ADVANCEMENTS IN THE FIELD OF CARDIOVASCULAR DISEASE

PHARMACOGENETICS
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PHARMACOGENETICS

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ABSTRACT

Background and Objectives: Pharmacogenetics has the potential to maximize drug efficacy and minimize adverse effects of cardiovascular disease (CVD) but its translation into clinical practice has been slow. However, recent advancements in genotyping and statistical methodologies have now provided robust evidence in the support of personalized medicine. This thesis addresses how the advancements in pharmacogenetics may help to gain novel insights into existing drug targets, inform and guide clinical decision-making and validate potential disease target pathways.

Methods: This was achieved by exploring whether the COX-2 genetic variant (rs20417) is associated with a decreased risk of CVD outcomes, assessing whether bile acid sequestrants (BAS) are associated with a reduced the risk of coronary artery disease (CAD) using the principles of Mendelian Randomization and investigating whether genetic variants associated with dysglycaemia are associated with an increased risk of CAD.

Results: We demonstrated that COX-2 carrier status was associated with a decreased risk of major cardiovascular outcomes. Furthermore, we also showed that BAS appear to be associated with a reduced risk of CAD and genetic variants associated with HbA1c and diabetes were associated with an increased risk of CAD.

Conclusions: The convergence of technological and statistical advancements in pharmacogenetics have led to a more high-quality and cost-effective means of assessing the effect of CVD therapeutic agents.
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**LIST OF ABBREVIATIONS**

CVD = Cardiovascular disease

NGS = Next generation sequencing

CAD = Coronary artery disease

GWAS = Genome-wide association studies

COX-2 = Cyclo-oxygenase-2

SNP = single nucleotide polymorphism

RCT = randomized controlled trials

ABCG5/8= ATP-binding cassette gene 5 and 8

PK = Pharmacokinetics

PD = Pharmacodynamics

OR = Odds ratio

CI = Confidence interval

INR = International Normalized Ratio

CYP = cytochrome P450

VKORC1= Vitamin K epoxide reductase complex, subunit 1 gene

HCV = Hepatitis C virus

SVR = Sustained virological response

HLA-C = Human leukocyte antigen

KIR = Killer immunoglobulin-like receptors

AHS = Abacavir Hypersensitivity Syndrome

HLA-B = Histocompatibility complex class I allele
PREDICT-1 = Prospective Randomized Evaluation of DNA screening in a Clinical Trial
COAG = Clarification of Optimal Anticoagulation through Genetics Study
STRENGTH = Statin Response Examined by Genetic Haplotype Markers Study
LDL-C = low-density lipoprotein cholesterol
LD = Linkage disequilibrium
PCSK9 = Proprotein convertase subtilisin/kexin type 9 gene
DNA = Deoxyribonucleic acid
FDR = false discovery rate
ACTIVE-A = Atrial Fibrillation Clopidogrel Trial With Irbesartan for Prevention of Vascular Events Study
CURE = Clopidogrel in Unstable Angina to Prevent Recurrent Events (CURE) Study
epiDREAM/DREAM = Diabetes Reduction Assessment with Ramipril and Rosiglitazone Medication (DREAM) Study/ epiDREAM
ONTARGET = Ongoing Telmisartan Alone and in combination with Ramipril Global Endpoint Trial
RE-LY = Randomized Evaluation of Long-Term Anticoagulation Therapy (RE-LY) Study
WGHS = Women Genome Health Study
MARS = Mechanisms of Aspirin Resistance (MARS Study) Study
BAS = Bile acid sequestrants
CARDIoGRAMplusC4D = Coronary ARtery DIsease Genome-wide Replication and Meta-analysis Consortium study
HDL-C = High density lipoprotein cholesterol
TC = Total cholesterol

apoA = Apolipoprotein A-1

apoB = Apolipoprotein B

MI = Myocardial infarction

CTT = Cholesterol Treatment Trialists' Collaboration

LRCCPPT = Lipid Research Clinics Coronary Primary Prevention Trial

HbA1c= Glycated hemoglobin

FG = Fasting glucose

MAGIC = Meta-Analyses of Glucose and Insulin-related traits Consortium study

DIAGRAM = DIAbetes Genetics Replication and Meta-analysis Consortium study

SBP = Systolic blood pressure

DBP = Diastolic blood pressure

ICBP = International Consortium for Blood Pressure GWAS

BMI = Body mass index

GIANT = Genetic Investigation of ANthropometric Traits GWAS

ERFC = Emerging Risk Factor Collaboration

HR = Hazard ratio

MeRP = Mendelian Randomization Pipeline
DECLARATION OF ACADEMIC ACHIEVEMENT

I was the main contributor and the first author for all studies included in this thesis. The details of all contributing authors’ are provided at the end of each study.
CHAPTER 1

Introduction

Cardiovascular disease (CVD) is one of the most common causes of mortality and morbidity worldwide. It is widely recognized that there is much intra-individual variability in the response to CVD drug therapies (1). Several factors contribute to this variability, including drug adherence, drug interactions and genetic polymorphisms (2;3). Pharmacogenetics is the study of genetic variability in drug response. The primary goal of pharmacogenetics is to use the effect of genetic polymorphisms to optimize drug therapy in order to reduce the risk of adverse cardiovascular events. Therefore a better understanding of the genetic determinants of cardiovascular medications may ultimately lead to personalized tailoring of these therapeutic agents.

The adoption of pharmacogenetics into clinical practice has been slow due to the lack of replication amongst previous published studies (4;5). Although there are several statistical and methodological explanations for these sometimes inconsistent findings (1), the lack of replication is primarily due to inadequate sample sizes and the selection of genetic variants used in candidate gene studies. Pharmacogenetic studies require large sample sizes because they must be powered to detect common variants with relatively low effect sizes or rare variants with large effect sizes (6;7). Furthermore, the candidate genetic polymorphism should have a known functional effect on the metabolism, transportation or targeting of the therapeutic agent and it should be strongly associated with a clinical response or outcome as documented in animal
model studies or in pharmacokinetic and pharmacodynamics studies. Thus by ensuring adequate sample sizes and a strong understanding of the molecular mechanisms of the underlying drug response allows for more precise estimation of the pharmacogenetic association.

Over the past decade there has been a wide scale accumulation of genetic data, owing to decreasing genotyping costs and new developments in high throughput technologies, such as exome sequencing and Next generation sequencing (NGS). These technologies allow for faster, cost-effective and more targeted sequencing of the whole genome with the potential for the discovery of novel and low-frequency genes associated with CVD(8). In addition, large biobanks and national and international data consortia have been developed to provide freely available genetic datasets, which consist of harmonized phenotypes and genotype variables from several prospective cohort studies. For example, the CARDIoGRAMplusC4D performed a meta-analysis of 63,746 cases of coronary artery disease (CAD) and 130,681 controls to identify genetic variants associated with the risk of CAD and myocardial infarction(9). These large datasets provide enough power to detect genetic associations that may have not reached genome-wide significance in underpowered genome-wide association studies (GWAS), as well as the ability to identify additional loci in pathways underlying the pathogenesis of CAD and potential drug targets.

In light of the accumulation of information from genetic data consortia, researchers now have a greater ability to account for the conflicting findings among published pharmacogenetic studies. Initially, many reported pharmacogenetic associations have not been replicated in larger patient populations with the use of stringent statistical parameters(10;11). It is most likely that these
initial pharmacogenetic studies overestimated the effect size of the genetic variant due to limited sample size or poorly characterized functional genetic variants (7). For instance, the effect of cyclo-oxygenase-2 (COX-2) enzyme in CVD is uncertain owing to the adverse effects reported in randomized controlled trials (RCTs) of the selective COX-2 inhibitor (14;15) and conflicting reports from animal model studies (12;13). However, by using data from 49,232 participants to assess the effect of the rs20417 single nucleotide polymorphism (SNP) (COX-2), which encodes for the COX-2 enzyme and has been associated with a decrease in COX-2 activity and reduced risk of CVD outcomes, the protective role of COX-2 was confirmed. Therefore improved access to large datasets with high genome coverage provides enough statistical power to confirm previously reported drug targets. Furthermore, revisiting these associations helps to enhance our understanding of how these genetic variants contribute to the pathogenesis of CVD and identifying potential drug targets.

As a result of the vast amount of data generated from genetic consortia, there is now a need to develop novel statistical methodologies in order to analyze and interpret these datasets. For instance, genetic variants may provide a useful instrument in assessing potential causal relationships reported in observational studies. Specifically, Mendelian Randomization analyses use genetic associations to explore the effects of modifiable exposures on outcomes based on the principle that genetic variants are randomly allocated at meiosis and are not influenced by factors that may bias observational associations, such as confounding and reverse causation (16). Thus by adopting the principles of Mendelian Randomization analyses enables us to infer the strength of the causal association reported in observational studies.
The principles of Mendelian Randomization may also be applied for drug target validation in order to guide treatment decisions in the absence of evidence from randomized trials. This approach helps to strengthen the rationale for conducting an RCT (16) because it is highly cost-effective due to the availability of genetic data through large-scale biobanks and data consortia. Furthermore, the random allocation of genetic variants replicates the double-blinded randomization process used in RCTs and carriers of an effect allele represent lifelong differences in exposure levels in comparison to their non-carriers counterparts unlike RCTs, where the length of the exposure is typically restricted to several years of follow-up. Drug target validation may be achieved by using functional alleles of a gene within a drug target pathway to extrapolate the effects of a therapeutic agent (17;18). For instance, bile acid sequestrants (BAS) are a widely prescribed intestinal cholesterol absorption agent yet the effects of BAS on the risk of CAD in uncertain. By applying the principles of Mendelian Randomization to explore the effect of BAS on the risk of CAD, we assessed whether the rs4299376 SNP (ABCG5/8) was associated with CVD outcomes. Thus Mendelian Randomization analysis may help to distinguish on-target and off-target actions of therapeutic interventions.

Another advantage of using a Mendelian Randomization analysis is it may resolve conflicting finding between observational studies and clinical trials and helps to identify potential disease target pathways. For instance, observational studies have shown that diabetes is associated with increased risk of CAD while elevated levels of glycated hemoglobin (HbA1c) and fasting glucose are modestly associated with CAD (19;20). In contrast, many glucose lowering clinical trials have not showed an effect on the risk of CAD. Thus we used data from large genetic epidemiology studies and applied the principles of Mendelian randomization to confirm the role of
dysglycemia in CAD. We reported that SNPs associated with diabetes and HbA1c appeared to be associated with the risk of CAD. Thus Mendelian Randomization studies help to determine which biomarkers are causally associated with CVD, and in turn, help to inform which disease pathways should be targeted.

The objectives of this thesis was to assess whether the recent advancements in pharmacogenetics allows for the confirmation of existing drug targets, informs clinical decision-making and validates potential drug target pathways. This will be achieved by:

1. Conducting a review to identify the potential clinical implications of pharmacogenetics and to describe outstanding methodological and statistical issues among these studies (Chapter 2);

2. Exploring whether the COX-2 genetic variant (rs20417) is associated with a decreased risk of CVD outcomes (Chapter 3);

3. Testing whether BAS are associated with a reduced risk of cardiovascular outcomes using the principles of Mendelian Randomization (Chapter 4); and

4. Investigating whether SNPs associated with dysglycaemia are associated with an increased risk of CAD (Chapter 5).
REFERENCES


CHAPTER 2

Promises and Challenges of Pharmacogenetics: An overview of study design, methodological and statistical issues

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ABSTRACT

Pharmacogenetics is the study of inherited variation in drug response. The goal of pharmacogenetics is to develop novel ways to maximize drug efficacy and minimize toxicity for individual patients. Personalized medicine has the potential to allow for a patient’s genetic information to predict optimal dosage for a drug with a narrow therapeutic index, to select the most appropriate pharmacologic agent for a given patient, and to develop cost-effective treatments. Although there is supporting evidence in favor of pharmacogenetics, its adoption in clinical practice has been slow because of sometimes conflicting findings among studies. This failure to replicate findings may result from a lack of high-quality pharmacogenetic studies, as well as unresolved methodological and statistical issues. The objective of this review is to discuss the benefits of incorporating pharmacogenetics in clinical practice. We will also address outstanding methodological and statistical issues that may lead to heterogeneity among reported pharmacogenetic studies and how they may be addressed.
Introduction

It is widely recognized that there is interindividual variability in drug response, where subgroups of patients experience either adverse drug reactions or do not respond properly to treatment \(^1\). While the definition of individualized response to drug treatment is not yet fully understood\(^2\) and there is uncertainty as to whether certain patients are consistent non-responders or simply inconsistent responders, this variability may be attributed to biological factors (i.e. age, sex, nature of disease), behavioral factors (i.e. smoking, drug interactions) or genetic factors (i.e. genetic variants). Furthermore, the lack of patient adherence is also recognized as an important contributor to variability of response. For example, the discontinuation of antiplatelet therapy is the strongest risk factor for stent thrombosis in percutaneous coronary intervention\(^3\). Nonetheless, it is estimated that genetic factors can account for 20% to 95% of individual variation in drug response\(^4\); however, the amount of explained variation depends on the class of drugs.

The wide variability in drug response emphasizes the need for a more “personalized” approach to medical treatment. It is possible that pharmacogenetics can address this need by providing a better understanding of how genetic variants influence drug response \(^5\). This review will focus primarily on pharmacogenetics, which assesses how genetic variants influences drug metabolism and effect. The ultimate goal of pharmacogenetics is to develop novel ways to minimize harmful drug effects and to optimize care for individual patients. More specifically, a patient’s genetic information may be used to predict the optimal dosage for a drug with a narrow therapeutic
index, to select the most appropriate pharmacologic agent, and to develop cost-effective treatment plans.

Despite the promise of personalized medicine, there has been little methodological consistency among pharmacogenetic studies. This may be due to modest effect sizes, heterogeneity among study designs and patient populations, as well as a lack of standardization among biological and phenotypic measures\(^6\)–\(^8\). Holmes et al. (2009) performed a systematic review and a field synopsis of pharmacogenetic studies\(^9\). They reported that the lack of consistency among studies may be a result of the preponderance of reviews over primary research, small sample sizes, a mainly candidate gene approach, surrogate markers, an excess of nominally positive to truly positive associations and paucity of meta-analyses. Therefore there is an urgent need for properly designed pharmacogenetic studies to advance the discovery and development of medical strategies for individualized treatment. The objective of this review is to discuss the potential benefits of incorporating pharmacogenetics into clinical practice, as well as methodological and statistical challenges faced in pharmacogenetic studies. In this review, we will first identify potential clinical applications of pharmacogenetic and illustrate these with promising contemporary examples. In the second part, we will summarize some of the major methodological challenges facing pharmacogenetic studies.

**Potential Applications Pharmacogenetics**

Personalized medicine has the potential to improve drug safety and efficacy for a specific individual. Adoption of pharmacogenetics in clinical practice promises more effective decision-making with regard to diagnostic testing, drug selection and dosing. In this section we will
describe some future applications of pharmacogenetics and provide contemporary examples that reflect how these topics may be applied to a clinical setting. It should be noted by readers, that in many instances further research is needed to unequivocally recommend pharmacogenetic testing. It is widely believed that a better understanding of the genetic mechanisms in drug response has the potential to help clinicians predict an individualized drug dosage; however, to date, there are few examples that illustrate this hypothesis with improved clinical outcomes.

**Individualized Drug Dosage**

The genetic variants that influence the observed differences in drug response can be classified into two groups: pharmacokinetics (PK) and pharmacodynamics (PD). The genes that influence the PK properties of a drug affect the mechanisms of how the drug is absorbed, distributed, metabolized and excreted by the body. The genes that influence the PD of a drug affect the mechanism of the drug’s target and how it impacts the body. One underlying principle of individualized drug dosage is that it must be faster and more effective than use of a PK or PD assay alone. In other words, genetic testing may not be required if the therapeutic level of the drugs or a surrogate can be measured, and it is rapidly available and widespread, such as the case with certain antibiotics (not withstanding the genetic susceptibility of the pathologic agent).

For example warfarin has a narrow therapeutic index, and inadequate or excessive anticoagulation can lead to an increased risk of adverse cardiovascular events or bleeding complications. Thus warfarin therapy dosage is complicated by individual variability and requires regular monitoring to achieve proper anticoagulation effects. Initial warfarin therapy is administered by a fixed dosage, or by an estimated regimen based on the patient’s clinical
characteristics with further adjustments based on the patient’s anticoagulation response measured by laboratory assays, such as the International Normalized Ratio (INR). However, it may be more beneficial to use both genetic factors and clinical covariates as opposed to frequent INR monitoring because genetic polymorphisms account for 30-35% of the variability in warfarin metabolism and clinical factors account for 17-21% of variation in warfarin dosing. Therefore an algorithm that incorporates a combination of these factors would ultimately improve the time required to establish a stable maintenance dose.

The principle genes involved in the metabolism of warfarin are the cytochrome P450 (CYP) 2C9 enzyme and the vitamin K epoxide reductase complex, subunit 1 (VKORC1) gene. Carriers of at least one or more variant alleles of the CYP2C9 genotype are associated with overcoagulation and an increased risk of bleeding while on warfarin therapy; whereas, those who possess the variant VKORC1 genotype experience warfarin treatment resistance and an increased risk of adverse cardiac events. The International Warfarin Pharmacogenetics Consortium developed a pharmacogenetic algorithm for an appropriate warfarin dosage. The study reported that among 5000 participants the pharmacogenetic algorithm identified a larger proportion of patients who required a lower dose (≤ 21 mg per week) of warfarin and those who required a higher dose (≥ 49 mg per week) to maintain stable therapeutic anticoagulation. The genetically-guided treatment benefited 46.2% of the entire cohort, specifically those for whom the standard dosage of warfarin would not be appropriate. It is important to properly identify this proportion of patients because some (i.e. who require ≤ 21 mg per week) are at risk for excessive anticoagulation, whereas others who require a higher dose of warfarin (i.e. ≥ 49 mg/wk) are at risk of inadequate
anticoagulation. Data on adverse events such as thromboembolic events or bleeding were not collected for this study.

In another study patients who were treated using a pharmacogenetic algorithm had 28% less hospitalizations after six months of warfarin therapy compared to a control group (18.5% vs. 25.5%, p < 0.001)\(^6\). The ability to increase the accuracy of dose prediction may help to enhance drug efficacy and drug safety associated with under-dosing or over-dosing patients. Although promising, it should be emphasized that the implementation of pharmacogenetic testing ultimately depends on clear evidence of improved clinical outcomes.

*Individualized Drug Selection*

Personalized medicine can help to guide individualized treatment when the clinical effect of a drug is expected to vary according to genotype. Under these conditions, the risk-benefit balance of a drug might depend on the variant allele carrier status. This balance can be affected by pharmacogenetic effects on safety, efficacy, or both. For example the incidence of adverse clinical events may differ according to genotypic groups, if for instance slow metabolizers accumulate a toxic metabolite. Thus prior knowledge of a patient’s genotype may be used to guide clinical decision-making because the patient may benefit from an alternative pharmacological regiment, such that they receive a reduced dose of a standard therapy or a different drug altogether. Conversely, patients who are classified as fast metabolizers may experience increased drug efficacy from a higher dose if their genetic status results in accelerated clearance of the active metabolite.
Chronic infection with hepatitis C virus (HCV) is treated with a combined therapy of peginterferon-α-2a (PegIFN-α-2a) or PegIFN-α-2b and ribavirin. However, less than half of treated patients achieve a sustained virological response (SVR)\(^\text{17}\). A genome-wide association study of 1671 chronic HCV patients reported that a genetic polymorphism in the \(IL28B\) gene (rs12979860) region was strongly associated with SVR\(^\text{18}\). The authors reported that the polymorphism was associated with a two-fold change in treatment response among Caucasians (\(P=1.06\times10^{-25}\)) and African Americans (\(P=2.06\times10^{-3}\)). Interestingly, the differences in allelic frequency of the \(IL28B\) genetic variant may explain about half of the difference in treatment response between these two ethnic groups.

Another study assessed whether accounting for the human leukocyte antigen C (HLA-C), and the killer immunoglobulin-like receptors (KIR) improved the predictive value of the \(IL28B\) genotype\(^\text{19}\). The authors found that the carriers of the variant \(IL28B\) genotype were associated with absence of treatment-induced HCV infection clearance and absence of spontaneous HCV infection clearance. Furthermore carriers of the variant \(HLA-C\) genotype were associated with failed treatment but not spontaneous HCV infection clearance. Thus the prediction of treatment failure among HCV patients was improved from 66% using the \(IL28B\) genotype to 80% with the use of the \(IL28B\) and \(HLA-C\) genotypes. Incorporating this information can help clinicians to improve the clinical management of patients infected with chronic HCV because they will be able to better predict those who will respond the best to PegIFN treatment, which will help to reduce the adverse side effects associated with this treatment.
Pharmacoeconomy

Pharmacogenetics has the potential to reduce the costs associated with inappropriate drug treatments or serious adverse drug reactions that require hospitalization\(^{20}\). Pharmacoeconomic considerations are especially important given the moderate effects of genetic determinants typically reported in pharmacogenetic studies. In other words, if a more expensive drug has a slightly decreased benefit in individuals with a certain genotype, then careful evaluation of the costs associated with an alternative therapy or the cost of genotyping is necessary before recommending further pharmacogenetic testing.

One example of utilizing genetic testing to improve cost-effectiveness is the treatment of HIV positive patients with abacavir, a nucleotide reverse-transcriptase inhibitor. Abacavir Hypersensitivity Syndrome (AHS) is a potentially lethal side effect affecting 5-8% of patients in the first six weeks of treatment\(^{21}\). It presents with a constellation of symptoms such as fever and rash; and rechallenge with abacavir, after initial therapy, may result in worsening AHS symptoms with an increased risk of mortality\(^{22}\). Patients who experience AHS are strongly associated with the variant histocompatibility complex class I allele (HLA-B)*5701 genotype, which is present in 2-6% of Caucasians\(^{23}\).

Mallal et al (2008) observed that, in a double-blind prospective randomized study, Prospective Randomized Evaluation of DNA screening in a Clinical Trial (PREDICT-1), selective abacavir use informed by HLA-B*5701 testing reduced the risk of AHS\(^{23}\). The authors of this study reported that screening eliminated AHS (0% in the prospective-screening group vs. 2.7% in the control group, \(P<0.001\)), and had a negative predictive value of 100% and a positive predictive
value of 47.9%. This led to the recommendation that prospective *HLA-B*\(^*5701\) screening should be adopted in clinical care\(^{24,25}\).

Furthermore several studies have evaluated the cost of prospective *HLA-B*\(^*5701\) screening\(^{26-28}\). Kauf et al analyzed the cost-effectiveness of *HLA-B*\(^*5701\) screening by assessing the cost of prior genetic screening and the cost of using an alternative medication, tenofovir, within short-term and lifetime models\(^{28}\). The authors reported that the short-term costs of prospective screening were dependent on the cost of the genetic test, the cost associated with AHS treatment and screening performance. The lifetime models showed that genetically-guided abacavir treatment was more effective and less costly than alternative treatment with tenofovir. Furthermore, as of 2009, the patent for abacavir has expired in the United States. Thus the cost-effectiveness of *HLA-B*\(^*5701\) screening prior to abacavir-based treatment is now highly dependent on the prevalence of the *HLA-B*\(^*5701\) genotype, the cost of prescribing a generic medication compared to a non-generic one, screening costs and the method of health care funding.

**Methodological Issues in Pharmacogenetics**

Although pharmacogenetics has the potential to address variability in drug response and improve drug efficacy and safety, the adoption of pharmacogenetics in clinical practice has been slow. This resistance may stem from sometimes conflicting findings among pharmacogenetic studies. The failure to replicate these findings may result from a lack of high-quality studies and unresolved methodological issues. In this section, we will address methodological issues
pertaining to pharmacogenetic study design and provide specific examples of pharmacogenetic studies that illustrate potential challenges the reader may encounter.

**Study Design**

Table 2.1 provides a brief description of each study design.

