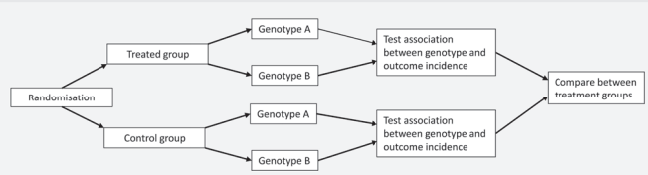
Various studies are available and appropriate to answer different types of pharmacogenetic research questions, depending on the stage of drug development. Here we focus on those suitable to evaluate the effect of genetic variation on treatment efficacy and adverse drug reactions, questions which will typically be asked after a drug has already been approved for clinical use. In addition to post-hoc subgroup analyses within a randomized controlled trial (RCT), all traditional population-based epidemiological studies can be used in this phase. However, all study designs come with underlying assumptions and limitations, and may not be able to answer all relevant questions (**Table 1**).

Our discussion here focuses mostly on sources of bias general to all epidemiology. However, a source of confounding specific to genetic epidemiology concerns population stratification (17). If there exist subgroups of individuals within the study population which differ in terms of genotype frequency and disease risk, spurious associations may arise if this is not taken into account. Typically, this can occur when individuals from different ethnic backgrounds with limited admixture are included in the same analysis (18). However, even apparently homogenous populations may contain genetically distinct subgroups (19). As larger samples will likely be more heterogeneous, population stratification will be a larger problem here (17). This should be of particular concern to researchers involved in the field of drug-gene interaction, where large studies are typically necessary to find promising signals.

Outcome-based designs

The case-control design is perhaps the most common approach for pharmacogenetic investigations into clinical effects, often focusing on adverse drug reactions. Sampling is based on the outcome, with individuals who did (cases) develop the outcome of interest being compared to those who did not (controls), with regard to drug exposure prevalence and genotype frequencies. Case-control studies can be used to assess both main effects of the genetic variant and drug exposure on the outcome, but may also assess interaction on the additive and multiplicative scale (20) (**Table 2**).

Table 1. Popular epidemiological study designs suitable for pharmacogenetic research questions on clinical effects of drug therapy

Design	Graphical representation
Outcome-based designs	
Case-control	
Treated-only case-control	
Case-only, nested within RCT	
Cohort-based designs	
Cohort	
Treated-only cohort	
Trial-based design	
Subgroup analyses within RCT	

RCT denotes randomised controlled trial

Key assumptions for gene-treatment interaction	Advantages	Limitations
Valid control selection	Cost-effective; can evaluate rare events caused by rare variants; can assess both main and interaction effects	Prone to selection/ information bias and confounding due to observational design
As case-control; no association between genotype and outcome in untreated group	Genotyping untreated individuals not needed	See case-control; can only assess interaction on multiplicative scale
No association between genotype and drug exposure in source population	More efficient than case-control in evaluating interaction effects; genotyping controls not needed	See case-control; can only assess interaction on multiplicative scale; gene-treatment independence assumption unlikely to hold in non-randomised cohort
-	Repeated measures; can study multiple outcomes and rare exposures; can evaluate both main and interaction effects, can assess population-attributable risk	Subject-driven assignment of treatment; resource-intensive; prone to differential loss-to-follow up (selection bias); prone to information bias and confounding; inefficient for rare outcomes
No association between genotype and outcome in untreated group	Avoids issue of confounding by contraindication; more efficient than cohort study in evaluating interaction effects	See cohort; can only assess interaction effects; prior knowledge necessary to make key assumption for gene-treatment interaction
Valid randomization procedure	Random allocation of treatment assures comparability at baseline; regression-to-the-mean can be taken into account; allows for blinding	Resource-intensive; limited generalizability; inefficient for rare outcomes

There also exist case-control studies which solely include individuals with known drug exposure, in which the analysis is limited to comparing genotype frequency between cases and controls. For the purpose of simplicity we will assume throughout the manuscript and tables that a particular susceptibility genotype is classified as being either present or absent. If it can be assumed that genotype does not associate with the outcome of interest in the absence of drug exposure, potential differences in disease occurrence between genotype groups can be interpreted as gene-treatment interactions (21). Whether this assumption is valid is highly dependent on the outcome of interest and the observation window chosen to assess this outcome. For example, this assumption is likely to hold for LDL-C reduction after statin treatment, since genetic variants are unlikely to lead to such acute (i.e. within days/weeks) and significant LDL-C changes (~30%) in absence of the drug treatment. In contrast, a treated-only case-control study on the occurrence of coronary artery disease after statin use is likely to also turn up genetic variants affecting risk in absence of statin treatment, as the underlying atherosclerotic process has a much slower onset than statin-induced LDL-C reduction.

Major benefits of the case-control design are its cost-effectiveness compared to large cohort studies, but more importantly that it is highly suited for rare (drug) outcomes. For severe adverse drug reactions, it may sometimes even form the only realistic approach to examine genetic contributions. When the outcome of interest has a continuous distribution, sampling individuals from the extremes of the outcome distribution (e.g. comparing high- with non-responders in LDL-reduction after starting statin treatment) may greatly increase statistical power when faced with budgetary restrictions for genotyping (22). However, as shown for non-responders to statin therapy in the PROspective Study of Pravastatin in the Elderly at Risk (PROSPER) trial, issues of treatment non-adherence are especially important to consider here (23). This strategy may also be promising when rare variants are investigated, as their prevalence may be greater on the extreme ends of the outcome spectrum (24).

There are some notable challenges in performing case-control studies, the first and foremost being the selection of an appropriate control group. The control group should be representative of the source population in terms of exposure distribution and genetic ancestry (e.g. European, Asian or African ancestry), and should ideally consist of individuals who would be classified as cases if they had developed the outcome of interest. In other words, controls should meet the eligibility requirements for cases except for their outcome status (20). Preferably, a geographically defined population should be the source of sampling, so the

Table 2. Comparison of effect estimators from outcome-based study designs

Case-control setting (frequency data complete)				
Drug (E)	Genotype (G)	Cases	Controls	Effect estimator
-	-	a	b	
-	+	c	d	OR_G = b*c / a*d
+	-	e	f	OR_E = b*e / a*f
+	+	g	h	OR_{GE} = b*g / a*h
To assess for interaction on the multiplicative scale: OR_{GE} / (OR_G * OR_E)				
Treated-only case-control setting (subset of frequency data)				
Drug (E)	Genotype (G)	Cases	Controls	Treated-only case-control OR = f*g / e*h
-	-	n/a	n/a	If the genetic variant G is not associated with the outcome among untreated individuals (OR_G=1), the treatment-only case-control OR will estimate the assessment of interaction on the multiplicative scale from the case-control setting.
-	+	n/a	n/a	
+	-	e	f	
+	+	g	h	
Case-only setting (subset of frequency data)				
Drug (E)	Genotype (G)	Cases	Controls	Case-only OR = a*g / c*e
-	-	a	n/a	If the drug treatment E and genetic variant G are not associated among controls (i.e. source population), the case-only OR will estimate the assessment of interaction on the multiplicative scale from the case-control setting.
-	+	c	n/a	
+	-	e	n/a	
+	+	g	n/a	

OR denotes odds ratio. While the above table denotes genotype as the presence of absence of a certain susceptibility genotype, it will equally hold for more complex situations, including combinations of alleles at multiple loci.

entire at-risk population can be enumerated. For hospital- or clinic-based case-control studies it may be difficult to identify this source population, as it does not correspond to a specific geographical area. For example, trauma victims referred to the hospital could live nearby or have been flown in by helicopter. In general, the catchment area for a hospital or clinic is likely to differ for different diseases, which will need to be considered when sampling controls. Similarly, as the cases of outcome-based studies on adverse drug reactions are often identified through databases it may be difficult to recruit an appropriate control group, especially since these events are often underreported (25, 26). Case-control studies nested within an existing cohort may fare better in this regard.

A further risk is that cases with short survival times may be underrepresented if collection of (genetic) data occurs sometime after the event of interest.

An alternative outcome-based design is the case-only study, wherein the analysis is restricted to cases (**Table 1**). This simple approach, which can evaluate gene-treatment interaction on the multiplicative scale, assumes that genotype and drug treatment are not correlated in the population that gave rise to the cases. Under this assumption this design increases power for the test of interaction, thereby lowering the number of cases needed to be genotyped (27). Not having or being able to find a suitable control group is another reason why this may be an attractive alternative to the conventional case-control study (28). If nested in a RCT the distributions of gene and treatment can be assumed to be independent by virtue of randomization, making the case-only odds ratio a valid measure of gene-treatment interaction (**Table 2**). The calculated odds ratio may however (slightly) differ between case-control and case-only studies, as case-control studies estimate different population parameters (odds-, rate-, or risk-ratio), depending on how the controls were sampled (29). An example of the case-only approach in the field of statin pharmacogenetics is that by Schiffman and colleagues, who performed a genome-wide association study on coronary heart disease risk reduction when being treated with pravastatin therapy (30). In the discovery phase they solely included coronary heart disease cases from the Cholesterol and Recurrent Events (CARE) trial and the West of Scotland Coronary Prevention Study (WOSCOPS) trial, finding that 79 common genetic variants were nominally ($P<10^{-4}$) associated with differential event reduction by the therapy. To validate these results, these variants were then genotyped in an additional placebo-controlled pravastatin trial, and in all remaining patients from CARE and WOSCOPS (with or without event) (30). This study thereby exemplified how the case-only approach could be utilized as a cost-saving measure, by first screening the genome for promising signals, before including controls.

Nesting a case-only study within a cohort study can be problematic, as it is possible that genetic factors could influence the ability to tolerate therapy. Therefore, independence between genotype and treatment may not be a valid assumption. While this could also occur within an RCT, this experimental study design is more likely to have information on, and be able to include in the analysis, enrolled individuals who did not respond or had severe side effects. It has been argued that tests of gene-treatment association in controls may indicate whether genotype and treatment are truly independent in the source population, if the outcome is sufficiently rare (31). If however the assumption of gene-treatment independence is violated and ignored, the case-only approach will provide a

biased interaction effect and lead to increased false-negative results (32). Another limitation of the case-only design is that main effects of either genetic or drug treatment on the outcome cannot be estimated, and inference is limited to examining interaction on the multiplicative scale. More generally, all outcome-based designs which cannot approximate risk ratios (rare disease assumption) or risk differences (due to knowing sampling fractions) are unable to examine interaction on the additive scale, which is often of greater public health relevance (33). Due to their observational nature, outcome-based studies are additionally highly prone to confounding, selection bias (i.e. that the association between (drug) exposure and disease differs for participants who were and were not included in the study) and information bias (i.e. systematic error in the approach adopted for measuring or collecting data from a study) (20). For the last category, especially recall bias can pose an issue, which will not apply to genotype but might to drug history.

Cohort-based designs

Cohort-based designs include the cohort and treated-only designs (**Table 1**). Typically, the rate of occurrence (or recurrence) is compared between individuals with different drug exposures levels. Increasingly, population-based cohort studies are undertaken, in which an ideally random sample or even the entirety of a defined population is included in which multiple hypotheses can be evaluated. Though these relatively expensive and time-consuming studies aim to answer the same questions of causality that outcome-based designs do, the extensive and repeated phenotyping and follow-up allows for more flexibility in investigating multiple outcomes and recent, prior and repeated drug exposure (21). In addition, studying a cohort representative of a defined population allows for the calculation of population attributable risks. While this type of study typically includes more participants than outcome-based studies, it is unlikely that a single study would be able to overcome the power and sample size issues associated with genome-wide testing. Considerations of sample size are discussed in detail in a separate section below. As cohort-based designs do not typically allow for blinding of researchers and participants, it is very likely that observer effects will not be equal between the treatment groups. In addition, if genetic testing was not undertaken close to commencement of treatment, selection bias may occur when non-responders or those with severe side effects are absent from the population.

Of greater issue is that the assignment of drug therapy is likely to have been subject driven. This means that the prognoses of the treated and untreated subjects will generally not be alike. In addition to this previously discussed

confounding by (contra)indication, the issue of regression-to-the-mean may be problematic here. This occurs because the group of subjects at the extremes of the response distribution at baseline not just consists of those who consistently have more extreme values compared to the population average, but also those who simply by chance had an extreme value at baseline. Subsequent measurements of those who fall in the second category will therefore tend to be closer to the population mean thereof. Observed phenotypic changes over time may thus (partially) represent this regression-to-the-mean, which can occur when participants and/or treatment are selected on phenotypic cut-offs at baseline. This statistical phenomenon has been demonstrated for a wide range of biological measures, including lipid levels (34). Therefore, in non-randomized studies, it should be considered to combine multiple baseline measurements to reduce measurement error when selecting subjects, or to use suitable statistical methods (35, 36).

The treated-only design essentially tries to limit the issue of confounding by contraindication whilst improving statistical efficiency (37). As the name suggests, this design limits the analysis to those exposed to the drug, thereby leaving out the subjects who might have had a pertinent contraindication to treatment. This contrasts with cohorts which do include an untreated control group, in which confounding by (contra)indication is more commonly addressed through statistical adjustment, although applying stricter inclusion criteria at enrolment may also limit this issue (14). A clear benefit of the treated-only approach is that less individuals are required for the analysis, which can be highly advantageous when genotyping study participants. As noted for the treated-only outcome-based design, the central assumption for inferring gene-treatment interaction effects here is that the genetic variant is unlikely to explain change in outcome in absence of the drug exposure (21). A clear drawback are that the main effects of genetic variants on the outcome are inseparable from drug-treatment interaction effects. Observed loci may thus be associated with the natural course of the disease (37). In these cases, leveraging publicly available data from genome-wide association studies (GWAS) may help to substantiate the claim of absence of a main effect of a genetic variant on the outcome of interest. This approach will however require these GWAS to have taken into account possible effects of drug treatment and to have a similar outcome definition.

Of special note, an increasing number of researchers are utilizing (singular or repeated) cross-sectional data from cohort studies to perform genome-wide gene-treatment interaction analyses for quantitative traits (38). These efforts have largely been motivated by the issue that the design of many cohorts is not

ideal for measuring longitudinal drug-induced changes. Specifically, assessment may be problematic when drug exposures are rare, when large intervals of time separate repeat drug exposure assessment, and when outcome phenotypes are not collected at each study visit. Therefore, the use of repeated exposure cross-sections allows for more cohorts to contribute, noting that increases in power from including more participants has been shown to be larger than the modest increase in power from making use of repeat cross-sectional measures in the same participants (39). To date, this approach has particularly been applied to questions of gene-treatment interaction for different drug classes on electrocardiography-markers (39, 40). Similar research efforts are currently underway for the field of statin pharmacogenetics.

As study information on exposure and outcome is typically determined at the same time, or at least analyzed without regard for differences in time, the temporal relationship between exposure and outcome remains unclear in these cross-sectional analyses. In fact, making a distinction between exposure and outcome will generally not be possible, unless a well-established drug response phenotype is available (20). Furthermore, aside from the issues discussed previously concerning the use of a single on-treatment measurement, care must be taken to differentiate effects from those on off-treatment values. Therefore, formal comparison with an untreated group is to be advised. Alternative explanations for detected associations between genotype and outcome may be differences in number and duration of previous treatment(s) and differences in severity of disease. Using data from established cohorts may greatly facilitate the execution of these investigations. Nonetheless, due to their inherent limitations, cross-sectional studies are most suitable as hypothesis-generating tools for slowly developing diseases without sharp onset times, rather than for making solid pharmacogenetic inferences of gene-treatment interaction.

Randomized Controlled Trial

While similar in design to a cohort with a control group, the key difference for the RCT is that drug treatment is randomly allocated. As this ensures that the predictors of the outcome are equally distributed between the treated and untreated group, we can assume that: “the treated, had they remained untreated, would have experienced the same average outcome as the untreated did, and vice versa” (41). In addition, this strategy enables blinding of researcher and participant, which aims to prevent subsequent differential co-interventions or biased assessment of outcomes (42). As previously noted, if the trial is of adequate size the distributions of genotype and exposure will be independent. Due to these

study characteristics, it is possible to either avoid or account for regression-to-the-mean, confounding by (contra)indication, and selection bias. Consequently, it is possible to make more firm conclusions regarding the underlying treatment effects than is possible in non-randomized studies (**Figure 2**). While reducing the likelihood of selection bias is a major appeal of RCTs, it should be noted that genotyping in blood samples taken after study completion may still introduce this problem.

Subgroup analyses in trials have also been criticized (43), but "breaking" the randomization will typically only occur if researchers condition on a variable that occurs after treatment, which will not apply to genotype. Though RCTs are considered the gold standard to estimate unbiased drug-SNP interaction effects, a variety of reasons exist which explain why researchers may prefer observational study settings instead. Trials will typically have included a select number of participants, thus leading to reduced statistical power compared to large observational cohorts. In addition, the relative limited number and narrow definition of exposures and outcomes under investigation may allow for less flexibility for pharmacogenetic enquiries. For example, both drug exposures and outcomes may be more clinically meaningful when examined as classes not envisioned when designing the trial. Other considerations include concerns of generalizability due to RCTs often having strict exclusion criteria, and that the RCT approach is even less suited than the cohort-based designs to investigate rare adverse outcomes. This results from individuals with relevant co-morbid conditions or with severe side effects typically being excluded before randomization (e.g. during a run-in phase), in addition to trials often not having adequate follow-up to investigate outcomes which can occur long after the invention (44).

An approach analogous to that of the RCT, known as Mendelian randomization, is increasingly being used in the context of pharmacogenetics and pharmacovigilance. These investigations, in which the causal effect of an exposure on an outcome is assessed by using a genetic proxy (e.g. one or multiple genetic variants) instead of the exposure (45), have been applied to a range of different types of questions. For example, summary level statistics from a large-scale pharmacogenetic meta-analysis of GWAS of statin-induced lipid response were recently used to demonstrate that genetic predisposition for increased LDL-C levels may decrease efficacy of statin therapy if effects on off-treatment lipid levels are taken into account (46). Mendelian randomization might alternatively be used to predict unintended drug effects. For example, Swerdlow and colleagues used SNPs in the HMGCR (i.e. the enzyme targeted by

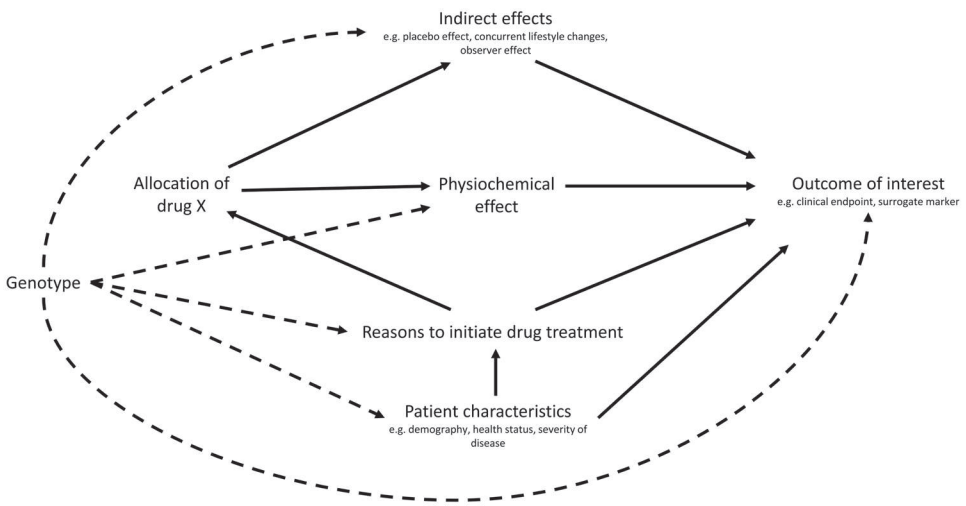


Figure 2. Non-randomized study on treatment response. The observed treatment response to drug X depends not just on the underlying physiochemical response and natural course of the disease process, but also on secondary effects of being allocated drug X. Moreover, confounding by (contra)indication may occur if reasons to initiate or refrain from drug treatment also associate with the outcome of interest. Pharmacogenetic research aims to answer which, if any, inherited genetic factors explain variation in the outcome of interest in the presence of a certain (drug) treatment (i.e. drug-gene interaction effects), distinguishing these effects from direct (i.e. main genetic effects) on the outcome.

statins) gene to demonstrate that the increase in new-onset type 2 diabetes risk is "at least partially" explained by HMGCR inhibition (47). In theory, Mendelian randomization investigations could reveal these effects prior to drugs licensing, potentially preventing exposure of large groups of patients to unnecessary risks (48). Lastly, stratifying Mendelian randomization analyses could provide evidence which subpopulations are likely to derive greater benefit from a drug, which could guide future RCTs (49).

CONSIDERATIONS OF SAMPLE SIZE

A major issue in pharmacogenetic research has been the poor reproducibility of promising signals, likely in part due to underestimation of the sample sizes necessary to examine gene-treatment interaction. It has previously been demonstrated that study sizes for investigations into interaction on the multiplicative scale should be over four times as large as those necessary to detect main effects of the same magnitude (50). Given the relatively small

effect sizes involved, it should therefore not come as a surprise that necessary sample sizes can run into the tens of thousands when genome-wide strategies are considered, where one must not just account for multiple testing but also consider the necessity of replicating ones results (51). Programs for sample size and power calculations for gene-treatment interaction have also been used to estimate sample size requirements for investigations into clinical effects of statin therapy (5). In addition to study design, researchers must consider the expected sizes of both the genetic effect and the drug response, the size of their interaction effects, allele frequencies, mode of inheritance, and the prevalence of the drug treatment and outcome. Moreover, studies are likely to genotype variants in linkage disequilibrium with the true causal variant, which will also influence sample size requirements (52).

In recent years, data from mega-biobanks have been become increasingly available, which will provide unprecedented possibilities for pharmacogenetic enquiries. It should however be noted that participation rates have been relatively low, which will pose unique challenges when interpreting results. For example, only 5.2% of the 9.2 million individuals invited to enter the population-based UK Biobank actually participated in the baseline assessment (53). Similarly, in mid-2015 the Million Veterans Program estimated their response rate at 13.2% of the first 3 million invited individuals (54). In addition, it is highly questionable whether signals which can only be detected under these increased sample sizes will actually translate into clinically meaningful results.

Further considerations must be made when multiple study designs are incorporated in the same analysis via a meta-analytic approach. In the next section we will examine some of these considerations, taking the largest pharmacogenetic meta-analysis of genome-wide association studies of statin-induced LDL-C changes as an example (55).

GENOMIC INVESTIGATION OF STATIN THERAPY (GIST) CONSORTIUM

A major limitation of previously performed individual pharmacogenetic studies of statins was the lack of statistical power to detect small pharmacogenetics effects. To overcome this problem, a large meta-analysis of all available data on statin response was initiated, in which the investigators aimed to combine results from statin trials and large-scale cohorts. For their meta-analysis on differential response in LDL-C to statin therapy, the GIST consortium included 6 statin-trials (n=8,421) and 10 observational studies (n=10,175) for the discovery

stage. Thereafter, the most promising signals were validated in a further 22,318 subjects. Within this large GWAS effort, four loci were found to be associated with LDL-C lowering response to statin therapy. The most significant association was for a SNP on chromosome 6, at *LPA* (rs10455872, minor allele frequency (MAF)=0.08, beta=0.052, standard error (s.e.)=0.004, $P=7.41x10^{-44}$), indicating that carriers of the rs10455872 SNP respond to statins with a 5.2% smaller LDL-C lowering effect per minor allele compared with non-carriers. The second strongest was a SNP at *APOE* on chromosome 19 (rs445925, MAF=0.11, beta=-0.051, s.e.=0.005, $P=8.52x10^{-29}$), indicating an additional 5.1% increase per allele in LDL-C lowering effect compared to non-carriers. In addition, SNPs at two novel GWAS loci were shown to be significantly associated with statin response: *SORT1*/*CELSR2*/*PSRC1* at chromosome 1 (rs646776, MAF=0.22, beta=-0.013, s.e.=0.002, $P=1.05x10^{-9}$) and *SLCO1B1* at chromosome 12 (rs2900478, MAF=0.16, beta=0.016, s.e.=0.003, $P=1.22x10^{-9}$).

Notably, the consortium solely included statin-users, which made it possible to compare associations found in trials with those of observational studies. In addition, this approach made it possible to gather large enough numbers, given the necessity to account for multiple testing. To mimic the trial setting as close as possible, only incident statin users with a pre- and post-measurement were included from observational studies.

As discussed previously, the central assumption for inferring gene-treatment interaction effects via this treated-only approach is that genotype should be unlikely to significantly correlate with the response in absence of drug exposure. Given that the underlying disease course (i.e. LDL-C levels) can be assumed to be quite stable in absence of lipid-lowering treatment, this assumption may very well be valid. In addition, placebo- and observer-effects will likely be near absent for statin-induced LDL-reduction, which will exist for more subjective complaints such as those seen within the field of psychiatric pharmacogenetics (56). The suitability of this approach was reinforced by the large homogeneity of estimates when RCTs and observational studies were separately considered.

A major point of discussion however surrounded the question how to account for the possible effect of genetic variants on off-treatment values, which cannot simply be accounted for by taking the (fractional) difference between on- and off-treatment levels as the outcome. In the end, the researchers solely included participants with on- and off-treatment LDL-C levels. Each study independently performed a GWAS on the difference between the natural log-transformed LDL-C levels on- and off-treatment which can be interpreted as the fraction of differential LDL-C lowering in carriers versus non-carriers of a genetic variant.

These analyses were then adjusted for natural log-transformed off-treatment values to try to distinguish drug-treatment interaction effects from genetic effects on off-treatment LDL-C levels, a strategy for which there exists extensive debate, particularly for non-randomized studies (57, 58) By performing additional analyses, the researchers were however able to validate this approach. These included calculating formal gene-treatment interaction terms within a trial not involved in the first-stage meta-analysis for the genetic variants found to be genome-wide significant, but also by adjusting for the measurement error and intra-individual variation in off-treatment values in the only study which had multiple baseline measurements available (59).

The main limitation of the analysis is the large degree of clinical heterogeneity. This is evidenced not only by differences in eligibility criteria of the original studies, leading to the inclusion of different patient groups, but also by differences in statin types (n=8) and dosages. While adjustment for statin dose was achieved by dividing the dose by the statin-specific dose equivalent based on daily dosages required to achieve mean 30% LDL-C reduction, changes in dose during follow-up could not be taken into account. Nonetheless, the project remains a clear example that if certain assumptions can be realistically met, inherent limitations to pharmacogenetic inference may be overcome.

CONCLUSION

Pharmacogenetic research is an expanding field, whose relevance is slowly becoming visible. While post-hoc subgroup comparisons in RCTs are still considered the gold standard in pharmacogenetic research of treatment efficacy, there exist many research questions for which RCTs cannot provide the solution. As all study designs and response phenotypes have their merits and problems, authors should be vigilant to avoid making conclusions which their methodology cannot back up. In particular, the assumptions needed to make inferences on gene-treatment interaction must be carefully considered, especially when case-only or treated-only strategies are employed. These challenges to inference remain ever relevant as new avenues of pharmacogenetic investigations emerge, including those using epigenetics or mRNA, as these studies will typically be performed in similar research settings.

REFERENCE LIST

1 Vogel F. Moderne Probleme der Humangenetik. Ergebn. Inn. Med. Kinderheilkd. 1959; 12: 52-125.

2 Lander ES, Linton LM, Birren B, et al. Initial sequencing and analysis of the human genome. Nature. 2001;409:860-921.

3 Sim SC, Altman RB, Ingelman-Sundberg M. Databases in the area of pharmacogenetics. Hum Mutat. 2011;32:526-531.

4 Zhang G, Zhang Y, Ling Y, et al. Web resources for pharmacogenomics. Genomics Proteomics Bioinformatics. 2015;13:51-54.

5 Leusink M, Onland-Moret NC, De Bakker PI, et al. Seventeen years of statin pharmacogenetics: a systematic review. Pharmacogenomics. 2016;17:163-180.

6 Sathasivam S, Lecky B. Statin induced myopathy. BMJ. 2008;337:a2286.

7 Steiner DL. Breaking up is hard to do: the heartbreak of dichotomizing continuous data. Can J Psychiatry. 2002;47:262-266.

8 Hill AB. The environment and disease: association or causation? Proc R Soc Med. 1965;58:295-300.

9 de Rotte MC, Luime JJ, Bulatovic M, et al. Do snapshot statistics fool us in MTX pharmacogenetic studies in arthritis research? Rheumatology (Oxford). 2010;49:1200-1201.

10 Lennernäs H, Fager G. Pharmacodynamics and pharmacokinetics of the HMG-CoA reductase inhibitors. Similarities and differences. Clin Pharmacokinet. 1997;32:403-425.

11 Hansen KE, Hildebrand JP, Ferguson EE, et al. Outcomes in 45 patients with statin-associated myopathy. Arch Intern Med. 2005;165:2671-2676.

12 Collins R, Reith C, Emberson J, et al. Interpretation of the evidence for the efficacy and safety of statin therapy. Lancet. 2016;388:2532-2561.

13 Lubsen J, de Lang R. Klinisch geneesmiddelenonderzoek Utrecht: Bunge, 1987.

14 Grobbee DE, Hoes AW. Clinical Epidemiology: Principles, Methods, and Applications for Clinical Research. Jones & Bartlett Learning, 2009.

15 Grobbee DE, Hoes AW. Confounding and indication for treatment in evaluation of drug treatment for hypertension. BMJ. 1997;315:1151-1154.

16 Vanderweele TJ, Mukherjee B, Chen J. Sensitivity analysis for interactions under unmeasured confounding. Stat Med. 2012;31:2552-2564.

17 Marchini J, Cardon LR, Phillips MS, et al. The effects of human population structure on large genetic association studies. Nat Genet. 2004;36:512-517.

18 Wang Y, Localio R, Rebbeck TR. Evaluating bias due to population stratification in epidemiologic studies of gene-gene or gene-environment interactions. Cancer Epidemiol Biomarkers Prev. 2006;15:124-132.

19 Davey Smith G, Lawlor DA, Timpson NJ, et al. Lactase persistence-related genetic variant: population substructure and health outcomes. Eur J Hum Genet. 2009;17:357-367.

20 Rothman KJ. Modern Epidemiology. 1st Edition. Boston, MA: Little, Brown and Company. 1986.

21 Little J, Sharp L, Khoury MJ, et al. The epidemiologic approach to pharmacogenomics. Am J Pharmacogenomics. 2005;5:1-20.

22 Nebert DW. Extreme discordant phenotype methodology: an intuitive approach to clinical pharmacogenetics. Eur J Pharmacol. 2000;410:107-120.

23 Trompet S, Postmus I, Slagboom PE, et al. Non-response to (statin) therapy: the importance of distinguishing non-responders from non-adherers in pharmacogenetic studies. Eur J Clin Pharmacol. 2016;72:431-437.

24 Auer PL, Lettre G. Rare variant association studies: considerations, challenges and opportunities. Genome Med. 2015;7:16.

25 Jorgensen AL, Williamson PR. Methodological quality of pharmacogenetic studies: issues of concern. Stat Med. 2008;27:6547-6569.

26 Eland IA, Belton KJ, van Grootheest AC, et al. Attitudinal survey of voluntary reporting of adverse drug reactions. Br J Clin Pharmacol. 1999;48:623-627.

27 Piegorsch WW, Weinberg CR, Taylor JA. Non-hierarchical logistic models and case-only designs for assessing susceptibility in population-based case-control studies. Stat Med. 1994;13:153-162.

28 Khoury MJ, Flanders WD. Nontraditional epidemiologic approaches in the analysis of gene-environment interaction: case-control studies with no controls! Am J Epidemiol. 1996;144:207-213.

29 Schmidt S, Schaid DJ. Potential Misinterpretation of the Case-Only Study to Assess Gene-Environment Interaction. Am J Epidemiol. 1999;150:878-885.

30 Schiffman D, Trompet S, Louie JZ, et al. Genome-wide study of gene variants associated with differential cardiovascular event reduction by pravastatin therapy. PLoS One. 2012;7:e38240.

31 Dennis J, Hawken S, Krewski D, et al. Bias in the case-only design applied to studies of gene-environment and gene-gene interaction: a systematic review and meta-analysis. Int J Epidemiol. 2011;40:1329-1341.

32 Albert PS, Ratnasinghe D, Tangrea J, et al. Limitations of the case-only design for identifying gene-environment interactions. Am J Epidemiol. 2001;154:687-693.

33 Kalilani L, Atashili J. Measuring additive interaction using odds ratios. Epidemiol Perspect Innov. 2006;3:5.

34 Forrow L, Calkins DR, Allshouse K, et al. Evaluating cholesterol screening. The importance of controlling for regression to the mean. Arch Intern Med. 1995;155:2177-2184.

35 Barnett AG, van der Pols JC, Dobson AJ. Regression to the mean: what it is and how to deal with it. Int J Epidemiol. 2005;34:215-220.

36 Chiolero A, Paradis G, Rich B, et al. Assessing the Relationship between the Baseline Value of a Continuous Variable and Subsequent Change Over Time. Front Public Health. 2013;1:29.

37 Avery CL, Der JS, Whitsel EA, et al. Comparison of study designs used to detect and characterize pharmacogenomic interactions in nonexperimental studies: a simulation study. Pharmacogenet Genomics. 2014;24:146-155.

38 Sitlani CM, Rice KM, Lumley T, et al. Generalized estimating equations for genome-wide association studies using longitudinal phenotype data. Stat Med. 2015;34:118-130.

39 Avery CL, Sitlani CM, Arking DE, et al. Drug-gene interactions and the search for missing heritability: a cross-sectional pharmacogenomics study of the QT interval. Pharmacogenomics J. 2014;14:6-13.

40 Noordam R, Sitlani CM, Avery CL, et al. A genome-wide interaction analysis of tricyclic/tetracyclic antidepressants and RR and QT intervals: a pharmacogenomics study from the Cohorts for Heart and Aging Research in Genomic Epidemiology (CHARGE) consortium. J Med Genet. 2017;54:313-323.

41 Hernán MA, Robins JM. Causal Inference. Boca Raton: Chapman & Hall/CRC, forthcoming. 2017.

42 Karanicolas PJ, Farrokhyar F, Bhandari M. Practical tips for surgical research: blinding: who, what, when, why, how? Can J Surg 2010;53:345-348.

43 Sun X, Ioannidis JP, Agoritsas T, et al. How to use a subgroup analysis: users' guide to the medical literature. JAMA. 2014;311:405-411.

44 Rothwell PM. External validity of randomised controlled trials: "To whom do the results of this trial apply?" Lancet. 2005;365:82-93.

45 Davey Smith G, Hemani G. Mendelian randomization: genetic anchors for causal inference in epidemiological studies. Hum Mol Genet. 2014;23:R89-98.

46 Smit RAJ, Postmus I, Trompet S, et al. Rooted in risk: genetic predisposition for low-density lipoprotein cholesterol level associates with diminished low-density lipoprotein cholesterol response to statin treatment. Pharmacogenomics. 2016;17:1621-1628.

47 Swerdlow DI, Preiss D, Kuchenbaecker KB, et al. HMG-coenzyme A reductase inhibition, type 2 diabetes, and bodyweight: evidence from genetic analysis and randomised trials. Lancet 2015;385:351-361.

48 Walker VM, Davey Smith G, Davies NM, et al. Mendelian randomization: a novel approach for the prediction of adverse drug events and drug repurposing opportunities. Int J Epidemiol. 2017;46:2078-2089.

49 Tardif JC, Rhéaume E, Lemieux Perreault LP, et al. Pharmacogenomic determinants of the cardiovascular effects of dalcetrapib. Circ Cardiovasc Genet. 2015;8:372-382.

50 Smith PG, Day NE. The design of case-control studies: the influence of confounding and interaction effects. Int J Epidemiol. 1984;13:356-365.

51 Kraft P, Zeggini E, Ioannidis JP. Replication in genome-wide association studies. Stat Sci. 2009;24:561-573.

52 Bromley CM, Close S, Cohen Nm et al. Designing pharmacogenetic projects in industry: practical design perspectives from the Industry Pharmacogenomics Working Group. Pharmacogenomics J. 2009;9:14-22.

53 Fry A, Littlejohns TJ, Sudlow C, et al. Comparison of Sociodemographic and Health-Related Characteristics of UK Biobank Participants with the General Population. Am J Epidemiol. 2017;186:1026-1034.

54 Gaziano JM, Concato J, Brophy M, et al. Million Veteran Program: A mega-biobank to study genetic influences on health and disease. J Clin Epidemiol. 2016;70:214-223.

55 Postmus I, Trompet S, Deshmukh HA, et al. Pharmacogenetic meta-analysis of genome-wide association studies of LDL cholesterol response to statins. Nat Commun. 2014;5:5068.

56 Noordam R, Avery CL, Visser LE et al. Identifying genetic loci affecting antidepressant drug response in depression using drug–gene interaction models. Pharmacogenomics. 2016;17:1029-1040.

57 Lord EM. A paradox in the interpretation of group comparisons. Psychological Bulletin. 1967;68:304-305.

58 Glymour MM, Weuve J, Berkman LF et al. When is baseline adjustment useful in analyses of change? An example with education and cognitive change. Am J Epidemiol. 2005;162:267-278.

59 Deshmukh HA, Colhoun HM, Johnson T et al. Genome-wide association study of genetic determinants of LDL-c response to atorvastatin therapy: importance of Lp(a). J Lipid Res. 2012;53:1000-1011.

CHAPTER 4

Meta-analysis of genome-wide association studies of HDL cholesterol response to statins

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ABSTRACT

Background: In addition to lowering low density lipoprotein-cholesterol (LDL-C), statin therapy also raises high density lipoprotein-cholesterol (HDL-C) levels. Inter-individual variation in HDL-C response to statins may be partially explained by genetic variation.

Methods and Results: We performed a meta-analysis of genome-wide association studies (GWAS) to identify variants with an effect on statin-induced HDL-C changes. The 123 most promising signals with P<5x10-4 from the 16,769 statin-treated participants in the first analysis stage were followed up in an independent group of 10,951 statin-treated individuals, providing a total sample size of 27,720 individuals. The only associations of genome-wide significance (P<5x10-8) were between minor alleles at the CETP locus and greater HDL-C response to statin treatment.

Conclusion: Based on results from this study that included a relatively large sample size, we suggest that CETP may be the only detectable locus with common genetic variants that influence HDL-C response to statins substantially in individuals of European descent. Although CETP is known to be associated with HDL-C, we provide evidence that this pharmacogenetic effect is independent of its association with baseline HDL-C levels.

INTRODUCTION

The drug class of 3-hydroxymethyl-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, better known as “statins”, are widely prescribed and effective for the prevention and management of cardiovascular disease (CVD) (1). While the major CVD benefit of statins is due to reduction in plasma low density lipoprotein cholesterol (LDL-C) (2), statins also produce moderate increases, ranging from 4 to 10%, in levels of high density lipoprotein cholesterol (HDL-C) (3, 4). This is of particular interest since HDL-C levels are inversely related to CVD risk in the general population and in patients treated with statins (5, 6). However, a causal role of low HDL-C as a determinant of increased CVD risk is controversial (7).

The increase in HDL-C after statin therapy varies among individuals (3). This might be partly due to genetic variation. Previous studies that have investigated associations between genotype and statin-induced changes in HDL-C (8-10) have focused primarily on variants within the *CETP* gene that are known to affect circulating HDL-C levels (11) and risk of coronary artery disease (12). To address whether additional loci have an effect on statin-induced changes in HDL-C levels, we conducted a large-scale meta-analysis of genome-wide association studies (GWAS) using datasets from both randomized controlled trials (RCTs) and cohort studies in the large Genomic Investigation of Statin Therapy (GIST) consortium that previously identified four loci associated with LDL-C response to statins (13).

METHODS

Study populations

The GIST consortium assembled data from seven RCTs and eleven prospective population-based studies. The initial analysis (first stage) was performed in 16,769 statin-treated individuals; 8,506 individuals from six RCTs (ASCOT UK, CARDS, CAP, PRINCE, PROSPER, and TNT) and 8,263 statin-treated individuals from ten observational studies (AGES, ARIC, ASCOT UK-observational, BioVU, CHS, FHS, Health ABC, HVH, MESA, and the Rotterdam Study). Further investigation (second stage) was performed in 10,951 statin-treated individuals from two RCTs (ASCOT Scandinavia and JUPITER) and two observational studies (ASCOT Scandinavia – observational and GoDARTS), which were used to test for replication of findings from the first stage. Details of the first and second stage studies, including their genotyping and quality control (QC) information,

can be found in the **Supplementary Notes 1, 2 and 3** and **Supplementary tables 1 and 2**.

Subjects

Response to statin treatment was principally studied in statin-treated individuals only. Those treated with placebo were excluded from the analyses of RCTs and those not treated with statins were excluded from observational studies. HDL-C measurements were obtained before and after start of statin treatment. Only subjects with non-missing phenotypes and covariates were included. Those of reported or suspected non-European ancestry were excluded.

Outcome measurements

The response to statin treatment was defined as the difference between the natural log-transformed on- and off-treatment HDL-C levels (ln (on-treatment HDL-C) – ln (off-treatment HDL-C)). The corresponding linear regression coefficients thus reflect the fraction of differential HDL-C increase (relative increase) per copy of the coded allele in the additive genetic model. For observational studies, on-treatment HDL-C levels were calculated for all different prescribed statins, at any dosage, for any indication, and for any treatment episode extending at least four weeks prior to on-treatment HDL-C measurement. Characteristics of on- and off-treatment HDL-C levels and statins used in each study are shown in **Supplementary Table 2**. For each individual, at least one off-treatment HDL-C measurement and at least one on-treatment measurement were required. Subjects with missing on- or off-treatment measurements were excluded, with the exception of the GoDARTS study for which missing off-treatment HDL-C levels were estimated using imputation methods, as described previously (14). When multiple on- or off-treatment measurements were available, the mean of the measurements was used.

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 3**. In all studies, genotyping was performed using either Illumina, Affymetrix, or Perlegen genotyping arrays. Genotype data from each study had been imputed to the HapMap phase 2 reference panel (15), except for JUPITER which was imputed to the 1000genomes pilot data,

using either MACH, Impute, or BIMBAM software [16-18], resulting in a total of approximately 2.5 million SNPs for analysis.

GWAS analysis

Each study independently performed the GWAS on the difference between natural log-transformed on- and off-treatment HDL-C levels, according to a common, central analysis protocol. To reduce confounding by possible association with off-treatment HDL-C levels, analyses were adjusted for the natural log-transformed off-treatment HDL-C levels. Linear regression was used, with SNPs represented by an additive genetic model and with imputed SNPs represented by expected allele dosage. Analyses were additionally adjusted for age, sex, and study specific covariates (e.g. ancestry principal components (PCs), site, or country). FHS made use of a linear mixed effects model considering the kinship matrix in the analysis, hereby accounting for familial correlations within FHS. Analyses in the observational studies were, if the information was available, additionally adjusted for the time interval between on- and off-treatment HDL-C measures (mean follow-up times per study are provided in **Supplementary Table 2**) and for the natural logarithm of the statin dose equivalent, as defined in **Supplementary Table 4**. This table shows the dose for different statins for the LDL-C response; dividing the statin dosage for an individual drug by its dose equivalent shown in **Supplementary Table 4** gives the standardized statin dosage.

Quality control and Meta-analysis

Within each study, SNPs with minor allele frequency <1% or imputation quality <0.3 were excluded from the analysis. QQ-plots were assessed for each study to check that there were no between study differences nor evidence for systematic bias within studies (**Supplementary Figure 1**). The software package METAL was used to perform the meta-analysis (19). A fixed effects, inverse variance weighted approach was used. To correct for possible inflation of the test statistic, e.g. due to small amounts of potential population sub-structure, 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 $<1x10^{-4}$ in the first stage meta-analyses were selected for further investigation in the second stage. A maximum of two SNPs per locus (with a maximum 100 kB distance between SNPs) were selected, with the choice

based on statistical significance. A total of 123 SNPs in 83 loci were selected for the second stage, which was performed in the GoDARTS study, the JUPITER trial, and the RCT and observational arm of the ASCOT Scandinavia study. GWAS data and response to statin treatment were available for these studies. Analysis was performed as for the first stage. Results of the first and second stage were combined using a fixed effects, inverse variance weighted meta-analysis using METAL.

Interaction analysis

The interaction effect of the lead *CETP* SNP rs247616 with the binary treatment indicator for statin versus placebo allocation was assessed in five of the participating RCTs (ASCOT Scandinavia, ASCOT UK, CARDS, JUPITER, and PROSPER). For these analyses, placebo treated individuals in the RCTs were included. The total sample size was 17,857, with 8,978 statin treated individuals and 8,879 placebo treated individuals. Regression models were applied to the combined population of statin and placebo treated subjects by adding to the model extra terms including treatment (statin (=1) or placebo (=0)) allocation and the product of treatment allocation with SNP minor allele dose (20). Interaction coefficients of the five studies were combined in a fixed effects, inverse variance weighted meta-analysis using METAL.

