Genetics of coronary atherosclerosis with an emphasis on blood lipids

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Thesis for the degree of Philosophiae Doctor

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Erfðir kransæðasjúkdóms með áherslu á þátt blóðfitu

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Ágrip

Kransæðasjúkdómur dregur fleiri til dauða á hverju ári en nokkur annar sjúkdómur á heimsvísu. Sjúkdómurinn einkennist af framvindu æðakölkunar, sem er flókið og margþætt ferli. Á undanförnum 14 árum hafa rannsóknir með víðtækri erfðamengisleit leitt til stórstígra framfara í skilningi manna á hlutverki erfðaþátta í framgangi æðakölkunar. Sem dæmi hafa nýlegar erfðarannsóknir undirstrikað orsakasamband milli lípóprótína sem innihalda apólípóprótín B og aukinnar áhættu á kransæðasjúkdómi.

Doktorsritgerð þessi byggir á fjórum birtum rannsóknum. Rannsóknirnar voru unnar við Íslenska erfðagreiningu og byggja á yfirgripsmiklum gagnagrunni sem geymir arfgerðar- og svipgerðarupplýsingar yfir 160.000 Íslendinga. Hver einstaklingur var arfgerðargreindur með DNA-örflögum en auk þess voru erfðamengi allt að 50.000 einstaklinga heilraðgreind. Arfgerðarupplýsingar sem fengust með heilraðgreiningu voru síðan notaðar til að áætla arfðgerðir í heildarúrtakinu með líkindafræðilegum hætti.

Fyrsta rannsóknin skoðaði áhrif 50 algengra erfðaþátta kransæðasjúkdóms á útbreiðslu sjúkdómsins við kransæðaþræðingu hjá yfir 8.600 einstaklingum. Erfðafræðilegt áhættuskor sem tekur saman áhrif þessara breytileika hafði fylgni við aukna útbreiðslu kransæðasjúkdóms. Niðurstöðurnar sýndu að samanlögð áhrif algengra erfðaþátta kransæðasjúkdóms spáðu ekki aðeins fyrir um áhættu, heldur einnig útbreiðslu sjúkdómsins við kransæðaþræðingu.

Önnur rannsóknin skoðaði hvort erfðaþættir sem hafa þekkt tengsl við blóðfitu hafi áhrif á útbreiðslu kransæðasjúkdóms meðal yfir 17.000 einstaklinga sem höfðu gengist undir kransæðaþræðingu eða mat á kalki í kransæðum með tölvusneiðmyndun. Erfðaskor fyrir mismunandi blóðfitutegundir voru notuð til þess að kanna fylgni við útbreiðslu kransæðasjúkdóms og meta hvort orsakasamhengi sé til staðar, með aðferð sem nefnist á ensku Mendelian randomization. Þessi rannsókn undirstrikaði orsakaáhrif kólesteróls sem bundið er í lípóprótínum sem innihalda apólípóprótín B (þ.e. ekki-HDL kólesteról) á þróun æðakölkunar í kransæðum.

Þriðja rannsóknin miðaði að því að skoða algengi og afleiðingar arfbundinnar kólesterólhækkunar meðal 160.000 einstaklinga. Bornar voru saman tvenns konar skilgreiningar sem byggja annars vegar á arfgerð (þ.e. að sjúkdómsvaldandi stökkbreyting sé til staðar) og hins vegar á klínískri svipgerð sem samræmist sjúkdóminum (samkvæmt Dutch Lipid Clinic Network skilmerkjunum). Tuttugu meinvaldandi stökkbreytingar fundust, en tólf þeirra voru áður óþekktar í íslensku þýði. Samanlagt algengi stökkbreytinganna var u.þ.b. 1 af 800 í heildarúrtakinu. Arfbundin

kólesterólhækkun sem skilgreind er út frá klínískri svipgerð var hins vegar mun algengari (2,2% meðal fullorðinna einstaklinga með kólesterólmælingar) og í minnihluta tilfella mátti rekja hana til undirliggjandi stökkbreytingar. Báðir hópar með arfbundna kólesterólhækkun voru mjög vanmeðhöndlaðir með kólesteróllækkandi lyfjum.

Fjórða rannsóknin segir frá uppgötvun afar sjaldgæfrar stökkbreytingar í geni lágþéttni-lípóprótína (LDL) viðtakans (*LDLR*), sem veldur aukinni virkni prótínsins. Stökkbreytingin er 2,5 kílóbasa brottfelling á 3’ stýrisvæði (e. 3’ untranslated region) sem fannst í sjö einstaklingum innan sömu fjölskyldunnar. Arfberar höfðu yfir 70% lægra gildi lágþéttni-LDL kólesteróls í blóði og yfirborðstjáning LDL viðtakans í eitilfrumum þeirra var nærri tvöföld á við þá sem ekki bera stökkbreytinguna. Þetta er fyrsta stökkbreytingin í *LDLR* sem vitað er að auki verulega tjáningu viðtakans og veldur þar með mikilli lækkun LDL kólesteróls í blóði. Greining á áhrifum stökkbreytingarinnar varpar ljósi á þátt 3’ stýrisvæðisins í tjáningu LDL viðtakans og sýnir að stytting þess getur aukið tjáningu viðtakans verulega.

Saman varpa þessar rannsóknir ljósi á áhrif algengra og sjaldgæfra arfbreytileika á meingerð kransæðasjúkdóms, með sérstaka áherslu á þátt blóðfitu.

Efnisorð: erfðafræði, kransæðasjúkdómur, æðakölkun, blóðfitur, kólesteról.

Abstract

Coronary artery disease (CAD) is a common disease that remains the leading cause of death worldwide. CAD is characterized by the development of coronary atherosclerosis, a complex and multifaceted process. Over the last 14 years, genome-wide association studies have greatly improved our understanding of the contribution of DNA sequence variants to the development of coronary atherosclerosis. For example, genetic studies have underscored that circulating apolipoprotein B (apoB)-containing lipoproteins such as low-density lipoproteins (LDL) are causally related to the development of CAD.

This thesis is based on four genetic studies. The studies were carried out at deCODE genetics, Reykjavík, Iceland, and are based on an extensive database that holds the genotypes and phenotypes of over 160,000 Icelandic participants. Each participant was chip-genotyped and up to 50,000 participants additionally underwent whole-genome sequencing (WGS); the sequence variants identified using WGS were then imputed into the entire genotyped sample.

The first study examined the effects of 50 common risk variants for CAD on the extent of coronary atherosclerosis using angiographic data from over 8,600 genotyped participants. A genetic risk score that combines the effects of these variants was associated with increased extent of angiographic CAD, thus demonstrating that the cumulative burden of these variants is not only associated with increased risk of CAD, but also its angiographic extent.

The second study assessed the contribution of many established lipid-associated variants with the extent of coronary atherosclerosis in a total of over 17,000 participants who had undergone coronary angiography or coronary artery calcium scanning. Genetic scores for different lipid traits were used in a Mendelian randomization analysis to evaluate the causal effects of different lipid traits on the extent of coronary atherosclerosis. This study highlighted the causal role of non-HDL cholesterol in the development of coronary atherosclerosis.

The third study sought to characterize monogenic familial hypercholesterolemia (FH) and clinically defined FH (using the Dutch Lipid Clinic Network criteria) in over 160,000 genotyped Icelanders. Twenty FH mutations were identified in about 50,000 individuals in a WGS subsample, 12 of which had not been described in Iceland previously. The prevalence of these mutations was approximately 1 in 800 in the overall sample. Clinical FH was much more common than monogenic FH (prevalence 2.2% in adults with available cholesterol measurements) and rarely explained by

monogenic FH. Both monogenic FH and clinical FH were severely undertreated, as guideline-directed goals for lipid-lowering treatment were rarely achieved.

The fourth study describes the discovery of an extremely rare gain-of-function mutation in the LDL receptor gene (*LDLR*). This mutation is a 2.5 kilobase deletion involving the 3' untranslated region (UTR) of the gene, found in seven individuals within a single four generation family in Iceland. The mutation was associated with over 70% reduction in blood levels of LDL cholesterol and almost two-fold higher surface expression of the LDL receptor in lymphoblast cell lines derived from the carriers. This mutation is the first known high-impact gain-of-function mutation in *LDLR*. The functional consequences of this mutation highlight the role of negative regulation by the 3' UTR on *LDLR* mRNA expression and demonstrate the 3' UTR shortening via alternative polyadenylation is a powerful modulator of LDL receptor expression.

In summary, these studies demonstrate the influence of common and rare sequence variants on the development of coronary atherosclerosis with a particular emphasis on the role of blood lipids.

Keywords: genetics, coronary artery disease, atherosclerosis, lipids, cholesterol.

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Contents

List of abbreviations

apoA	apolipoprotein A
apoB	apolipoprotein B
CABG	coronary artery bypass grafting
CAC	coronary artery calcium
CAD	coronary artery disease
CNV	copy number variant
CT	computed tomography
CTCA	computed tomography coronary angiography
DLCN	Dutch Lipid Clinic Network
EAS	European Atherosclerosis Society
EBV	Epstein-Barr virus
ESC	European Society of Cardiology
FCH	familial combined hypolipidemia
FH	familial hypercholesterolemia
FHBL	familial hypobetalipoproteinemia
GRS	genetic risk score
GWA	genome-wide association
HDL	high density lipoprotein
IDL	intermediate density lipoprotein
LDL	low density lipoprotein
LDLR	LDL receptor gene
LPA	lipoprotein(a) gene
Lp(a)	lipoprotein(a)
MR	Mendelian randomization
PBMC	peripheral blood mononuclear cell
PCI	percutaneous coronary intervention
PCR	polymerase chain reaction
PCSK9	proprotein convertase subtilisin/kexin type 9
PRS	polygenic risk score
RACE	rapid amplification of complementary DNA ends
SNP	single-nucleotide polymorphism
TGL	triglyceride-rich lipoproteins
UTR	untranslated region
VLDL	very low density lipoprotein
WGS	whole-genome sequencing

List of figures

List of tables

List of original papers

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals:

I. Bjornsson E, Gudbjartsson DF, Helgadottir A, Gudnason T, Gudbjartsson T, Eyjolfsson K, Patel RS, Ghasemzadeh N, Thorleifsson G, Quyyumi AA, Thorsteinsdottir U, Thorgeirsson G, Stefansson K. **Common Sequence Variants Associated With Coronary Artery Disease Correlate With the Extent of Coronary Atherosclerosis**. *Arterioscler Thromb Vasc Biol.* 2015;35:1526–1531.

II. Björnsson E, Thorleifsson G, Helgadóttir A, Guðnason T, Guðbjartsson T, Andersen K, Grétarsdóttir S, Ólafsson Í, Tragante V, Ólafsson ÓH, Jónsdóttir B, Eyjólfsson GI, Sigurðardóttir Ó, Thorgeirsson G, Guðbjartsson DF, Thorsteinsdóttir U, Hólm H, Stefánsson K. **Association of Genetically Predicted Lipid Levels with the Extent of Coronary Atherosclerosis in Icelandic Adults**. *JAMA Cardiol.* 2020;5:13–20.

III. Björnsson E, Thorgeirsson G, Helgadóttir G, Thorleifsson G, Sveinbjörnsson G, Kristmundsdóttir S, Jónsson H, Jónasdóttir A, Jónasdóttir Á, Sigurðsson Á, Guðnason T, Ólafsson Í, Sigurðsson EL, Sigurðardóttir Ó, Viðarsson B, Baldvinsson M, Bjarnason R, Danielsen R, Matthíasson SE, Thórarinsson BL, Grétarsdóttir S, Steinthórsdóttir V, Halldórsson BV, Andersen K, Arnar DO, Jónsdóttir I, Guðbjartsson DF, Hólm H, Thorsteinsdóttir U, Sulem P, Stefánsson K. **Large-Scale Screening for Monogenic and Clinically Defined Familial Hypercholesterolemia in Iceland.** *Arterioscler Thromb Vasc Biol.* 2021;41:2616-2628.

IV. Bjornsson E, Gunnarsdottir K, Halldorsson GH, Sigurdsson A, Arnadóttir GA, Jonsson H, Olafsdóttir EF, Niehus S, Kehr B, Sveinbjörnsson G, Gudmundsdottir S, Helgadottir A, Andersen K, Thorleifsson G, Eyjolfsson GI, Olafsson I, Sigurdardottir O, Saemundsdottir J, Jonsdottir I, Magnusson OTh, Masson G, Stefansson H, Gudbjartsson DF, Thorgeirsson G, Holm H, Halldorsson BV, Melsted P, Norddahl GL, Sulem P, Thorsteinsdottir U, Stefansson K. **Lifelong Reduction in LDL (Low-Density Lipoprotein) Cholesterol due to a Gain-of-Function Mutation in LDLR**. *Circ Genom Precis Med.* 2021;14(1):e003029.

Declaration of contribution

Papers I-IV. In each paper, I had a central role in designing the study, analyzing and interpreting the data, preparing results for presentation, creating figures, writing and revising the manuscript and handling correspondence in the peer-review process. This was done in close cooperation and with supervision by members of the doctoral committee, along with valuable contributions from other co-authors.

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1 Introduction

Here, I will introduce several main concepts related to this thesis. The first two chapters relate to coronary artery disease (CAD) and techniques for assessment of the extent of coronary atherosclerosis (**paper I** and **paper II**). Chapter 1.3 introduces common genetic risk factors for CAD and how they influence the extent of coronary atherosclerosis (**paper I**). Chapter 1.4 provides an overview of blood lipids (**papers II-IV**). Chapter 1.5 discusses common genetic variants that influence blood lipids and how they relate to CAD (**paper II**). Finally, chapter 1.6 introduces two monogenic disorders of low density lipoprotein (LDL) metabolism (**paper III** and **paper IV**).

1.1 Coronary artery disease

Ischemic heart disease, or CAD, remains the most common cause of death worldwide, accounting for 16% of total deaths (WHO Global Health, 2020). The underlying pathogenesis of this disease is progressive atherosclerosis of the coronary arteries which leads to coronary narrowing and predisposition to myocardial infarction (Libby, 2008).

Atherosclerosis is a complex process that is characterized by chronic inflammation and the accumulation of lipoproteins in the arterial intima (Libby & Hansson, 2019) (**Figure 1**). Dysfunction of the vascular endothelium due to various causes is commonly thought to be an inciting factor in atherogenesis (Gimbrone & García-Cardeña, 2016). This promotes influx and retention of apolipoprotein B (apoB) lipoproteins such as LDL in the subendothelial space, which is recognized as a key driver of atherogenesis (Borén et al., 2020; Mundi et al., 2018). The details of transendothelial transport of lipoproteins are not fully understood, but recent evidence in mice shows that LDL may be actively transported across the endothelium by scavenger receptor class B type 1 (Huang et al., 2019; Jang et al., 2020). Once in the intima, apoB lipoproteins undergo modification with oxidation and hydrolysis, where they are engulfed by macrophages that become foam cells, inciting an inflammatory response (Moore & Tabas, 2011). The ensuing induction of various maladaptive immune system responses eventually evolves into a chronic inflammatory state, which further amplifies apoB lipoprotein retention and modification, a process that over time drives plaque progression (Hansson & Hermansson, 2011; Moore & Tabas, 2011).

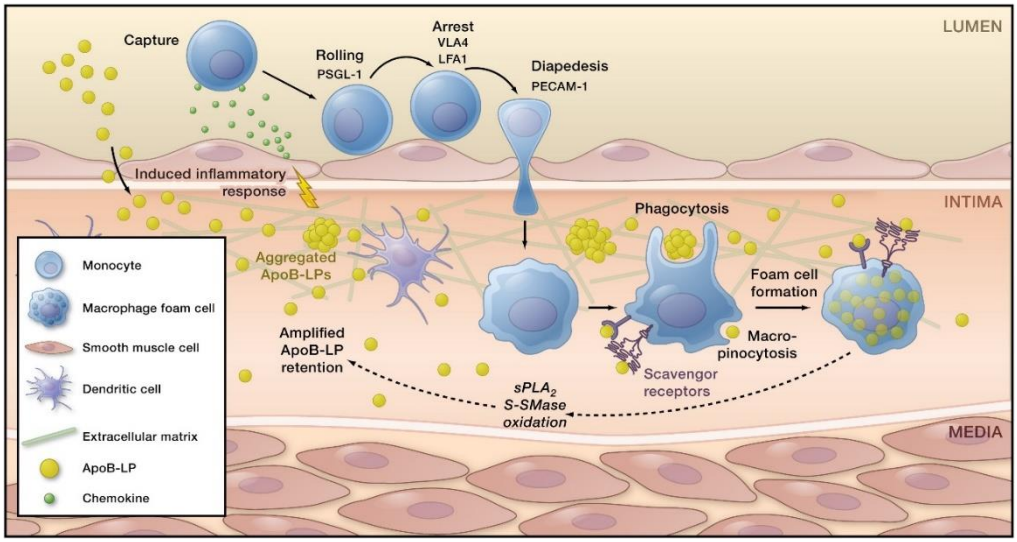

Figure 1. ApoB lipoprotein retention and immune response in atherosclerosis

ApoB lipoproteins (apoB-LPs) cross the endothelium, bind to proteoglycans, and undergo various modifications (e.g. oxidation and hydrolysis), thus becoming retained in the intima. This incites an inflammatory response characterized by cytokine release and recruitment of immune cells such as monocytes, which differentiate into macrophages that engulf modified apoB-LPs and become foam cells. Inflammation in the vessel wall contribute to further retention and modification of apoB-LPs, further driving atherosclerosis. Reproduced from Moore & Tabas, 2011, with permission (©Elsevier).

In coronary arteries, progression of atherosclerosis over decades may manifest clinically as angina pectoris (due to lesions that limit coronary blood flow) or acute ischemia and myocardial infarction (most commonly due to plaque rupture and thrombosis) (Falk et al., 2013; Libby, 2008). Well-established clinical risk factors for CAD include increasing age, male sex, dyslipidemia, hypertension, diabetes mellitus, cigarette smoking, obesity, physical inactivity and family history of premature CAD (Dzau et al., 2006).

1.2 Imaging techniques to assess coronary atherosclerosis

Several imaging techniques have been developed to detect and quantify the extent and burden of coronary atherosclerosis. In clinical practice, these techniques are used in diagnosis, estimation of prognosis and to inform management decisions (Gruettner et al., 2012). The most common methods to assess the coronary vasculature are invasive coronary angiography and cardiac computed tomography (CT) (**Figure 2**). Other techniques such as magnetic resonance imaging and positron emission tomography are in rapid development but are rarely used in clinical practice and will not be covered here (Adamson & Newby, 2019).