**Randomized Controlled Trials**

Randomized controlled trials (RCTs) remain the “gold standard” in epidemiological study design. In the field of pharmacogenetics, there are two ways in which RCTs can be used to establish pharmacogenetic determinants of drug safety and efficacy. First, patients can be randomized to a genetically-guided therapy versus standard care. While this design offers the best level of evidence to support the use of genetic data, it may be impractical in some situations. For example the speed of genotyping may cause delays in treatment or randomization for trials that require known pharmacogenetic determinants. Alternatively, if the genotype of interest is rare and the aim of the study is to compare response between two or more therapeutic regimen among carriers, participants may be stratified based on their genotype and then randomized to the intervention or control group.

Substudies within RCTs can be used to determine the impact of genetic variants in response to drug outcomes. In these studies stored biological samples from pre-existing clinical trials are genotyped with power comparable to that of a prospectively planned pharmacogenetic cohort study. This appears to be an optimal design to discover and characterize pharmacogenetic determinants prior to an evaluation of gene-guided therapy versus standard care.
Pharmacogenetic RCTs are able to measure the independent effects of the genotype, the drug response and the gene-drug interaction in the active drug and placebo/control groups. With this approach it is then possible to distinguish the differences between simple markers of disease progression and true pharmacogenetic markers, whose effect on disease progression is only seen in the presence of a drug. This can also be assessed by developing a “gene score” (i.e. combining information from many SNPs) and testing for a drug-gene interaction.

One major limitation of pharmacogenetic RCTs is the cost and time required to conduct the study. These studies require a large sample size to be powered enough to detect a modest effect size. Furthermore, a post-hoc analysis of a RCT may be inappropriate for a pharmacogenetic study because the initial cohort was designed using a specific null hypothesis, estimated effect size and study power and may underestimate the true gene-drug interaction.

An example of a genetically-guided RCT is the Clarification of Optimal Anticoagulation through Genetics (COAG) trial \(^{29}\). The COAG trial is a randomized, double-blinded clinical trial that compares genotype-guided dosing and clinical guided dosing for the initiation of warfarin treatment. The objective of the trial is to determine whether genetic information improves drug treatment. This trial is ongoing and final results of the study are yet to be published.

Another example of a pharmacogenetic RCT is the Statin Response Examined by Genetic Haplotype Markers (STRENGTH) Study \(^{30}\). The purpose of the STRENGTH Study was to explore the association between genetic polymorphisms and low-density lipoprotein cholesterol (LDLc) lowering in statin-treated patients. The STRENGTH Study was a 16-week, randomized,
open-label study of 3 statins in 509 outpatients with hypercholesterolemia. Study participants were initially randomized to 8 weeks of 10 mg/day atorvastatin, 20 mg/day simvastatin, or 10 mg/day pravastatin followed by 8 weeks of 80 mg/day atorvastatin, 80 mg/day simvastatin, and 40 mg/day pravastatin. Voora et al reported that carriers of the \textit{ABCA1} variant (rs12003906) were associated with a reduced LDLc lowering effect and carriers of the loss-of-function \textit{SLCO1B1} allele were associated with increased risk of statin therapy discontinuation \cite{30,31}. The use of pharmacogenetic RCTs will be instrumental in the understanding of how genetic variants contribute to drug therapy and lay a solid foundation for tailored medical therapy.

Another recent RCT example is the effect of the \textit{CYP2C19} genotype on the safety and efficacy of clopidogrel. Dual antiplatelet therapy of clopidogrel and aspirin has been shown to reduce adverse vascular events among patients with acute coronary syndromes \cite{32,33}. Several studies have observed that carriers of the loss-of-function allele are associated with a reduced response to clopidogrel and an increased risk of adverse cardiovascular outcomes \cite{34,35}. Based on these findings, in 2010 the FDA put a boxed warning for the prescription of clopidogrel which may require dose adjustment or use of a different drug \cite{36}. However, a genotyped subgroup from the CURE study showed that carrier status of the loss-of-function \textit{CYP2C19} allele did not differ in the safety and efficacy of clopidogrel \cite{37}. These findings were also replicated in a subgroup of the ACTIVE A trial. While patients in the CURE study were mostly non-invasively managed, another distinguishing feature of the analysis is the inclusion of the placebo group. The addition of the placebo group provides evidence of the efficacy of the experimental treatment. It also helps to reduced sources of confounding, such as potential pleiotropic genetic effects and population stratification. The results of this study have also been confirmed by a systematic
review and meta-analysis consisting of 32 studies and 42,016 patients. The authors reported a significant association between loss-of-function carrier status and risk of CVD events using “treatment-only” studies. However, the authors failed to report a significant association when using “effect-modification” studies or studies with more than 200 cardiovascular events. These analyses shows the importance of using large RCTs with both placebo and drug arms to guide validate recommendations on pharmacogenetic findings and medication use.

**Prospective Cohort Studies**

Prospective cohort studies follow a group of participants who are self-selected into a drug treatment group and assess how genetic distribution corresponds to the risk of developing the study outcome. Prospective cohort studies are able to examine causality through the temporal affects of drug exposure and genetic variants on disease risk. However, prospective cohort designs are expensive and time-consuming because they often require a large sample size to detect a relatively modest drug-gene interaction. Moreover, this study design is more subject to confounding because the assignment of drug therapy is subject-driven rather than randomly allocated.

Selection bias occurs in prospective cohort studies if loss to follow-up is differential by drug exposure or by genotype. For example loss to follow-up and drug use may vary by age, and loss to follow-up and genetic polymorphisms may vary by ethnic group. Furthermore, if individuals who were lost to follow-up tended to have different risks associated with the study outcome as compared to those who remained for the entire length of the study then the overall incidence estimates would be biased.
Prospective cohort studies are more subject to nondifferential misclassification as compared to case-control studies. Nondifferential misclassification occurs when exposure measurement errors are independent of the outcome and result in dilution of the measure of association and bias estimates toward the null. This may occur if drug use is not collected at multiple time points throughout the study. During the study participants may begin a new medication or discontinue their current treatment because of adverse drug events. An increase in data collection over the study period will help to ensure improved accuracy of patient behavior and improved measurements.

As mentioned previously, subgroups of participants from prospective cohorts can be analyzed in nested case-control study studies. These studies select participants who experienced the study outcome and compare them to randomly selected controls from the original study cohort. The advantages of using this design are the cases are compared to the same comparison group, which helps to reduce bias and confounding. Furthermore this design allows researchers to use small sample sizes and allows for a more cost-effective approach.

One such example in pharmacogenetics is the examination of the CYP2D6*4 allele in tamoxifen treated patients from the Rotterdam Study 40. The CYP2D6 gene is involved in the formation of endoxifen from tamoxifen, which is used for the treatment of estrogen receptor-positive breast cancer within post-menopausal women 41. The objective of the study assessed the association between carriers of the CYP2D6*4 allele and breast cancer mortality among all incident users of tamoxifen. The study reported that the hazard ratio of breast cancer mortality in patients with the
*4/*4 genotype was 4.1 (95% CI 1.1–15.9; p=0.041) compared to those with the wild-type genotype. Although these results are subject to potentially more bias as the exposed and unexposed groups were not randomized, there is greater generalizability in this study as compared to an RCT. This represents a trade-off between optimal internal validity in the RCT design compared to external applicability in the prospective cohort design. It would be wiser to report the more robust estimates of the RCT and use subsequent studies to explore the generalizability of these findings than rely on the estimates from a prospective cohort study.

Case-Control Studies

Case-control studies are the most common study design in pharmacogenetics. Under this model, cases are defined as those who have had a specific adverse drug event or a poor therapy outcome. The genetic variant frequencies in the cases are compared to the controls who have a comparable level of drug exposure but are also free of the study outcome. These studies are able to measure the effect of the gene-drug interaction but the independent effects of the genotype and drug response cannot be ascertained.

Case-controls studies can be performed quickly and they are more cost-effective than large prospective studies. Case-control studies may be the only feasible study design when it is not possible to conduct an RCT. For example it may not be possible to use a prospective study design to assess rare adverse drug outcomes or rare variants because they require a very large sample size. Furthermore, it is unethical to conduct a RCT with a priori unequivocal knowledge of severe drug-gene interaction, in which carriers of a variant allele are known to be susceptible to adverse events.
The retrospective design of case-control studies makes it be more prone to confounding, selection bias and information bias. Selection bias is the product of inappropriate choice of study controls and differential participation rates between cases and controls. Ideally, controls should represent cases with respect to potential exposures and have the same risk of developing the outcome phenotype. For example pooled hospital-based controls may include participants whose allelic frequencies correspond to another underlying disease, which will distort the exposure-disease association. Selection bias may also result from differential nonparticipation among cases and controls if those who failed to participate were related to genotype or drug exposure.

Information bias in case-control studies is most likely to result from differential misclassification. Differential misclassification occurs when there is systematic error in the degree of misclassification between cases and controls, which will distort the true magnitude of association in any direction. One common type of information bias in case-control studies is recall bias. Recall bias occurs when cases remember past exposures differently than controls. For example cases may recall past drug exposures better than those who did not experience the outcome because they have more motivation to identify possible causes of their disease. It is important to note that there is no recall bias with genetic exposure because participant’s genotypes are fixed.
**Genetic Epidemiology Considerations**

*Phenotype definition*

In pharmacogenetic studies the selection of the study endpoint and the patient response phenotype are crucial for interpreting drug efficacy. However, since many pharmacogenetic studies use data from prospective studies, the study endpoints and patient population may not be precise enough to identify functional genes that are associated with the drug response. For example it may be more appropriate to measure clinical outcomes, such as adverse bleeding events, when studying the association between safety measures and genetic markers.

Nevertheless physiological and biochemical measures may be more appropriate phenotypes to represent the underlying gene function in the drug-gene interaction, such as platelet count or clotting time. These phenotypes represent stronger biological or causal evidence of the functional activity of the gene or protein in question.

However, across studies, there is great heterogeneity in the biological measurement and definitions of outcomes or phenotypes. For example the reported prevalence of aspirin resistance ranges from 5-45%, which is thought to result from small sample sizes and heterogeneity within the methodologies used to measure the biochemical and functional components of aspirin resistance. Goodman et al (2008) performed a systematic review of all the genetic studies of aspirin resistance, and observed that the effect of the $PIA1/PIA2$ polymorphism in the GPIIIa receptor appears to differ according to the technique used to measure aspirin resistance. The lack of standardization among laboratory tests leads to imprecise effect estimates of the polymorphism and drug response. Therefore to decrease heterogeneity among studies and for
more reliable estimates of pharmacogenetic associations there must be consistent and functionally relevant phenotypic definitions.

*Genetic Polymorphisms*

The associated genetic variants are either directly functional or they are indirectly correlated with another variant that is the actual cause of the drug-response. Linkage disequilibrium (LD) “is the tendency for a pair of alleles at two linked loci to be associated with each other in the population more than would be expected by chance” 49. LD is useful in genetic association studies because high LD allows for a smaller subset of markers single nucleotide polymorphisms (SNPs) to be genotyped while capturing most of the genetic information. However, LD varies among ethnic populations and this may affect cross subpopulation comparisons when causal SNPs are not directly genotyped but rather captured by “proxy” SNPs 50, 51.

*Population Stratification*

A source of confounding within population-based pharmacogenetic studies can result from population stratification 52. Population stratification occurs when ethnic subpopulations within the entire study population differ in terms of genotype frequency and risk of disease 53. Population stratification confounds pharmacogenetic associations when differences in the prevalence of an allele parallels the incidence of study outcomes 52 and may bias both the strength of the association and estimates of precision of the genetic variant-outcome association. In other words, clinical outcomes might vary among genetically distinct populations for reasons other than the variant being tested and thus bias pooled drug-gene interaction effects 54. Stratification can also occur in apparently homogeneous populations, for example Davey Smith
et al observed an increasing north-south gradient in the frequency of the variant allele for lactase persistence across Britain 55.

One approach to minimizing the confounding effects of population stratification is to match participants based on geographical region and by markers of ethnic origin 56. Stratifying the study sample by ethnic groups allows for fair comparisons among homogenous groups; however, depending on the amount of stratification, too many groups will decrease the power able to detect an effect within each stratum. Genetic principal components are also widely used to minimize confounding by stratification. This method corrects for spurious associations in traits that differ among populations and have different allelic frequencies for the genotype of interest. Most differences in allelic frequencies are thought to have occurred because of genetic drift and may not represent functional variants 57. Thus the principle component technique is used to detect and correct for the population heterogeneity to minimize false positive associations 58. Variance component methods have also been recently developed to adjust for population stratification 59. Importantly, randomized studies are immune to this bias since equal numbers of individuals of each population strata will be randomized to the drug of interest or placebo group.

**Genetic Pleiotropy**

Genetic pleiotropy is the phenomenon in which a single gene is responsible for a number of distinct and seemingly unrelated phenotypic traits 60. This phenomenon is of special importance to pharmacogenetics because it may confound the pharmacogenetic association. For instance if the gene of interest is associated with multiple outcomes or intermediate phenotypes, the reported drug-gene interaction may be a result of the underlying gene mechanism and not a
product of the drug response \(^6\). For example the SH2B3 gene has been associated with multiple phenotypic traits, such as blood pressure \(^6^2, 6^3\), blood eosinophil number \(^6^4\), myocardial infarction \(^6^4\), celiac disease \(^6^5\), type I diabetes \(^6^6\), LDL-cholesterol \(^6^7\), asthma \(^6^4\), blood platelet number \(^6^8\), hemoglobin concentration \(^6^9\) and hematocrit \(^6^9\).

Several large trials have observed that lowering low-density lipoprotein cholesterol (LDLc) levels decreases the risk of atherosclerosis events, which can be achieved through statin therapy \(^7^0\). The proprotein convertase subtilisin/kexin type 9 (PCSK9) gene degrades the LDL receptor, which helps to increase the clearance of LDLc from circulation. Gain-of-function carriers of the PCSK9 genotype are associated with mild to severe hypercholesterolemia, while loss-of-function carriers are associated with decreased LDLc and decreased risk of cardiovascular events \(^7^1, 7^2\). The loss-of-function carriers are also associated with more pronounced decrease in LDLc with statin therapy \(^7^3\), and it is difficult to determine to which extent the observed relationship is driven by a pharmacogenetic effect or by the gene effect. Therefore to distinguish if there is an independent relationship and true effect modification it is essential to use a RCT design with a control group to see if the effect occurs in the treatment group alone.

**Statistical Issues in Pharmacogenetics**

A major issue in pharmacogenetics is the lack of replication amongst population-based studies. Possible explanations for the sometime inconsistent findings are modest effect sizes, small sample sizes and multiple hypothesis testing. In this section we will discuss sample size and multiple testing issues, and how to address them.
Sample Size

The ability to determine whether there is a clinically significant difference between groups is dependent on the study sample size. Pharmacogenetic studies must be large in order to have enough statistical power to detect a gene effect, a treatment effect and a drug-gene interaction. The power to detect a statistical interaction depends on the number of SNPs, the allelic frequencies of each SNP, and the type of study design. It is unlikely that a common genetic variant will have a large effect in a complex trait, such as drug response. Studies should thus be powered to detect a common or rare variant with a modest or very large effect size, respectively.

Table 2.2 shows the approximate sample sizes needed to detect a significant gene-drug interaction (assuming 80% power and $\alpha = 0.05$) by effect size and allelic frequency (among controls). Under these conditions, it is assumed that the genetic variant is causal; however, it is possible that the variant allele is in LD with the actual causal variant, which may require a larger sample size. If a rare genetic variant is anticipated with a small or modest effect, a sample size of more than 900,000 participants would be required. However, if a common variant with a large effect was expected, then a sample size of approximately 900 participants is needed. These results suggest that the majority of pharmacogenetic studies are underpowered, which may give rise to false-negative or false-positive estimates.

For some pharmacogenetic questions, the required sample sizes may be difficult to obtain. The need for large datasets has lead to the creation of international consortia where data between investigators is pooled or analyzed together, or large population-based biobanks, which store
biological materials (i.e. blood or DNA) and demographic information, including drug use. In addition, RCTs now incorporate genetic add-on studies which has the same high internal and external validity and large sample size of the parent RCT, while remaining cost-effective.

**Multiple Testing**

Multiple testing refers to the repeated use of a statistical test and the risk of an overall type I error. Multiple testing arises when there are multiple comparisons in statistical models that contain multiple genes, multiple exposures and multiple interactions. Within these models it is inappropriate to use the standard p-value of 0.05 because as the number of tests increases so does the frequency of type I errors.

The most common approach to correct for multiple testing is to use the Bonferroni correction, in which the p-value that is used for one test is divided by the total number of tests in the analysis. However, the use of the Bonferroni correction may be considered too conservative because many SNPs are in LD, which may mask their effects and increase type II errors. Furthermore, since many of the pharmacogenetic studies are underpowered to detect a drug-gene interaction, the Bonferroni correction may null the study results. Another possible approach to adjust for multiple testing is to use the false discovery rate (FDR), which is less conservative than the Bonferroni correction. The FDR estimates the expected proportion of false positives among associations that are declared significant, which is expressed as a q-value.
Conclusions

Pharmacogenetic studies offer both a promising future yet have a challenging present.

Personalized medicine has the potential to maximize drug efficacy and minimize the toxic effects; however, there are many issues in study design and analysis that need to be addressed.

Large collaborative efforts across biostatisticians, epidemiologists, pharmacologists and clinicians is needed to provide robust evidence to support individualized treatment for improved drug efficacy and safety.
DECLARATIONS

Competing interests

GP reports receiving consulting and speaker fees from Sanofi-Aventis, Bristol-Myers Squibb and Boehringer-Ingelheim, and research grant support from Bristol-Myers-Squibb and Sanofi-Aventis. SSA reports receiving lecture fees from Bristol-Myers Squibb.

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Ethical approval

Not applicable

Guarantor

GP

Contributorship

SR and GP wrote the first draft. SSA and PJ provided comments on all drafts and supplied additional content and relevant references.

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None
REFERENCES


Ref Type: Online Source


TABLES

Table 2.1: Study designs for pharmacogenetic studies and their main strengths and limitations

<table>
<thead>
<tr>
<th>Study Design</th>
<th>Description</th>
<th>Strength</th>
<th>Limitation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Randomized Controlled Trial</td>
<td>Participants are stratified by genotype and randomized to treatment groups</td>
<td>Evidence of a drug-gene interaction; Evidence of causality; Assess multiple outcomes</td>
<td>Requires large sample size; High cost; Unable to assess rare events</td>
</tr>
<tr>
<td>Prospective Cohort Study</td>
<td>Participants are followed over time and disease outcome is compared with drug and genotype subgroups</td>
<td>Prospective nature; Assess multiple outcomes</td>
<td>Selection bias (loss-to-follow-up); Information bias (nondifferential); Confounding; Unable to assess rare events</td>
</tr>
<tr>
<td>Case-Control Study</td>
<td>The genotype frequency and drug response outcome is compared among cases and controls</td>
<td>Requires small sample size; Low cost; Assess rare events</td>
<td>Selection bias; Information bias (differential); Confounding; Unable to assess rare events</td>
</tr>
</tbody>
</table>
Table 2.2: Sample size required to detect a drug-gene interaction in a pharmacogenetic study based on minor allele frequency*.

<table>
<thead>
<tr>
<th>Prevalence of variant allele carriers among controls</th>
<th>Odds ratio*</th>
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<td>0.15</td>
<td>71330</td>
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<tr>
<td>0.20</td>
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</table>

*Sample sizes have been calculated based on a drug-gene interaction assuming an additive genetic model. These estimates assume a type-I error rate of 0.05, a power of 80% and a baseline risk of an adverse drug reaction among exposed subjects to be 10%. Sample sizes were calculated using QUANTO78.
CHAPTER 3

Association of cyclooxygenase-2 genetic variant with cardiovascular disease

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WORD COUNT: 4,942
ABSTRACT

Aims: A genetic variant (rs20417) of the PTGS2 gene, encoding for COX-2, has been associated with decreased COX-2 activity and a decreased risk of cardiovascular disease (CVD). However, this genetic association and the role of COX-2 in CVD remains controversial.

Methods and Results: The association of rs20417 with CVD was prospectively explored in 49,232 subjects (ACTIVE-A, CURE, epiDREAM/DREAM, ONTARGET, RE-LY, and WGHS) and the effect of potentially modifiable risk factors on the genetic association was further explored in 9,363 INTERHEART participants. The effect of rs20417 on urinary thromboxane and prostacyclin metabolite concentrations were measured in 119 healthy individuals. Carriage of the rs20417 minor allele was associated with a decreased risk of major CVD outcomes (OR=0.78, 95% CI: 0.70 - 0.87; P=1.2x10^{-5}). The genetic effect was significantly stronger in aspirin users (OR: 0.74, 95% CI: 0.64–0.84; P=1.20x10^{-5}) than non-users (OR: 0.87, 95% CI: 0.72–1.06; P=0.16) (interaction p-value: 0.0041). Among patients with previous coronary artery disease (CAD), rs20417 carriers had a stronger protective effect on risk of major adverse events as compared to individuals without previous CAD (interaction p-value: 0.015). Carriers had significantly lower urinary levels of thromboxane (P=0.02) and prostacyclin (P=0.01) metabolites as compared to noncarriers.

Conclusion: The rs20417 polymorphism is associated with a reduced risk of major cardiovascular events and lower levels of thromboxane and prostacyclin. Our results suggest that a genetic decrease in COX-2 activity may be beneficial with respect to CVD risk, especially in higher risk patients on aspirin.

KEYWORDS: pharmacogenetics, genetics, aspirin, myocardial infarction, stroke
INTRODUCTION

Cyclooxygenase (COX) enzymes are responsible for converting arachidonic acid into prostaglandin (PG) H2, which acts as a metabolic precursor of prostaglandins, prostacyclin and thromboxane. Three isoforms of the COX enzyme have been identified (COX-1, COX-2, COX-3), but only the COX-1 and COX-2 isoforms are functional. The COX-1 enzyme is constitutively expressed in most tissues, including platelets, where it is involved in the formation of thromboxane A2 through an intermediate. Low dose aspirin decreases platelet activity by irreversibly acetylating COX-1 and inhibiting the production of platelet-derived thromboxane A2. COX-2 is an inducible enzyme that is expressed by cells involved in inflammation (i.e. endothelial cells, monocytes, and macrophages). It is believed to have cardioprotective effects by facilitating the production of prostacyclin, which is a potent vasodilator and inhibits platelet activation and smooth muscle cell proliferation.

The role of COX-2 in atherothrombosis remains controversial. Some animal studies suggest that genetic inhibition of the COX-2 enzyme decreases the risk of atherosclerosis, whereas others demonstrate an increased risk of thrombosis. Most clinical studies have linked pharmacologic inhibition by selective COX-2 inhibitors with an increased risk of CV events, presumably because COX-2 inhibition leads to unopposed COX-1 dependent thromboxane production and subsequent platelet activation and vasoconstriction. Additionally, higher doses of aspirin with shorter dosing intervals are required to inhibit the COX-2-dependent pathways since nucleated cells rapidly resynthesize this enzyme. In contrast with these results, a genetic polymorphism (rs20417) in the promoter of the PTGS2 gene (COX-2) has been associated with lower COX-2
activity in atherosclerotic plaque and a decreased risk of myocardial infarction and stroke\(^9\). Furthermore, an interaction between aspirin use and carriage of the COX-2 polymorphism has been reported, whereby the genetic effect is stronger in aspirin users than non-users\(^{10,11}\). However, previous studies mostly had small sample sizes, and only some\(^{10-14}\) but not all\(^{15-19}\) have replicated these findings. Thus confirmation and characterization of the genetic association between rs20417 and major adverse cardiovascular outcomes may provide greater insights into the biological role of COX-2 in CVD, and may also improve risk stratification of CVD patients.

Given the contradictory evidence on the role of COX-2 and CVD, we undertook to (1) test the association of the rs20417 polymorphism with CVD, (2) examine whether the genetic association is modified by aspirin use, or the presence or absence of major CVD risk factors, and (3) explore the functional mechanisms of the polymorphism by examining its impact on thromboxane and prostacyclin urine levels.

**METHODS**

**Study Populations Overview**

Events were classified according to definitions from each parent study. Our primary outcome was major adverse vascular events, defined, unless otherwise specified, as the composite of CVD death, non-fatal myocardial infarction, or non-fatal stroke.