Effect of genetic determinants of HDL-C levels on statin-induced HDL-C response

We performed a look-up in our GWAS results for all known genome-wide significant genetic variants associated with HDL-C levels, obtained from the most recent Global Lipids Genetics Consortium (GLGC) paper (11). Of the 80 variants, 78 were available in our GWAS on statin induced HDL-C response. Subsequently, we examined whether a multi-SNP genotypic risk score constructed from these GLGC variants was associated with the level of statin induced HDL-C response, using publicly available summary level data from the GLGC (http://csg.sph.umich.edu//abecasis/public/lipids2013/). The joint effect of the 78 genetic variants on statin-induced HDL-C response was examined by means of a data-driven inverse-variance weighted approach, described previously by Dastani *et al* (21), and accomplished through the gtx-package (22) (Genetics ToolboX, http://cran.r-project.org/web/packages/gtx) in the R statistical software environment (23). Analogous to deriving a pooled estimate from the results of individual studies in conventional meta-analysis, this approach combines the causal estimates of multiple genetic variants, defined as the ratio of their association with statin response to their association with HDL-c levels.

Conditional analysis

Conditional analysis was conducted within GCTA software (24), using the *–cojo* method, which performs conditional and joint analysis with model selection. The genome-wide meta-analysis summary statistics from the combined analysis of both first-stage and second-stage data were used as the input data. Analysis was restricted to chromosome 16, containing the only genome-wide significant result from the meta-analysis, in order to determine whether the *CETP* region contains more than one independent signal of association. Within the GCTA analysis, MAF was restricted to ≥1% and a p-value cut-off of $5x10^{-7}$ was used as the selection threshold. LD was calculated between pairwise SNPs, but any SNPs further than 10 Mb apart were assumed to be in linkage equilibrium.

Variance explained

Two secondary analyses were performed to investigate the heritability of this pharmacogenetic trait. Firstly, the genome-wide heritability was calculated in GCTA (24) by estimating h^2 using GREML analysis, according to all HapMap SNPs with MAF ≥ 1%, with reference to the genomic relatedness matrix generated within GCTA. Secondly, the percentage variance explained of the HDL response

adjusted for baseline HDL-C to statins trait was calculated specifically for the lead *CETP* SNP rs247616 using R software (23), by including the dosage data for this SNP as a continuous predictor variable within the model. The R^2 calculated from the fitted linear regression model was used to estimate the percentage of the trait variance explained. Both analyses were performed using the ASCOT-UK dataset only, as individual level raw genotype data are required. The combination of both the RCT and observational sub-cohorts of ASCOT-UK gave a total sample size of N = 2,055 statin-treated patients. The linear regression model used exactly the same data and covariates as from the primary GWAS analysis, including the top 10 PCs.

RESULTS

First-stage meta-analysis

In the first stage of this analysis, six randomized controlled trials (n=8,506 statin recipients) and ten observational studies (n=8,263 statin recipients) were included (**Supplementary Notes 1 and 2** and **Supplementary Tables 1 and 2**). Three SNPs at the *CETP* locus (chromosome 16) were identified as genome-wide significant ($P<5x10^{-8}$) for their association with HDL-C response to statin treatment (**Figures 1 and 2** and **Table 1**). The most significant association was for SNP rs247616 (MAF=0.324, ß=0.011, SE=0.002, $P=5.95x10^{-10}$) (**Figure 3**), indicating that carriers of the minor allele of this SNP respond to statins with a 1.1% greater per-allele increase in HDL-C compared with non-carriers. This 1.1% per-allele increase in HDL-C is equivalent to a 0.014 mmol/L increase. We found no other loci associated with HDL-C response to statin treatment at a genome-wide significant level at this first stage.

Second-stage meta-analysis

We selected 123 SNPs from 83 loci with $P<1x10^{-4}$ in the first stage meta-analysis for further investigation in the second stage, which included 10,951 statin-treated individuals from two RCTs and two observational studies (**Supplementary Note 3** and **Supplementary Tables 1 and 2**). The second stage meta-analysis confirmed the significant association between genetic variants within

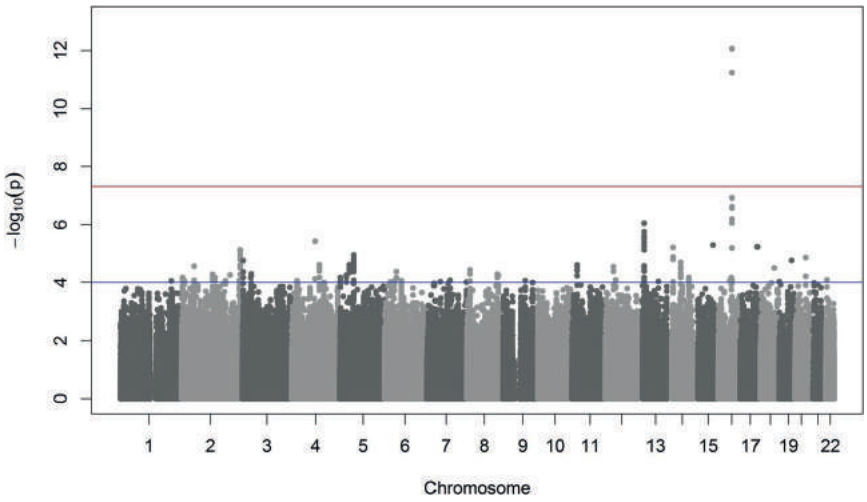

Figure 1. Results of the GWAS meta-analysis. Manhattan plot presenting the $-\log_{10}$ P-values from the combined stage 1 and 2 meta-analysis on HDL-C response to statin treatment. The top (red) line represents the P-value $5x10^{-8}$, the second (blue) line represents the P-value $1x10^{-4}$, the threshold for moving SNPs to the second stage.

Table 1. Association of *CETP* SNP rs247616 (chromosome 16, bp 55547091) with HDL-C response after statin treatment in the stage 1, stage 2, and combined GWAS meta-analyses.

Phase	N	Coding allele	Non-coding allele	Frequency coding allele	Beta*	SE	% extra increase#	P-value
Stage 1	14693	T	C	0.324	0.011	0.002	1.1	$5.95x10^{-10}$
Stage 2	10961	T	C	0.327	0.005	0.001	0.5	$1.59x10^{-5}$
Combined	25654	T	C	0.326	0.007	0.001	0.7	$8.52x10^{-13}$

*Beta for difference between the natural log transformed on- and off-treatment HDL-C levels, adjusted for natural log transformed off-treatment HDL-C, age, sex, and study specific covariates. The beta reflects the fraction of differential HDL-C lowering in carriers vs. non-carriers of the SNP; a positive beta indicates a better statin response (larger HDL-C increase). #This percentage reflects the % extra HDL-C increase in carriers vs. non-carriers of the SNP.

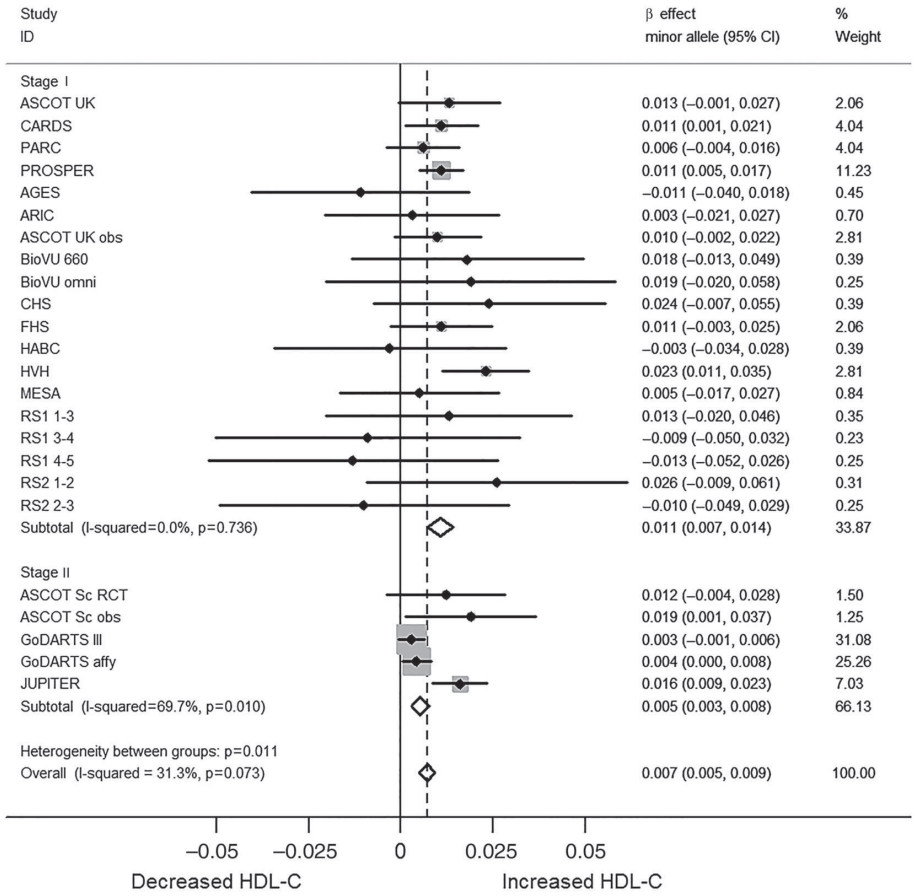

Figure 2. Forest plot showing the association in each study and overall association of the lead *CETP* SNP rs247616 with HDL-C response to statin treatment. Beta represents fractional HDL-C change for each copy of the minor allele.

the *CETP* loci and HDL-C response from the first stage meta-analysis (rs247616: MAF=0.327, ß=0.005, SE=0.001, $P=1.59x10^{-5}$) as $P<6x10^{-4}$, the Bonferroni p-value threshold for testing 123 SNPs (**Table 1**, **Figure 2**, and **Supplementary Table 5**). The combined effect from the first and second stage meta-analysis for the *CETP* rs247616 SNP was genome-wide significant (MAF=0.326, ß=0.007, SE=0.001, $P=8.52x10^{-13}$) (**Table 1**, **Figure 2**, and **Supplementary Table 5**). No other locus reached statistical significance ($P<4x10^{-4}$) in the second stage meta-analysis or in the combined meta-analysis ($P<5x10^{-8}$) for association with HDL-C response to statin treatment (**Figure 1** and **Supplementary Table 5**). Indeed, **Supplementary Table 5** (ordered by the combined meta-analysis p-values)

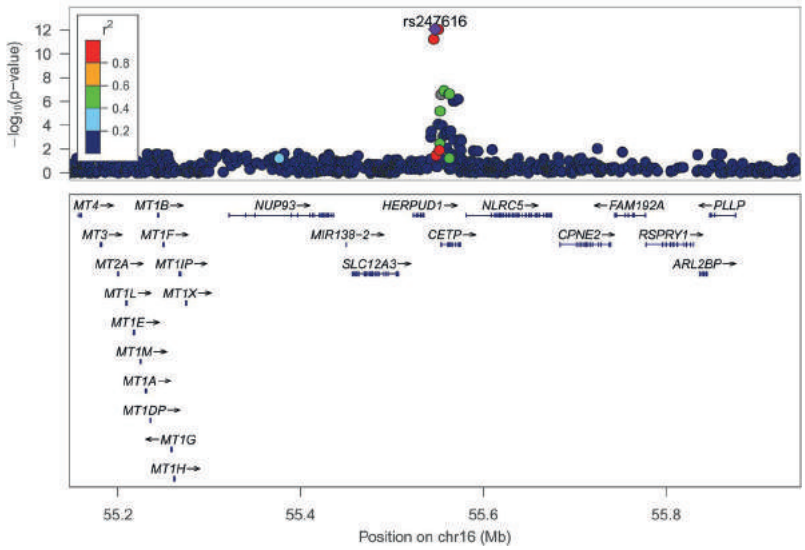

Figure 3. Regional association plot of the *CETP* region that was genome-wide significant for association with HDL-C response to statin treatment, using the results of the combined meta-analysis (generated using LocusZoom (37)). The color of each SNP is based on the LD (r^2) with the lead SNP rs247616 (shown in purple). The RefSeq genes in the region are shown in the lower panel.

shows that the three SNPs within *CETP* which were genome-wide significant in the first stage, were the only SNPs that reached Bonferroni significance in the second stage and genome-wide significance in the combined meta-analysis

Interaction analysis

To exclude the possibility of confounding in the association between *CETP* and HDL-C response to statin treatment, we tested for interaction between the *CETP* lead SNP rs247616 and randomized statin or placebo allocation using data from five of the participating RCTs. **Table 2** shows a significant P-value for interaction ($P\text{-}3.52x10^{-3}$, ß=0.007, SE=0.002) for the *CETP* SNP, indicating that genetic effects of *CETP* on baseline HDL-C contribute at most only in part to genetic effects on HDL-C response in the statin-treated group, as the genetic effect is modified by the use of statin treatment.

Table 2. Interaction between *CETP* rs247616 and statin vs. placebo allocation on HDL-C response.

SNP	N	Coding allele	Non-coding allele	Frequency coding allele	Interaction Beta	Interaction SE	Interaction P-value
rs247616	17857	T	C	0.341	0.007	0.002	$3.52x10^{-3}$

Meta-analysis of data from 5 RCTs. Interaction beta and SE refer to statistics from linear regression modelling the difference between the natural log transformed on- and of-treatment HDL-C levels adjusted for natural log transformed off-treatment HDL-C, age, sex, and study specific covariates, and including an interaction term between SNP and statin or placebo allocation. The interaction p-value refers to the significance of the SNP-by-statin or placebo allocation interaction term in the regression model.

Effect of genetic determinants of HDL-C levels on statin-induced HDL-C response

SNPs previously shown to be associated with HDL-C levels (n=78)[11] were assessed for their association with statin-induced HDL-C response in our meta-analysis. After Bonferroni correction, rs3764261 (*CETP)* was the sole genetic variant associated with statin-induced HDL-C response amongst the 78 examined variants (**Supplementary Table 5**). Joint analysis of the HDL-C associated variants demonstrated that predisposition to high HDL-C levels is associated with increased statin-induced HDL-C response (**Figure 4**). This

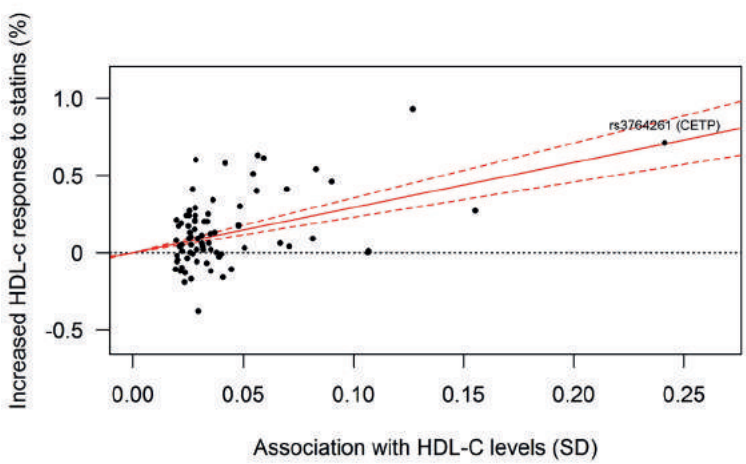

Figure 4. Plot of the per-allele association of genetic variants with HDL-C levels (x-axis, per allele in SD units, as reported by Willer *et al.* (11)) against the association with HDL-C response to statin treatment (y-axis, percentage) (generated using (22)). The regression line shows the linear relationship between these two, with 95% confidence boundaries.

amounted to a 2.9% fractional increase (ß=0.029, SE=0.003, $P=1x10^{-19}$) in statin-induced HDL-C response per SD increase in genetically raised HDL-C levels. Excluding the *CETP* SNP (rs3764261) from the model did not materially change the results (ß=0.029, SE=0.005, $P=1x10^{-8}$). Testing for heterogeneity did not reveal any indication of pleiotropic effects (P=0.64).

Conditional analysis

The conditional analysis within GCTA resulted in only one remaining SNP selected in the model, namely the lead SNP rs247616 within the *CETP* locus, with a joint p-value of $9.96x10^{-10}$ and joint ß=0.0104, equal to its unconditional effect size estimate. As can be seen from the locus zoom plot in **Figure 3**, the other two genome-wide significant hits are in high LD with the lead SNP, and after conditioning on the lead SNP, the GCTA conditional analysis results show that no other SNPs within chromosome 16 have significant residual association, with the minimum conditional p-value being $p\sim3x10^{-5}$. Hence we conclude that there is only one independent signal within the *CETP* association.

Variance explained

From genome-wide data of the ASCOT-UK datasets, the trait heritability for HDL-C response to statins was estimated as h^2 = 17.8% (SE = 0.154). The trait variance explained by the lead *CETP* SNP rs247616 alone was calculated to be 7.8%.

DISCUSSION

In this study we have performed a meta-analysis of GWAS including over 27,700 statin-treated individuals, investigating genetic variants associated with variation in HDL-C response to statin treatment. We identified three genetic variants in the *CETP* locus that were highly significantly associated with a larger HDL-C response to statin treatment. No other SNPs met the genome-wide criterion for association of HDL-C change with statin use.

CETP plays an important role in HDL-C metabolism by promoting the exchange of cholesteryl esters in HDL particles with triglycerides in apolipoprotein B-containing particles, leading to increased HDL catabolism and lower HDL-C levels. Increases in HDL-C levels after statin treatment are probably partly the result of a reduction in CETP mediated lipid transfer (25). Statin treatment decreases CETP activity up to 30% (26, 27). Previously it has been shown that genetic variants within *CETP* are associated with differences in CETP

concentration (28). The three SNPs associated with HDL-C response to statins in the present study are located 2.5-7 kb upstream of the *CETP* gene and are in high linkage disequilibrium (**Figure 3**) (29). The minor alleles of these SNPs have been shown to be associated with lower *CETP* mRNA expression levels in liver tissue and with higher HDL-C levels (29, 30).

Previous studies investigating the association between SNPs in the *CETP* locus and the HDL-C response to statin treatment have yielded inconsistent results. Several studies showed associations with a greater HDL-C response (8, 10), whereas others showed no significant associations (12, 31-33). These discrepancies could be explained by limited sample sizes and by the investigation of different genetic variants in these studies. An alternative explanation could be the fact that the effect of statins on HDL-C response is relatively small and depends on statin dose and type (3, 4). Since the power to detect genetic effects on these small variations is low in single studies, the results from the present large meta-analysis, with replication, provide strong evidence that genetic variation at the *CETP* locus is associated with HDL-C response.

The results of six randomized clinical trials and ten observational studies were combined in the first stage of the current study. Different statins were investigated in the trials and used within the observational studies, resulting in combining several types of statins in our analysis. This and the variation in statin dosages during follow-up for an individual are a limitation of the current study, since the pharmacogenetic impact might be dependent on specific statin types and dose. To address this possible limitation, the individual study analyses were adjusted for statin equivalent dose based on effect on LDL-C levels, making the different statin types likely more comparable with respect to clinical effectiveness on HDL-C levels. Combining RCTs and observational cohort might also result in heterogeneity between the study types. However no heterogeneity was observed between the groups within the current study ($p=0.761$).

Another possible limitation of the current study is the association of the identified genetic variant with baseline HDL-C concentration. As shown in previous large GWAS studies, the *CETP* SNP rs3764261 is strongly associated with HDL-C levels (11, 30). In pharmacogenetic studies investigating lipid responses to drug exposure, it is important to eliminate the effect of the association between baseline lipid levels and the investigated genetic variants (13). To reduce the impact of these possible confounding effects, our response to treatment analyses were adjusted for baseline HDL-C levels. In addition, interaction analyses in five of the RCTs, with direct modeled comparison with a random assignment to a placebo group, suggested little or no influence of the association between the *CETP*

SNPs and baseline HDL-C levels on the genetic effect on HDL-C response to statin treatment. It is, however, possible that mechanisms underlying the effects of CETP on HDL-C levels are also involved in mediating statin effects on HDL-C.

All genetic data in the current study was imputed up to 2.5 million autosomal SNPs based on data from the HapMap project (15). Imputation based on the more recent 1000 Genomes project might have revealed more associations with less common genetic variants (34). In addition, in our analysis we excluded genetic variants with a minor allele frequency <1%, restricting our analysis to common genetic variants. Future studies using exome sequencing data and investigating rare variants may identify new association between genetic variants and statin-induced HDL-C response.

The implications of the present findings regarding genetic effects on the efficacy of statins for reductions in risk of CVD are uncertain. Based on the strong inverse relationship of HDL-C with CVD, the greater statin-induced increase in HDL-C among carriers of the minor vs. major alleles of the three *CETP* SNPs reported here may confer a greater protective effect of statins on CVD in patients carrying the minor allele. However, a recent study employing Mendelian randomization found that genotypes associated with plasma HDL-C levels were not associated with the impact on CVD risk that would be predicted by the magnitude of the genotypic effects on HDL-C (7). Moreover, two large clinical trials have failed to show reduction of CVD events by nicotinic acid-induced increases in HDL-C in patients with well-controlled LDL-C levels (35, 36). Hence, whether greater genetically-mediated HDL-C increases with statin treatment confer increased protection from CVD remains unknown.

In conclusion, this study is the largest meta-analysis of GWAS for HDL-C response to statin treatment conducted to date. The findings suggest that *CETP* may be the only locus in which common genetic variants are significantly associated with a substantial HDL-C response to statin treatment in individuals of European descent.

Supporting information

The Supplementary Material for this article can be found online at:
https://jmg.bmj.com/content/53/12/835.long

REFERENCES

1 Davidson MH, Toth PP. Comparative effects of lipid-lowering therapies. Prog. Cardiovasc.Dis. 2004;47:73-104.

2 Baigent C, Blackwell L, Emberson J, et al. Efficacy and safety of more intensive lowering of LDL cholesterol: a meta-analysis of data from 170,000 participants in 26 randomised trials. Lancet. 2010;376:1670-81.

3 McTaggart F, Jones P. Effects of statins on high-density lipoproteins: a potential contribution to cardiovascular benefit. Cardiovasc.Drugs Ther. 2008;22:321-38.

4 Nicholls SJ, Tuzcu EM, Sipahi I, et al. Statins, high-density lipoprotein cholesterol, and regression of coronary atherosclerosis. JAMA. 2007;297:499-508.

5 Di AE, Sarwar N, Perry P, et al. Major lipids, apolipoproteins, and risk of vascular disease. JAMA. 2009;302:1993-2000.

6 Boekholdt SM, Arsenault BJ, Hovingh GK, et al. Levels and changes of HDL cholesterol and apolipoprotein A-I in relation to risk of cardiovascular events among statin-treated patients: a meta-analysis. Circulation. 2013;128:1504-12.

7 Voight BF, Peloso GM, Orho-Melander M, et al. Plasma HDL cholesterol and risk of myocardial infarction: a mendelian randomisation study. Lancet 2012;380:572-80.

8 Anagnostopoulou K, Kolovou G, Kostakou P, et al. Pharmacogenetic study of cholesteryl ester transfer protein gene and simvastatin treatment in hypercholesterolaemic subjects. Expert Opin Pharmacother. 2007;8:2459-63.

9 Bercovich D, Friedlander Y, Korem S, et al. The association of common SNPs and haplotypes in the CETP and MDR1 genes with lipids response to fluvastatin in familial hypercholesterolemia. Atherosclerosis. 2006;185:97-107.

10 Leusink M, Onland-Moret NC, Asselbergs FW, et al. Cholesteryl ester transfer protein polymorphisms, statin use, and their impact on cholesterol levels and cardiovascular events. Clin.Pharmacol.Ther. 2014;95:314-20.

11 Willer CJ, Schmidt EM, Sengupta S, et al. Discovery and refinement of loci associated with lipid levels. Nat Genet. 2013;45:1274-83.

12 Boekholdt SM, Sacks FM, Jukema JW, et al. Cholesteryl ester transfer protein TaqIB variant, high-density lipoprotein cholesterol levels, cardiovascular risk, and efficacy of pravastatin treatment: individual patient meta-analysis of 13,677 subjects. Circulation. 2005;111:278-87.

13 Postmus I, Trompet S, Deshmukh HA, et al. Pharmacogenetic meta-analysis of genome-wide association studies of LDL cholesterol response to statins. Nat Commun. 2014;5:5068.

14 Donnelly LA, van Zuydam NR, Zhou K, et al. Robust association of the LPA locus with low-density lipoprotein cholesterol lowering response to statin treatment in a meta-analysis of 30 467 individuals from both randomized control trials and observational studies and association with coronary artery disease outcome during statin treatment. Pharmacogenet Genomics. 2013;23:518-25.

15 The International HapMap Project. Nature. 2003;426:789-96.

16 Li Y, Willer CJ, Ding J, et al. MaCH: using sequence and genotype data to estimate haplotypes and unobserved genotypes. Genet Epidemiol. 2010;34:816-34.

17 Marchini J, Howie B, Myers S, et al. A new multipoint method for genome-wide association studies by imputation of genotypes. Nat Genet. 2007;39:906-13.

18 Servin B, Stephens M. Imputation-based analysis of association studies: candidate regions and quantitative traits. PLoS Genet. 2007;3:e114.

19 Willer CJ, Li Y, Abecasis GR. METAL: fast and efficient meta-analysis of genomewide association scans. Bioinformatics. 2010;26:2190-91.

20 Chasman DI, Giulianini F, Macfadyen J, et al. Genetic Determinants of Statin Induced LDL-C Reduction: The JUPITER Trial. Circ Cardiovasc Genet. 2012;5:257-64.

21 Dastani Z, Hivert MF, Timpson N, et al. Novel loci for adiponectin levels and their influence on type 2 diabetes and metabolic traits: a multi-ethnic meta-analysis of 45,891 individuals. PLoS Genet. 2012;8:e1002607.

22 Johnson T. Efficient calculation for multi-SNP genetic risk scores. Technical report, The Comprehensive R Archive Network, 2013. http://cran.r-project.org/web/packages/gtx/vignettes/ashg2012.pdf. Last accessed 28 September 2015.

23 R Core Team: A language and environment for statistical computing. (2015) R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org, 2015.

24 Yang J, Ferreira T, Morris AP, et al. Conditional and joint multiple-SNP analysis of GWAS summary statistics identifies additional variants influencing complex traits. Nat Genet. 2012;44:369-3.

25 Rensen PC, Havekes LM. Cholesteryl ester transfer protein inhibition: effect on reverse cholesterol transport? Arterioscler Thromb Vasc Biol. 2006;26:681-84.

26 Ahnadi CE, Berthezene F, Ponsin G. Simvastatin-induced decrease in the transfer of cholesterol esters from high density lipoproteins to very low and low density lipoproteins in normolipidemic subjects. Atherosclerosis. 1993;99:219-28.

27 Guerin M, Egger P, Soudant C, et al. Dose-dependent action of atorvastatin in type IIB hyperlipidemia: preferential and progressive reduction of atherogenic apoB-containing lipoprotein subclasses (VLDL-2, IDL, small dense LDL) and stimulation of cellular cholesterol efflux. Atherosclerosis. 2002;163:287-96.

28 Kuivenhoven JA, Jukema JW, Zwinderman AH, et al. The role of a common variant of the cholesteryl ester transfer protein gene in the progression of coronary atherosclerosis. The Regression Growth Evaluation Statin Study Group. N Engl J Med. 1998;338:86-93.

29 Papp AC, Pinsonneault JK, Wang D, et al. Cholesteryl Ester Transfer Protein (CETP) polymorphisms affect mRNA splicing, HDL levels, and sex-dependent cardiovascular risk. PLoS One. 2012;7:e31930.

30 Teslovich TM, Musunuru K, Smith AV, et al. Biological, clinical and population relevance of 95 loci for blood lipids. Nature. 2010;466:707-13.

31 Barber MJ, Mangravite LM, Hyde CL, et al. Genome-wide association of lipid-lowering response to statins in combined study populations. PLoS One. 2010;5:e9763.

32 Li J, Zhang L, Xie NZ, et al. Relationship between the cholesterol ester transfer protein TaqIB polymorphism and the lipid-lowering effect of atorvastatin in patients with coronary atherosclerotic heart disease. Genet Mol Res. 2014;13:2140-48.

33 Singer JB, Holdaas H, Jardine AG, et al. Genetic analysis of fluvastatin response and dyslipidemia in renal transplant recipients. J Lipid Res. 2007;48:2072-78.

34 Abecasis GR, Auton A, Brooks LD, et al. An integrated map of genetic variation from 1,092 human genomes. Nature. 2012;491:56-65.

35 Boden WE, Probstfield JL, Anderson T, et al. Niacin in patients with low HDL cholesterol levels receiving intensive statin therapy. N Engl J Med. 2011;365:2255-67.

36 Landray MJ, Haynes R, Hopewell JC, et al. Effects of extended-release niacin with laropiprant in high-risk patients. N Engl J Med. 2014;371:203-12.

37 Pruim RJ, Welch RP, Sanna S, et al. LocusZoom: regional visualization of genome-wide association scan results. Bioinformatics. 2010;26:2336-37.

CHAPTER 5

Rooted in risk; genetic predisposition for low-density lipoprotein cholesterol level associates with diminished low-density lipoprotein cholesterol response to statin treatment

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ABSTRACT

Aim: To utilize previously reported lead SNPs for LDL-c levels to find additional loci of importance to statin response, and examine whether genetic predisposition to LDL-c levels associates with differential statin response.

Patients/methods: We investigated effects on statin response of 59 LDL-c SNPs, by combining summary level statistics from the Global Lipids Genetics and Genomic Investigation of Statin Therapy consortia.

Results: Lead SNPs for APOE, SORT1, and NPC1L1 were associated with a decreased LDL-c response to statin treatment, as was overall genetic predisposition for increased LDL-c levels as quantified with 59 SNPs, with a 5.4% smaller statin response per standard deviation increase in genetically raised LDL-c levels.

Conclusion: Genetic predisposition for increased LDL-c level may decrease efficacy of statin therapy.

INTRODUCTION

HMG-CoA reductase inhibitors, also known as statins, have proven themselves as a highly effective treatment option in the management and prevention of cardiovascular disease, both in research and clinical settings (1, 2). Their effect is thought to primarily result from reducing low-density lipoprotein cholesterol (LDL-c) levels by up to 50% (3), thereby achieving a 20-30% reduction of cardiovascular events. However, substantial interindividual variability exists in the LDL-c response to statins, in part due to genetic factors, which influences their efficacy in reducing the occurrence of major adverse events.

Recently, through the largest pharmacogenomic meta-analysis for differential LDL-c response to statin therapy to date, the Genomic Investigation of Statin Therapy (GIST) consortium identified four loci (APOE, LPA, SORT1/CELSR2/PSRC1, and SLCO1B1) at a genome-wide significant level, whose effect on statin response was independent of off-treatment LDL-c levels (4). With the exception of SLCO1B1, these loci have previously been independently reported to associate with LDL-c levels by the Global Lipids Genetics consortium (GLGC) (5). As loci associated with LDL-c homeostasis are strong mechanistic candidates for differential LDL-c response to statin therapy, we performed a look-up of the previously reported lead SNPs for loci associated with LDL-c levels by the GLGC in the GIST consortium, to examine whether additional loci of importance to differential LDL-c statin response could be identified. Furthermore, we examined whether overall genetic predisposition to higher LDL-c levels (i.e. having more alleles associated with higher LDL-c levels) is associated with differential LDL-c response to statins, by combining summary level statistics from our GIST consortium with publicly available data from the GLGC for all lead SNPs through an inverse-variance weighted approach.

METHODS

Selection of Single Nucleotide Polymorphisms (SNPs) associated with LDL-c levels

In the most recent and largest genome-wide association study (GWAS) for blood lipid levels, which examined up to 188,577 European-ancestry individuals, 157 nearly independent loci ($r^2 < 0.10$) were found to associate with lipid levels at p-values lower than 5×10^{-8} (5). Of the reported 157 lead SNPs, 60 were associated with LDL-c levels (**Supplementary Table 1**). Summary level data of the associations of these 60 lead SNPs with LDL-c levels was downloaded from

the University of Michigan GLGC webpage (http://csg.sph.umich.edu//abecasis/public/lipids2013/). Effects on lipid levels were reported in standard deviations. We excluded rs9411489 (ABO) from our analyses, as the genotype could not be imputed in our populations, and therefore included the remaining 59 lead SNPs in our analyses. To further isolate the effects on LDL-c levels from those of other lipids, we repeated all analyses with a restricted SNP list, excluding the 17 variants which also associated with either high-density lipoprotein cholesterol (HDL-c) or triglycerides (TG) levels at a genome-wide significant level. Of these, 5 associated solely with HDL-c, 4 solely with TG, and 8 with both lipid traits. As LDL-c is closely linked to total cholesterol (TC), we did not exclude variants which also associated with TC at a genome-wide significant level. The restricted list therefore included the remaining 42 LDL-c specific SNPs.

Description of pharmacogenetic meta-analysis

The GIST consortium included 6 randomized controlled statin trials (ASCOT, CARDS, CAP, PRINCE, PROSPER, and TNT) and 10 prospective, population-based studies (AGES, ARIC, BioVU, CHS, FHS, GoDARTS I, GoDARTS II, Health ABC, HVH, MESA) for the first stage, comprised of up to 18,596 statin recipients. In addition, 246 SNPS with $p<5x10^{-4}$ were further investigated in three additional studies (HPS, JUPITER, Rotterdam Study), contributing up to 22,318 additional statin-treated subjects to the meta-analysis. Of the 59 lead SNPs for LDL-c levels reported by the GLGC, only one (rs4420638, APOE) was included amongst these 246 SNPs. The GWAS was performed on the difference between natural log-transformed on- and off-treatment LDL-c levels, adjusting for the natural log-transformed off-treatment LDL-c level to control for possible mediation through off-treatment genetic effects. The beta of the corresponding regression therefore represents the fraction of differential LDL lowering in carriers versus non-carriers of each SNP. For observational studies, this meant that subjects with missing on- or off-treatment measurements were excluded, with the exception of the GoDARTS cohorts for which off-treatment LDL-C levels were imputed. In addition, analyses in the observational studies were, if available, additionally adjusted for statin dose through the use of the natural logarithm of the equivalent dose taken from the literature. Details on included studies, genotyping and GWAS analyses have been described previously (4).

Look-up of single SNPs

We performed a look-up of all 59 candidate LDL-c markers within the pharmacogenetic meta-analysis performed by the GIST consortium, assessing their effect on differential LDL-c response to statin therapy adjusted for off-treatment LDL-c values. Adjusted unstandardized beta-coefficients are given for the LDL-c-increasing alleles reported by the GLGC. Multiple testing was taken into account by means of a Bonferroni-corrected p-value threshold of $8.5x10^{-4}$ (i.e. 0.05/59).

Summary data methods for overall effect of LDL-c predisposition

Next, we investigated whether overall genetic predisposition for LDL-c levels was associated with statin response, making use of summary level data from both the GLGC and GIST consortia. All analyses were carried out separately for the full (n=59) and restricted (n=42) SNP lists. Analogous to pooling estimates from different studies in conventional meta-analysis using inverse-variance weighting (IVW), we pooled the causal estimates from the different genetic variants, defined as the ratio of each SNP's per-allele effect on response to statin therapy to its per-allele effect on LDL-c levels. The average of these ratio estimates was weighted by the inverse of the variance of the per-allele effect on response to statin therapy, and can be visualized as a regression line constrained to pass through the origin (6, 7). As this approach may be biased by the inclusion of genetic variants violating the underlying assumptions of instrumental variable (IV) methods (8), most notably by the presence of unbalanced pleiotropic effects on phenotypes other than LDL-c, we performed two additional analyses which should be considered as sensitivity analyses for Mendelian randomization (MR) investigations with multiple genetic variants (9).

We first employed the recently published MR-Egger method (10), which provides a formal test of the presence of directional (i.e. unbalanced) pleiotropy from separate genetic variants by introducing an intercept term to the IVW method and determining whether this term deviates significantly from zero. Based on the Egger test (11), which assesses the presence of small study bias in meta-analysis, this intercept term can be interpreted as the average pleiotropic effect across the genetic variants. After taking these effects into account, the Egger-regression slope reflects the strength of any residual dose-response relationship. Under the assumption that the strength of the association of each variant with LDL-c levels is independent of the pleiotropic effects of the variant

(i.e. not via LDL-c), MR-Egger regression gives a valid causal effect estimate even when all the genetic variants are invalid instrumental variables (10).

Secondly, we calculated the weighted median estimator, defined as the 50% weighted percentile of the distribution of causal estimates given weights proportional to the inverse of their variance (9). As the median of any distribution is less susceptible to outliers, this method provides a consistent causal estimate under the assumption that over 50% of the weight in the analysis is due to valid instruments. We also provide the penalized weighted median estimate, which severely limits the contribution of heterogeneous (i.e. outlying) variants, which are more likely to represent invalid instrumental variables. This penalty is based on the heterogeneity between estimates as quantified by Cochran's Q statistic. We considered p-values of 0.05 or smaller statistically significant for these summary data methods.

Finally, to examine whether the use of epidemiological cohort data by the GIST consortium might have introduced imprecision to the causal estimates, we repeated the summary data methods whilst solely including the data from the randomized controlled trials participating in the first-stage GIST meta-analysis. All analyses were performed with R software version 3.1.1. (12), utilizing the R code provided by the corresponding methodology papers on MR-Egger and median-based methods (9, 10).

RESULTS

Look-up of single SNPs

After correction for multiple testing, three SNPs were found to have attained a statistically significant association with LDL-c response to statins (all p-values $< 8.5 \times 10^{-4}$, **Table 1**). The results indicate that carriers of these SNPs have a smaller LDL-c response to statin therapy when compared with non-carriers. The magnitudes of these per-allele proportional decreases were 2.5% (APOE, 95% CI: 1.8-3.1), 1.5% (SORT1, 95% CI: 0.9-2.1), and 1.8% (NPC1L1, 95% CI: 0.8-2.7) respectively. When restricting the SNP list to those 42 variants primarily associated with LDL-c, which did not include the lead SNPs for APOE and SORT1, NPC1L1 was the sole statistically significant finding ($p=2.1 \times 10^{-4}$), also after adjusting the Bonferroni-corrected p-value threshold to 1.2×10^{-3} (i.e. 0.05/42).

Table 1. Candidate markers significantly associated with LDL-c response to statin therapy

SNP	Locus	Chr	Global Lipids Genetics consortium (GLGC)					Genomic Investigation of Statin Therapy (GIST) consortium		
			EA	EA freq. (1000G)	Beta (SE)*	p-value	Other lipids	EA freq.	Beta (SE)†	p-value
rs4420638	APOE	19	G	0.19	0.225 (0.008)	$2x10^{-178}$	HDL-c, triglycerides, TC	0.17	0.025 (0.003)	$3.9x10^{-15}$
rs629301	SORT1	1	T	0.79	0.167 (0.005)	$5x10^{-241}$	HDL-c, TC	0.77	0.015 (0.003)	$9.4x10^{-7}$
rs2072183	NPC1L1	7	C	0.24	0.039 (0.004)	$7 x10^{-16}$	TC	0.24	0.018 (0.005)	$2.1x10^{-4}$

Listed variants are those with p-values smaller than the Bonferroni-corrected threshold of $8.5x10^{-4}$ (i.e. 0.05/59) for the association with statin response.
Chr, chromosome; EA, effect allele for increased LDL-c levels from the GLGC consortium; HDL-c, high-density lipoprotein cholesterol; TC, total cholesterol
* Beta for effect on LDL-c levels, in standard deviations
† Beta for difference between the natural log-transformed on- and off-treatment LDL-c levels adjusted for natural log-transformed off-treatment LDL-c, age-, sex- and study-specific covariates. A negative beta indicates a better statin response, a positive beta a worse statin response.

Summary results for overall effect of LDL-c predisposition

As shown in **Figure 1** and **Table 2**, the conventional inverse-variance weighted method revealed strong evidence that overall genetic predisposition for higher LDL-c levels associates with a decreased LDL-c response to statin therapy. For the full list (all LDL-c associated variants), this amounted to a 5.4% (95% CI: 4.2-6.7, $p=8.4x10^{-12}$) smaller response per standard deviation increase in genetically raised LDL-c levels. Despite the effect being slightly reduced, the direction of the association was similar for the restricted list (excluding HDL-c and TG-associated variants), showing a 3.2% (95% CI: 1.2-5.1, $p=2.1x10^{-3}$) decreased response per standard deviation increase in genetically raised LDL-c levels.

Results from both sensitivity analyses were largely consistent with those seen for the IVW approach, with regard to magnitude and direction of the association, especially for the restricted SNP-list (**Table 2**). The MR-Egger results indicated the presence of unbalanced pleiotropy for the full list of variants, as the intercept deviated significantly from zero ($p=7.6x10^{-5}$), which was not present when analyses were restricted to those variants primarily associated with LDL-c (p=0.40). Though inconclusive, further attempts to disentangle the influence of HDL-c and TG-associated variants suggested that the variants associated with HDL-c were especially influential with regard to possible unbalanced pleiotropic effects on statin response, as their exclusion led to the greatest decrease in the MR-Egger intercept term (**Supplemental Table 2**). Of the median-based methods, the penalized estimator was the most consistent with the IVW-estimate, for both SNP lists. As shown in **Supplemental Table 3**, there was large homogeneity between the causal estimates obtained from the full sample and when restricting the analyses to the data from the randomized controlled trials participating in the first-stage GIST meta-analysis.

Table 2. IVW, MR-Egger, and median-based estimators for the association between LDL-c levels and proportional LDL-c response to statin therapy

	Full list of 59 variants		42 variants primarily associated with LDL-c	
Analysis method	**Beta (SE)**	**p-value**	**Beta (SE)**	**p-value**
Inverse-variance weighted	0.054 (0.006)	**$8.4x10^{-12}$**	0.032 (0.010)	**$2.1x10^{-3}$**
MR-Egger: slope	0.089 (0.010)	**$1.0x10^{-11}$**	0.044 (0.018)	**$1.7x10^{-2}$**
MR-Egger: intercept	-0.003 (0.001)	**$7.6x10^{-5}$**	-0.001 (0.001)	0.40
Weighted median	0.070 (0.011)	**$4.2x10^{-8}$**	0.043 (0.015)	**$6.4x10^{-3}$**
Penalized weighted median	0.051 (0.011)	**$2.8x10^{-5}$**	0.043 (0.015)	**$6.8x10^{-3}$**

Beta's (SE) given as differential LDL-c response to statin therapy per standard deviation increase in LDL-c levels. The MR-Egger intercept term provides a formal test of directional pleiotropy. P-values in bold reflect statistically significant results, using a p-value threshold of 0.05.

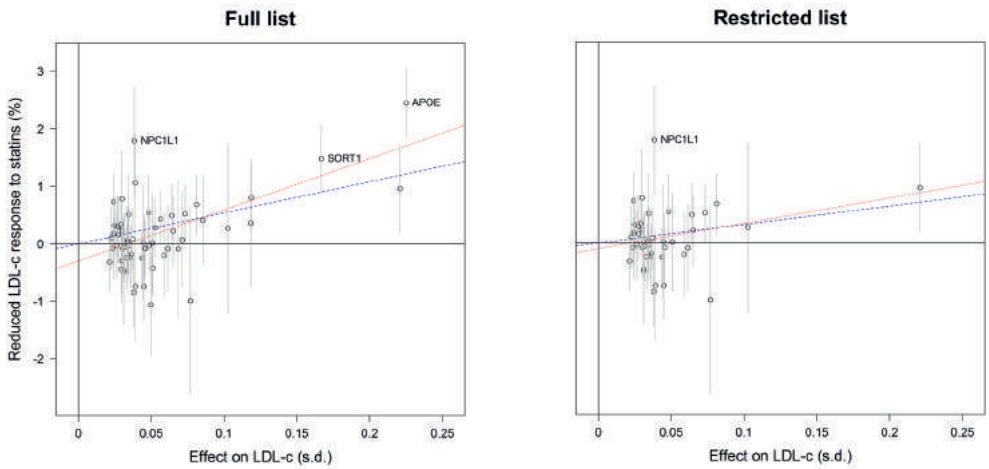

Figure 1. Scatter plots of the genetic associations with LDL-c against genetic associations with differential LDL-c response to statin therapy, both plotted as per-allele effects. In addition, 95% CI's are presented for the genetic associations with statin response. The blue (dashed) and red (dotted) line correspond to the inverse-variance weighted and MR-Egger estimators respectively, and are shown for the full (59 SNPs) and restricted (42 SNPs) lists, with a positive slope reflecting a worse statin response.