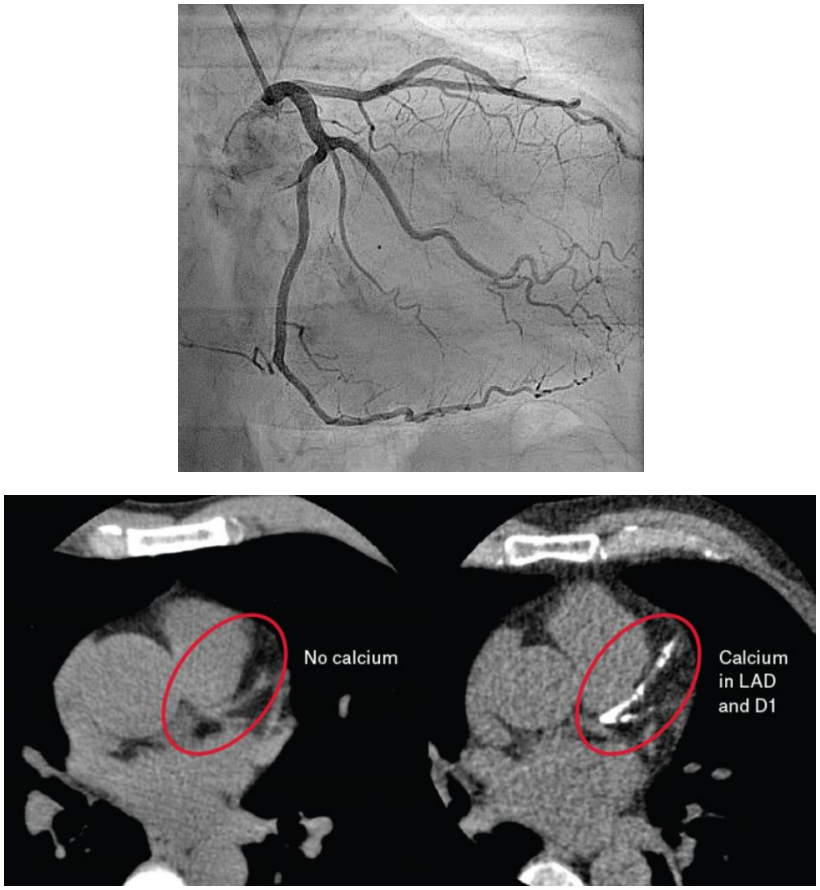

Figure 2. Assessment of extent of coronary atherosclerosis

Top: Coronary angiography showing normal left coronary arteries (reproduced from Kočka 2015, ©Elsevier). Bottom: Coronary artery calcium on cardiac computed tomography (reproduced from Chua et al., 2020, ©The Royal Australian College of General Practitioners).

1.2.1 Coronary angiography

Percutaneous coronary angiography is considered the gold standard for quantification of the extent of coronary atherosclerosis (Braunwald, 2012). In recent two decades, although cardiac CT has emerged as an important non-invasive alternative in the stable patient, coronary angiography remains the diagnostic method of choice in the setting of a suspected acute coronary event. In coronary angiography, intravascular contrast is delivered into the coronary arteries via catheter in order to assess for luminal stenoses indicative of coronary disease. In the case of a clinically significant obstructive lesion (usually at least 50-70% luminal stenosis by visual estimation), percutaneous coronary intervention (PCI) with stenting may be indicated and can be carried out at the time of angiography (Iqbal & Serruys, 2014).

Several methods have been developed to quantify the extent of coronary atherosclerosis as assessed by coronary angiography (Neeland et al., 2012). The simplest and likely most commonly used method is a vessel score, defined as the number of major epicardial coronary arteries that are obstructed (commonly at least 50-70% luminal stenosis). The vessel score is easily obtained from the routine coronary angiogram and ranges from 0 to 3 (or 4 if the left main coronary artery is counted as a separate coronary artery) (Neeland et al., 2012; Ringqvist et al., 1983). The main limitation of the vessel score is that it provides only rough categorization of CAD extent and is insensitive to non-obstructive atherosclerosis (Topol & Nissen, 1995). Despite these limitations, the vessel score is an established marker of the total burden of coronary atherosclerosis, carries important prognostic information and is widely used to guide treatment decisions (e.g. invasive vs medical management alone, or PCI vs coronary artery bypass grafting for extensive disease) (Neeland et al., 2012). Other scoring systems have been developed, notably the Gensini score, but these will not be covered here.

1.2.2 Cardiac CT

Cardiac CT represents noninvasive imaging of the coronary arteries using a cardiac-gated multidetector CT scan. Two main imaging modalities have been developed for cardiac CT: coronary artery calcium (CAC) scanning and CT coronary angiography.

CAC scanning is used for the detection and quantification of coronary calcification, which is a marker of atherosclerosis (Adamson & Newby, 2019). CAC scanning does not require intravenous contrast and the radiation exposure is low (<1 mSv). Total CAC is most commonly reported in Agatston units, a semi-quantitative measure that provides an approximation of the overall plaque burden and is correlated with risk of cardiovascular events (Agatston et al., 1990). In clinical practice, CAC scanning is most commonly used for risk assessment in intermediate-risk individuals without known coronary disease, who are either asymptomatic or have a history of suspected angina (Adamson & Newby, 2019). A CAC score (also known as Agatston score) of >0 indicates the presence of coronary atherosclerosis. CAC is commonly graded into groups based on this score: 1-100 indicating mild CAC, 101-400 moderate CAC, and 400+ extensive CAC (Budoff et al., 2009). These CAC groups correlate with risk of coronary events and offer better risk prediction than age/sex/race-specific percentiles (Budoff et al., 2009). The main limitation of CAC scanning is that it has limited spatial resolution (commonly 1.5-3.0 mm) and does not detect non-calcified plaques. Thus a CAC score of 0 does not rule out the possibility of non-calcified plaques.

CT coronary angiography (CTCA) uses intravenous contrast to visualize the coronary arterial lumen and provides a non-invasive alternative to conventional coronary angiography for the assessment of coronary stenoses in select patients (Adamson & Newby, 2019). Like invasive coronary angiography, CTCA provides prognostic information by the burden and distribution of coronary plaques (Min et al.,

2007). A key advantage of CTCA over conventional angiography, however, is that it allows better detection of non-obstructive atheroma and enables assessment of plaque characteristics that may indicate high-risk vulnerable plaques (Thomsen & Abdulla, 2016). The diagnostic accuracy of CTCA is dependent on image quality, which can be poor in the setting of high heart rates (motion artifacts), dense calcifications and coronary stents (Adamson & Newby, 2019).

1.3 Common genetic variation and coronary atherosclerosis

The heritable component of CAD has been estimated between 40% and 50% (Marenberg et al., 1994; Zdravkovic et al., 2002). Since the first genome-wide association (GWA) studies on CAD risk in 2007 (Helgadottir et al., 2007; McPherson et al., 2007; Samani et al., 2007; Wellcome et al., 2007), DNA sequence variants at over 160 loci have been associated with risk of CAD (van der Harst & Verweij, 2018). While some are related to known risk factors such as dyslipidemia, hypertension, diabetes, obesity and inflammation, the majority of the identified risk variants appear to exert their effect through other biological pathways or via genetic interactions that are not fully understood (van der Harst & Verweij, 2018). Most GWA studies have been performed using genotyping arrays that genotype from 500,000 to 1 million single-nucleotide polymorphisms (SNPs) scattered throughout the genome. These arrays, often called SNP chips, have good coverage for common SNPs (allele frequency at least 5%) but poorer coverage of rarer SNPs (e.g. allele frequency below 1%) (Zuk et al., 2014). In addition to SNPs genotyped with SNP chips, GWA studies commonly include millions of additional SNPs that are not themselves genotyped directly, but inferred based on a haplotype (or genotype) reference panel, such as the 1000 Genomes reference panel (The 1000 Genomes Project Consortium, 2012).

The vast majority of SNPs shown to be associated with risk of CAD are common (van der Harst & Verweij, 2018). Typically, each individual SNP modulates CAD risk by only about 5% to 10% per allele, with a corresponding odds ratio (OR) between 1.05 and 1.10 (0.95 and 0.90 for protective variants). Notable exceptions of variant with a larger effect magnitude include variants within the 9p21 locus (*CDKN2A-CDKN2B*), the first locus shown to associate with CAD in GWA studies (Helgadottir et al., 2007; McPherson et al., 2007), where the risk allele confers increased risk by about 20% (OR 1.20 for rs4977574, allele frequency 48%); the lipoprotein(a) gene (*LPA*) (OR 1.36 for rs55730499, allele frequency 7%); and the gene for proprotein convertase subtilisin/kexin type 9 (*PCSK9*) (OR 0.79 for rs11591147, allele frequency 1.6%) (van der Harst & Verweij, 2018).

Due to the small increase in risk conferred by each individual SNP, their effects are often combined into a genetic score, known as a genetic risk score (GRS) (Assimes & Roberts, 2016). Most commonly, a GRS is calculated as the weighted sum of the number at-risk alleles for a given individual, where the effect size (log OR) for each SNPs is used as weight. Most studies using GRSs for CAD have included only

common SNPs that have reached genome-wide significance in GWA studies (most commonly defined as $P < 5 \times 10^{-8}$). Recent studies have relaxed this threshold and included up to millions of SNPs in order to increase predictive power (Abraham et al., 2016; Khera et al., 2018). Such scores are more commonly referred to as polygenic risk scores (PRSs). Although PRSs generally include a greater number of variants and are usually not restricted to a threshold of genome-wide significance, the terms GRS and PRS are not formally differentiated and are generally interchangeable (Wand et al., 2021). To date, GRSs for CAD have been robustly associated with risk of CAD (in cross-sectional analyses) and incident coronary events (in prospective analyses), largely independent of traditional risk factors and family history (Assimes et al., 2017). Despite these associations, the clinical utility of GRSs in cardiovascular risk prediction remains unclear (Assimes & Roberts, 2016).

By the time **paper I** was published (early 2015), case-control GWA studies had yielded 50 chromosomal loci associated with risk of CAD (Roberts, 2014). By that time, GRSs for CAD had been shown to associate with incident cardiovascular events in prospective studies (Davies et al., 2010; Ganna et al., 2013; Hughes et al., 2012; S. Ripatti et al., 2010; Thanassoulis et al., 2012; Tikkanen et al., 2013; Vaarhorst et al., 2012), as well as other atherosclerotic phenotypes such as peripheral artery disease (Tragante et al., 2013), carotid intima-media thickness (Hamrefors et al., 2012) and CAC (Thanassoulis et al., 2012). Variants within the 9p21 locus and *LPA* had recently been found to not only to associate with risk of CAD but also the extent of coronary atherosclerosis as assessed by coronary angiography (Dandona et al., 2010; Helgadottir et al., 2012; Patel et al., 2010). These findings indicated that risk variants at 9p21 and *LPA* not only predisposed to the onset of CAD but also contributed to a greater atherosclerotic burden in patients with established disease, thus supporting their involvement in the atherosclerotic process. In **paper I**, we extended these findings and assessed the associations of all 50 known risk loci for CAD with the extent of coronary atherosclerosis as assessed by coronary angiography, both individually and when combined into a single GRS.

1.4 Blood lipids and lipoproteins

Over the last 100 years, the study of how circulating lipoproteins and their lipid contents influence the development of atherosclerosis has led to monumental progress in prevention and treatment of cardiovascular disease. Genetic studies have been instrumental on this path, from the identification of the LDL receptor and its gene in 1973 to GWA studies and Mendelian randomization (MR) analyses of the last 15 years. Despite this, we still do not have complete understanding of how lipoproteins contribute to atherosclerosis and how to best quantify that risk.

1.4.1 Lipid molecules: cholesterol and triglycerides

Cholesterol

Cholesterol is one of the most intensively studied molecule in the history of biochemistry and medicine (Goldstein & Brown, 2015). Cholesterol is essential to cells of the human body, where its most important role is to maintain integrity and function of cell membranes (Gurr et al., 2016). In addition, cholesterol provides the raw material for the production of adrenal and gonadal steroid hormones, vitamin D and bile acids. Cholesterol exists in two main forms: unesterified ("free") and esterified. Unesterified cholesterol is amphipathic (with hydrophilic and hydrophobic domains) and is most common in cell membranes, while esterified cholesterol is strongly hydrophobic and is the dominant form when cholesterol is stored or transported in the bloodstream within lipoproteins. In the body, cholesterol homeostasis is a complex process involving the regulation of *de novo* synthesis, absorption of dietary cholesterol and excretion from the body (biliary and intestinal) (Luo et al., 2020).

All mammalian cells are capable of *de novo* cholesterol synthesis from acetyl-CoA, a process that is tightly regulated in response to intracellular cholesterol concentrations (Luo et al., 2020). In fact, most cholesterol in the body is endogenous (50% of which is synthetized in the liver) with a smaller contribution from dietary cholesterol (about 25%) (Luo et al., 2020; Lecerf & de Lorgeril, 2011). Within cells, the main sources of cholesterol are *de novo* biosynthesis and cholesterol derived from circulating LDLs taken up via LDL receptors (Luo et al., 2020).

Lipoproteins provide the means of transportation of cholesterol and other lipids from one tissue to another, as cholesterol and other lipids are insoluble in water. Any measurement of plasma cholesterol reflects the amount of cholesterol found within lipoproteins. As discussed below, plasma cholesterol is commonly classified according to the lipoprotein fraction from which it is measured, e.g. LDL cholesterol.

Triglycerides

Triglycerides, or triacylglycerol, are lipids consisting of three fatty acid molecules that are linked to a glycerol backbone via esters (Gurr et al., 2016). Triglycerides are the main constituent in natural fats and oils. In the body, triglycerides are the major storage form of energy and an important source of free fatty acids for energy production and storage, production of phospholipids and other lipid-derived molecules. Most of dietary fats are in the form of triglycerides (90-95%) with a smaller contribution from cholesterol and other sterols and phospholipids. Triglycerides are synthesized mainly in the small intestine and the liver and are transported to peripheral tissues within lipoproteins, mostly within chylomicrons (from the small intestine) or very low density lipoproteins (VLDLs, from the liver). The triglycerides within these lipoproteins are hydrolyzed within the capillary beds of muscles and adipose tissue, releasing free fatty

acids. The triglyceride-deplete ‘remnant’ particles (of chylomicrons and VLDLs) are then mostly cleared from the circulation by the liver.

1.4.2 Lipoproteins and lipid transport

Lipoproteins are spherical macromolecules that generally consist of an inner hydrophobic core containing triglycerides and cholesteryl esters, surrounded by an outer layer composed of phospholipids, unesterified cholesterol and proteins known as apolipoproteins (Ramasamy, 2014). Apolipoproteins provide stability to the particle and influence its biochemical properties. Apolipoproteins may function as ligands for lipoprotein receptors or cofactors in enzymatic processes. There are several types of apolipoproteins, e.g. apolipoprotein A (apoA) (subtypes A-I, A-II, A-IV and A-V), apoB (subtypes B-48 and B-100), apoC (subtypes C-I, C-II and C-III), apoD and apoE (Gurr et al., 2016). Lipoproteins vary greatly in size, composition and biochemical attributes, but all have the common role of transporting lipids in the bloodstream. An overview of lipoprotein metabolism is shown in **Figure 3**.

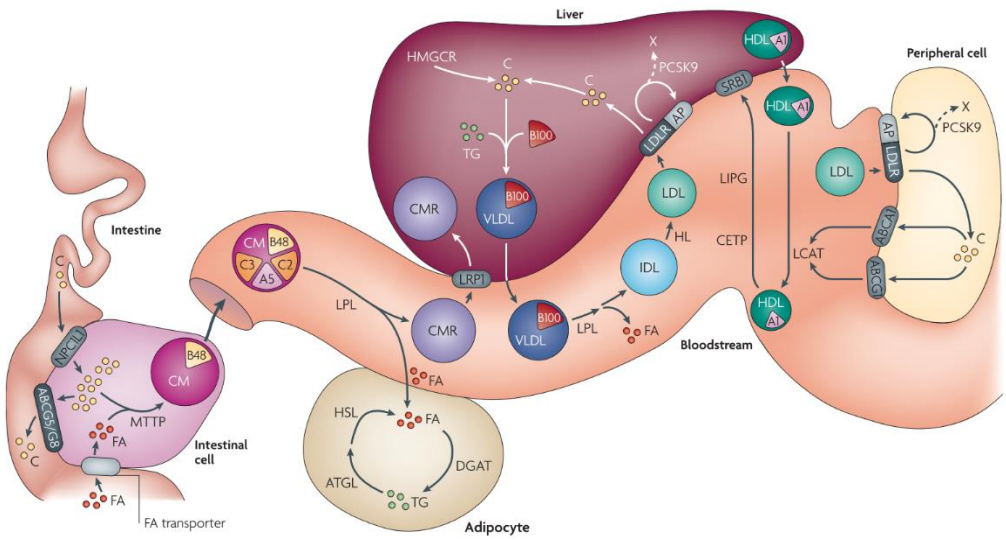

Figure 3. Overview of lipoprotein metabolism

Refer to main text for details. Main abbreviations not introduced in the main text: C, cholesterol; CM, chylomicrons; CMR, chylomicron remnants; FA, fatty acids; TG, triglycerides. Reproduced from Hegele, 2009, with permission (©Springer Nature).

Classes of lipoproteins

Lipoproteins are commonly classified according to their dominant apolipoprotein (either apoB or apoA-I) and their density on analytical ultracentrifugation (Gurr et al., 2016). The apoB lipoproteins include (from lowest to highest density): chylomicrons, VLDLs, intermediate-density lipoproteins (IDLs), LDLs and lipoprotein(a) (Lp(a)). The apoA-I

lipoproteins are high density lipoproteins (HDLs), which have the highest density of all lipoproteins when separated using analytical centrifugation. The composition and properties of the major lipoprotein classes are shown in **Table 1**. In general, the ratio of triglycerides to protein is the highest in the chylomicrons and falls with increasing density. Among the apoB lipoproteins, the cholesterol content rises with increasing density and is the highest in LDL, where cholesterol accounts for about half of particle mass. It is important to keep in mind that each lipoprotein class represents a spectrum of particles that vary in size and composition.

Table 1. Composition and characteristics of the major plasma lipoproteins

	Chylomicrons	VLDL	LDL	HDL
Protein (%)	2	7	20	50
Triglycerides (%)	83	50	10	8
Cholesterol (%)	8	22	48	20
Phospholipids (%)	7	20	22	22
Particle mass (Daltons)	$100\text{-}1000\times10^6$	$10\text{-}100\times10^6$	$2\text{-}3.5\times10^6$	$1.75\text{-}3.6\times10^5$
Density range (g/ml)	<0.95	0.95-1.006	1.019-1.063	1.063-1.210
Diameter (nm)	80-1000	30-90	18-22	5-12
Apolipoproteins	A-I, A-II, A-IV, (A-V), B-48, C-I, C-II, C-III, E	(A-V), B-100, C-I, C-II, C-III, E	B-100	A-I, A-II, A-IV, (A-V), C-I, C-II, C-III, D, E

Not included: IDL and Lp(a).