Further details of the study population characteristics, genotyping and imputation are described in the Supplementary Methods. In brief, ACTIVE-A was a randomized, double-blind, placebo-controlled trial comparing clopidogrel (75mg/d) with placebo in patients with high-risk atrial
fibrillation (AF). CURE was a randomized, double-blind, placebo-controlled trial comparing clopidogrel (75 mg per day) with placebo in patients with ACS without ST-segment elevation. DREAM was a randomized, double-blind trial with a 2-by-2 factorial design that assigned participants at high risk for or who had diabetes to receive either ramipril (15 mg/day) vs. placebo or rosiglitazone (8 mg/day) vs. placebo. The EpiDREAM trial was an epidemiological arm of the DREAM trial and is comprised of participants who were either screened for eligibility to enter the DREAM clinical trial but were not eligible or who did not want to enter the trial but agreed to long term prospective follow-up. ONTARGET was a randomized, double-blind, parallel trial comparing the effects of ramipril (10 mg per day), telmisartan (80 mg per day), and combination therapy in patients with vascular disease or high-risk diabetes patients. RE-LY was a prospective, open-label, randomized trial that compared two fixed doses of dabigatran (110 mg or 150 mg twice daily) with open-label use of warfarin in patients with high risk AF. The WGHS Study is a subset of the Women’s Health Study (WHS), which consists of healthy female participants who were randomized either to an aspirin intervention arm (100 mg of aspirin every other day) or placebo. In addition, the INTERHEART study was a large, international, standardized case-control study consisting of non-fatal acute myocardial infarction cases and controls from 52 countries. Finally, MARS was an open-label, two phase case-control study of individuals with CVD; however, for the purposes of this analysis only healthy controls were considered.
Statistical analysis

Deviation from Hardy-Weinberg equilibrium was tested in each ethnic group for each study (P>0.05 for all). Due to the limited number of individuals homozygous for the minor allele of rs20417, a dominant genetic model was used throughout, whereby individuals carrying either 1 or 2 minor alleles were pooled together, and thereafter referred as “carriers” (unless otherwise specified). Logistic regression models were used for each individual study, with adjustment for age, sex, randomization status (when appropriate), and self-reported ethnicity. Results from each study were then combined using fixed-effect meta-analysis. Effect of rs20417 on outcomes was also assessed using Cox proportional hazard regression, without further adjustment. Association of rs20417 carrier status with urinary 11-dehydro thromboxane B₂ and 2,3-dinor-6-keto PGF₁α concentrations were performed using a non-parametric Kruskal-Wallis test. The statistical significance threshold was set at 0.05 (two-sided) for all analyses. All analyses were performed using R.

RESULTS

Characteristics of study populations

The baseline demographics of the prospective study populations (ACTIVE-A, CURE, epiDREAM/DREAM, ONTARGET, RE-LY, and WGHS) are presented in Table 3.1. The baseline demographics of the INTERHEART and MARS study populations are presented in Supplementary Table 1 and Table 2.
Association of the rs20417 polymorphism with major adverse cardiovascular events

Overall, 29.6% of participants were carriers of at least one rs20417 minor allele. Among the six prospective study populations (ACTIVE-A, CURE, epiDREAM/DREAM, ONTARGET, RE-LY, and WGHS), rs20417 carrier status was significantly associated with a reduced risk of major cardiovascular outcomes (OR=0.78, 95% CI: 0.70 - 0.87; P=1.20x10^{-5}), vascular death (OR=0.76, 95% CI: 0.63 - 0.90; P=0.0017), myocardial infarction (OR=0.78, 95% CI: 0.67 - 0.92; P=0.003) and stroke (OR=0.83, 95% CI: 0.70 – 1.00; P=0.04) (Figure 3.1). Heterogeneity across study populations was observed for major adverse cardiovascular events (heterogeneity P=0.0017), as well as vascular death (heterogeneity P=0.025) and myocardial infarction (heterogeneity P=0.047). However, once CURE was removed from the pooled analyses there was no longer evidence of heterogeneity (heterogeneity P>0.05 for all). No interaction with randomized treatment was observed in each trial (ACTIVE-A, CURE, DREAM, ONTARGET and RE-LY; P>0.05 for all).

Effect modification by ASA use

Due to the close biological relationship between COX-2 and ASA, and previous reports of a genetic interaction with ASA use, we tested rs20417 carrier status for association with cardiovascular events stratified by aspirin use. Aspirin use appeared to modify the relationship between rs20417 carrier status and risk of major cardiovascular events (heterogeneity P: 0.0041) (Figure 3.2). Among participants using ASA, carrier status was associated with a lower risk of CVD outcomes (OR: 0.74, 95% CI: 0.64–0.84; P=1.20x10^{-5}) while this relationship was attenuated in aspirin non-users (OR: 0.87, 95% CI: 0.72–1.06; P=0.16). We also observed significant heterogeneity among the pooled estimate of aspirin users, which points to differences
between studies (heterogeneity \( P = 0.0053 \)). As most patients on ASA have established coronary artery disease (CAD), we also assessed the genetic association between rs20417 carrier status for adverse CVD events stratified by previous CAD (Figure 3.3). Previous CAD was defined as established acute coronary syndromes or angina. Among patients with previous CAD, carriage of the rs20417 polymorphism had a stronger effect on risk of major adverse events (OR: 0.69, 95% CI: 0.58−0.81; \( P = 1.10 \times 10^{-5} \)) as compared to individuals without previous CAD (OR: 0.86, 95% CI: 0.74−1.00; \( P = 0.06 \)) with an overall interaction \( P \)-value of 0.015.

**Genetic association in relation to other major CVD risk factors**

To evaluate the strength of the rs20417 carrier status association with CVD in relation to conventional risk factors, we utilized a multivariate analysis including rs20417 carrier status and other major CVD risk factors. Analyses were performed in ACTIVE-A and CURE because both studies were significantly associated with rs20417 carrier status in a univariate analyses and both had all participants on ASA by design. Kaplan-Meier survival curves for ACTIVE-A and CURE are shown in Supplementary Figure 1, along with hazard ratios calculated using unadjusted Cox proportional hazard models. For ease of interpretation, individuals with two rs20417 risk alleles were compared to individuals with one or more protective allele, such that two rs20417 risk alleles is presented as a risk factor (Table 3.2). No conventional risk factors were consistently associated with a larger effect estimate than carrying two rs20417 risk alleles. In fact, carrying two rs20417 risk alleles had the largest effect size of all risk factors (excluding age) among participants in CURE (OR=1.90, 95% CI: 1.46−2.48; \( P = 2.10 \times 10^{-6} \)).
Effect modification by major CVD risk factors

To explore whether the presence of specific CVD risk factors modified the association of rs20417 carrier status with cardiovascular outcomes, we performed sub-group analyses within the prospective populations (Supplementary Figure 2). WGHS was excluded as there were no males and the prevalence of many risk factors (e.g. diabetes, advanced age) were low in this study of apparently healthy middle-aged women. None of the risk factors tested, showed a significant interaction with carriage of the rs20417 minor allele (P>0.05 for all). We also explored the association of rs20417 with myocardial infarction and its relation with major CVD risk factors in INTERHEART. Overall, 32.3% of INTERHEART participants were carriers of the rs20417 alternate allele (Supplementary Table 1). There was no association between carrier status and non-fatal myocardial infarction when using a dominant genetic model (OR=0.93, 95% CI: 0.85-1.02; P=0.115) but a weak and consistent association was observed using an additive genetic model (OR=0.92, 95% CI: 0.85-0.99, P=0.02). There was a marginally significant interaction between apolipoprotein A1 (apoA1) levels and carrier status (interaction p= 0.017); whereby, carrier status was significantly associated with CVD among individuals with lower than median ApoA1 levels (OR=0.82, 95% CI: 0.73-0.93, P=0.002) but not among those with greater than median ApoA1 levels (OR=1.05, 95% CI 0.93-1.20, P=0.42) (Supplementary Figure 3). None of the other modifiable risk factors or clinical characteristics showed a significant interaction with carrier status (P>0.05 for all). We also assessed whether the rs20417 polymorphism was associated with CVD risk factors using a model adjusted for age, sex and ethnicity. None of the risk factors were significantly associated with carrier status (P>0.05 for all) (Supplementary Table 3).
Association of rs20417 with urinary metabolites of thromboxane and prostacyclin

Finally, we tested whether rs20417 carrier status was associated with COX-1 and COX-2 derived urinary 11-dehydrothromboxane B2 and urinary 2,3-dinor-6-keto PGF$_{1\alpha}$ in 119 healthy European participants (not taking aspirin or other cyclooxygenase inhibitors). Minor allele carriers were shown to have decreased 11-dehydrothromboxane B2 urine concentration (P=0.02), with median values of 97.0 ng/mmol creatinine and 125.5 ng/mmol creatinine in carriers (N=32) and noncarriers (N=87), respectively. In addition, minor allele carriers were shown to have decreased urinary 2,3-dinor-6-keto PGF$_{1\alpha}$ concentration (P=0.01), with median values of 3335.8 pg/mg creatinine and 4702.0 pg/mg creatinine in carriers (N=32) and noncarriers (N=87), respectively (Supplementary Figure 4).

DISCUSSION

We found that the COX-2 (PTGS2) genetic variant rs20417 is associated with a decreased risk of major cardiovascular events (OR= 0.78, 95% CI: 0.70-0.87, P=1.20 x 10^{-5}), with consistent effects for cardiovascular death, myocardial infarction and stroke. We also observed significant interactions with aspirin use (P heterogeneity: 0.0041) and previous CAD (P heterogeneity: 0.015). Indeed, in ACTIVE-A and CURE, the magnitude of CVD risk associated with non-carriage of the rs20417 polymorphism was similar to that of other traditional risk factors (e.g. age, sex, diabetes, smoking status, high blood pressure and obesity). Our urinary metabolite results corroborate reports of lower COX-2 activity in rs20417 carriers.

Similar to previous reports, we observed an interaction between the nonselective COX inhibitor ASA and rs20417 carrier status$^{10, 11}$. While the possibility of a biological interaction is
compelling, this association may also be confounded since aspirin users are also more likely to represent those with established CVD and thereby reflect a stronger genetic effect in higher risk populations\textsuperscript{29} rather than an interaction with ASA use. Indeed, the benefit of ASA parallels the baseline risk of study populations, with a 12\% proportional reduction in CVD events in primary prevention population\textsuperscript{30}, 19\% in secondary prevention\textsuperscript{30}, and 23\% relative reduction in death in the acute ACS setting\textsuperscript{31}. Consistent with this hypothesis, previous CAD appeared to enhance the association between rs20417 carrier status and risk of CVD outcomes (P heterogeneity: 0.015). Similarly, we observed a strong association between rs20417 and CVD risk in ACS patients enrolled in CURE but no association in WGHS where the reported benefit of ASA was modest\textsuperscript{27}. However, it should also be noted that all CURE participants were assigned a standard dose aspirin (75 – 325 mg daily) while WGHS participants were randomly allocated to treatment with low dose aspirin (100 mg every other day) or placebo. Alternatively, this interaction may also reflect a stronger effect of ASA in rs20417 carriers as compared to noncarriers. Finally, with the exception of ApoA1 (interaction p-value: 0.017), we did not observe any interactions among the tested risk factors in INTERHEART. However, the interaction was modest and should be considered exploratory since it would not withstand adjustment for multiple testing.

In agreement with previous work showing that rs20417 minor allele is linked to decreased COX-2 expression\textsuperscript{9,19}, we demonstrated that minor allele carriers had lower levels of both urinary 11-dehydrothromboxane B\textsubscript{2} and urinary 2,3,2,3-dinor-6-keto PGF\textsubscript{1\alpha} excretion. We posit that tissue-specific effects of the genetic variant could explain the apparent discrepancy between genetic and pharmacological inhibition of COX-2 activity with respect to cardiovascular risk. Indeed, Cipollone et al (2004) demonstrated that the rs20417 genotype modified COX-2 activity in
carotid plaques, whereby carrier status was associated with lower levels of COX-2 expression in plaque-derived macrophages; however, carrier status did not influence COX-2 activity in endothelial tissue\(^9\). Furthermore macrophage-specific COX-2 knock-out mice models have decreased atherosclerosis\(^4\); whereas, deletion of COX-2 in endothelial cells and vascular smooth muscle cells in double knock-out mice leads to an increased risk of thrombosis\(^5\). This may also explain the apparent heterogeneity among studies, since participants with more advanced stages of CVD are likely to have complex, macrophage-rich plaques, and hence be more susceptible to the COX-2 inhibiting effect of rs20417.

Several factors likely contributed to the previously inconsistent reports of an association between rs20417 and risk of CVD \(^{15-19}\). For instance the sample size among several studies exploring the rs20417 association ranged from 220\(^{15}\) to 4,994\(^{11}\), which indicates that some of these studies may not have had enough statistical power to detect a genetic effect. Also, case-control studies may have underestimated the reported association between rs20417 minor allele carriers and CVD outcomes since these studies are more likely to include COX-2 carriers who experienced non-fatal vascular events as opposed to those who experienced vascular death. Indeed, inclusion of a large number of individuals from prospective studies strengthens our meta-analysis. Finally, it is possible that the genetic effect varies according to study population and ASA exposure such that heterogeneous estimates reflect true genetic risks.

A few limitations of our study warrant discussion. First, tissue-specific gene expression would ideally be needed to delineate the effects of rs20417 on COX-2 gene expression in relevant tissues such as endothelial cells, atherosclerosis plaques and macrophages. Second, rs20417 was
not directly genotyped in RE-LY or in ONTARGET and could not be imputed such that a proxy was used. However, use of a proxy should bias our results towards the null without invalidating our conclusions. Third, while the genetic effect was consistent across ethnic groups in both the epiDREAM/DREAM and INTERHEART patient populations, our study populations were predominantly European and further studies will be needed to confirm in other ethnic groups. Finally, although our results suggest an interaction between apoA1 levels and carriers of the rs20417 genotype with CVD, this was an exploratory analysis and further work is needed.

In summary, we confirmed the protective effect of the minor allele of the COX-2 (PTGS2) SNP, providing a genetic link between COX-2 activity and cardiovascular risk. In particular, our data suggest that decreased COX-2 activity is not universally deleterious in humans with respect to risk of adverse CVD outcomes, and taken together with observations in human atherosclerosis plaques and model systems implies that inhibition of COX-2 in macrophages could be beneficial. Additionally, the biologically compelling interaction between rs20417 carrier status and aspirin use suggests that widely prescribed non-selective COX inhibitors may be more beneficial among rs20417 carriers. Our results also highlight the complex genetic epidemiology of CVD and argue that genetic determinants may have different effect sizes according to study population characteristics, such as ASA use or presence of vascular disease. Further research will be needed to fully delineate the clinical, epidemiological and pathophysiological implications of the observed genetic association.
ACKNOWLEDGEMENTS

We are thankful to all the participants having agreed to contribute to this project.

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REFERENCES


FIGURE LEGENDS

Figure 3.1: Association of rs20417 carrier status with major cardiovascular events in six prospective patient populations.
Analyses were adjusted for age, sex, randomization status (when appropriate) and self-reported ethnicity. ACTIVE-A, CURE, epiDREAM/DREAM, ONTARGET, RE-LY and WGHS data were included in the meta-analysis. DREAM represents epiDREAM/DREAM. Hetero. P. represents heterogeneity p-value.

Figure 3.2: Analysis of association of rs20417 carrier status with major cardiovascular events stratified by aspirin use in six prospective patient populations.
Analyses were adjusted for age, sex, randomization status (when appropriate) and self-reported ethnicity. ACTIVE-A, CURE, epiDREAM/DREAM, ONTARGET, RE-LY and WGHS data were included in the meta-analysis. DREAM represents epiDREAM/DREAM. Hetero. P. represents heterogeneity p-value.

Figure 3.3: Analysis of association of rs20417 carrier status with major cardiovascular events stratified by previous coronary artery disease in six prospective patient populations.
Analyses were adjusted for age, sex, randomization status (when appropriate) and self-reported ethnicity. ACTIVE-A, CURE, epiDREAM/DREAM, ONTARGET, RE-LY and WGHS data were included in the meta-analysis. DREAM represents epiDREAM/DREAM. Hetero. P. represents heterogeneity p-value.
### Table 3.1: Baseline characteristics of prospective study populations

<table>
<thead>
<tr>
<th></th>
<th>ACTIVE-A</th>
<th>CURE</th>
<th>epiDREAM/DREAM</th>
<th>ONTARGET</th>
<th>RE-LY</th>
<th>WGHS</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>1061</td>
<td>4662</td>
<td>14104</td>
<td>3610</td>
<td>2501</td>
<td>23294</td>
</tr>
<tr>
<td>Mean age (SD)</td>
<td>71.0 (9.9)</td>
<td>63.6 (11)</td>
<td>52.0 (11)</td>
<td>67.0 (7.3)</td>
<td>71.9 (7.4)</td>
<td>54.2 (7.1)</td>
</tr>
<tr>
<td>Female (%)</td>
<td>483 (45.5)</td>
<td>1921 (41.2)</td>
<td>8589 (60.9)</td>
<td>998 (27.6)</td>
<td>804 (32.1)</td>
<td>23,294 (100)</td>
</tr>
<tr>
<td>BMI (kg/m²) (SD)</td>
<td>29.1 (5.6)</td>
<td>27.7 (4.2)</td>
<td>30.0 (5.8)</td>
<td>29.8 (5.2)</td>
<td>29.2 (5.5)</td>
<td>25.9 (5.0)</td>
</tr>
<tr>
<td>Previous CAD (%)</td>
<td>314 (29.6)</td>
<td>466 (2)</td>
<td>1535 (10.9)</td>
<td>2843 (78.8)</td>
<td>727 (29.1)</td>
<td>11617 (50)</td>
</tr>
<tr>
<td>Diabetes (%)</td>
<td>222 (20.9)</td>
<td>994 (21.3)</td>
<td>1842 (13.1)</td>
<td>1909 (52.9)</td>
<td>495 (19.8)</td>
<td>586 (2.5)</td>
</tr>
<tr>
<td>High Blood Pressure (%)</td>
<td>908 (85.6)</td>
<td>2852 (61.2)</td>
<td>2049 (14.5)</td>
<td>2710 (11.6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Current Smoking (%)</td>
<td>81 (7.6)</td>
<td>1048 (22.5)</td>
<td>393 (10.9)</td>
<td>5730 (24.6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aspirin use (%)</td>
<td>1061 (100)</td>
<td>4662 (100)</td>
<td>1535 (10.9)</td>
<td>11617 (50)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs20417 carrier status, No. (%)</td>
<td>318 (30.0)</td>
<td>1233 (26.4)</td>
<td>4802 (34)</td>
<td>727 (29.1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carriers</td>
<td>743 (70.0)</td>
<td>3429 (73.6)</td>
<td>9301 (66)</td>
<td>11617 (50)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median Follow-up (years)</td>
<td>3.5</td>
<td>0.8</td>
<td>3.6</td>
<td>2.1</td>
<td>10.2</td>
<td></td>
</tr>
<tr>
<td>Major cardiovascular events</td>
<td>247</td>
<td>456</td>
<td>131</td>
<td>565</td>
<td>87</td>
<td>518</td>
</tr>
<tr>
<td>N events</td>
<td>7.3 (6.3-8.3)</td>
<td>16.2 (14.7-17.7)</td>
<td>0.3 (0.2-0.3)</td>
<td>1.7 (1.3-2.1)</td>
<td>0.2 (0.2-0.2)</td>
<td></td>
</tr>
<tr>
<td>Events per 100 person-years (95% CI)</td>
<td>4.8 (4.0-5.5)</td>
<td>7.7 (6.7-8.8)</td>
<td>0.05 (0.08-94.5)</td>
<td>0.58 (0.48-0.68)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vascular death</td>
<td>165</td>
<td>239</td>
<td>20</td>
<td>228</td>
<td>46</td>
<td>135</td>
</tr>
<tr>
<td>N events</td>
<td>43</td>
<td>255</td>
<td>86</td>
<td>269</td>
<td>43</td>
<td>217</td>
</tr>
<tr>
<td>Events per 100 person-years (95% CI)</td>
<td>1.2 (0.8-1.6)</td>
<td>9.5 (8.3-10.7)</td>
<td>0.2 (0.1-0.2)</td>
<td>0.8 (0.5-1.1)</td>
<td>0.09 (0.08-0.11)</td>
<td></td>
</tr>
<tr>
<td>Myocardial infarction</td>
<td>105</td>
<td>55</td>
<td>48</td>
<td>157</td>
<td>46</td>
<td>270</td>
</tr>
<tr>
<td>N events</td>
<td>3.1 (2.5-3.8)</td>
<td>2.0 (1.4-2.5)</td>
<td>0.1 (0.08-0.1)</td>
<td>0.8 (0.5-1.1)</td>
<td>0.12 (0.10-0.13)</td>
<td></td>
</tr>
</tbody>
</table>
Table 3.2: Association of rs20417 carrier status and conventional risk factors with major cardiovascular events in ACTIVE-A and CURE.

Odds ratios of conventional risk factors for the risk of adverse events in CVD risk patient populations from multivariate models also including randomization status (when appropriate) and self-reported ethnicity.

<table>
<thead>
<tr>
<th>Risk factor</th>
<th>CURE OR (95% CI)</th>
<th>P</th>
<th>ACTIVE-A OR (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carriage of two rs20417 risk alleles</td>
<td>1.90 (1.46-2.48)</td>
<td>2.10E-06</td>
<td>1.63 (1.16-2.30)</td>
<td>5.10E-03</td>
</tr>
<tr>
<td>10 Years of age</td>
<td>1.62 (1.45-1.81)</td>
<td>1.10E-17</td>
<td>1.84 (1.53-2.21)</td>
<td>1.10E-10</td>
</tr>
<tr>
<td>Male sex</td>
<td>1.52 (1.23-1.89)</td>
<td>1.20E-04</td>
<td>1.15 (0.84-1.57)</td>
<td>0.38</td>
</tr>
<tr>
<td>Presence of diabetes</td>
<td>1.54 (1.23-1.93)</td>
<td>1.50E-04</td>
<td>2.05 (1.44-2.91)</td>
<td>6.70E-05</td>
</tr>
<tr>
<td>Current smoking</td>
<td>1.02 (0.76-1.35)</td>
<td>0.90</td>
<td>1.66 (0.92-2.98)</td>
<td>0.09</td>
</tr>
<tr>
<td>High blood pressure</td>
<td>1.31 (1.05-1.64)</td>
<td>0.02</td>
<td>0.99 (0.65-1.52)</td>
<td>0.98</td>
</tr>
<tr>
<td>Obesity (BMI&gt;30)</td>
<td>0.94 (0.74-1.19)</td>
<td>0.59</td>
<td>0.89 (0.64-1.24)</td>
<td>0.50</td>
</tr>
</tbody>
</table>
Figure 3.1: Association of rs20417 carrier status with major cardiovascular events in six prospective patient populations.