DISCUSSION

Within the present study, we aimed to examine whether additional loci of importance to LDL-c response to statin therapy could be identified by focusing our efforts on previously reported lead SNPs explaining variation in LDL-c levels. In addition to reconfirming the previously described associations of APOE and SORT1 with LDL-c response to statin therapy, we found suggestive evidence that NPC1L1 is of importance to statin pharmacogenetics. Of note, our previously reported association of LPA with statin response was not among these results, reflecting the different lead SNP reported by the GLGC, which also explains why the association with statin response was not genome-wide significant for SORT1. Consistent with the results for the individual lead SNPs, we found strong evidence that overall genetic predisposition for higher LDL-c levels is associated with a decreased LDL-c statin response, and robustly quantified this association using summary level data from the largest and most recent GWA studies on lipid levels and LDL-c response to statin therapy. In addition, MR-Egger and median-based estimators showed largely consistent results, both in direction and magnitude, thereby strengthening the findings of the IVW approach.

Localized to gastrointestinal tract epithelial cells as well as hepatocytes, the Niemann-pick C1-like 1 (NPC1L1) protein is a key regulator of cholesterol absorption (13), and is the drug target of ezetimibe (14). Shown to associate with interindividual variation in response to ezetimibe treatment (15, 16), genetic variation in NPC1L1 has also been previously linked to LDL-c response to statin therapy in smaller studies. In 37 men with central obesity, Chan and colleagues found that subjects with the NPC1L1 2/2 haplotype had a greater reduction in LDL-c levels than non-2/2 haplotype subjects, independent of their higher baseline LDL-c levels (17). Moreover, in the PROSPER trial, the NPC1L1 -133A>G variant was found to associate with greater 6-month change in lipid levels in pravastatin-treated individuals, but also with higher baseline LDL-c levels, which were not adjusted for in the analyses (18).

In contrast, our findings are unlikely to be explained by differences in off-treatment LDL-c levels, as these were statistically accounted for in the GIST meta-analysis. Rather, the genetic associations with LDL-c levels reflect lifelong effects on lipid metabolism, which we now show may influence the efficacy of clinical interventions later in life. Unfortunately, our use of summary level data precludes providing more detailed mechanistic insights, though there exists some evidence that statin therapy efficacy interacts with cholesterol synthesis and absorption, possibly in part through changes in intestinal expression of NPC1L1 (19, 20).

While the MR-Egger test did not show evidence for directional pleiotropy after excluding variants associated with HDL-c or TG at a genome-wide significant level, it is possible that the remaining variants are not solely of importance to LDL-c homeostasis, as meaningful sub-threshold associations may exists for HDL-c or TG. Similarly, we cannot be certain that the associations with HDL-c and TG of the excluded genetic variants reflected true biological pleiotropy, or merely downstream effects of LDL-c on other phenotypic traits (lipid or otherwise), which are specifically the effects of interest in MR investigations (21). However, by creating a restricted list we attempted to isolate variants more specific to LDL-c levels, as has previously been done when constructing genetic risk scores consisting of large numbers of genetic variants (22). In line with this, the consistency of the different methods for the restricted score indicates that this score is less likely to contain invalid instruments. Furthermore, the relatively large difference in mean estimates between the MR-Egger and median-weighted methods for the full list of variants possibly reflects violation of MR-Egger's underlying assumptions, as variants associated with LDL-c levels might be proportionally associated with HDL-c and TG levels.

As we included summary level data from partially overlapping data sources, our findings may have been influenced by weak IV bias (24). More specifically, of the 10 prospective, population-based studies which contributed to the first-stage meta-analysis of GIST, 6 (AGES, ARIC, CHS, FHS, Go-DARTS I, Go-DARTS II) also contributed to the GLGC meta-analysis. With the exception of rs4420638 (APOE), which was validated in additional populations in the second-stage meta-analysis of GIST, this means that up to 43% of GIST participants included in the first-stage meta-analysis were possibly also included in the GLGC analyses. However, the median F-statistic of our instruments for LDL-c levels was 58.35 (IQR 42.51-118.59), making it unlikely to have substantially influenced our results, as instruments with F-statistics over 10 are generally considered sufficiently strong (24). The homogeneity between the causal estimates obtained from the full GIST sample and those generated when solely including data from the GIST randomized controlled trials strengthens this claim, as these trials were not included in the GLGC meta-analysis.

In summary, we investigated whether 59 lead SNPs known to associate with LDL-c levels also associate with differential LDL-c response to statin therapy. After taking multiple testing into account, we found that three lead SNPs (for APOE, SORT1, and NPC1L1) were associated with smaller LDL-c response to statin treatment, thereby identifying one new locus of importance to statin response, namely NPC1L1. In addition, our findings indicate that individuals with overall genetic predisposition for high LDL-c levels are less likely to respond well to statins.

FUTURE PERSPECTIVE

To date, pharmacogenetic research on statin therapy has identified genetic variants with only modest effect sizes and therefore limited clinical utility (25). Recently, Leusink et al. generated a genetic risk score based on previously reported genetic variants of importance to statin response in ABCG2, LPA and APOE. However, the small effect size (roughly 2% of average LDL-C reduction) suggests that the applicability of this score in clinical practice would be limited (25). While the main aim of our study was to examine whether overall predisposition to LDL-C level associates with statin response, our results suggest that risk stratification based on a LDL-c genetic risk score might identify individuals most likely to benefit from combination therapy of statin and non-statin lipid-lowering medication, as genetic predisposition to higher LDL-c levels may not affect their efficacy to the same degree. However, as the various summary method

effect estimates observed in our study varied between 3 and 9% reduced LDL-C response to statin therapy per standard deviation increase in genetically raised LDL-C levels, clinical utility will likely be limited. If genetic information becomes available, large experimental studies such as the recently completed IMPROVE-IT trial (26) would be most suited to determine possible clinical significance. In addition, pharmacogenetic studies of non-statin LDL-lowering therapies should also consider examining the role of genetic predisposition for higher LDL-c levels. Finally, it would be of great interest for future studies to examine whether NPC1L1-dependent compensatory mechanisms to lipid-lowering treatment exist, which could add to the rationale behind combination therapy with ezetimibe.

EXECUTIVE SUMMARY

Background

- There exists substantial interindividual variation in low-density lipoprotein cholesterol (LDL-c) response to statin treatment, in part due to genetic factors. Several genetic loci have been found to associate with differential LDL-c response to statins, independent of off-treatment LDL-c levels.
- The majority of these loci have additionally been found to associate with LDL-c levels. LDL-c level-associated loci may therefore represent strong candidates for pharmacogenetic studies on statin therapy.

Patients & methods

- To identify additional loci of importance to statin response, we performed a look-up of 59 lead SNPs for LDL-c levels in the pharmacogenetic meta-analysis of the GIST consortium.
- We further examined whether overall genetic predisposition for higher LDL-c levels associates with statin response, by combining summary statistics from the GLGC and GIST consortia for 59 lead SNPs for LDL-c levels from the GLGC, through an inverse-variance weighted approach. MR-Egger regression and median-based methods were then performed as sensitivity analyses.

Results: main findings

- Lead SNPs for APOE, SORT1, and NPC1L1 were associated with diminished statin response, as was overall genetic predisposition for increased LDL-c level.

REFERENCE LIST

Papers of special note have been highlighted as: * of interest; ** of considerable interest

1 Cholesterol Treatment Trialist' (CTT) Collaborators, Mihaylova B, Emberson J, et al. The effects of lowering LDL cholesterol with statin therapy in people at low risk of vascular disease: meta-analysis of individual data from 27 randomised trials. Lancet. 2012;380:581-90.

2 Cholesterol Treatment Trialists' (CTT) Collaboration, Baigent C, Blackwell L, et al. Efficacy and safety of more intensive lowering of LDL cholesterol: a meta-analysis of data from 170 000 participants in 26 randomised trials. Lancet. 2010;376:1670-81.

3 Davidson MH, Toth PP. Comparative effects of lipid-lowering therapies. Prog Cardiovasc Dis. 2004;47:73-104.

4 Postmus I, Trompet S, Deshmukh HA, et al. Pharmacogenetic meta-analysis of genome-wide association studies of LDL cholesterol response to statins. Nat Commun. 2014;5:5068.

** Largest pharmacogenetic meta-analysis on LDL-c response to statin therapy

5 Global Lipids Genetics Consortium, Willer CJ, Schmidt EM, et al. Discovery and refinement of loci associated with lipid levels. Nat Genet. 2013;45:1274-83.

** Largest GWAS on blood lipid levels

6 Johnson T. Efficient calculation for multi-SNP genetic risk scores. Technical report, The Comprehensive R Archive Network, 2013. http://cran.r-project.org/web/packages/gtx/vignettes/ashg2012.pdf.

7 Burgess S, Butterworth A, Thompson SG. Mendelian randomization analysis with multiple genetic variants using summarized data. Genet Epidemiol. 2013;37:658-65.

8 Greenland S. An introduction to instrumental variables for epidemiologists. Int J Epidemiol. 2000;29:722-9.

9 Bowden J, Davey Smith G, Haycock PC, et al. Consistent Estimation in Mendelian Randomization with Some Invalid Instruments Using a Weighted Median Estimator. Genet Epidemiol. 2016;40:304-14.

*Methodology paper on median-based methods

10 Bowden J, Davey Smith G, Burgess S. Mendelian randomization with invalid instruments: effect estimation and bias detection through Egger regression. Int J Epidemiol. 2015;44:512-25.

*Methodology paper on MR-Egger method

11 Egger M, Davey Smith G, Schneider M, et al. Bias in meta-analysis detected by a simple, graphical test. BMJ. 1997;315:629-34.

12 R Core Team. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria (2016).

13 Jia L, Betters JL, Yu L. Niemann-pick C1-like 1 (NPC1L1) protein in intestinal and hepatic cholesterol transport. Annu Rev Physiol. 2011;73:239-59.

14 Garcia-Calvo M, Lisnock J, Bull HG, et al. The target of ezetimibe is Niemann-Pick C1-Like 1 (NPC1L1). Proc Natl Acad Sci U S A. 2005;102:8132-7.

15 Hegele RA, Guy J, Ban MR, et al. NPC1L1 haplotype is associated with inter-individual variation in plasma low-density lipoprotein response to ezetimibe. Lipids Health Dis. 2005;4:16.

16 Simon JS, Karnoub MC, Devlin DJ, et al. Sequence variation in NPC1L1 and association with improved LDL-cholesterol lowering in response to ezetimibe treatment. Genomics. 2005;86:648-56.

17 Chan DC, Watts GF, Wang J, et al. Variation in Niemann-Pick C1-like 1 gene as a determinant of apolipoprotein B-100 kinetics and response to statin therapy in centrally obese men. Clin Endocrinol (Oxf). 2008;69:45-51.

18 Polisecki E, Peter I, Simon JS, et al. Genetic variation at the NPC1L1 gene locus, plasma lipoproteins, and heart disease risk in the elderly. J Lipid Res. 2010;51:1201-7.

19 Qi Y, Liu J, Ma C, et al. Association between cholesterol synthesis/absorption markers and effects of cholesterol lowering by atorvastatin among patients with high risk of coronary heart disease. J Lipid Res. 2013;54:3189-97.

20 Tremblay AJ, Lamarche B, Lemelin V, et al. Atorvastatin increases intestinal expression of NPC1L1 in hyperlipidemic men. J Lipid Res. 2011;52:558-65.

21 Davey Smith G, Hemani G. Mendelian randomization: genetic anchors for causal inference in epidemiological studies. Hum Mol Genet. 2014;23:R89-98.

22 Holmes MV, Asselbergs FW, Palmer TM, et al. Mendelian randomization of blood lipids for coronary heart disease. Eur Heart J. 2014;36:539-50.

23 Pierce BL, Burgess S. Efficient design for Mendelian randomization studies: subsample and 2-sample instrumental variable estimators. Am J Epidemiol. 2013;178:1177-84.

24 Stock J, Yogo M. Testing for Weak Instruments in Linear IV Regression. New York: Cambridge; 2005.

25 Leusink M, Maitland-van der Zee AH, Ding B, et al. A genetic risk score is associated with statin-induced low-density lipoprotein cholesterol lowering. Pharmacogenomics. 2016;17:583-91.

26 Cannon CP, Blazing MA, Giugliano RP, et al. Ezetimibe Added to Statin Therapy after Acute Coronary Syndromes. N Engl J Med. 2015;372:2387-97.

Supplemental Table 1. Candidate markers known to associate with LDL-c levels at a genome-wide significant level ($p<5x10^{-8}$)

SNP	Chr	Locus	Other lipids*	Included in restricted list	P-value GLGC*	P-value GIST
rs4420638	19	APOE	HDL-c, TG, TC		1.5×10^{-178}	3.9×10^{-15}
rs629301	1	SORT1	HDL-c, TC		5.4×10^{-241}	9.4×10^{-7}
rs2072183	7	NPC1L1	TC	x	7.1×10^{-16}	0.0002099
rs314253	17	DLG4	TC	x	3.4×10^{-10}	0.003703
rs3757354	6	MYLIP	TC	x	2.1×10^{-17}	0.006422
rs1367117	2	APOB	HDL-c, TG, TC		9.5×10^{-183}	0.008034
rs4299376	2	ABCG5/8	TC	x	3.9×10^{-72}	0.0123
rs11136341	8	PLEC1	TC	x	7.1×10^{-12}	0.01421
rs6511720	19	LDLR	TC	x	3.9×10^{-262}	0.01442
rs4722551	7	MIR148A	TG, TC		4.0×10^{-14}	0.01664
rs12748152	1	PIGV-NR0B2	HDL-c, TG		3.2×10^{-12}	0.02199
rs12916	5	HMGCR	TC	x	7.8×10^{-78}	0.04682
rs2328223	20	SNX5	none	x	5.6×10^{-09}	0.07138
rs2479409	1	PCSK9	TC	x	2.5×10^{-50}	0.08195
rs2954029	8	TRIB1	HDL-c, TG, TC		2.1×10^{-50}	0.08733
rs492602	19	FLJ36070	TC	x	9.4×10^{-14}	0.1075
rs174546	11	FADS1-2-3	HDL-c, TG, TC		1.6×10^{-39}	0.1083
rs1564348	6	LPA	TC	x	2.8×10^{-21}	0.1144
rs1883025	9	ABCA1	HDL-c, TC		6.1×10^{-11}	0.1315
rs7640978	3	CMTM6	TC	x	9.8×10^{-09}	0.1333
rs12670798	7	DNAH11	TC	x	4.8×10^{-14}	0.1591
rs2030746	2	LOC84931	TC	x	8.6×10^{-09}	0.2212
rs364585	20	SPTLC3	none	x	4.3×10^{-10}	0.2212
rs5763662	22	MTMR3	none	x	1.2×10^{-8}	0.2358
rs11065987	12	BRAP	HDL-c, TC		1.2×10^{-11}	0.2694
rs3764261	16	CETP	HDL-c, TG, TC		2.2×10^{-34}	0.2844
rs2902940	20	MAFB	TC	x	1.7×10^{-11}	0.2857
rs2255141	10	GPAM	HDL-c, TG, TC		1.3×10^{-13}	0.2869
rs964184	11	APOA1	HDL-c, TG, TC		2.0×10^{-26}	0.296
rs6029526	20	TOP1	TC	x	4.8×10^{-18}	0.3202
rs4253776	22	PPARA	TC	x	3.4×10^{-8}	0.3304
rs12027135	1	LDLRAP1	TC	x	2.3×10^{-14}	0.3605
rs2642442	1	MOSC1	TC	x	5.3×10^{-11}	0.3942
rs267733	1	ANXA9-CERS2	none	x	5.3×10^{-9}	0.4107

SNP	Chr	Locus	Other lipids*	Included in restricted list	P-value GLGC*	P-value GIST
rs4942486	13	BRCA2	none	x	2.3×10^{-11}	0.4742
rs514230	1	IRF2BP2	TC	x	9.2×10^{-12}	0.4914
rs2000999	16	HPR	TC	x	4.2×10^{-41}	0.4944
rs4530754	5	CSNK1G3	TC	x	3.6×10^{-12}	0.5247
rs10401969	19	CILP2	TG, TC		2.7×10^{-54}	0.5301
rs1250229	2	FN1	none	x	3.1×10^{-8}	0.5373
rs11220462	11	ST3GAL4	TC	x	6.6×10^{-21}	0.5911
rs1801689	17	APOH-PRXCA	none	x	9.8×10^{-12}	0.7205
rs6818397	4	LRPAP1	TC	x	1.7×10^{-08}	0.7226
rs6882076	5	TIMD4	TG, TC		3.3×10^{-31}	0.7504
rs8017377	14	NYNRIN	TC	x	2.5×10^{-15}	0.7597
rs1169288	12	HNF1A	TC	x	6.4×10^{-21}	0.7684
rs2710642	2	EHBP1	none	x	6.1×10^{-9}	0.7684
rs3177928	6	HLA	TC	x	3.1×10^{-17}	0.791
rs1800562	6	HFE	TC	x	8.3×10^{-14}	0.8186
rs10102164	8	SOX17	TC	x	3.7×10^{-11}	0.8474
rs17404153	3	ACAD11	none	x	1.8×10^{-09}	0.8474
rs9987289	8	PPP1R3B	HDL-c, TC		8.5×10^{-24}	0.8715
rs1800961	20	HNF4A	HDL-c, TC		6.0×10^{-10}	0.8834
rs10128711	11	SPTY2D1	TC	x	9.2×10^{-13}	0.891
rs2131925	1	ANGPTL3	TG, TC		3.0×10^{-32}	0.9087
rs7570971	2	RAB3GAP1	TC	x	6.3×10^{-11}	0.9208
rs11563251	2	UGT1A1	TC	x	4.5×10^{-8}	0.928
rs3780181	9	VLDLR	TC	x	1.8×10^{-9}	0.9689
rs10490626	2	INSIG2	TC	x	1.7×10^{-12}	0.982

*As reported in the Global Lipids Genetics consortium summary files at http://csg.sph.umich.edu//abecasis/public/lipids2013/

This list does not include rs9411489 (ABO), as genotype could not be imputed.

GIST, Genomic Investigation of Statin Therapy consortium; LDL-c, low-density lipoprotein cholesterol; HDL-c, high-density lipoprotein cholesterol; TG, triglycerides; TC, total cholesterol

Supplemental Table 2. IVW, MR-Egger, and median-based estimators after separately excluding HDL-C or triglyceride-associated SNPs

	Restricted to SNPs not associated with HDL-c at $p<5x10^{-8}$ (n=46)		Restricted to SNPs not associated with triglycerides at $p<5x10^{-8}$ (n=47)	
Analysis method	**Beta (SE)**	**p-value**	**Beta (SE)**	**p-value**
Inverse-variance weighted	0.031 (0.009)	**$1.6x10^{-3}$**	0.042 (0.008)	**$6.5x10^{-6}$**
MR-Egger: slope	0.042 (0.017)	**$1.8x10^{-2}$**	0.065 (0.014)	**$3.1x10^{-5}$**
MR-Egger: intercept	-0.001 (0.001)	0.43	-0.002 (0.001)	**$4.5x10^{-2}$**
Weighted median	0.040 (0.015)	**$1.1x10^{-2}$**	0.044 (0.014)	**$2.4x10^{-3}$**
Penalized weighted median	0.040 (0.015)	**$1.1x10^{-2}$**	0.044 (0.016)	**$9.9x10^{-3}$**

Beta's (SE) given as differential LDL-c response to statin therapy per standard deviation increase in genetically raised LDL-c levels. The MR-Egger intercept term provides a formal test of directional pleiotropy. While largely overlapping, these lists differed with regard to 9 SNPs (5 solely associated with HDL-c, 4 solely associated with TG). P-values in bold reflect statistically significant results, using a p-value threshold of 0.05.

Supplemental Table 3. IVW, MR-Egger, and median-based estimators when solely including data from the randomized controlled trials participating in GIST

	Full GIST sample		Stage 1 RCT's	
Analysis method	**Beta (SE)**	**p-value**	**Beta (SE)**	**p-value**
Inverse-variance weighted	0.054 (0.006)	**8.4×10^{-12}**	0.037 (0.008)	**1.3×10^{-5}**
MR-Egger: slope	0.089 (0.010)	**1.0×10^{-11}**	0.078 (0.014)	**3.2×10^{-7}**
MR-Egger: intercept	-0.003 (0.001)	**7.6×10^{-5}**	-0.003 (0.001)	**3.6×10^{-4}**
Weighted median	0.070 (0.011)	**4.2×10^{-8}**	0.045 (0.012)	**3.7×10^{-4}**
Penalized weighted median	0.051 (0.011)	**2.8×10^{-5}**	0.044 (0.012)	**4.4×10^{-4}**

Beta's (SE) given as differential LDL-c response to statin therapy per standard deviation increase in genetically raised LDL-c levels. The MR-Egger intercept term provides a formal test of directional pleiotropy. P-values in bold reflect statistically significant results, using a p-value threshold of 0.05.
GIST, Genomic Investigation of Statin Therapy consortium

CHAPTER 6

Statin-induced LDL cholesterol response and type 2 diabetes: a bidirectional two-sample summary data Mendelian randomization study

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Submitted

ABSTRACT

Background: Following observations that statin treatment appears to modestly increase the risk of type 2 diabetes (T2D), various studies have shown that genetic predisposition to low LDL cholesterol (LDL-C) levels associates with increased T2D risk. However, it remains unclear whether the increased risk seen in statin users is due to low LDL-C concentrations, or due to the statin-induced proportional change in LDL-C. In addition, whether the greater absolute cardiovascular disease risk reduction benefit from statin therapy observed in T2D is due to differential LDL-C lowering has not been investigated using genetic tools.

Methods and results: We assessed the genome-wide genetic correlation between statin-induced LDL-C response and T2D using LD score regression, and performed a two-sample bidirectional Mendelian randomization analysis by combining summary level statistics from the Genomic Investigation of Statin Therapy (GIST, n_{max}=40,914) and DIAGRAM (n_{max}=159,208) consortia. We found a positive genetic correlation between LDL-C statin response and T2D using LD score regression ($r_{genetic}$=0.36, s.e.=0.13). The Mendelian randomization analysis results did not provide support for statin response having a causal effect on T2D risk (OR 1.00 (95%CI: 0.97,1.03) per 10% increase in statin response), nor that liability to T2D has a causal effect on statin-induced LDL-C response (0.20% increase in response (95%CI: -0.40,0.80) per doubling of odds of liability to T2D).

Conclusions: Liability to T2D is unlikely to influence LDL-C response to a statin. Although we found no evidence to suggest that proportional statin response influences T2D risk, a definitive assessment should be made in populations comprised exclusively of statin-users.

INTRODUCTION

3-Hydroxy-3-methylglutaryl–coenzyme A (HMG-CoA) reductase inhibitors, also known as statins, have demonstrated consistent benefits to cardiovascular disease risk reduction, while being safe and well-tolerated for most people.[1] also known as statins, have demonstrated consistent benefits to cardiovascular disease risk reduction, while being safe and well-tolerated for most people (1). However, statin treatment has been linked to a modestly increased risk of new-onset type 2 diabetes (T2D), an observation first noted in the JUPITER trial (2) which has since been replicated in large-scale meta-analyses of randomized controlled trials (3-5). As promising novel strategies for lowering low-density lipoprotein cholesterol (LDL-C) such as proprotein convertase subtilisin–kexin type 9 (PCSK9) inhibitors emerge, the safety of lipid-modifying treatments with regard to diabetes risk remains an important question.

In recent years, genetic epidemiology has started to untangle the complex link between LDL-C lowering and T2D risk. For example, analyses of patients with familial hypercholesterolemia have shown that the prevalence of T2D is significantly lower than among unaffected relatives, with variability by underlying mutation type (6). Furthermore, apparent causal effects on T2D have been shown both for overall genetic predisposition to lower LDL-C concentrations (7-9) as well as for *HMGCR*-, *NPC1L1*-, and *PCSK9*-gene specific (i.e. on target) mechanisms of lowering LDL-C (9-13). These findings, and recent reanalysis of statin trial data using Egger regression (14), suggest that statin-related dysglycaemia might be mediated largely through LDL-C lowering mechanisms rather than through proposed pleiotropic mechanisms of statins (15).

However, meta-regression approaches modeling heterogeneity among treatment effects from statin trials have produced conflicting results as to whether statin-induced proportional change of LDL-C influences T2D risk (3-5). Previous Mendelian randomization (MR) studies have been unable to directly answer this question, as genetic instruments solely proxying lifelong lower levels of LDL-C have been utilized. In addition, genetic instruments have not been proposed before to examine whether the greater absolute cardiovascular disease (CVD) risk reduction conferred by statin therapy in individuals with T2D could result from greater proportional statin-induced LDL-C lowering. Findings from the largest pharmacogenomic meta-analysis for differential LDL-C response to statin therapy to date by the Genomic Investigation of Statin Therapy (GIST) consortium might be used to investigate these questions. We therefore aimed to use these data to examine the causal direction of the relationship between proportional statin response and T2D using a bidirectional two-sample MR approach.

METHODS

To assess the likelihood of a shared etiology between statin response and T2D we assessed their genetic correlation using cross-trait linkage disequilibrium (LD) score regression. Furthermore, to detect potential direct causality we performed a bidirectional two-sample MR analysis, combining summary level statistics from the GIST (16) and DIAbetes Genetics Replication And Meta-analysis (DIAGRAM) (17) consortia (**Figure 1**), to estimate: (1) the causal effect of statin-induced LDL-C response on T2D risk, and (2) the causal effect of liability to T2D on statin-induced LDL-C response. We refer to liability to T2D in this second analysis as it is not possible to determine whether individuals in the GIST dataset have been diagnosed with T2D.

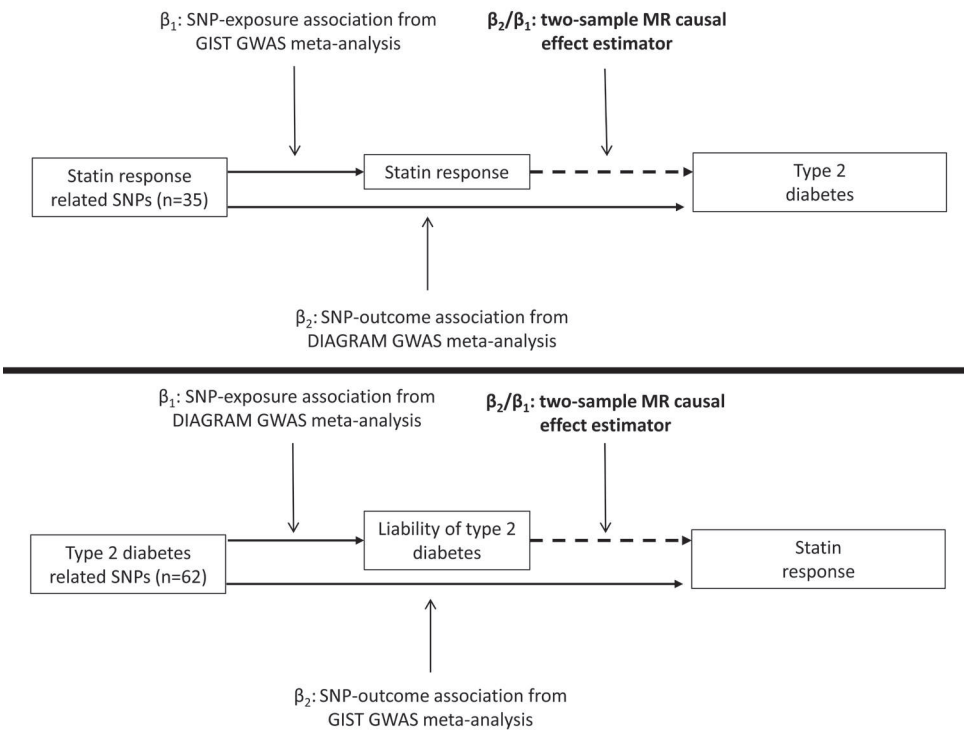

Figure 1. Overview of two-sample Mendelian randomization (MR) study on the bidirectional association between statin-induced LDL cholesterol response and type 2 diabetes (T2D). Top panel shows direction of statin response to T2D, bottom panel liability to T2D to statin response. Layout of figure based upon the work by Taylor et al. 2016 (PMID 27215954).

Causal effect of statin-induced LDL-C response on T2D risk

The GIST consortium's 2014 meta-analysis on statin-induced LDL-C response included up to 18,596 statin-treated subjects in the discovery stage, of whom 9,064 (48.7%) were known to have a history of diabetes (type unspecified). The most promising signals (n=246) were taken forward to a subsequent replication phase, to be validated in an additional 22,318 statin recipients (16). Statin response had been defined as the difference between natural log-transformed on- and off-treatment LDL-C levels. Linear regression analyses using this statin response phenotype as dependent variable and genetic variant as independent variable were adjusted for natural log-transformed off-treatment LDL-C level, age, sex, and study-specific covariates including principal components of ancestry. Observational studies had additionally adjusted for statin type-specific equivalent dose in their regression models. The resulting regression coefficient thus approximates the fraction of differential LDL lowering in carriers versus non-carriers of the SNP. While lead variants for four independent loci (*APOE*, *LPA*, *SLCO1B1*, *SORT1*/*CELSR2*/*PSRC1*) were presented as the top genome-wide significant hits for statin response in the GIST paper[16], 63 correlated variants attained a p-value lower than $5x10^{-5}$ in a combined meta-analysis of the discovery and replication stage results.

To assess the effects of these instruments on T2D, we extracted discovery stage summary statistics for these 63 variants from DIAGRAM's 2017 meta-analysis of genome-wide association data from 26,676 T2D case and 132,532 control subjects of European ancestry after imputation using the 1000 Genomes all ancestries reference panel (March, 2012 release) (17). Contributing studies had performed logistic regression association analysis of T2D against each genetic variant, adjusted for age, sex, and principal components of ancestry. The summary statistics were extracted from the publicly available summary statistics dataset on the DIAGRAM website (http://www.diagram-consortium.org/). All variants were available in the DIAGRAM dataset, except two, for which we could not find suitable (i.e. high-LD) proxies. We subsequently LD clumped the set of variants using 0.001 as the maximum LD r2 value to ensure that the remaining instruments were essentially independent. This reduced the set of statin response instruments from 61 to 35 (**Supplemental Table 1**). We separately examine the effects of the full set of 35 statin response instruments and of the four top hits together.

Causal effect of liability to T2D on statin-induced LDL-C response

As candidate genetic instruments for liability of T2D we selected 128 genetic instruments at 113 loci. These 128 variants represent the established loci from the literature before the DIAGRAM's 2017 publication as well as the novel signals detected therein, with 42 being genome-wide significant ($p<5x10^{-8}$) in this DIAGRAM dataset. Discovery stage regression coefficients and standard errors for a total of 128 single nucleotide polymorphisms (SNPs) at 113 loci were extracted from the publicly available summary statistics dataset on the DIAGRAM website (http://www.diagram-consortium.org/).

Next, we extracted summary statistics for the identified T2D liability instruments from GIST's 2014 genome-wide meta-analysis on statin-induced LDL-C response. Of note, none of the identified T2D liability instruments were among those SNPs carried forwards to the replication stage of the GIST meta-analysis. In total, 78 of the 128 instruments were available in the discovery GIST dataset. We subsequently LD clumped the set of variants, again using 0.001 as the maximum LD r2 value. This reduced the set of instruments from 78 to 62 (including 24 genome-wide significant instruments), which include 19 proxies with an $r2\geq0.8$ with the original variant in 1000 Genomes European samples (**Supplemental Table 2**). To tease out possible bias from using weaker instruments, we aimed to examine the combined effects of the T2D liability instruments before and after restricting the analysis to the genome-wide significant instruments.

Sample overlap

Of the ten prospective, population-based studies that contributed to the discovery-stage meta-analysis of GIST, four (Atherosclerosis Risk in Communities Study (ARIC), Framingham Heart Study, Genetics of Diabetes Audit and Research in Tayside Scotland (GoDARTS) I and II) also contributed to the DIAGRAM meta-analysis. Of the studies contributing to the replication-stage meta-analysis of GIST, one (Rotterdam Study) also contributed to the DIAGRAM meta-analysis. We were unable to precisely determine the overlap between the datasets. However, if we assume that all participants from these five studies contributed to both analyses, up to 5% (with respect to the larger dataset, i.e. DIAGRAM) of overlap in participants may be present for our analyses.

Statistics - LD score regression

In essence, MR studies use variants significantly associated with an exposure to interrogate genetic correlation (i.e. genetic overlap) between the exposure

and an outcome. If present, this is then interpreted as evidence for a causal relationship. A complementary, non-directional approach is to estimate genetic correlation between the exposure and outcome traits of interest by considering variants across the whole genome, including those that do not reach a certain significance threshold. Therefore, the genome-wide summary-level datasets of both GIST (discovery stage, including only variants with N>5000, and without genome control-correction) and DIAGRAM's 2012 GWAS[18] were used to estimate the genetic correlation of statin-induced LDL-C proportional response with T2D through LD score regression[19] using the LD Hub platform (http://ldsc.broadinstitute.org/ldhub/).[20] After QC, 1,039,702 genetic variants which overlapped between the two GWAS datasets were included for this analysis.

Statistics – MR analysis

Partial F-statistics were calculated per instrument as measure of instrument strength (21). For each of the four sets of instruments separately (**i.** statin response-all (n=35), **ii.** statin response-restricted (n=4 genome-wide significant instruments), **iii.** T2D liability-all (n=62), **iv.** T2D liability-restricted (n=24 genome-wide significant instruments) a MR analysis was performed using an inverse-variance weighted (IVW) linear regression, with instrument-outcome associations as dependent variable, instrument-exposure associations as independent variable, and with the intercept constrained to zero (22). Estimates of the causal effect of statin response on T2D are presented as odds ratio for T2D per 10% increase in statin response. For examining the effects of liability to T2D on statin response we rescaled effect estimates such that they represent increase in statin response (% extra lowering of LDL-C) per doubling of the odds of liability to T2D in the population, by multiplying the causal estimate by 0.693 (i.e. $\log_e 2$) prior to exponentiating.

Instrument-outcome associations were plotted against instrument-exposure associations to visualize the resulting regression line from the IVW analysis using the full and restricted sets of instruments. Furthermore, causal effect estimates for the individual instruments (i.e. Wald ratios) were plotted against the inverse of their standard error to facilitate visual detection of possible horizontal pleiotropy (i.e. a direct effect on the outcome rather than via the exposure). We subsequently performed three complementary sensitivity analyses which relax the assumption of no horizontal pleiotropy amongst the genetic variants. First, MR-Egger regression, of which the intercept formally tests for the presence of unbalanced horizontal pleiotropy, and the slope reflects the causal effect estimate after adjusting for this pleiotropy by adding an intercept to the IVW method (23).

Additional approaches that are similarly more robust to potential violations of the instrumental variable assumptions than the conventional (i.e. IVW) MR analysis were the weighted median- and the weighted mode-based estimator (24, 25), which respectively use the weighted median of, and the highest density of, the ratio estimates across the individual instruments as estimate of the true causal effect. Finally, as several of the proposed instruments for statin-induced LDL-C response are known to independently associate with fasting LDL-C levels, we performed a multivariable MR analysis for the analysis of statin-response to T2D, adjusted for effects on fasting LDL-C levels (26). This multivariable analysis included 32 out of the 35 statin response instruments, and 64 instruments for fasting LDL-C concentrations from the Global Lipids Genetics Consortium 2013 GWAS on blood lipid levels (27). The number of instruments used for the multivariable MR analysis differ from the other MR-analyses due to not all instruments being available in all three GWAS datasets. All MR-analyses were carried out in R version 3.4.2 (28), using the TwoSampleMR R-package which accompanies the MR-base analytical platform, and the sample code provided by the methodology paper on multivariable MR (26, 29).

RESULTS

We found a statistically significant positive genetic correlation of statin-induced LDL-C response with type 2 diabetes ($r_{genetic}$ (s.e.) = 0.36 (0.13), p = 0.0071) using LD score regression.

The median F-statistic (25, 75th percentile) was 23.2 (16.3, 37.8) for the full set of statin response instruments, and 18.1 (16.2, 20.9) for the full set of T2D liability instruments. These respectively increased to 44.8 (35.5, 70.7) and 73.1 (38.8, 120.3) for the restricted sets of instruments. As shown in **Figure 2** and the **Table**, we did not find statistical evidence that statin-induced differential LDL-C response has a causal effect on T2D risk, nor that liability to T2D has a causal effect on statin-induced differential LDL-C response. This held true for both the full and restricted sets of instruments, and results from all sensitivity analyses were consistent with these findings. More specifically, our results for the full sets of instruments indicate the OR of T2D is 1.00 (95% CI: 0.97, 1.03) per 10% increase in statin-induced LDL-C response, and that statin-induced LDL-C response is increased by 0.20% (95% CI: -0.40, 0.80) per doubling of the odds of T2D liability. Evidence of unbalanced horizontal pleiotropy was present only for the restricted set of statin response instruments, as indicated by the MR-Egger intercept (intercept (95% CI): 1.04 (1.01, 1.07)) and **Figure 3**, but

this is likely an artefact of including such a small number of instruments (n=4). Finally, a multivariable MR analysis where we adjust for effects of all SNPs on fasted LDL-C did not lead to different conclusions regarding the effect of statin response on T2D risk (OR 1.02 (95% CI: 0.97, 1.07 per 10% increase in statin-induced LDL-C response).

DISCUSSION

Using LD score regression we found a positive genetic correlation of proportional statin response with T2D using genome-wide data, pointing to shared genetic determinants between these traits.

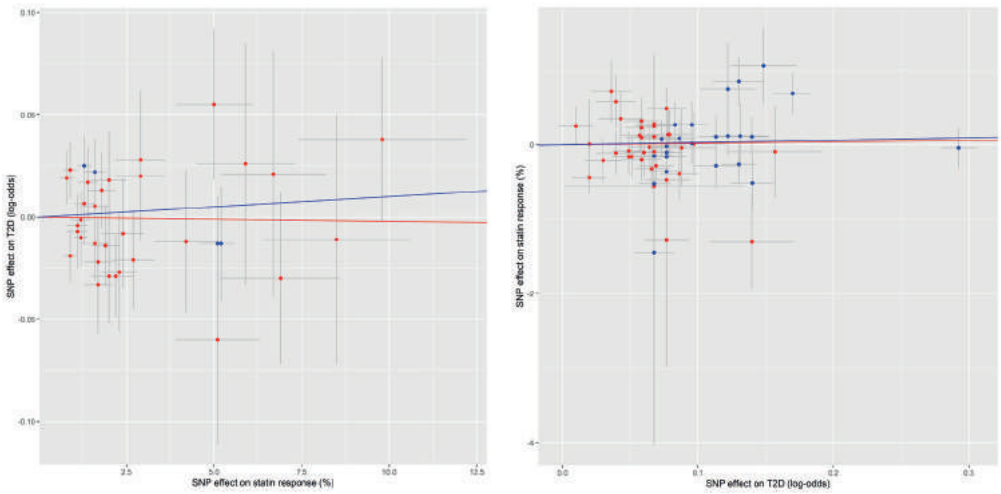

Figure 2. Scatter plots of instrument-outcomes (y-axis) against individual instrument-exposure (x-axis) per-allele effects, shown separately for statin response (left panel) and liability of type 2 diabetes instruments (T2D, right panel). The blue dots correspond to the restricted lists of variants (see text), while the full set included both the red and blue dots. The lines correspond to the inverse-variance weighted combined MR estimator, for the restricted (blue line) and full (red line) set of instruments.

Table. Mendelian randomization (MR) estimators for the bidirectional association between type 2 diabetes (T2D) and statin-induced LDL-C response

	Number of instruments	F-statistic, median (25th, 75th percentile)	Estimate (IVW method, 95% CI)	Sensitivity analyses			
				Weighted mode estimate (95% CI)	Weighted median estimate (95% CI)	MR-Egger estimate (95% CI)	MR-Egger intercept (95% CI)
Statin response ➔ T2D*	35	18.1 (16.2, 20.9)	1.00 (0.97, 1.03)	0.99 (0.93, 1.04)	0.98 (0.94, 1.02)	0.99 (0.94, 1.04)	1.02 (0.92, 1.14)
	4	73.1 (38.8, 120.3)	1.01 (0.93, 1.09)	0.98 (0.91, 1.05)	0.98 (0.91, 1.05)	0.91 (0.81, 1.01)	1.04 (1.01, 1.07)
T2D liability ➔ Statin response†	62	23.2 (16.3, 37.8)	0.20 (-0.40, 0.80)	0.11 (-0.95, 1.18)	0.03 (-0.97, 1.02)	0.62 (-0.55, 1.80)	-0.04 (-0.15, 0.06)
	24	44.8 (35.5, 70.7)	0.32 (-0.47, 1.12)	0.04 (-1.07, 1.14)	0.08 (-0.99, 1,15)	1.24 (-0.67, 3.15)	-0.12 (-0.36, 0.11)

IVW denotes inverse-variance weighted. The MR-Egger intercept provides a formal test of the presence of horizontal pleiotropy. The different MR estimators can be interpreted as *: odds ratio for T2D per 10% increase in proportional statin response, and †: the effect on proportional statin response (%) per doubling in the odds of liability to T2D, respectively. A positive statin response value corresponds to an increased LDL cholesterol lowering effect of statin therapy. F-statistics were approximated per instrument.

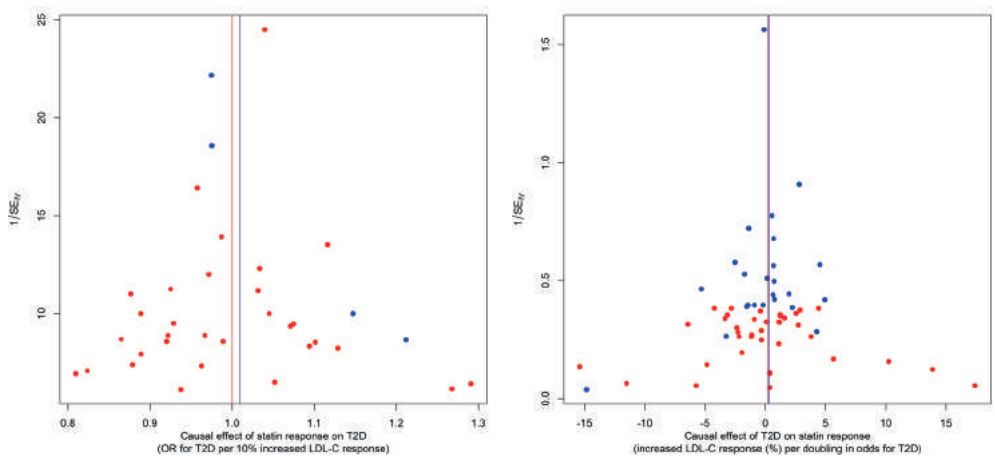

Figure 3. Funnel plots of individual causal effect estimates (Wald ratios) for statin response on type 2 diabetes (T2D, left panel), and liability to T2D on statin response (right panel). The blue dots correspond to the restricted lists of variants (see text), while the full set included both the red and blue dots. The lines correspond to the inverse-variance weighted combined MR estimator, for the restricted (blue line) and full (red line) set of instruments.

However, our bi-directional MR analyses did not provide evidence of direct causal mechanisms of either statin-induced LDL-lowering on risk for T2D, nor liability to T2D on statin response. Sensitivity analyses of these MR analyses showed consistent results, suggesting that the issue of horizontal pleiotropy is unlikely to substantially influence our results.

The findings from the MR analyses suggest that statin-induced proportional change of LDL-C is unlikely to influence T2D risk. If true, this would indicate that it is not the degree of proportional lowering of LDL-C levels in response to statin therapy which increases the risk for diabetes, but the low levels in an absolute sense which may result from this transition. Indeed, results from the JUPITER trial have shown that achieving LDL-C concentrations <30 mg/dl with high-intensity statin therapy was associated with more physician-reported diabetes (30), which was not observed when a threshold of <50 mg/dl was considered (31). Of further note here is a recent comparison of the risk of T2D in a large electronic health record database between individuals with low and normal LDL-C levels, which showed that LDL-C levels below 60 mg/dl occurring in absence of statin therapy are also associated with higher T2D risk (32). Moreover, also in line with

our results, researchers using data from 129,170 participants free from T2D at baseline from 20 statin trials did not observe evidence of a clinically relevant association between LDL-C proportional lowering at 1 year and within-trial odds ratios for new-onset type 2 diabetes (log-odds per 1% reduction in LDL-C: 0.004 (95% CI -0.001, 0.009)) (10).