Adapted from Lipids: Biochemistry, Biotechnology and Health (6th ed., Wiley Blackwell) (Gurr et al., 2016).

ApoB lipoproteins

ApoB is unique among the apolipoproteins in that it does not circulate in a lipid-free form and remains tightly bound to its lipoprotein throughout its lifetime (one molecule of apoB per particle) (Gurr et al., 2016). Other apolipoproteins such as apoA and apoE, in contrast, are soluble and may exchange between lipoprotein particles. ApoB lipoproteins are secreted by the liver or small intestine. Chylomicrons from the small intestine carry the apoB-48 isoform (so named because it contains 48% of the complete amino acid sequence of apoB-100) and VLDLs from the liver carry the apoB-100 isoform (Ramasamy, 2014). Naturally, the metabolic derivatives of VLDL such as VLDL remnants, IDL and LDL carry also a single molecule of apoB-100. ApoB-100 has a half-life in the circulation of about 3 days and 90-95% is found in LDLs (Ramasamy, 2014). An overview of the major apoB lipoproteins is shown in **Figure 4**.

All apoB lipoproteins are considered to be atherogenic (Borén & Williams, 2016). With the exception of chylomicrons and very large VLDLs, apoB lipoproteins are

generally smaller than 70 nm in diameter and can readily cross the endothelium (Borén et al., 2020). Various factors modulate the propensity of apoB lipoproteins to cross into the arterial intima, to undergo modification and become retained and thus drive the atherosclerotic process (Borén et al., 2020). The cholesterol-rich LDL particles account for the vast majority of circulating atherogenic apoB lipoproteins.

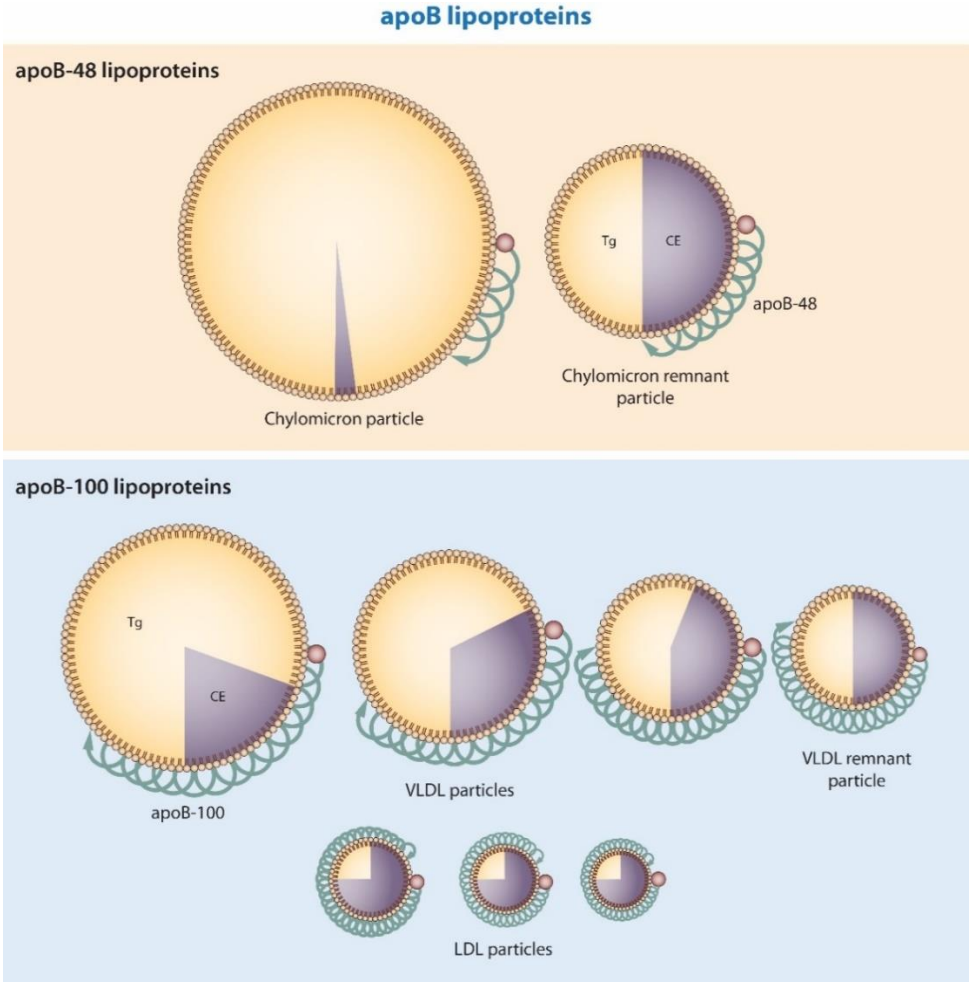

Figure 4. Overview of apoB lipoproteins

The relative content of triglycerides (Tg) and cholesteryl esters (CE) is shown. Lipoprotein(a) is not depicted. Adapted and modified from Sniderman et al., 2010, with permission (©Springer Nature).

Chylomicrons – Chylomicrons are the largest and least dense of the lipoproteins, with a very high triglyceride content (close to 80% of particle mass) (Gurr et al., 2016). Chylomicrons provide the main route for the transport of dietary lipids (90-95% triglycerides) from the small intestine to the liver. Chylomicrons are assembled in epithelial cells of the small intestine and secreted into circulation via the lymphatic

system. They are assembled around a single molecule of apoB-48, which is unique to chylomicrons. In addition to apoB-48, chylomicrons carry various other apolipoprotein classes, e.g. apoA (I, II, IV, V), apoC (I, II, III) and apoE (Gurr et al., 2016).

In the circulation, the triglyceride content of chylomicrons is rapidly hydrolyzed by the endothelial-bound enzyme lipoprotein lipase (LPL) with apoC-II serving as an essential co-factor, thereby releasing free fatty acids to muscle and adipose tissue (Gurr et al., 2016). This is accompanied by transfer of free cholesterol, phospholipids, apoC-II and apoC-III from chylomicrons to HDL. These changes result in reduction in size, producing remnant particles. The chylomicron remnants are relatively depleted of triglycerides and enriched in cholesteryl esters. The remnants are rapidly removed from the circulation by hepatocytes, partly by the interaction of apoE with LDL receptors, VLDL receptors and LDL receptor-related proteins (LRPs) (Ramasamy, 2014). Chylomicrons are short-lived in the circulation and are usually cleared from the circulation in a matter of few hours.

Very low density lipoprotein (VLDL) – In the liver, endogenous and dietary-derived lipids are packaged and secreted into the circulation as VLDL (Gurr et al., 2016). VLDL is assembled around a single molecule of apoB-100, which wraps around the surface of the particle. In addition to apoB-100, VLDLs contains apoC-I, C-II, C-III and apoE in variable amounts (Ramasamy, 2014). Similar to chylomicrons, VLDL is rich in triglycerides and its assembly requires adequate loading of triglycerides, a process involving microsomal triglyceride transfer protein (MTP) (Ramasamy, 2014). Within the circulation, like chylomicrons, VLDLs undergo successive interactions with LPL resulting in depletion of its triglyceride content, yielding sequentially smaller particles with lower triglyceride content. At the same time, they become enriched in cholesteryl esters due to the action of cholesteryl ester transfer protein (CETP) (Ramasamy, 2014). In this way, VLDLs are transformed from large triglyceride-rich particles to the more cholesterol-rich and smaller VLDL remnant particles. Some VLDL remnants are cleared directly from the circulation by the liver through apoB-100 and apoE-mediated interaction with LDL receptors, VLDL receptors and LRPs, while others undergo further transformation to become IDLs and ultimately, LDLs (Ruiz et al., 2005). This process is rapid, with a half-life of circulating VLDLs being about 5 hours (Pownall et al., 2016).

Intermediate density lipoprotein (IDL) – In essence, IDL is a VLDL remnant that has lost most of its triglycerides content and apolipoproteins and is enriched with HDL-derived apoE (Gurr et al., 2016). IDLs are short-lived (half-life of about 1-2 hours) and mostly cleared from the circulation by the liver, with less than half going on to transform into LDL (with further hydrolysis and eventual loss of apoE) (Ramasamy, 2014).

Low density lipoprotein (LDL) – All circulating LDL is metabolically derived from VLDL produced by the liver. LDL particles are cholesterol-rich, with cholesteryl esters comprising about half of their mass, and they generally carry about two-thirds of the

total cholesterol in plasma (Gurr et al., 2016). LDL is by far the most common apoB lipoprotein, containing 90-95% of plasma apoB (Gurr et al., 2016). This is mainly due to their relatively long half-life of 2-3 days, as compared to the short-lived VLDLs and IDLs. As apoE is lost during transformation of IDL to LDL and apoB-100 thus remains as the only apolipoprotein, the rate of clearance from the circulation is decreased (apoE binds to the LDL receptor with higher affinity than apoB-100) (Ruiz et al., 2005). Uptake of LDL is mediated primarily by LDL receptors, a process closely regulated to maintain cellular cholesterol homeostasis (Luo et al., 2020). Most tissues express LDL receptors. However, the majority of LDL is cleared from the circulation by the liver where excess cholesterol is excreted via bile (Gurr et al., 2016). Genetic defects in the LDL receptor gene (*LDLR*) cause familial hypercholesterolemia (see chapter 1.6). Like other lipoproteins, LDL comprises a spectrum of particles varying in size and density. LDL particles can be subdivided according to their density on ultracentrifugation, e.g. into large buoyant LDLs (density 1.019-1.044 g/ml) and small dense LDLs (density 1.044-1.063 g/ml) (Gurr et al., 2016).

Lipoprotein(a) – Although not considered a separate density class, lipoprotein(a) or Lp(a) deserves special mention due to its role in atherogenesis (Kronenberg & Utermann, 2013). Lp(a) is an LDL-like lipoprotein that is characterized by an additional apolipoprotein, apoprotein(a), that is covalently bound to apoB-100. It has a density that is intermediate between small dense LDL and HDL_2. Levels of Lp(a) are determined by genetic variation in the lipoprotein(a) gene (*LPA*) gene and remain constant throughout life (Gudbjartsson et al., 2019; Kronenberg & Utermann, 2013). Among the apoB lipoproteins, Lp(a) is particularly atherogenic, a property that is likely related to its additional cargo of proinflammatory oxidized phospholipids and prothrombotic properties (Gudbjartsson et al., 2019; Tsimikas et al., 2018).

ApoA-I lipoproteins

High density lipoprotein (HDL) – Unlike apoB lipoproteins, HDLs are not secreted directly by the liver. Rather, HDL particles are assembled within the circulation from its basic components, apoA-I and phospholipids (Gurr et al., 2016). By particle count, HDL is by far the commonest lipoprotein and more common than all others combined. HDLs can be further subdivided according to density, e.g. into HDL_2 (density 1.063-1.125 g/ml) and the smaller HDL_3 (density 1.125-1.21 g/ml).

HDL has a key role in trafficking cholesterol from peripheral tissues to the liver and other organs, a process commonly known as *reverse cholesterol transport* (Ouimet et al., 2019). Here, this process is simplified into 4 steps: (i) the immature and lipid-poor HDL particle acquires unesterified cholesterol and phospholipids through interaction with cell membranes in tissues (most often occurring in the liver or small intestine), largely by the action of ATP-binding cassette transporter member 1 (ABCA1); (ii) the HDL particle swells and grows larger as it acquires more cholesterol; (iii) the now cholesterol-rich HDL particle unloads its cholesterol content in two principal ways: (a)

through interaction with cells in the liver, small intestine and steroidogenic tissues such as the adrenal gland and the ovaries; or (b) through interaction with triglyceride-rich lipoproteins (e.g. chylomicrons and VLDLs), where cholesteryl esters from the HDL particle are exchanged for triglycerides by the action of CETP; (iv) the now lipid-deplete HDL particle remains in the circulation, and the cycle is repeated. It must be noted that the process of cholesterol efflux from cells and reverse cholesterol transport is immensely more complex than outlined here and involves many other enzymes, receptors and transfer proteins (reviewed by Ouimet et al., 2019).

1.4.3 Commonly measured lipid traits – the standard lipid profile

Blood lipids are routinely assessed in clinical practice and play an important part in cardiovascular risk stratification (Langlois et al., 2020). Most commonly, a standard lipid panel includes measurements of total cholesterol, HDL cholesterol and triglycerides, from which non-HDL cholesterol is calculated and LDL cholesterol is estimated (most commonly using the Friedewald equation). Here, I will briefly review these lipid measures, how they relate to lipoproteins and their relationship with cardiovascular risk.

Total serum cholesterol – Total cholesterol is a measure of all cholesterol found within the plasma lipoproteins. The largest contribution is from LDLs (about 2/3) with smaller contributions from HDLs (about 1/4) and triglyceride-rich lipoproteins (chylomicrons, VLDLs and their remnants), IDLs and Lp(a). Due to their short-lived postprandial nature and low cholesterol content, the contribution of chylomicrons to total cholesterol is most often negligible (Gurr et al., 2016).

In epidemiological studies, ever since the first results of the Framingham study in the 1960s (Kannel et al., 1961), elevated total cholesterol has proved a robust marker of higher cardiovascular risk. Because total cholesterol also contains HDL cholesterol, which was found to have a robust and *inverse* relationship with cardiovascular risk in the 1960s (Gofman et al., 1966; Gordon et al., 1977), subsequent research into cholesterol and its role in cardiovascular disease has focused not on levels of total cholesterol but rather its individual components, most prominently HDL cholesterol, LDL cholesterol and non-HDL cholesterol.

HDL cholesterol – HDL cholesterol is a measure of the cholesterol content of HDL particles. In the 1960s and 1970s, higher levels of HDL particles (measured by ultracentrifugation) and levels of HDL cholesterol were found to have a robust and *inverse* relationship with cardiovascular risk (Gofman et al., 1966; Gordon et al., 1977). Ever since, low HDL cholesterol has proved to be a consistent marker of increased cardiovascular risk (Gordon et al., 1988). Due to the strong epidemiologic observations and the recognized role in mediating cholesterol efflux and reverse cholesterol transport, HDL cholesterol was thought to be protective and anti-atherogenic (Ouimet et al., 2019). However, intervention trials involving agents that raise HDL cholesterol

have generally failed to show benefit (Rader & Hovingh, 2014) and large genetic studies have not supported the notion that HDL cholesterol may causally modulate the risk of cardiovascular disease (Do et al., 2013; Helgadottir et al., 2016, 2018; Voight et al., 2012). Although somewhat unclear, the association of reduced HDL cholesterol with lower incidence of cardiovascular disease may be related to its association with elevated levels of apoB lipoproteins and non-HDL cholesterol (Rosenson et al., 2018). In addition, HDL cholesterol is a poor marker of HDL function and its role in cholesterol efflux and reverse cholesterol transport from arterial walls (Rohatgi et al., 2014). Although HDL cholesterol itself is not causative factor, the potentially protective role of HDLs and reverse cholesterol transport in the development of atherosclerosis remains an active field of study (Ouimet et al., 2019).

Triglycerides – Plasma triglycerides are mostly found within the triglyceride-rich lipoproteins: chylomicrons and their remnants and VLDLs and their remnants. As outlined above, triglycerides are important source of energy and their transportation within the circulation vital. Elevated levels of triglycerides are well-known to correlate with increased cardiovascular risk and are commonly observed in the metabolic syndrome, insulin resistance and diabetes (Nordestgaard & Varbo, 2014). This is explained by the fact that triglyceride levels reflect levels of triglyceride-rich lipoproteins, whose atherogenicity is chiefly due to their content of apoB and cholesterol (Nordestgaard, 2016). Plasma triglycerides *per se* are not thought to have major role in atherosclerosis and trials of triglyceride-lowering therapies have produced variable results (Sandesara et al., 2018). However, certain triglyceride-related mechanisms may play some role in the pathogenesis of coronary atherosclerosis (Borén et al., 2020).

LDL cholesterol – LDL cholesterol is the cholesterol carried within circulating LDLs, representing about two thirds of plasma total cholesterol. Seminal observations in the 1950s and 1960s identified elevated LDL, as measured by ultracentrifugation, as a risk factor for coronary disease (Gofman et al., 1954, 1966). In the 1970s, the development of the Friedewald equation enabled LDL cholesterol to be estimated from the routinely measured lipid traits: total cholesterol, HDL cholesterol and triglycerides. The Friedewald equation is: LDL cholesterol = total cholesterol – HDL cholesterol – triglycerides/2.2 (to estimate VLDL cholesterol), all units in mmol/L (Friedewald et al., 1972). Calculated LDL cholesterol has been repeatedly proven to be one of the strongest lipid markers of cardiovascular risk and remains the most common method to assess LDL cholesterol (Ference et al., 2017). Calculated LDL cholesterol also estimates cholesterol carried by IDLs and Lp(a). In the setting of high serum triglycerides or very low levels of LDL, the Friedewald equation underestimates LDL cholesterol and a direct measurement of LDL cholesterol (using chemical-based methods) is more accurate (Martin et al., 2013). Multiple lines of evidence, including consistent findings from genetic studies and interventional trials of LDL-lowering

medications (e.g. statins, ezetimibe and PCSK9 inhibitors) have firmly established elevated plasma LDL as a causal driver of atherosclerosis (Ference et al., 2017).

Non-HDL cholesterol – Non-HDL cholesterol is defined as all cholesterol that is not contained within HDL. That is, total cholesterol minus HDL cholesterol. In other words, non-HDL cholesterol is the sum of all cholesterol carried by the apoB lipoproteins (Miller et al., 2008). Normally, the vast majority of non-HDL cholesterol is found within LDLs. In certain conditions, however, such as in the setting of hypertriglyceridemia, insulin resistance and diabetes; a substantial contribution may come from VLDLs and their remnants (which is not reflected in calculated or directly measured LDL cholesterol) (Ramasamy, 2014). Accumulating evidence indicates that all apoB lipoproteins are atherogenic and central to the development and progression of atherosclerosis (Borén & Williams, 2016). Markers that reflect the entire spectrum of apoB lipoproteins, such as levels of non-HDL cholesterol and apoB, have proven superior to LDL cholesterol alone as a marker of cardiovascular risk (Arsenault et al., 2009; Boekholdt et al., 2012; Pischon et al., 2005; Sniderman et al., 2011; The Emerging Risk Factors Collaboration, 2009).