<table>
<thead>
<tr>
<th>Event</th>
<th>Carriers</th>
<th>Noncarriers</th>
<th>OR (95% CI)</th>
<th>P</th>
<th>Hetero P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Primary Composite Endpoint</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACTIVE-A</td>
<td>18.2%(58/318)</td>
<td>25.4%(189/743)</td>
<td>0.62(0.44–0.86)</td>
<td>5.10E–03</td>
<td></td>
</tr>
<tr>
<td>CURE</td>
<td>6.0%(74/1233)</td>
<td>11.1%(382/3429)</td>
<td>0.53(0.41–0.68)</td>
<td>1.40E–06</td>
<td></td>
</tr>
<tr>
<td>DREAM</td>
<td>0.7%(34/4802)</td>
<td>1.0%(97/9301)</td>
<td>0.67(0.45–1.00)</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>ONTARGET</td>
<td>14.7%(126857)</td>
<td>15.9%(439/2753)</td>
<td>0.90(0.73–1.12)</td>
<td>0.35</td>
<td></td>
</tr>
<tr>
<td>RE–LY</td>
<td>2.4%(14/581)</td>
<td>3.8%(73/1920)</td>
<td>0.62(0.35–1.12)</td>
<td>0.11</td>
<td></td>
</tr>
<tr>
<td>WGHS</td>
<td>2.2%(147/6794)</td>
<td>2.2%(371/16490)</td>
<td>0.98(0.81–1.18)</td>
<td>0.8</td>
<td></td>
</tr>
<tr>
<td><strong>Summary</strong></td>
<td>3.1%(453/14585)</td>
<td>4.5%(1551/34636)</td>
<td>0.78 (0.70–0.87)</td>
<td>1.20E–05</td>
<td>0.002</td>
</tr>
<tr>
<td><strong>Cardiovascular Death</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACTIVE-A</td>
<td>12.6%(40/318)</td>
<td>16.8%(125/743)</td>
<td>0.67(0.45–1.00)</td>
<td>0.05</td>
<td></td>
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<tr>
<td>CURE</td>
<td>2.8%(35/1233)</td>
<td>5.9%(204/3429)</td>
<td>0.49(0.34–0.71)</td>
<td>1.38E–04</td>
<td></td>
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<tr>
<td>DREAM</td>
<td>0.2%(10/4802)</td>
<td>0.1%(10/9301)</td>
<td>1.80(0.74–4.41)</td>
<td>0.2</td>
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</tr>
<tr>
<td>ONTARGET</td>
<td>5.3%(45/857)</td>
<td>6.6%(183/2753)</td>
<td>0.77(0.55–1.08)</td>
<td>0.13</td>
<td></td>
</tr>
<tr>
<td>RE–LY</td>
<td>1.5%(9/581)</td>
<td>1.9%(37/1920)</td>
<td>0.77(0.37–1.62)</td>
<td>0.49</td>
<td></td>
</tr>
<tr>
<td>WGHS</td>
<td>0.6%(41/6794)</td>
<td>0.6%(94/16490)</td>
<td>1.08(0.75–1.56)</td>
<td>0.66</td>
<td></td>
</tr>
<tr>
<td><strong>Summary</strong></td>
<td>1.2%(180/14585)</td>
<td>1.9%(653/34636)</td>
<td>0.76 (0.63–0.90)</td>
<td>1.70E–03</td>
<td>0.025</td>
</tr>
<tr>
<td><strong>Myocardial Infarction</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACTIVE-A</td>
<td>2.8%(9/318)</td>
<td>4.6%(34/743)</td>
<td>0.58(0.27–1.23)</td>
<td>0.16</td>
<td></td>
</tr>
<tr>
<td>CURE</td>
<td>3.4%(42/1233)</td>
<td>6.2%(213/3429)</td>
<td>0.55(0.39–0.77)</td>
<td>5.90E–04</td>
<td></td>
</tr>
<tr>
<td>DREAM</td>
<td>0.4%(20/4802)</td>
<td>0.7%(66/9301)</td>
<td>0.59(0.36–0.99)</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>ONTARGET</td>
<td>7.6%(65/857)</td>
<td>7.4%(204/2753)</td>
<td>1.02(0.76–1.36)</td>
<td>0.90</td>
<td></td>
</tr>
<tr>
<td>RE–LY</td>
<td>1.0%(6/581)</td>
<td>1.9%(37/1920)</td>
<td>0.53(0.22–1.26)</td>
<td>0.15</td>
<td></td>
</tr>
<tr>
<td>WGHS</td>
<td>0.9%(60/6794)</td>
<td>1.0%(157/16490)</td>
<td>0.94(0.70–1.26)</td>
<td>0.67</td>
<td></td>
</tr>
<tr>
<td><strong>Summary</strong></td>
<td>1.4%(202/14585)</td>
<td>2.1%(711/34636)</td>
<td>0.76 (0.67–0.92)</td>
<td>3.00E–03</td>
<td>0.047</td>
</tr>
<tr>
<td><strong>Stroke</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACTIVE-A</td>
<td>7.5%(24/318)</td>
<td>10.9%(81/743)</td>
<td>0.64(0.39–1.03)</td>
<td>0.07</td>
<td></td>
</tr>
<tr>
<td>CURE</td>
<td>0.6%(8/1233)</td>
<td>1.4%(47/3429)</td>
<td>0.47(0.22–1.00)</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>DREAM</td>
<td>0.3%(14/4802)</td>
<td>0.4%(34/9301)</td>
<td>0.75(0.40–1.42)</td>
<td>0.38</td>
<td></td>
</tr>
<tr>
<td>ONTARGET</td>
<td>4.3%(37/857)</td>
<td>4.4%(120/2753)</td>
<td>0.99(0.68–1.44)</td>
<td>0.95</td>
<td></td>
</tr>
<tr>
<td>RE–LY</td>
<td>1.7%(10/581)</td>
<td>1.9%(36/1920)</td>
<td>0.90(0.44–1.83)</td>
<td>0.78</td>
<td></td>
</tr>
<tr>
<td>WGHS</td>
<td>1.1%(72/6794)</td>
<td>1.2%(198/16490)</td>
<td>0.90(0.69–1.17)</td>
<td>0.43</td>
<td></td>
</tr>
<tr>
<td><strong>Summary</strong></td>
<td>1.1%(165/14585)</td>
<td>1.5%(516/34636)</td>
<td>0.83 (0.70–1.00)</td>
<td>0.04</td>
<td>0.45</td>
</tr>
</tbody>
</table>

Odds Ratio

72
Figure 3.2: Analysis of association of rs20417 carrier status with major cardiovascular events stratified by aspirin use in six prospective patient populations.

<table>
<thead>
<tr>
<th>Study Name</th>
<th>Carriers</th>
<th>Noncarriers</th>
<th>Odds Ratio (95% CI)</th>
<th>P</th>
<th>Hetero. P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Aspirin users</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACTIVE-A</td>
<td>18.2% (58/318)</td>
<td>25.4% (189/743)</td>
<td>0.62 (0.44–0.86)</td>
<td>5.10E-03</td>
<td></td>
</tr>
<tr>
<td>CURE</td>
<td>6.0% (74/1233)</td>
<td>11.1% (382/3429)</td>
<td>0.53 (0.41–0.68)</td>
<td>1.40E-06</td>
<td></td>
</tr>
<tr>
<td>DREAM</td>
<td>2.3% (11/487)</td>
<td>1.4% (15/1048)</td>
<td>1.46 (0.65–3.29)</td>
<td>0.36</td>
<td></td>
</tr>
<tr>
<td>ONTARGET</td>
<td>13.5% (90/667)</td>
<td>15.0% (326/2176)</td>
<td>0.89 (0.69–1.15)</td>
<td>0.36</td>
<td></td>
</tr>
<tr>
<td>RE-LY</td>
<td>1.6% (3/187)</td>
<td>4.3% (23/540)</td>
<td>0.35 (0.10–1.19)</td>
<td>0.09</td>
<td></td>
</tr>
<tr>
<td>WGHS</td>
<td>2.1% (71/3388)</td>
<td>2.3% (185/8224)</td>
<td>0.94 (0.71–1.23)</td>
<td>0.64</td>
<td></td>
</tr>
<tr>
<td><strong>Summary</strong></td>
<td>4.9% (307/6280)</td>
<td>6.9% (1120/16160)</td>
<td>0.74 (0.64–0.84)</td>
<td>1.20E-05</td>
<td>0.005</td>
</tr>
<tr>
<td><strong>Aspirin non-users</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DREAM</td>
<td>0.5% (23/4314)</td>
<td>1.0% (82/8252)</td>
<td>0.53 (0.33–0.85)</td>
<td>8.90E-03</td>
<td></td>
</tr>
<tr>
<td>ONTARGET</td>
<td>18.9% (36/190)</td>
<td>19.6% (113/577)</td>
<td>0.91 (0.60–1.39)</td>
<td>0.67</td>
<td></td>
</tr>
<tr>
<td>RE-LY</td>
<td>2.8% (11/394)</td>
<td>3.6% (50/1380)</td>
<td>0.78 (0.40–1.52)</td>
<td>0.47</td>
<td></td>
</tr>
<tr>
<td>WGHS</td>
<td>2.3% (76/3388)</td>
<td>2.2% (186/8224)</td>
<td>1.01 (0.78–1.32)</td>
<td>0.92</td>
<td></td>
</tr>
<tr>
<td><strong>Summary</strong></td>
<td>1.8% (146/8286)</td>
<td>2.3% (431/18433)</td>
<td>0.87 (0.72–1.06)</td>
<td>0.16</td>
<td>0.13</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>3.1% (453/14566)</td>
<td>4.5% (1551/34593)</td>
<td>0.78 (0.70–0.87)</td>
<td>1.20E-05</td>
<td>0.004</td>
</tr>
</tbody>
</table>
Figure 3.3: Analysis of association of rs20417 carrier status with major cardiovascular events stratified by previous coronary artery disease in six prospective patient populations.

<table>
<thead>
<tr>
<th>Study Name</th>
<th>Carriers</th>
<th>Noncarriers</th>
<th>Odds Ratio (95% CI)</th>
<th>P</th>
<th>Hetero. P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Previous CAD</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACTIVE–A</td>
<td>26.7% (24/90)</td>
<td>32.1% (72/224)</td>
<td>0.76 (0.43–1.33)</td>
<td>0.33</td>
<td></td>
</tr>
<tr>
<td>CURE</td>
<td>6.0% (74/1233)</td>
<td>11.1% (382/3429)</td>
<td>0.53 (0.41–0.68)</td>
<td>1.40E–06</td>
<td></td>
</tr>
<tr>
<td>DREAM</td>
<td>2.6% (1/39)</td>
<td>2.7% (2/75)</td>
<td>1.17 (0.046–29.66)</td>
<td>0.92</td>
<td></td>
</tr>
<tr>
<td>ONTARGET</td>
<td>14.6% (98/669)</td>
<td>16.2% (358/2213)</td>
<td>0.88 (0.69–1.13)</td>
<td>0.31</td>
<td></td>
</tr>
<tr>
<td>RE–LY</td>
<td>3.8% (7/183)</td>
<td>7.6% (47/616)</td>
<td>0.48 (0.21–1.09)</td>
<td>0.08</td>
<td></td>
</tr>
<tr>
<td><strong>Summary</strong></td>
<td>9.2% (204/2214)</td>
<td>13.1% (861/6557)</td>
<td>0.69 (0.58–0.81)</td>
<td>1.10E–05</td>
<td>0.062</td>
</tr>
<tr>
<td><strong>No previous CAD</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACTIVE–A</td>
<td>14.9% (34/228)</td>
<td>22.5% (117/519)</td>
<td>0.55 (0.36–0.85)</td>
<td>6.70E–03</td>
<td></td>
</tr>
<tr>
<td>DREAM</td>
<td>0.7% (33/4763)</td>
<td>1.0% (95/9226)</td>
<td>0.67 (0.45–1.00)</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>ONTARGET</td>
<td>14.9% (28/188)</td>
<td>15.0% (81/540)</td>
<td>0.97 (0.61–1.56)</td>
<td>0.91</td>
<td></td>
</tr>
<tr>
<td>RE–LY</td>
<td>1.8% (7/398)</td>
<td>2.0% (26/1304)</td>
<td>0.86 (0.37–1.99)</td>
<td>0.72</td>
<td></td>
</tr>
<tr>
<td>WGHS</td>
<td>2.2% (147/6794)</td>
<td>2.2% (371/16490)</td>
<td>0.98 (0.81–1.18)</td>
<td>0.8</td>
<td></td>
</tr>
<tr>
<td><strong>Summary</strong></td>
<td>2.0% (249/12371)</td>
<td>2.5% (690/28079)</td>
<td>0.86 (0.74–1.00)</td>
<td>0.06</td>
<td>0.11</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>3.1% (453/14585)</td>
<td>4.5% (1551/34636)</td>
<td>0.78 (0.70–0.87)</td>
<td>1.30E–05</td>
<td>0.015</td>
</tr>
</tbody>
</table>
CHAPTER 4

The effect of bile acid sequestrants on the risk of cardiovascular events: A Mendelian Randomization analysis

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6. Department of Cardiovascular Sciences, University of Leicester, Leicester, UK
7. National Institute for Health Research Leicester Cardiovascular Biomedical Research Unit, Glenfield Hospital, Leicester, UK
8. Department of Medicine, University of Ottawa, Ottawa, ON, Canada
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10. Thrombosis & Atherosclerosis Research Institute, Hamilton Health Sciences & McMaster University, Hamilton, ON, Canada

*Authors of the CARDIoGRAMplusC4D Consortium are included in the Supplemental Material

WORD COUNT: 6,475

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Email: pareg@mcmaster.ca
ABSTRACT

Background: Statins are used to lower low density lipoprotein cholesterol (LDL-C) but they may be ineffective or not well tolerated. Bile acid sequestrants (BAS) act to reduce the intestinal absorption of cholesterol but previous trials were underpowered to demonstrate an effect on clinical outcomes.

Methods and Results: We conducted a systematic review and meta-analysis of randomized controlled trials (RCTs) to assess the effect of two approved BAS, cholestyramine and colesevelam, compared to a placebo on plasma lipid levels. We then applied the principles of Mendelian Randomization to estimate the effect of BAS on reducing the risk of CAD. First, we quantified the effect of rs4299376 (ABCG5/ABCG8), which affects the intestinal cholesterol absorption pathway targeted by BAS, on both LDL-C and CAD, and then we used these estimates to predict the effect of BAS on CAD. Nineteen RCTs with a total of 7,021 study participants were included. Cholestyramine 24g/d was associated with a reduction in LDL-C of 23.5 mg/dL (95% CI: -26.8,-20.2; N=3,806) and a trend towards reduced risk of CAD (OR: 0.81, 95% CI: 0.70-1.02; P=0.07; N=3,806) while colesevelam 3.75g/d was associated with a reduction in LDL-C of 22.7 mg/dL (95% CI: -28.3,-17.2; N=759). Based on genetic findings demonstrating that rs4299376 was associated with a 2.75 mg/dL decrease in LDL-C and a 5% decrease in risk of CAD outcomes, we estimated that cholestyramine may be associated with an OR for CAD of 0.63 (95% CI: 0.52 - 0.77; P= 6.3x10⁻⁶; N=123,223) and colesevelam with an OR of 0.64 (95% CI: 0.52-0.79, P: 4.3x10⁻⁵). These estimates were not statistically different from previously reported trends from BAS clinical trials (P>0.05).

Conclusions: The cholesterol lowering effect of BAS can be expected to translate into a clinically relevant reduction in the risk of CAD.
Keywords: cholesterol-lowering drugs, coronary artery disease, genetics, lipids, Mendelian randomization
INTRODUCTION

Elevated plasma levels of low-density lipoprotein cholesterol (LDL-C) are a well-established risk factor of cardiovascular disease (CVD)\(^1\). Current guidelines recommend that statin therapy should be used in select groups of patients with atherosclerotic CVD in primary and secondary prevention settings\(^2\). However, statins may not be fully effective in lowering LDL-C\(^3,4\) or well tolerated\(^5\), and therefore patients may require additional or alternative lipid-lowering treatments.

Bile acid sequestrants (BAS) are large polymers that bind to bile salts in the small intestine, preventing their reabsorption into the enterohepatic circulation pathway. The resulting depletion of bile acids leads to increased hepatic metabolism of cholesterol for bile salt synthesis, thereby lowering plasma LDL-C levels\(^6\). Three BAS have been approved for clinical use: cholestyramine and colestipol (first generation) and colesevelam hydrochloride (colesevelam) (second generation). Colesevelam was developed to overcome gastrointestinal intolerance associated with the first-generation BAS\(^7,9\). Three randomized controlled trials (RCTs) have evaluated the efficacy of cholestyramine for cardiovascular prevention but results have been inconclusive\(^8,10,11\). Although most of these trials have demonstrated that treatment with cholestyramine reduces LDL-C levels, only one trial has shown a modest reduction in the risk of CVD events (OR: 0.81 (95% CI: 0.70 -1.02); \(P=0.07\))\(^8\). To date, there are no adequately powered trials exploring the effects of colesevelam or colestipol on the risk of major cardiovascular events. Thus the efficacy of BAS in the prevention of CVD is uncertain.
Mendelian Randomization analyses use genetic variants with a known biological function to explore the effects of a modifiable exposure on an outcome. Genetic variants are useful instruments for assessing causality because they are randomly allocated and they are independent of many factors that may confound observational associations. Thus, in the absence of evidence from randomized trials, the principles of Mendelian Randomization can be applied for drug target validation as functional alleles of a gene within a drug target pathway can be used to extrapolate the effects of the pharmacological intervention. This approach can strengthen the rationale for conducting an RCT because it is highly cost-effective due to the availability of genetic data through large-scale biobanks and data consortia.

The ATP-binding cassette (ABC) genetic subfamily forms active membrane transporters that regulate the delivery and disposal of intestinal cholesterol and affects the same pathway that is targeted by BAS. The ATP-binding cassette sub-family G member 5 (ABCG5) and ABCG8 genes are mainly expressed in hepatocytes and enterocytes. In the liver, these transporter genes are responsible for increased biliary cholesterol secretion, while in the intestine, they recycle free cholesterol from the enterocyte back into the intestine lumen and promote the fecal excretion of biliary sterols. The rs4299376 single nucleotide polymorphism (SNP) is an intronic variant of ABCG5/8 that has been associated with altered plasma LDL-C levels and risk of coronary artery disease (CAD) in the CARDIoGRAMplusC4D Consortium. The same variant is in perfect linkage disequilibrium (r^2=1) with the rs6544713 SNP, which is also known to be associated with CAD. This genetic polymorphism represents a potential proxy for the mechanism-based effect of BAS on LDL-C and the risk of CVD.
In order to test whether BAS has the potential to reduce the risk of cardiovascular outcomes, we first conducted a systematic review and meta-analysis to assess the effect of BAS on plasma lipid levels and major cardiovascular outcomes. We then applied principles of Mendelian Randomization to predict the effect of BAS on CAD using the known genetic association of the \textit{ABCG5/ABCG8} polymorphism rs4299376 with lipids\textsuperscript{23} and CAD\textsuperscript{22}.

**METHODS**

\textit{Search strategy and study selection of clinical trials}

A structured search of RCTs evaluating the effects of BAS on markers of cardiovascular risk or clinical outcomes was conducted in the PubMed database. The following terms were used to search all clinical trial registries and databases: colesevelam; cholestyramine; colestipol; placebo; and randomized controlled trials. Only studies with a double-blinded, placebo-controlled trial design in adults aged 18 years that assessed the effect of BAS (i.e. cholestyramine, colestipol and colesevelam) in comparison with a placebo were included. Refer to the Supplemental Methods for more details.

\textit{Global Lipids Genetics Consortium}

Data on the genetic association between the rs4299376 SNP and plasma lipid levels were obtained from a previously published genome-wide association study (GWAS). In brief, Teslovich et al (2011) performed a meta-analysis of 46 lipid GWAS assessing common variants associated with serum lipids (LDL-C, high density lipoprotein cholesterol (HDL-C), total cholesterol (TC), and triglycerides)\textsuperscript{23}. A total of 46 studies and 91,285 individuals of European
descent were analyzed for the genetic association with LDL-C, while data from 95,708, 95,992 and 92,410 individuals were available for HDL-C, TC and triglycerides, respectively.

**CARDIoGRAMplusC4D Consortium**

Data on the genetic association between the rs4299376 SNP (ABCG5/8) and the risk of CAD was obtained from the CARDIoGRAMplusC4D Consortium. Briefly, the CARDIoGRAMplusC4D Consortium performed a meta-analysis of 63,746 cases of CAD and 130,681 controls\(^2^2\). CAD outcomes were defined as one of the following: myocardial infarction (MI), > 50% stenosis in at least one coronary vessel at angiography, history of percutaneous transluminal coronary angioplasty or coronary artery bypass graft surgery, angina or death due to CAD\(^2^4\). For the association between the rs4299376 SNP and CAD outcomes the lipid-lowering allele was used as reference throughout the manuscript.

**Cholesterol Treatment Trialists' (CTT) Collaboration**

As a sensitivity analysis, we confirmed the predicted effect of BAS on CAD using data from the Cholesterol Treatment Trialists' (CTT) Collaboration\(^2^5\). Briefly, the CTT assessed the association between change in LDL-C with statin therapy and reduction in risk of CVD. The CTT was a prospective meta-analysis from 169,138 individuals from 26 statin RCTs. Over a period of 5 years, there were a total of 24,323 major vascular events, which was defined as the first occurrence of coronary death or non-fatal MI, coronary revascularization, or stroke.
Statistical Analysis

To calculate the effect of BAS on plasma lipids levels, the mean change-from-baseline of plasma lipids in the 24 g/d cholestyramine treatment group and the 3.75 g/d colesvelam group were compared to the mean differences in the placebo group. Meta-analyses were performed using an inverse variance random effect meta-analysis. Refer to the Supplemental Methods for further details.

Simulations were performed to predict the effect of 24 g/d cholestyramine on plasma lipid profiles (HDL-C, TC, and triglycerides) using the known genetic associations of rs4299376 SNP with lipids fractions. To do so, we adapted the method from Sofat et al\textsuperscript{14} to match the genetic effects to the effect of cholestyramine 24 g/d on LDL-C, taking into account the uncertainty of both the genetic and drug effect estimates. Refer to the Supplemental Methods for more information. In order to validate whether the rs4299376 SNP had a similar effect on plasma lipid profiles as cholestyramine, the predicted effects of cholestyramine on plasma levels of HDL-C, TC and triglycerides were estimated using genetic data. These predicted estimates were then compared to known effects of cholestyramine on the same lipids fractions from clinical data. Next, the predicted effect of cholestyramine on the risk of cardiovascular outcomes was projected using data from the genetic association of rs4299376 with CAD. This was then compared to the effect of cholestyramine on CAD from the only outcome trial of cholestyramine, Lipid Research Clinics Coronary Primary Prevention Trial (LRCCPPT)\textsuperscript{8}. As a sensitivity analysis, the predicted effect of cholestyramine on CAD was also estimated using data from the CTT\textsuperscript{25}. This estimate was similarly compared to the cardiovascular outcomes reported in the LRCCPPT in order to compare the predicted effect of BAS with statin use. The same analyses
were performed for 3.75 g/d colesvelam. Refer to the Supplemental Methods for more information. All statistical analyses were performed using R.

RESULTS

Study Selection
The structured literature search of PubMed databases derived a total of 420 citations and 19 studies were identified for inclusion in this review. Supplemental Figure 1 contains a flow diagram of the study selection process. Owing to the lack of reported data from clinical trials the results of the colestipol meta-analysis are described in the Supplemental Methods.

Randomized Controlled Trials of Cholestyramine
We identified a total of six RCTs comprising 4,598 hyperlipidemia participants (mean age 49.5 years, 4.8% women)\(^8;10;11;26-28\) (Table 4.1). In the pooled analysis of plasma lipid levels, three RCTs evaluated the effect of 24 g of cholestyramine daily dose compared to matching placebo in 4,002 hyperlipidemia patients (Figure 4.1). The pooled estimates indicate that cholestyramine treatment resulted in a mean decrease of LDL-C by 53.4 mg/dL (95% CI: -91.8, -15.0) and a decrease of TC by 50.7 mg/dL (95% CI: -89.9, -11.5). There was significant heterogeneity among the pooled changes in LDL-C (I\(^2\): 93.3% and P for heterogeneity: 5.4x10\(^{-6}\)) and TC (I\(^2\): 93.5% and P for heterogeneity: 9.1x10\(^{-6}\)). Two pooled studies (196 participants) demonstrated a nonsignificant effect in the change of HDL-C and triglycerides (2.6 mg/dL (95% CI: -1.2, 6.5) and 3.1 mg/dL (95% CI: -15.5, 21.7), respectively). One study (80 participants) reported a significant decrease of apoB by 44.0 mg/dL (95% CI: -61.7, -26.3) and a nonsignificant effect in the change of apoA (10.0 mg/dL (95% CI: -3.9, 23.9)). One RCT reported the effect of
cholestyramine (24 g/d) on cardiovascular outcomes⁸, randomizing 3,806 patients, 342 of whom experienced an event. Cholestyramine did not significantly reduce the composite of cardiovascular death or MI (OR: 0.81, 95% CI: 0.65 - 1.02, P=0.07), cardiovascular mortality (OR: 0.78, 95% CI: 0.48-1.27, P=0.322) or MI (OR: 0.81, 95% CI: 0.63-1.03, P=0.082).