However, we cannot exclude the possibility that a direct causal effect of statin-induced proportional LDL-C response on T2D may exist, but was diluted by the presence of non-statin users in the DIAGRAM dataset. This is because proposed genetic instruments for pharmacological response phenotypes can only exert their effect in the presence of (i.e. are conditional on) drug usage. More intuitively, if this type of instrument were to associate with an outcome in a population which includes no relevant drug users, this must reflect an alternative pathway unrelated to that specific drug response (i.e. horizontal pleiotropy), or at least a shared genetic etiology between the two traits. Therefore, assessment of any causal effect of instruments derived from pharmacogenetic studies should ideally also be examined in populations composed solely of individuals using the drug of interest. An analogous dilemma has been described in the context of smoking heaviness, where a SNP which strongly predicts cigarettes per day was detected in a GWAS sample including daily smokers, and found to exert an effect only after a person has become an established smoker (33, 34). Due to our use of summary statistics, we were unable to stratify our analyses on statin use. In addition, it was not possible to weight for the prevalence of statin use, as this is unknown for the DIAGRAM consortium, where statin use is additionally likely to be differential by case/control status. Therefore, given our null results, it is more appropriate to conclude that our MR analyses did not provide evidence for a shared genetic etiology between statin-induced proportional LDL-C response and T2D.

Furthermore, our observation that liability to T2D does not associate with LDL-C response resulting from statin treatment is consistent with previous studies showing that, while individuals with type 2 diabetes are likely to gain greater clinical benefit from statin therapy in terms of absolute CVD risk reduction, this does not result from differential lowering of their LDL-C concentrations when compared with non-diabetics (35).

While an important strength of our analyses is the use of large-scale GWAS data, increasing the power of our investigations for both directions of causality, we purposely included instruments which did not attain genome-wide significance in their corresponding GWAS to increase the number of instruments. Though instrument-exposure associations will have been estimated with less precision

for the weaker (i.e. sub-threshold) instruments, we considered this issue analogous to possible misspecification of weights in allele scores, which causal estimates have been shown to be generally robust to (36). However, we cannot exclude the possibility of weak instrument bias, particularly for the full set of T2D liability instruments, which included several instruments with an individual F-statistic below 10 (21). Given the relatively small overlap between the GWAS datasets, it is likely that any weak instrument bias would be towards the null (37). However, the analysis using the restricted list of strong instruments reassuringly showed similar results.

In conclusion, our results suggest that liability to T2D is unlikely to influence LDL-C response to a statin, but provided some evidence of a shared genetic etiology between statin-induced LDL-C response and T2D. Future studies should make a definitive assessment of direct causal effects of statin-induced proportional LDL-C response on T2D in populations of statin users. This analysis could employ novel two-sample MR methods which allow for the inclusion of even weaker instruments than we currently considered (38).

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REFERENCE LIST

1. Adhyaru BB, Jacobson TA. Safety and efficacy of statin therapy. Nat Rev Cardiol. 2018;15:757-769.
2. Ridker PM, Danielson E, Fonseca FA, et al. Rosuvastatin to Prevent Vascular Events in Men and Women with Elevated C-Reactive Protein. N Engl J Med. 2008;359:2195-207.
3. Sattar N, Preiss D, Murray HM, et al. Statins and risk of incident diabetes: a collaborative meta-analysis of randomised statin trials. Lancet. 2010;375:735-742.
4. Preiss D, Seshasai SR, Welsh P, et al. Risk of incident diabetes with intensive-dose compared with moderate-dose statin therapy: a meta-analysis. JAMA. 2011;305:2556-64.
5. Navarese EP, Buffon A, Andreotti F, et al. Meta-analysis of impact of different types and doses of statins on new-onset diabetes mellitus. Am J Cardiol. 2013;111:1123-30.
6. Besseling J, Kastelein JJ, Defesche JC, et al. Association between familial hypercholesterolemia and prevalence of type 2 diabetes mellitus. JAMA. 2015;313:1029-36.
7. Fall T, Xie W, Poon W, et al. Using Genetic Variants to Assess the Relationship Between Circulating Lipids and Type 2 Diabetes. Diabetes. 2015;64:2676-84.
8. White J, Swerdlow DI, Preiss D, et al. Association of Lipid Fractions With Risks for Coronary Artery Disease and Diabetes. JAMA Cardiol. 2016;1:692-9.
9. Liu DJ, Peloso GM, Yu H, et al. Exome-wide association study of plasma lipids in >300,000 individuals. Nat Genet. 2017;49:1758-1766.
10. Swerdlow DI, Preiss D, Kuchenbaecker KB, et al. HMG-coenzyme A reductase inhibition, type 2 diabetes, and bodyweight: evidence from genetic analysis and randomised trials. The Lancet. 2015;385:351-361.
11. Schmidt AF, Swerdlow DI, Holmes MV, et al. PCSK9 genetic variants and risk of type 2 diabetes: a mendelian randomisation study. The Lancet Diabetes & Endocrinology. 2017;5:97-105.
12. Lotta LA, Sharp SJ, Burgess S, et al. Association Between Low-Density Lipoprotein Cholesterol-Lowering Genetic Variants and Risk of Type 2 Diabetes: A Meta-analysis. JAMA. 2016;316:1383-1391.
13. Ference BA, Robinson JG, Brook RD, et al. Variation in PCSK9 and HMGCR and Risk of Cardiovascular Disease and Diabetes. N Engl J Med. 2016;375:2144-2153.
14. Labos C, Brophy JM, Smith GD, et al. Evaluation of the Pleiotropic Effects of Statins: A Reanalysis of the Randomized Trial Evidence Using Egger Regression-Brief Report. Arterioscler Thromb Vasc Biol. 2018;38:262-265.
15. Zhou Q, Liao JK. Pleiotropic Effects of Statins. Circulation Journal. 2010;74:818-826.
16. Postmus I, Trompet S, Deshmukh HA, et al. Pharmacogenetic meta-analysis of genome-wide association studies of LDL cholesterol response to statins. Nat Commun. 2014;5:5068.
17. Scott RA, Scott LJ, Mägi R, et al. An Expanded Genome-Wide Association Study of Type 2 Diabetes in Europeans. Diabetes. 2017;66:2888-2902.

18. Morris AP, Voight BF, Teslovich TM, et al. Large-scale association analysis provides insights into the genetic architecture and pathophysiology of type 2 diabetes. Nat Genet. 2012;44:981-90.
19. Bulik-Sullivan B, Finucane HK, Anttila V, et al. An atlas of genetic correlations across human diseases and traits. Nat Genet. 2015;47:1236-41.
20. Zheng J, Erzurumluoglu AM, Elsworth BL, et al. LD Hub: a centralized database and web interface to perform LD score regression that maximizes the potential of summary level GWAS data for SNP heritability and genetic correlation analysis. Bioinformatics. 2017;33:272-279.
21. Stock J, Yogo M. Testing for Weak Instruments in Linear IV Regression. Economics. 2005.
22. Burgess S, Butterworth A, Thompson SG. Mendelian randomization analysis with multiple genetic variants using summarized data. Genet Epidemiol. 2013;37:658-65.
23. Bowden J, Davey Smith G, Burgess S. Mendelian randomization with invalid instruments: effect estimation and bias detection through Egger regression. Int J Epidemiol. 2015;44:512-25.
24. Bowden J, Davey Smith G, Haycock PC, et al. Consistent estimation in Mendelian randomization with some invalid instruments using a weighted median estimator. Genet Epidemiol. 2016;40:304-14.
25. Hartwig FP, Davey Smith G, Bowden J. Robust inference in summary data Mendelian randomization via the zero modal pleiotropy assumption. Int J Epidemiol. 2017;46:1985-1998.
26. Burgess S, Thompson SG. Multivariable mendelian randomization: the use of pleiotropic genetic variants to estimate causal effects. Am J Epidemiol. 2015;181:251-60.
27. Willer CJ, Schmidt EM, Sengupta S, et al. Discovery and refinement of loci associated with lipid levels. Nat Genet. 2013;45:1274-83.
28. R Core Team. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria. 2017.
29. Hemani G, Zheng J, Elsworth B, et al. The MR-Base platform supports systematic causal inference across the human phenome. eLife. 2018;7:pii: e34408.
30. Everett BM, Mora S, Glynn RJ, et al. Safety profile of subjects treated to very low low-density lipoprotein cholesterol levels (<30 mg/dl) with rosuvastatin 20 mg daily (from JUPITER). Am J Cardiol. 2014;114:1682-9.
31. Hsia J, MacFadyen JG, Monyak J, et al. Cardiovascular event reduction and adverse events among subjects attaining low-density lipoprotein cholesterol <50 mg/dl with rosuvastatin. The JUPITER trial (Justification for the Use of Statins in Prevention: an Intervention Trial Evaluating Rosuvastatin). J Am Coll Cardiol. 2011;57:1666-75.
32. Feng Q, Wei WQ, Chung CP, et al. Relationship between very low low-density lipoprotein cholesterol concentrations not due to statin therapy and risk of type 2 diabetes: A US-based cross-sectional observational study using electronic health records. PLoS Med. 2018;15:e1002642.

33. Gage SH, Jones HJ, Taylor AE, et al. Investigating causality in associations between smoking initiation and schizophrenia using Mendelian randomization. Sci Rep. 2017;7:40653.
34. Ware JJ, van den Bree MB, Munafo MR. Association of the CHRNA5-A3-B4 gene cluster with heaviness of smoking: a meta-analysis. Nicotine Tob Res. 2011;13:1167-75.
35. Cholesterol Treatment Trialist' (CTT) Collaborators, Kearney PM, Blackwell L, et al. Efficacy of cholesterol-lowering therapy in 18 686 people with diabetes in 14 randomised trials of statins: a meta-analysis. Lancet. 2008;371:117-125.
36. Burgess S, Thompson SG. Use of allele scores as instrumental variables for Mendelian randomization. Int J Epidemiol. 2013;42:1134-44.
37. Pierce BL, Burgess S. Efficient design for Mendelian randomization studies: subsample and 2-sample instrumental variable estimators. Am J Epidemiol. 2013;178:1177-84.
38. Zhao Q, Wang J, Hemani G, et al. Statistical inference in two-sample summary-data Mendelian randomization using robust adjusted profile score. arXiv:180109652v2 [statAP]. 2018.

CHAPTER 7

Survival bias in Mendelian randomization studies: a threat to causal inference

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Submitted

ABSTRACT

It has been argued that survival bias may distort results in Mendelian randomization studies in older populations. Through simulations of a simple causal structure we investigate which factors influence the extent of this bias in the context of exposures which affect survival. We observed that selecting on survival may decrease instrument strength and will, for exposures with directionally concordant effects on survival (and the outcome), introduce bias towards the null for the instrument-outcome association if the true causal effect is not equal to null, and bias from the null if the true causal effect is null. Stronger selection effects and higher ages at study inclusion generally increased this bias when the true causal effect was not equal to null. Moreover, the impact of this bias may differ depending on the distribution of the exposures. The bias in the estimated exposure-outcome relation depended on whether Mendelian randomization estimation was conducted in the one- or two-sample setting. Finally, we discuss how survival bias may be detected in epidemiological cohorts, and which statistical approaches might help to alleviate this and other types of selection bias.

INTRODUCTION

An increasing number of studies are proposing genetic instruments to examine the causal effect of (typically modifiable) exposures on health states or disease. This approach is known as Mendelian randomization. The basic idea is that a genetic marker (polymorphism or haplotype) serves as a proxy for a particular exposure, under the assumption that the potential effect of the genetic marker on the outcome of interest is only through this exposure. Given the continued methodological developments in the field of Mendelian randomization (1), and that summary level statistics from genome-wide association studies (GWAS) are increasingly made publicly available, it is expected that this trend will continue for the foreseeable future. Considerable efforts are being made to facilitate and standardize this advance in Mendelian randomization studies (2-4).

Although often assumed to give a valid causal estimate in contexts where observational evidence might be biased due to residual confounding or reverse causation, Mendelian randomization studies can give biased results when analyses are performed in selected subgroups, as spurious associations may emerge when selection is performed on a common effect of two variables – "one of which is either the treatment or a cause of treatment, and the other is either the outcome or a cause of the outcome" (5). Formally known as collider-stratification bias in causal graph theory, this specific form of selection bias has been suggested to contribute to several counterintuitive phenomena in the clinical literature. These include observations that maternal smoking is associated with lower infant mortality amongst low birthweight infants (the 'birthweight paradox') (6, 7), that obesity is associated with greater survival in individuals with certain chronic diseases (the 'obesity paradox') (8), and that higher levels of serum cholesterol and blood pressure appear protective in the oldest old (9-12). The latter examples are thought to exemplify a subtype of selection bias, known as survival bias, caused by only recruiting or analyzing the non-random subset of the population who have survived long enough to be included.

It has been argued that in Mendelian randomization studies in older populations, survival bias may distort results (13, 14). While this issue has received limited attention in the literature, some researchers have recognized this potential source of bias. For example, Østergaard and colleagues noted that the protective associations of systolic blood pressure with Alzheimer's disease observed in their Mendelian randomization study might arise as a result of differential survival bias (15). Another notable discussion of survival bias followed the observation that variants known to increase BMI associated with a lower risk of Parkinson's disease (16), which contrasted with the null effect

observed in a large meta-analysis of cohort studies on the topic (17). We aimed to investigate the impact of survival bias on Mendelian randomization analyses through a simulation study. In this paper, we will describe which factors influence the extent of this bias. We will also discuss how to determine whether survival bias is present in epidemiological cohorts, and which (statistical) approaches may help to minimize or address this bias.

METHODS

Review of the theory

We define *X* as the exposure and *Y* as the outcome of interest (**Figure 1**). Drawing valid conclusions from a Mendelian randomization analysis requires using a genetic instrument *G* (e.g. a single-nucleotide polymorphism) that meets three key assumptions: i.) *G* explains variation in exposure *X*, ii.) *G* is independent of the (known and unknown) confounders *U* of the association between *X* and the outcome *Y*, and iii.) *G* is independent of *Y* given *X* and *U* (18). In addition, in order to obtain a point estimate of a causal estimate, a fourth assumption is required. This may either be the assumption of homogeneity, or the sometimes more plausible, alternative assumption of monotonicity (19). If these assumptions hold, a causal effect of *X* on *Y* can be reliably estimated, as the association between *G* and *Y* should be essentially free from reverse causality and residual confounding (20).

Consider the following example where we are interested in a causal effect of *X* (e.g. cholesterol) on a continuous outcome *Y* (e.g. cognitive test performance) (**Figure 2**). The inherent concept of Mendelian randomization, that alleles are randomly assigned at conception, would normally ensure that the association measure between *G* and *Y* can be attributed solely to the effect of the exposure *X* on *Y*. However, we must consider that for older populations the study population is restricted by design, including only those who have survived until a certain age.

In this situation, survival until study inclusion (*S*) is influenced by the exposure of interest *X* and a second exposure *R* (e.g. smoking) (**Figure 2A)**. For the purpose of simplicity we assume that these two exposures are uncorrelated in the unselected population. However, if we condition on a common effect of *X* and *R*, i.e. survival (*S*=1), we induce an association between *X* and *R*, and therefore also between *G* and *R*. More intuitively, if someone survives until study inclusion with risk factor *R* (i.e. smokes), they are less likely to also have high levels of risk factor *X* (i.e. hypercholesterolemia), and in extension less likely to have

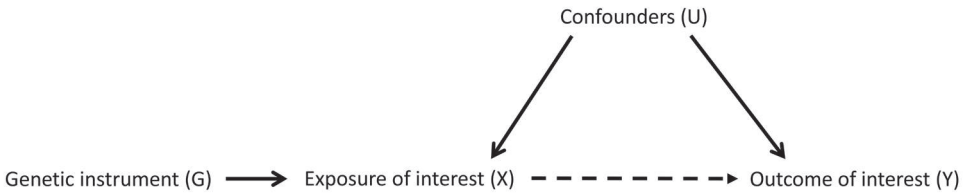


Figure 1. Schematic outline of the Mendelian randomization approach

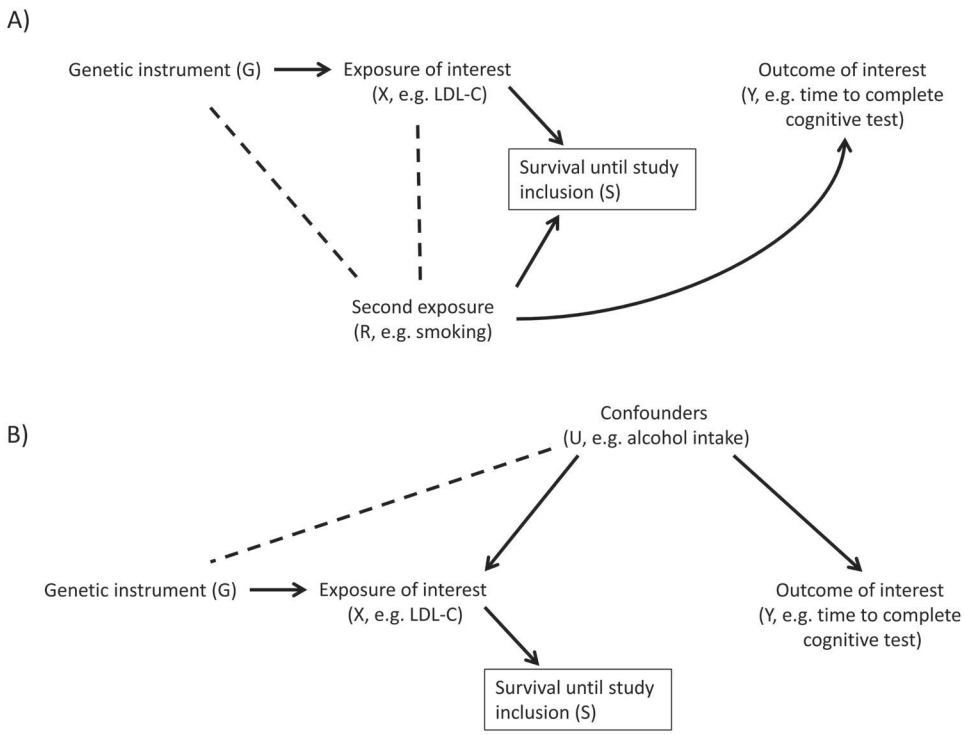


Figure 2. In the example of two exposures affecting probability of survival, conditioning on survival (*S*) may induce an association between previously uncorrelated risk factors *X* (and its genetic proxy *G*) and *R* (dashed lines shown in A). More intuitively, if you are a smoker and still alive at study inclusion, you are less likely to also have a high level of LDL-cholesterol (LDL-C), and vice versa. Additionally, conditioning on survival may induce an association between the genetic instrument G and any confounders *U* of the *X*-*Y* association (dashed line shown in B), even in the absence of risk factor *R*. In both situations, the association between the genetic instrument and the outcome of interest might thus become biased. Please note that while we did not include a line from *X* to *Y* in either causal structure, we also simulate scenarios where *X* does have a causal effect on *Y*. Adapted from Boef AG, et al. (13)

inherited trait *G* that causes hypercholesterolemia. We therefore expect that the previously uncorrelated, competing variables will become negatively associated when restricting the analyses to the 'survivors'. It follows that the estimated *G*-*Y* association can therefore no longer be solely attributed to the effect of exposure *X* on outcome *Y* (i.e. will become biased), as conditioning on the common effect of *X* and *R* has opened an indirect path from *G* to *Y* going through *R* (21).

In the example above we have assumed no confounders exist of the *X*-*Y* association. However, their presence may be problematic (**Figure 2B**). This is because restricting the analysis to survivors means that entry into the study becomes conditional upon the value of *X*. As *X* in turn depends both on the genetic instrument *G* and the confounder *U* (e.g. alcohol intake), *G* and *U* may become correlated.

In essence, the third assumption described at the start of this paragraph has become violated through survival bias in both examples. While our simulations will primarily focus on the causal structure shown in **Figure 2A**, we present simulations on **Figure 2B** and on a combination of these causal structures in the supplemental material.

Data generation

All simulation scenarios assume the basic causal structure shown in **Figure 2A**. All causal associations between variables are chosen such that an increase in cause will lead to an increase in the consequence, except for the effect on survival where higher values in exposure, confounder or risk factor correspond to lower survival times. In addition, our simulations assumed constant treatment effects. For each scenario we generated a dataset of 10 million observations with multiple randomly generated variables: a binary genetic instrument (*G*), a continuous exposure (*X*) influenced by *G*, a second exposure (*R*), a continuous outcome (*Y*) principally influenced by *R* and in later scenarios also by *X*, and finally an age of death influenced by both *X* and *R*. In secondary analyses we additionally generate a continuous confounder (*U*) with equal effects on *X* and *Y* (appendix). All simulations were performed separately for binary and continuously distributed *R*'s.

Details of data generation and parameters values are presented in **Table 1**. Of note, *X* was standardized to have a mean of 0 and standard deviation of 1. The effect of *G* on *X* was chosen such that the corresponding strength of the instrument, measured by the partial R^2, equaled 1, 5, 10, and 15%. In addition, while the per-unit effect size was the same for the two types of *R*, the different scales of measurement (dichotomous (e.g. presence or absence

of hypercholesterolemia) against per standard deviation increase) means their impact on other variables will differ.

Table 1. Parameters values and details of data generation

Parameter (scale)	Data generation and alternative values	Standard value
G (binary)	Prevalence of 25, 50, 75%	50%
X (continuous)	Normally distributed with mean 0 and var(X|G)=1, with varying contribution of G (and if applicable U)	
Variance of X explained by G	1, 5, 10, 15% of X	5%
U (continuous)	Normally distributed with μ=0, σ=1. Only included in scenarios in appendix	
Effect of U on X	Increase of 0.5 per one unit increase in U	None
R (binary)	Prevalence of 12.5, 25, 50%	25%
R (continuous)	Normally distributed with μ=0, σ=1	
Age of death	Gompertz distributed with baseline parameters $a=4.59053\times10^{-5}$ and $b=8.76978320\times10^{-2}$, with varying (additional) contribution of X and R	
Effects of X on age of death	HR of 1.1, 1.25, or 1.5 per one unit increase in X	HR 1.25
Effects of R on age of death	HR of 1.25, 1.5, 2, or 4 if R=1 (binary R) or per one unit increase in R (continuous R)	HR 1.5
S (binary)	Indicates whether age of death is larger than age at inclusion	
Y (continuous)	Normally distributed with mean 0 and variance(Y|X,R)=1, with varying contribution of X and R (and if applicable U)	
Effects of X on Y	Increase of 0, 0.5, 1, or 2 per one unit increase in X	0
Effects of R on Y	Increase of 0.25, 0.5, or 1 if R=1 (binary R) or per one unit increase in R (continuous R)	0.5
Effect of U on Y	Increase of 0.5 per one unit increase in U	None
Number of observations	10.000.000 in all scenarios	

S.D. denotes standard deviation.

To generate survival time we obtained the 2016 mortality data of the United States from the Human Mortality Database (22). Using the *MortalityLaws* R-package (23) we estimated the parameters of the Gompertz model (24) within

this real-world dataset (**eFigure 1**), which were subsequently used to generate survival times for our simulated population. Effects of both *X* and *R* on age of death were modelled as hazard ratios, with having higher levels of *X* and/or *R* translating into an earlier death (on average), and lower levels of *X* and/or *R* in a later death (on average). Subsequently, we considered different age boundaries for study inclusion, from 75-95 years, thereby steadily decreasing the number of surviving participants (*S*=1). We used R (version 3.4.1) for all data generation and analyses (25). Sample code is provided as supplemental material.

Effects on instrument strength

Firstly, we examined whether selecting on survival may influence the strength of instrument *G*, reflected by the squared correlation between *G* and *X* (R^2), which indicates how much variance of *X* is explained by *G*. Given that selecting on survival will yield smaller data sets, and that the F-statistic strongly depends on sample size, we did not consider the F-statistic as a measure of instrument strength (26). We chose different strengths of the instrument, while all other parameter values were kept fixed at a standard value given in **Table 1**. No effect of *X* on *Y* was assumed.

Effects on association between the genetic instrument G and exposure R

Secondly, we considered the effect of different parameters on the induced correlation between *G* and *R* within an increasingly selected population. Effects of changing the following parameters were considered:

i. variance of *X* explained by *G* (R^2);
ii. effects of *X* on age at death;
iii. effects of *R* on age at death;
iv. effects of *R* on *Y*;
v. prevalence of *G*;
vi. prevalence of *R* (for dichotomous *R*).

In each simulation, the other parameters were held at their standard values, and no effect of *X* on *Y* was assumed. Accompanying confidence intervals for the correlation between *G* and *R* were calculated using Fisher's z-transformation. (27)

Effects on association between genetic instrument G and outcome of interest Y

Thirdly, we examined how this induced correlation between *G* and *R* influences the *Y*~*G* association, estimated with linear regression. Different true effects of *X* on *Y* were assumed (**Table 1**). Other parameters were again held at their standard value.

Effects on instrumental variable (IV) estimators

Finally, we considered how the induced correlation between *G* and *R* might influence an IV-estimator. In its simplest form this estimator equals the ratio of regression coefficients, known as the Wald ratio (28), defined for our continuous outcome *Y* as

$$Wald\ ratio = \frac{coefficient\ of\ G\ in\ regression\ of\ outcome\ Y\ on\ G}{coefficient\ of\ G\ in\ regression\ of\ exposure\ X\ on\ G} = \frac{\hat{\beta}(Y \sim G)}{\hat{\beta}(X \sim G)}$$

The Wald ratio thus quantifies the causal effect of the exposure on the outcome and estimates the mean increase in outcome per unit increase in exposure. Increasingly, summarized data (coefficients and standard errors) from large genome-wide association study (GWAS) consortia are made publicly available, which enable researchers to perform two-sample Mendelian randomization even if their own study does not allow for estimation of both coefficients necessary to calculate the Wald ratio (29). These external datasets are generally more likely to have primarily included middle-aged participants (30-32), and thus less likely to be affected by survival bias. Therefore, under the assumption of no age-related effect modification, we not only considered the scenario where both coefficients are estimated with linear regression in the same increasingly selected dataset (i.e. 'internal' estimation), but also what happens if the association measure between *G* and *X* were to be taken from an external dataset not selected on survival (i.e. 'external' estimation, by taking the fixed value of our total population). Confidence intervals for the internally estimated Wald ratio were calculated using the *tsls* function from the *sem* R-package (33).

RESULTS

Instrument strength

As shown in the main plot of **Figure 3**, the variance explained in exposure *X* by *G* decreases when higher ages-at-inclusion are considered. The decline in R^2 between age 75 and 95 years is greater in absolute terms, but comparable in relative terms, for stronger genetic instruments. For example, for the instrument explaining 1% of variance in X in the unselected (i.e. entire) sample R^2 declined from 0.99% at 75 years to 0.90% at 95 years, set against a decline from 14.75% to 13.35% for the instrument originally explaining 15% of variance in X. Shown in the figure's insets are the **A)** the change in prevalence of *G* and **B)** the survival curve for the population from 75 years until 95 years. The prevalence of *G* was observed to decline from 0.49 at age 75 years to 0.46 at age 95. Furthermore, of the population alive at 75 years, 15.6% was still alive at 95 years. Results for the continuously distributed R were comparable (**eFigure 2**).

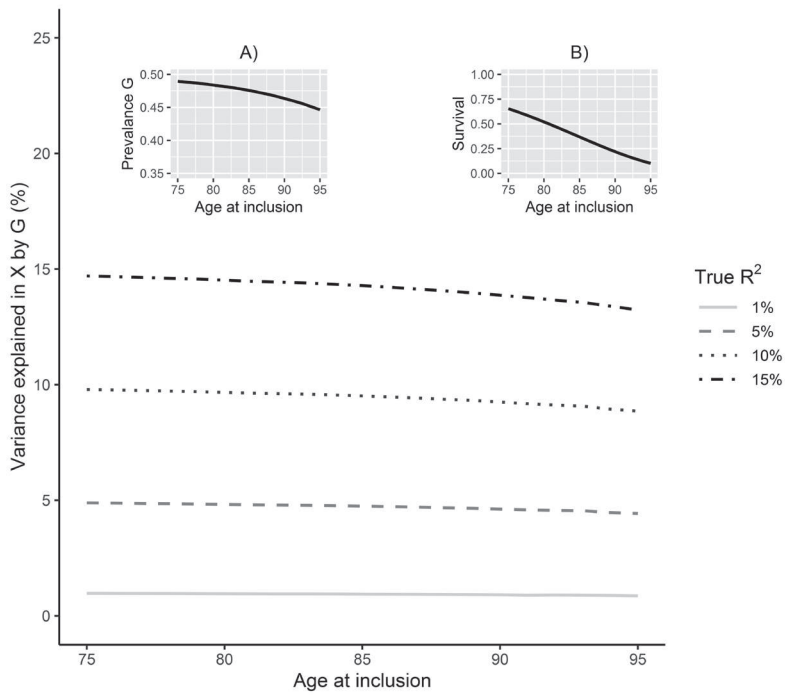


Figure 3. Variance explained in the exposure of interest *X* by its genetic proxy *G* for an increasingly selected population, when incorporating a binary *R*. Shown in the insets are A) the prevalence of *G* and B) the accompanying survival curve, both with the true (i.e. unselected) R^2 set at 5%.

Correlation between G and R

The induced negative correlation due to selection on age between the causally independent variables *G* and *R* across different simulation scenarios is shown in **Figure 4**. Keeping all other parameters constant, the correlation becomes more negative as **i.** the instrument is stronger (i.e. more variance in *X* is explained by *G)* (**4A,B**), **ii.** *X* has greater effects on age at death (**4C,D**), **iii.** *R* has greater effects on age at death (**4E,F**), and **iv.** as *R*'s prevalence becomes greater (dichotomous *R)* (**4K**). However, once the prevalence of *R* exceeds 0.5 the induced correlation between *G* and *R* decreases again. In contrast, the correlation remains constant for different effects of *R* on *Y* (**4G,H**), and is largely unchanged by changing the prevalence of G (**4I,J**). Of note, the association between age-at-inclusion and the induced *G~R* correlation attenuates at higher ages when the deleterious effect of *R* on *S* corresponds to an hazard ratio of 4 (**4E**), with the nadir of the curve occurring between 80 and 85 years of age. This specific example likely results from the rapid depletion of the *R*-carrying participant pool, an effect also visible but less extreme for the simulations incorporating a continuous *R* (**4F**).

Bias to Y ~ G association

Varying the true underlying effect of *X* on *Y* reveals how the association between *G* and *Y* is biased by selecting on *S*=1 (**Figure 5**). In cases where the true effect ≠ 0, a bias towards the null is seen, underestimating the true effect. While this bias is greater in absolute terms, in relative terms we observe a slight attenuation across different effects of *X* on *Y* when considering a dichotomous *R* (at 95 years: 12.3% underestimation for true effect of 0.5 (**5C**) versus 10.1% for true effect of 2 (**5E**). A different pattern was observed for the situation where the true effect of *X* on *Y* is null. In this case, where the statistical association between the genetic variant and the outcome of interest is completely due to bias, the resulting association becomes nominally negative (**5A**). The same, but slightly exaggerated pattern occurs for a continuously distributed *R*. The *Y~G* association namely moves away from the null to a considerably greater extent when the true effect of *X* on *Y* is null (**5B**), and greater attenuation of the bias towards the null occurs for greater effects of *X* of *Y* when the true effect is not equal to 0 (**5D,F**).

Bias to IV estimator

The IV estimator is influenced by survival bias, where magnitude and direction of the bias are dependent on **i.** whether the association measure between *G* and *X* is estimated within the same selected dataset as the association measure

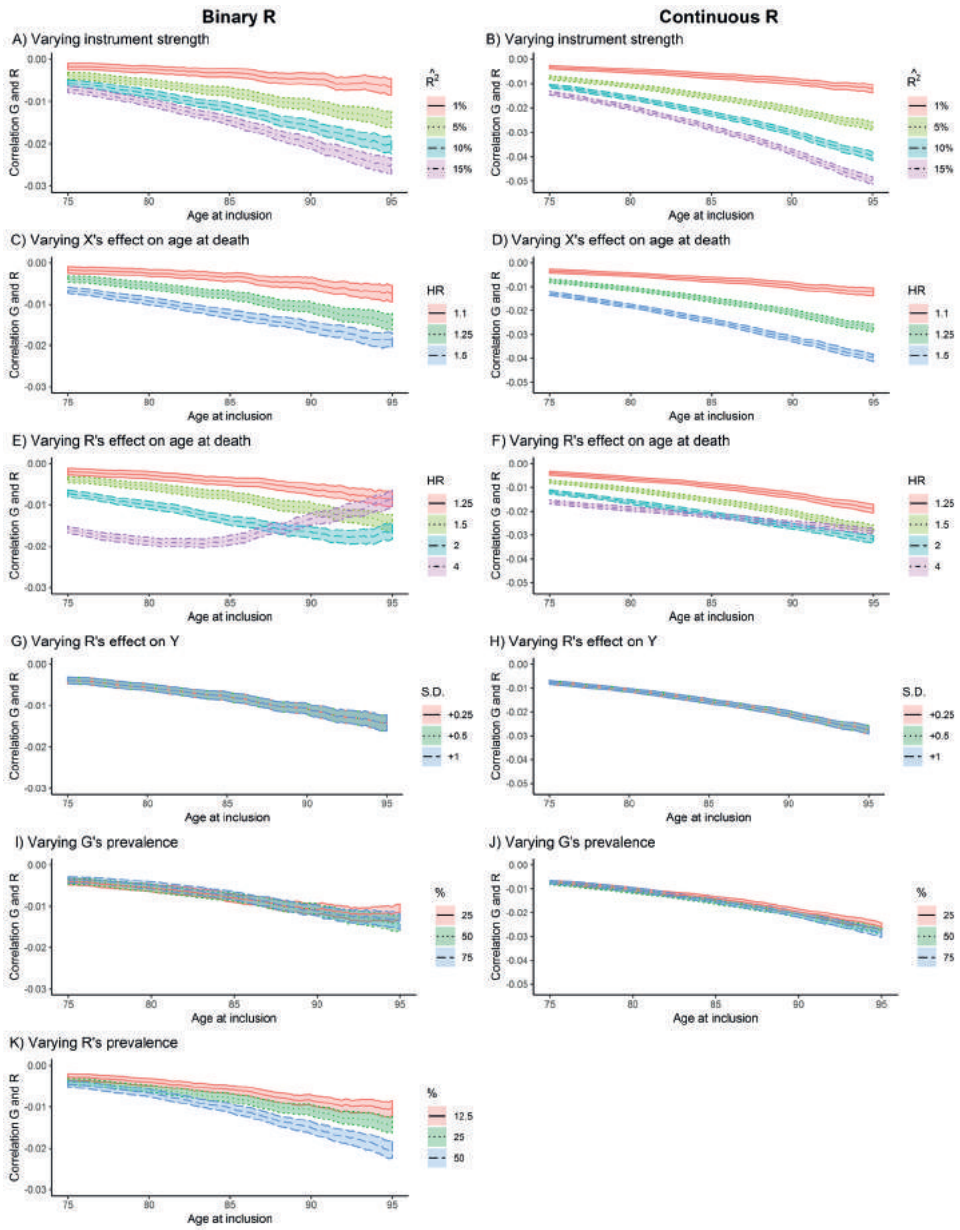


Figure 4. Effect of varying different parameters on the induced correlation (95% CI) between the genetic instrument *G* and the second exposure *R* for an increasingly selected population. Shown for binary (left column) and continuously (right column) distributed *R*. S.D. denotes standard deviation.

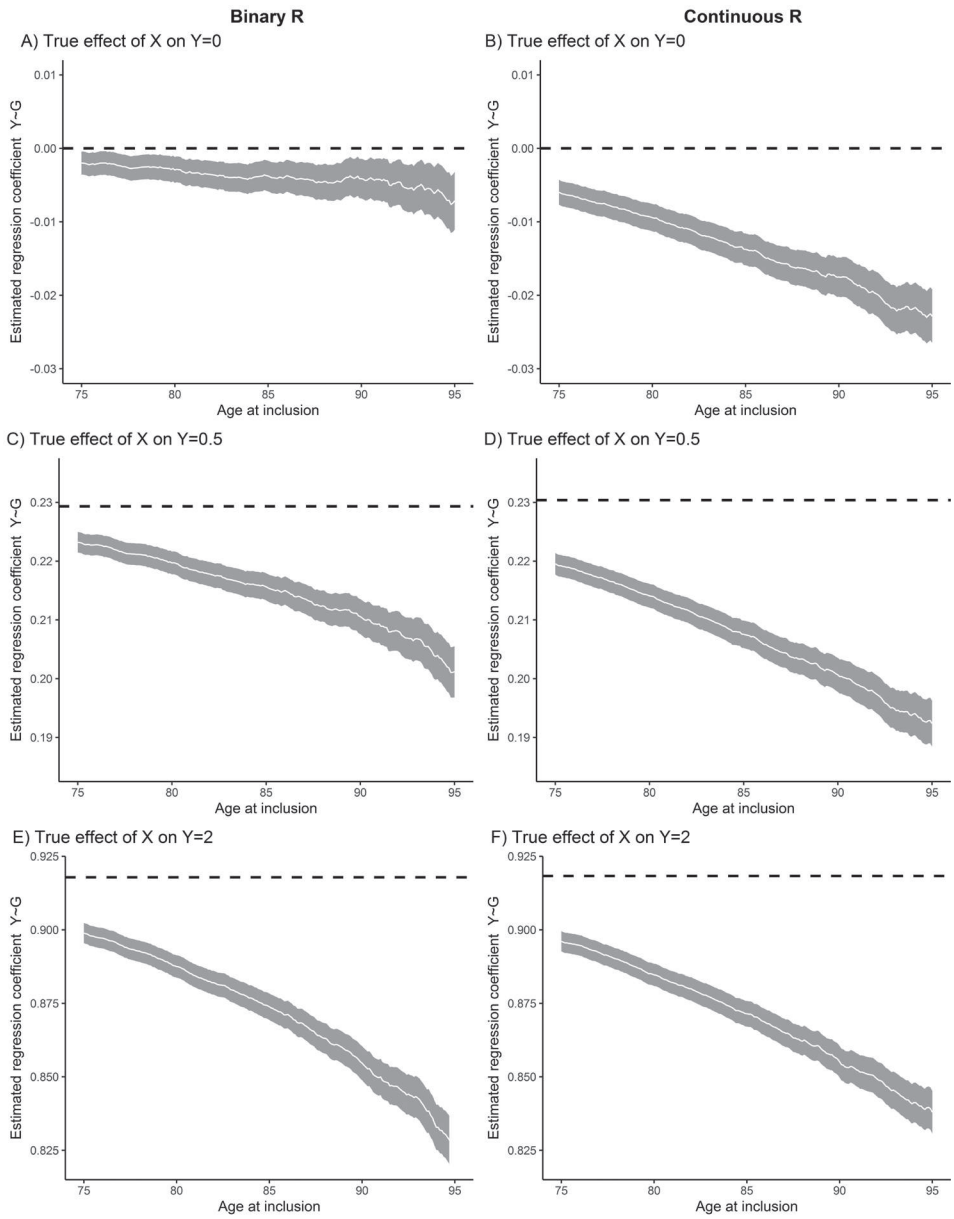


Figure 5. Effect of survival bias on the association between the genetic instrument G and the outcome of interest *Y*, for different true effects of exposure *X* on *Y*. Data are presented as regression coefficients (95% CI) estimated with linear regression. The true (i.e. unselected) regression coefficient for *G* on *Y* is shown as a dashed line in each plot. Shown for binary (left column) and continuously (right column) distributed *R*.

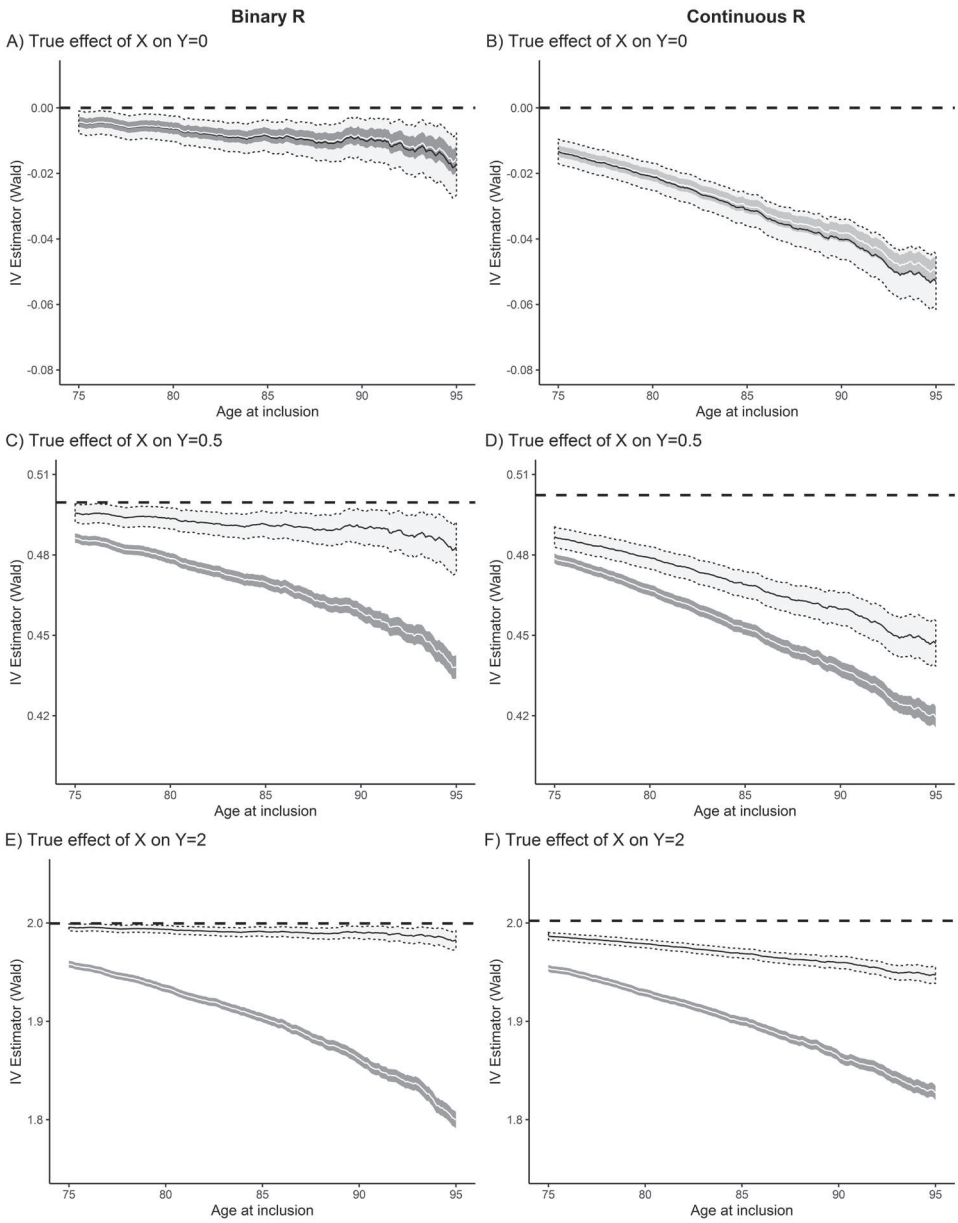


Figure 6. Wald ratios (95% CI) based on internally (white ribbon) versus externally (grey ribbon) estimated association between exposure *X* and the outcome *Y*, for different true effects of exposure *X* on *Y*. Shown for binary (left column) and continuously (right column) distributed *R*. Dashed lines denote the true (i.e. unselected) Wald ratio, which equals the true causal effect of *X* on *Y*.

between *G* and *Y* was, or within an external source not selected on age, and **ii.** whether the true effect of *X* on *Y* is null or not (**Figure 6**). When both the numerator (*Y~G*) and denominator (*X~G*) of the Wald ratio (i.e. our causal effect estimate) are taken from the same selected dataset, we observe that they will be similarly biased. Taking the ratio of these two therefore seemingly cancels out much of the bias to the IV estimator, compared to the situation where only the numerator is taken from a population selected on survival. In this latter situation, the relative degree of the bias equals that seen for the association measure between *G* and *Y*. The two IV estimators diverge more strongly as stronger true effects of *X* on *Y* are considered. This is more clearly observed when a dichotomous *R* is considered. For a continuously distributed *R*, selection bias partially persists for the internally estimated IV estimator (**6B,D,F**).

Alternative causal structures

Simulation results for the causal structure depicted under **Figure 2B**, and for the combination of **2A** and **2B**, did not show markedly different results (**eFigures 4-10**).