1.5 Common genetic variation and blood lipids

In the last 15 years, GWA studies have revealed numerous biological insights into the regulation of lipid levels. Before the GWA era, genetic studies of dyslipidemias were mostly limited to small case-control or cohort studies, relying on classical linkage analysis and candidate gene approach (Hegele, 2009). Many lipid traits were suspected to have a polygenic basis, being influenced by a number of common genetic variants but the details of their genetic makeup were very limited (Hegele, 2009). The first wave of GWA studies of lipid levels came in 2008 (Kathiresan et al., 2008; Sandhu et al., 2008; Willer et al., 2008). Using SNP microarrays that enabled detection of up to 500,000 SNPs in a tens of thousands of participants, these initial studies identified common SNPs (allele frequency typically >5%) that associated with lipid levels at 18 genomic loci. Twelve of these loci were located in proximity to genes with a known role in lipid regulation (*ABCA1*, the *APOA5-APOA4-APOC3-APOA1* and *APOE-APOC* clusters, *APOB*, *CETP*, *GCKR*, *HMGCR*, *LDLR*, *LPL*, *LIPC*, *LIPG* and *PCSK9*) while six loci pointed to the involvement of genes with no previous known role in lipid metabolism (*CELSR2*/*PSCR1*/*SORT1*, *CILP2*/*PBX4*, *GALNT2*, *TBL2*/*MLXIPL*, *TRIB1*, *ANGPTL3*) (Kathiresan et al., 2008). Since then, with the formation of ever larger consortiums (most notably the Global Lipids Genetics Consortium) and advances in genotyping and imputation methods, the number of loci associated with lipid levels has steadily increased (Liu et al., 2017; Teslovich et al., 2010; Willer et al., 2013). Most recently, published in 2018, a GWA study involving data from over 600,000 individuals has brought the total number of lipid-associated loci up to 306 (Klarin et al., 2018).

1.5.1 Genetic scores for lipid traits

The vast majority of SNPs identified in GWA studies of lipid traits to date have only small to moderate effect sizes (generally below 0.3 standard deviations per allele) (Klarin et al., 2018). When assessed individually, each SNP accounts for very little of the phenotypic variance within the population. In aggregate, however, they may explain 8.8-12.3% of the phenotypic variance in lipid levels (Klarin et al., 2018). A common method to assess the aggregate effects of many SNPs is to combine their genotypes into a single score analogous to a genetic risk score (GRS) for CAD (see chapter 1.3). Instead of the term GRS, which implies a binary outcome (e.g. CAD), the more general term “genetic score” is often used for non-binary traits such as lipid levels (Wand et al., 2021). Here, the term “genetic score” will be used for that purpose. For each individual, a genetic score is calculated as the weighted sum of the number of SNP alleles, where the effect size for each SNPs (in standard deviations) is used as weight. Genetic scores constructed in this way have been shown to be powerful predictors of lipid levels (Helgadottir et al., 2016; Kathiresan et al., 2008; Teslovich et al., 2010). Genetic scores may also be constructed from much larger sets of SNPs that have not necessarily been previously associated with the phenotype of interest, often several millions of SNPs. These are generally called polygenic risk scores (PRS, see chapter 1.3) and have been assessed in the context of lipid traits (Natarajan et al., 2018; Ripatti et al., 2020), but further discussion is beyond the scope of this work. One potential use of genetic scores for lipid traits is for assessment of causality in MR analyses (Burgess & Thompson, 2013).

1.5.2 Mendelian randomization (MR) for lipid traits

In MR, genetic information is used to assess causal relationships between an exposure and outcome in observational data (Ebrahim & Davey Smith, 2008). For example, a MR study may aim to assess whether iron overload (exposure) causally influences the risk of liver cancer (outcome). This could be performed by selecting a genetic variant that has a robust association with hemochromatosis (hereditary iron overload) and using that variant as a proxy for iron overload. An association between the variant (known as an instrumental variable) and risk of liver cancer in observational data would then suggest a causal role of iron overload, if the assumptions of MR are met. Valid inferences in MR rely on three main assumptions (Sheehan et al., 2008):

1. The instrumental variable must have a reliable association with the exposure, ideally through a single, known biological pathway;

2. The instrumental variable must not associate with confounders that may influence the relationship between the exposure and the outcome;

3. The instrumental variable must not have an association with the outcome that is independent of the exposure.

Ideally, each assumption should be met for a valid inference of causality. In practice, however, the instrumental variable assumptions are often violated, commonly due to pleiotropic associations of the genetic variants used as instrumental variables (Bowden et al., 2015; Burgess & Thompson, 2015; Slob & Burgess, 2020). Importantly, several robust methods have been developed to reach valid inferences in MR analyses even when the instrumental variable assumptions are violated (Bowden et al., 2015; Burgess & Thompson, 2015; Slob & Burgess, 2020)

MR studies have assessed the causality of blood lipids in the development of coronary disease by using lipid-associated genetic variants as instrumental variables (Holmes et al., 2017). MR analyses of lipid traits are complicated by the fact that most lipid-associated SNPs have considerable pleiotropic effects. That is, most influence more than one lipid trait (and potentially other traits as well). Therefore, in order to assess causality of a given lipid trait through the analysis of many lipid-associated SNPs, one must carefully account for the effects on other lipid traits (Burgess & Thompson, 2015). Using genetic scores, one may account for the effects of pleiotropy by adjusting the association of a genetic score for one lipid trait by accounting for genetic scores for other lipid traits, as demonstrated previously by our group (Helgadottir et al., 2016)

MR studies of lipid traits have consistently highlighted that apoB lipoproteins and its main components, LDL and the triglyceride-rich lipoproteins are causally related to risk of coronary disease (Crosby et al., 2014; Dewey et al., 2016; Do et al., 2013, 2015; Helgadottir et al., 2016; Holmes et al., 2014; Jørgensen et al., 2014; Khera et al., 2017; Myocardial Infarction Genetics and CARDIoGRAM Exome Consortia Investigators et al., 2016; Richardson et al., 2020). In contrast, MR studies have consistently showed that HDL cholesterol is not likely causally related to risk of coronary disease (Do et al., 2013; Helgadottir et al., 2016, 2018; Voight et al., 2012).

1.5.3 Causal relationship of non-HDL cholesterol with CAD risk

In a study published in 2016, our group performed MR analyses to assess the causality of commonly measured lipid traits with respect to CAD risk in the Icelandic population (Helgadottir et al., 2016). The study used genetic scores constructed from 212 SNPs (rare and common) as instrumental variables, one genetic score for each lipid trait. Within each genetic score, each SNP was weighted according to its effect size for the given lipid trait in the Icelandic population (in up to 119,000 genotyped individuals). When assessed individually, the genetic scores for non-HDL cholesterol, LDL cholesterol, triglycerides and HDL cholesterol were all associated with CAD risk (among 15,575 CAD cases and 88,556 controls). In joint analyses, however, the genetic score for non-HDL cholesterol was found to fully capture the associations of the other genetic scores. The association for the non-HDL cholesterol genetic score remained significantly associated with CAD risk after adjustment for the LDL cholesterol genetic score. Thus, these findings highlighted non-HDL cholesterol as a

causal risk factor for CAD, providing predictive information over that of LDL cholesterol. This is in line with multiple lines of evidence showing that all apoB lipoproteins are atherogenic, not just LDL (Borén et al., 2020). Among the commonly measured lipid traits, non-HDL cholesterol is thus the best marker of the cardiovascular risk associated with atherogenic apoB lipoproteins.

1.5.4 Blood lipids and the extent of coronary atherosclerosis

Prior to the publication of **paper II** in 2020, two studies (Tsao et al., 2012; van Setten et al., 2015) had tested genetic scores for lipid levels for association with extent of coronary atherosclerosis as estimated by CAC. A genetic score for LDL cholesterol was associated with higher CAC in one study (van Setten et al., 2015) but not the other (Tsao et al., 2012). In addition, one of the studies assessed genetic scores for HDL cholesterol and triglyceride levels but did not find significant associations with CAC (van Setten et al., 2015). In **paper II** we performed MR analyses to assess the causal influence of lipid traits on the extent and severity of coronary atherosclerosis as estimated by coronary angiography and CAC.

1.6 Monogenic disorders of LDL metabolism

In this section, we depart from polygenic influences of blood lipids and focus on two autosomal dominant genetic disorders with a strong and opposite impact on LDL levels: familial hypercholesterolemia (FH) and familial hypobetalipoproteinemia (FHBL). **Paper III** is dedicated to a comprehensive characterization of FH in Iceland, while **paper IV** describes a novel deletion in *LDLR* that causes a FHBL-like phenotype.

1.6.1 Familial hypercholesterolemia

FH is one of the most common monogenic disorders, and is characterized by markedly elevated levels of LDL cholesterol from childhood, leading to premature cardiovascular disease and death (Defesche et al., 2017). Despite receiving considerable attention in recent years, FH remains underdiagnosed and undertreated in most countries (Nordestgaard et al., 2013).

FH was first described by the Norwegian physician Carl Müller in 1938. He described a hereditary condition characterized by frequent co-occurrence of cholesterol-rich deposits in tendons and skin, severe hypercholesterolemia, coronary disease and sudden death (Müller, 1938, 1939). Through his careful study of affected families, Müller correctly assumed the condition was transmitted in an autosomal dominant manner. In the 1950s and 1960s it became known that the source of excess plasma cholesterol in FH was elevated levels of LDL (Goldstein & Brown, 2009). In the 1970s and 1980s, through their work on FH, Goldstein and Brown discovered the LDL receptor and elucidated the LDL receptor pathway as a key component in cellular cholesterol homeostasis (Brown & Goldstein, 1986). They demonstrated that FH is caused by a defect in the LDL receptor, resulting in impaired clearance of circulating LDLs by the liver and thus higher plasma levels of LDL cholesterol (Brown & Goldstein,

1986). In 1987, Grundy and colleagues identified a second gene involved in FH, the gene for apolipoprotein B (*APOB*) (Innerarity et al., 1987). And in 2003, the third FH gene was discovered, the gene for proprotein convertase subtilisin/kexin type 9 (*PCSK9*) (Abifadel et al., 2003).

To date, mutations in three major genes are known to cause FH: *LDLR*, *APOB* and *PCSK9*. Over 2000 pathogenic mutations have been described in *LDLR* and are thought to explain about 80-85% of cases of autosomal dominant FH (Berberich & Hegele, 2019). Rarer causes of FH include mutations in the apolipoprotein E gene (*APOE*) and the extremely rare recessive forms due to mutations in the genes for LDL receptor adaptor protein 1 (*LDLRAP1*), adenosine triphosphate-binding cassette transporters G5 and G8 (*ABCG5* and *ABCG8*) and lysosomal acid lipase (*LIPA*) (Defesche et al., 2017). Apart from the rare recessive forms, FH is an autosomal dominant, monogenic disease. This means that having single mutation allele (i.e. heterozygous state) is generally enough to produce the phenotype. The penetrance of FH mutations is generally high, although they may exhibit some phenotypic variance (Defesche et al., 2017). Homozygous state in autosomal dominant FH is an extremely rare and severe form of FH and is outside the scope of this introduction (Sjouke et al., 2015).

In 1973, Goldstein et. al estimated the population prevalence of heterozygous FH to lie somewhere between 1 in 1000 (0.1%) and 1 in 500 (0.2%), based their meticulous study of survivors of myocardial infarctions and their families in Seattle, Washington (Goldstein et al., 1973). Other studies in the 1970s, using mathematical estimates based on prevalence homozygous FH or cholesterol screening in infants, pointed to a prevalence in the range of 1 in 500 to 1 in 200 (Carter et al., 1971; Leonard et al., 1976; Tsang et al., 1974). In the following years, the estimate of 1 in 500 became widely acknowledged as the most likely prevalence of heterozygous FH in most populations (Goldstein & Brown, 1979; Goldstein et al, 2001).

In recent years, genotyping of large population samples has enabled researchers to estimate the population prevalence of heterozygous FH based on the frequency of FH mutations, irrespective of the phenotype. Several such studies in European and North American populations have indicated that the prevalence of FH mutations may be higher than the traditional estimate 1 in 500, potentially as high as 1 in 250 to 1 in 200 (Abul-Husn et al., 2016; Benn et al., 2016; Grzymski et al., 2020; Khera et al., 2016; Trinder et al., 2020).

A unifying mechanism underlying the pathogenesis of FH is severely reduced LDL receptor-mediated clearance of LDL from the circulation, leading to elevated plasma levels of LDL (Defesche et al., 2017) (summarized in **Figure 5**). FH mutations in *LDLR* most commonly cause an amino acid change (missense mutations) that impairs the function of LDL receptors, e.g. through impaired intracellular transport, impaired binding to LDL, impaired clustering of LDL receptors in clathrin-coated pits or impaired recycling of the LDL receptor up to the cellular surface after endocytosis (Brown & Goldstein, 1986). Mutations in *LDLR* may also cause complete failure of receptor synthesis (loss-of-function or nonsense mutations). Mutations in *APOB* occur in the

LDL receptor binding domain and impair binding of apoB-100 (and thus LDL) to the LDL receptor, thereby decreasing LDL clearance. Mutations in *PCSK9* are gain-of-function mutations that lead to increased activity of the enzyme PCSK9, causing increased catabolism of LDL receptors and thus reduced clearance of LDL from the circulation.

The principal clinical characteristics of heterozygous FH are: (i) severely elevated levels of LDL cholesterol (generally above 5 mmol/L) from early age, generally with normal levels of triglycerides and HDL cholesterol; (ii) premature CAD (4- to 5-fold increased risk of premature CAD); (iii) family history of hypercholesterolemia, premature CAD and sudden death; and (iv) tendon xanthomata and xanthelasma (late presentation, rarely seen today) (Defesche et al., 2017).

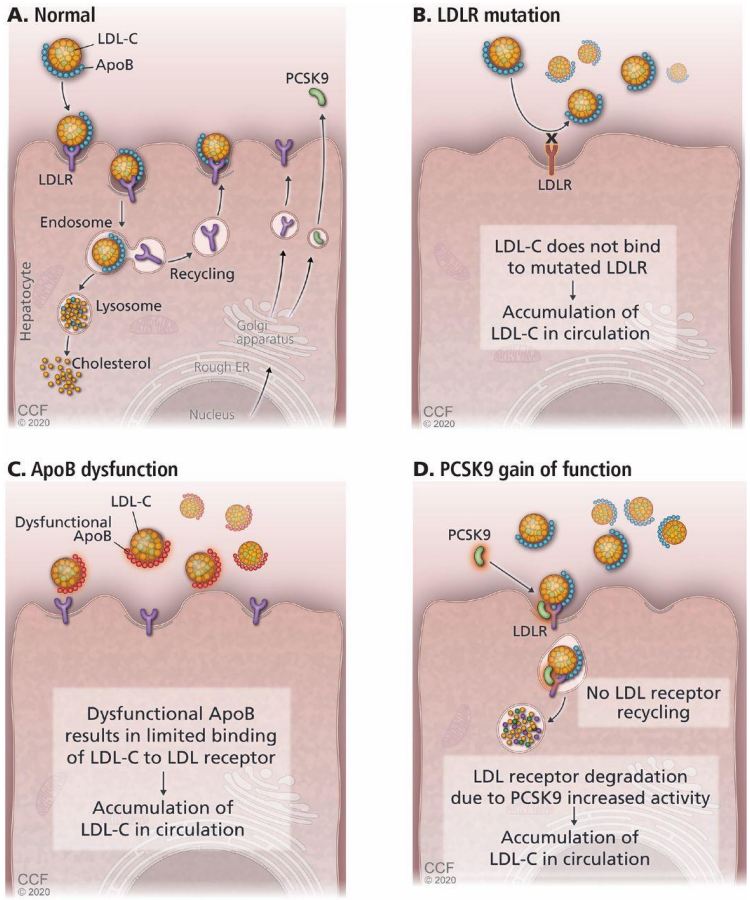

Figure 5. Molecular causes of monogenic familial hypercholesterolemia

Reproduced from Shah et al., 2020, with permission (©The Cleveland Clinic Foundation).

Several scoring systems have been developed to estimate the probability of heterozygous FH based on clinical characteristics, often also taking into account genetic information when available (McGowan et al., 2019). Among them, the Dutch

Lipid Clinic Network (DLCN) criteria are most commonly used (described below). These scoring systems are often used to diagnose FH in the clinical setting and confirmation by genetic testing, although recommended, is not required for diagnosis (Sturm et al., 2018). Upon genetic testing, over half of individuals with clinically diagnosed FH may not carry a causative mutation (Mariano et al., 2020; Talmud et al., 2013; J. Wang et al., 2016). This means that, in general, a clinical diagnosis of FH does not equate to having a single causative mutation. In fact, many of those who have clinically diagnosed FH are likely to have polygenic basis for their condition, rather than a single causative mutation (Mariano et al., 2020; Talmud et al., 2013; J. Wang et al., 2016).

In clinical practice and in scientific literature, the term 'FH' may refer to a phenotype, genotype or both (Fahed et al., 2011). Hereafter, in order to avoid confusion, I will use the term 'monogenic FH' to refer explicitly to a genetic definition of FH (i.e. a causative mutation is present), and 'clinically defined FH' or 'clinical FH' to refer to the phenotypic diagnosis of FH (irrespective of underlying cause).

Recent studies on monogenic FH

In recent years, several large studies have applied a genetic approach to estimate a population prevalence and the phenotypic consequences of monogenic FH (Abul-Husn et al., 2016; Benn et al., 2016; Grzymski et al., 2020; Khera et al., 2016; Trinder et al., 2020). This approach entails genotyping (including rare variants) of a large population sample and defining a group of individuals considered to have monogenic FH based on their genotype. Most commonly, variants are defined as causative of FH based on previous reports of pathogenicity (e.g. in the ClinVar database (Landrum et al., 2016)), predicted loss-of-function effects in *LDLR* (e.g. exonic deletions, frameshift, stop-gain or essential splice site mutations) and *in-silico* prediction of pathogenicity of missense mutations based on their expected impact on protein function (using various algorithms such as PolyPhen-2, SIFT, MutationTaster and PROVEAN; reviewed by Ghosh et al., 2017). Although the American College of Medical Genetics and Genomics (ACMG) has released guidelines on the classification and interpretation of potentially pathological sequence variants (Richards et al., 2015), there is no consensus on how FH mutations should be defined in large-scale screening studies.

In Denmark, among 98,097 participants in the Copenhagen General Population Study, the overall prevalence of monogenic FH was estimated 1 in 217 (Benn et al., 2016). In this study, only 4 of the most common FH mutations in Denmark were genotyped directly and their combined prevalence (1 in 564) used to estimate the overall prevalence of monogenic FH based on their yield in clinical genetic services (the 4 mutations accounted for 38.7% of identified FH mutations, thus the estimate: (1/564)/0.387 = 1/217. Within the Geisinger Health System in Pennsylvania, United States, 50,726 individuals underwent whole-exome sequencing and the overall prevalence of monogenic FH was estimated at 1 in 222 (Abul-Husn et al., 2016). In another study of 20,485 multiethnic participants in 12 clinical studies, who all underwent whole-exome sequencing, the combined estimated prevalence of monogenic FH was 1 in 211 (Khera et al., 2016). In the Healthy Nevada Project in

Nevada, United States, among 26,906 whole-exome sequenced participants, 1 in 260 was found to carry an FH mutation (Grzymski et al., 2020). In the UK Biobank, among 48,741 whole-exome sequenced participants, 1 in 176 was found to carry a predicted FH mutation (Trinder et al., 2020). Taken together, these studies indicate that the prevalence of monogenic FH in various, mostly white/European populations, may be twice as common than the traditional estimate of 1 in 500.