**Randomized Controlled Trials of Colesevelam**

We identified 10 trials with a total of 1,142 participants with hyperlipidemia and 883 participants with type two diabetes mellitus²⁰;²⁹-³⁷ (mean age 50.2 years, 51% women) (Table 4.1). Seven RCTs comprising 767 study participants evaluating the effect of colesevelam 3.75 g daily compared to matching placebo were used in the primary analysis (Figure 4.2). Treatment with colesevelam resulted in a mean decrease of LDL-C by 22.7 mg/dL (95% CI: -28.3,-17.2) with significant heterogeneity among the pooled change in LDL-C (I²: 56.95% and P for heterogeneity: 0.032). Colesevelam treatment was also associated with a decrease in TC by 19.2 mg/dL (95% CI: -24.4,-14.0) while the effect was attenuated in HDL-C and triglycerides (0.30 mg/dL (95% CI: -0.14, 2.0) and 9.8 mg/dL (95% CI: -1.8, 21.4), respectively). Five pooled studies (628 participants) demonstrated a decrease of apoB by 14.0 mg/dL (95% CI: -17.7,-10.3) and had a nonsignificant effect in the change of apoA (1.8 mg/dL (95% CI: -0.8, 4.5)). We were unable to conduct subgroup analyses in order to explore the presence of heterogeneity among pooled estimates owing to a lack of data.

**Predicted effects of BAS on plasma lipids using genetic data**

Teslovich et al (2010) confirmed the association between the rs4299376 SNP and plasma lipid levels²³. The rs4299376 polymorphism was significantly associated with a decrease in LDL-C of
2.75 mg/dL per allele (95% CI: -3.14, -2.36) (P=1.73x10^{-47}), a decrease in TC of 3.01 (95% CI: -3.44, -2.58) mg/dL per allele (P=4.0x10^{-45}), a decrease in triglycerides of 1.08 (95% CI: -1.80, -0.36) mg/dL per allele (P=0.003) and had a null effect on HDL-C levels (0.05 mg/dL per allele, 95% CI: -0.09, 0.19; P=0.212). We also explored whether the rs4299376 SNP had potential pleiotropic effects on the risk of diabetes or on the change in glycated hemoglobin (HbA1c), fasting glucose, systolic blood pressure, diastolic blood pressure and body mass index using data from the DIAGRAM^{38}, MAGIC^{39,40}, GIANT^{41} and ICBP^{42} consortia. We did not observe any significant changes among these traits (P > 0.05 for all) (Supplemental Table 2). Next, we sought to determine whether the predicted effect of BAS using genetic data had a similar effect on plasma lipids levels as compared to the reported pharmacological effect. To do so, we adjusted the per-allele genetic effect to match the LDL-C reducing effect of 24 g/d cholestyramine, as reported in the LRCCPPT trial^{8} (the only BAS outcome trial available). We then predicted the effect of cholestyramine on TC using genetic data, and compared it to the known effect of cholestyramine. The predicted reduction of TC was 25.8 mg/dL (95% CI: -32.3, -19.4), which was not statistically different from the reported trial estimate (P for difference > 0.05).

We performed a similar analysis using the effect of colesevelam 3.75 g/d on LDL-C as the reference for the genetic effect (Figure 4.3). The predicted reduction of TC by colesevelam was estimated at 25.0 mg/dL (95% CI: -33.0, -16.9), which was not different (P>0.05) from results of our meta-analysis. The predicted effect on HDL was null (0.42 mg/dL, 95% CI: -0.78, 1.61) and was consistent with the reported effect of colesevelam (P for difference >0.05). The predicted effect of colesevelam was associated with a modest decrease in triglycerides (8.94 mg/dL (95%
CI: -15.5, -2.32) and was statistically different from the observed drug effect (P for difference: 0.001).

**Predicted effects of BAS on cardiovascular outcomes using genetic data**

Data from the CARDIoGRAMplusC4D Consortium was obtained to assess the association of rs4299376 with risk of CAD. The minor allele (LDL-C decreasing) of rs4299376 was associated with a modest yet significant decrease in risk of CAD (OR: 0.95, 95% CI: 0.93 – 0.97; P=2.85x10^{-7}). We then derived the predicted effect of 24g/d cholestyramine on risk of CAD based on the association of the ABCG5/8 rs4299376 polymorphism on CAD, adjusting the per-allele genetic effect to match the LDL-C reducing effect of 24 g/d cholestyramine. Cholestyramine 24g/d was predicted to significantly reduce the risk of CAD (OR= 0.63, 95% CI: 0.52 - 0.77; P=6.3x10^{-6}). The predicted estimate was not significantly different from the effect observed in the only outcome trial of cholestyramine, LRCCPPT (P for difference>0.05) (Figure 4.4). The effect of rs4299376 was also matched to the LDL-C reducing effect of 3.75 g/d colesvelam, leading to a predicted CAD reduction of OR=0.64 (95% CI: 0.52-0.79; P=4.3x10^{-5}) with colesvelam 3.75 g/d (P for difference>0.05) (Figure 4.4).

**Predicted effect of BAS on cardiovascular outcomes based on CTT data**

As a sensitivity analysis, we estimated the predicted effect of 24g/d cholestyramine on CVD outcomes using data from the CTT, a large meta-analysis evaluating the effect of cholesterol reduction on CVD. The change in LDL-C levels from 24g/d cholestyramine was predicted to significantly decrease the risk of major vascular events (OR: 0.86, 95% CI: 0.85 - 0.87; P=6.6x10^{-83}) (Figure 4.4). This estimate was not significantly different from observed effect of
cholestyramine from clinical trial\textsuperscript{8} (LRCCPPT; P for difference $> 0.05$). Similarly, the effect of 3.75g/d colesevelam was also predicted to significantly reduce the risk of cardiovascular events (OR: 0.90, 95% CI: 0.87 - 0.93; $P=1.3\times10^{-13}$; P for difference $> 0.05$).

**DISCUSSION**

Mendelian Randomization analyses utilize the random allocation of alleles in order to replicate the randomization process in double-blinded clinical trials and to reduce the potential effects of reverse causation and confounding factors. The results of our Mendelian Randomization analysis suggest that BAS may be effective in the prevention of CAD. Thus, when given in currently recommended doses, our data demonstrates that cholestyramine and colesevelam were associated with a reduced risk of CAD. Furthermore, our projections concerning the effect of BAS on clinical outcomes were consistent with estimates obtained from the cholestyramine LRCCPPT trial and the CTT.

The predicted effects of BAS on cardiovascular outcomes were based on robust genetic data derived from the CARDIOGRAMplusC4D and the Global Lipids Genetics Consortia, which collectively involved 388,353 individuals from prospective cohort and case-control studies. Leveraging already available genetic data is highly cost-effective and has the added advantage of providing estimates that reflect lifelong difference in plasma LDL-C levels between carriers and non-carriers of the rs4299376 allele. In contrast, randomized trials are complex, expensive and are generally restricted to several years of follow-up, which limits the ability to assess the long-term effects of BAS on clinical outcomes.
Our findings have important clinical implications. Although BAS monotherapy may not be as effective as statin therapy, our results suggest that BAS are likely to be an effective second-line therapy. In contrast, adequately powered randomized trials have failed to show a benefit of Niacin and CETP inhibitors \(^{43-45}\). Additionally, there has been a shift in clinical guidelines, where patients are more likely to be prescribed with high dose statin therapy to reduce the risk of CAD irrespective of meeting specific LDL-C targets\(^2\). However, statin therapy may not be well-tolerated or effective in all patients, and the addition of BAS in combination with statin therapy may further prevent the risk of CAD. Even though there is clinical evidence demonstrating that cholestyramine effectively reduces LDL-C levels, as well as suggestive evidence that it decreases the risk of CAD events, its use is hampered by poor patient tolerability and adverse side effects \(^6\). Colesevelam is much better tolerated \(^{46,47}\), has other potential benefits, such as reducing fasting blood glucose levels\(^{48}\), and in our Mendelian Randomization analysis produced a similar reduction in CAD to that of cholestyramine. Thus our study demonstrates that there is a need for well-designed clinical trials to fully understand the clinical efficacy and safety of BAS, especially colesevelam.

The \(ABCG5/8\) genes and BAS act through related biological mechanisms. BAS bind to intestinal bile acids and are excreted through the feces, thus impeding the enterohepatic circulation of bile acid. This leads to an increase in bile acid synthesis and a subsequent decrease in plasma LDL-C levels\(^{49}\). Animal models have demonstrated that hepatic \(ABCG5/8\) transporters are responsible for secreting multiple sterols in the bile while intestinal transporters limit cholesterol absorption from the lumen and thus promote fecal excretion\(^{50,51}\). Overexpression of \(ABCG5/8\) genes in transgenic mice resulted in an increase in biliary cholesterol secretion, reduced cholesterol
absorption, and increased hepatic cholesterol synthesis \(^{50}\), leading to a significant reduction in plasma cholesterol levels and atherosclerotic lesions. In addition, treatment with BAS has also been associated with reduced levels of fasting plasma glucose \(^{48}\). Although the underlying mechanism is unknown, it has been suggested that the binding of BAS to bile acids alters the GI tract glucose absorption \(^{52}\). However, we observed that the rs4299376 SNP was not associated with the changes in the levels of fasting glucose or HbA\(_{1c}\) and diabetes using data from the MAGIC and DIAGRAM Consortia \((P>0.05 \text{ for all})^{38,39}\). Furthermore, genetic mutations of \(ABCG5/8\) have been associated with sitosterolemia, a rare genetic disorder resulting in increased intestinal absorption, decreased biliary excretion of dietary sterols, hypercholesterolemia and atherosclerosis. BAS treatment lowers blood levels of dietary sterols \(^{53,54}\) and is recommended for patients with sitosterolemia. Teupser et al (2010) also reported that common \(ABCG5/8\) polymorphisms lower phytosterol levels as well as CVD risk \(^{55}\), again confirming the similarity between BAS treatment and the effect of rs4299376. Taken together, these results confirm the similarity between BAS treatment and the effect of rs4299376. Therefore our genetic results illustrate that inhibition of intestinal cholesterol absorption may provide a valuable therapeutic target for the prevention of CVD.

A few limitations of our study warrant discussion. First, we were unable to assess the overall effect of colestipol on plasma lipid levels, the effect of colesevelam and colestipol on cardiovascular outcomes or the predicted effect of BAS on apoA, apoB and adverse side effects due to the lack of reported data. Second, we found that the effect of colesevelam on triglycerides predicted by genetic data was statistically different from the pharmacological effect. Nonetheless, the predicted effect was weak \((8.94 \text{ mg/dL (95\% CI: 15.5, 2.32)})\) and should not
affect CAD risk estimates since the effect size of triglycerides is modest in comparison with other CAD risk factors. Furthermore, our meta-analysis may have been underpowered to detect any change because triglycerides are highly clinically variable. However, the effects on TC and HDL-C predicted from genetic data were consistent with estimates from the meta-analysis. Third, the protective effect of BAS on CAD was larger in the Mendelian Randomization analysis as compared to the reported trend from LRCCPPT and estimates derived from the CTT. Although the differences in estimates were not statistically different, this may be due to the observation that rs4299376 carriers have a lifelong exposure to lower levels of LDL-C. Fourth, there may also be a possibility of pleiotropic effects whereby either the rs4299376 SNP or BAS influence yet unknown pathways unrelated to lipids. For instance, both are involved in the absorption of dietary sterol which may be a key mediator of their CAD protective effect. Fifth, the predicted side effects of BAS therapy using a Mendelian Randomization analysis have not been addressed and further research may be required.

In summary, this systematic review, meta-analysis and large-scale Mendelian Randomization analysis illustrates that pharmacological inhibition of intestinal cholesterol absorption may reduce the risk of major cardiovascular events. Comparison of genetic association studies and clinical trials of coleselvam supports the potential use of BAS as a second line therapy to reduce LDL-C in the prevention of CAD. Our results point to the need for large-scale randomized trials to fully assess the efficacy and safety of BAS treatment on CVD, as well as their effect when combined with other lipid lowering agents such as statins.
ACKNOWLEDGMENTS

We are thankful to all the participants having agreed to contribute to this project. Data on CAD have been contributed by CARDIoGRAMplusC4D investigators and have been downloaded from www.CARDIOGRAMPLUSC4D.ORG. Data on plasma lipid levels have been contributed by Global Lipids Genetic Consortium investigators and have been downloaded from http://www.sph.umich.edu/csg/abecasis/public/lipids2010/.

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DISCLOSURES

No conflicts of interest were reported.
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(34) Hunninghake D, Insull W, Jr., Toth P, Davidson D, Donovan JM, Burke SK.


Ph.D. Thesis – S. Ross; McMaster University – Clinical Epidemiology & Biostatistics


mutations involving sterolin-1 and sterolin-2, encoded by ABCG5 and ABCG8, respectively. *Am J Hum Genet* 2001;69:278-90.


FIGURE LEGENDS

Figure 4.1: Forest plot of the association of 24 g/d of cholestyramine treatment and the mean difference of LDL-C, HDL-C, total cholesterol, triglycerides, apoA and apoB.

Het P refers to the heterogeneity p-value.

Figure 4.2: Forest plot of the association of 3.75 g/d of colesevelam treatment and the mean difference of LDL-C, HDL-C, total cholesterol, triglycerides, apoA and apoB.

Het P refers to the heterogeneity p-value.

Figure 4.3: Predicted effects of BAS using genetic data and the effect of 3.75g/d colesevelam on LDL-C, HDL-C, TC and triglycerides.

Figure 4.4: Predicted effects of BAS using genetic data and the effects of 24g/d cholestyramine and 3.75 g/d colesevelam on the risk of CAD outcomes.

CAD is defined as one of the following: MI, > 50% stenosis in at least one coronary vessel at angiography, history of percutaneous transluminal coronary angioplasty or coronary artery bypass graft surgery, angina or death due to CAD\textsuperscript{24}. Diff P represents the statistical difference between the predicted BAS effect on CAD outcomes as compared to the pharmacological effect on CAD outcomes.
TABLES

Table 4.1: Studies contributing to the BAS meta-analysis

<table>
<thead>
<tr>
<th>Author &amp; Date</th>
<th>Patient Population</th>
<th>Follow-Up</th>
<th>Intervention</th>
<th>Comparison</th>
<th>Total Sample size</th>
<th>Age</th>
<th>Women</th>
<th>LDL-C (mg/dL)*</th>
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<tr>
<td></td>
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<td></td>
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<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
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<td>(NR)</td>
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**CHOLESTRYAMINE**

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<tr>
<th>Author &amp; Date</th>
<th>Patient Population</th>
<th>Follow-Up</th>
<th>Intervention</th>
<th>Comparison</th>
<th>Total Sample size</th>
<th>Age</th>
<th>Women</th>
<th>LDL-C (mg/dL)*</th>
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<tr>
<td>Betteridge 1992</td>
<td>Hyperlipidemia</td>
<td>12 weeks</td>
<td>Pravastatin (20 mg bid); Cholestyramine (16-24 g/d)</td>
<td>Placebo</td>
<td>128</td>
<td>18-70</td>
<td>36(28)</td>
<td>295 (8.9)</td>
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<td>LRCCPPT 1984</td>
<td>Hyperlipidemia</td>
<td>7.4 years</td>
<td>Cholestyramine (24 g/d)</td>
<td>Placebo</td>
<td>3806</td>
<td>47.8</td>
<td>0 (0)</td>
<td>215.6 (NR)</td>
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<td>NHLBI Type II Coronary Intervention Study 1984</td>
<td>Hyperlipidemia</td>
<td>5 years</td>
<td>Cholestyramine (24 g/d)</td>
<td>Placebo</td>
<td>143</td>
<td>46.3 (0.55)</td>
<td>28 (20)</td>
<td>241.8 (6.5)</td>
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<td>Pravastatin Multicenter Study Group II 1993</td>
<td>Hyperlipidemia</td>
<td>8 weeks</td>
<td>Pravastatin (20 mg/bid); Pravastatin (40 mg/bid); Cholestyramine (12 g/bid); Pravastatin (20 mg bid) &amp; Cholestyramine (12 g bid)</td>
<td>Placebo</td>
<td>311</td>
<td>51.9</td>
<td>95 (31)</td>
<td>236(6.6)</td>
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<td>Watts 1992</td>
<td>Hyperlipidemia</td>
<td>3.5 months</td>
<td>Diet &amp; Cholestyramine (8 g/day); Diet</td>
<td>Placebo</td>
<td>90</td>
<td>50.8(4.7)</td>
<td>0 (0)</td>
<td>203.4(8.5)</td>
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<td>Wiklund 1990</td>
<td>Hyperlipidemia</td>
<td>12 weeks</td>
<td>Pravastatin (10-20 mg/bid); Cholestyramine (24 g/d to highest dose)</td>
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<td>120</td>
<td>50.6 (13)</td>
<td>60 (50)</td>
<td>304.6(68.0)</td>
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**COLESEVELAM**

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<th>Author &amp; Date</th>
<th>Patient Population</th>
<th>Follow-Up</th>
<th>Intervention</th>
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<th>Women</th>
<th>LDL-C (mg/dL)*</th>
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<td>Bays 2008</td>
<td>Diabetes</td>
<td>26 weeks</td>
<td>Colesevelam (3.75 g/d) with DM drugs</td>
<td>Placebo with DM drugs</td>
<td>316</td>
<td>56.3(9.6)</td>
<td>152(48)</td>
<td>105.6(33.8)</td>
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<td>Davidson 1999</td>
<td>Hyperlipidemia</td>
<td>6 weeks</td>
<td>Colesevelam (1.5 g/d; 2.25 g/d; 3.0 g/d; or 3.75 g/d)</td>
<td>Placebo</td>
<td>147</td>
<td>56.0(11)</td>
<td>82(56)</td>
<td>202(26)</td>
</tr>
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<td>Davidson 2001</td>
<td>Hyperlipidemia</td>
<td>4 weeks</td>
<td>Colesevelam (2.3 g/d); Lovastatin (10 mg/d); Colesevelam (2.3 g/d) &amp; Lovastatin (10 mg/d)</td>
<td>Placebo</td>
<td>135</td>
<td>57.8(13.4)</td>
<td>72(53)</td>
<td>172(5)</td>
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<tr>
<td>Author &amp; Date</td>
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<td>Intervention</td>
<td>Comparison</td>
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<td>Age</td>
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<td>LDL-C (mg/dL)*</td>
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<td>Endpoint</td>
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<td>Devaraj 2006[^2]</td>
<td>Hyperlipidemia</td>
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<td>Colesevelam (3.75 g/d)</td>
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<td>48</td>
<td>NR</td>
<td>NR</td>
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<tr>
<td>Handelsman 2010[^3]</td>
<td>Diabetes</td>
<td>16 weeks</td>
<td>Colesevelam (3.75 g/d)</td>
<td>Placebo</td>
<td>216</td>
<td>54.5 (11.7)</td>
<td>149 (69)</td>
<td>132.8(23.9)</td>
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<td>Hunninghake 2001[^4]</td>
<td>Hyperlipidemia</td>
<td>4 weeks</td>
<td>Colesevelam (3.8 g/day); Atorvastatin (10 mg/day); Colesevelam (3.8 g/day) &amp; Atorvastatin (10 mg/day); or Atorvastatin (80 mg/day)</td>
<td>Placebo</td>
<td>94</td>
<td>57.2(11.4)</td>
<td>37(39)</td>
<td>184(5)</td>
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<tr>
<td>Insull 2001[^5]</td>
<td>Hyperlipidemia</td>
<td>24 weeks</td>
<td>Colesevelam (2.3 g/day; 3.0 g/day; 3.8 g/day; or 4.5 g/day)</td>
<td>Placebo</td>
<td>467</td>
<td>56 (12)</td>
<td>235 (50)</td>
<td>155(17)</td>
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<tr>
<td>Knapp 2001[^9]</td>
<td>Hyperlipidemia</td>
<td>6 weeks</td>
<td>Colesevelam (3.8 g/d); Simvastatin (10 mg/d); Colesevelam (3.8 g/d) &amp; Simvastatin (10 mg/d); Colesevelam (2.3 g/d); Simvastatin (20 mg/d); or Colesevelam (2.3 g/d) &amp; Simvastatin (20 mg/d)</td>
<td>Placebo</td>
<td>251</td>
<td>54.7(12.4)</td>
<td>118 (47)</td>
<td>198(39)</td>
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<td>Rosenstock 2010[^8]</td>
<td>Diabetes</td>
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<td>Colesevelam (3.75 g/d) with DM drugs</td>
<td>Placebo with DM drugs</td>
<td>286</td>
<td>53.3(10.8)</td>
<td>161 (56.3)</td>
<td>130(NR)</td>
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<tr>
<td>Zieve 2007[^7]</td>
<td>Diabetes</td>
<td>12 weeks</td>
<td>Colesevelam (3.75 g/d)</td>
<td>Placebo</td>
<td>65</td>
<td>56.2 (9.3)</td>
<td>29 (44.6)</td>
<td>122.6(32.7)</td>
</tr>
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</table>

*Refers to the highest single BAS dose reported in the study; NR: not reported
Figure 4.1: Forest plot of the association of 24 g/d of cholestyramine treatment and the mean difference of LDL-C, HDL-C, total cholesterol, triglycerides, apoA and apoB.

Het P refers to the heterogeneity p-value.
Figure 4.2: Forest plot of the association of 3.75 g/d of colesevelam treatment and the mean difference of LDL-C, HDL-C, total cholesterol, triglycerides, apoA and apoB.

Het P refers to the heterogeneity p-value.
Figure 4.3: Predicted effects of BAS using genetic data and the effect of 3.75g/d colesevelam on LDL-C, HDL-C, TC and triglycerides.
Figure 4.4: Predicted effects of BAS using genetic data and the effects of 24g/d cholestyramine and 3.75 g/d colesevelam on the risk of CAD outcomes.

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>OR (95% CI)</th>
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<th>Diff P</th>
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<td><strong>Cholestyramine 24g/d dose</strong></td>
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<tr>
<td>Effect using LRCCPPT RCT data</td>
<td>3806</td>
<td>0.81 (0.70–1.02)</td>
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<td>Predicted effect using genetic data</td>
<td>123223</td>
<td>0.63 (0.52–0.77)</td>
<td>6.30E–06</td>
<td>0.095</td>
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<td>Predicted effect using CTT data</td>
<td>169138</td>
<td>0.86 (0.85–0.87)</td>
<td>6.50E–83</td>
<td>0.629</td>
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<td><strong>Colesevelam 3.75 g/d dose</strong></td>
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<tr>
<td>Predicted effect using genetic data</td>
<td>123223</td>
<td>0.64 (0.52–0.79)</td>
<td>4.03E–05</td>
<td>0.128</td>
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<tr>
<td>Predicted effect using CTT data</td>
<td>169138</td>
<td>0.90 (0.87–0.93)</td>
<td>1.30E–13</td>
<td>0.385</td>
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CHAPTER 5

Mendelian randomization analysis supports the causal role of dysglycemia and diabetes in the risk of coronary artery disease

AUTHOUR(S): Stephanie Ross, MSc\textsuperscript{1,2,3}; Hertzel C. Gerstein, MD\textsuperscript{1,4}; John Eikelboom, MBBS, MSc\textsuperscript{1,4}; Sonia S. Anand MD, PhD, FRCPC\textsuperscript{1,2,3,4}; and Guillaume Paré, MD, MSc\textsuperscript{1,2,3,5,6}

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WORD COUNT: 2,661

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ABSTRACT

INTRODUCTION: Type 2 diabetes is a strong risk factor for coronary artery disease (CAD). However, the absence of a clear reduction in CAD by intensive glucose lowering in randomized controlled trials (RCTs) has fuelled uncertainty regarding the causal role of dysglycemia and CAD.

OBJECTIVE: To assess whether Mendelian randomization supports a causal role of dysglycemia and diabetes for risk of CAD.