DISCUSSION

In this paper we show that previously uncorrelated, competing risk factors may become associated due to selection on survival, consequently biasing estimates from Mendelian randomization studies. More specifically we observed that, if the effect of the exposure of interest on the outcome of interest is genuinely non-null and the selection-related exposures have directionally concordant effects on the outcome, the association measure between genetic proxies of that exposure and the outcome will become biased towards the null. Of further importance is the observation that as the population size decreases instrument strength also weakens, as measured by R^2. The combination of a smaller population size with a weaker instrument strength will be detrimental to statistical power in hypothesis testing. It should be noted here that the decrease in instrument strength not just results from the decreasing prevalence of the genetic instrument, but also due to the genetic instrument becoming associated with the random noise contributing to the exposure (**eFigure 3**). We additionally observed that the induced correlation between *G* and *R* is greater for stronger instruments. However, as bias amplification is smaller for stronger instruments, we expect that instrument strength will not substantially affect the degree of bias of either *Y~G* or IV estimators.

A fundamental assumption in inferring causality using Mendelian randomization is that the genetic instrument should not independently associate with traits of aetiological significance to the outcome other than the exposure of interest. In the simple causal structure considered in our simulations, we observe that this assumption is violated by selection on survival. While we solely explored scenarios with one genetic instrument, this problem will also occur for any combination of genetic instruments for exposures which jointly influence the probability of surviving until study inclusion. In essence, quasi-pleiotropic effects are induced by conditioning on survival till study inclusion. More specifically, given that these pleiotropic effects are unlikely to average to zero across a combination of genetic instruments proxying the same exposure, survival bias is equivalent to introducing directional pleiotropy into Mendelian randomization analyses. To our knowledge it has not been examined whether robust analysis methods specifically aimed at correcting for bias due to unbalanced directional pleiotropy, such as MR Egger regression (34), would be able to cope with this problem. Of particular interest would be whether sets of polygenic instruments, whose individual metabolic pathways to the intermediate phenotype may differ, might be differentially affected by survival bias.

While our simulations specifically examined age-related selection, researchers with data on (younger) populations selected on alternative characteristics (e.g. disease status) will similarly have to consider the possible influence of selection bias in genetic analyses, including genome-wide association testing (35-37). This also holds for investigations within increasingly popular mega-biobanks such as population-based UK Biobank and the Million Veterans Program, both of which have had relatively limited response rates (38-40). Alternative causal structures which might give rise to selection bias in Mendelian randomization studies have been presented elsewhere (40).

There exist several ways for researchers to substantiate the claim that survival bias may be present in their study population, most of which require individual level data. One approach is to examine the associations between the genetic instrument(s) and confounders of the association between exposure and outcome of interest (*X* and *Y*), and/or with variables upon which the population was selected. A key point here is that no association should be present in younger, less-selected populations. Theoretically, if no trends across age are found, it is unlikely that the genetic variant significantly influences mortality. However, this approach will generally only be feasible if large-scale data across different age groups is available on a variety of phenotypic traits, or if the population is strongly enriched or depleted for the trait of interest (35). Leveraging summary

statistics from genome-wide testing performed in large-scale population-based studies may make it possible to differentiate between survival bias-induced associations and alternative pleiotropic mechanisms. Alternatively, researchers can examine whether the strength of the instrument (i.e. the explained variance in the exposure of interest by the instrument) is significantly lower in older than that reported in younger populations. In extension, allele frequencies of high-risk variants are likely to decline in an age-dependent manner, as observed in our simulations, as individuals with a substantially deleterious genetic predisposition will gradually be phased out of the population. This is in line with previously described observations of a large-scale genetic risk score for low-density lipoprotein cholesterol decreasing with increasing age (41). However, it should be noted there does not exist a failsafe method of ruling out survival bias, nor were the above approaches developed for the IV-context under bias amplification. In addition, these methods assume that cohort effects are not present, with younger and older populations coming from the same source population.

Recent work by Canan and colleagues suggests that for the causal structure under investigation in our simulations, selection bias may be corrected via inverse probability weighting (14). In general, we expect that if the selection gradient solely depends on measured variables which are available for the entire original study population (i.e. also for those individuals who are not selected in the study sample), and assuming a constant treatment effect, both inverse probability weighting and multiple imputation could be suitable solutions for selection bias. If data are only available for the selected individuals, but a sufficient set of selection-related variables are precisely measured, then inclusion of these selection-related variables in multivariable regression models may resolve the bias if the models are well-specified. The value of representative cohorts with little selection (e.g. birth cohorts) cannot be overstated in this context (40, 42), though genotyping genetically informative family members may hold promise as well (43). Alternative strategies have been proposed in the context of hazard models (44-46), which may fare better when selection depends on (partially) unobserved variables. In addition, methods of using covariate balance to detect dependent censoring in longitudinal studies exist, though these approaches have not been extended to IV-analysis where bias amplification may occur (47, 48).

We must acknowledge several limitations of our study. In our simulations we made a number of assumptions, due to which caution must be taken in making generalizations. These include that exposures but also genotypes had constant effects during life, ignoring possible antagonistic pleiotropy (49), and that

survival bias would similarly affect different components of the causal structure (e.g. both the numerator and denominator of the Wald ratio). In addition, we solely considered one commonly occurring genetic instrument and uncorrelated exposures with directionally concordant effects on survival (and the outcome of interest). *R* could however be considered a combined vector for many possible competing causes of death before study inclusion. Furthermore, we did not consider a binary outcome of interest, to avoid the issue of non-collapsibility, and restricted our investigations to a linear instrument-exposure association. We also did not examine the effect of possible effect modification between the two exposures, which might lead to stronger correlations between the genetic instrument and exposure *R* and therefore increased bias (50). These choices were aimed at examining the basic underpinnings of survival bias in the context of Mendelian randomization studies, in absence of real-world complexities.

In conclusion, using a simple causal structure we were able to demonstrate that survival bias may lead to biased estimates in Mendelian randomization studies. It will be of interest to examine more detailed simulations in the future, using greater numbers of instruments and exposures to derive bias formulas (as others have done for collider bias in binary variable structures (51)), ideally coupled with comparing the performance of the possible correction methods for survival bias described above. Finally, future work should explore the implications of using different instrumental variable assumptions such as monotonicity, instead of the assumption of homogenous treatment effects of our simulations.

REFERENCES

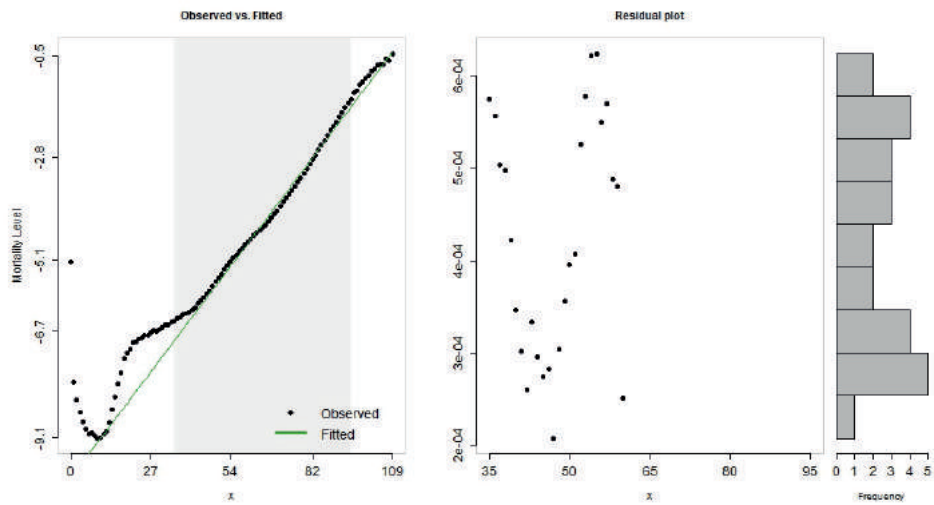
1. Zheng J, Baird D, Borges MC, et al. Recent Developments in Mendelian Randomization Studies. Curr Epidemiol Rep. 2017;4:330-345.
2. Boef AG, Dekkers OM, Le Cessie S, et al. Reporting Instrumental Variable Analyses. Epidemiology. 2013;24:937-8.
3. Hemani G, Zheng J, Elsworth B, et al. The MR-Base platform supports systematic causal inference across the human phenome. eLife. 2018;7:pii: e34408.
4. Haycock PC, Burgess S, Wade KH, et al. Best (but oft-forgotten) practices: the design, analysis, and interpretation of Mendelian randomization studies. Am J Clin Nutr. 2016;103:965-78.
5. Hernán MA, Robins JM. Causal Inference. Boca Raton: Chapman & Hall/CRC, forthcoming. 2018.
6. Yerushalmy J. The relationship of parents' cigarette smoking to outcome of pregnancy--implications as to the problem of inferring causation from observed associations. Am J Epidemiol. 1971;93:443-56.
7. VanderWeele TJ. Commentary: Resolutions of the birthweight paradox: competing explanations and analytical insights. Int J Epidemiol. 2014;43:1368-73.
8. Sperrin M, Candlish J, Badrick E, et al. Collider Bias Is Only a Partial Explanation for the Obesity Paradox. Epidemiology. 2016;27:525-30.
9. Schatz IJ, Masaki K, Yano K, et al. Cholesterol and all-cause mortality in elderly people from the Honolulu Heart Program: a cohort study. The Lancet. 2001;358:351-355.
10. Oates DJ, Berlowitz DR, Glickman ME, et al. Blood pressure and survival in the oldest old. J Am Geriatr Soc. 2007;55:383-8.
11. Molander L, Lövheim H, Norman T, et al. Lower systolic blood pressure is associated with greater mortality in people aged 85 and older. J Am Geriatr Soc. 2008;56:1853-9.
12. Weverling-Rijnsburger AWE, Blauw GJ, Lagaay AM, et al. Total cholesterol and risk of mortality in the oldest old. The Lancet. 1997;350:1119-1123.
13. Boef AG, Le Cessie S, Dekkers OM. Mendelian randomization studies in the elderly. Epidemiology. 2015;26:e15-16.
14. Canan C, Lesko C, Lau B. Instrumental Variable Analyses and Selection Bias. Epidemiology. 2017;28:396-398.
15. Østergaard SD, Mukherjee S, Sharp SJ, et al. Associations between Potentially Modifiable Risk Factors and Alzheimer Disease: A Mendelian Randomization Study. PLoS Med. 2015;12:e1001841.
16. Noyce AJ, Kia DA, Hemani G, et al. Estimating the causal influence of body mass index on risk of Parkinson disease: A Mendelian randomisation study. PLoS Med. 2017;14:e1002314.
17. Wang YL, Wang YT, Li JF, et al. Body Mass Index and Risk of Parkinson's Disease: A Dose-Response Meta-Analysis of Prospective Studies. PLoS One. 2015;10:e0131778.

18. Greenland S. An introduction to instrumental variables for epidemiologists. Int J Epidemiol. 2000;29:722-9.
19. Labrecque J, Swanson SA. Understanding the Assumptions Underlying Instrumental Variable Analyses: a Brief Review of Falsification Strategies and Related Tools. Curr Epidemiol Rep. 2018;5:214-220.
20. Ebrahim S, Davey Smith G. Mendelian randomization: can genetic epidemiology help redress the failures of observational epidemiology? Hum Genet. 2008;123:15-33.
21. Greenland S, Pearl J, Robins JM. Causal diagrams for epidemiologic research. Epidemiology. 1999;10:37-48.
22. Human Mortality Database. University of California, Berkeley (USA), and Max Planck Institute for Demographic Research (Germany). Available at www.mortality.org or www.humanmortality.de (data downloaded on July 30 2018).
23. Pascariu MD. MortalityLaws: Parametric Mortality Models, Life Tables and HMD. 2018 R package version 1.5.0. https://github.com/mpascariu/MortalityLaws.
24. Gompertz B. On the Nature of the Function Expressive of the Law of Human Mortality, and on a New Mode of Determining the Value of Life Contingencies. Philosophical Transactions of the Royal Society of London. 1825;115:513-585.
25. R Core Team. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria. 2016.
26. Stock J, Yogo M. Testing for Weak Instruments in Linear IV Regression. Cambridge, 2005.
27. Fisher RA. Frequency distribution of the values of the correlation coefficient in samples from an indefinitely large population. Biometrika. 1915;10:507-521.
28. Wald A. The fitting of straight lines if both variables are subject to error. Ann Math Stat. 1940;11:284-300.
29. Burgess S, Butterworth A, Thompson SG. Mendelian randomization analysis with multiple genetic variants using summarized data. Genet Epidemiol. 2013;37:658-65.
30. Locke AE, Kahali B, Berndt SI, et al. Genetic studies of body mass index yield new insights for obesity biology. Nature. 2015;518:197-206.
31. Willer CJ, Schmidt EM, Sengupta S, et al. Discovery and refinement of loci associated with lipid levels. Nat Genet. 2013;45:1274-83.
32. Ehret GB, Munroe PB, Rice KM, et al. Genetic variants in novel pathways influence blood pressure and cardiovascular disease risk. Nature. 2011;478:103-9.
33. Fox J, Byrnes J, Culbertson M, et al. "Package 'sem'," in http://cran.r-project.org/web/packages/sem/sem.pdf. 2018.
34. Bowden J, Davey Smith G, Burgess S. Mendelian randomization with invalid instruments: effect estimation and bias detection through Egger regression. Int J Epidemiol. 2015;44:512-25.
35. Yaghootkar H, Bancks MP, Jones SE, et al. Quantifying the extent to which index event biases influence large genetic association studies. Hum Mol Genet. 2017;26:1018-1030.

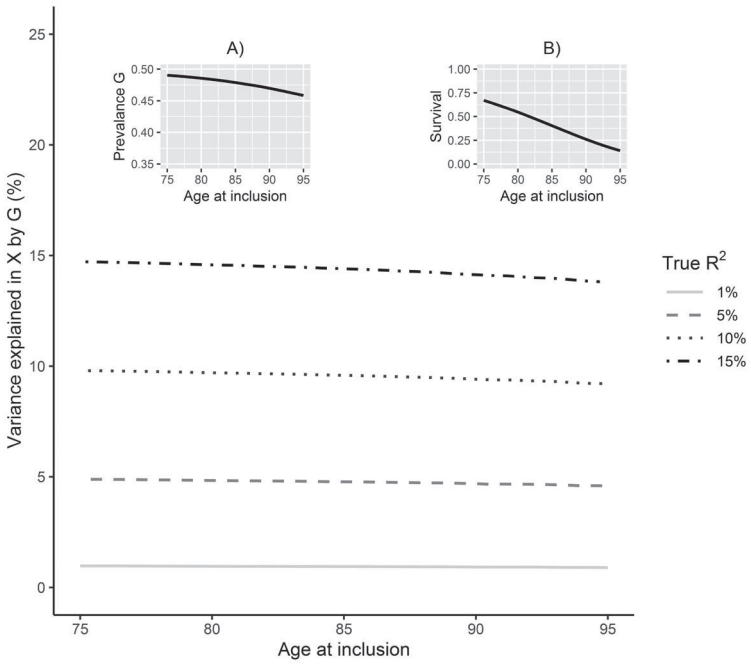
36. Anderson CD, Nalls MA, Biffi A, et al. The effect of survival bias on case-control genetic association studies of highly lethal diseases. Circ Cardiovasc Genet. 2011;4:188-96.
37. Hu YJ, Schmidt AF, Dudbridge F, et al. Impact of Selection Bias on Estimation of Subsequent Event Risk. Circ Cardiovasc Genet. 2017;10.
38. Fry A, Littlejohns TJ, Sudlow C, et al. Comparison of Sociodemographic and Health-Related Characteristics of UK Biobank Participants with the General Population. Am J Epidemiol. 2017;186:1026-1034.
39. Gaziano JM, Concato J, Brophy M, et al. Million Veteran Program: A mega-biobank to study genetic influences on health and disease. J Clin Epidemiol. 2016;70:214-23.
40. Munafo MR, Tilling K, Taylor AE, et al. Collider scope: when selection bias can substantially influence observed associations. Int J Epidemiol. 2017;47:226-235.
41. Postmus I, Deelen J, Sedaghat S, et al. LDL cholesterol still a problem in old age? A Mendelian randomization study. Int J Epidemiol. 2015;44:604-12.
42. Domingue BW, Belsky DW, Harrati A, et al. Mortality selection in a genetic sample and implications for association studies. Int J Epidemiol. 2017;46:1285-1294.
43. Chen L, Weinberg CR, Chen J. Using family members to augment genetic case-control studies of a life-threatening disease. Stat Med. 2016;35:2815-30.
44. Vansteelandt S, Dukes O, Martinussen T. Survivor bias in Mendelian randomization analysis. Biostatistics. 2018;19:426-443
45. Stensrud MJ. Handling survival bias in proportional hazards models: A frailty approach. arXiv:1701.06014v4 [stat.ME] (preprint). 2017.
46. Carlin CS, Solid CA. An approach to addressing selection bias in survival analysis. Stat Med. 2014;33:4073-86.
47. Zubizarreta JR. Stable Weights that Balance Covariates for Estimation with Incomplete Outcome Data. Journal of the American Statistical Association. 2015;110:910-922.
48. Jackson JW. Diagnostics for Confounding of Time-varying and Other Joint Exposures. Epidemiology. 2016;27:859-69.
49. Toupance B, Godelle B, Gouyon PH, et al. A model for antagonistic pleiotropic gene action for mortality and advanced age. Am J Hum Genet. 1998;62:1525-34.
50. Hernán MA, Hernández-Díaz S, Robins JM. A Structural Approach to Selection Bias. Epidemiology. 2004;15:615-625.
51. Nguyen TQ, Dafoe A, Ogburn EL. The Magnitude and Direction of Collider Bias for Binary Variables. arXiv:1609.00606 [stat.ME] (preprint). 2017.

Supplemental Material

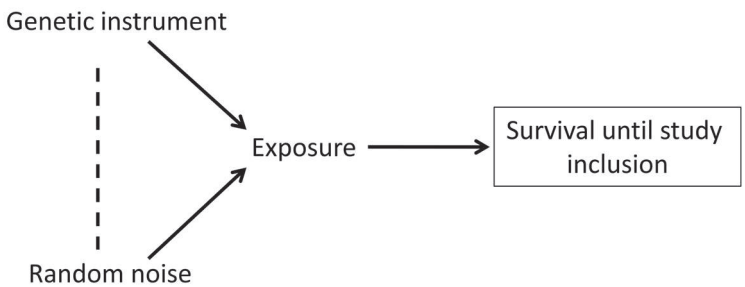
eFigure 1. Results of fitting Gompertz-model using mortality-data (USA 2016)
eFigure 2. Variance explained in X by G for continuously distributed R, for causal structure presented under Figure 2A (main text)
eFigure 3. Genetic instruments and noise in X
eFigure 4: Causal structure also presented in Figure 2B (main text)
eFigures 5-6: Results of simulations for causal structure shown in eFigure 4
eFigure 7: Causal structure combining those presented in Figure 2 (main text)
eFigures 8-10: Results of simulations for causal structure shown in eFigure 7



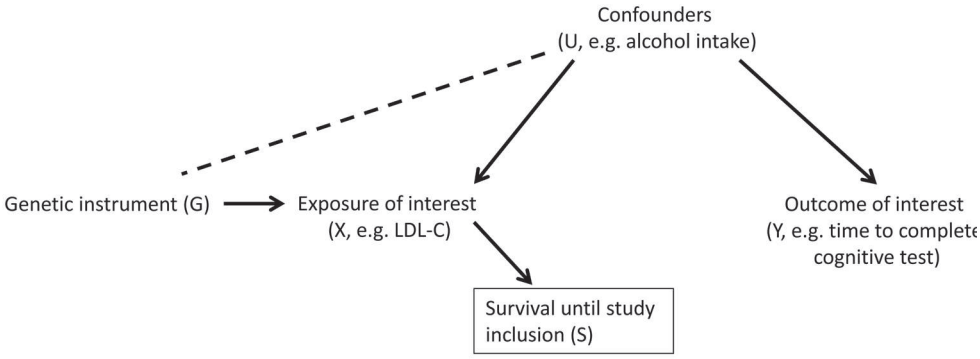
eFigure 1. Results from fitting the Gompertz-model ($a*e^{b*age}$) onto the 2016 mortality data of the United States (age range 35-95) obtained from the Human Mortality Database (www.mortality.org), using the *MortalityLaws* R-package. Estimated model parameters: a, 0.0000459053; b, 0.0876978320.



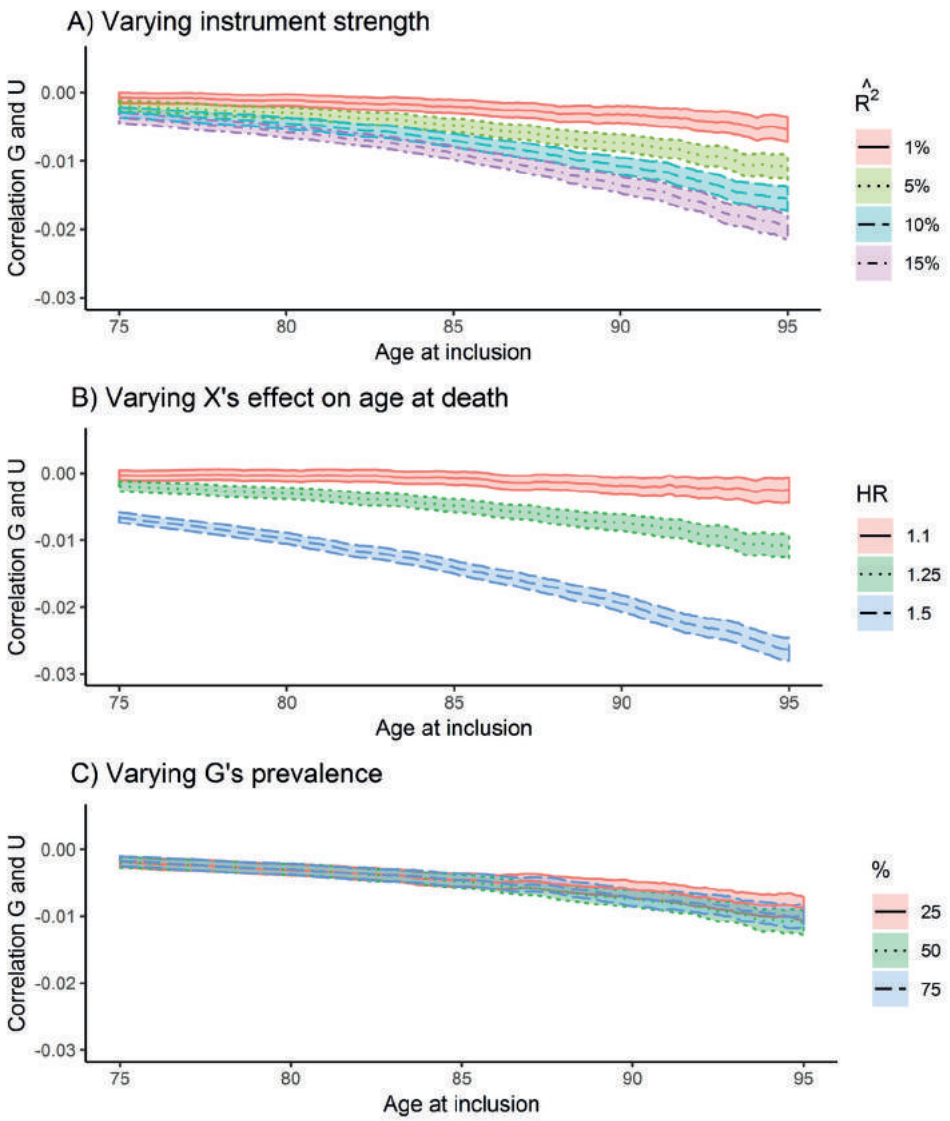
eFigure 2. Variance explained in the exposure of interest *X* by its genetic proxy *G* for an increasingly selected population, for a continuously distributed *R*. Shown in the insets are A) the prevalence of *G* and B) the accompanying survival curve, both with the true (i.e. unselected) R^2 set at 5%.



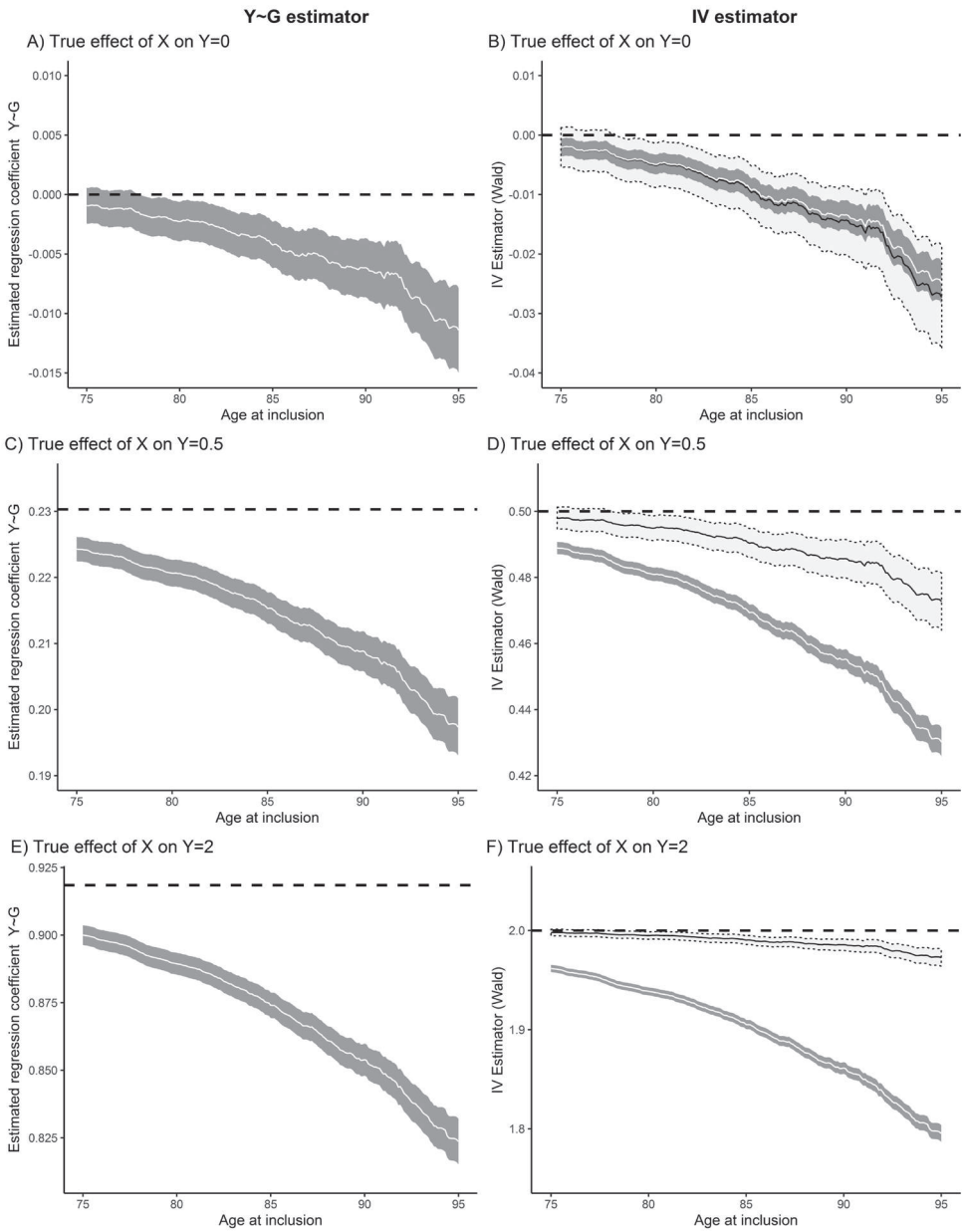
eFigure 3. Genetic instruments for exposures which affect the likelihood of surviving until study inclusion will become weaker if only due to becoming increasingly associated with the random noise in the exposure.



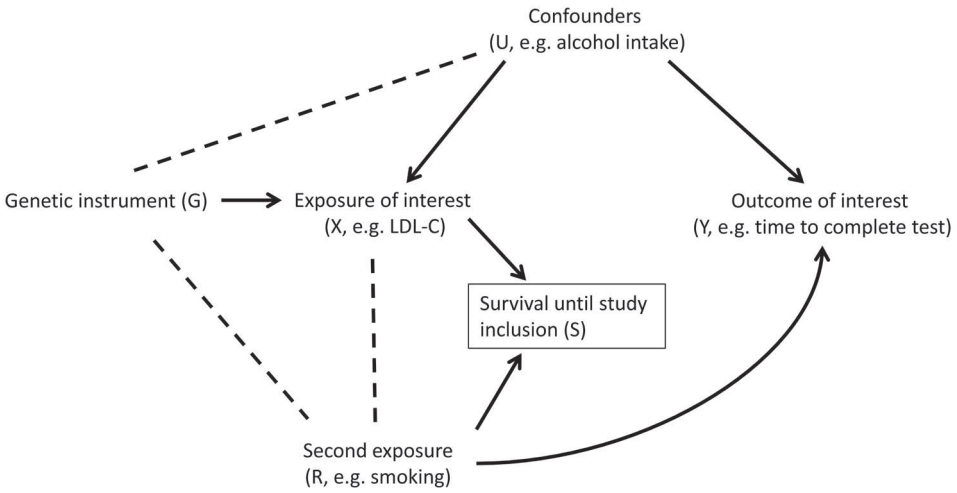
eFigure 4. Causal structure in which selection bias occurs in the presence of confounder U. Conditioning on survival S induces an assocation between genetic instrument G and confounder U. Also presented in Figure 2B.



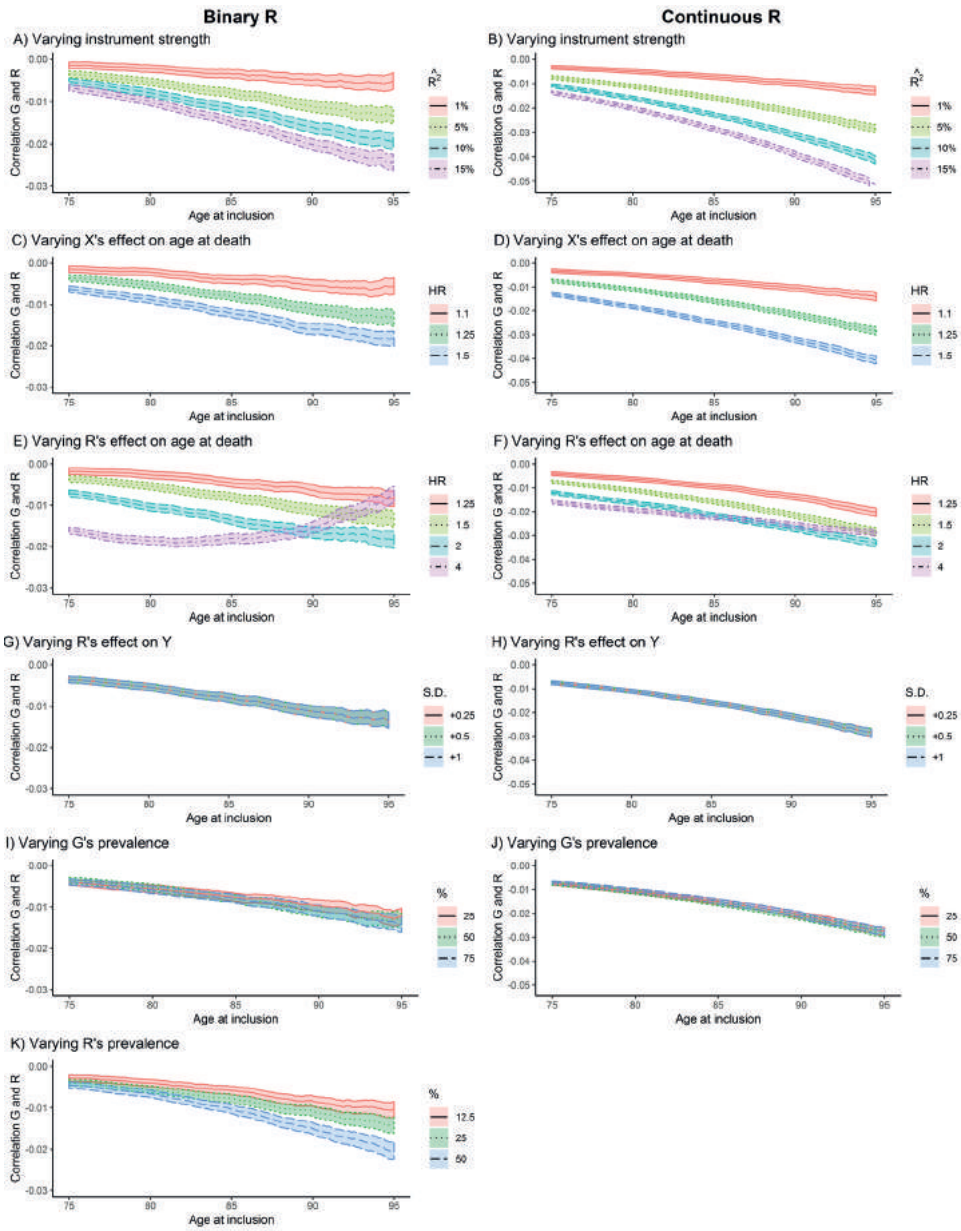
eFigure 5. Effect of varying different parameters on the induced correlation (95% CI) between the genetic instrument *G* and the confounder *U* for an increasingly selected population.



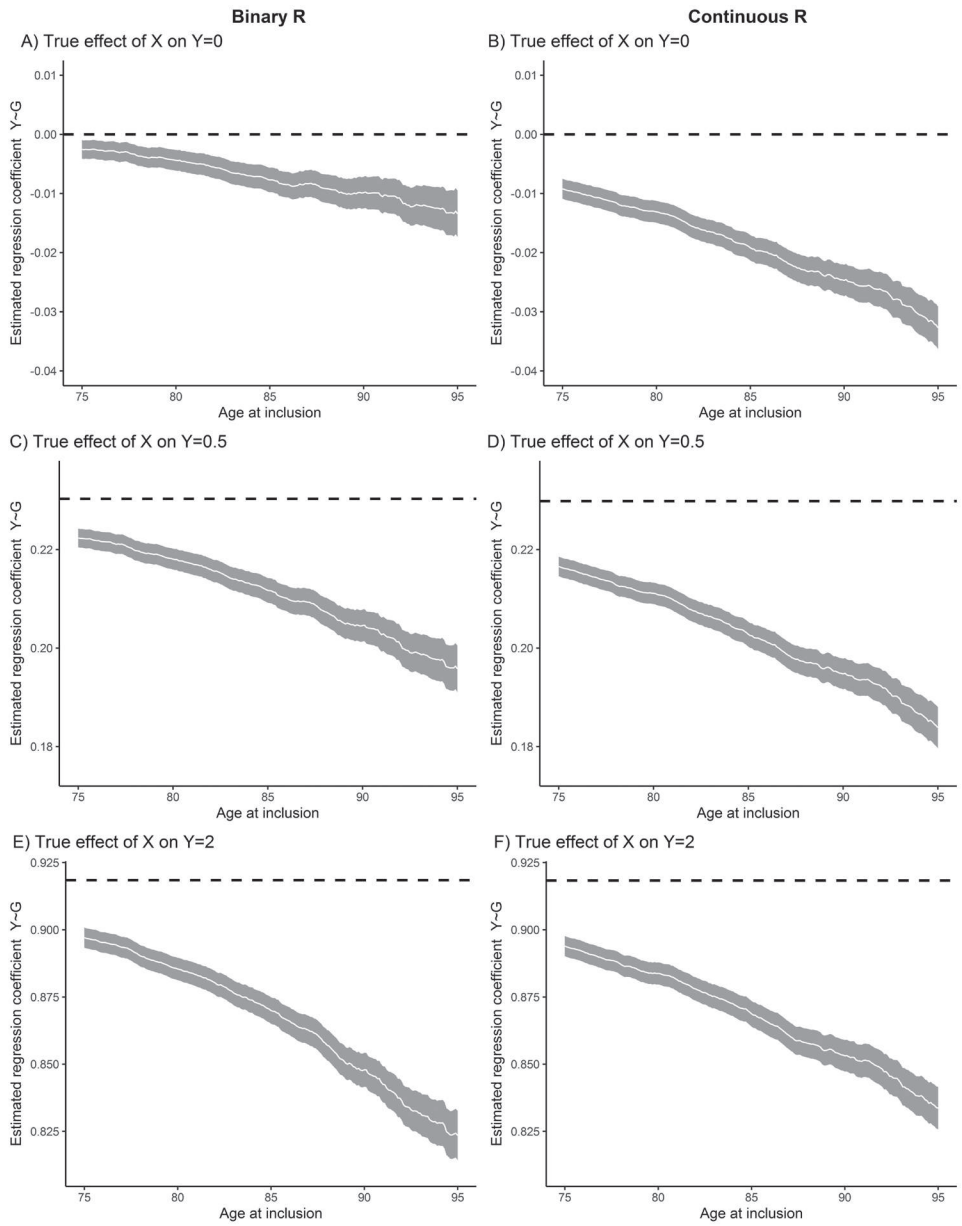
eFigure 6. Effect of survival bias on the association between the genetic instrument G and the outcome of interest *Y* (left panels), and on the Wald ratio IV-estimator (right panels), for different true effects of exposure *X* on *Y*. The true (i.e. unselected) regression coefficient for *G* on *Y*, and of true (i.e. unselected) Wald ratio, are shown as a dashed line in each plot.



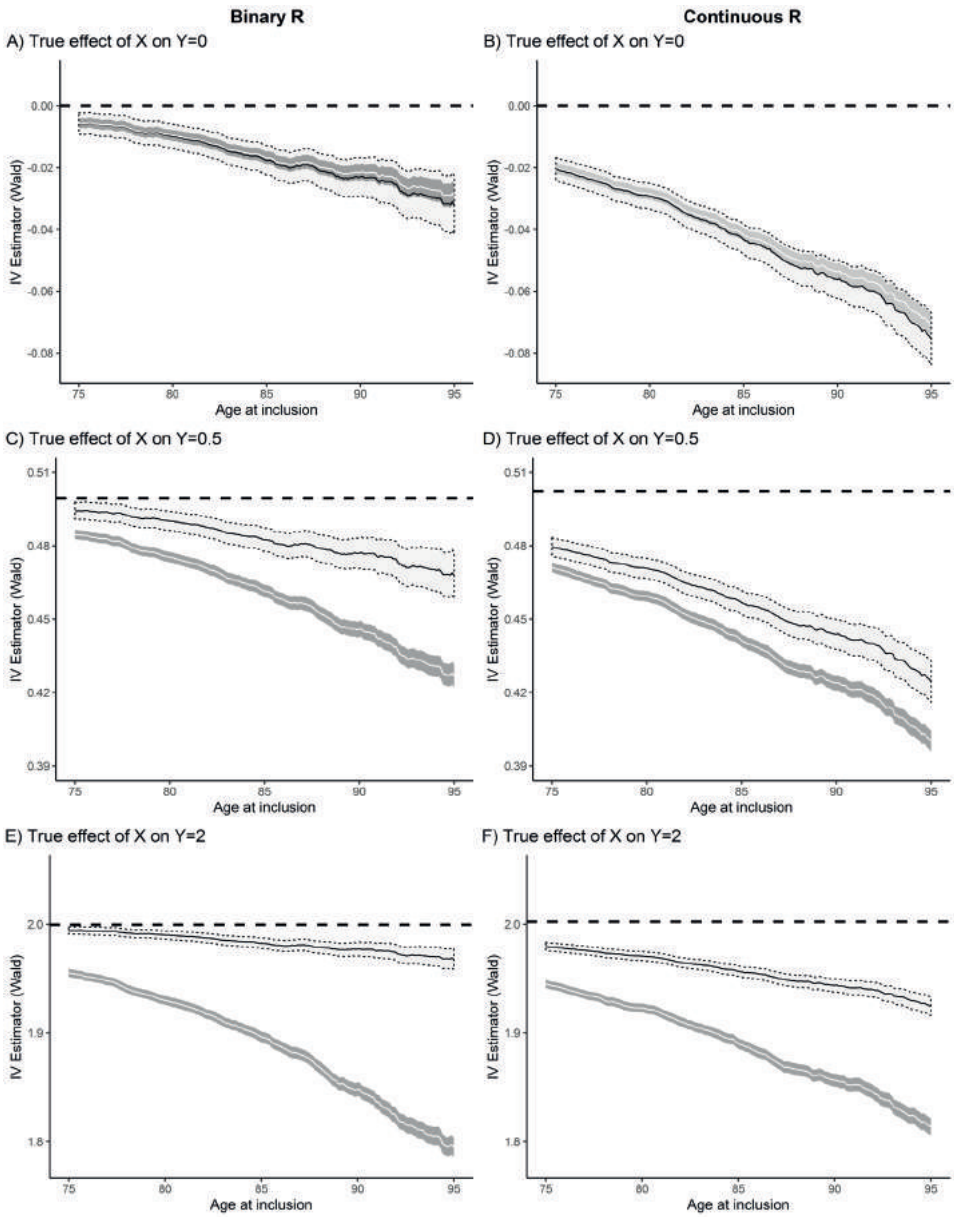
eFigure 7. Causal structure in which selection bias occurs in the context of both a second exposure R and confounder U.



eFigure 8. Effect of varying different parameters on the induced correlation (95% CI) between the genetic instrument *G* and the second exposure *R* for an increasingly selected population. Shown for binary (left column) and continuously (right column) distributed *R*. S.D. denotes standard deviation.



eFigure 9. Effect of survival bias on the association between the genetic instrument G and the outcome of interest *Y*, for different true effects of exposure *X* on *Y*. Data are presented as regression coefficients (95% CI) estimated with linear regression. The true (i.e. unselected) regression coefficient for *G* on *Y* is shown as a dashed line in each plot. Shown for binary (left column) and continuously (right column) distributed *R*.



eFigure 10. Wald ratios (95% CI) based on internally (white ribbon) versus externally (grey ribbon) estimated association between exposure *X* and the outcome *Y*, for different true effects of exposure *X* on *Y*. Shown for binary (left column) and continuously (right column) distributed *R*. Dashed lines denote the true (i.e. unselected) Wald ratio, which equals the true causal effect of *X* on *Y*.

7

PART III

Statins and visit-to-visit lipid variability

CHAPTER 8

Higher visit-to-visit low-density lipoprotein cholesterol variability is associated with lower cognitive performance, lower cerebral blood flow, and greater white matter hyperintensity load in older subjects

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ABSTRACT

Background: Recently it was shown that intra-individual variation in low-density lipoprotein cholesterol (LDL-c) predicts both cerebro- and cardiovascular events. We aimed to examine whether this extends to cognitive function, and examined possible pathways by using an MRI substudy.

Methods and results: We investigated the association between LDL-c variability and four cognitive domains at month thirty in 4428 participants of the PROspective Study of Pravastatin in the Elderly at Risk (PROSPER). Additionally, we assessed the association of LDL-c variability with neuroimaging outcomes in a subset of 535 participants. LDL-c variability was defined as the intra-individual standard deviation over four post-baseline LDL-c measurements, and all analyses were adjusted for mean LDL-c levels and cardiovascular risk factors. We observed that higher LDL-c variability was associated with lower cognitive function in both the placebo and pravastatin treatment arm. Associations were present for selective attention, processing speed, and memory. Furthermore, higher LDL-c variability was associated with lower cerebral blood flow in both trial arms, and with greater white matter hyperintensity load in the pravastatin arm. No evidence was found for interaction between LDL-c variability and pravastatin treatment for both cognitive and MRI outcomes.

Conclusions: We found that higher visit-to-visit variability in LDL-c, independent of mean LDL-c levels and statin treatment, is associated with lower cognitive performance, lower cerebral blood flow, and greater white matter hyperintensity load.

INTRODUCTION

Over eighty-five years ago, Cannon hypothesized that loss of physiological homeostasis, for instance through disease or the ageing process, would lead to disturbances in intrinsic variability (1). This intra-individual variability in various physiological measures has become of increasing interest in recent years, as both lowered heart rate variability and increased blood pressure variability have been repeatedly linked to adverse outcomes such as vascular events, impaired cognition, and mortality (2-6). However, little is known about cholesterol variability, which may be considerable even on a day-to-day basis (7, 8). Recent evidence indicates that, in subjects with coronary artery disease, greater visit-to-visit variability in low-density lipoprotein cholesterol (LDL-c) is associated with higher risks of coronary and other cardiovascular events, stroke, and mortality, independent of mean LDL-c levels (9). Whether visit-to-visit variability in LDL-c is associated with cognitive performance is currently unknown.