Estimates of monogenic FH prevalence are naturally dependent on which mutations are deemed causative of FH. On one hand, estimates will be inflated if non-pathogenic mutations are considered causative, and on the other hand, failure to include truly pathogenic mutations will lead to underestimation of the prevalence of monogenic FH. Below is an illustrative example of prevalence overestimation in two of the aforementioned studies due to the inclusion of a non-pathogenic variant as a causative mutation:

The missense mutation Arg3558Cys in *APOB* was the second most common FH mutation identified in the Geisinger Health System study (Abul-Husn et al., 2016), and the most common in a study of 20,485 multiethnic participants (Khera et al., 2016). In these two studies, Arg3558Cys accounted for approximately 20% and 10% of cases of monogenic FH, respectively. Although this mutation was annotated as “Pathogenic” in ClinVar at the time, it has now been shown that Arg3558Cys is not associated with an increase in LDL cholesterol that is compatible with an FH mutation (adjusted increase 0.26 mmol/l [$P = 6.3 \times 10^{-7}$] based on 366 carriers among 331,107 participants in the Million Veteran Program (Sun et al., 2018)). This mild effect was also observed in the Geisinger Health System study, where carriers of Arg3558Cys had only mildly elevated levels of LDL cholesterol (mean, 4.0 mmol/l vs. 3.4 mmol/l in non-carriers) (Abul-Husn et al., 2016). Also consistent with non-pathogenicity, Arg3558Cys is now annotated in ClinVar as a variant of “uncertain significance” based on 12 submissions (ClinVar, accessed May 10, 2021). Thus, the prevalence estimates in the two studies were inflated due to the inclusion of Arg3558Cys. In the Geisinger Health System study data, the exclusion of Arg3558Cys yields a prevalence estimate of 1 in 277 instead of 1 in 222.

The above example demonstrates how the prevalence of monogenic FH may be overestimated by the erroneous inclusion of a non-pathogenic variant. This example highlights the importance of using high-quality annotations of pathogenicity (preferably from multiple sources) and how lipid associations in a sufficiently large sample can help ‘rule in’ or ‘rule out’ a variant as a pathogenic FH mutation.

Prevalence of clinically defined FH

Three large studies have estimated the prevalence of the clinical phenotype of FH using the DLCN criteria (Abul-Husn et al., 2016; Benn et al., 2016; De Ferranti et al., 2016). In these studies, genotype information was excluded from the DLCN criteria. In brief, each individual is assigned a score based on family history of hypercholesterolemia or premature cardiovascular disease (max. 2 points), personal history of premature cardiovascular disease (max. 2 points) and levels of LDL

cholesterol (max. 8 points), as shown in **Table 2**. Most often, physical examination findings of tendinous xanthomata or arcus cornealis (max. 6 points) are unavailable in electronic health records and therefore excluded. Based on the sum of points, an individual is considered to have clinical FH if the score indicates 'probable FH' (scores 6-8) or 'definite FH' (score >8).

Table 2. The modified Dutch Lipid Clinic Network (DLCN) criteria

Criteria	Points
A. Family history (max. 2 points)	
First-degree relative with premature CAD, stroke or PAD	1
First-degree relative with hypercholesterolemia (LDL-C ≥ 95th percentile)	1
Children under 18 with LDL-C ≥ 95th percentile	2
B. Clinical history (max. 2 points)	
Premature CAD (<55 years for men, <60 years for women)	2
Other premature cardiovascular disease (stroke or PAD)	1
C. LDL cholesterol levels (max. 8 points)	
8.5 mmol/l or higher	8
6.5 mmol/l to 8.4 mmol/l	5
5.0 mmol/l to 6.4 mmol/l	3
4.0 mmol/l to 4.9 mmol/l	1
Not included: Physical examination and DNA analysis*	
Diagnosis (based on the sum of points**)	
Definite FH	>8
Probable FH	6-8
Possible FH	3-5
Unlikely FH	<3

CAD indicates coronary artery disease; PAD, peripheral artery disease.

* In the unmodified criteria, physical examination findings may give up to 6 points (6 points for tendinous xanthomata, 4 points for arcus cornealis prior to age 45) and a causative mutation in LDLR, APOB or PCSK9 gives 8 points.

** Sum of highest number of points scored within each category: A (max. 2) + B (max. 2) + C (max. 8) = (max. 12).

Two of the studies that performed population screening for monogenic FH (see above) also assessed prevalence of clinical FH: In the Copenhagen study, the prevalence of clinical FH was 0.35% (out of 98,000) (Benn et al., 2016); whereas in the Geisinger Health System study, the prevalence was 1.2% (out of 46,285) (Abul-Husn et al., 2016). In a representative population sample from the United States (n = 42,471), using data from the 1999 to 2014 National Health and Nutrition Examination Survey (NHANES), the prevalence of clinical FH was estimated at 0.47% (De Ferranti et al., 2016). The relatively higher estimate in the Geisinger study (1.2%) may be related to: (i) the use of electronic health record data to define personal and family history variables (thus reducing possible recall bias); (ii) availability of longitudinal LDL cholesterol measurements (allowing for selection of the highest LDL cholesterol measurement for the DLCN criteria); (iii) possible selection bias, as reflected in high overall statin use in the sample (54%, compared to 12% in the Copenhagen study and <20% in the NHANES study). A recent meta-analysis, including these studies and several other smaller studies, estimated the population prevalence of clinical FH by the DLCN criteria at 0.22%, or about 1 in 450 (Beheshti et al., 2020).

It is important to note that prevalence estimates of clinical FH, such as presented above, are inevitably reliant on the availability and quality of the clinical data. For example, self-reported information such as family history of premature cardiovascular disease or hypercholesterolemia is strongly dependent on an individual's awareness of his own family history (relying on communication between relatives), the accuracy of that information and eventually recall of that information when asked. In the above studies, recall bias is an important factor that may have led to underestimation of the estimated prevalence of clinical FH.

FH in Iceland

The first mention of FH in Icelandic print was likely in a 1961 article titled "Coronary artery disease: prevalence and causes" [my translation] in Læknablaðið – the Journal of the Icelandic Medical Association (Samúelsson, 1961). However, it is unclear from the text whether any individuals had at that time been diagnosed with FH in Iceland. A decade later, in 1973, the first case report of an individual with FH is published in Læknablaðið (Ólafsson & Sigfússon, 1973). There it is mentioned that FH had been recognized in at least three Icelandic families. Since the late 1970s, individuals with hypercholesterolemia could be referred to an outpatient clinic run by Landspítali – The National University Hospital in close collaboration with the non-profit organization Hjartavernd – the Icelandic Heart Association. This facilitated the study of individuals with FH, as mentioned in the first review article on FH published in Icelandic in 1980 (Sigurðsson, 1980). This work resulted in the first molecular characterization of FH in Iceland (Taylor et al., 1989). In that study, individuals from 17 unrelated FH families were studied using restriction fragment length polymorphisms in the *LDLR* gene. In one family, they identified a 2 kb deletion of two exons (9 and 10) in *LDLR*, the first FH

mutation identified in Icelanders. Further, they identified at least four other haplotypes likely harboring unknown FH mutations (Taylor et al., 1989). In 1997, a second causative FH mutation was discovered. This mutation, a splice donor mutation in *LDLR* (c.694+2T>C, also known as I4T+2C), was found to explain FH in 10 of the 18 families studied (Gudnason et al., 1997). The authors concluded that this mutation was a founder mutation in Iceland that accounted for >60% of FH cases in Iceland (Gudnason et al., 1997). In the following years, systematic cascade screening in families carrying the founder mutation was carried out at Hjartavernd (Thorsson et al., 2003). In 2020, Hjartavernd collaborated with Phosphorus Diagnostics to search for FH mutations in a sample of 62 individuals from 23 FH families and additional 315 individuals with hypercholesterolemia (total cholesterol ≥95th percentile) from the REFINE Reykjavik study (Kellogg et al., 2020). Using a targeted next generation sequencing panel, they identified several novel mutations, bringing the total of FH mutations identified in Icelanders to nine. All are located in *LDLR*.

Paper III describes the first comprehensive analysis of monogenic and clinical FH in a large sample of Icelanders, with an emphasis on characterization of genetic causes, phenotypic impact and current treatment with cholesterol-lowering therapies in Iceland.

1.6.2 Familial hypobetalipoproteinemia

Familial hypobetalipoproteinemia (FHBL) can be thought of as being the opposite of FH. FHBL is a monogenic, autosomal dominant condition characterized by very low plasma concentrations of apoB lipoproteins (<5th percentile for age and sex) (Welty, 2014). FHBL is caused by mutations in *APOB* that lead to premature truncation or absence of apoB, resulting in decreased production and increased catabolism of apoB lipoproteins (Elias et al., 1999; Welty et al., 1997). Thus VLDL levels are low, leading to low levels of its metabolic derivatives, the most important being LDL. In heterozygous FHBL, the resulting lipid derangement is very low LDL cholesterol (about 25-50% of normal; usually between 0.5 and 1.3 mmol/l) which is accompanied by low levels of triglycerides (50-60% lower than in controls) (Di Costanzo et al., 2017; Linton et al., 1993; Peloso et al., 2019). Impaired production of VLDL leads to decreased triglyceride export from the liver, predisposing to the development of nonalcoholic fatty liver disease (Tanoli et al., 2004). Thus, the main adverse clinical manifestation in heterozygous FHBL is hepatic steatosis, often reflected by a mild elevation of liver enzymes (Welty, 2014). In some cases, FHBL-related hepatic steatosis may progress to cirrhosis and hepatocellular carcinoma (Cefalù et al., 2013). The homozygous form of FHBL is a more severe, abetalipoproteinemia-like condition and is outside the scope of this work (Ramasamy, 2016).

Homozygous loss-of-function of *PCSK9* causes a phenotype similar to FHBL (Ramasamy, 2016). Here, absent or near-absent activity of the PCSK9 enzyme leads to reduced catabolism of LDL receptors and thus increased overall activity of the LDL receptor pathway. Unlike FHBL due to *APOB* mutations, where the production of apoB

lipoproteins is impaired, the FHBL-like phenotype due to *PCSK9* loss-of-function is the result of increased clearance of LDL through an increase in the number of LDL receptors (Horton et al., 2007). Levels of VLDL are minimally affected and the primary lipid derangement is very low LDL cholesterol with normal triglyceride levels. *PCSK9* nonsense mutations are relatively common in individuals of African ancestry (about 2%) but are very rare in Europeans (<0.1%) (Ramasamy, 2016). Only two complete human knockouts for *PCSK9* have been described, one homozygote for C679X and a compound heterozygote for Y142X and ΔR97 (Hooper et al., 2007; Zhao et al., 2006). These individuals were of African ancestry, young (32 and 21 years old) and had LDL cholesterol levels around 0.40 mmol/l. In Europeans, the *PCSK9* missense variant R46L (allele frequency about 1.6%) is often described as a loss-of-function mutation (Benn et al., 2010). However, R46L causes only partially reduced function of PCSK9 and does not cause a FHBL-like phenotype in the homozygous state (Benn et al., 2010). Recently, a rare deletion causing complete loss-of-function of PCSK9 was identified in Icelanders (allele frequency 0.037%) (Beyter et al., 2021). Heterozygotes for the mutation had lower LDL cholesterol by 0.93 mmol/l, on average, but no homozygous carriers were identified.

Familial combined hypolipidemia (FCH) is sometimes considered to be a subtype of FHBL (known as type 2 FHBL, or FHBL2) (Ramasamy, 2016). In contrast to apoB-related FHBL, FCH is a recessive condition and is caused by homozygosity for loss-of-function mutations in the gene for angiopoietin-like protein 3 (ANGPTL3). ANGPTL3 is a protein that inhibits the activity of lipoprotein lipase and endothelial lipase, thereby modulating catabolism of triglyceride-rich lipoproteins (mainly VLDL and its remnants) and HDL (Ramasamy, 2016). In FCH there is total deficiency of ANGPTL3, resulting in very low levels of LDL cholesterol (about 0.8 mmol/l), triglycerides (about 0.2-0.3 mmol/l) and HDL cholesterol (about 0.5 mmol/l) (Musunuru et al., 2010; Noto et al., 2012). Heterozygosity for ANGPTL3 inactivating mutations yields an intermediate phenotype, but with unaffected HDL levels (Ramasamy, 2016). In clinical practice, FCH can be distinguished from FHBL by the presence of low HDL cholesterol levels (normal in FHBL). FCH is not thought to be associated with an adverse effect on health (Minicocci et al., 2012).

Depending on their protein consequences, mutations in *APOB* and *PCSK9* have the demonstrated potential to cause either an extreme elevation of LDL (as in FH), or extreme reduction in LDL (as in FHBL). **Paper IV** describes the discovery of the first gain-of-function mutation in *LDLR*, causing extreme hypocholesterolemia (a FHBL-like phenotype) due to increased LDL receptor expression.

2 Aims

This thesis is based on four published papers. The unifying aim of these studies was to evolve our understanding of the genetic architecture of coronary atherosclerosis and blood lipid levels, with a special focus on monogenic causes of extremely high or extremely low levels of LDL cholesterol. The specific aims of each study were as follows:

Paper I. The aim of this study was to assess the impact of known, common risk variants for CAD on the angiographic extent of coronary atherosclerosis. In other words, we aimed to assess whether risk variants for CAD also affect the severity of the disease.

Paper II. The aim of this study was to assess the individual contributions of different blood lipids on the extent of coronary atherosclerosis in a genetic MR analysis.

Paper III. The aim of this study was to characterize monogenic and clinically defined FH in a large sample from the Icelandic population. We performed a comprehensive assessment of causes, prevalence, phenotypic impact and treatment of FH in Iceland.

Paper IV. The aim of this study was to search for novel variants that have a large impact on levels of LDL cholesterol. We describe the discovery and characterization of a rare deletion in *LDLR* that results in gain-of-function and a large reduction in plasma LDL cholesterol.

3 Materials and methods

3.1 Study population

The studies on which this thesis is based (**papers I-IV**) were genetic studies carried out at deCODE genetics in Reykjavík, Iceland. All participants had signed an informed consent and donated a biological sample for genotyping at deCODE genetics. Phenotypic information was accessed in the extensive deCODE genetics phenotypic database or were obtained on a study-specific basis as described below.

In brief, the overall study population in **papers I-IV** comprises Icelanders who have voluntarily participated and consented to genotyping in one or more of deCODE genetics' studies, ever since its founding in 1996 (Gudbjartsson et al, 2015). Throughout the years, the size of the overall genotyped sample has grown with the participation of ever more Icelanders. The characteristics of the overall sample in 2020 is presented in **Table 3** (reproduced from paper III). Shown are general characteristics, availability of LDL cholesterol measurements and the prevalence of several cardiovascular diseases. The statistics are stratified by genotyping method (see below).

3.1.1 General description of the deCODE genetics phenotypic database

The deCODE genetics phenotype database contains extensive information on clinical phenotypes in the Icelandic population, for example disease diagnoses and biochemical traits (Gudbjartsson et al., 2015; Nioi et al., 2016). These data have been obtained mostly through collaboration with Icelandic medical institutions and clinicians within each field, since 1996. As with genotype data, all phenotypic data is stored under encrypted personal identifiers.

Examples of phenotype information available in this database include diagnoses and detailed phenotyping of cardiovascular diseases (myocardial infarction, coronary artery disease, peripheral artery disease, atrial fibrillation, sick sinus syndrome and stroke), metabolic disorders (obesity, diabetes and metabolic syndrome), psychiatric disorders (schizophrenia, bipolar disorder, anxiety and depression), addictions (nicotine, alcohol), inflammatory diseases (rheumatoid arthritis, lupus, and asthma), musculoskeletal disorders (osteoarthritis, osteoporosis), eye diseases (glaucoma), kidney diseases (kidney stones, kidney failure) and various types of cancer. Routinely measured biochemical traits including, for example, sodium, potassium, bicarbonate, calcium, phosphate, creatinine, blood cell counts, hemoglobin, hematocrit, immunoglobulins, iron, vitamins, blood lipids and liver function tests have been obtained from clinical laboratories at Landspítali – The National University Hospital in Reykjavík and other medical institutions in Iceland. In addition, the database includes the phenotype data obtained through the deCODE health study.

Table 3. Characteristics of the overall study sample

Characteristics	All	Whole-genome sequencing and chip-genotyping	Chip-genotyping only
N	166,281	49,962	116,319
Year of birth, mean	1960	1956	1962
Alive as of 2020, %	83.2	75.0	86.7
Mean age (alive), years	55.3	56.8	54.6
Men, %	45.8	45.8	45.8
LDL-C measurement available, %	63.3	74.7	58.4
Maximum LDL-C (mmol/l), mean	3.94	4.03	3.90
Coronary artery disease (CAD), %	12.2	16.1	10.5
Early-onset CAD, %	2.2	3.0	1.8
Ischemic stroke, %	3.2	4.4	2.7
Heart failure, %	8.2	11.9	6.6
Atrial fibrillation, %	9.0	12.6	7.4
Hypertension, %	25.9	33.1	22.8
Diabetes mellitus type 2, %	7.0	9.7	5.8

The deCODE health study

Since 2016, the deCODE health study (Icelandic: *Heilsurannókn Íslenskrar erfðagreiningar*) has actively recruited individuals for comprehensive phenotyping. By design, the participant sample is enriched for known and possible carriers of rare and potentially high impact mutations. Information on general health, lifestyle and medication use is obtained via an online questionnaire and verbal interviews. Participants undergo a range of physical measurements involving cardiopulmonary function (including blood pressure, resting heart rate, electrocardiogram, pulse oximetry, spirometry and cardiopulmonary exercise testing), bone density and body composition (e.g. fat distribution), grip strength, hearing, peripheral nerve conduction, physical characteristics (e.g. height, weight, body-mass index), ophthalmological tests and various tests of cognitive function. All participants sign an informed consent and provide a blood sample used for isolation of nucleic acids (DNA and RNA), plasma and serum for various laboratory measurements and peripheral blood mononuclear cells (PBMC). All persons phenotyping the participants are blind to genotype.

As described in **paper IV**, carriers of the *LDLR* 3' UTR deletion and their close family members were invited to participate in the deCODE health study in an effort to obtain in-depth phenotypic information.