METHODS: Effect size estimates of common genetic variants associated with fasting glucose, glycated hemoglobin (HbA1c) and diabetes were obtained from the MAGIC and DIAGRAM consortia. The corresponding effect estimates of these SNPs on the risk of CAD were then evaluated in CARDIOGRAMplusC4D.

RESULTS: SNPs associated with HbA1c and diabetes were associated with an increased risk of CAD. Using information from 59 genetic variants associated with diabetes, the causal effect of diabetes on the risk of CAD was estimated at an odds ratio (OR) of 1.62 (95% CI: 1.23-2.07; P=0.002). On the other hand, nine genetic variants associated with HbA1c was associated with an OR of 1.53 per % increase (95% CI: 1.14-2.05; P=0.023) in the risk of CAD. No significant differences were observed when categorizing genetic loci according to their effect on either β cell function or insulin resistance.

CONCLUSIONS: These Mendelian randomization analyses support a causal role for diabetes and its associated high glucose levels on CAD, and suggest that long-term glucose lowering may reduce CAD events.

KEY WORDS: genetic variants, dysglycemia, diabetes, coronary artery disease
INTRODUCTION

Large prospective observational studies have reported that type 2 diabetes increases the risk of cardiovascular events by approximately 2-fold following the adjustment for other risk factors\(^1\). These and other studies have also reported a progressive relationship between various measures of glycemia, including fasting glucose (FG) and glycated hemoglobin (HbA\(_{1c}\)), and cardiovascular outcomes, both in people with diabetes and in people without a history of diabetes or cardiovascular events\(^1,2\). Conversely, large randomized controlled trials (RCTs) assessing the effect of glucose lowering have also yielded mixed results. For instance, meta-analyses have demonstrated a modest 9% reduction in the composite cardiovascular outcome and a 15% reduction in coronary artery disease (CAD) \(^3,4,5\). Moreover, at least one analysis suggests that this effect on CAD is due to the effect of the intervention on HbA\(_{1c}\)\(^6\).

The conflicting reports from epidemiological studies and clinical trials regarding the potential effects of dysglycaemia on cardiovascular outcomes have fuelled uncertainty regarding the etiologic relationship between dysglycemia and CAD. However, Mendelian randomization analyses may help to clarify the relationship between glucose traits, diabetes and risk of CAD. This approach uses genetic associations to explore the effects of modifiable exposures on outcomes. It is based on the principle that genetic variants are randomly allocated at birth and this distribution is independent of many factors that may bias observational associations\(^7\), such as confounding factors and reverse causation\(^8\). However, this approach does not rule out the possibility that genetic variants associated with dysglycaemia may also be correlated with other CAD risk factors such as dyslipidemia, blood pressure elevation or weight gain \(^7\). This limitation
can be circumvented by adopting the method proposed by Do et al (2013) to adjust Mendelian randomization analyses for genetic effects on these other risk factors. This approach allows for the dissection of causal influences for the risk of CAD among sets of correlated glucose traits and CAD risk factors.

To explore the relationship between dysglycaemia-related indices (i.e. FG, HbA1C and diabetes) and the risk of CAD, we identified genetic variants associated with these three indices and then confirmed whether their genetic effect supports a causal association with CAD. We also explored whether genetic variants that modify β cell function have a different relationship to CAD than variants that modify insulin resistance.

**METHODS**

*Data Sources*

Effect size estimates for SNPs associated with glucose traits (FG and HbA1C) were obtained from the Meta-Analyses of Glucose and Insulin-related traits Consortium (MAGIC) study, a genome-wide association study (GWAS) consisting of more than 133,010 of European descent without diabetes. Genetic data for the association of diabetes was obtained from the DIAbetes Genetics Replication and Meta-analysis (DIAGRAM) Consortium study, a GWAS of 34,840 cases and 114,981 controls of European descent. Genetic data for the association of the risk of CAD was obtained from the Coronary ARtery DIsease Genome-wide Replication and Meta-analysis (CARDIoGRAMplusC4D) Consortium study, a two-stage GWAS of 63,746 cases of CAD and 130,681 controls. When not available in CARDIoGRAMplusC4D, effect estimates were obtained from the CARDIoGRAM, which is a meta-analysis of 22 GWAS studies of
22,233 cases and 64,762 controls\textsuperscript{15}. CAD outcomes were defined as one of the following: myocardial infarction (MI), > 50% stenosis in at least one coronary vessel at angiography, history of percutaneous transluminal coronary angioplasty or coronary artery bypass graft surgery, angina or death due to CAD\textsuperscript{14}. Genetic data on the association of low density lipoprotein (LDL), high density lipoprotein (HDL), total cholesterol (TC) and triglycerides (TG) were obtained from the Global Lipids Genetics Consortium study, a GWAS of 188,577 individuals from 60 studies\textsuperscript{16}. Genetic data for the SNP associated with systolic blood pressure (SBP) and diastolic blood pressure (DBP) were obtained from the International Consortium for Blood Pressure (ICBP) GWAS in more than 200,000 European individuals\textsuperscript{17}. Genetic data for the association of body mass index (BMI) was obtained from the Genetic Investigation of ANthropometric Traits (GIANT) GWAS in more than 133,154 European individuals\textsuperscript{18}. Further details of each consortium are included in the Supplementary Methods.

\textit{SNP Selection}

SNPs were selected if they were associated with at least one of the two glucose traits (FG and HbA\textsubscript{1c}) or diabetes at genome-wide level significance of $P < 5 \times 10^{-8}$. For duplicate SNPs and SNPs associated with glucose trait or diabetes that were less than 500kb apart we obtained the linkage disequilibrium (LD) estimates using data from the 1000 Genomes Pilot 1\textsuperscript{19} and then assigned a lead SNP based on the strength of association with either glucose traits or diabetes (Supplementary Figure 1).
Statistical Analysis

We first tested FG, HbA$_{1c}$ and diabetes separately for the risk of CAD. This was done by using the effect estimates of SNPs associated with FG, HbA$_{1c}$ or diabetes with their corresponding genetic effect estimates on CAD using data from the MAGIC, DIAGRAM and CARDIOGRAMplusC4D consortia, respectively. Figure 5.1 represents the schematic representation of the Mendelian Randomization design. Linear regressions (without intercept) were performed using the effect estimates of SNPs on FG, HbA$_{1c}$ and diabetes as the independent variables and the genetic effect sizes on CAD as the dependent variable. To account for putative pleiotropic effects, we developed multivariate models to adjust for the effects of LDL, HDL, TC, TG, SBP, DBP and BMI, including effect size estimates of SNPs on these latter traits as independent variables in regression models. Throughout the manuscript, the effect of the SNPs associated with diabetes on the risk of CAD was expressed as the relative risk of CAD among diabetic individuals as compared to individuals without diabetes. Here, literature estimates of the prevalence of diabetes and the prevalence of CAD in nondiabetic individuals were obtained $^{20,21,22}$, and these estimates were applied to derive the relative risk of CAD associated with diabetes (further details in Supplementary Methods). Next, the causal effects of glucose traits and diabetes on the risk of CAD derived from Mendelian randomization were compared to estimates obtained from observational studies to determine if there was a similar magnitude of effect. Estimates of FG available in units of mg/dL were converted to mmol/L using a multiplication factor of 0.055. Comparison of magnitude of effect was achieved through random simulations (N=10,000) of the effect of SNPs on glucose traits, diabetes and CAD, sampling from the known effect size distribution of SNPs on these traits (i.e. published mean and standard error of genetic associations). The causal effect of glucose traits and diabetes on risk of
CAD was then calculated for each simulation, providing a confidence interval of the estimated causal effect on CAD risk. The genetic effect was then compared to the effect on the risk of CAD from observational studies using a z-test. We also derived power estimates of the causal effect of SNPs associated with glucose traits and diabetes on the risk of CAD using simulations. To do so, we first calculated the predicted effect of each SNP on CAD by matching their effect on glycaemia or diabetes with expected effect on CAD based on estimates from observational studies. We then performed 10,000 random simulations, regressing the predicted effect of each SNP on CAD on their known effect on glucose traits and diabetes. Finally, loci were categorized according to their effect on “β cell dysfunction” or “insulin resistance” to determine if association with CAD could be ascribed to either hypo- or hyper-insulinemia, respectively. Only SNPs with an unequivocal effect on “β cell dysfunction” or “insulin resistance” based on literature reviews were included in this analysis, with all SNPs of unknown function or with conflicting reports excluded. All statistical analyses were performed using R.

RESULTS

Association of glucose levels and diabetes with risk of CAD

Thirty SNPs were associated with FG \(^{10}\), nine associated with HbA\(_1c\) \(^{12}\) and 59 associated with diabetes \(^{13}\). Further details on the risk alleles, associated loci, and sample sizes for the trait specific SNPs are presented in Supplementary Table 1. To investigate the consistency and directional effect of SNPs association with glucose traits and CAD, we plotted the effect of SNPs on FG, HbA\(_1c\) and diabetes with their corresponding effect on risk of CAD (Figure 5.2). Next, to explore whether SNPs associated with glucose traits and diabetes predict the risk of CAD, we performed linear regression analyses for each trait using the respective effect sizes of SNPs on...
FG, HbA1c and diabetes as the independent variables with the corresponding effects sizes for CAD as the dependent variables (Figure 5.3). SNPs associated with HbA1c and diabetes were significantly associated with an increased risk of CAD (OR: 1.53 per % increase in HbA1c, 95% CI: 1.14 – 2.05; P=0.023 and OR: 1.57, 95% CI: 1.16 – 2.05; P=0.008, respectively) while SNPs associated with FG were not associated with risk of CAD (P> 0.05; Figure 5.3). When regression models for HbA1c and diabetes were adjusted for potential effects on other CAD risk factors (i.e. LDL, HDL, TC, TG, SBP, DBP and BMI), only SNPs associated with diabetes remained significantly associated with CAD (OR: 1.62, 95% CI: 1.23 – 2.07; P=0.002) while association with HbA1c was non-significant (P> 0.05).

Comparison with literature estimates from observational studies

To date, the largest prospective meta-analysis to assess the effects of glucose traits and diabetes on the risk of CVD is the Emerging Risk Factor Collaboration (ERFC)20,21. The authors reported that diabetes was associated with an increased risk of CAD (hazard ratio (HR): 2.00, 95% CI: 1.83 – 2.19) in 698,782 individuals from 102 prospective studies. They also observed similar trends for FG (HR: 1.02 per mmol/L, 95% CI: 1.02 – 1.03), and HbA1c (HR: 1.43 per %, 95% CI: 1.07-1.91) in 294,998 individuals without diabetes or CAD from 73 prospective studies. We sought to determine whether causal effects of glycaemia and diabetes on the risk of CAD derived from Mendelian randomization were consistent with estimates obtained from the ERFC (Figure 5.4). We observed that CAD risk estimates derived from diabetes SNPs were statistically different from the risk estimates obtained from the ERFC (P for difference=9.60x10⁻⁵) while there were no statistical differences for glucose traits SNPs and the corresponding literature estimates (P for difference >0.05 for all). Using reported risk estimates from observational
studies, we estimated power to detect a genetic association between CAD and diabetes at 100%, 72.2% for HbA1C and 5.6% for FG.

**Effect of gene function on CAD risk estimates**

We also explored whether the causal association of diabetes with CAD differed between sets of genes known to influence either β cell function or insulin resistance. We thus stratified diabetes SNPs according to their known biological function, namely: "β cell dysfunction" or "insulin resistance" (Figure 5.5). Among the 59 SNPs associated with diabetes, there were 26 loci associated with β cell dysfunction and 11 loci associated with insulin resistance. Loci influencing β cell dysfunction and insulin resistance were both associated with an increased risk in CAD (OR 1.83, 95% CI: 1.19-2.62; P=0.015 and OR: 2.35, 95% CI: 1.46-3.53; P=0.01, respectively).

**DISCUSSION**

Using genetic information from 59 SNPs with known association with diabetes, our Mendelian randomization analysis supports a causal role of diabetes for CAD. We demonstrated that SNPs associated with HbA1C and diabetes were associated with an increased risk of CAD, which is consistent with findings from large observational studies²¹,²⁰. Furthermore, consistent results were obtained when restricting the analysis to genes affecting either β cell dysfunction or insulin resistance, suggesting that the therapeutic interventions that act through these different pathways have the potential to reduce CAD irrespective of their mechanism of action. Although our estimates of the effect of diabetes on CAD appeared to be more modest in comparison to observational studies²¹,²⁰ this may be explained by residual confounding or bias among these studies. SNPs associated with HbA1C were also associated with an increased risk of CAD but the
effect was attenuated after adjustment for other CAD risk factors. SNPs associated with FG were not associated with the risk of CAD but we had limited power to detect an effect for FG (5.6%).

These analyses have several strengths. First, the random allocation of genetic variants acts to reduce the potential effects of confounding and reverse-causation observed in epidemiological studies. Furthermore, unlike other Mendelian randomization analyses that have assessed the effect of glucose traits on the risk of CVD outcomes\textsuperscript{23}, we were able to control for genetic effects on other CAD risk factors such as blood lipids, blood pressure and obesity. We also had very robust estimates from the CARDIOGRAMplusC4D, DIAGRAM, GIANT, GLGC, ICBP and MAGIC consortia which collectively included a total of 881,875 individuals. The differences we observed between carriers and non-carriers of genetic variants represent lifelong effects on HbA\textsubscript{1C} and diabetes. Indeed, in the UKPDS trial, the authors reported a nonsignificant reduction in the risk of myocardial infarction among patients randomized to intensive or conventional glucose lowering strategies\textsuperscript{24}. In addition, after 8.5 years of post-trial observations, those originally randomized to the active arm experienced a 15\% reduction in myocardial infarction (P=0.01) and 13\% reduction in all-cause mortality (P=0.007)\textsuperscript{25}. Similar trends were also observed in the DCCT/EDIC trial\textsuperscript{26}. Thus the genetic properties of our analysis provide further support that long-term treatment with glucose-lowering agents may be beneficial. Also, loci involved in β cell dysfunction and insulin resistance were both associated with CAD. Taken together, these results suggest that long-term treatment with glucose-lowering agents, regardless of the mechanism of action, may be required before the effects of glycemic intervention on CVD events may be observed.
There are several limitations in our study. First, the definition of diabetes used in the DIAGRAM consortium was specific to each cohort, which might introduce heterogeneity into results. Second, estimates obtained from the DIAGRAM and CARDIOGRAMplusC4D consortia consist of both incident and prevalent cases from prospective and case-control studies. Third, owing to a lack of genetic data we were only able to explore the genetic effect of glucose traits and diabetes in predominantly European populations and we were also unable to account for other confounders that might influence the effect of SNPs associated with glycaemia traits and diabetes on the risk of CAD, such as smoking and waist-to-hip ratio. Fourth, due to the lack of statistical power, we were limited in our ability to evaluate associations with FG. Fifth, we were unable to assess whether there was a non-linear trend for glycaemia traits and the risk of CAD using Mendelian Randomization since we did not have individual level data. Finally, we were not able to assess the mechanism of action for all SNPs included in this analysis owing to a lack of functional data in the literature.

In summary, our genetic analysis provides further insight into the causal role of glucose levels, diabetes and the risk of CAD. Our results support that diabetes has an independent and causal effect on the risk of major cardiovascular events. Thus improved glycemic control among diabetic patients and prevention of diabetes may reduce the risk of CAD outcomes. Our results emphasize the need to further explore the benefits of long-term glucose lowering on CAD.
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FIGURE LEGENDS

Figure 5.1: Schematic representation of the Mendelian Randomization design.

Figure 5.2: Effect of SNPs associated with levels of FG, HbA\textsubscript{1c} and diabetes on the risk of coronary artery disease.

Each black dot represents a SNP associated with a glycemic trait (fasting glucose, diabetes or HbA\textsubscript{1c}) with a $P < 5 \times 10^{-8}$. The association of each SNP with CAD ($\beta$ value) is represented by the y-axis while association with glycemic trait is represented by the x-axis. The blue line illustrates regression of CAD effects on glycemic effects. The p-value for the association of FG SNPs with the risk of CAD was 0.102, HbA\textsubscript{1c} SNPs with the risk of CAD was 0.023, diabetes SNPs with the risk of CAD was 0.008.

Figure 5.3: Genetic estimates of association of diabetes, HbA\textsubscript{1c} and FG with risk of CAD.

Analyses were adjusted for the potential pleiotropic effects of LDL, HDL, TC, TG, SBP, DBP and BMI. Adjusted analyses are only presented for diabetes because the number of FG and HbA\textsubscript{1c} SNPs was insufficient to perform statistical adjustment.

Figure 5.4: Comparison of estimated effects of glycemia and diabetes on CAD derived from genetic analysis with estimates from observational studies

Figure 5.5: Subgroup analysis of loci influencing $\beta$ cell dysfunction or insulin resistance on risk of CAD.
Figure 5.1: Schematic representation of the Mendelian Randomization design.
Figure 5.2: Effect of SNPs associated with levels of FG, HbA1c and diabetes on the risk of coronary artery disease.
Figure 5.3: Genetic estimates of association of diabetes, HbA1c and FG with risk of CAD.

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<th>Estimates</th>
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<tr>
<td>Fasting Glucose per mmol/L</td>
<td>1.18 (0.97-1.42)</td>
<td>0.102</td>
</tr>
<tr>
<td>HbA1c per %</td>
<td>1.53 (1.14-2.05)</td>
<td>0.023</td>
</tr>
<tr>
<td>Diabetes</td>
<td>1.57 (1.16-2.05)</td>
<td>0.008</td>
</tr>
<tr>
<td><strong>Adjusted</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetes</td>
<td>1.62 (1.23-2.07)</td>
<td>0.002</td>
</tr>
</tbody>
</table>
Figure 5.4: Comparison of estimated effects of glycemia and diabetes on CAD derived from genetic analysis with estimates from observational studies

<table>
<thead>
<tr>
<th>Estimates</th>
<th>OR (95% CI)</th>
<th>P for difference</th>
<th>Power</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasting glucose per mmol/L</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Observational</td>
<td>1.02 (1.02–1.03)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genetic</td>
<td>1.18 (0.97–1.42)</td>
<td>0.140</td>
<td>5.60%</td>
</tr>
<tr>
<td>HbA1c per %</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Observational</td>
<td>1.43 (1.07–1.91)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genetic</td>
<td>1.53 (1.14–2.05)</td>
<td>0.750</td>
<td>72.2%</td>
</tr>
<tr>
<td>Diabetes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Observational</td>
<td>2.00 (1.83–2.19)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genetic</td>
<td>1.62 (1.23–2.07)</td>
<td>9.60E-05</td>
<td>100%</td>
</tr>
</tbody>
</table>
Figure 5.5: Subgroup analysis of loci influencing β cell dysfunction or insulin resistance on risk of CAD.

<table>
<thead>
<tr>
<th>Estimate</th>
<th>OR (95% CI)</th>
<th>P</th>
<th>Het P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beta cell dysfunction</td>
<td>1.83 (1.19–2.62)</td>
<td>0.015</td>
<td></td>
</tr>
<tr>
<td>Insulin resistance</td>
<td>2.35 (1.46–3.53)</td>
<td>0.010</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>2.02 (1.49–2.66)</td>
<td>5.12E-05</td>
<td>0.4</td>
</tr>
</tbody>
</table>

OR (95% CI)
CHAPTER 6

Conclusion

Advancements in Pharmacogenetics

Overall, we have demonstrated that pharmacogenetics has the potential to maximize drug efficacy and minimize adverse effects. However, its translation into clinical practice been slow due to a lack of replication among previously published studies. Yet, large collaborative efforts that incorporate genetic and clinical data have provided robust evidence in the support of personalized medicine. These advancements have allowed researchers to gain novel insights into existing drug targets, inform and guide clinical decision-making and validate potential disease target pathways. In this section we will briefly summarize the main pharmacogenetic research papers presented in this thesis, as well as the potential research implications and limitations of these studies, and the future directions of pharmacogenetics.

Existing Drug Targets

In our analysis of the effect of the rs20417 SNP (COX-2) on the risk of CVD, we confirmed that COX-2 carrier status was associated with a decreased risk of major cardiovascular outcomes among 49,232 participants from the ACTIVE-A, CURE, epiDREAM/DREAM, ONTARGET, RE-LY, and WGHS studies (1). We also observed that aspirin use and previous CAD appeared to modify the association between rs20417 carrier status and the risk of CVD outcomes. Furthermore, carriers had significantly lower urinary levels of thromboxane and prostacyclin metabolites as compared to noncarriers.
Inform treatment decision

In our meta-analysis and Mendelian Randomization analysis of the effect of BAS on the risk of cardiovascular events, we observed that BAS appeared to be associated with a reduced risk of CAD (Chapter 4). First, our systematic review and meta-analysis showed that 24g/d of cholestyramine was associated with a reduction in LDL-C levels and a modest reduction in the risk of CAD while 3.75 g/d coleselevam was associated with a reduction in LDL-C. We also showed that the predicted effects of 24 g/d of cholestyramine and 3.75 g/d of coleselevam using genetic data were associated with a reduced risk of CAD and these estimates were not statistically different from previously reported trends in clinical trials.

Validate targeting of disease pathways

Our Mendelian Randomization analysis of the effects of dysglycemia on the risk of CAD outcomes demonstrated that SNPs associated with HbA₁c and diabetes appeared to be associated with an increased risk of CAD (Chapter 5). Our results were consistent with reports from observational studies (2;3), which suggest that dysglycemia may have a causal role in the risk of CAD. Furthermore, our results also indicate that therapeutic interventions targeting either insulin resistance or β cell dysfunction pathways may potentially decrease the risk of CAD.

Implications of Research

There are several clinical and research implications for using pharmacogenetics in order to validate existing drug targets. In our analysis of the effect of COX-2 carrier status on the risk of CVD, we proposed that the COX-2 genetic variant may have tissue-specific effects, which indicates that selective targeting of the COX-2 genetic variant may have greater beneficial effects
in secondary prevention patients. Thus it would appear that COX-2 inhibition may not be universally deleterious in all patients; however, there needs to be further research is required to better understand the biological role of COX-2 genetic variant in CVD and how this variant may influence drug response. We also observed that the association between COX-2 carrier status and the risk of CVD was modified by aspirin use. Despite the potential confounding issues between the interaction of aspirin use and COX-2 carrier status with the risk of CVD, our results suggest that widely prescribed non-selective COX inhibitors, such as aspirin, may have a greater beneficial effect among COX-2 carriers as compared to noncarriers. However, more research is required to address this compelling biological interaction because most all high-risk patients are appropriately prescribed aspirin and we can only speculate as to the cardiovascular and bleeding risk of aspirin-naïve secondary prevention patients. Therefore by revisiting previously reported pharmacogenetic associations using large genetic data sources provides a better understanding of the pathology of CVD and advances the knowledge of how genetic variants influence drug response.

There were also implications of using pharmacogenetics to help inform clinical decision-making. In our analysis of the effect of BAS on the risk of CAD, we suggested that BAS therapy may provide an appropriate second-line therapy in the prevention of CAD among patients where statin therapy is not well tolerated or effective. Although the clinical evidence shows that cholestyramine reduces LDL-C levels and it modestly decreases the risk of CAD events, its use has been hampered by poor patient tolerability (4). In contrast, colesevelam is better tolerated (5;6) and the predicted effect of 3.75 g/d colesevelam using genetic data on the risk of CAD was similar that of cholestyramine. In addition, the results our analysis confirmed the similarity
between BAS treatment and the effect of rs4299376 (ABCG5/8). Therefore the inhibition of intestinal cholesterol absorption may provide a valuable therapeutic target for the prevention of CVD. Thus there is still a need for a well-designed double-blinded RCT to assess the effects of colselevam on the risk of clinical outcomes since there is a lack of available data.

There are also implications of using pharmacogenetics to validate potential disease target pathways. In our analysis of the effects of dysglycemia on the risk of CAD, we showed, through the utilization of genetic variants, that long-term reduction in glucose may be required to observe a protective effect against CVD. Furthermore, the genetic properties of our analysis provided further evidence that long-term treatment with glucose-lowering agents may be beneficial. We also demonstrated that therapeutic agents targeting insulin secretion and insulin resistance pathways may reduce the risk of diabetes and CAD. Thus long-term treatment with glucose-lowering agents, regardless of the mechanism of action, may provide a valuable therapeutic intervention in the prevention of CVD. However, more adequately powered, long-term RCTs that target different glucose pathways will help to explore the effects of glucose reduction on the risk of CVD.