Here, we assessed whether visit-to-visit variability in LDL-c is associated with cognitive function, independent of mean LDL-c levels, in 4428 participants of the PROspective Study of Pravastatin in the Elderly at Risk (PROSPER). Additionally, to assess potential mechanisms behind this association, we examined the link between LDL-c variability and hippocampal volume, cerebral blood flow, and white matter hyperintensity load in an MRI substudy.

METHODS

Study population

All subjects were participants of the PROSPER study, of which the study design has been described in detail elsewhere (10). In short, this multicentre, randomized, placebo-controlled trial aimed to determine whether pravastatin reduces the risk of major cardio- and cerebrovascular events in participants aged 70-82 years with pre-existing vascular disease (coronary, cerebral, or peripheral) or at higher risk for developing vascular disease due to a history of hypertension, cigarette smoking or diabetes mellitus. To be eligible for enrolment, plasma total cholesterol was required to be 4.0-9.0 mmol/L, with triglyceride concentrations lower than 6.0 mmol/L. Participants were recruited in Scotland, Ireland, and the Netherlands. The study was approved by the institutional ethics review boards of each center, and all participants gave written informed consent. LDL-c variability and cognitive measures were available for 4428 participants. In addition, MRI measurements at end of study were available for 535 participants.

Assessment of LDL-c variability

Lipid levels were assessed after an overnight fast, and LDL-c was measured directly. Lipoprotein profiles were quantified at the Centre for Disease Control certified central lipoprotein laboratory in Glasgow. Visit-to-visit variability of LDL-c was calculated by means of the intra-individual standard deviation over each individual's measurements, using post-baseline measurements after 3, 6, 12 and 24 months. The coefficient of variation, another measure for LDL-c variability but standardized to the intra-individual mean LDL-c level over the same measurement period, was highly correlated with the intra-individual standard deviation (Pearson's $r = 0.87$). Baseline measurements were excluded to avoid including artificially induced variability from commencement of statin therapy or as an initial response to dietary and lifestyle advice given to all participants at baseline. Throughout the trial, subjects received nutritional advice and health counselling, and were stimulated to follow the National Cholesterol Education Program Step 1 diet or a local equivalent that provided <30% of total calories from fat (<10% as saturated fat) and a cholesterol intake of <300 mg/day.

Assessment of cognition

Subjects with poor cognitive function (Mini Mental State Examination (MMSE) score < 24) were excluded from enrolment in the main PROSPER study. Serving as outcome variables, cognitive function was evaluated through four cognitive measures (11). The Stroop-Colour-Word-Test (Stroop) was employed to test selective attention, with total number of seconds needed to complete the third test part used as the outcome parameter. The Letter-Digit Coding Test (LDT) assessed information processing speed, taking the number of correct digits filled in within 60 seconds, with higher scores denoting better performance. The Picture-Word Learning Test (PLT) was used as a verbal memory test, separately assessing immediate (number of recalled pictures over three learning trials) and delayed recall after twenty minutes, with higher scores denoting better performance. All cognitive outcomes were assessed at month thirty to maximize the availability of cognitive outcomes following the measurement of LDL-c variability.

Magnetic Resonance Imaging Substudy

Of the eligible Dutch participants of the main PROSPER study, 646 consented to participate in a nested MRI substudy, of which the methods and results have been published previously (12). Subjects with intraorbital vascular clips,

collagen disease, cardiac pacemakers, hearing implants, multiple sclerosis, or claustrophobia were excluded from participating. In the current study, we examined results from imaging performed after a mean±SD follow-up of 33±1.4 months. Data on visit-to-visit LDL-c variability and MRI outcomes were available for 535 participants.

A clinical MR-system operating at a field strength of 1.5 Tesla was employed for all imaging (Philips Medical Center, Best, the Netherlands). The Oxford Centre for Functional MRI of the Brain's integrated registration and segmentation tool (FIRST) was utilized to estimate the hippocampal volume (13). Using the phase contrast technique, cerebral blood flow was calculated by adding the flow from the left and right internal carotid arteries to the flow in both vertebral arteries, and was subsequently standardized to whole-brain parenchymal volume (12). Quantification of white matter hyperintensity load was performed using Software for Neuro-Image Processing in Experimental Research (SNIPER), an in-house developed fully automatic segmentation method combining information from proton density, T2-weighted and fluid-attenuated inversion recovery (FLAIR) images (14).

Demographic and clinical characteristics

Participant characteristics were assessed at baseline. These included age, education (age of leaving school), body-mass index, current smoking status (yes/no), alcohol intake (measured in units per week), and history of various clinical diseases.

Statistical analyses

All analyses were conducted separately for the placebo and pravastatin arm. Demographic and clinical characteristics are presented as numbers with percentage, means with standard deviations, or medians with interquartile range when appropriate. Participant characteristics were compared over tertiles of LDL-c variability using analysis of variance and Pearson's chi-square test. Using multivariable linear regression models, the association between post-baseline LDL-c variability and cognitive performance at month 30, and MRI measures at end of study, was determined. Subjects with a minimum of two out of four LDL-c measurements were included. While reporting mean (SE) cognitive scores and MRI measures over tertiles of LDL-c variability to gain insight into the underlying distribution of neurocognitive function, intra-individual variability was used as a continuous covariate in the linear regression models. Adjusted unstandardized

regression coefficients, 95% confidence intervals, and p-values were reported. Covariate adjustments were made based on their biological plausibility as potential confounders for the association between LDL-c variability and neurocognitive outcomes. These covariates consisted of diseases and factors that are known to influence LDL-c levels, and have been linked to either cognitive or neurovascular impairment. For the minimally adjusted model we included age, gender, country, education, average LDL-c, and cognitive test version and whole-brain parenchymal volume where appropriate. The fully adjusted model additionally included body-mass index, current smoking status, alcohol intake, and history of diabetes, hypertension, and vascular disease. Data on these baseline covariates was complete for all participants. Possible violations of the assumptions of multiple linear regression were examined by visually inspecting the distribution of residuals through both histograms and normal P-P plots. We further checked for deviations of linearity and homoscedasticity by visually inspecting scatterplots of standardized residuals by standardized predicted values. In addition, we assessed Variance Inflation Factors to examine the possibility of multicollinearity. We considered p-values of 0.05 or smaller statistically significant. All analyses were conducted using IBM SPSS Statistics version 20.0.

Sensitivity analyses

Several sensitivity analyses were conducted in order to measure how robust the findings were to different subsets of the data, and to elucidate possible mechanisms through which LDL-c variability might associate with cognitive function. First, the association with cognitive performance at end of study was assessed, rather than cognition at month thirty, whilst using the same exposure measurement period. On average, this meant cognitive performance was assessed 9 months later. A further consideration was possible influence of the number of lipid measurements. Therefore, we restricted our analyses to those participants with all four measurements. We additionally performed separate analyses excluding history of, and incident events of, cerebro- and cardiovascular disease. As both cancer and serious infection may influence levels of LDL-c, we also carried out analyses excluding these incident disease states. Furthermore, blood pressure variability has been shown to associate with cognition in recent years (4). As variability in LDL-c and blood pressure could arise from a common cause, we adjusted for systolic blood pressure (SBP) variability to distinguish effects of LDL-c variability from those mediated by blood pressure variability. SBP variability was defined as the intra-individual standard deviation over months 3-24, with blood pressure measured every three months, and these

analyses were additionally adjusted for mean SBP over the same measurement period. Further, it is possible that LDL-c variability reflects consistent trends over time rather than an undulating pattern, e.g. due to progressively reduced dietary intake in the context of overall decline in health status. Therefore, we carried out analyses whilst adjusting for the average slope of LDL-c during the measurement period. Finally, as concomitant medication usage may underlie differences in lipid variability, we performed analyses adjusting for baseline medication usage of diuretics, ACE I- or II-inhibitors, beta-blockers, calcium channel blockers, nitrates, anticoagulants, anti-arrhythmic medication, and glucose-lowering medication (insulin and non-insulin separately). For all sensitivity analyses, we report the results from the fully adjusted model only, which were similar to those seen for the minimally adjusted model.

RESULTS

Demographic and clinical characteristics

Participant characteristics are described in **Table 1**. In both the placebo and pravastatin arms, participants in higher tertiles of visit-to-visit LDL-c variability had a higher SBP variability (p=0.003, p=0.006, respectively), higher average LDL-c (both p<0.001), were more often female (p=0.001, p=0.002), and less likely to be Dutch rather than Scottish or Irish when compared to the other tertiles (p=0.047, p=0.014). However, the difference in the proportion of females and males disappeared after standardizing variability to the intra-individual mean LDL-c, by means of the coefficient of variation, in both trial arms (p=0.67, p=0.23, respectively). As shown in **Supplemental Table 1**, the participants of the MRI substudy were largely representative of the Dutch participants.

Effect of pravastatin on LDL-c

Statin therapy was associated with a reduction of both average LDL-c (-1.18 mmol/L, 95% CI: -1.14 to -1.22) and mean visit-to-visit LDL-c variability (-0.02 mmol/L, 95% CI: -0.01 to -0.04), as measured by the intra-individual standard deviation.

Association between LDL-c variability and cognitive performance

In both the placebo and pravastatin group, higher LDL-c variability was significantly associated with lower cognitive test scores (**Table 2**). While most consistent for the memory measures

Table 1. Baseline characteristics over tertiles of LDL-c variability

	Placebo (n=2226)			
	Lowest tertile n=742*	Middle tertile n=742†	Highest tertile n=742‡	p-value
Continuous variables (mean ± SD)				
Age (years)	75.3 ± 3.4	75.1 ± 3.2	74.9 ± 3.3	0.10
Education (age left school, years)	15.3 ± 2.3	15.0 ± 1.9	15.2 ± 2.0	0.15
Alcohol intake (units/month)	5.4 ± 8.7	5.1 ± 8.7	5.2 ± 9.6	0.79
Body mass index (kg/m^2)	26.9 ± 4.2	27.0 ± 4.4	27.1 ± 4.1	0.79
Mean SBP (mmHg)**	153.9 ± 16.0	153.4 ± 17.2	153.8 ± 16.1	0.82
SBP variability (mmHg)**	13.8 ± 5.0	14.2 ± 5.4	14.8 ± 14.8	**0.003**
Mean LDL cholesterol (mmol/L)**	3.5 ± 0.7	3.7 ± 0.7	3.9 ± 0.8	**<0.001**
Categorical variables (n, %)				
Female	350 (47.2)	368 (49.6)	420 (56.6)	**0.001**
History of hypertension	462 (62.3)	458 (61.7)	464 (62.5)	0.95
History of diabetes mellitus	88 (11.9)	84 (11.3)	70 (9.4)	0.29
History of stroke or TIA	87 (11.7)	82 (11.1)	72 (9.7)	0.44
History of myocardial infarction	101 (13.6)	104 (14.0)	91 (12.3)	0.58
History of vascular disease	320 (43.1)	301 (40.6)	330 (44.5)	0.30
Current smoker	190 (25.6)	187 (25.2)	189 (25.5)	0.98
Country of origin (n, %)				
Scotland	296 (39.9)	300 (40.4)	301 (40.6)	**0.047**
Ireland	261 (35.2)	288 (38.8)	301 (40.6)	
The Netherlands	185 (24.9)	154 (20.8)	140 (18.9)	

P-values calculated using analysis of variance and Pearson's chi-square test when appropriate. LDL-c denotes low-density lipoprotein cholesterol; SBP, systolic blood pressure; TIA, transient ischemic attack.
LDL-c variability ranges (mmol/L): *0.02-0.22, †0.22-0.35, ‡0.35-1.71, §0.00-0.18, ||0.18-0.30, #0.30-1.56; ** calculated over months 3 to 24, similar to LDL-c variability.

Pravastatin (n=2202)			
Lowest tertile n=734§	Middle tertile n=735‖	Highest tertile n=733#	p-value
75.4 ± 3.3	75.0 ± 3.4	75.1 ± 3.2	0.13
15.3 ± 2.2	15.3 ± 2.3	15.2 ± 2.1	0.76
4.8 ± 8.4	5.5 ± 8.4	5.9 ± 11.1	0.07
26.8 ± 1.8	26.8 ± 3.9	26.9 ± 4.0	0.89
153.0 ± 16.4	153.7 ± 17.4	153.7 ± 16.4	0.62
13.7 ± 5.3	14.0 ± 5.2	14.6 ± 5.5	**0.006**
2.3 ± 0.5	2.5 ± 0.6	2.8 ± 0.7	**<0.001**
364 (49.6)	360 (49.0)	419 (57.2)	**0.002**
480 (65.4)	450 (61.2)	467 (63.7)	0.25
83 (11.3)	76 (10.3)	58 (7.9)	0.08
86 (11.7)	78 (10.6)	72 (9.8)	0.50
84 (11.4)	101 (13.7)	91 (12.4)	0.41
310 (42.2)	342 (46.5)	323 (44.1)	0.25
172 (23.4)	170 (23.1)	192 (26.2)	0.32
287 (39.1)	296 (40.3)	311 (42.4)	**0.014**
259 (35.3)	278 (37.8)	290 (39.6)	
188 (25.6)	161 (21.9)	132 (18.0)	

Table 2. Cognitive function, at month thirty, over tertiles of LDL-c variability

		Lowest tertile	Middle tertile
Placebo (n=2226)			
Stroop card III, seconds needed	Model 1	62.25 (0.91)	65.16 (0.93)
	Model 2	65.06 (1.21)	68.00 (1.23)
LDT, digits coded correctly	Model 1	23.67 (0.24)	22.93 (0.24)
	Model 2	23.05 (0.32)	22.29 (0.32)
PLTi, pictures remembered	Model 1	9.72 (0.07)	9.51 (0.07)
	Model 2	9.60 (0.10)	9.38 (0.10)
PLTd, pictures remembered	Model 1	10.56 (0.10)	10.24 (0.10)
	Model 2	10.36 (0.13)	10.03 (0.13)
Pravastatin (n=2202)			
Stroop card III, seconds needed	Model 1	62.39 (0.90)	61.77 (0.88)
	Model 2	65.17 (1.21)	64.70 (1.20)
LDT, digits coded correctly	Model 1	23.35 (0.25)	23.80 (0.25)
	Model 2	22.41 (0.34)	22.81 (0.34)
PLTi, pictures remembered	Model 1	9.66 (0.07)	9.65 (0.07)
	Model 2	9.38 (0.10)	9.36 (0.09)
PLTd, pictures remembered	Model 1	10.54 (0.10)	10.42 (0.10)
	Model 2	10.14 (0.14)	10.00 (0.14)

Data are presented as mean cognitive test scores (SE). The adjusted unstandardized regression coefficient and p-value for trend were calculated using LDL-c variability (mmol/L) as a continuous measure.
LDT denotes Letter-Digit Coding test; PLTi, 15-Picture Learning test immediate; PLTd, 15-Picture Learning test delayed.
Model 1: adjusted for age, gender, country, education, mean LDL cholesterol, and test version where appropriate.
Model 2: as model 1, additionally for BMI, smoking status, alcohol intake, history of diabetes mellitus, hypertension, and vascular disease.

Highest tertile	Beta (95% CI)	p-value
65.12 (0.94)	6.24 (0.92, 11.56)	**0.021**
68.00 (1.23)	6.44 (1.13, 11.75)	**0.017**
22.99 (0.25)	-0.92 (-2.32, 0.48)	0.196
22.39 (0.32)	-0.91 (-2.30, 0.49)	0.204
9.48 (0.07)	-0.68 (-1.09, -0.27)	**0.001**
9.36 (0.10)	-0.66 (-1.07, -0.25)	**0.002**
10.16 (0.10)	-1.02 (-1.60, -0.44)	**0.001**
9.97 (0.13)	-1.00 (-1.58, -0.42)	**0.001**
64.66 (0.94)	3.94 (-0.88, 8.75)	0.109
67.54 (1.23)	3.89 (-0.92. 8.70)	0.113
22.69 (0.27)	-1.51 (-2.86, -0.15)	**0.030**
21.72 (0.34)	-1.51 (-2.86, -0.15)	**0.029**
9.37 (0.08)	-0.56 (-0.95, -0.16)	**0.006**
9.08 (0.10)	-0.55 (-0.94, -0.15)	**0.006**
9.87 (0.11)	-1.22 (-1.78, -0.66)	**<0.001**
9.46 (0.14)	-1.20 (-1.76, -0.64)	**<0.001**

(immediate recall: p=0.002, p=0.006; delayed recall: p=0.001, p=<0.001), statistically significant associations were also seen for Stroop (p=0.017, p=0.11) and LDT (p=0.20, p=0.029) test scores. These fully adjusted associations were essentially unchanged from those seen for the minimally adjusted model. We found no evidence for interaction between LDL-c variability and pravastatin treatment, for all cognitive outcomes (**Supplemental table 2**).

Sensitivity analyses for cognitive outcomes

As shown in **Figure 1,** the associations between LDL-c variability and cognitive performance were essentially unchanged by restricting the analyses to different subsets, c.q. adjusting for various possible common causes of LDL-c variability and cognitive performance, in both trial arms.

Association between LDL-c variability and MRI measures

We found no evidence for an association between LDL-c variability and hippocampal volume (p=0.779, p=0.864, respectively). However, higher LDL-c variability was associated with lower total cerebral blood blow in the fully adjusted model (**Table 3**), in both placebo and pravastatin group **(**p=0.031, p=0.050, respectively). Furthermore, higher LDL-c variability was associated with greater white matter hyperintensity load in the pravastatin group (p=0.046), but this association did not reach statistical significance in the placebo group (p=0.184). Additionally, no interaction was observed between LDL-c variability and pravastatin treatment, for all MRI measures (**Supplemental table 3**). Further adjustments for whole-brain, or grey-matter specific, atrophy did not markedly change any of the results (data not shown).

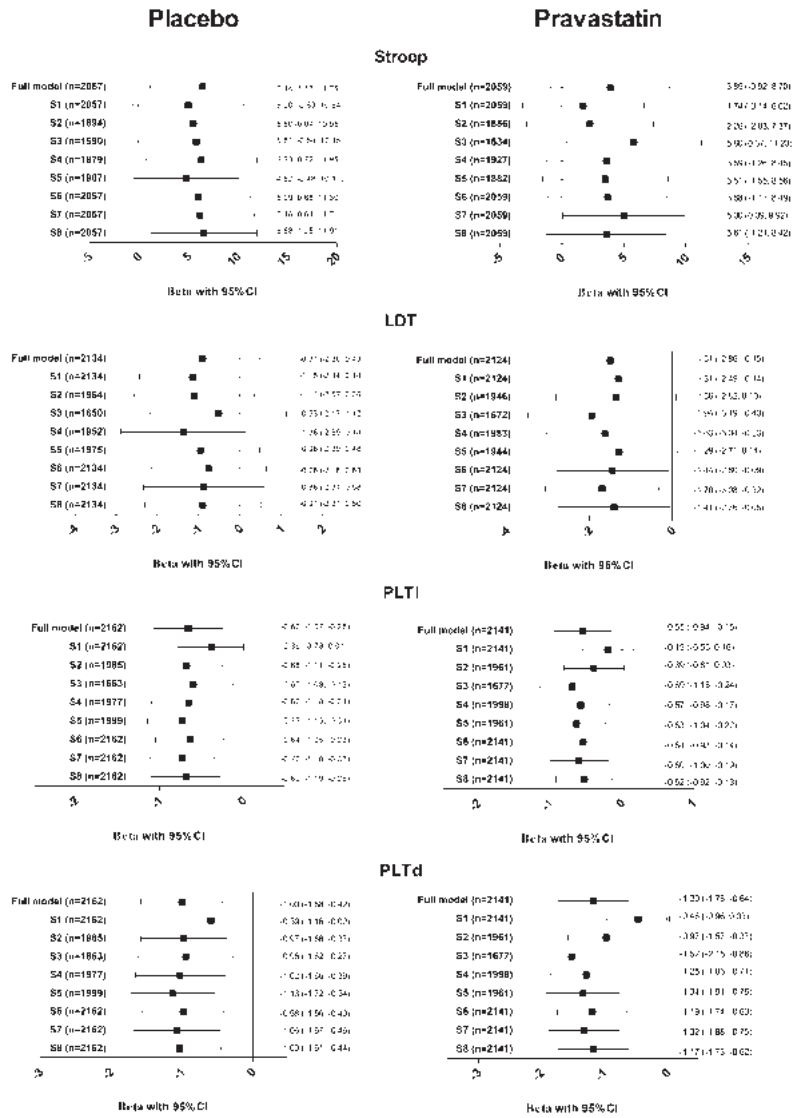

Figure 1. Sensitivity analyses of the association between LDL-c variability and cognitive performance. Consecutively listed, these are: (S1) assessing cognition at end of study, (S2) only subjects with four (complete) LDL-c measurements, (S3) excluding history of cerebro- and cardiovascular disease, (S4) excluding incident cerebro- and cardiovascular disease, (S5) excluding incident serious infection and cancer, (S6) adjusting for visit-to-visit systolic BP variability, (S7) adjusting for mean LDL-c slope during measurement period, (S8) adjusting for concomitant baseline medication usage. Results are presented as adjusted unstandardized regression coefficients with 95% confidence intervals. LDT denotes Letter-Digit Coding test; PLTi, 15-Picture Learning test immediate; PLTd, 15-Picture Learning test delayed.

Table 3. MRI measures, at end of study, over tertiles of LDL-c variability

		Lowest tertile	Middle tertile
Placebo (n=269)		n=89*	n=90†
Hippocampal volume (ml)	Model 1	9.21 (0.13)	9.26 (0.13)
	Model 2	9.14 (0.15)	9.08 (0.16)
Cerebral blood flow (ml/min/100 ml)	Model 1	48.09 (1.10)	48.43 (1.14)
	Model 2	46.95 (1.33)	47.21 (1.44)
WMHL (ml)	Model 1	7.79 (1.35)	7.06 (1.34)
	Model 2	7.76 (1.62)	7.10 (1.71)
Pravastatin (n=266)		n=88§	n=89‖
Hippocampal volume (ml)	Model 1	9.31 (0.13)	9.34 (0.12)
	Model 2	9.17 (0.17)	9.18 (0.16)
Cerebral blood flow (ml/min/100 ml)	Model 1	48.19 (1.05)	49.17 (1.02)
	Model 2	48.80 (1.38)	49.89 (1.46)
WMHL (ml)	Model 1	5.21 (1.30)	7.54 (1.22)
	Model 2	4.50 (1.69)	6.88 (1.71)

Data are presented as mean MRI measure (SE). The adjusted unstandardized regression coefficient and p-value for trend were calculated using LDL-c variability (mmol/L) as a continuous measure.
WMHL denotes white matter hyperintensity load.
Model 1: adjusted for age, gender, education, mean LDL cholesterol, and whole-brain parenchymal volume.
Model 2: as model 1, additionally for BMI, smoking status, alcohol intake, history of diabetes mellitus, hypertension, and vascular disease.
LDL-c variability ranges (mmol/L): *0.05-0.20, †0.20-0.32, ‡0.32-1.18, §0.03-0.16, ‖0.16-0.25, #0.25-1.52

Highest tertile	Beta (95% CI)	p-value
n=90‡		
9.15 (0.13)	0.19 (-0.57, 0.95)	0.622
9.07 (0.16)	0.11 (-0.66, 0.88)	0.779
45.30 (1.14)	-6.39 (-13.13, 0.34)	0.063
44.12 (1.40)	-7.66 (-14.61, -0.70)	**0.031**
8.56 (1.33)	4.74 (-3.33, 12.80)	0.249
8.75 (1.66)	5.69 (-2.72, 14.09)	0.184
n=89#		
9.36 (0.11)	-0.19 (-0.83, 0.45)	0.557
9.27 (0.15)	-0.06 (-0.72, 0.61)	0.864
46.93 (1.09)	-6.17 (-12.78, 0.44)	0.067
47.33 (1.40)	-6.82 (-13.63, -0.01)	**0.050**
8.47 (1.26)	5.62 (-1.50, 12.75)	0.121
8.43 (1.62)	7.42 (0.15, 14.69)	**0.046**

Multiple linear regression assumptions

We found no evidence of non-normality, curvilinearity, heteroscedasticity, or multicollinearity in any of our models. This held true for all cognitive tests and magnetic-resonance imaging outcomes.

DISCUSSION

We found that higher visit-to-visit variability in LDL-c is robustly associated with lower cognitive performance, independent of mean LDL-c levels. While most consistent for both immediate and delayed memory-related outcomes, similar trends were present for selective attention and processing speed. In addition, we observed that higher variability is associated with lower cerebral blood flow and greater white matter hyperintensity load within an MRI substudy. All associations were independent of clinically overt cerebro- and cardiovascular disease and comorbidities. Of particular importance is that these associations were present within both placebo and pravastatin treatment arm, with no evidence for interaction by pravastatin treatment. This advocates against increased LDL-c variability purely reflecting the known beneficial and harmful pleiotropic effects of statins, or behavioral factors which may undermine response to lipid lowering treatment, most notably non-adherence. Nonetheless, our findings that higher LDL-c variability associates with lower neurocognitive function highlight the need for further investigations into the potential influence of lipid-lowering treatment on LDL-c variability and consequent adverse events. While it should be noted that these events are uncommon, and the adverse event reporting not part of a systematic evaluation of neurocognitive function, currently available trial evidence suggests that neurocognitive adverse events may occur more frequently in individuals receiving proprotein convertase subtilisin-kexin type 9 (PCSK9) inhibitors, independent of on-treatment LDL levels (15). At the same time, high-dose monthly regimens of PCSK9 monoclonal antibodies are known to produce substantial fluctuations of LDL-c between doses (16). Based on our results, this increased variability could possibly contribute to the observed higher rate of neurocognitive events, and should be examined by currently ongoing PCSK9 trials. To our knowledge, this is the first study examining the association between lipid variability and cognitive performance, and provides further evidence that lipid variability could be of clinical significance. The implications of our findings are thus in line with those from the recently published results from the Treating to New Targets trial (9), but extending these to cognitive and neuroimaging outcomes.

Major strengths of the current study are its size, with over 4400 participants providing data on lipid variability and cognitive performance, and the opportunity to perform these analyses both in the presence, and absence, of lipid-lowering therapy. Moreover, due to the exclusion of participants with MMSE scores lower than 24 we were able to examine a fairly homogenous population with regard to cognitive function. A limitation of the current study is the observational nature of the data, due to which we are unable to infer causal relationships. Furthermore, our ability to look at cognitive performance at different time points and perform longitudinal analyses was limited by the number of, and varying time intervals between, post-baseline LDL-c measurements. In addition, we included a limited neurocognitive test battery, which did not provide information on various important cognitive domains such as visual-constructive function or language. A further possible limitation could be that we did not adjust for multiple testing. However, we did not consider our analyses to be hypothesis-free, as we included neurocognitive tests specifically known to be affected by neurovascular impairment, which are additionally known to be correlated. Applying multiple comparison methods like Bonferroni in this case would therefore yield too conservative results. Finally, though lipid levels were measured after an overnight fast, we did not have data on the exact nature and timing of last consumed meal. While this might have influenced our results, it is very likely that any potential dietary effect would be random in nature.

There are several explanations for our findings, which roughly fall within two categories. First, it is possible that LDL-c variability is causally related to cognition function. Histological studies have demonstrated that lipid-lowering treatments such as statins may lower the lipid content of human carotid plaques (17), with recent animal studies suggesting that complete atherosclerotic regression of early lesions is possible through the lowering of lipid levels (18). As such, varying levels of LDL-c could theoretically lead to fluctuations in the composition of atherosclerotic plaques, possibly inducing plaque instability and thereby increasing the risk of (sub)clinical cerebrovascular damage (19). Another pathway might be through endothelial dysfunction, which can be caused by many of the risk factors that predispose to atherosclerosis (20). As individuals with elevated serum markers of endothelial dysfunction are at higher risk for developing cognitive impairment (21), possibly through changes in cerebral blood flow (22, 23), increased LDL-c variability might lead to cognitive impairment. In line with this hypothesis, we observed that higher LDL-c variability associated with lower cerebral blood flow, but also with greater white matter hyperintensity load, which has been linked to endothelial (dys)function (24).

Explanations within the second category dismiss a causal role for LDL-c variability. Here, visit-to-visit variability would rather reflect other processes leading to cognitive dysfunction. For example, despite excluding participants with a diagnosis of cancer or serious infection from the analyses in a sensitivity analysis, undetected subclinical disease might have led both to increased lipid variability and cognitive impairment. This also holds true for liver disease, though participants with clinically significant liver damage were explicitly excluded from enrolling in the trial. Exploratory analyses with inflammatory markers (fibrinogen, IL-6, IL-10, CRP) measured at baseline did not reveal evidence of an association with LDL-c variability (all p-values > 0.1, data not shown). Furthermore, numerous drugs may have unintended effects on lipid levels (25). While adjusting for baseline medication usage did not materially change our findings, exact timing of new drug commencement, although known to be few, was unfortunately not available within our study, and it was therefore not possible to take this into account. The observation that the associations were independent of blood pressure variability might imply that loss of homeostatic function does not underlie our current findings. However, more likely, it may signify that the different regulatory systems involved in homeostasis may be affected through different pathological pathways. Finally, due to the cross-sectional design of our analyses we cannot rule out that subclinical cerebrovascular damage, for which cognitive dysfunction may be a marker, leads to increased LDL-c variability.

In conclusion, we showed for the first time that in older participants at risk for vascular disease, higher visit-to-visit LDL-c variability is associated with lower cognitive performance, lower cerebral blood flow and greater white matter hyperintensity load. Our findings underscore the potential of LDL-c variability being a useful prognostic marker for different clinical outcomes. Future replication studies are needed to corroborate these findings, and should ideally also employ longitudinal assessments of neuroimaging to further elucidate the possible relationship between LDL-c variability, cerebral blood flow, and white matter hyperintensities.

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REFERENCE LIST

1 Cannon WB. Organization for physiological homeostasis. Physiol Rev. 1929;9:399-431.

2 Tai C, Sun Y, Dai N, et al. Prognostic significance of visit-to-visit systolic blood pressure variability: a meta-analysis of 77,299 patients. J Clin Hypertens (Greenwich). 2015;17:107-15.

3 Rothwell PM, Howard SC, Dolan E, et al. Prognostic significance of visit-to-visit variability, maximum systolic blood pressure, and episodic hypertension. Lancet. 2010;375:895-905.

4 Sabayan B, Wijsman LW, Foster-Dingley JC, et al. Association of visit-to-visit variability in blood pressure with cognitive function in old age: prospective cohort study. BMJ. 2013;347:f4600.

5 Kim DH, Lipsitz LA, Ferrucci L, et al. Association between reduced heart rate variability and cognitive impairment in older disabled women in the community: Women's Health and Aging Study I. J Am Geriatr Soc. 2006;54:1751-7.

6 Tsuji H, Larson MG, Venditti FJ Jr, et al. Impact of Reduced Heart Rate Variability on Risk for Cardiac Events: The Framingham Heart Study. Circulation. 1996;94:2850-5.

7 Cooper GR, Myers GL, Smith SJ, et al. Blood lipid measurements. Variations and practical utility. JAMA. 1992;267:1652-60.

8 Smith SJ, Cooper GR, Myers GL, et al. Biological variability in concentrations of serum lipids: sources of variation among results from published studies and composite predicted values. Clin Chem. 1993;39:1012-22.

9 Bangalore S, Breazna A, DeMicco DA, et al. Visit-to-visit low-density lipoprotein cholesterol variability and risk of cardiovascular outcomes: insights from the TNT trial. J Am Coll Cardiol. 2015;65:1539-48.

10 Shepherd J, Blauw GJ, Murphy MB, et al. The design of a prospective study of Pravastatin in the Elderly at Risk (PROSPER). PROSPER Study Group. PROspective Study of Pravastatin in the Elderly at Risk. Am J Cardiol. 1999;84:1192-7.

11 Houx PJ, Shepherd J, Blauw GJ, et al. Testing cognitive function in elderly populations: the PROSPER study. PROspective Study of Pravastatin in the Elderly at Risk. J Neurol Neurosurg Psychiatry. 2002;73:385-9.

12 ten Dam VH, Box FM, de Craen AJ, et al. Lack of effect of pravastatin on cerebral blood flow or parenchymal volume loss in elderly at risk for vascular disease. Stroke. 2005;36:1633-6.

13 Patenaude B, Smith SM, Kennedy DN, et al. A Bayesian model of shape and appearance for subcortical brain segmentation. Neuroimage. 2011;56:907-22.

14 Admiraal-Behloul F, van den Heuvel DM, Olofsen H, et al. Fully automatic segmentation of white matter hyperintensities in MR images of the elderly. Neuroimage. 2005;28:607-17.

15 Swiger KJ, Martin SS. PCSK9 inhibitors and neurocognitive adverse events: exploring the FDA directive and a proposal for N-of-1 trials. Drug Saf. 2015;38:519-26.

16 Ballantyne CM, Neutel J, Cropp A, et al. Results of bococizumab, a monoclonal antibody against proprotein convertase subtilisin/kexin type 9, from a randomized,

placebo-controlled, dose-ranging study in statin-treated subjects with hypercholesterolemia. Am J Cardiol. 2015;115:1212-21.

17 Crisby M, Nordin-Fredriksson G, Shah PK, et al. Pravastatin treatment increases collagen content and decreases lipid content, inflammation, metalloproteinases, and cell death in human carotid plaques: implications for plaque stabilization. Circulation. 2001;103:926-33.

18 Björkegren JL, Hägg S, Talukdar HA, et al. Plasma cholesterol-induced lesion networks activated before regression of early, mature, and advanced atherosclerosis. PLoS Genet. 2014;10:e1004201.

19 Chen Z, Ichetovkin M, Kurtz M, et al. Cholesterol in human atherosclerotic plaque is a marker for underlying disease state and plaque vulnerability. Lipids Health Dis. 2010;9:61.

20 Davignon J, Ganz P. Role of endothelial dysfunction in atherosclerosis. Circulation. 2004;109:III27-32.

21 Quinn TJ, Gallacher J, Deary IJ, et al. Association between circulating hemostatic measures and dementia or cognitive impairment: systematic review and meta-analyzes. J Thromb Haemost. 2011;9:1475-82.

22 de la Torre JC. Is Alzheimer's disease a neurodegenerative or a vascular disorder? Data, dogma, and dialectics. Lancet Neurol. 2004;3:184-90.

23 Sabayan B, Westendorp RG, van der Grond J, et al. Markers of endothelial dysfunction and cerebral blood flow in older adults. Neurobiol Aging. 2014;35:373-7.

24 Hoth KF, Tate DF, Poppas A, et al. Endothelial function and white matter hyperintensities in older adults with cardiovascular disease. Stroke. 2007;38:308-12.

25 Mantel-Teeuwisse AK, Kloosterman JME, Maitland-van der Zee AH, et al. Drug-Induced lipid changes - a review of the unintended effects of some commonly used drugs on serum lipid levels. Drug Saf. 2001;24:443-56.

Supplemental table 1. Characteristics of study participants included in the whole group, Dutch subsample, and magnetic resonance imaging (MRI) substudy.

	Overall cohort (n=4428)	Dutch subsample (n=960)	MRI substudy (n=535)
Continuous variables (mean ± SD)			
Age (years)	75.2 (3.3)	75.1 (3.3)	75.0 (3.2)
Education (age left school, years)	15.2 (2.1)	15.4 (2.9)	15.5 (2.9)
Alcohol intake (units/month)	5.3 (9.2)	6.9 (8.3)	6.7 (8.2)
Body mass index (kg/m^2)	26.9 (4.1)	26.8 (3.8)	26.7 (3.7)
Mean SBP (mmHg)*	153.6 (16.6)	156.6 (16.8)	156.6 (17.4)
SBP variability (mmHg)*	14.2 (5.4)	13.2 (5.2)	13.2 (5.4)
Mean LDL cholesterol (mmol/L)*	3.1 (0.9)	3.2 (0.9)	3.2 (0.9)
LDL-c variability (mmol/L)*	0.31 (0.21)	0.28 (0.20)	0.28 (0.20)
Stroop card III, seconds needed †	64.5 (26.1)	55.2 (20.0)	54.8 (20.0)
LDT, digits coded correctly †	22.9 (7.8)	26.7 (7.3)	27.1 (7.1)
PLTi, pictures remembered †	9.5 (2.0)	10.2 (2.1)	10.3 (2.0)
PLTd, pictures remembered †	10.2 (2.9)	11.3 (2.8)	11.3 (2.8)
Categorical variables (n, %)			
Female	2281 (51.5)	461 (48.0)	233 (43.6)
History of hypertension	2781 (62.8)	619 (64.5)	339 (63.4)
History of diabetes mellitus	459 (10.4)	158 (16.5)	88 (16.4)
History of stroke or TIA	477 (10.8)	158 (16.5)	87 (16.3)
History of myocardial infarction	572 (12.9)	144 (15.0)	64 (12.0)
History of vascular disease	1926 (43.5)	407 (42.4)	234 (43.7)
Current smoker	1100 (24.8)	228 (23.8)	113 (21.1)

LDL-c denotes low-density lipoprotein cholesterol; SBP, systolic blood pressure; TIA, transient ischemic attack; LDT, Letter-Digit Coding test; PLTi, 15-Picture Learning test immediate; PLTd, 15-Picture Learning test delayed.
* calculated over months 3 to 24, † at month 30.

Supplemental Table 2. Cognitive function, at month thirty, over treatment-specific tertiles of LDL-c variability (n=4428)

	Lowest tertile	Middle tertile	Highest tertile	Beta (95% CI)	P_{trend}	$P_{interaction}$
Stroop card III, seconds	65.09 (0.85)	66.34 (0.86)	67.77 (0.86)	5.10 (1.59, 8.62)	**0.004**	0.504
LDT, digits coded	22.73 (0.23)	22.56 (0.23)	22.06 (0.23)	-1.26 (-2.22, -0.31)	**0.010**	0.549
PLTi, pictures remembered	9.39 (0.07)	9.38 (0.07)	9.22 (0.07)	-0.63 (-0.91, -0.35)	**<0.001**	0.730
PLTd, pictures remembered	10.26 (0.10)	10.03 (0.10)	9.72 (0.10)	-1.12 (-1.52, -0.73)	**<0.001**	0.790

Data are presented as mean cognitive test scores (SE). The adjusted unstandardized regression coefficient, p-value for trend, and p-value for interaction between treatment and LDL-c variability were calculated using variability (mmol/L) as a continuous measure. LDT denotes Letter-Digit Coding test; PLTi, 15-Picture Learning test immediate; PLTd, 15-Picture Learning test delayed.

Adjusted for age, gender, country, education, mean LDL cholesterol, pravastatin use, test version where appropriate, BMI, smoking status, alcohol intake, history of diabetes mellitus, hypertension, and vascular disease.

Supplemental Table 3. MRI measures, at end of study, over treatment-specific tertiles of LDL-c variability (n=535)

	Lowest tertile	Middle tertile	Highest tertile	Beta (95% CI)	P_{trend}	$p_{interaction}$
Hippocampal volume (ml)	9.16 (0.11)	9.14 (0.11)	9.22 (0.11)	0.11 (-0.38, 0.61)	0.646	0.848
Cerebral blood flow (ml/min/100 ml)	47.70 (0.96)	48.37 (1.01)	45.60 (0.97)	-6.13 (-10.80,-1.47)	**0.010**	0.746
WMHL (ml)	6.20 (1.15)	7.02 (1.18)	8.71 (1.13)	6.64 (1.36, 11.93)	**0.014**	0.840

Data are presented as mean MRI measure (SE). The adjusted unstandardized regression coefficient, p-value for trend, and p-value for interaction between treatment and LDL-c variability were calculated using variability (mmol/L) as a continuous measure. WMHL denotes white matter hyperintensity load. Adjusted for age, gender, education, mean LDL cholesterol, whole-brain parenchymal volume, pravastatin use, BMI, smoking status, alcohol intake, history of diabetes mellitus, hypertension, and vascular disease.

CHAPTER 9

Visit-to-visit lipid variability: clinical significance, effects of lipid-lowering treatment, and (pharmaco)genetics

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ABSTRACT

In recent years, visit-to-visit variability of serum lipids has been linked to both clinical outcomes and surrogate markers for vascular disease. In this article, we present an overview of the current evidence connecting this intra-individual variability to these outcome measures, discuss its interplay with lipid-lowering treatment, and describe the literature regarding genetic factors of possible interest. In addition, we undertook an explorative genome-wide association analysis on visit-to-visit variability of LDL-C and HDL-C, examining additive effects in 2,530 participants from the placebo-arm of the PROSPER trial. While we identified suggestive associations ($p<1x10-6$) at 3 different loci (KIAA0391, ACCN1, DKK3), previously published data from the GWAS literature did not suggest plausible mechanistic pathways. Given the large degree of both clinical and methodological heterogeneity in the literature, additional research is needed to harmonize visit-to-visit variability parameters across studies and to definitively assess the possible role of (pharmaco)genetic factors.

INTRODUCTION

There is a growing body of evidence showing that, in addition to average levels, fluctuations in various traditional risk factors may be of importance to cardiovascular risk assessment. For example, it is now well-established that higher intra-individual variability of blood pressure (BP) (1-3) and lower variability in heart rate (4, 5) associate with various adverse outcomes. However, lipid concentrations are also known to fluctuate substantially, even on a day-to-day basis (6, 7).

Modulated by a myriad of factors including biological, sampling, analytical, and clinical conditions (8), this measurement 'noise' may lead to uncertainty in clinical practice, making repeated lipid measurements necessary before determining that a patient is above a disease or risk threshold, or when evaluating the efficacy of lipid-level altering treatments.

Recent evidence suggests that visit-to-visit variability of lipids may independently associate with adverse outcomes. Here, we present an overview of the current literature linking this intra-individual variability of lipids to clinical outcomes, describe its relation to lipid-lowering treatment, and briefly summarize which genetic variants have previously been found to contribute to increased lipid variability. In addition, we present data from the first genome-wide association study (GWAS) on visit-to-visit variability of low-density lipoprotein cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C) levels, using data from the PROspective Study of Pravastatin in the Elderly at Risk for vascular disease (PROSPER).

CLINICAL SIGNIFICANCE

In 1960 an interesting collection of observations was published by Groover et al., who examined 177 military personnel over 5 years. Comparing cholesterol fluctuations over this period, it appeared that the group of individuals who had developed clinical manifestations of coronary artery disease had greater fluctuations in the preceding years (though no formal statistical testing was performed) (9). It wasn't until 34 years later that researchers from the Framingham study reported that greater long-term intra-individual variability in total cholesterol (TC) associates with all-cause mortality over a 24-year period in men, and with cardiovascular and coronary disease incidence and mortality in both sexes (10).

Only recently has an interest in the clinical impact of visit-to-visit variability of lipids re-emerged, with a number of studies showing that various metrics of higher

variability also associate with clinical outcomes over shorter periods of follow-up (**Table 1**). Of these, five studies have reported that higher intra-individual lipid variability is predictive of higher occurrence of adverse cardiovascular events. First, researchers from the Treating to New Targets (TNT) study found that variability of LDL-C is a predictor of cardiovascular events and mortality, independent of statin treatment, average LDL-C levels, and medication adherence as determined through pill count in individuals with stable coronary artery disease (11). These findings were recently replicated for measures of variability in HDL-C and triglycerides in the same population, additionally showing evidence that both LDL-C and triglyceride variability associate with incident diabetes (12). Similar findings between LDL-C variability and vascular events and all-cause mortality were shown in post-hoc analyses of the Incremental Decrease in End Points Through Aggressive Lipid-Lowering (IDEAL) trial of 8,658 patients with previous MI (13). In addition, Boey et al. observed that variability of LDL-C and HDL-C levels associated with 5-year occurrence of major adverse cardiac events after surviving ST-segment elevation myocardial infarction (14). Lastly, a recent large-scale investigation of over 3.5 million individuals from the Korean National Health Insurance System (NHIS) cohort without a history of MI and stroke showed that higher TC variability linearly associated with greater incidence of MI, stroke and all-cause mortality (15).

Visit-to-visit variability of lipids has also been demonstrated to associate with other outcomes. Chang et al. found that fluctuations of HDL-C, but not LDL-C, associate with a higher risk of diabetic nephropathy progression in type 2 diabetes patients (16). Both LDL-C and HDL-C variability have additionally been shown to associate with decline in glomerular filtration rate, but not with incidence of albuminuria (17). Findings from the Korean NIHS also suggest that lipid variability is related to change in kidney function, as analyses in almost 8.5 million individuals showed that increasing TC variability associated with progression to end-stage renal disease (18). Furthermore, higher variability of LDL-C was shown to cross-sectionally associate with lower cognitive test performance in four cognitive domains, lower cerebral blood flow, and greater white matter hyperintensity volume, in older individuals at high risk for vascular disease, independent of average LDL-C levels and statin treatment (19). In addition, relatively smaller studies have shown cross-sectional associations between higher LDL-C variability and obstructive sleep apnea (20) and maximum carotid intima-media thickness (21).