3.1.2 Assessment of the extent of coronary atherosclerosis

Coronary angiography datasets

We obtained clinical data on invasive coronary angiography procedures performed for various clinical indications at Landspítali – The National University Hospital, the only interventional cardiology center in Iceland. Computerized records were gathered from three main sources: (i) The Swedish Coronary Angiography and Angioplasty Registry (SCAAR), which holds records on all coronary angiographies and PCI procedures performed in Iceland since January 1, 2007 (Fröbert et al., 2013; T. Gudnason et al., 2013). (ii) A registry of all PCI procedures performed in Iceland between January 1, 1987 and December 31, 2006. (iii) A registry of coronary artery bypass grafting (CABG) procedures performed in Iceland, which holds a wealth of clinical and procedural data including the results of pre-procedural coronary angiography (Johannesdottir et al., 2017). Data on pre-procedural angiographies for CABG surgeries performed between January 1, 2001 and December 31, 2013 were used in **paper I** and **paper II**. In **paper I** and **paper II**, all three data sources were combined into a single dataset in which only the earliest record was used for each individual. In **paper III**, we used only the SCAAR database to study a period (2007-2017) where the coverage for all coronary angiography procedures performed in Iceland, including PCI and pre-procedural coronary angiographies for CABG, was complete.

Information on cardiovascular risk factors was obtained from these registries. In the combined dataset, hypertension, diabetes and hyperlipidemia were defined by a previous diagnosis of the respective condition or medical treatment at the time of angiography (with anti-hypertensive, anti-diabetic or lipid-lowering medication, respectively). Individuals with missing data were removed. **Table 4** summarizes the sample sizes and coronary angiography datasets used in **papers I-III**.

Table 4. Coronary angiography datasets used in papers I, II and III

Paper	Number of genotyped individuals	SCAAR	PCI	CABG
I	8,622	2007-2013	1987-2006	2001-2013
II	12,460	2007-2017	1987-2006	2001-2013
III	11,246	2007-2017	Not used	Not used

Emory biobank replication dataset. In **paper I**, we replicated the associations observed in the Icelandic dataset in a sample of 1853 Caucasian participants in the

Emory Cardiovascular Biobank Study (Helgadottir et al., 2012). All participants included in the analyses had significant angiographic CAD (≥50% diameter stenosis). Participants were enrolled at Emory University Hospital and its affiliated centers in Atlanta, Georgia, USA. All subjects provided written informed consent and the study was approved by the Institutional Review Board at Emory University, Atlanta, Georgia, USA.

Definitions of angiographic extent of CAD

We used measures of angiographic CAD extent that are based on counting the number of major coronary arteries with significant coronary disease (i.e. vessel score). This relatively simple method is one of the oldest methods of scoring CAD extent, provides important prognostic value and remains widely used in clinical practice to this day (see chapter 1.2.1) (Ringqvist et al., 1983). In the angiographic registries described above, the vessel score was recorded by the interventional cardiologist at the time of coronary angiography.

Obstructive or significant angiographic CAD (terms used synonymously in **papers I-III**) was defined as luminal diameter stenosis of at least 50% in any of the four major coronary arteries (the left main coronary artery, the left anterior descending artery, the circumflex artery or the right coronary artery). Thus, having obstructive CAD is the same as having a vessel score of at least 1. If obstructive CAD was not present, a vessel score of 0 was assigned. Multivessel disease was defined as having at least two major coronary arteries with at least 50% stenosis, i.e. a vessel score of at least 2. In **paper I**, left main disease was treated as an independent, single vessel and thus the vessel score ranged from 0 to 4 in that study. In **paper II** and **paper III**, left main disease were scored as multivessel disease irrespective of stenoses in other coronary arteries.

In the main analysis of **paper I**, we tested for association with the vessel score as a continuous variable (0, 1, 2, 3, 4). In **paper II** and **paper III**, we assessed CAD extent as dichotomous variables only, e.g. obstructive CAD vs. non-obstructive CAD and multivessel disease vs. single-vessel disease.

Coronary artery calcium (CAC)

In **paper II** and **paper III**, we used data on individuals who underwent cardiac-gated computed tomography for any indication at Röntgen Domus, the largest privately-operated medical imaging clinic in Iceland, between January 4, 2009 and October 31, 2017. The CAC score, also known as Agatston score (see chapter 1.2.2), was calculated by the reading radiologist and extracted from the radiology reports. CAC was assessed either as a log-transformed quantitative trait or as a dichotomized variable using commonly-used cut-points: 0 (no CAC detected), 1-100, 101-400 and 400+ (Budoff et al., 2009). For each individual, we used the earliest record only.

Information on cardiovascular risk factors, other than age at the time of procedure and sex, were not available from this resource.

3.1.3 Measurements of lipid traits

Measurement sites

Measurements of total cholesterol, HDL cholesterol and triglycerides were obtained from three of the largest clinical laboratories in Iceland: (i) Landspítali – The National University Hospital (hospitalized and ambulatory patients); (ii) the Laboratory in Mjódd, Reykjavík (ambulatory patients); and (iii) Akureyri Hospital, Regional Hospital in North Iceland, Akureyri (hospitalized and ambulatory patients). In addition, measurements of these lipid traits and others (directly measured LDL cholesterol, apolipoprotein A, apolipoprotein B and lipoprotein(a)) were obtained from the laboratory at deCODE genetics, the majority of which are samples from individuals recruited through the deCODE health study. Lipid measurements were obtained either in a fasting or non-fasting state.

non-HDL cholesterol and LDL cholesterol

Non-HDL cholesterol was calculated as total cholesterol – HDL cholesterol. Calculated LDL cholesterol levels were calculated using the Friedewald equation: LDL cholesterol = total cholesterol – HDL cholesterol – triglycerides/2.2 for triglyceride levels <4.00 mmol/L. LDL cholesterol was not calculated for higher triglyceride levels. Directly measured LDL cholesterol levels (used in **paper IV**) were obtained using a homogeneous enzymatic colorimetric assay (LDL-Cholesterol Gen.3) run on a Cobas automated analyzer (Roche Diagnostics) at deCODE genetics.

Adjustment for statin use. Levels of non-HDL cholesterol and calculated LDL cholesterol were adjusted for statin use in following manner: For individuals who were prescribed statins within one year prior to measurement, total cholesterol was divided by 0.8 and the modified value was used for calculation of non-HDL cholesterol and LDL cholesterol. The number 0.8 used for the denominator represents a crude approximation of the average reduction in total cholesterol on statin treatment (approximately 1.2 mmol/l in a large meta-analysis of statin trials (Mihaylova et al., 2012)) and is used by convention in many genetic studies (Khera et al., 2016; Liu et al., 2017). Directly measured LDL cholesterol was not adjusted for statin use (**paper IV**).

Apolipoproteins A-1, apolipoprotein B and lipoprotein(a)

Serum concentrations of apolipoproteins A-1, apolipoprotein B and lipoprotein(a) were quantified using immunoturbidimetric assays (Tina-quant Apolipoprotein A-1 ver.2, Tina-quant Apolipoprotein B ver.2 and Tina-quant Lipoprotein(a) Gen.2, respectively) run on a Cobas automated analyzer (Roche Diagnostics) at deCODE genetics.

The sample sizes for genotyped participants with available lipid measurements used in **papers II-IV** are summarized in **Table 5**.

Table 5. Sample sizes for lipid traits in papers II-IV

Lipid trait	Paper II	Paper III	Paper IV
Total cholesterol			113,355
non-HDL cholesterol	93,556		106,864
LDL cholesterol (calc.)	87,212	104,828	101,857
LDL cholesterol (meas.)			12,573
HDL cholesterol	93,746	110,076	107,036
Triglycerides	94,242	109,550	94,630
Apolipoprotein A			23,875
Apolipoprotein B			23,875
Lipoprotein(a)		24,257	23,823

3.1.4 Icelandic genealogical database

A comprehensive genealogical database for the Icelandic population, commonly known as *Íslendingabók* (English: Book of Icelanders), is kept at deCODE genetics and is described in ref (Gudbjartsson et al., 2015). The database dates back to 740 AD and contains information on over 820,000 individuals, of which about 471,000 were recorded to have been born in the 20th century. To build this database, genealogical information was obtained from many sources, primarily from church books, censuses (from the first census in Iceland carried out in 1703), Registers Iceland (*Þjóðskrá Íslands*), local records of inhabitants and other official documents, but also from various other sources including old manuscripts, letters, annals, books of Althingi, books of judgments, books of family pedigrees, registers of farmers, registers of professionals and lists of descendants (Gudbjartsson et al., 2015).

The genealogical database is a valuable resource for various aspects of genetic research in the Icelandic population by deCODE genetics (Gudbjartsson et al., 2015). For instance, it is used to improve calling and phasing of genotypes and to inform familial imputation (described below). Here, in particular, the genealogical database was used to identify families carrying rare sequence variants and to trace the origin of these variants (**paper III** and **paper IV**). In addition, the database was used to create family history variables based on identification of first-degree relatives (**paper I** and **paper III**).

3.1.5 Dutch Lipid Clinic Network (DLCN) criteria

In **paper III**, we used a modified version of the DLCN criteria to define clinical FH. This modified version excludes physical examination findings and genetic information and has been used previously by other groups (Benn et al., 2012, 2016). We applied these criteria to genotyped participants with at least one available LDL cholesterol measurement, restricting to those alive and between the approximate ages of 20 and 80 at the time of analysis. Variables were defined using available phenotypic data at deCODE genetics. The Icelandic genealogical database was used to identify first degree relatives of each participant and construct the relevant family history variables.

The modified DLCN criteria are shown in **Table 2** (chapter 1.6.1). Each participant was assigned a score based on family history (among first-degree relatives) of hypercholesterolemia (defined as LDL cholesterol above the 95th percentile) or premature cardiovascular disease (max. 2 points), personal history of premature cardiovascular disease (max. 2 points) and the maximum documented LDL cholesterol levels (max. 8 points). Based on the sum of points, each participant was classified as having unlikely (<3), possible (3-5), probable (score 6-8) or definite (score >8) FH.

3.2 Genotyping methods

The studies on which this thesis is based (**papers I-IV**) use the extensive database of DNA sequence variation in the Icelandic population that has been built at deCODE genetics. Since its foundation in 1996, deCODE genetics has genotyped biological samples donated by voluntary participants in its various genetic research projects. Over the years, the genetic database has continuously expanded with increasing number of participants. As of 2020, it contains genotype information for over 160,000 participants, of which roughly 50,000 participants have undergone whole-genome sequencing (WGS).

Table 6 shows information on the genotype datasets used in the studies. What follows is a general description of the main methods used to generate the genotype database at deCODE genetics. For further technical details, see Gudbjartsson et al., 2015 and Jónsson et al., 2017.

Table 6. Genotype datasets in papers I-IV

Paper	Year published	Chip-genotyped	Whole-genome sequenced (median depth)	Individuals with genotypes based on familial imputation
I	2015	95,085	2230 (20×)	296,526
II	2020	155,250	28,075 (37×)	Not used
III	2021	166,281	49,962 (39×)	Not used
IV	2021	155,250	43,202 (38×)	Not used

3.2.1 SNP array genotyping

SNP microarrays or 'chips' provide an accurate and low-cost platform to genotype up to two million (or even more) SNPs that are interspersed throughout the genome. All participants at deCODE genetics are genotyped using SNP chips. Various chips have been used for this purpose, including Illumina HumanHap300, HumanCNV370, HumanHap610, HumanHap1M, HumanHap660, Omni1, Omni2.5 and OmniExpress-24 bead chips. In addition to other quality control measures, rare SNPs with minor allele frequency below 1% are excluded in the imputation. Thus, for the imputation purpose, only common genotyped SNPs are used.

3.2.2 Long-rage phasing

The SNP genotypes are then *phased*, in which each SNP is assigned a maternal or paternal origin. The algorithm used for genotype phasing in deCODE genetics data is known as long-range phasing (Kong et al., 2008). Long-range phasing is a computation method which detects shared SNP haplotypes inherited from a common ancestor (identity-by-descent segments) and uses that information to assign a maternal or paternal origin to each SNP. This method is facilitated by the genetic homogeneity and relatively high relatedness in the Icelandic population.

3.2.3 Whole-genome sequencing

A subsample of participants in deCODE genetics studies has been selected for whole-genome sequencing (WGS) in addition to chip-genotyping. The number of participants who have undergone WGS has grown rapidly in recent years. The genotype set used in **paper I** (published in 2015) included 2230 individuals who underwent WGS whereas the most recent study (**paper III**, published in 2021) included 49,962 (**Table 6**). WGS at deCODE genetics is carried out using Illumina next-generation sequencing technology which is based on rapid and massively parallel sequencing of randomly fragmented DNA using paired-end *sequencing by synthesis* (Bentley et al., 2008).

The workflow for the generation of WGS data at deCODE genetics is roughly as follows (see detailed description in Gudbjartsson et al., 2015 and Jónsson et al., 2017): First, paired-end libraries for sequencing are prepared from DNA samples (derived from blood or buccal swabs) using Illumina preparation kits. Second, paired-end sequencing-by-synthesis is performed on Illumina sequencers (in order of oldest to most recent): Genome Analyzer II_x, HiSeq 2000/2500, HiSeq X and NovaSeq. Third, the sequenced reads (varying in length from 2 × 76 bp to 2 × 150 bp, depending on the instrument and/or sequencing kit used) are aligned to the human reference genome using a Burrows–Wheeler aligner algorithm. Fourth, after a single Binary Alignment Map (BAM) has been generated for each individual, the BAM files are then merged across all individuals (using SAMtools) for variant calling. Fifth, sequence variants (SNPs and insertions-deletions [indels] up to about 60bp in size) are then called using a Genome Analysis Toolkit (GATK) unified genotype caller (McKenna et al., 2010).

Finally, information on haplotype sharing is used to improve genotype quality and generate phased genotypes (using a iterative algorithm based on the IMPUTE HMM model (Marchini et al., 2007) and long-range phased haplotypes). For a WGS sample of 15,220 individuals, this results in calling of about 39 million autosomal variants (Jónsson et al., 2017).

The WGS dataset is enriched for individuals with extreme phenotypes as they have been chosen for WGS as part of various projects at deCODE genetics. For example, the WGS dataset is enriched for individuals at the extremes of the distribution of LDL cholesterol (Björnsson et al., 2021). This is of particular relevance with respect to identification of rare variants with a large effect on LDL cholesterol, such as those described in **paper III** and **paper IV**.

3.2.4 Imputation

Genotype imputation is a well-established technique in which the genotype set obtained using SNP chips is expanded substantially by inferring genotypes based on haplotype sharing in a larger reference set (Li et al., 2009). Here, this method is applied in which individuals that have been genotyped with SNP chips only are assigned genotype probabilities for millions of additional variants based on haplotype sharing with individuals that have also undergone WGS. This imputation method is based on the IMPUTE HMM model (Marchini et al., 2007) and long-range phased haplotypes and is described in detail in Gudbjartsson et al., 2015.

3.2.5 Calling of structural variants

Structural variation such as deletions, insertions, duplications and other copy-number variants (CNVs) that are larger than about 50bp in size are not identified in WGS data using standard calling methods (Guan & Sung, 2016). In **paper IV**, we searched specifically for rare CNVs in the *LDLR* gene using several different algorithms as described below.

We searched for deletions involving *LDLR* in WGS data from 43,202 participants using a newly developed algorithm called PopDel. PopDel identifies deletions from about 500 to 10,000 bp in size by searching for deviations in insert size across thousands of WGS samples simultaneously (Niehus et al., 2021). In addition, we searched for additional structural variants in *LDLR* using several other algorithms: DELLY (Rausch et al., 2012), Graphtyper (Eggertsson et al., 2017) and Manta (Chen et al., 2016) (WGS based) and PennCNV (Wang et al., 2007) (SNP data), in addition to examining long-read sequences of 3,622 Icelanders who had undergone PromethION sequencing (Oxford Nanopore Technologies) (Beyter et al., 2021).

3.2.6 Familial imputation

Genealogical information may be used to infer the genotypes of over 280,000 Icelanders who are first- or second-degree relatives of genotyped participants but have

not themselves been genotyped (Gudbjartsson et al., 2015). In this way, the overall genotype set may be expanded substantially to increase statistical power in association analyses. Familial imputation genotypes are not stored, in accordance with regulations of the Icelandic Data Protection Authority. Genotypes derived from familial imputation were used in **paper I**, but not in **papers II-IV**.

3.3 Laboratory methods

In **paper III** and **paper IV**, we used several laboratory methods that are briefly described below. These procedures were carried out by experienced laboratory personnel at deCODE genetics.

3.3.1 Sanger sequencing

Sanger sequencing is a robust method for determining the nucleotide sequence of a particular segment of DNA, flanked by two known primers, using a chain-termination polymerase chain reaction (PCR) (Sanger et al., 1977). Here, Sanger sequencing was used to confirm genotypes for FH mutations in **paper III**, as well as genotypes for the 2.5kb *LDLR* 3' UTR deletion in **paper IV.** The general method is described in Styrkarsdottir et al., 2014.

3.3.2 3' rapid amplification of complementary DNA ends (3' RACE)

3' RACE is a method used to determine the sequence of a messenger RNA (mRNA) between a defined internal site and the 3' end of the mRNA (Frohman et al., 1988). One use of 3' RACE is to determine the length of the 3' UTR of mRNA, which may differ as a result of alternative polyadenylation (Tian & Manley, 2016). In **paper IV**, we used 3' RACE to confirm the presence of a short 3' UTR in a carrier of the *LDLR* 3' UTR deletion. Please refer to the online supplement to **paper IV** for further details.

3.3.3 LDL receptor expression in Epstein-Barr virus (EBV)-transformed lymphocytes

In **paper IV**, we studied the impact of the *LDLR* 3' UTR deletion on LDL receptor expression in cells from Epstein-Barr virus (EBV)-transformed lymphoblastoid cell lines that were derived from PBMCs collected from deletion carriers and non-carriers as controls. EBV-transformation was carried out using a conventional protocol. Surface LDL receptor expression was quantified using a fluorescence-activated cell sorter (Attune Nxt, Thermo Fisher Scientific). In addition, we assessed *LDLR* mRNA expression in the EBV-transformed lymphocytes using RNA sequencing. Preparation of poly(A)+ cDNA sequencing libraries and RNA sequencing were carried out as described previously (Rafnar et al., 2018). Please refer to the online supplement to **paper IV** for further details.

3.4 Statistical analyses

3.4.1 Lipid traits

Before association testing, continuous lipid traits were adjusted for various confounding variables by first obtaining adjusted residuals from a linear regression model. In general, the procedure was as follows: First, all measurements (one or more per individual, adjusted for statin use where possible [see chapter 3.1.3]) were entered as the outcome variable (in original units, e.g. mmol/l) in a linear regression model that included the covariates: year of birth, age at measurement, age at measurement squared, sex, site of measurement (factor) and county of birth (factor). Second, residuals were obtained from the model. These residuals (one per measurement) have mean of 0 and a unit that is the same as the original unit (e.g. mmol/l). For individuals with multiple measurements, the mean of the residuals was used in the subsequent step. Third, the residuals (one per individual) were inverse-normal transformed to yield a standard normal distribution with a mean of 0 and a standard deviation of 1. The standardized adjusted residuals were then used for association analyses.