Limitations

Nevertheless, there are some limitations when considering our analysis of the effect of the COX-2 on the risk of CVD. This analysis lacked tissue-specific COX-2 gene expression data from endothelial tissue, atherosclerosis plaques and macrophage tissue. In addition, a proxy SNP was used in the RE-LY and ONTARGET patient populations. A proxy SNP provides an appropriate surrogate measure because it is in high linkage disequilibrium with the candidate SNP ($r^2 >$
0.80)(7). Although the proxy SNP may account for the lower allelic frequencies in RE-LY and ONTARGET, its use should bias our results towards the null without invalidating our conclusions.

Mendelian Randomization provides a unique tool for assessing causality. However, this analysis requires that a series of criteria be met, which include: the genetic variant must be associated with the exposure of interest, the genetic variant must also be independent of confounders, and the genetic variant is independent of the outcome given the exposure and confounding factors(8). Not only must these assumptions be met but there are other potential limitations that threaten the validity of these analyses. First, the presence of genetic heterogeneity may violate the assumptions of the Mendelian Randomization analysis. For instance, there might be effects of pleiotropy, where the genetic variant may be associated with the exposure, as well as the confounding factors or the outcome of interest(9). Additionally, there may also be effects of linkage disequilibrium, where the selected genetic variant is highly correlated with a polymorphism associated with the outcome of interest or an intermediate variable within the disease pathway(10). Second, population stratification may violate the assumptions of the Mendelian Randomization analysis. Population stratification results from differing genotype frequencies and risk of disease in ethnic subpopulations(11) and may bias the analysis if the prevalence of the variant allele parallels the incidence of the study outcomes(12). Third, the Mendelian Randomization may also be limited by canalization, which is a developmental compensation where a phenotype is selected in a population despite the genetic variability(13). Finally, weak instrumental bias may also limit the Mendelian Randomization analysis(14). This occurs when the selected genetic variant has a modest association with the modifiable exposure.
The weak instrument reduces the power to detect an effect and thus more genetic variants or a larger sample size is required.

Based on these aforementioned limitations, there are some issues to address in our analysis of the effects of BAS on the risk of CAD. We were unable to address the effect of colestipol or colescevelam on the risk of clinical outcomes due to a lack of clinical data. Furthermore, the predicted effect of BAS on the risk of CAD using genetic data appeared to have a stronger effect size than the estimates from LRCCPPT and the CTT. This may be a result of the fact that rs4299376 allele carriers have a lifelong exposure to lower levels of LDL-C. Finally, there may also be a possibility of pleiotropic effects whereby either the rs4299376 SNP or BAS influence yet unknown pathways unrelated to lipids.

In addition, there are also limitations in our analysis of the effect of the dysglycemia on the risk of CAD. There was limited power to detect the effect of FG on the risk of CAD. Furthermore, owing to a lack of available genetic data, we were unable to account for other confounders that might influence the effect of SNPs associated with dysglycaemia traits on the risk of CAD, such as smoking and waist-to-hip ratio. Finally, we were unable to assess whether there was a non-linear trend for glycaemia traits and the risk of CAD using Mendelian Randomization since we did not have individual patient level data.

**Future Directions**

Despite the advancements in pharmacogenetics, there is still a need to develop a systematic evidence-based framework to assess the quality of pharmacogenetic studies. First, there is a need
for more high-quality evidence from well-designed RCTs, as well as reports obtained from meta-analyses of pharmacogenetic studies, high-quality prospective cohort studies or case-control studies. However, it is also important that future pharmacogenetic studies use larger sample sizes in order to have enough power to detect drug-gene interactions. In other fields of genetic epidemiology, it is common to conduct meta-analyses using more than 100,000 individuals to explore the underlying genetic determinants of disease (15-17). Thus large collaborative efforts across many disciplines are still required to guide and validate recommendations for the use of pharmacogenetics. In addition, the genetic polymorphisms used in these pharmacogenetic studies should have either a direct or indirect functional effect on the mechanism of the drug-response. Thus by assessing pharmacogenetic associations with stringent standards should strengthen the reproducibility of these results and enhance the clinical relevance of pharmacogenetics.

The recent advancements in exome sequencing have provided a unique opportunity to identify rare mutations and novel genetic variants, which was not possible using chip-based technologies(18;19). Although rare variants do not occur frequently within the population(19), these rare mutations may result in individual alteration of the function of the drug target protein(20;21). Thereby, the characterization of rare variants will help to inform the clinical application of pharmacogenetics by identifying molecular mechanisms and prompt the search for potential therapies for CVD. Furthermore, despite the recent adoption of throughput new technologies in pharmacogenetics, there is still very limited data on the effect of epigenetic modifications, metabolomics, proteomics and microRNAs on the risk of CVD(22-24). A better understanding of these circulating metabolic and protein traits that alter genome structure and function will provide a more accurate method of identification and validation of drug targets and
biomarkers for designing personalized therapies(25;26). In order to do so, future research should systematically test whether genetic variants associated with metabolomics and proteomic traits are causally related to CVD or whether they represent markers of disease.

Methodological developments have also greatly expanded the applicability of Mendelian Randomization analyses. In these analyses, the genetic variant should only be related to the exposure and independent of any confounding factors and the given outcome. However, in many instances, the genetic variants may be associated with multiple intermediate phenotypes. Thus Do et al (2013) proposed building large statistical frameworks of common SNPs in order to adjust for these confounding factors, and demonstrated that a causal relationship between triglycerides, HDL-C and LDL-C in relation to the risk CAD(27). Another development in Mendelian Randomization is hypothesis-free testing, where multiple genetic variant instruments representing multiple exposure phenotypes are created to assess the effects on several clinical outcomes using larger, well-characterized genetic datasets(28;29). For example, Yin et al (2014) developed a Mendelian Randomization Pipeline (MeRP) to facilitate the rapid assessment of Mendelian Randomization analyses using freely available public data(30). This method provides a powerful tool for efficiently screening the effects multiple exposures and outcomes using large networks of phenotypes, which may help to guide the discovery of future drug targets or strengthen the causal inference of CVD epidemiological associations. Furthermore, the majority of Mendelian Randomization analyses use linear models to explore causal associations; however, this approach may not be appropriate for all observational associations. For instance, some exposure-outcome relationships will demonstrate a nonlinear association, such as salt intake with the risk of blood pressure(31) and body mass index with the risk of mortality(32). Several
authors have proposed methods to explore these non-linear trends\(^{(33;34)}\). Silverwood et al (2014) described a method to assess nonlinear trends for binary genotypes using data from the Alcohol-ADH1B Consortium \(^{(33)}\). Adopting these methods allows for better interpretation of nonlinear exposure-outcome relationships and provides a more appropriate assessment of the given causal associations.

The convergence of genotyping technologies and clinical datasets has created a large, high-quality and cost-effective resource to assess the effect of therapeutic agents using genetic variants. Genetic variants provide a useful tool because they independent of many confounding factors and they represent lifelong follow-up. Furthermore, the advancements in genotyping technologies have relieved a large number of unique pharmacogenetic markers, which allows us to better validate existing drug targets and discover new potential targets. Thus the implementation of a computational infrastructure and stringent methodological guidelines may help to ease the transition of pharmacogenetic research into clinical practice.
REFERENCE


Supplemental Appendices
Supplementary Appendix 1

Association of Cyclooxygenase-2 genetic variant with cardiovascular disease

AUTHORS: Stephanie Ross; John Eikelboom; Sonia Anand; Niclas Eriksson; Hertzel Gerstein; Shamir Mehta; Stuart Connolly; Lynda Rose; Paul M. Ridker; Lars Wallentin; Daniel Chasman; Salim Yusuf; and Guillaume Paré.
METHODS

Study Population Overview

The Institutional Review Board independently approved each study, and all patients provided written informed consent. Only those patients who also consented to participate in genetic studies were eligible for this analysis.

Atrial Fibrillation Clopidogrel Trial With Irbesartan for Prevention of Vascular Events (ACTIVE-A) Study

The design and results of the ACTIVE-A study have been described previously\(^1\).\(^2\). ACTIVE-A was a randomized, double-blind, placebo-controlled trial comparing clopidogrel (75mg/d) with placebo for stroke prevention in 7,554 in patients with atrial fibrillation (AF) and at least one additional risk factor for stroke who were not eligible for warfarin therapy. Regardless of treatment group, all study participants received a recommended daily dose of aspirin (75 to 100 mg). Major bleeding was defined as major hemorrhage, where any overt bleeding requiring transfusion of at least 2 units of blood or any overt bleeding meeting the criteria for severe hemorrhage. Results are presented for individuals of European (N=1,016) ancestry only. Genotyping was performed using Sequenom iPlex technology.

Clopidogrel in Unstable Angina to Prevent Recurrent Events (CURE) Study

The design and results of the CURE trial have been described previously\(^3\)-\(^5\). CURE was a randomized, double-blind, placebo-controlled trial comparing clopidogrel (75 mg per day) with placebo in 12,562 patients with ACS without ST-segment elevation. All study participants were
on a background of aspirin (recommended dose, 75 to 325 mg, daily). Major bleeding was defined as substantially disabling bleeding, intraocular bleeding leading to the loss of vision, or bleeding necessitating the transfusion of at least 2 units of blood. Results are presented only for individuals of European (N=4,014) and Latin American (N=648) ancestry. Individuals from other ethnic groups were excluded because of small numbers (N=99 for the next largest group) and concerns about the potential for population stratification. Genotyping was performed using Sequenom iPLEX technology.

**Diabetes Reduction Assessment with Ramipril and Rosiglitazone Medication (DREAM) Study/ epiDREAM**

The study design and results of the DREAM\textsuperscript{6-8} and epiDREAM\textsuperscript{9} trials have been described previously. DREAM was a randomized, double-blind trial with a 2-by-2 factorial design that assigned 5,269 participants without CVD but with impaired fasting glucose levels or impaired glucose tolerance to receive either ramipril (15 mg/day) vs. placebo or rosiglitazone (8 mg/day) vs. placebo. EpiDREAM was an epidemiological arm of the DREAM trial and is comprised of 18,990 participants who were either screened for eligibility to enter the DREAM clinical trial but were not eligible or who did not want to enter the trial but agreed to long term prospective follow-up\textsuperscript{9}. Aspirin use among study participants was self-reported. Of the 18,486 individuals who provided a DNA sample and met quality control criteria\textsuperscript{9}, 14,104 were prospectively followed and included in the current study. These included 6,236 participants of European ancestry, 3,269 of Latin American, 2,744 of South Asian, 1,162 of African, 479 of Native North American and 214 Asian ancestries. All ancestry assignments were self-reported and further
confirmed using principal component analysis. Genotyping was performed using the CVD chip described in details elsewhere\textsuperscript{10}.

**Ongoing Telmisartan Alone and in combination with Ramipril Global Endpoint Trial (ONTARGET)**

The design and results of the ONTARGET trial have been described previously\textsuperscript{11,12}. ONTARGET was a randomized, double-blind, parallel trial comparing the effects of ramipril (10 mg per day), telmisartan (80 mg per day), and combination therapy among 25,620 patients with coronary, peripheral, or cerebrovascular disease or high risk diabetes with evidence of end-organ damage. Aspirin use among study participants was self-reported. Genotyping was performed using Illumina’s metabochip. Genetic data was available only for individuals of European (N=3,610) ancestry. Since the rs20417 genotype was not available on this chip and could not be imputed, the best available proxy rs2066826 (D\textsuperscript{1}=1.00 and r\textsuperscript{2}=0.943 in Caucasians according to 1000 Genomes data\textsuperscript{13}) was used.

**Randomized Evaluation of Long-Term Anticoagulation Therapy (RE-LY) Study**

The study design\textsuperscript{14} and results\textsuperscript{15} have been described previously. RE-LY was a prospective, open-label, randomized trial that compared two fixed doses of dabigatran (110 mg or 150 mg twice daily) administered in a blinded manner, with open-label use of warfarin in 18,113 patients who had AF and at least one additional risk factor for stroke. Concomitant use of aspirin (at a dose of <100 mg per day) and other antiplatelet agents were permitted. Major bleeds were defined as a reduction in the hemoglobin level of at least 20 g per liter, transfusion of at least 2 units of blood, or symptomatic bleeding in a critical area or organ. Only participants of self-
reported European (N=2,501) ancestry, as confirmed through principle component analysis, were used for the genetic analysis. Genotyping was performed using the Illumina Human610-quad DNA analysis beadchip. Since the rs20417 genotype was not available on this chip and could not be imputed, the best available proxy rs6672638 (D^2=1.00 and r^2=0.943 in Caucasians according to 1000 Genomes data^{13}) was used.

**Women Genome Health Study (WGHS)**

The WGHS^{16} is a subset of the Women’s Health Study (WHS), which consists of North American female health professionals with no prior history of CVD or other major chronic diseases who provided a baseline blood sample at the time of study enrollment^{17}. WHS study participants who were randomized to the aspirin intervention arm received 100 mg of aspirin every other day. 23,294 individuals of self-reported Caucasian ancestry, confirmed with genetic analysis^{16}, were used for this analysis. Genotyping of rs20417 was performed using either the HumanHap300 Duo-Plus chip or the combination of the HumanHap300 Duo and I-Select.

**INTERHEART**

The design and results of the INTERHEART Study have been described previously^{18}. INTERHEART was a large, international, standardized case-control study designed to determine the association between various risk factors and non-fatal acute myocardial infarction in a total of 15,152 cases and 14,820 controls from 52 countries. Cases were defined as those who were admitted to a coronary care unit or equivalent cardiology ward within 24 hours of clinical characteristics of new myocardial infarction. Controls were matched by age and sex, had no
previous diagnosis of heart disease and were recruited from hospital or community-based settings. Aspirin use was self-reported. Immunoturbidimetric assays were used to measure apolipoprotein concentrations (Roche/Hitachi 917 analyser with Tina-quant ApoB version 2 and ApoA1 version 2 kits; Roche Diagnostics, Mannheim, Germany). Of the 9,602 individuals who provided a DNA sample and met quality control criteria, 9,363 were included in this study. These included 2,051 participants of European, 1,484 of South East Asian, 1,918 of South Asian, 1,433 of Arab, 1,668 of Latin American, 809 of African ancestries. All ancestry assignments were self-reported and further confirmed using principal component analysis. Genotyping was performed using the Illumina VeraCode GoldenGate Genotyping Kit using the BeadXpress.

**Mechanisms of Aspirin Resistance (MARS Study) Study**

The MARS study was an open-label, two phase, case-control study of individuals with CVD; however, for the purposes of assessing the association of rs20417 with urinary 11-dehydro thromboxane B$_2$ and urinary 2,3-dinor-6-keto PGF$_{1\alpha}$ concentrations only healthy controls were considered in order to measure levels before the aspirin intervention. Briefly, controls were eligible for inclusion if they met the following criteria: (i) age at least 30 years; (ii) ankle-brachial index (ABI) >0.9; (iii) no known vascular disease. Urine was collected into preservative free tubes for measurement of 11-dehydro thromboxane B$_2$ and prostacyclin using standard method (Cayman Chemical, Ann Arbor, MI). Of the participants who provided a DNA sample, 119 participants of European ancestry were included in this analysis. Genotyping was performed using the Illumina VeraCode GoldenGate Genotyping using the BeadXpress.
Table 1: Baseline characteristics of INTERHEART participants

<table>
<thead>
<tr>
<th></th>
<th>Cases (n = 4465)</th>
<th>Controls (n = 4898)</th>
<th>Total (n = 9363)</th>
</tr>
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<tbody>
<tr>
<td>Mean age (SD)</td>
<td>56.5 (12.0)</td>
<td>55.5 (12.0)</td>
<td>56 (12.1)</td>
</tr>
<tr>
<td>Female, No. (%)</td>
<td>3529 (79.0)</td>
<td>3866 (81.0)</td>
<td>7395 (79.0)</td>
</tr>
<tr>
<td>Self-reported ethnicity, No. (%)</td>
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<td></td>
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</tr>
<tr>
<td>European</td>
<td>1042 (23.3)</td>
<td>1009 (20.6)</td>
<td>2051 (21.9)</td>
</tr>
<tr>
<td>Chinese and other Asian</td>
<td>723 (16.2)</td>
<td>761 (15.5)</td>
<td>1484 (15.8)</td>
</tr>
<tr>
<td>South Asian</td>
<td>993 (22.2)</td>
<td>925 (18.9)</td>
<td>1918 (20.5)</td>
</tr>
<tr>
<td>Arab</td>
<td>569 (12.7)</td>
<td>864 (17.6)</td>
<td>1433 (15.3)</td>
</tr>
<tr>
<td>Latin American</td>
<td>835 (18.7)</td>
<td>833 (17.0)</td>
<td>1668 (17.8)</td>
</tr>
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<td>Black African and Coloured African</td>
<td>303 (6.8)</td>
<td>506 (10.3)</td>
<td>809 (8.6)</td>
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<tr>
<td>Obesity Tertiles*, No. (%)</td>
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<td></td>
</tr>
<tr>
<td>Obesity Tertile 1</td>
<td>751 (17.8)</td>
<td>1381 (28.7)</td>
<td>2132 (23.6)</td>
</tr>
<tr>
<td>Obesity Tertile 2</td>
<td>1235 (29.3)</td>
<td>1673 (34.8)</td>
<td>2908 (32.3)</td>
</tr>
<tr>
<td>Obesity Tertile 3</td>
<td>2222 (52.8)</td>
<td>1755 (36.5)</td>
<td>3977 (44.1)</td>
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<td>Diabetes, No. (%)</td>
<td>891 (20.3)</td>
<td>423 (8.7)</td>
<td>1314 (14.2)</td>
</tr>
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<td>High Blood Pressure, No. (%)</td>
<td>1692 (38.5)</td>
<td>1066 (21.8)</td>
<td>2758 (29.7)</td>
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<td>Current Smoking, No. (%)</td>
<td>1974 (45.9)</td>
<td>1393 (29.0)</td>
<td>3367 (37.0)</td>
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<tr>
<td>Aspirin use, No. (%)</td>
<td>659 (14.8)</td>
<td>284 (5.8)</td>
<td>943 (10.1)</td>
</tr>
<tr>
<td>Median apolipoprotein A1 g/L (IQR)</td>
<td>1.1 (1.0, 1.2)</td>
<td>1.2 (1.0, 1.4)</td>
<td>1.1 (1.0, 1.3)</td>
</tr>
<tr>
<td>Median apolipoprotein B g/L (IQR)</td>
<td>1.0 (0.8, 1.2)</td>
<td>0.9 (0.8, 1.1)</td>
<td>1.00 (0.8, 1.1)</td>
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<td>Diet score, No. (%)</td>
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<td></td>
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<tr>
<td>Neither daily</td>
<td>850 (19.9)</td>
<td>783 (16.2)</td>
<td>1633 (17.9)</td>
</tr>
<tr>
<td>Fruit or vegetables daily</td>
<td>1842 (43.2)</td>
<td>2077 (43.0)</td>
<td>3919 (43.1)</td>
</tr>
<tr>
<td>Both daily</td>
<td>1573 (36.9)</td>
<td>1973 (40.8)</td>
<td>3546 (39.0)</td>
</tr>
<tr>
<td>Life stress, No. (%)</td>
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</tr>
<tr>
<td>None</td>
<td>970 (22.9)</td>
<td>1235 (25.9)</td>
<td>2205 (24.5)</td>
</tr>
<tr>
<td>Some periods</td>
<td>1959 (46.1)</td>
<td>2415 (50.6)</td>
<td>4374 (48.5)</td>
</tr>
<tr>
<td>Several periods</td>
<td>904 (21.3)</td>
<td>865 (18.1)</td>
<td>1769 (19.6)</td>
</tr>
<tr>
<td>Permanent stress</td>
<td>412 (9.7)</td>
<td>258 (5.4)</td>
<td>670 (7.4)</td>
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<td>rs20417 carrier status, No. (%)</td>
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<td></td>
</tr>
<tr>
<td>Carriers</td>
<td>1386 (31.0)</td>
<td>1640 (33.5)</td>
<td>3026 (32.3)</td>
</tr>
<tr>
<td>Noncarriers</td>
<td>3079 (69.0)</td>
<td>3258 (66.5)</td>
<td>6337 (67.7)</td>
</tr>
</tbody>
</table>

*Obesity tertiles represent waist/hip ratio measures. Tertiles were calculated separately for men and women based on the overall control data. Among men, the tertiles cutoffs were 0.90 and 0.95 and 0.83 and 0.90 in women.
Table 2: Baseline characteristics for MARS in healthy controls only

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>MARS</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>119</td>
</tr>
<tr>
<td>Mean age (SD)</td>
<td>47.2(11.0)</td>
</tr>
<tr>
<td>Female, No. (%)</td>
<td>100 (84.0)</td>
</tr>
<tr>
<td>Mean weight (kg) (SD)</td>
<td>76.3(21.0)</td>
</tr>
<tr>
<td>Mean height (cm) (SD)</td>
<td>165.7(12.7)</td>
</tr>
<tr>
<td>Diabetes, No. (%)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>High Blood Pressure, No. (%)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Current Smoking No. (%)</td>
<td>59 (49.6)</td>
</tr>
<tr>
<td>Aspirin use, No. (%)</td>
<td>2 (1.7)</td>
</tr>
<tr>
<td>Median urinary 11-dehydro thromboxane B2 pg/mg creatinine (IQR)</td>
<td>112.0 (87.5,164.5)</td>
</tr>
<tr>
<td>Median urinary 2,3-dinor-6-keto PGF1α pg/mg creatinine (IQR)</td>
<td>4244.7 (3022.0,6407.0)</td>
</tr>
<tr>
<td>rs20417 carrier status, No. (%)</td>
<td></td>
</tr>
<tr>
<td>Carriers</td>
<td>32 (26.9)</td>
</tr>
<tr>
<td>Noncarriers</td>
<td>87 (73.1)</td>
</tr>
</tbody>
</table>
Table 3: Association of rs20417 carrier status and major cardiovascular risk factors in control participants from the INTERHEART study

Analyses were adjusted for age, sex, and self-reported ethnicity.

<table>
<thead>
<tr>
<th>Risk Factors</th>
<th>Adjusted Dominant OR (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Measured ApoA (g/L)</td>
<td>0.81 (0.64-1.03)</td>
<td>0.091</td>
</tr>
<tr>
<td>Measured ApoB (g/L)</td>
<td>0.80 (0.63-1.03)</td>
<td>0.079</td>
</tr>
<tr>
<td>Current Smoker</td>
<td>1.02 (0.89-1.17)</td>
<td>0.77</td>
</tr>
<tr>
<td>Hypertension</td>
<td>0.88 (0.76-1.02)</td>
<td>0.096</td>
</tr>
<tr>
<td>Diabetes</td>
<td>0.87 (0.70-1.07)</td>
<td>0.183</td>
</tr>
<tr>
<td>Aspirin Use</td>
<td>0.86 (0.66-1.11)</td>
<td>0.251</td>
</tr>
<tr>
<td>Obesity Tertiles*</td>
<td>1.06 (0.98-1.15)</td>
<td>0.133</td>
</tr>
<tr>
<td>Diet Score</td>
<td>0.98 (0.90-1.07)</td>
<td>0.646</td>
</tr>
<tr>
<td>Life Stress</td>
<td>1.01 (0.93-1.09)</td>
<td>0.879</td>
</tr>
</tbody>
</table>

*Obesity tertiles represent waist/hip ratio measures. Tertiles were calculated separately for men and women based on the overall control data. Among men, the tertiles cutoffs were 0.90 and 0.95 and 0.83 and 0.90 in women.
Figure 1: Kaplan-Meier major cardiovascular event-free survival according to rs20417 carrier status in ACTIVE-A and CURE.