Several hypotheses have been put forward to explain these observational findings. On the one hand, lipid variability might simply be a risk marker for

distinct pathological processes leading to adverse outcomes. These include (sub)clinical disease (e.g. inflammation, cancer, kidney or liver disease), but also use of, or non-adherence to, various types of medication (22). If so, interventions specifically aimed at reducing variability are not likely to be effective. On the other hand, lipid variability might represent a novel modifiable risk factor. In the past, intermittent high-fat diets have been used to induce atherosclerotic lesions in animals (23, 24). Moreover, it has recently been shown that lipid lowering treatment in both animal models and humans may lead to changes of the cholesterol content of plaques (25, 26), which may have consequences for plaque stability (27, 28). These studies provide circumstantial evidence that fluctuations in lipid levels could also causally lead to a higher occurrence of adverse events.

Current knowledge on lipid variability has important limitations. As recently argued for research on visit-to-visit variability of BP (29), standardized definitions should be developed to facilitate comparisons across studies and assess whether reduction of variability will improve outcomes. Much of the evidence in favour of clinical significance of lipid variability stems from post-hoc analysis of trials, or from research with participants at high risk for vascular disease. However, the recent studies performed within the nationwide Korean NIHS suggest that these relationships might also hold for the general population, and may even be more pronounced within low-risk groups (e.g. younger age, or in absence of comorbidities such as obesity and diabetes) (15, 18). To date, all studies have solely examined mid- to long-term lipid variability (i.e. months to years). While these studies have consistently shown that higher lipid variability associates with worse clinical outcomes, these investigations are largely incomparable due to the heterogeneity in chosen outcomes of interest and metrics of variability. More specifically, five different metrics have been used, though all are known to be susceptible to either trend effects or mean levels in a repeated measurements setting (**Supplemental Table 1**). Moreover, there exist large differences in source population and study design, fasting status, number and regularity of lipid measurements, and selection of covariates. In addition, we should acknowledge the likely presence of submission and publication bias, as evidenced by the substantial publication time gaps between the Air Force and Framingham articles and the more recent publications. It therefore remains to be seen whether lipid variability truly reflects a reproducible phenomenon, and whether more short-term (e.g. daily or weekly) fluctuations also hold promise for clinical risk assessment.

Table 1. Chronologically listed studies which have reported on associations between visit-to-visit lipid variability and (sub)clinical outcomes

CLINICALLY OVERT CARDIOVASCULAR DISEASE			
First author (year)	**Study population/design**	**Lipid traits**	**Variability metric(s)**
Groover[9] (1960)	177 men aged 40 to 60 years, comparison between individuals who did (n=16) and did not develop CAD, cross-sectional analysis	TC (non-fasted)	% difference between highest and average of measurement(s)
Kreger[10] (1994)	1,505 women and 1,407 men aged 30 to 62 years, population-based cohort, follow-up of 24 years	TC (non-fasted)	RMSE
Bangalore[11] (2015)#	9,572 patients aged 35 to 75 years with known CAD, post-hoc analysis from RCT comparing atorvastatin 80 versus 10 mg/day, median follow-up of 4.9 years	LDL-C (fasted)	s.d., ASV, CV, cVIM
Boey[14] (2016)	130 patients aged 54.1 ± 9.3 years with ST-segment elevation myocardial infarction and surviving to discharge, mean follow-up of 62.4 ± 30.5 months	LDL-C, HDL-C (non-fasted)	s.d., CV, cVIM
Bangalore[13] (2017)	8,658 patients aged 62 ± 9.5 years with previous MI, post-hoc analysis from RCT comparing atorvastatin 80 mg/day versus simvastatin 20 mg/day, median follow-up 4.8 years	LDL-C (fasted)	s.d., ASV, CV, cVIM

Number of, and time between, measurements	Model covariates	Main results
≥6 yearly measurements for 5 consecutive years, time intervals unspecified	None (no formal statistical testing)	Greater deviations from 5-year average within CAD group
6 biennial measurements	Age, average slope of TC, mean TC	Higher variability associated with all-cause mortality and cardiovascular and coronary incidence and mortality in both sexes
At week 12, at 12 months, thereafter annual, minimum of 2 post-baseline measurements	Age, adherence (pill count), mean LDL-C, treatment arm	Higher variability associated with higher incidence of any coronary or cardiovascular event, all-cause mortality, MI, and stroke
9.1 ± 4.5 LDL-C measurements, 9.3 ± 4.5 HDL-C measurements, minimum of 3 from two months after discharge, with variable measurement schedules	Mean lipid levels, diabetes mellitus	Higher variability in both LDL-C and HDL-C associated with higher risk of major adverse cardiac event (death, MI, stroke, unplanned revascularization, heart failure admission)
At week 12, 24, year 1, thereafter yearly	Demographics, treatment arm, cardiovascular comorbidities, mean LDL-C	Higher variability associated with risk of any coronary or cardiovascular event, all-cause mortality, and MI

Table 1. Continued

First author (year)	Study population/design	Lipid traits	Variability metric(s)
CLINICALLY OVERT CARDIOVASCULAR DISEASE			
Kim[15] (2017)*	3,656,648 individuals aged 44.9 ±12.6 years without history of MI and stroke, population-based cohort, median follow-up of 8.3 years	TC (fasted)	s.d., CV, VIM
Waters[12] (2017)#	9,572 patients aged 35 to 75 years with known CAD, post-hoc analysis from RCT comparing atorvastatin 80 versus 10 mg/day, median follow-up of 4.9 years	LDL-C, HDL-C, TG (fasted)	s.d., ASV, CV, cVIM
OTHER OUTCOMES			
Chang[16] (2013)	864 type 2 diabetic patients aged 62.7 ± 11.8 years, mean follow-up of 3.8 years	TC, LDL-C, HDL-C, TG (fasted)	s.d.
Smit[19] (2016)	4,428 patients aged 70 to 82 years at high risk of vascular disease, post-hoc analysis from placebo-controlled RCT of pravastatin 40 mg/day, with MRI substudy of 535 participants, cross-sectional analyses stratified by treatment arm	LDL-C (fasted)	s.d.
Ng[20] (2017)	190 patients aged 54.0 ± 8.8 years with known CAD, cohort followed up after overnight sleep study, cross-sectional analyses	LDL-C (fasted)	cVIM

Number of, and time between, measurements	Model covariates	Main results
3-6 measurements during 6 years (4.2±1.2), time intervals unspecified	Demographics, cardiovascular comorbidities, baseline and/or mean TC, lipid-lowering treatment	Higher variability linearly associated with incidence of MI, stroke and all-cause mortality
At week 12, at 12 months, thereafter annual, minimum of 2 post-baseline measurements	Demographics, cardiovascular comorbidities, mean lipid levels, treatment arm, change in lipid levels	Higher variability in each lipid trait associated with incidence of coronary and cardiovascular events. In addition, LDL-C and TG variability associated with incident diabetes.
8.5 ± 1.5 measurements, measured either quarterly or every 6 months	Demographics, smoking, disease duration, kidney function, ACEI/ARB, lipid-lowering treatment	Higher HDL-C variability associated with higher risk of diabetic nephropathy progression
4 post-baseline measurements at months 3, 6, 12, 24 (92% with all 4)	Demographics, cardiovascular comorbidities, mean LDL-C	Higher variability associated with worse cognitive performance at month 30 for selective attention, processing speed, immediate and delayed recall, and with lower cerebral blood flow and greater white matter hyperintensity load at end of study, in both treatment arms
8.1 ± 4.2 (minimum of 3) measurements during 53.2 ± 25.3 months, time intervals unspecified	Diabetes mellitus, hyperlipidemia	Higher scores on apnea-hypopnea index associated with greater visit-to-visit variability

Table 1. Continued

OTHER OUTCOMES			
First author (year)	**Study population/design**	**Lipid traits**	**Variability metric(s)**
Takenouchi[21] (2017)	162 type 2 diabetic patients aged 62 ± 10 years, cross-sectional analyses	LDL-C (fasted)	s.d.
Ceriello[17] (2017)	Type 2 diabetes patients, 2 cohorts: 4,231 with median age of 67.4 (IQR: 60.3-73.4) and normoalbuminuria, 7,560 aged 65.0 (58.5-71.3) with eGFR ≥ 60 mL/min/1.73 m^2, median follow-up 3.4 years (range 1.7-4.2)	TC, LDL-C, HDL-C, TG (fasting status unspecified)	s.d.
Kim[18] (2017)*	8,493,277 individuals aged 48.5 ± 13.8 years and free from ESRD, population-based cohort, median follow-up 6.1 years	TC (fasted)	s.d., CV, VIM

#/*: complete/partial overlap in study populations. RCT denotes randomized clinical trial; CAD, coronary artery disease; MI, myocardial infarction; eGFR, estimated glomerular filtration rate; ESRD, end-stage renal disease; TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; TG, triglycerides; RSME, square root of mean squared error ; s.d., standard deviation; ASV, average successive variability; CV, coefficient of variation; (c)VIM, (corrected) variation independent of mean; ACEI/ARB, angiotensin converting enzyme inhibitor or angiotensin receptor blocker.

Number of, and time between, measurements	Model covariates	Main results
94% had 6 measurements measured during 12 month period, time intervals unspecified	Age, sex	Higher variability associated with maximum carotid intima-media thickness
≥5 measurements over 3 years, time intervals unspecified	Demographics, baseline lipid levels/ blood pressure/kidney function, glucose- and lipid-lowering treatment, ACEI/ARB, duration of diabetes	No associations with incident albuminuria. However, higher variability in LDL-C and HDL-C associated with increased risk for decline in eGFR below 60 mL/min/1.73 m^2
3-5 measurements over 6 years (3.5 ± 0.8), time intervals unspecified	Demographics, cardiovascular comorbidities, baseline and/or mean TC, lipid-lowering treatment, baseline kidney function	Graded association between higher variability with incident ESRD

Nonetheless, if it can be shown that appraisal of lipid variability could benefit risk assessment, this might influence ordering patterns of lipid levels in clinical practice. Researchers working with large-scale data from the Korean NHIS have recently shown that incorporating variability of different cardiovascular disease risk factors (including intra-individual variability of total cholesterol) substantially improved cardiovascular risk predictability compared with single measurement values or taking the average of repeated measurements (30), though this was not examined separately for lipid variability. These findings are in line with a previous simulation study showing that blood pressure and cholesterol variability may lead to substantial misclassification when cardiovascular risk assessment is based on single measurements (31), and with increasing evidence that incorporating repeated measurements can improve cardiovascular risk prediction (32). Based on the current literature it is however not yet possible to make recommendations on the necessity of repeated lipid measurements in clinical practice either before or after starting lipid lowering treatment, beyond which is already viewed as necessary to overcome short-term fluctuations in lipid levels.

INTERPLAY WITH LIPID-LOWERING TREATMENT

To date, few studies have systematically examined the effects of lipid-lowering treatment on intra-individual variability of lipids. Commencement of statin treatment has been shown to lead to a minor decline in absolute values of visit-to-visit lipid variability in clinical trials (19), as measured by the intra-individual standard deviation, with more intensive statin treatment leading to even more stable LDL-C levels (11, 13). While these dose-dependent results are not always seen in observational studies, this may be due to different prescription patterns (14). It is currently unknown whether drug-class effects exist, which have been described in research on visit-to-visit BP variability (33, 34), though a cross-over study in 26 individuals with type 2 diabetes suggests that these might depend on the methods of measuring and calculating lipid profiles (35, 36).

Despite this absolute decrease, results (**Table 2**) from our PROSPER study suggest that statin therapy may also lead to an increase in relative measures of lipid variability. This likely occurs because declines in average levels of lipids will generally be larger than declines in variability, which will influence relative metrics such as the coefficient of variation. However, it is expected that absolute declines will be of greater importance in clinical settings, offsetting any relative increase.

Another treatment-related factor contributing to intra-individual variability of lipids is non-adherence (37), as has similarly been shown for antihypertensive

medication and visit-to-visit variability of BP (38). While combined pharmacological treatment modalities may reduce adherence-associated variability (39), adjusting for non-adherence is often difficult due to the absence of reliable assessment methods (40, 41), which may limit which studies are best suited to investigate effects of visit-to-visit variability in absence of non-adherence. However, studies which have performed analyses stratified by use of lipid-lowering agents have shown either highly comparable (19) or more pronounced (15, 18) associations between variability and clinical outcomes in individuals not using lipid-lowering medication. It is therefore unlikely that, at least in those studies, the findings can be explained solely by non-adherence. Dosing schedules can also influence variability. While high-dose monthly dosing of PCSK9-inhibitors are known to produce substantial fluctuations of LDL levels in between injections (42, 43), there exists tentative trial evidence that adverse neurocognitive events may be more prevalent, independent of on-treatment lipid levels (44). It will therefore be of interest for PCSK9-trials to examine the possible influence of lipid variability on cognitive test performance in greater detail.

Table 2. Demographic characteristics and lipid parameters for the PROSPER study

	Placebo (n=2,530)	Pravastatin (n=2,504)	p-value
Age at randomization	75.31 ± 3.35	75.33 ± 3.35	-
Females (%)	1309 (51.7)	1300 (51.9)	-
Lipid parameters at baseline (mmol/L)			
LDL-C	3.79 ± 0.78	3.80 ± 0.81	-
HDL-C	1.28 ± 0.34	1.29 ± 0.36	-
Lipid parameters during follow-up (mmol/L)*			
No. of measurements	4.39 ± 0.82	4.39 ± 0.81	0.98
Average LDL-C	3.70 ± 0.76	2.56 ± 0.65	<0.001
LDL-C variability (standard deviation)	0.33 ± 0.21	0.32 ± 0.24	0.02
LDL-C variability (coefficient of variation)	0.09 ± 0.06	0.13 ± 0.13	<0.001
Average HDL-C	1.33 ± 0.36	1.40 ± 0.38	<0.001
HDL-C variability (standard deviation)	0.12 ± 0.08	0.13 ± 0.08	0.001
HDL-C variability (coefficient of variation)	0.09 ± 0.05	0.09 ± 0.05	0.53

Unless otherwise specified, data are presented as mean ± standard deviation. P-values calculated using Student t-test and Pearson's chi-square test when appropriate.

LDL-C denotes low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol

* calculated per-individual, over months 3 to 36

GENETIC BASIS OF VISIT-TO-VISIT VARIABILITY OF LIPIDS

While over 157 loci associated with blood lipid levels have been identified and annotated through large-scale efforts (45), little is known about the genetic predisposition for intra-individual variability of lipids. The same applies to variability of other physiological measures. For example, to date just one GWAS has been published on visit-to-visit variability of BP (46), which many consider the poster child of intra-individual variability.

Previously, Pereira et al. assessed the association between 11 genetic polymorphisms involved in lipid metabolism and intra-individual variability of total cholesterol and HDL-C in up to 458 men and women from 27 feeding or supplement trials designed to change serum cholesterol (47). The authors found evidence that two polymorphisms may increase the variability of total cholesterol (ApoA4 -347 (0.015 mmol/l higher geometric mean of the intra-individual standard deviations for genotype 12/22 versus genotype 11, p=0.02); MTP -493 (0.017 mmol/l higher for genotype 11 versus genotype 12/22, p=0.004)). In a study of 117 men with peripheral arterial disease, it was reported that those heterozygous for the ApoB EcoRI polymorphism had higher within-individual variation of total serum cholesterol concentration over a period of 5-10 years using annual lipid measurements (48). Furthermore, Porkka et al. examined the influence of selected genetic markers on long-term variability of serum lipids in up to 320 subjects aged 3-18 years at baseline over 3-year intervals during a 6-year follow-up period (49). They found that ApoB XbaI genotypes significantly influenced variability of TC and LDL-C levels in both sexes, and variability of triglycerides in males only. Moreover, ApoAI/CIII genotype influenced variability of TC and LDL-C levels but again, only in males. Finally, by comparing within-pair differences in monozygotic twins, possible 'variability gene effects' on lipid levels of genes in the Kidd blood group locus and of the TaqIB polymorphism in the CETP gene have been demonstrated by Berg and colleagues (50, 51).

As no other studies have examined whether commonly occurring genetic variants are of importance to visit-to-visit variability of lipids, we undertook an explorative genome-wide association study on intra-individual variability of LDL-C and HDL-C, as fluctuations in specifically these two lipid traits have recently been shown to associate with clinical outcomes.

GWAS

We included 2,530 individuals from the placebo-arm of the PHArmacogenetic study of Statin in the Elderly at risk (PHASE) (52, 53). Genotyping was conducted using Illumina 660-Quad beadchips and imputation with MACH imputation

software based on the Hapmap built II release 23. We excluded variants with a minor allele frequency below 1%, and those with an imputation quality below 0.3. Lipid levels were assessed after an overnight fast. LDL-C was directly measured, and visit-to-visit variability of both LDL-C and HDL-C was defined as the intra-individual standard deviation over each individual's lipid measurements at 3, 6, 12, 24, and 36 months after randomization.

The association analyses were conducted using PROBABEL software (http://www.genabel.org/). For both LDL-C and HDL-C variability, an additive linear regression model was used. Given the negligible difference in absolute values of visit-to-visit variability between the two trial arms, we did not undertake genome-wide association analyses on the interaction terms with statin treatment. However, as non-adherence to pravastatin might influence the degree of visit-to-visit lipid variability, the analyses presented here were conducted solely in the placebo group. All analyses were adjusted for age, gender, principal components of ancestry (n=4), and mean intra-individual lipid level during follow-up. The p-value threshold for genome-wide significance was set at $5x10^{-8}$.

Known host genes for variants of note found in the GWAS were located via the SCAN database (http://www.scandb.org/) (54). Furthermore, we searched Phenoscanner (http://www.phenoscanner.medschl.cam.ac.uk) (55), a curated database holding publicly available results from large-scale GWAS, for evidence of plausible mechanistic pathways for these three variants. In addition, we examined our GWAS results for the lead SNPs for loci previously found to associate with either LDL-C or HDL-C levels at a genome-wide significant level in the largest lipid GWAS to date (45). As some lead SNPs were associated with both traits this list comprised 124 different lead SNPs. To account for multiple testing, the p-value threshold for statistical significance was set at 0.0002 (i.e. 0.05/248 tests).

RESULTS

We did not observe any genome-wide significant associations for additive effects on lipid variability (**Figure**). However, we did detect two suggestive ($p<1x10^{-6}$) signals for LDL-C variability (KIAA0391 and Amiloride-sensitive cation channel 1 neuronal (ACCN1)) and one for HDL-C variability (Dickkopf WNT Signaling Pathway Inhibitor 3 (DKK3)), as shown in **Table 3**. Q-Q plots did not reveal evidence of systematic bias (**Supplementary Figure**).

In order to examine possible mechanistic pathways leading to lipid variability, we queried the three suggestive lead SNPs shown in **Table 3** in the Phenoscanner database. However, with the exception of nominal associations with body-mass

index and height (p-values between 0.05 and 0.001), no traits were shared by multiple variants (data not shown).

Finally, as shown in **Supplemental Table 2** and **3**, no previously reported lead SNPs for loci associated with either LDL-C or HDL-C levels attained statistical significance after correction for multiple testing.

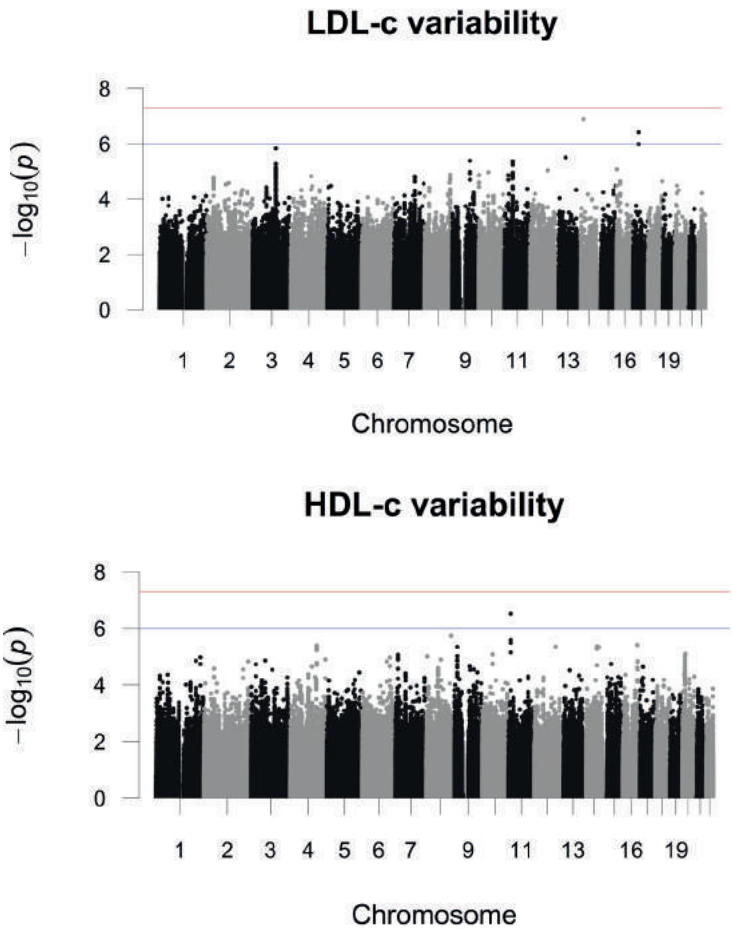


Figure. Genome-wide Manhattan plots for visit-to-visit variability of low-density lipoprotein cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C), as measured by the intra-individual standard deviation, in the placebo group (n=2,530) of the Prospective Study of Pravastatin in the Elderly at Risk (PROSPER). Individual $-\log_{10}$ p-values are plotted against their genomic position. Adjusted for age, gender, mean intra-individual lipid level during follow-up, and principal components of ancestry (n=4).

Table 3. Genetic variants independently associated with lipid variability at $p < 1x10^{-6}$ (n=2,530)

Trait	Lead SNP	Chr.	Position	Gene*	Coding allele (CA)	Noncoding allele	Freq. CA	Beta †	s.e.	p-value
LDL-C variability	rs2295463	14	34806024	KIAA0391	C	T	0.98	-0.115	0.022	1.3×10^{-7}
	rs11867369	17	29243349	ACCN1	C	T	0.09	0.050	0.010	3.9×10^{-7}
HDL-C variability	rs4757730	11	11971832	DKK3	G	T	0.90	0.016	0.003	3.0×10^{-7}

Chr., chromosome; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol

* As reported by the SCAN database (available at http://www.scandb.org).

† Beta for per-allele additive effect on lipid variability (intra-individual standard deviation, mmol/L), adjusted for age, sex, mean intra-individual lipid level, and principal components of ancestry (n=4).

DISCUSSION

In this narrative review we have presented the literature on visit-to-visit lipid variability to date. While the exact role of lipid lowering treatment remains to be elucidated, it is evident that the substantial clinical and methodological heterogeneity among studies impedes drawing strong conclusions regarding possible clinical significance. Furthermore, our current genome-wide association results suggest that most genetic variants, including those that influence mean LDL-C or HDL-C levels, are not associated with intra-individual variability of lipids, or that their effects are too small to detect with our current sample size. Replication studies will therefore be necessary to determine whether these explorative findings reflect true associations or merely statistical noise. Given the negligible difference in absolute values of lipid variability between the two PROSPER trial arms, it appears unlikely or at least doubtful that clinically relevant pharmacogenetic-guided interventions will be based on common genetic variants.

The major limitations of our association analysis were the relatively small sample size, though not dissimilar to the sole GWAS study on visit-to-visit variability of BP, and the inclusion of exclusively European-descent participants. Future studies on (pharmaco)genetic effects on intra-individual lipid variability should carefully consider issues of non-adherence. In addition, the influence of number of visits, the effect of duration of time between measurements, and the proximity of lipid measurements to drug administration may be important to consider (47, 56, 57).

It should further be noted that intra-individual lipid variability will presumably vary within and among populations due to varying genetic and environmental factors, which could limit the generalizability of any given study (47). For example, it is likely that genetic factors of importance will differ between younger and older populations, as age- or clinical disease-related disturbances to homeostatic mechanisms will be of little significance to younger populations. This is supported by research on the heritability of intra-individual BP variability, as researchers from the Twins UK cohort found that environmental factors were responsible for over 80% of the variance in variability in older age groups, versus over 50% for twin pairs younger than 51 years (58). However, given that age-related loss of physiological homeostasis would presumably lead to greater overall intrinsic variability (59), there might exist genetic variants of importance to visit-to-visit variability of multiple physiological measures in older populations.

Future studies could focus on overall genetic predisposition to lipid levels in greater detail, by examining loci previously found to associate with lipid

metabolism (45), as those individuals genetically predisposed to certain lipid levels might be less likely to vary from visit-to-visit. In addition, factoring in lipid-lowering treatment may enhance power for the detection of genes of importance to intra-individual variability of lipids, especially if genetic loci have a differential effect conditional on the treatment. Gene-environment-wide interaction studies (GEWIS) using a joint meta-analysis (JMA) approach may therefore provide further insight into the (pharmaco)genetic background of visit-to-visit variability of lipids (60). While these methods are promising, there remains ample room for the development of methodology and statistical software packages to detect genetic loci affecting visit-to-visit variability, which account for phenotypic variability across individuals (61).

In summary, while visit-to-visit variability could be a novel prognostic marker for clinical practice, additional efforts are needed to harmonize phenotype definitions across different studies, and replication studies are required to definitively assess the possible importance of (pharmaco)genetic factors.

REFERENCE LIST

1. Tai C, Sun Y, Dai N, et al. Prognostic significance of visit-to-visit systolic blood pressure variability: a meta-analysis of 77,299 patients. J Clin Hypertens (Greenwich). 2015;17:107-115.
2. Rothwell PM, Howard SC, Dolan E, et al. Prognostic significance of visit-to-visit variability, maximum systolic blood pressure, and episodic hypertension. Lancet. 2010;375:895-905.
3. Sabayan B, Wijsman LW, Foster-Dingley JC, et al. Association of visit-to-visit variability in blood pressure with cognitive function in old age: prospective cohort study. BMJ. 2013;347:f4600.
4. Kim DH, Lipsitz LA, Ferrucci L, et al. Association between reduced heart rate variability and cognitive impairment in older disabled women in the community: Women's Health and Aging Study I. J Am Geriatr Soc. 2006;54:1751-1757.
5. Tsuji H, Larson MG, Venditti FJJ, et al. Impact of Reduced Heart Rate Variability on Risk for Cardiac Events: The Framingham Heart Study. Circulation. 1996;1:2850-2855.
6. Smith SJ, Cooper GR, Myers GL, et al. Biological variability in concentrations of serum lipids: sources of variation among results from published studies and composite predicted values. Clin Chem. 1993;39:1012-1022.
7. Nazir DJ, Roberts RS, Hill SA, et al. Monthly intra-individual variation in lipids over a 1-year period in 22 normal subjects. Clin Biochem. 1999;32:381-389.
8. Cooper GR, Myers GL, Smith SJ, et al. Blood lipid measurements. Variations and practical utility. JAMA. 1992;267:1652-1660.
9. Groover ME, Jernigan JA, Martin CD. Variations in serum lipid concentration and clinical coronary disease. Am J Med Sci. 1960;239:133-139.
10. Kreger BE, Odell PM, D'Agostino RB, et al. Long-term intraindividual cholesterol variability: natural course and adverse impact on morbidity and mortality--the Framingham Study. Am Heart J. 1994;127:1607-1614.
11. Bangalore S, Breazna A, DeMicco DA, et al. Visit-to-visit low-density lipoprotein cholesterol variability and risk of cardiovascular outcomes: insights from the TNT trial. J Am Coll Cardiol. 2015;65:1539-1548.
12. Waters DD, Bangalore S, Fayyad R, et al. Visit-to-Visit Variability of Lipid Measurements As Predictors of Cardiovascular Events. J Clin Lipidol. 2017. http://dx.doi.org/10.1016/j.jacl.2017.12.003.
13. Bangalore S, Fayyad R, Messerli FH, et al. Relation of Variability of Low-Density Lipoprotein Cholesterol and Blood Pressure to Events in Patients With Previous Myocardial Infarction from the IDEAL Trial. Am J Cardiol. 2017;119:379-387.
14. Boey E, Gay GM, Poh KK, et al. Visit-to-visit variability in LDL- and HDL-cholesterol is associated with adverse events after ST-segment elevation myocardial infarction: A 5-year follow-up study. Atherosclerosis. 2016;244:86-92.
15. Kim MK, Han K, Kim HS, et al. Cholesterol variability and the risk of mortality, myocardial infarction, and stroke: a nationwide population-based study. Eur Heart J. 2017;38:3560-3566.

16. Chang YH, Chang DM, Lin KC, et al. High-density lipoprotein cholesterol and the risk of nephropathy in type 2 diabetic patients. Nutr Metab Cardiovasc Dis. 2013;23:751-757.
17. Ceriello A, De Cosmo S, Rossi MC, et al. Variability in HbA1c, blood pressure, lipid parameters and serum uric acid, and risk of development of chronic kidney disease in type 2 diabetes. Diabetes Obes Metab. 2017;19:1570-1578.
18. Kim MK, Han K, Koh ES, et al. Variability in Total Cholesterol Is Associated With the Risk of End-Stage Renal Disease: A Nationwide Population-Based Study. Arterioscler Thromb Vasc Biol. 2017;37:1963-1970.
19. Smit RAJ, Trompet S, Sabayan B, et al. Higher visit-to-visit LDL-c variability is associated with lower cognitive performance, lower cerebral blood flow, and greater white matter hyperintensity load in older subjects. Circulation. 2016;134:212-221.
20. Ng G, Boey E, Frampton C, et al. Obstructive sleep apnea is associated with visit-to-visit variability in low-density lipoprotein-cholesterol in patients with coronary artery disease. Sleep Breath. 2017;21:271-278.
21. Takenouchi A, Tsuboi A, Kitaoka K, et al. Visit-to-Visit Low-Density Lipoprotein Cholesterol Variability Is an Independent Determinant of Carotid Intima-Media Thickness in Patients With Type 2 Diabetes. J Clin Med Res. 2017;9:310-316.
22. Mantel-Teeuwisse AK, Kloosterman JME, Maitland-van der Zee AH, et al. Drug-Induced Lipid Changes - A Review of the Unintended Effects of Some Commonly Used Drugs on Serum Lipid Levels. Drug Safety. 2001;24:443-456.
23. Constantinides P, Booth J, Carlson G. Production of advanced cholesterol atherosclerosis in the rabbit. Arch Pathol. 1960;70:712-724.
24. Bullock BC, Wagner WD, Bond MG, et al. The effect of intermittent hypercholesterolemia on aortic atherosclerosis of rhesus monkeys [Abstract]. Am J Pathol. 1976;82:82-83A.
25. Crisby M, Nordin-Fredriksson G, Shah PK, et al. Pravastatin treatment increases collagen content and decreases lipid content, inflammation, metalloproteinases, and cell death in human carotid plaques: implications for plaque stabilization. Circulation. 2001;103:926-933.
26. Bjorkegren JL, Hagg S, Talukdar HA, et al. Plasma cholesterol-induced lesion networks activated before regression of early, mature, and advanced atherosclerosis. PLoS Genet. 2014;10:e1004201.
27. Chen Z, Ichetovkin M, Kurtz M, et al. Cholesterol in human atherosclerotic plaque is a marker for underlying disease state and plaque vulnerability. Lipids Health Dis. 2010;9:61.
28. Vedre A, Pathak DR, Crimp M, et al. Physical factors that trigger cholesterol crystallization leading to plaque rupture. Atherosclerosis. 2009;203:89-96.
29. Hussein WF, Chang TI. Visit-to-Visit Variability of Systolic Blood Pressure and Cardiovascular Disease. Curr Hypertens Rep. 2015;17:14.
30. Cho IJ, Sung JM, Chang HJ, et al. Incremental Value of Repeated Risk Factor Measurements for Cardiovascular Disease Prediction in Middle-Aged Korean Adults: Results From the NHIS-HEALS (National Health Insurance System-National Health Screening Cohort). Circ Cardiovasc Qual Outcomes. 2017;10:pii: e004197.

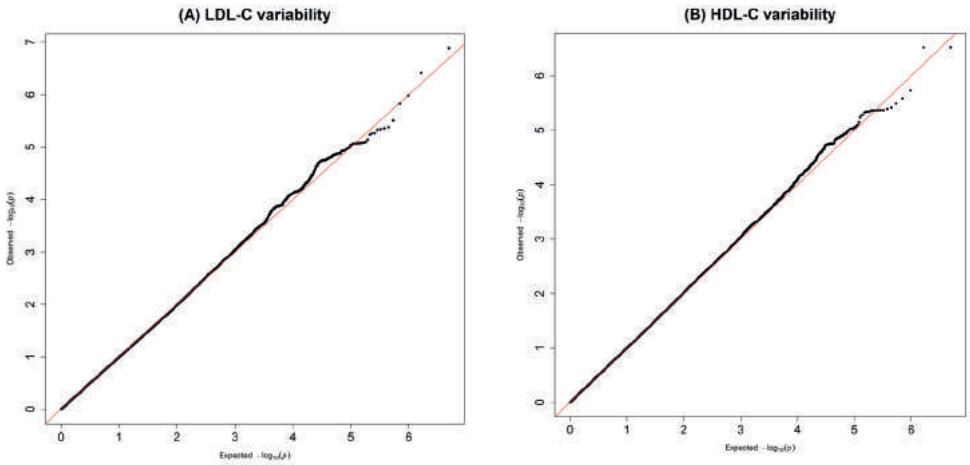
31. Marshall T. The effect of blood pressure and cholesterol variability on the precision of Framingham cardiovascular risk estimation: a simulation study. J Hum Hypertens. 2010;24:631-638.
32. Paige E, Barrett J, Pennells L, et al. Use of Repeated Blood Pressure and Cholesterol Measurements to Improve Cardiovascular Disease Risk Prediction: An Individual-Participant-Data Meta-Analysis. Am J Epidemiol. 2017;186:899-907.
33. Rothwell PM, Howard SC, Dolan E, et al. Effects of beta blockers and calcium-channel blockers on within-individual variability in blood pressure and risk of stroke. Lancet Neurol. 2010;9:469-480.
34. Smith TR, Drozda JP, Jr., Vanslette JA, et al. Medication class effects on visit-to-visit variability of blood pressure measurements: analysis of electronic health record data in the “real world”. J Clin Hypertens (Greenwich). 2013;15:655-662.
35. Sathyapalan T, Atkin SL, Kilpatrick ES. Low density lipoprotein-cholesterol variability in patients with type 2 diabetes taking atorvastatin compared to simvastatin: justification for direct measurement? Diabetes Obes Metab. 2010;12:540-544.
36. Sathyapalan T, Atkin SL, Kilpatrick ES. LDL cholesterol variability in patients with Type 2 diabetes taking atorvastatin and simvastatin: a comparison of two formulae for LDL-C estimation. Ann Clin Biochem. 2015;52:180-182.
37. Mann DM, Glazer NL, Winter M, et al. A pilot study identifying statin nonadherence with visit-to-visit variability of low-density lipoprotein cholesterol. Am J Cardiol. 2013;111:1437-1442.
38. Kronish IM, Lynch AI, Oparil S, et al. The Association Between Antihypertensive Medication Nonadherence and Visit-to-Visit Variability of Blood Pressure: Findings From the Antihypertensive and Lipid-Lowering Treatment to Prevent Heart Attack Trial. Hypertension. 2016;68:39-45.
39. Baber U, Halperin JL. Variability in low-density lipoprotein cholesterol and cardiovascular risk: should consistency be a new target? J Am Coll Cardiol. 2015;65:1549-1551.
40. Liu H, Golin CE, Miller LG, et al. A comparison study of multiple measures of adherence to HIV protease inhibitors. Ann Intern Med. 2001;134:968-977.
41. Trompet S, Postmus I, Slagboom PE, et al. Non-response to (statin) therapy: the importance of distinguishing non-responders from non-adherers in pharmacogenetic studies. Eur J Clin Pharmacol. 2016;72:431-437.
42. Ballantyne CM, Neutel J, Cropp A, et al. Results of bococizumab, a monoclonal antibody against proprotein convertase subtilisin/kexin type 9, from a randomized, placebo-controlled, dose-ranging study in statin-treated subjects with hypercholesterolemia. Am J Cardiol. 2015;115:1212-1221.
43. Kastelein JJ, Nissen SE, Rader DJ, et al. Safety and efficacy of LY3015014, a monoclonal antibody to proprotein convertase subtilisin/kexin type 9 (PCSK9): a randomized, placebo-controlled Phase 2 study. Eur Heart J. 2016;37:1360-1369.
44. Swiger KJ, Martin SS. PCSK9 inhibitors and neurocognitive adverse events: exploring the FDA directive and a proposal for N-of-1 trials. Drug Saf. 2015;38:519-526.
45. Willer CJ, Schmidt EM, Sengupta S, et al. Discovery and refinement of loci associated with lipid levels. Nat Genet. 2013;45:1274-1283.

46. Yadav S, Cotlarciuc I, Munroe PB, et al. Genome-wide analysis of blood pressure variability and ischemic stroke. Stroke. 2013;44:2703-2709.
47. Pereira MA, Weggemans RM, Jacobs DR, Jr., et al. Within-person variation in serum lipids: implications for clinical trials. Int J Epidemiol. 2004;33:534-541.
48. Monsalve MV, Robinson D, Woolcock NE, et al. Within-individual variation in serum cholesterol levels: association with DNA polymorphisms at the apolipoprotein B and AI-CIII-AIV loci in patients with peripheral arterial disease. Clin Genet. 1991;39:260-273.
49. Porkka KV, Taimela S, Kontula K, et al. Variability gene effects of DNA polymorphisms at the apo B, apo A I/C III and apo E loci on serum lipids: the Cardiovascular Risk in Young Finns Study. Clin Genet. 1994;45:113-121.
50. Berg K. Variability gene effect on cholesterol at the Kidd blood group locus. Clin Genet. 1988;33:102-107.
51. Berg K, Kondo I, Drayna D, et al. "Variability gene" effect of cholesteryl ester transfer protein (CETP) genes. Clin Genet. 1989;35:437-445.
52. Trompet S, de Craen AJ, Postmus I, et al. Replication of LDL GWAs hits in PROSPER/PHASE as validation for future (pharmaco)genetic analyses. BMC Med Genet. 2011;12:131.
53. Shepherd J, Blauw GJ, Murphy MB, et al. The design of a prospective study of Pravastatin in the Elderly at Risk (PROSPER). PROSPER Study Group. PROspective Study of Pravastatin in the Elderly at Risk. Am J Cardiol. 1999:1192-1197.
54. Gamazon ER, Zhang W, Konkashbaev A, et al. SCAN: SNP and copy number annotation. Bioinformatics. 2010;26:259-262.
55. Staley JR, Blackshaw J, Kamat MA, et al. PhenoScanner: a database of human genotype-phenotype associations. Bioinformatics. 2016; 32:3207-3209.
56. Rotterdam EP, Katan MB, Knuiman JT. Importance of time interval between repeated measurements of total or high-density lipoprotein cholesterol when estimating an individual's baseline concentrations. Clin Chem. 1987;33:1913-1915.
57. Choudbury N, Wall PM, Truswell AS. Effect of time between measurements on within-subject variability for total cholesterol and high-density lipoprotein cholesterol in women. Clin Chem. 1994;40:710-715.
58. Menni C, Mangino M, Zhang F, et al. Heritability analyses show visit-to-visit blood pressure variability reflects different pathological phenotypes in younger and older adults: evidence from UK twins. J Hypertens. 2013;31:2356-2361.
59. Cannon WB. Organization for physiological homeostasis. Physiol Rev. 1929;9:399-431.
60. Aschard H, Hancock DB, London SJ, et al. Genome-wide meta-analysis of joint tests for genetic and gene-environment interaction effects. Hum Hered. 2010;70:292-300.
61. Rönnegård L, Valdar W. Recent developments in statistical methods for detecting genetic loci affecting phenotypic variability. BMC Genet. 2012;13:doi: 10.1186/1471-2156-1113-1163.

Supplemental Table 1. Metrics of lipid visit-to-visit variability used in the literature

Measure	Formula	Properties
Square root of mean squared error (RSME)	$\sqrt{\frac{\sum_{i=1}^{n}(x_i\text{-}\hat{x}_i)^2}{n-2}}$ With $\hat{x}_i$ obtained from fitting *x* against time	Takes (assumed to be linear) trend of repeated measurements into account, but is susceptible to differences in mean follow-up levels
Standard deviation (s.d)	$\sqrt{\frac{\sum_{i=1}^{n}(x_i\text{-}\bar{x})^2}{(n-1)}}$	Dependent on mean follow-up levels, and susceptible to trend across measurements
Coefficient of variation (CV)	$\frac{s.d.}{\bar{x}}$	Largely independent of mean follow-up levels, but susceptible to trend effects
Average successive variability (ASV)	$\frac{\sum_{i=1}^{n-1}\lvert x_{i+1}\text{-}x_i\rvert}{n-1}$	Largely independent of trend effects, but susceptible to differences in mean follow-up levels
Corrected variation independent of mean (cVIM)	$VIM = \frac{s.d.}{\bar{x}^{beta}}$ With *beta* obtained from fitting s.d. on $\bar{x}$, after natural log-transformation. $cVIM = \frac{(VIM \times \overline{CV})}{VIM}$	Independent of mean follow-up levels, but susceptible to trend effects

x_i denotes the *i*-th measurement of a set of n-measurements



Supplemental Figure. Q-Q plots for the two genome-wide association analyses. Corresponding λ's: (A) 0.995; (B) 1.002

Supplemental Table 2. Lead SNPs for previously reported loci for LDL-C levels.

SNP	Chr.	Locus	LDL-C var.	HDL-C var.
rs10102164	8	SOX17	0.03	0.66
rs10128711	11	SPTY2D1	0.56	0.56
rs10401969	19	CILP2	0.35	0.24
rs10490626	2	INSIG2	0.66	0.29
rs11065987	12	BRAP	0.51	0.89
rs11136341	8	PLEC1	0.92	0.55
rs11220462	11	ST3GAL4	0.9	0.21
rs11563251	2	UGT1A1	0.11	0.47
rs1169288	12	HNF1A	0.07	0.85
rs12027135	1	LDLRAP1	0.61	0.56
rs1250229	2	FN1	0.11	0.5
rs12670798	7	DNAH11	0.62	0.73
rs12748152	1	PIGV-NR0B2	0.12	0.44
rs12916	5	HMGCR	0.75	0.26
rs1367117	2	APOB	0.89	0.54
rs1564348	6	LPA	0.43	0.19

Supplemental Table 2. Continued

SNP	Chr.	Locus	LDL-C var.	HDL-C var.
rs17404153	3	ACAD11	0.54	0.27
rs174546	11	FADS1-2-3	0.25	0.55
rs1800562	6	HFE	0.55	0.49
rs1800961	20	HNF4A	0.03	0.57
rs1883025	9	ABCA1	0.39	0.78
rs2000999	16	HPR	0.76	0.31
rs2030746	2	LOC84931	0.85	0.26
rs2072183	7	NPC1L1	0.68	0.77
rs2131925	1	ANGPTL3	0.13	0.57
rs2255141	10	GPAM	0.42	0.38
rs2328223	20	SNX5	0.69	0.74
rs2479409	1	PCSK9	0.21	0.34
rs2642442	1	MOSC1	0.5	0.17
rs267733	1	ANXA9-CERS2	0.51	0.62
rs2710642	2	EHBP1	0.85	0.46
rs2902940	20	MAFB	0.28	0.3
rs2954029	8	TRIB1	0.05	0.72
rs314253	17	DLG4	0.94	0.43
rs3177928	6	HLA	0.05	0.63
rs364585	20	SPTLC3	0.16	0.72
rs3757354	6	MYLIP	0.42	0.93
rs3764261	16	CETP	0.05	0.48
rs3780181	9	VLDLR	0.65	0.06
rs4253776	22	PPARA	0.7	0.48
rs4299376	2	ABCG5/8	0.18	0.02
rs4420638	19	APOE	0.79	0.97
rs4530754	5	CSNK1G3	0.5	0.92
rs4722551	7	MIR148A	0.83	0.72
rs492602	19	FLJ36070	0.68	0.28

SNP	Chr.	Locus	LDL-C var.	HDL-C var.
rs4942486	13	BRCA2	0.85	0.17
rs514230	1	IRF2BP2	0.16	0.1
rs5763662	22	MTMR3	0.41	0.92
rs6029526	20	TOP1	0.93	0.92
rs629301	1	SORT1	0.26	0.78
rs6511720	19	LDLR	0.38	0.61
rs6818397	4	LRPAP1	0.2	0.87
rs6882076	5	TIMD4	0.19	0.55
rs7570971	2	RAB3GAP1	0.34	0.65
rs7640978	3	CMTM6	0.42	0.57
rs8017377	14	NYNRIN	0.39	0.43
rs964184	11	APOA1	0.003	0.37
rs9987289	8	PPP1R3B	0.06	0.47

Data are presented as p-values for additive effects on visit-to-visit lipid variability as measured by the intra-individual standard deviation. Lead SNPs as reported by Willer CJ, Schmidt EM, Sengupta S, et al. Discovery and refinement of loci associated with lipid levels. *Nat Genet.* 2013;45:1274-1283.