In **paper III**, we tested for association with the highest documented levels of LDL cholesterol in the main analyses. To generate this variable, we selected only the highest documented measurement of LDL cholesterol for each individual, adjusted for statin use, and used that for calculation of adjusted residuals as described above. The *raw* adjusted residuals were used directly for association testing and thus this variable was not standardized using inverse-normal transformation. This was done to preserve the information from outliers with very high LDL cholesterol, which is of particular relevance in FH as carriers of FH mutations often have extreme values of LDL cholesterol.

3.4.2 Calculation of genetic scores

We used individual-level genetic scores as a means of combining the effects of multiple SNPs on a particular trait into a single variable. The genetic scores were weighted according to the effect sizes of the SNPs for the respective trait. Thus, for each participant, the genetic score was defined as the weighted sum of the SNP alleles:

$$\text{genetic score} = \sum_{j=1}^{m} X_j \, \beta_j$$

where X_j is the number of alleles and β_j is the weight (log-odds ratio for CAD, standard deviations for lipid traits) for SNP *j.* Weights were derived from external sources, in most cases from large meta-analyses for CAD (**paper I**) or plasma lipid levels (**paper II** and **paper III**).

In **paper I**, we created a GRS for the risk CAD based on 50 SNPs that had been shown to be associated with risk of CAD at genome-wide significance in at least one of

several large meta-analyses published prior to 2014: CARDIoGRAM Consortium (Schunkert et al., 2011), C4D Consortium (The Coronary Artery Disease (C4D) Genetics Consortium, 2011), IBC 50K CAD Consortium (The IBC 50K CAD Consortium, 2011) and CARDIoGRAMplusC4D Consortium (Deloukas et al., 2013). For each SNP, the natural logarithm of the published odds ratio for CAD was used as a weight.

In **paper II**, we created genetic scores for non-HDL cholesterol, LDL cholesterol, HDL cholesterol and triglycerides. Each of the genetic scores were based on a set of 345 SNPs that were associated with at least one lipid trait (total cholesterol, LDL cholesterol, HDL cholesterol or triglycerides) in an exome-wide association study of lipid traits in over 300,000 individuals published in 2017 (Liu et al., 2017). For calculation of the genetic scores for LDL cholesterol, HDL cholesterol and triglycerides, the published effect sizes (given in standard deviations) were used as weights. For non-HDL cholesterol, the effect sizes (weights) were estimated based on published effect sizes for total cholesterol and HDL cholesterol using the formula:

$$\text{non-HDL cholesterol} = \text{total cholesterol} \times 0.979 - \text{HDL cholesterol} \times 0.354$$

as effect sizes for non-HDL cholesterol were not reported in ref (Liu et al., 2017). The formula was derived based on genetic associations in the Icelandic data and provides an accurate estimate of effect sizes for non-HDL cholesterol, as described in detail in **paper II**.

In **paper III**, we used the same genetic score for LDL cholesterol as the one used in **paper II**.

3.4.3 Mendelian randomization in the context of lipid traits

In **paper II**, we used a Mendelian randomization approach to attempt to disentangle the individual contributions of commonly measured blood lipids on the extent of coronary atherosclerosis (see chapter 1.5.2). We used genetic scores for non-HDL cholesterol, LDL cholesterol, HDL cholesterol and triglycerides (see above) as instrumental variables. Due to the pleiotropy of lipid-associated variants (where one variant often has associations with more than one lipid trait) and thus intercorrelation of the genetic scores, we attempted to account for these pleiotropic effects by analyzing the genetic scores in a joint model. Here the association of a given genetic score (e.g., for LDL cholesterol) is adjusted for genetic scores for other lipid levels (e.g., for HDL cholesterol and triglycerides) by including them as covariates in the model. This approach was applied in a previous publication by our group (Helgadottir et al., 2016).

3.4.4 Association testing

In general, genetic associations were performed using linear regression for quantitative traits and logistic regression for dichotomous traits. In **paper III** and **paper IV**, a generalized form of linear regression that accounts for the relatedness between

individuals and potential population stratification was used to test for associations with quantitative traits and diseases. Relevant potential confounding variables were included as covariates in the regression models, as appropriate. Please refer to **papers I-IV** for details.

Statistical analyses were generally performed using R software (The R Foundation for Statistical Computing). For single comparisons, $P < 0.05$ was considered to be statistically significant. In **paper I**, for single-SNP associations, we accounted for multiple comparisons and considered $P < 0.001$ as statistically significant (Bonferroni threshold for the testing of 50 SNPs: 0.05/50 = 0.001).

3.5 Ethics statement

All participating individuals, or their guardians, gave their informed consent before donating biological samples for genotyping. All sample identifiers were encrypted with a third-party encryption system as provided by the Data Protection Authority of Iceland. The studies were approved by the National Bioethics Committee and the Icelandic Data Protection Authority.

4 Summary of results

In this section, the results of **papers I-IV** are summarized. For brevity and readability, some analyses have been omitted and statistical details minimized. For full details, please refer to the original publications in the Appendix.

4.1 Genetic variation and the extent of coronary atherosclerosis

In **paper I** and **paper II**, we assessed the effects of common sequence variants on the extent of coronary atherosclerosis.

4.1.1 Paper I: Common risk variants for CAD

In this study, we assessed the association of established risk variants for CAD with the extent of CAD in a sample of 8,622 Icelandic patients who had undergone coronary angiography and had obstructive CAD (defined as at least 50% stenosis in an epicardial coronary artery). The findings were replicated in an independent sample of 1853 patients from the Emory Biobank study. When testing each of the 50 SNPs individually, only rs1333049 at 9p21 and rs10455872 at the lipoprotein(a) gene (*LPA*) associated significantly with the number of coronary arteries with at least 50% stenosis. When the 50 variants were combined into a GRS, we observed an association between the GRS and the number of diseased coronary arteries that was stronger than for any single variant. The association was independent of traditional cardiovascular risk factors and family history of CAD. Compared to the bottom quintile of the genetic score, patients in the top quintile were 1.67-times more likely to have multivessel disease, as shown in **Figure 6**. The GRS associated also with younger age at angiography. The associations of the GRS with CAD extent persisted after removing variants at the loci most strongly associated with CAD extent (1 variant at 9p21 and 2 variants at *LPA*). Thus, we concluded that established risk variants for CAD are likely to have an aggregate effect on the development and progression of atherosclerosis, not only contributing toward higher CAD risk but also greater severity and extent of angiographic CAD among those with the disease.

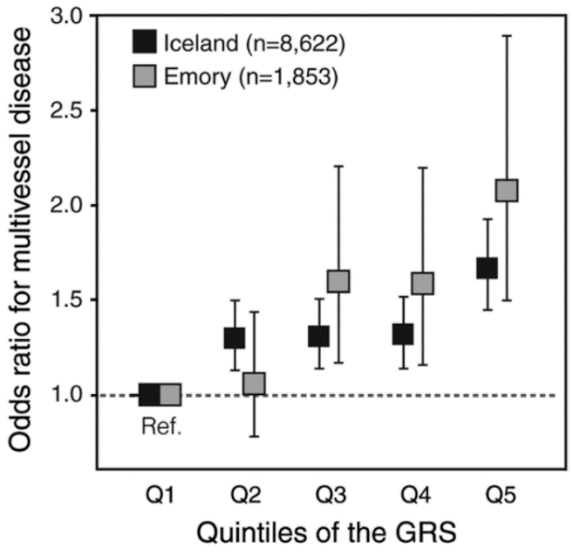

Figure 6. Genetic risk score for CAD and risk of multivessel disease

Adjusted odds ratios for multivessel disease by quintiles of the genetic score for CAD risk (genetic risk score, GRS). The Icelandic sample is indicated in black. The replication sample from the Emory Biobank is indicated in gray. Odds ratios are calculated with reference to the bottom quintile and are presented with 95% confidence intervals. Reproduced from Bjornsson et al., 2015 (©American Heart Association).

4.1.2 Paper II: Common variants that influence blood lipid levels

In this study, we applied MR to estimate the causal contribution of commonly measured lipid traits toward the extent of coronary atherosclerosis. We calculated a separate genetic score for each lipid trait and tested for associations with markers of CAD extent in a sample of 12,460 Icelandic individuals who had undergone coronary angiography and 4837 individuals who had undergone assessment of CAC.

When tested individually, the genetic scores for non-HDL cholesterol, LDL cholesterol and triglycerides associated significantly with increased risk of obstructive CAD, 3-vessel disease (as compared to 1- or 2-vessel disease) (**Table 7**) and higher CAC score. The non-HDL cholesterol genetic score had the most significant associations with CAD extent in all cases. After accounting for the non-HDL cholesterol genetic score, none of the other genetic scores remained associated with CAD extent (**Table 7**). This indicates that the associations of the genetic scores for LDL cholesterol and triglycerides with CAD extent are fully explained by the non-HDL cholesterol genetic score. Also, the associations of the non-HDL cholesterol genetic score with obstructive CAD and CAC score remained significant even after accounting for the LDL cholesterol genetic score.

The findings of this study extended previous findings by our group that had showed similar results in the context of CAD risk (Helgadottir et al., 2016). Thus, we concluded that non-HDL cholesterol is causally related to greater extent of coronary atherosclerosis and that it is likely the best marker of atherogenic apoB lipoproteins among the commonly measured lipid traits.

Table 7. Genetic scores for lipid levels and angiographic extent of CAD

Table 2. Genetic Scores for Lipid Levels and Angiographic Extent of Coronary Artery Disease

	Obstructive Coronary Artery Disease in Overall Sample (N = 12 460)		Patients With Obstructive Coronary Artery Disease (n = 8984)			
			Multivessel Disease		3-Vessel Disease	
Covariates (Genetic Scores)[a]	Odds Ratio (95% CI)	*P* Value	Odds Ratio[b] (95% CI)	*P* Value	Odds Ratio[b] (95% CI)	*P* Value
Non-HDL-C	1.83 (1.63-2.07)	2.8×10^{-23}	1.26 (1.11-1.44)	4.1×10^{-4}	1.47 (1.26-1.72)	9.2×10^{-7}
HDL-C	1.74 (1.54-1.98)	3.7×10^{-18}	1.26 (1.10-1.45)	6.5×10^{-4}	1.46 (1.24-1.72)	4.1×10^{-6}
HDL-C and triglycerides	1.75 (1.52-2.01)	3.2×10^{-15}	1.31 (1.13-1.52)	4.6×10^{-4}	1.44 (1.21-1.73)	5.8×10^{-5}
LDL-C	2.13 (1.47-3.10)	6.4×10^{-5}	1.01 (0.68-1.50)	.97	1.49 (0.93-2.38)	.10
LDL-C	1.73 (1.54-1.95)	6.4×10^{-20}	1.28 (1.12-1.45)	1.9×10^{-4}	1.43 (1.23-1.67)	3.8×10^{-6}
HDL-C and triglycerides	1.63 (1.44-1.84)	3.0×10^{-15}	1.27 (1.11-1.45)	3.5×10^{-4}	1.39 (1.19-1.63)	4.1×10^{-5}
Non-HDL-C	0.85 (0.59-1.23)	.40	1.27 (0.86-1.87)	.24	0.99 (0.62-1.58)	.96
HDL-C	0.71 (0.62-0.80)	3.0×10^{-8}	0.94 (0.82-1.07)	.35	0.87 (0.75-1.02)	.09
LDL-C and triglycerides	0.83 (0.72-0.96)	.01	0.98 (0.85-1.13)	.77	0.99 (0.83-1.17)	.89
Non-HDL-C and triglycerides	0.83 (0.72-0.96)	.011	0.98 (0.85-1.13)	.78	0.99 (0.83-1.18)	.90
Triglycerides	1.86 (1.51-2.29)	6.4×10^{-9}	1.12 (0.89-1.39)	.34	1.45 (1.12-1.89)	.005
LDL-C and HDL-C	1.35 (1.06-1.71)	.014	1.01 (0.78-1.29)	.96	1.28 (0.95-1.73)	.10
Non-HDL-C and HDL-C	0.99 (0.76-1.29)	.94	0.87 (0.66-1.15)	.32	1.05 (0.75-1.46)	.78

4.2 Paper III: Familial hypercholesterolemia in Iceland

In this study, we carried out a comprehensive analysis of the prevalence and phenotypic characteristics of FH in Iceland. We considered two partially overlapping definitions of FH based on genotype (monogenic FH) or phenotype (clinically defined FH), and compared these two groups. Definitions of FH were entirely based on genotypic and phenotypic data available at deCODE genetics and were independent of any diagnoses of FH made in a clinical setting.

Monogenic FH

We identified 20 FH mutations in the WGS dataset of 49,962 Icelanders (**Table 8**). Twelve of the mutations had not been reported in the Icelandic population previously. Genotype imputation and Sanger sequencing to confirm the genotypes of all identified carriers (followed by re-imputation) yielded an overall sample of 166,281 individuals with information on these mutations. We identified 199 carriers of FH mutations in the overall sample, corresponding to a monogenic FH prevalence of 1 in 836. Monogenic FH was associated with 3.4 mmol/l higher levels of highest documented LDL cholesterol, 5-fold increased risk of premature coronary artery disease, over 3-fold increased risk of aortic valve stenosis and shorter lifespan than non-carriers. In

addition, individuals with monogenic FH had greater burden of coronary atherosclerosis upon coronary angiography or CAC scanning. Statins had been prescribed to 76% of individuals with monogenic FH and 55% had received a high potency statin (2003-2018). As shown in **Figure 7**, individuals with monogenic FH were markedly undertreated with cholesterol-lowering therapies as only 11% had attained an LDL cholesterol target below 2.6 mmol/l, as suggested by the 2016 ESC/ EAS Guidelines for the management of dyslipidemia for primary prevention in FH (Catapano et al., 2016). None had LDL cholesterol levels below 1.8 mmol/l, as suggested by the more recent 2019 ESC/EAS guidelines (Mach et al., 2020).

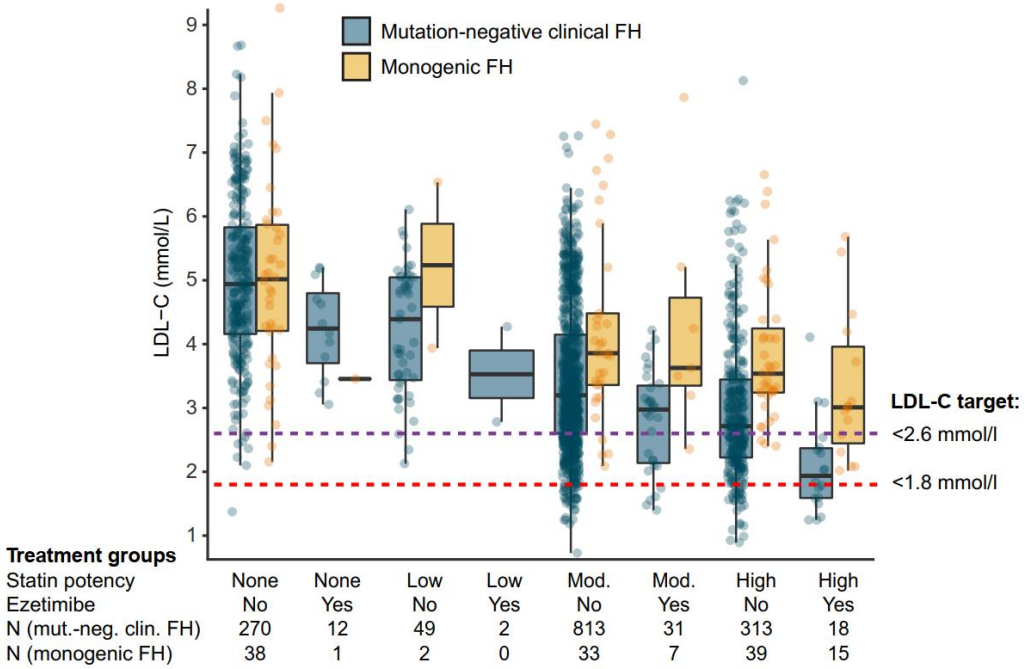

Figure 7. Achieved levels of LDL cholesterol by treatment group

Latest available LDL cholesterol measurement (years 2004-2018) for alive individuals with monogenic FH (yellow) and mutation-negative clinical FH (blue), separated by statins potency and use of ezetimibe during the preceding year. Horizontal lines indicate the recommended LDL cholesterol goals for primary prevention in FH according to the ESC/EAS guidelines from 2016 (purple, <2.6 mmol/l) and 2019 (red, <1.8 mmol/l). Reproduced from Björnsson et al., 2021 (©American Heart Association).

Table 8. FH mutations identified in 166,281 genotyped Icelanders

Gene	Position (hg38)	Alleles*	Mutation	Type	Carriers (N)	Freq (%)**	Previously identified in Iceland
PCSK9	chr1:55043921	C/T	Arg96Cys	missense	3	9.0×10^{-4}	
	chr1:55044020	G/A	Asp129Asn	missense	1	3.0×10^{-4}	
	chr1:55052398	G/A	Arg215His	missense	1	3.0×10^{-4}	
APOB	chr2:21006288	G/A	Arg3527Gln	missense	10	3.0×10^{-3}	
LDLR	chr19:11089397	C/T	c.-152C>T	promoter	21	6.3×10^{-3}	
	chr19:11102772	A/T	Asp100Val	missense	1	3.0×10^{-4}	
	chr19:11105315	G/A	Gly137Ser	missense	2	6.0×10^{-4}	
	chr19:11105599	C/A	Cys231Ter	loss-of-function	5	1.5×10^{-3}	Yes
	chr19:11105602	T/C	c.694+2T>C	loss-of-function	80	0.024	Yes
	chr19:11106640	G/A	Arg257Gln	missense	2	6.0×10^{-4}	
	chr19:11107493	G/A	Asp307Asn	missense	20	6.0×10^{-3}	Yes
	chr19:11111577	A/G	Tyr375Cys	missense	3	9.0×10^{-4}	Yes
	chr19:11113337	C/T	Arg416Trp	missense	1	3.0×10^{-4}	
	chr19:11113398	T/C	Val436Ala	missense	5	1.5×10^{-3}	
	chr19:11116125	G/A	Ala540Thr	missense	20	6.0×10^{-3}	Yes
	chr19:11116198	A/G	Asn564Ser	missense	1	3.0×10^{-4}	
	chr19:11116880	A/C	Tyr576Ser	missense	11	3.3×10^{-3}	Yes
	chr19:11120502	A/T	Asp707Val	missense	5	1.5×10^{-3}	Yes
	chr19:11112202-11114606		Ex9-10DEL	loss-of-function (deletion)	5	1.5×10^{-3}	Yes
	chr19:11129598	C/G	Asn825Lys	missense	2	6.0×10^{-4}	

PCSK9 indicates the proprotein convertase subtilisin/kexin type 9 gene; *APOB*, apolipoprotein B gene; *LDLR*, LDL receptor gene.