A) ACTIVE-A

B) CURE

No. at Risk

<table>
<thead>
<tr>
<th>Carriers Clopidogrel</th>
<th>Noncarriers Clopidogrel</th>
<th>Carriers Placebo</th>
<th>Noncarriers Placebo</th>
</tr>
</thead>
<tbody>
<tr>
<td>146</td>
<td>374</td>
<td>172</td>
<td>369</td>
</tr>
</tbody>
</table>

Days After Randomization

<table>
<thead>
<tr>
<th>Carriers Clopidogrel</th>
<th>Noncarriers Clopidogrel</th>
<th>Carriers Placebo</th>
<th>Noncarriers Placebo</th>
</tr>
</thead>
<tbody>
<tr>
<td>193</td>
<td>333</td>
<td>152</td>
<td>316</td>
</tr>
</tbody>
</table>

Freedom from Primary Efficacy Endpoint

According to rs20417 Carrier Status

- Clopidogrel only: HR=0.63, 95% CI 0.39–1.02, P=0.06
- Placebo only: HR=0.71, 95% CI 0.49–1.00, P=0.072
- Combined: HR=0.69, 95% CI 0.51–0.92, P=0.012

- Clopidogrel only: HR=0.60, 95% CI 0.41–0.86, P=0.0061
- Placebo only: HR=0.48, 95% CI 0.34–0.67, P=1.9e-05
- Combined: HR=0.53, 95% CI 0.41–0.68, P=5.8e-07
Figure 2: Subgroup analysis of association of rs20417 carrier status with major cardiovascular events in five prospective patient populations.

Analyses were adjusted for age, sex, randomization status (when appropriate) and self-reported ethnicity. ACTIVE-A, CURE, epiDREAM/DREAM, ONTARGET, and RE-LY data were included in the meta-analysis. Hetero. P. represents heterogeneity p-value.

<table>
<thead>
<tr>
<th>Subgroup</th>
<th>Carriers</th>
<th>Noncarriers</th>
<th>Odds Ratio (95% CI)</th>
<th>P</th>
<th>Hetero P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Advanced Age</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>7.4% (255/3457)</td>
<td>10.8% (989/9155)</td>
<td>0.71 (0.61-0.82)</td>
<td>4.90E-06</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>4.3% (152/4514)</td>
<td>6.9% (744/10804)</td>
<td>0.69 (0.58-0.82)</td>
<td>2.90E-05</td>
<td></td>
</tr>
<tr>
<td>Summary</td>
<td>5.6% (447/7971)</td>
<td>8.7% (1733/19959)</td>
<td>0.70 (0.63-0.78)</td>
<td>6.00E-10</td>
<td></td>
</tr>
<tr>
<td>Male Vs Female</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>5.3% (150/3706)</td>
<td>6.2% (773/9436)</td>
<td>0.72 (0.61-0.85)</td>
<td>1.00E-04</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>4.3% (152/4514)</td>
<td>6.9% (744/10804)</td>
<td>0.69 (0.58-0.82)</td>
<td>2.90E-05</td>
<td></td>
</tr>
<tr>
<td>Summary</td>
<td>4.7% (388/8220)</td>
<td>7.5% (1517/20240)</td>
<td>0.71 (0.63-0.80)</td>
<td>1.90E-08</td>
<td></td>
</tr>
<tr>
<td>Diabetes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>4.7% (49/1048)</td>
<td>8.3% (207/2505)</td>
<td>0.60 (0.42-0.85)</td>
<td>4.10E-03</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>4.3% (152/4514)</td>
<td>6.9% (744/10804)</td>
<td>0.69 (0.58-0.82)</td>
<td>2.90E-05</td>
<td></td>
</tr>
<tr>
<td>Summary</td>
<td>4.3% (241/5562)</td>
<td>7.1% (951/13309)</td>
<td>0.67 (0.58-0.79)</td>
<td>5.20E-07</td>
<td></td>
</tr>
<tr>
<td>Current Smoker</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>2.6% (26/1057)</td>
<td>4.9% (116/2346)</td>
<td>0.56 (0.35-0.85)</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>4.3% (152/4514)</td>
<td>6.9% (744/10804)</td>
<td>0.69 (0.58-0.82)</td>
<td>2.90E-05</td>
<td></td>
</tr>
<tr>
<td>Summary</td>
<td>3.9% (218/5571)</td>
<td>6.5% (860/13150)</td>
<td>0.68 (0.57-0.79)</td>
<td>1.80E-06</td>
<td></td>
</tr>
<tr>
<td>Hypertension</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>4.2% (136/3209)</td>
<td>7.0% (518/7446)</td>
<td>0.63 (0.51-0.76)</td>
<td>1.30E-05</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>4.3% (152/4514)</td>
<td>6.9% (744/10804)</td>
<td>0.69 (0.58-0.82)</td>
<td>2.90E-05</td>
<td></td>
</tr>
<tr>
<td>Summary</td>
<td>4.2% (327/7723)</td>
<td>6.9% (1262/19250)</td>
<td>0.67 (0.58-0.76)</td>
<td>1.90E-09</td>
<td></td>
</tr>
<tr>
<td>Obesity</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>3.4% (110/3201)</td>
<td>5.9% (422/7183)</td>
<td>0.74 (0.59-0.94)</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>4.3% (152/4514)</td>
<td>6.9% (744/10804)</td>
<td>0.69 (0.58-0.82)</td>
<td>2.90E-05</td>
<td></td>
</tr>
<tr>
<td>Summary</td>
<td>3.9% (302/7715)</td>
<td>6.5% (1166/17987)</td>
<td>0.71 (0.62-0.82)</td>
<td>1.20E-06</td>
<td></td>
</tr>
</tbody>
</table>
Figure 3: Sub-group analysis of association of rs20417 carrier status with non-fatal myocardial infarction in patients from the INTERHEART study.

Analyses were adjusted for age, sex and self-reported ethnicity. Hetero. P. represents heterogeneity p-value.
Figure 4: Boxplots of urinary 11-dehydro thromboxane B$_2$ and urinary 2,3-dinor-6-keto PGF$_{1\alpha}$ concentrations according to rs20417 carrier status in healthy individuals (N=119) from the MARS study.

A) Urinary 11-dehydro thromboxane B$_2$

B) Urinary 2,3-dinor-6-keto PGF$_{1\alpha}$
SUPPLEMENTAL REFERENCES


Supplementary Appendix 2

The effect of bile acid sequestrants on the risk of cardiovascular events: A Mendelian Randomization analysis

SUPPLEMENTAL METHODS

Systematic Review and Meta-Analysis

Eligibility Criteria

Types of studies: Randomized, double-blinded, placebo controlled clinical trials (RCTs) that compared bile acid sequestrant (BAS) treatment with placebo. There were no restrictions based on publication status or publication date; however, only studies published in English were considered.

Type of patients: Only patients aged ≥ 18 years were considered for this review.

Type of Intervention: RCTs that compared the effects of BAS (i.e. 24 g daily cholestyramine, 5 g/d colestipol, and 3.75 g/d coleselam) with placebo or no treatment. There were no restrictions based on the frequency, dosage, length or duration of the BAS intervention.

Types of Outcome Measures:

Primary outcome measures include:

1. Cardiovascular mortality;
2. Myocardial infarction (MI); and
3. Baseline and endpoint mean values or the absolute treatment difference in the intervention and placebo arms for the change in low density lipoprotein cholesterol (LDL-C) levels.

Studies with at least one of these primary outcomes were considered.

Secondary outcome measures include:

1. Baseline and endpoint mean values or the absolute treatment difference in the intervention and placebo arms for the change in high-density lipoprotein cholesterol
(HDL-C), total cholesterol (TC), triglycerides, apolipoprotein A1 (apoA), and apolipoprotein B (apoB).

Information sources

A structured literature search was performed by identifying studies through electronic databases, hand searching reference lists, consulting with field experts and pharmaceutical companies, and scanning trial registries. This search was applied to PubMed (1946 to 2014 in Ovid).

Search

The following terms were used to search all clinical trial registries and databases: cholestyramine; colestipol; colesevelam HCl; placebo; and randomized controlled trials. Where possible, authors of relevant publications were contacted to provide additional information and details about outstanding issues.

Study Selection and Data Items

Based on the results of the search strategy, titles and abstracts for each reference were examined independently by two reviewers (MD and SR). Relevant studies obtained from the full-text screening phase were reviewed for methodological quality and disagreements were resolved through discussion or consultation with a clinician (GP). The following information was extracted from each included trial: (1) characteristics of the study participants (i.e. age, sex, patient population); (2) characteristics of the study (i.e. study design, sample size, median follow-up period); (3) characteristics of the intervention (i.e. dose and frequency of the intervention); and (4) characteristics of the outcome measures (including cardiovascular mortality, MI, and mean change in LDL-C, HDL-C, TC, triglycerides, apoA and apoB).

Data collection process
The two reviewers independently extracted data from the included studies using data collection forms. When methodological information could not be obtained from a publication, the author was contacted for further comment. All forms used in this systematic review were subject to pilot-testing using ten randomly selected studies. Data entry was performed independently by one reviewer (SR) and cross-referenced by the other reviewer (MD). Any discrepancies between the two reviewers were documented and the forms were changed accordingly.

**Summary measures**

For continuous traits, studies that reported median values were converted to an equivalent mean value and the corresponding standard deviation values were calculated by dividing the interquartile range by 1.35. If studies did not report the standard deviation, it was calculated by multiplying the standard error by the square root of the sample size. Where data for LDL-C, HDL-C and TC were available in units of mmol/L, they were converted to mg/dL using a multiplication factor of 38.66. Triglycerides, and apoA and apoB were similarly converted using a multiplication factor of 88.6 and 100, respectively. The mean change-from-baseline in plasma lipid levels in the BAS intervention group were compared to the mean differences in the placebo group with the 95% confidence interval (CI) and p-value as a measure of uncertainty. For binary outcomes, the treatment effect was expressed as an odds ratio (OR) with the 95% CI and p-value. Meta-analyses were performed using an inverse variance random effect meta-analysis.

**Synthesis of results**

Heterogeneity was assessed using the chi-square statistic ($\chi^2$) and inconsistency ($I^2$) was measured by assessing the percentage of total variation of the effects of BAS across studies due to heterogeneity. A low p-value (p<0.10) or $I^2$ test statistic of > 30% provided evidence of
heterogeneity of intervention effects. If these estimates gave rise to sufficient evidence of heterogeneity than attempts were made to explain these differences.

**Additional Analyses**

To explain any evidence of heterogeneity, subgroup analyses were conducted based on the characteristics of the participants (i.e. presence of hyperlipidaemia or type 2 diabetes mellitus) and the study interventions (i.e. length of follow-up). Sensitivity analyses were pre-specified and were used to test the robustness of the pooled results. Unless otherwise specified, a correlation coefficient ($r$) of 0.5 for the difference in the mean change from baseline was assumed for all analyses. Thus the $r$ was varied by 0.3 and 0.7 for all the relevant studies to determine if this altered the reported estimates.

**Simulation Statistical Analysis**

Simulations were performed to predict the effect of 24 g/d cholestyramine on plasma lipid profiles (HDL-C, TC, triglycerides, apoA and apoB) using the known genetic associations of rs4299376 SNP with lipids fractions. To do so, we adapted the method from Sofat et al\(^2\) to match the genetic effects to the effect of cholestyramine 24 g/d on LDL-C, taking into account the uncertainty of both the genetic and drug effect estimates. Random numbers were selected from the normal distributions of the change in LDL-C for the pharmacological and genetic effect (i.e. fixing the mean and standard deviation of each distribution to their respective estimated values). In order to validate whether the rs4299376 SNP had a similar effect on plasma lipid profiles as cholestyramine, the predicted effects of cholestyramine on plasma levels of HDL-C, TC and triglycerides were estimated using genetic data. These predicted estimates were then compared to known effects of cholestyramine on the same lipids fractions from clinical data. 10,000 simulations were performed to generate the distribution of HDL-C, TC and triglycerides.
assuming each allele has the same predicted effect as cholestyramine on LDL-C, and the mean effect and 95% CI were calculated. The p-value for the difference between the predicted effect of cholestyramine and the observed effects of BAS on lipid levels were calculated by comparing the randomly generated point estimate of the effect of cholestyramine to the randomly generated point estimate of the predicted effect of the drug. Next, the effect of 24 g/d cholestyramine on the risk of cardiovascular outcomes was predicted using data on genetic association of rs4299376 with CAD and compared to the effect of cholestyramine on CAD from the only outcome trial of cholestyramine, LRCCPPT\(^3\). The predicted drug effect was compared to the observed effect of a comparable dose of cholestyramine on the risk of CVD outcomes using a z-test. As a sensitivity analysis, the predicted effect of cholestyramine on CAD was also estimated using data from the CTT\(^4\). This estimate was similarly compared to the cardiovascular outcomes reported in the LRCCPPT in order to compare the predicted effect of BAS with statin use using a z-test. These analyses were also repeated using the summary effect of 3.75 g/d of colesevelam.

**Results**

**Study Selection**

A total of 19 studies were identified for inclusion in this review. The structured literature search of PubMed databases derived a total of 420 citations. Of these, 360 studies were discarded because after reviewing the abstracts it appeared that these papers clearly did not meet our inclusion criteria. The full-text of the remaining 60 citations were examined in more detail. It appeared that 40 articles did not meet the inclusion criteria. Of the included articles, there were six cholestyramine RCTs\(^3, 5-9\), three colestipol RCTs\(^10-12\) and 10 colesevelam RCTs\(^13-21\) with a
total of 7,021 study participants. Supplemental Figure 1 illustrates the flow diagram of the study selection process.

**Randomized Controlled Trials of Colestipol**

We identified three RCTs with a total of 398 participants with hyperlipidemia (mean age 54 years, 44% women)\(^{10-12}\) (Supplemental Table 1). Owing to the lack of reported data and differences in study dose, we did not pool the reported effect of colestipol on plasma lipid levels.

**Additional Analyses**

We were unable to conduct subgroup analyses in order to explore the presence of heterogeneity among the pooled estimates of 24 g/d cholestyramine and 3.75 g/d colesevelam on the mean change in plasma lipid levels due to a lack of reported data. Therefore, to account for the high degree of heterogeneity in the pooled estimates of cholestyramine, the effect estimates of the mean change in LDL-C and TC from the LRCCPPT trial\(^3\) will be used as a surrogate since it was the only outcome trial.

To test the robustness of the main findings, the \(r\) of the mean change from baseline in the 24 g/d cholestyramine and the 3.75 g/d colesevelam meta-analyses were varied. Assuming an \(r\) of 0.3 and 0.7 did not demonstrate any difference in the reported treatment effects of cholestyramine (Supplemental Figure 2 and 3) or colesevelam (Supplemental Figure 4 and 5). However, assuming an \(r\)=0.3 within the cholestyramine meta-analysis resulted in a reduction of the high degree of heterogeneity in the pooled LDL-C estimates (\(P\) for heterogeneity =1.70x10\(^{-4}\)) while an \(r\)=0.7 significantly increased the presence of heterogeneity (heterogeneity \(P\)-value:2.10x10\(^{-9}\)). Similar results were also obtained for the treatment effects of colesevelam.
SUPPLEMENTAL TABLES

**Supplemental Table #1:** Studies contributing to the colestipol meta-analysis

<table>
<thead>
<tr>
<th>Author &amp; Date</th>
<th>Patient Population</th>
<th>Follow-Up</th>
<th>Intervention</th>
<th>Comparison</th>
<th>Sample size</th>
<th>Age</th>
<th>Women</th>
<th>LDL-C (mg/dL)*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>COLESTIPOL</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hunninghake 1995&lt;sup&gt;10&lt;/sup&gt;</td>
<td>Hyperlipidemia</td>
<td>8 weeks</td>
<td>Colestipol (2 g; 4 g; 8 g; 16 g)</td>
<td>Placebo</td>
<td>196</td>
<td>56.2</td>
<td>(NR)</td>
<td>190.0(NR)</td>
</tr>
<tr>
<td>Simons 1992&lt;sup&gt;11&lt;/sup&gt;</td>
<td>Hyperlipidemia</td>
<td>18 weeks</td>
<td>Colestipol (5 g); Colestipol (10 g) &amp; each with 6 weeks of placebo; 6 weeks of simvaslatin (20 mg); 6 weeks of simvaslatin (40 mg)</td>
<td>Placebo with 6 weeks of placebo; 6 weeks of simvaslatin (20 mg); 6 weeks of simvaslatin (40 mg)</td>
<td>61</td>
<td>45.3</td>
<td>(19)</td>
<td>303.1(77.7)</td>
</tr>
<tr>
<td>Superko 1992&lt;sup&gt;12&lt;/sup&gt;</td>
<td>Hyperlipidemia</td>
<td>12 weeks</td>
<td>Colestipol (5g/d; 10g/d; 15g/d)</td>
<td>Placebo</td>
<td>141</td>
<td>49(12)</td>
<td>49 (35)</td>
<td>168.0(12.0)</td>
</tr>
</tbody>
</table>

*Refers to the highest single BAS dose reported in the study; NR: not reported

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Supplemental Table #2: The association of rs4299376 SNP (ABCG5/8) and the risk of diabetes, glyced hemoglobin (HbA1c), fasting glucose, systolic blood pressure (SBP), diastolic blood pressure (DBP) and body mass index (BMI).

<table>
<thead>
<tr>
<th>Trait</th>
<th>Effect Allele</th>
<th>Other Allele</th>
<th>Effect Estimate</th>
<th>Standard Error</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasting glucose</td>
<td>G</td>
<td>T</td>
<td>0.00088</td>
<td>0.0026</td>
<td>0.737689</td>
</tr>
<tr>
<td>HbA1c</td>
<td>T</td>
<td>G</td>
<td>-0.0051</td>
<td>0.004</td>
<td>0.199</td>
</tr>
<tr>
<td>Diabetes</td>
<td>G</td>
<td>T</td>
<td>-0.00738</td>
<td>0.016336</td>
<td>0.65164</td>
</tr>
<tr>
<td>SBP</td>
<td>G</td>
<td>T</td>
<td>0.024683</td>
<td>0.112445</td>
<td>0.826253</td>
</tr>
<tr>
<td>DBP</td>
<td>G</td>
<td>T</td>
<td>0.00435</td>
<td>0.070956</td>
<td>0.951115</td>
</tr>
<tr>
<td>BMI</td>
<td>T</td>
<td>G</td>
<td>-0.0054</td>
<td>0.0064</td>
<td>0.4</td>
</tr>
</tbody>
</table>
SUPPLEMENTAL FIGURES

Supplemental Figure #1: Study flow diagram.
Supplemental Figure #2: Forest plot of the association of 24 g/d of cholestyramine treatment and the summary mean difference of LDL-C, HDL-C, total cholesterol, triglycerides, apoA and apoB assuming a correlation coefficient 0.3.

Het P refers to the heterogeneity p-value.
Supplemental Figure #3: Forest plot of the association of 24 g/d of cholestyramine treatment and the summary mean difference of LDL-C, HDL-C, total cholesterol, triglycerides, apoA and apoB assuming a correlation coefficient 0.7.

Het P refers to the heterogeneity p-value.
Supplemental Figure #4: Forest plot of the association of 3.75 g/d of colesevelam treatment and the summary mean difference of LDL-C, HDL-C, total cholesterol, triglycerides, apoA and apoB assuming a correlation coefficient 0.3.

Het P refers to the heterogeneity p-value.
Supplemental Figure #5: Forest plot of the association of 3.75 g/d of colesevelam treatment and the summary mean difference of LDL-C, HDL-C, total cholesterol, triglycerides, apoA and apoB assuming a correlation coefficient 0.7.

Het P refers to the heterogeneity p-value.
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Supplementary Appendix 3

Mendelian randomization analysis supports the causal role of dysglycemia and diabetes in the risk of coronary artery disease

AUTHORS: Stephanie Ross; Hertzel C. Gerstein; John Eikelboom; Sonia S. Anand; and Guillaume Paré.

Methods

Supplementary Table #1: SNPs associated with FG, HbA₁c and diabetes.

Supplementary Figure #1: Flow chart of SNPs included in analysis.
METHODS

Data Sources

Data on the genetic associations with fasting plasma glucose was obtained from the Meta-Analyses of Glucose and Insulin-related traits Consortium. MAGIC was a genome wide association study (GWAS) to identify the genetic determinants of glycemic and metabolic traits. The association between genetic variants and the change in FPG (mmol/L) was assessed in 133,010 and 42,854 non-diabetic European individuals\textsuperscript{1}. The association between genetic variants and the change in hba1c (%) was assessed among 46,368 non-diabetic European individuals\textsuperscript{2}. Genotyping was performed using the Metabochip.

Data on the genetic associations with plasma lipid levels were obtained from the Global Lipids Consortium (GLCC)\textsuperscript{3}. In brief, the GLCC performed a meta-analysis of 46 lipid GWAS assessing common variants associated with serum lipids (LDL-C, high density lipoprotein cholesterol (HDL-C), total cholesterol (TC), and triglycerides). A total of 46 studies and 91,285 individuals of European descent were analyzed for the genetic association with LDL-C, while data from 95,708, 95,992 and 92,410 individuals were available for HDL-C, TC and triglycerides, respectively. Genotyping was performed using commercially available Affymetrix or Illumina genotyping arrays or custom Perlegen arrays.

Data on the genetic association with the risk of CAD was obtained from the CARDIoGRAMplusC4D Consortium. Briefly, the CARDIoGRAMplusC4D Consortium performed a meta-analysis of 63,746 cases of CAD and 130,681 controls\textsuperscript{4}. Genotyping was performed using the Metabochip, which is a custom iSELECT chop (Illumina). In addition,
estimates were also obtained from the CARDIoGRAM Consortium, which was a meta-analysis of 22,233 cases of CAD and 64,762 controls from 22 GWAS.5

Data on the genetic associations with diabetes was obtained from the DIAGRAM Consortium. Briefly, the DIAGRAM Consortium performed a meta-analysis of 22,669 cases of diabetes and 58,119 controls of European descent and 1,178 cases and 2,472 controls of Pakistani descent (PROMIS).6 Genotyping was performed using the Metabochip.

Data on the genetic associations with body mass index (BMI) were obtained from the Genetic Investigation of ANthropometric Traits (GIANT) consortium. In brief, GIANT performed a meta-analysis of 51 GWAS assessing common variants associated with BMI in over 170,000 individuals of European descent. Genotyping was performed using commercially available Affymetrix or Illumina genotyping arrays or custom Perlegen arrays.

Data on the genetic associations with systolic blood pressure (SBP) and diastolic blood pressure (DBP) were obtained from the International Consortium for Blood Pressure Genome-Wide Association Studies (ICBP) consortium.8 In brief, ICBP performed a meta-analysis of 82 GWAS assessing common variants associated with blood pressure in over 200,000 individuals of European descent. Genotyping was performed using commercially available Affymetrix or Illumina genotyping arrays.

**The relative risk of CAD associated with diabetes**

The procedure to derive genetic estimates of CAD odds ratio is outlined below. Given:
(A) Prevalence of diabetes

(B) Prevalence of CAD in individuals without diabetes

(C) Odds ratio for CAD associated with diabetes

(D) Regression coefficient of SNP associations with CAD as function of SNP association with diabetes (where genetic effects are expressed as log(OR) per allele for both CAD and diabetes)

The following can be calculated:

(E) Calculated population prevalence of CAD: \( (A \times B \times C) + (1 - A) \times B \)

(F) Estimated prevalence of CAD in individuals with a theoretical increase in risk of diabetes of \( e = 2.72 \) fold: \( (A \times \exp(1) \times B \times C) + (1 - A \times \exp(1)) \times B \)

(G) Estimated odds ratio for CAD per \( e = 2.72 \) fold increase in risk of diabetes: \( F/E \)

It results that:

(H) \( \exp(D) = G = \frac{F}{E} = \frac{(A \times \exp(1) \times B \times C) + (1 - A \times \exp(1)) \times B}{(A \times B \times C) + (1 - A) \times B} \)

As \( C \) is the only unknown variable, the odds ratio for CAD associated with diabetes as estimated from genetic data can be calculated using algebraic transformations.
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Supplementary Table #1: SNPs associated with FG, HbA1c and diabetes.

SNPs associated with FG

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