Supplemental Table 3. Lead SNPs for previously reported loci for HDL-C levels.

SNP	Chr.	Locus	LDL-C var.	HDL-C var.
rs10019888	4	C4orf52	0.19	0.2
rs11065987	12	BRAP	0.51	0.89
rs1121980	16	FTO	0.29	0.51
rs11246602	11	OR4C46	0.69	0.53
rs11613352	12	LRP1	0.48	0.43
rs11869286	17	STARD3	0.56	0.77
rs12145743	1	HDGF-PMVK	0.99	0.97
rs12328675	2	COBLL1	0.13	0.76
rs12678919	8	LPL	0.31	0.17
rs12748152	1	PIGV-NR0B2	0.12	0.44
rs12801636	11	KAT5	0.07	0.19
rs12967135	18	MC4R	0.41	0.27

Supplemental Table 3. Continued

SNP	Chr.	Locus	LDL-C var.	HDL-C var.
rs13076253	3	ACAD11	0.98	0.23
rs13107325	4	SLC39A8	0.54	0.01
rs13326165	3	STAB1	0.84	0.25
rs1367117	2	APOB	0.89	0.54
rs1532085	15	LIPC	0.14	0.78
rs1689800	1	ZNF648	0.04	0.84
rs16942887	16	LCAT	0.15	0.56
rs17145738	7	MLXIPL	0.65	0.78
rs17173637	7	TMEM176A	0.1	0.34
rs174546	11	FADS1-2-3	0.25	0.55
rs17695224	19	HAS1	0.55	0.9
rs1800961	20	HNF4A	0.03	0.57
rs181362	22	UBE2L3	0.33	0.63
rs1883025	9	ABCA1	0.39	0.78
rs1936800	6	RSPO3	0.42	0.86
rs2013208	3	RBM5	0.06	0.52
rs2255141	10	GPAM	0.42	0.38
rs2290547	3	SETD2	0.18	0.45
rs2293889	8	TRPS1	0.62	0.61
rs2412710	15	CAPN3	0.78	0.31
rs2602836	4	ADH5	0.04	0.91
rs2606736	3	ATG7	0.4	0.27
rs2652834	15	LACTB	0.62	0.69
rs2814982	6	C6orf106	0.89	0.22
rs2923084	11	AMPD3	0.09	0.88
rs2925979	16	CMIP	0.38	0.79
rs2954029	8	TRIB1	0.05	0.72
rs2972146	2	IRS1	0.59	0.05
rs3136441	11	LRP4	0.24	0.22

SNP	Chr.	Locus	LDL-C var.	HDL-C var.
rs3764261	16	CETP	0.05	0.48
rs3822072	4	FAM13A	0.46	0.58
rs386000	19	LILRA3	0.72	0.12
rs4129767	17	PGS1	0.52	0.35
rs4142995	7	SNX13	0.25	0.87
rs4148008	17	ABCA8	0.31	0.92
rs4420638	19	APOE	0.79	0.97
rs442177	4	KLHL8	0.8	0.25
rs4650994	1	ANGPTL1	0.69	0.27
rs4660293	1	PABPC4	0.49	0.06
rs4731702	7	KLF14	0.64	0.81
rs4759375	12	SBNO1	0.02	0.61
rs4765127	12	ZNF664	0.83	0.31
rs4846914	1	GALNT2	0.13	0.003
rs4917014	7	IKZF1	0.92	0.81
rs4983559	14	ZBTB42-AKT1	0.63	0.41
rs499974	11	MOGAT2-DGAT2	0.46	0.78
rs581080	9	TTC39B	0.94	0.88
rs605066	6	CITED2	0.45	0.27
rs6065906	20	PLTP	0.73	0.57
rs629301	1	SORT1	0.26	0.78
rs6450176	5	ARL15	0.55	0.8
rs645040	3	MSL2L1	0.65	0.95
rs6805251	3	GSK3B	0.97	0.39
rs702485	7	DAGLB	0.95	0.38
rs7134375	12	PDE3A	0.6	0.59
rs7134594	12	MVK	0.05	0.34
rs7241918	18	LIPG	0.52	0.96

Supplemental Table 3. Continued

SNP	Chr.	Locus	LDL-C var.	HDL-C var.
rs7255436	19	ANGPTL4	0.83	0.55
rs731839	19	PEPD	0.54	0.46
rs737337	19	ANGPTL8	0.16	0.81
rs7941030	11	UBASH3B	0.84	0.48
rs838880	12	SCARB1	0.27	0.95
rs964184	11	APOA1	0.003	0.37
rs9686661	5	MAP3K1	0.37	0.78
rs970548	10	MARCH8-ALOX5	0.54	0.49
rs998584	6	VEGFA	0.42	0.52
rs9987289	8	PPP1R3B	0.06	0.47

Data are presented as p-values for additive effects on visit-to-visit lipid variability as measured by the intra-individual standard deviation. Lead SNPs as reported by Willer CJ, Schmidt EM, Sengupta S, et al. Discovery and refinement of loci associated with lipid levels. *Nat Genet.* 2013;45:1274-1283.

9

PART IV

Summary

CHAPTER 10

Main findings and discussion

The overall aim of this thesis was to describe how both genetic and more conventional epidemiological endeavors may complement research into effects of statin therapy. Without question, the introduction of statins has provided health care providers with an effective and generally well-tolerated pharmacological intervention to reduce excess cardiovascular risk due to hypercholesterolemia. However, given the widespread use of statins, even small unintended drug-effects may lead to a significant number of adverse events at the population level (1). It therefore remains of importance to examine the validity and relevance of purported safety risks due to statin therapy.

A research approach which is increasingly used to help disentangle questions of causality – when randomized clinical trials are either impractical, unethical, or unlikely to demonstrate uncommon unintended effects of drugs – is Mendelian randomization (MR). However, as described in **Chapter 2**, there are key assumptions which must be considered when interpreting results from this type of study. The same holds true when drawing conclusions from pharmacogenetic studies, where different study designs and response phenotypes come with implicit underlying assumptions. In **Chapter 3** we highlighted popular study designs for inferring gene-treatment interaction effects of clinical effects of drug treatment and the challenges accompanying the use of case-only (which assumes that the genotype and drug treatment are not correlated in the population that gave rise to the cases) or treated-only (which assumes that the genotype will not cause changes in the phenotype in absence of the treatment) study designs. We also discussed that a major issue in pharmacogenetic research has been the poor reproducibility of promising signals, with low statistical power being a likely contributing factor. This underscores the need to collaborate with other research groups when contemplating genome-wide approaches to pharmacogenetic research. Therefore, to identify novel genetic loci for statin-induced proportional HDL-C response, we participated in a large-scale initiative by the GIST consortium. As described in **Chapter 4**, this effort included up to 27,720 statin-treated individuals from 20 studies, with results indicating that *CETP* is likely the only detectable locus with common variants of importance to HDL-C response to statin therapy in individuals of European descent. While we provided evidence that *CETP*'s effect is independent of baseline HDL-C levels, we also showed that genetic predisposition to HDL-C concentrations positively associates with HDL-C response to statin therapy. In essence, those genetically predisposed to having higher HDL-C levels respond more favourably to statin therapy, with regard to attaining higher HDL-C levels. In contrast, we showed in **Chapter 5** that those genetically predisposed to higher LDL-C concentrations tend to have

a lower differential LDL-C response, independent of their baseline LDL-C levels. Specifically, we estimated the effect to be a 5.4% (95%CI: 4.2–6.7) smaller statin response per standard deviation increase in genetically raised LDL-C concentrations. However, we should recognise that, given the low-cost threshold of remeasuring LDL-C concentrations and the relative ease of adjusting statin dosage, it is unlikely that this observation will alter current clinical practises. In **Chapter 6** we provided evidence that the presence of type 2 diabetes is unlikely to alter ones proportional LDL-C response to statin therapy. In addition, although we found no evidence to suggest that the proportional statin response influences the risk of developing new-onset type 2 diabetes, definitive assessment should be made in a population composed of statin-users.

It has furthermore been argued that MR studies might also be affected by selection bias. In **Chapter 7** we focused specifically on survival bias, a subtype of selection bias, which may occur when studies include older individuals who must necessarily have survived until study inclusion. By simulating simple causal structures, we showed that selecting on survival may decrease instrument strength. In addition, we observed that the results from MR studies may become increasingly biased when the selection gradient is larger. To what degree may however depend on both the distribution of the exposures as well as whether one- or two-sample MR approaches are undertaken.

In **Chapter 8** we examined whether the previously reported associations between visit-to-visit variability in LDL-C concentrations and vascular events also extend to neurocognitive outcomes. We observed that those individuals with greater intra-individual LDL-C variability are also those who tend to have worse cognitive test performance, lower cerebral blood flow, and greater white matter hyperintensity load. Of note, these associations did not markedly differ between statin and non-statin users. It is therefore likely that factors other than either non-adherence to statin therapy or known pleiotropic effects of statins can explain the previously reported findings in the literature. However, as demonstrated in **Chapter 9**, there still remain a great number of unanswered questions regarding the clinical relevance of visit-to-visit lipid variability. While circumstantial evidence suggests that lipid variability may in itself have causal effects on clinical outcomes, it is equally likely to be a risk marker for underlying disease processes. In addition, we should recognise that the current literature is highly heterogeneous with regard to the populations investigated and variability measures utilized. It also remains unclear whether genetic factors may be of importance to intra-individual lipid variability, though our explorative GWAS did not provide evidence of common variants of importance.

FUTURE PERSPECTIVES

The scope of genetic epidemiology is constantly shifting due to ongoing methodological development and ever-greater sample sizes, in part facilitated by ever-decreasing costs of genome-wide genotyping and whole-genome and -exome sequencing. The reference panels from the 1000 Genomes Project (2) and Haplotype Reference Consortium (3) have additionally allowed for denser genotype imputation of previously measured genome-wide SNP microarrays. Several projects using these reference panels are currently underway within the GIST consortium. However, while novel hits may be detected and previously detected signals refined, it has become clear that pharmacogenetic guidance of statin therapy is unlikely to be useful in clinical practice (4), though exploring possible pleiotropic effects of statins such as changes in CRP levels may still hold promise.

We are currently witnessing a near-exponential growth in MR studies in the literature, particularly of those taking a two-sample approach. This trend can be attributed to several factors, perhaps most significantly the increased availability of publicly available summary level datasets from large-scale GWAS combined with the development of methods to use this aggregated data in MR studies (5). Furthermore, while effects of common variants detected by GWAS tend to be small, increasing GWAS' sample sizes keep uncovering additional potential instruments, of which the combined strength may dwarf those of single instruments. National mega-biobanks such as the UK Biobank (6) and the China Kadoorie biobank (7) also offer tremendous possibilities to perform research with genotypic information on large numbers of participants. In addition, association studies using traits derived from next generation characterization and quantification of pools of biological molecules (e.g. proteomics (8), metabolomics (9, 10)) are providing candidate instruments whose biological function can more readily be interpreted due to the molecules being fairly proximal consequences of natural genetic variation (11).

There now exists an active community of methodologists, statisticians, and epidemiologists developing and applying novel extensions to, and sensitivity analyses for, the basic MR design (12). In recent years much of this effort has focused on the issue of pleiotropy (13-15), with several of these methods also being showcased in this thesis. A particularly laudable effort has been the creation of MR-base, a platform which links a curated database of publicly available GWAS results to both a web-app and R-packages that automate two-sample MR studies (16). Together with **i.** recent initiatives providing guidance to the undertaking and interpretation of MR studies (17-19), **ii.** the perspective

of future methods which might allow the inclusion of many hundreds of weak candidate instruments (20), and **iii.** the biennial MR conference in Bristol (https://www.mendelianrandomization.org.uk/), it is likely that the upsurge in MR studies will continue undiminished.

While these developments are promising, this thesis highlights potential problems and limitations of pharmacogenetic research, the MR approach, and applying MR research strategies to pharmacogenetic questions. It is particularly important that researchers recognize **i.** the implicit assumptions of pharmacogenetic study methodology lest they be violated, **ii.** that we often deal with selected study populations which may also affect the results of MR studies, and **iii.** that MR studies proposing genetic instruments for response phenotypes, as identified in pharmacogenetic research, are likely to only produce valid results under very specific conditions.

In addition, the recent developments in MR-methodology have also shone some light on commonly under-recognized assumptions of instrumental variable analyses with genetic instruments which already go beyond the findings and limitations described in this thesis. For instance, examination of the oft-made analogy between MR studies and randomized trials has shown that these designs differ in regards to key aspects. For example, the intervention under investigation in a randomized trial is often implemented for a short-term period at a particular point in the life course, e.g. rarely before adulthood in the case of statins, while MR studies aim to estimate causal effects of life-long interventions which theoretically 'switch on' at conception (21, 22). This also means that estimating period-specific effects in mid- or late-life are beyond the possibilities of MR, as effects of genotype on outcome will always represent the average effect from conception to end of follow-up. There is also increasing awareness that bias in MR studies may arise due to exposures varying over time (23). This is also relevant for statins, where dosage adjustment may be needed based on renal function or drug interactions. Moreover, it has been argued that instrumental variable analyses can only provide bounds when estimating a causal effect, with point estimates requiring additional unverifiable assumptions, and the relative ease with which assumptions of homogeneity and monotonicity are likely be violated in the context of MR studies (24). Finally, causal inference using data from large-scale biobanks of seemingly homogenous groups of individuals is likely to be more challenging than previously imagined (25, 26). Combined, these issues show that ample room exists for future methodological but also applied research in the field of MR, which will no doubt also benefit research into questions of causality for statin-associated outcomes.

REFERENCES

1. Rose G. Sick individuals and sick populations. Int J Epidemiol. 1985;14:32-38.
2. Genomes Project C, Auton A, Brooks LD, et al. A global reference for human genetic variation. Nature. 2015;526:68-74.
3. McCarthy S, Das S, Kretzschmar W, et al. A reference panel of 64,976 haplotypes for genotype imputation. Nat Genet. 2016;48:1279-1283.
4. Leusink M, Maitland-van der Zee AH, Ding B, et al. A genetic risk score is associated with statin-induced low-density lipoprotein cholesterol lowering. Pharmacogenomics. 2016;17:583-91.
5. Johnson T. Efficient calculation for multi-SNP genetic risk scores. Technical report, The Comprehensive R Archive Network, 2013. http://cran.r-project.org/web/packages/gtx/vignettes/ashg2012.pdf.
6. Fry A, Littlejohns TJ, Sudlow C, et al. Comparison of Sociodemographic and Health-Related Characteristics of UK Biobank Participants with the General Population. Am J Epidemiol. 2017;186:1026-1034.
7. Chen Z, Chen J, Collins R, et al. China Kadoorie Biobank of 0.5 million people: survey methods, baseline characteristics and long-term follow-up. Int J Epidemiol. 2011;40:1652-1666.
8. Sun BB, Maranville JC, Peters JE, et al. Genomic atlas of the human plasma proteome. Nature. 2018;558:73-79.
9. Shin SY, Fauman EB, Petersen AK, et al. An atlas of genetic influences on human blood metabolites. Nat Genet. 2014;46:543-550.
10. Kettunen J, Demirkan A, Wurtz P, et al. Genome-wide study for circulating metabolites identifies 62 loci and reveals novel systemic effects of LPA. Nat Commun. 2016;7:11122.
11. Swerdlow DI, Kuchenbaecker KB, Shah S, et al. Selecting instruments for Mendelian randomization in the wake of genome-wide association studies. Int J Epidemiol. 2016;45:1600-1616.
12. Zheng J, Baird D, Borges MC, et al. Recent Developments in Mendelian Randomization Studies. Curr Epidemiol Rep. 2017;4:330-345.
13. Bowden J, Davey Smith G, Burgess S. Mendelian randomization with invalid instruments: effect estimation and bias detection through Egger regression. Int J Epidemiol. 2015;44:512-525.
14. Hartwig FP, Davey Smith G, Bowden J. Robust inference in summary data Mendelian randomization via the zero modal pleiotropy assumption. Int J Epidemiol. 2017;46:1985-1998.
15. Bowden J, Davey Smith G, Haycock PC, et al. Consistent Estimation in Mendelian Randomization with Some Invalid Instruments Using a Weighted Median Estimator. Genet Epidemiol. 2016;40:304-314.
16. Hemani G, Zheng J, Elsworth B, et al. The MR-Base platform supports systematic causal inference across the human phenome. eLife. 2018. doi: https://doi.org/10.7554/eLife.34408.
17. Davies NM, Holmes MV, Davey Smith G. Reading Mendelian randomisation studies: a guide, glossary, and checklist for clinicians. BMJ. 2018;362:k601.

18. Haycock PC, Burgess S, Wade KH, et al. Best (but oft-forgotten) practices: the design, analysis, and interpretation of Mendelian randomization studies. Am J Clin Nutr. 2016;103:965-978.
19. Boef AG, Dekkers OM, Le Cessie S, et al. Reporting Instrumental Variable Analyses. Epidemiology. 2013;24:937-938.
20. Zhao Q, Wang J, Hemani G, et al. Statistical inference in two-sample summary-data Mendelian randomization using robust adjusted profile score. arXiv:1801.09652v2 [stat.AP]. 2018.
21. Swanson SA, Tiemeier H, Ikram MA, et al. Nature as a Trialist?: Deconstructing the Analogy Between Mendelian Randomization and Randomized Trials. Epidemiology. 2017;28:653-659.
22. Holmes MV, Ala-Korpela M, Davey Smith G. Mendelian randomization in cardiometabolic disease: challenges in evaluating causality. Nat Rev Cardiol. 2017;14:577-590.
23. Labrecque J, Swanson SA. Interpretation and potential biases of Mendelian randomization estimates with time-varying exposures. Am J Epidemiol. 2018. doi: 10.1093/aje/kwy204.
24. Swanson SA, Hernán MA, Miller M, et al. Partial Identification of the Average Treatment Effect Using Instrumental Variables: Review of Methods for Binary Instruments, Treatments, and Outcomes. Journal of the American Statistical Association. 2018;113:933-947.
25. Haworth S, Mitchell R, Corbin L, et al. Common genetic variants and health outcomes appear geographically structured in the UK Biobank sample: Old concerns returning and their implications. BioRxiv. 2018.
26. Munafo MR, Tilling K, Taylor AE, et al. Collider scope: when selection bias can substantially influence observed associations. Int J Epidemiol. 2018; 47:226-235.

APPENDICES

NEDERLANDSE SAMENVATTING

Een belangrijk doel van dit proefschrift was het beschrijven hoe zowel genetische als meer conventionele epidemiologische inspanningen onze kennis over effecten van statinetherapie kunnen aanvullen. Ongetwijfeld heeft de introductie van statines zorgverleners voorzien van een effectieve, en over het algemeen goed verdragen, farmacologische interventie om overmatig cardiovasculair risico als gevolg van hypercholesterolemie te verminderen. Gezien het wijdverspreide gebruik van statines kunnen echter kleine onbedoelde bijwerkingen leiden tot een aanzienlijk aantal ongewenste voorvallen op populatieniveau. Het blijft daarom van belang om de validiteit en relevantie van vermeende veiligheidsrisico's als gevolg van statinetherapie te onderzoeken.

Een onderzoeksbenadering die in toenemende mate wordt gebruikt om vraagstukken van causaliteit te ontrafelen - wanneer gerandomiseerde klinische onderzoeken ofwel onpraktisch of onethisch zijn, of maar moeilijk zeldzame bijwerkingen van medicatie kunnen aantonen - is Mendeliaanse randomisatie (MR). Zoals beschreven in **hoofdstuk 2** zijn er echter belangrijke assumpties waarmee rekening moet worden gehouden bij het interpreteren van resultaten van dit type onderzoek. Hetzelfde geldt voor het trekken van conclusies uit farmacogenetische studies, waarin verschillende onderzoeksontwerpen en responsfenotypen impliciete onderliggende assumpties hebben. In **Hoofdstuk 3** hebben we onderzoeksontwerpen besproken die populair zijn om gen-behandelings effect-modificatie met betrekking tot klinische effecten van medicamenteuze behandeling aan te tonen, en de uitdagingen besproken die volgen uit het gebruik van *case-only* (waarin wordt verondersteld dat genotype en de medicamenteuze behandeling niet gecorreleerd zijn in de populatie die aanleiding gaf tot de cases) en *treated-only* (waarbij wordt aangenomen dat het genotype geen veranderingen in het fenotype zal veroorzaken in afwezigheid van de behandeling) onderzoeksontwerpen. We bespraken daarnaast dat een groot probleem in farmacogenetisch onderzoek de slechte reproduceerbaarheid is, waaraan een tekort aan statistische power veelal bijdraagt. Dit onderstreept de noodzaak om samen te werken met andere onderzoeksgroepen bij genoom-brede benaderingen van farmacogenetisch onderzoek. Om deze reden namen we deel aan een grootschalig initiatief van het GIST-consortium om genetische loci voor statine-geïnduceerde proportionele HDL-C-respons te identificeren. Zoals beschreven in **hoofdstuk 4** includeerde dit project tot wel 27,720 met statine behandelde personen uit 20 studies. De resultaten tonen aan dat *CETP* waarschijnlijk de enige detecteerbare locus is

met veel voorkomende varianten die van belang zijn voor de HDL-C respons op statinetherapie bij personen van Europese afkomst. Hoewel we bewijs leveren dat het *CETP*-effect onafhankelijk is van baseline HDL-C concentraties tonen we ook aan dat genetische predispositie voor HDL-C concentraties positief associeert met de HDL-C respons op statinetherapie. In essentie reageren diegenen die een genetische aanleg hebben voor hogere HDL-C concentraties gunstiger op statinetherapie, wat betreft het bereiken van hogere HDL-C concentraties. In **Hoofdstuk 5** laten we zien dat degenen met een genetische aanleg voor hogere LDL-C concentraties een lagere proportionele LDL-C-respons hebben, onafhankelijk van hun LDL-C waarden op baseline. Onze beste schatting van dit effect was een 5.4% (95% CI: 4.2-6.7) kleinere statine-respons per standaarddeviatie toename in genetisch verhoogde LDL-C concentraties. We moeten echter erkennen dat, gezien het relatieve gemak van het opnieuw meten van LDL-C concentraties en bijstellen van de statinedosering, het onwaarschijnlijk is dat deze waarneming de huidige klinische praktijk zal veranderen. In **Hoofdstuk 6** hebben we aangetoond dat het onwaarschijnlijk is dat de aanwezigheid van type 2 diabetes de proportionele LDL-C respons op statine-therapie zal beïnvloeden. Alhoewel we geen aanwijzingen vonden dat de proportionele statine-respons een effect heeft op het risico op het ontwikkelen van diabetes type 2, moet een definitieve beoordeling plaatsvinden in een populatie die volledig is samengesteld uit statine-gebruikers.

Verder is aangevoerd dat MR-studies ook beïnvloedt kunnen worden door selectiebias. In **Hoofdstuk 7** hebben we ons specifiek gericht op overlevingsbias, een subtype van selectiebias, wat kan voorkomen wanneer studies oudere personen includeren die noodzakelijkerwijs moeten hebben overleefd tot de studie is gestart. Door eenvoudige causale structuren te simuleren hebben we aangetoond dat selecteren op overleving de instrumentsterkte kan verminderen. Daarnaast hebben we vastgesteld dat de resultaten van MR-studies mogelijk meer vertekend worden wanneer de selectiegradiënt groter is. De ernst van deze vertekening is echter zowel afhankelijk van de verdeling van de onderzochte risicofactoren als van de keuze voor de één- of twee-sample setting.

In **Hoofdstuk 8** hebben we onderzocht of de eerder gerapporteerde associaties tussen intra-individuele variabiliteit in LDL-C concentraties en vasculaire events zich ook generaliseren tot neurocognitieve uitkomsten. We hebben vastgesteld dat die personen met een grotere intra-individuele LDL-C variabiliteit ook degenen zijn die slechter presteren op cognitieve testen, een lagere cerebrale bloedstroom hebben en meer witte stof afwijkingen hebben. Van belang is dat

deze associaties niet verschilden tussen statine- en niet-statine-gebruikers. Het is daarom waarschijnlijk dat andere factoren dan therapie(on)trouw of pleiotrope effecten van statines de eerder gerapporteerde bevindingen in de literatuur kunnen verklaren. Zoals echter aangetoond in **hoofdstuk 9** zijn er nog steeds een groot aantal onbeantwoorde vragen met betrekking tot de klinische relevantie van intra-individuele variabiliteit van lipiden. Hoewel indirect bewijs suggereert dat deze variabiliteit causale effecten op klinische uitkomsten kan hebben, is het even waarschijnlijk een risicomarker voor onderliggende ziekteprocessen. Bovendien moeten we erkennen dat de huidige literatuur zeer heterogeen is wat betreft de onderzochte populaties en maten van variabiliteit. Het blijft daarnaast onduidelijk of genetische factoren van belang kunnen zijn voor intra-individuele variabiliteit van lipiden, hoewel onze exploratieve GWAS geen bewijs leverde dat veel voorkomende genetische varianten van belang zijn.

Toekomstperspectieven

De genetische epidemiologie is continu in beweging als gevolg van de voortdurende methodologische ontwikkeling en toenemende studiegroottes, deels gefaciliteerd door de alsmaar dalende kosten van genoom-brede genotypering en whole-genome en -exome sequencing. De referentiepanels van het 1000 Genomes Project en het Haplotype Reference Consortium hebben bovendien een dichtere genotype-imputatie van eerder gemeten genoom-brede SNP-microarrays mogelijk gemaakt. Verschillende projecten die gebruik maken van deze referentiepanels zijn momenteel lopend binnen het GIST-consortium. Hoewel dit onderzoekers toestaat nieuwe hits te identificeren en eerder gedetecteerde signalen te verfijnen, is het duidelijk geworden dat “farmacogenetische sturing” van statinetherapie niet nuttig is in de klinische praktijk. Dit neemt niet weg dat het onderzoeken van mogelijke pleiotrope effecten van statines zoals veranderingen in CRP-niveaus nog steeds veelbelovend kan zijn.

We zijn momenteel getuige van een bijna exponentiële groei van MR-onderzoek in de literatuur, grotendeels bestaande uit studies die een twee-sample benadering volgen. Deze trend kan worden toegeschreven aan verschillende factoren. Wellicht de meest significante factor is de toegenomen beschikbaarheid van publiekelijk beschikbare datasets van grootschalige GWAS, in combinatie met de ontwikkeling van methoden om deze geaggregeerde gegevens in MR-studies te gebruiken. Hoewel de effecten van veelvoorkomende varianten die in GWAS worden gevonden doorgaans klein zijn leveren de toenames in studiegrootte nog steeds additionele potentiële instrumenten op, waarvan de gecombineerde sterkte die van afzonderlijke instrumenten dikwijls overtreft. Nationale mega-

biobanken zoals de UK Biobank en de China Kadoorie Biobank bieden ook enorme mogelijkheden om onderzoek met genotypische informatie uit te voeren bij grote aantallen deelnemers. Associatiestudies gebruikmakend van *next-generation* karakterisering en kwantificatie van biologische moleculen (bijv. proteomics, metabolomics) leveren tevens potentiele instrumenten op waarvan de biologische functie makkelijker kan worden geïnterpreteerd, omdat de moleculen proximale gevolgen van natuurlijke genetische variatie zijn.

Er bestaat nu een actieve gemeenschap van methodologen, statistici en epidemiologen die uitbreidingen en sensitiviteitsanalyses ontwikkelen voor het basis MR-studieontwerp. In de afgelopen jaren is veel van hun inspanningen gericht geweest op de kwestie van pleiotropie. Verschillende van deze methoden zijn ook belicht in dit proefschrift. Een bijzonder lovenswaardige inspanning was de oprichting van MR-base, een platform dat een database van publiek beschikbare GWAS-resultaten koppelt aan zowel een web-app als R-pakketten die two-sample MR-analyses automatiseren. Tezamen met **i.** recente initiatieven die richting geven aan de uitvoering en interpretatie van MR-studies, **ii.** het perspectief op toekomstige methoden die het gebruik van vele honderden zwakke kandidaat-instrumenten mogelijk maken, en **iii.** de tweejaarlijkse MR-conferentie in Bristol (https://www.mendelianrandomization.org.uk/), is het aannemelijk dat de opleving van MR-studies onverminderd zal doorgaan.

Hoewel deze ontwikkelingen veelbelovend zijn, worden in dit proefschrift mogelijke problemen en beperkingen van farmacogenetisch onderzoek, de MR-benadering en MR-onderzoeksstrategieën voor farmacogenetische vragen behandeld. Het is bijzonder belangrijk dat onderzoekers zich bewust worden van **i.** de impliciete assumpties van farmacogenetische studiemethoden, **ii.** dat we vaak te maken hebben met geselecteerde onderzoekspopulaties die ook de resultaten van MR-studies kunnen beïnvloeden, en **iii.** dat MR-studies die genetische instrumenten voor responsfenotypen gebruiken, zoals geïdentificeerd in farmacogenetisch onderzoek, waarschijnlijk alleen onder zeer specifieke omstandigheden valide resultaten zullen opleveren.

Daarnaast hebben de recente ontwikkelingen in MR-methoden ook licht geworpen op veelal onderkende aannames van instrumentele variabele analyses met genetische instrumenten die reeds verder gaan dan de bevindingen en beperkingen die in dit proefschrift worden beschreven. Zo heeft onderzoek naar de vaak gemaakte analogie tussen MR-studies en RCT's aangetoond dat deze ontwerpen verschillen met betrekking tot belangrijke aspecten. Interventies die in gerandomiseerde studies worden onderzocht worden vaak geïmplementeerd voor een korte periode op een bepaald moment in de levensloop, zelden voor de

middelbare leeftijd in het geval van statines, terwijl MR-studies gericht zijn op het schatten van de causale effecten van levenslange interventies die theoretisch 'aangezet worden' bij de conceptie. Dit betekent ook dat het schatten van periode-specifieke effecten in het midden of het late leven de mogelijkheden van MR te boven gaat, omdat geschatte effecten van genotype op een uitkomst altijd het gemiddelde effect zullen weergeven van de conceptie tot het einde van de follow-up. Er is ook een toenemend besef dat bias in MR-studies kan ontstaan als blootstellingen in de loop van de tijd veranderen. Dit is ook relevant voor statines, waar dosisaanpassing nodig kan zijn op basis van de nierfunctie of geneesmiddelinteracties. Bovendien is beargumenteerd dat instrumentele variabele analyses alleen grenzen (bounds) kunnen geven bij het schatten een causaal effect, doordat puntschattingen extra niet-verifieerbare assumpties vereisen, en het relatieve gemak waarmee assumpties van homogeniteit en monotoniciteit waarschijnlijk worden geschonden in de context van MR-studies. Ten slotte is causale inferentie op basis van gegevens van grootschalige biobanken van schijnbaar homogene groepen individuen waarschijnlijk een grotere uitdaging dan eerder werd gedacht. Tezamen tonen deze kwesties aan dat er ruimte is voor toekomstig methodologisch maar ook toegepast onderzoek op het gebied van MR, wat ongetwijfeld het onderzoek naar statine gerelateerde uitkomsten ook ten goede zal komen.

LIST OF PUBLICATIONS

1 **Smit RAJ**, Coebergh JA, Treurniet FE, Kuiters RR, Koppen H. A missing arch of the atlas vertebra in a patient struck by a tram. Neurol Sci. 2013;34(10):1855-6.

2 **Smit RAJ**, Trompet S, de Craen AJ, Jukema JW. Using genetic variation for establishing causality of cardiovascular risk factors: overcoming confounding and reverse causality. Neth Heart J. 2014;22(4):186-9.

3 **Smit RAJ**, Treurniet FE, Koppen H. Trigeminal autonomic headaches caused by a multinodular goiter--a case report. Headache. 2014;54(8):1369-70.

4 Moonen JE, Bertens AS, Foster-Dingley JC, **Smit RAJ**, van der Grond J, de Craen AJ, de Ruijter W, van der Mast RC. Lower blood pressure and apathy coincide in older persons with poorer functional ability: the Discontinuation of Antihypertensive Treatment in Elderly People (DANTE) Study Leiden. J Am Geriatr Soc. 2015;63(1):112-7.

5 Trompet S, Postmus I, Slagboom PE, Heijmans BT, **Smit RAJ**, Maier AB, Buckley BM, Sattar N, Stott DJ, Ford I, Westendorp RG, de Craen AJ, Jukema JW. Non-response to (statin) therapy: the importance of distinguishing non-responders from non-adherers in pharmacogenetic studies. Eur J Clin Pharmacol. 2016;72(4):431-7.

6 **Smit RAJ**, Trompet S, Sabayan B, le Cessie S, van der Grond J, van Buchem MA, de Craen AJ, Jukema JW. Higher Visit-to-Visit Low-Density Lipoprotein Cholesterol Variability Is Associated With Lower Cognitive Performance, Lower Cerebral Blood Flow, and Greater White Matter Hyperintensity Load in Older Subjects. Circulation. 2016;134(3):212-21.

7 Ogliari G, **Smit RAJ**, Westendorp RG, Jukema JW, de Craen AJ, Sabayan B. Visit-to-visit blood pressure variability and future functional decline in old age. J Hypertens. 2016;34(8):1544-50.

8 Yaghootkar H, Lotta LA, Tyrrell J, **Smit RAJ**, Jones SE, Donnelly L, Beaumont R, Campbell A, Tuke MA, Hayward C, Ruth KS, Padmanabhan S, Jukema JW, Palmer CC, Hattersley A, Freathy RM, Langenberg C, Wareham NJ, Wood AR, Murray A, Weedon MN, Sattar N, Pearson E, Scott RA, Frayling TM. Genetic Evidence for a Link Between Favorable Adiposity and Lower Risk of Type 2 Diabetes, Hypertension, and Heart Disease. Diabetes. 2016;65(8):2448-60.

9 **Smit RAJ**, Postmus I, Trompet S, Barnes MR, Warren H, Arsenault BJ, Chasman DI, Cupples LA, Hitman GA, Krauss RM, Li X, Psaty BM, Stein CM, Rotter JI, Jukema JW. Rooted in risk: genetic predisposition for low-density lipoprotein cholesterol level associates with diminished low-density lipoprotein cholesterol response to statin treatment. Pharmacogenomics. 2016;17(15):1621-1628.

10 Noordam R, **Smit RAJ**, Postmus I, Trompet S, van Heemst D. Assessment of causality between serum gamma-glutamyltransferase and type 2 diabetes mellitus using publicly available data: a Mendelian randomization study. Int J Epidemiol. 2016;45(6):1953-1960.

11 Postmus I, Warren HR, Trompet S, Arsenault BJ, Avery CL, Bis JC, Chasman DI, de Keyser CE, Deshmukh HA, Evans DS, Feng Q, Li X, **Smit RAJ**, Smith AV, Sun F, Taylor KD, Arnold AM, Barnes MR, Barratt BJ, Betteridge J, Boekholdt SM, Boerwinkle E, Buckley BM, Chen YI, de Craen AJ, Cummings SR, Denny JC, Dubé MP, Durrington PN, Eiriksdottir G, Ford I, Guo X, Harris TB, Heckbert SR, Hofman A, Hovingh GK,

Kastelein JJ, Launer LJ, Liu CT, Liu Y, Lumley T, McKeigue PM, Munroe PB, Neil A, Nickerson DA, Nyberg F, O'Brien E, O'Donnell CJ, Post W, Poulter N, Vasan RS, Rice K, Rich SS, Rivadeneira F, Sattar N, Sever P, Shaw-Hawkins S, Shields DC, Slagboom PE, Smith NL, Smith JD, Sotoodehnia N, Stanton A, Stott DJ, Stricker BH, Stürmer T, Uitterlinden AG, Wei WQ, Westendorp RG, Whitsel EA, Wiggins KL, Wilke RA, Ballantyne CM, Colhoun HM, Cupples LA, Franco OH, Gudnason V, Hitman G, Palmer CN, Psaty BM, Ridker PM, Stafford JM, Stein CM, Tardif JC, Caulfield MJ, Jukema JW, Rotter JI, Krauss RM. Meta-analysis of genome-wide association studies of HDL cholesterol response to statins. J Med Genet. 2016;53(12):835-845.

12 de Vries MA, Trompet S, Mooijaart SP, **Smit RAJ**, Böhringer S, Castro Cabezas M, Jukema JW. Complement receptor 1 gene polymorphisms are associated with cardiovascular risk. Atherosclerosis. 2017;257:16-21.

13 Ogliari G, **Smit RAJ**, van der Spoel E, Mari D, Torresani E, Felicetta I, Lucchi TA, Rossi PD, van Heemst D, de Craen AJ, Westendorp RG. Thyroid Status and Mortality Risk in Older Adults With Normal Thyrotropin: Sex Differences in the Milan Geriatrics 75+ Cohort Study. J Gerontol A Biol Sci Med Sci. 2017;72(4):554-559.

14 Bos MM, **Smit RAJ**, Trompet S, van Heemst D, Noordam R. Thyroid Signaling, Insulin Resistance, and 2 Diabetes Mellitus: A Mendelian Randomization Study. J Clin Endocrinol Metab. 2017;102(6):1960-1970.

15 Zewinger S, Kleber ME, Tragante V, McCubrey RO, Schmidt AF, Direk K, Laufs U, Werner C, Koenig W, Rothenbacher D, Mons U, Breitling LP, Brenner H, Jennings RT, Petrakis I, Triem S, Klug M, Filips A, Blankenberg S, Waldeyer C, Sinning C, Schnabel RB, Lackner KJ, Vlachopoulou E, Nygård O, Svingen GFT, Pedersen ER, Tell GS, Sinisalo J, Nieminen MS, Laaksonen R, Trompet S, **Smit RAJ**, Sattar N, Jukema JW, Groesdonk HV, Delgado G, Stojakovic T, Pilbrow AP, Cameron VA, Richards AM, Doughty RN, Gong Y, Cooper-DeHoff R, Johnson J, Scholz M, Beutner F, Thiery J, Smith JG, Vilmundarson RO, McPherson R, Stewart AFR, Cresci S, Lenzini PA, Spertus JA, Olivieri O, Girelli D, Martinelli NI, Leiherer A, Saely CH, Drexel H, Mündlein A, Braund PS, Nelson CP, Samani NJ, Kofink D, Hoefer IE, Pasterkamp G, Quyyumi AA, Ko YA, Hartiala JA, Allayee H, Tang WHW, Hazen SL, Eriksson N, Held C, Hagström E, Wallentin L, Åkerblom A, Siegbahn A, Karp I, Labos C, Pilote L, Engert JC, Brophy JM, Thanassoulis G, Bogaty P, Szczeklik W, Kaczor M, Sanak M, Virani SS, Ballantyne CM, Lee VV, Boerwinkle E, Holmes MV, Horne BD, Hingorani A, Asselbergs FW, Patel RS, Krämer BK, Scharnagl H, Fliser D, März W, Speer T. Relations between lipoprotein(a) concentrations, LPA genetic variants, and the risk of mortality in patients with established coronary heart disease: a molecular and genetic association study. Lancet Diabetes Endocrinol. 2017;5(7):534-543.

16 **Smit RAJ**, Jukema JW, Trompet S. Increasing HDL-C levels with medication: current perspectives. Curr Opin Lipidol. 2017;28(4):361-366.

17 Zhao W, Rasheed A, Tikkanen E, Lee JJ, Butterworth AS, Howson JMM, Assimes TL, Chowdhury R, Orho-Melander M, Damrauer S, Small A, Asma S, Imamura M, Yamauch T, Chambers JC, Chen P, Sapkota BR, Shah N, Jabeen S, Surendran P, Lu Y, Zhang W, Imran A, Abbas S, Majeed F, Trindade K, Qamar N, Mallick NH, Yaqoob Z, Saghir T, Rizvi SNH, Memon A, Rasheed SZ, Memon FU, Mehmood K, Ahmed N, Qureshi IH, Tanveer-Us-Salam, Iqbal W, Malik U, Mehra N, Kuo JZ, Sheu WH, Guo X, Hsiung CA, Juang JJ, Taylor KD, Hung YJ, Lee WJ, Quertermous T, Lee IT, Hsu CC, Bottinger EP, Ralhan S, Teo YY, Wang TD, Alam DS, Di Angelantonio

E, Epstein S, Nielsen SF, Nordestgaard BG, Tybjaerg-Hansen A, Young R, Benn M, Frikke-Schmidt R, Kamstrup PR, Jukema JW, Sattar N, **Smit RAJ**, Chung RH, Liang KW, Anand S, Sanghera DK, Ripatti S, Loos RJF, Kooner JS, Tai ES, Rotter JI, Chen YI, Frossard P, Maeda S, Kadowaki T, Reilly M, Pare G, Melander O, Salomaa V, Rader DJ, Danesh J, Voight BF, Saleheen D. Identification of new susceptibility loci for type 2 diabetes and shared etiological pathways with coronary heart disease. Nat Genet. 2017;49(10):1450-1457.

18 de Gelder J, Haenen E, Lucke JA, Klop HG, Blomaard LC, **Smit RAJ**, Mesri K, de Groot B, Fogteloo AJ, Anten S, Blauw GJ, Mooijaart SP. Optimising the ISAR-HP to screen efficiently for functional decline in older patients. Neth J Med. 2017;75(9):379-385.

19 **Smit RAJ**, Jukema JW, Postmus I, Ford I, Slagboom PE, Heijmans BT, Le Cessie S, Trompet S. Visit-to-visit lipid variability: Clinical significance, effects of lipid-lowering treatment, and (pharmaco) genetics. J Clin Lipidol. 2018;12(2):266-276.e3.

20 **Smit RAJ**, Noordam R, le Cessie S, Trompet S, Jukema JW. A critical appraisal of pharmacogenetic inference. Clin Genet. 2018;93(3):498-507.

21 Noordam R, Oudt CH, Bos MM, **Smit RAJ**, van Heemst D. High-sensitivity C-reactive protein, low-grade systemic inflammation and type 2 diabetes mellitus: A two-sample Mendelian randomization study. Nutr Metab Cardiovasc Dis. 2018 doi: 10.1016/j.numecd.2018.03.008.

22 Neeland IJ, Boone SC, Mook-Kanamori DO, Ayers C, **Smit RAJ**, Tzoulaki I, Karaman I, Boulange C, Vaidya D, Punjabi N, Allison M, Herrington DM, Jukema JW, Rosendaal FR, Lamb HJ, Willems van Dijk K, Greenland P, de Mutsert R. Metabolomics Profiling of Visceral Adipose Tissue: Results from the Multi-Ethnic Study of Atherosclerosis and Netherlands Epidemiology of Obesity Study. J Am Heart Assoc. 2019 [in press]

CURRICULUM VITAE

Roelof Adriaan Johan Smit was born on the 13th of June 1985 in Delft, the Netherlands. He is the eldest son of Herman Smit and Ans van Reuler, and brother of Erwin and Wouter. He lived abroad for much of his childhood (Nigeria (Lagos), New Zealand (New Plymouth), Brunei Darussalam (Seria)). After graduating from secondary school (Maerlant Lyceum, The Hague) in 2003 he started studying Psychology at Leiden University.

In 2013 he obtained his medical degree from Leiden University and started as a PhD student at the Departments of Cardiology and Gerontology and Geriatrics of the Leiden University Medical Center (LUMC), under supervision of Dr. S. Trompet, Dr. A.J.M. de Craen†, Prof. dr S. le Cessie and Prof. dr J.W. Jukema. This thesis describes the results of this PhD-project. During his PhD he was accepted to the SMBWO Epidemiology B training programme at the Department of Clinical Epidemiology of the LUMC (head: Prof. dr F.R. Rosendaal). In 2017 he received the Young Talent grant of the national ENERGISE consortium (primary applicant: Dr. ir R. de Mutsert).

In 2019 he was awarded the NWO/ZonMW Rubicon grant, which will enable him to gain research experience in the research group of Prof. dr Ruth Loos at the Icahn School of Medicine at Mount Sinai in New York City during a period of two years.

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