*Reference allele/alternative allele.

**Allele frequency in the overall sample of 166,281 genotyped individuals (49,962 of whom were also whole-genome sequenced).

4.2.1 Clinically defined FH

We defined clinical FH according to a modified version of the DLCN criteria, shown in **Table 2**. We applied these criteria to all genotyped participants that were alive between

the ages 20 and 80 years with at least one available LDL cholesterol measurement (n = 79,058 individuals). A total of 2.2% fulfilled the criteria for having clinical FH (*probable* or *definite FH*). As expected, individuals with clinical FH had high levels of maximum LDL cholesterol (mean, 7.1 mmol/l) and high burden of premature CAD (32%). We found that clinical FH was explained by monogenic FH in only about 5% of cases. Thus, about 95% with clinical FH had mutation-negative clinical FH. In analyses using a genetic score for LDL cholesterol (the same as used in **paper II**), we found that a substantial fraction of these individuals are likely to have a polygenic basis for hypercholesterolemia. To illustrate, 79% had values above the 50th percentile, 26% above the 90th percentile and 15% above the 95th percentile (compared to the expected 50%, 10% and 5%, respectively). As with monogenic FH, individuals with mutation-negative clinical FH were undertreated as only 25% and 5% attained an LDL cholesterol level below 2.6 mmol/l and 1.8 mmol/l, as endorsed by the 2016 and 2019 ESC/EAS guidelines, respectively (**Figure 7**).

Taken together, the main findings of this study were that (i) the prevalence of monogenic FH in Iceland may be close to 1 in 800, (ii) clinically defined FH is a common high-risk cardiovascular phenotype that is explained by monogenic FH in only a minority of cases, (iii) both monogenic FH and mutation-negative clinical FH are markedly undertreated with cholesterol-lowering agents in Iceland. These findings are summarized in **Figure 8**.

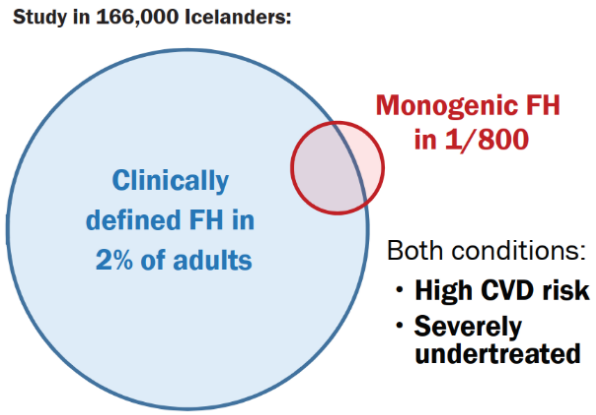

Figure 8. Graphical abstract for paper III

Reproduced from Björnsson et al., 2021 (©American Heart Association).

4.3 Paper IV: A novel deletion causes *LDLR* gain-of-function

In this study, we searched for rare sequence variants that have a large effect on levels of LDL cholesterol. We analyzed whole-genome sequencing data from 43,202 Icelanders and discovered a novel 2.5 kb deletion overlapping the 3' untranslated

region (UTR) of the LDL receptor gene (*LDLR*) (**Figure 9**). The deletion, hereafter referred to as del2.5, was identified using a newly developed algorithm named PopDel (Niehus et al., 2021) which is designed to detect medium-sized deletions in WGS data. Our analyses showed that del2.5 is confined to a single three-generation family, shown in **Figure 9**, that likely arose *de novo* in the father of the oldest carrier (individual I.2). All identified carriers with available LDL cholesterol measurements had extremely low LDL cholesterol (within the 1st percentile) with a mean of 0.87 mmol/l. In comparison, the mean level in 101,851 Icelandic non-carriers was 3.34 mmol/l (SD = 0.90 mmol/l). Thus, del2.5 associated with 74% lower LDL cholesterol, an adjusted difference of 2.48 mmol/l (P = 8.4 × 10^{-8}). Levels of triglycerides and HDL cholesterol were unaffected. Thus the carriers exhibited a FHBL-like phenotype (see chapter 1.6.2). The strong direction of effect toward lower LDL cholesterol suggested that del2.5 conferred gain-of-function of *LDLR*.

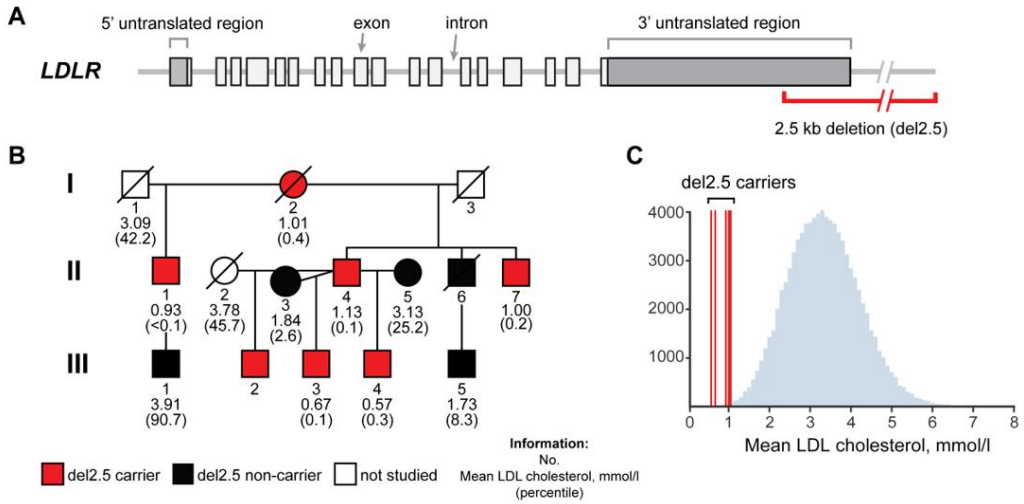

Figure 9. A novel gain-of-function mutation in *LDLR*

Panel A shows the location of the 2.5 kilobase deletion (del2.5) in *LDLR*. Del2.5 removes the distal part of the 3' untranslated region (UTR) and extends beyond the gene. Panel B shows the pedigree of the family. Red denotes carriers of del2.5 and black denotes genotyped non-carriers. The numbers presented below each individual are the individual's number (within each generation), mean level of LDL cholesterol in units of mmol/L, and age- and sex-adjusted percentile in the Icelandic population. In order to maintain anonymity, some non-participants and genotyped non-carriers were omitted from the pedigree. Panel C shows the distribution of mean levels of calculated LDL cholesterol in 101,857 genotyped Icelanders. Values for the six carriers with available measurements are indicated with red lines. Reproduced from Björnsson et al., 2020 (©American Heart Association).

In order to understand the functional consequence of del2.5, we performed 3' RACE in the blood of a carrier and generated cell lines of EBV-transformed lymphocytes from four carriers of del2.5. Using EBV-transformed lymphocytes, we performed RNA sequencing analyses and flow-cytometry-based protein expression analyses. Taken together, our results showed that by removing the distal end of the 3' UTR which contains the canonical polyadenylation signal, del2.5 leads to obligate use of an alternative polyadenylation site that is located proximally in the 3' UTR of *LDLR* mRNA (**Figure 10**). This leads to production of *LDLR* mRNA with a short 3' UTR. The short mRNA isoform was present in higher abundance than the wild-type long isoform in cells from del2.5 carriers and protein expression of the LDL receptor was 1.79-fold higher in cells from del2.5 carriers than in non-carriers (P = 0.0086). These results indicate that del2.5 causes a gain-of-function of *LDLR* through the production of a mRNA isoform with a short 3' UTR.

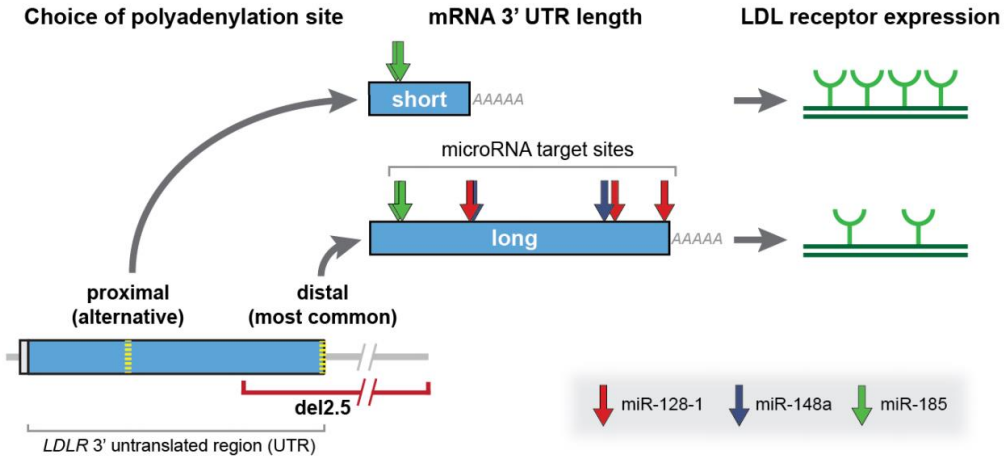

Figure 10. Schematic overview of the proposed mechanism for the gain-of-function effects of del2.5

Through removing the distal portion of the *LDLR* 3' UTR, which contains the canonical polyadenylation site (PAS), del2.5 causes an alternative, proximal PAS to be primarily used. This leads to production of mRNA with a short 3' UTR. The short 3' UTR lacks the majority of target sites for microRNAs that have been shown to negatively regulate *LDLR* mRNA stability and alter plasma LDL cholesterol in vivo. The short 3' UTR thus likely confers resistance to negative regulation, leading to greater stability of *LDLR* mRNA and increased expression of LDL receptor. This culminates in greater overall activity of the LDL receptor pathway, thereby leading to lower plasma levels of LDL cholesterol in carriers of del2.5. Reproduced from Björnsson et al., 2020 (©American Heart Association).

We assessed whether extremely low levels of LDL cholesterol that are sustained for decades may be harmful by studying the phenotypes of del2.5 carriers and comparing

them to non-carriers. Del2.5 carriers represent a unique human model to study the effects of very low LDL cholesterol as they sustain extremely low LDL levels throughout life as del2.5 is present from birth. We recruited del2.5 carriers and their family members for in-depth phenotyping through the deCODE health study (four carriers accepted to participate) and reviewed the medical records of all del2.5 carriers. Overall, we did not find evidence that del2.5 may have a harmful influence on the health of the carriers. However, these analyses were limited by the extreme rarity of del2.5 and thus we would only have been able to detect large and highly penetrant effects.

Taken together, we discovered an extremely rare deletion in *LDLR* that leads to a large reduction in plasma LDL through a gain-of-function effect. This is the first report of a large-effect gain-of-function mutation in *LDLR* and highlights the powerful impact of *LDLR* mRNA 3' UTR shortening via alternative polyadenylation on LDL receptor expression.

5 Discussion

The studies presented in this thesis investigated the role of common and rare genetic variation in the development of coronary atherosclerosis, with an emphasis on those that influence levels of blood lipids. The following discussion is structured around several key findings from these studies.

5.1 Risk variants for CAD influence atherogenesis

In **paper I**, we assessed the impact of risk variants for CAD (identified in GWA studies prior to 2015) on the extent of coronary atherosclerosis in two large samples of patients undergoing coronary angiography (over 10,000 individuals). Upon assessment of the individual and combined effects of the SNPs, we found that: (i) Their individual effects on CAD risk and angiographic extent of CAD were correlated, and (ii) the GRS associated significantly with greater extent of CAD. Furthermore, the association of the GRS remained significant after the exclusion of variants previously shown to correlate with CAD extent, at 9p21 (Dandona et al., 2010; Patel et al., 2010) and *LPA* (Helgadottir et al., 2012). Our findings thus indicated that risk variants for CAD identified prior to 2015 generally contributed toward accelerated development and progression of coronary atherosclerosis.

Our findings were in line with previous studies demonstrating associations between GRSs for CAD and subclinical carotid atherosclerosis and coronary artery calcification (Hamrefors et al., 2012; Thanassoulis et al., 2012). Since publication, our results have been replicated in two angiographic cohorts, where GRSs for CAD were found to be associated with the extent of angiographic CAD (Hindieh et al., 2016; Levin et al., 2018). Furthermore, recent studies have further established that polygenic scores for CAD associate with a greater burden of subclinical carotid and coronary atherosclerosis (Khera et al., 2016; Natarajan et al., 2016, 2017). Taken together, it is reasonable to conclude that common genetic variants that predispose to risk of CAD generally contribute to accelerated coronary atherosclerosis.

One attractive clinical application of genetic risk stratification based on genetic (or polygenic) scores is to help guide preventive screening for subclinical atherosclerosis and to identify subpopulations that could benefit from early and aggressive treatment with statins. This is supported by data from four randomized controlled trials of statins in primary (JUPITER and ASCOT) and secondary prevention (CARE and PROVE IT-TIMI 22), and a community-based cohort study (the Malmö Diet and Cancer Study), together comprising over 48,000 individuals, in which individuals within the highest polygenic risk were found to have the greatest clinical benefit from statin therapy (Mega et al., 2015). Similar findings were also observed in data from the WOSCOPS

randomized controlled trial of statins in primary prevention (Natarajan et al., 2017). Despite these prospects, however, genetic risk stratification has not been implemented in standard clinical care (Hadley et al., 2021). This is due to various factors such as costs of genotyping, lack of standardization in generation, validation and interpretation of polygenic scores, and the fact that the potential benefit of genetic risk prediction in clinical practice has not been assessed in prospective randomized clinical trials (Hadley et al., 2021; Wand et al., 2021). Taken together, genetic risk prediction may open up possibilities to improve prevention and treatment of cardiovascular disease, but further studies are needed before it can be implemented in clinical care (Hadley et al., 2021; Roberts et al., 2021).

5.2 non-HDL cholesterol is the best lipid marker of atherogenic apoB lipoproteins

In **paper II**, we assessed the impact of genetically predicted blood lipid traits on the extent of coronary atherosclerosis in over 17,000 individuals who underwent coronary angiography or CAC assessment. We found that the genetic score for non-HDL cholesterol was most strongly associated with extent of atherosclerosis and provided predictive information after accounting for the genetic score for LDL cholesterol. Overall, the non-HDL cholesterol genetic score effectively captured all associations of the genetic scores for LDL cholesterol, triglycerides and HDL cholesterol. These Mendelian randomization analyses extended our previous findings in the context of CAD risk (Helgadottir et al., 2016) (see chapter 1.5) and further support a causal relationship between non-HDL cholesterol and the development and progression of atherosclerosis.

In recent years, increasing evidence suggests that all apoB lipoproteins are atherogenic (Borén et al., 2020). As LDL accounts for the vast majority of apoB lipoproteins, LDL cholesterol is a useful marker of a large fraction of apoB lipoproteins (Langlois et al., 2020). LDL cholesterol, however, is a poor marker of larger apoB lipoproteins such as chylomicron remnants and VLDL and its remnants (collectively known as triglyceride-rich lipoproteins [TGLs] or remnant particles) (Langlois et al., 2020). In recent years, multiple genetic studies have supported a causal relationship between levels of TGLs and the risk of CAD (Crosby et al., 2014b; Dewey et al., 2016; Do et al., 2013; Helgadottir et al., 2016; Jørgensen et al., 2014; Khera et al., 2017; Myocardial Infarction Genetics and CARDIoGRAM Exome Consortia Investigators et al., 2016; Richardson et al., 2020; The Emerging Risk Factors Collaboration, 2009; Varbo et al., 2013). Our findings are consistent with a causative role of TGLs in the pathogenesis of coronary atherosclerosis, as genetically predicted non-HDL cholesterol remained a significant predictor of the extent of coronary atherosclerosis after accounting for LDL cholesterol (thus approximating remnant cholesterol). The atherogenic properties of TGLs are, however, most likely explained by their content of

apoB and cholesterol but not the triglycerides *per se*, although certain triglyceride-related mechanisms may play some role (Borén et al., 2020).

In clinical practice, a comprehensive assessment of risk associated with blood lipoproteins should not be limited to LDL, but rather consider the entire spectrum of apoB lipoproteins (including TGLs and Lp(a)) (Langlois et al., 2020). For this purpose, non-HDL cholesterol and apoB are useful biomarkers that reflect all circulating apoB lipoproteins. Non-HDL cholesterol reflects the cholesterol content of apoB lipoproteins, whereas apoB reflects their concentration (one apoB molecule per particle). Both non-HDL cholesterol and apoB consistently outperform LDL cholesterol in risk prediction, particularly when TGLs are disproportionally elevated (Arsenault et al., 2011). Few studies have compared directly the usefulness of non-HDL cholesterol and apoB for cardiovascular risk prediction. Among 2966 individuals in the Framingham Offspring Cohort, apoB was reported to improve cardiovascular risk prediction over both non-HDL cholesterol and LDL cholesterol (Pencina et al., 2015). However, in a recent study of 13,015 statin-treated patients from the Copenhagen General Population Study, although all-cause mortality was found to track more closely with apoB (over non-HDL cholesterol), prediction of myocardial infarction tracked more closely with non-HDL cholesterol (Johannesen et al., 2021). Taken together, non-HDL cholesterol and apoB are consistently better markers of cardiovascular risk than LDL cholesterol, but there is limited evidence in support of favoring either non-HDL cholesterol or apoB over the other. Due to its availability and lower cost, as it is readily calculated from the standard lipid panel, non-HDL cholesterol may be preferred for many applications. Currently, the European Atherosclerosis Society and the European Federation of Clinical Chemistry and Laboratory Medicine recommend either non-HDL cholesterol or apoB to be considered as an adjunct to the standard lipid profile, in order to better estimate the overall risk associated with atherogenic lipoproteins (Langlois et al., 2020).

5.3 The prevalence of monogenic FH in Iceland is relatively low

In **paper III**, we performed large-scale genetic screening for monogenic FH among >160,000 Icelanders. We identified 199 carriers of FH mutations, yielding an overall monogenic FH prevalence of 1 in 836. The prevalence was higher in the subsample of 50,000 participants who had been selected for WGS, or 1 in 515. The higher prevalence of monogenic FH in the WGS sample was expected due to the enrichment for individuals with high LDL cholesterol levels in that sample (approximately 2-fold enrichment for LDL cholesterol levels >99th percentile, compared to those who were not selected for WGS). Owing to several factors, including relatively strict criteria for inclusion of FH mutations that limit potential false-positives (e.g. we did not consider *in silico* predictions of pathogenicity) and the fact the WGS subsample (the 'discovery' sample used for identification of FH mutations) comprises only a third of the overall sample, it is likely that we have underestimated the prevalence of monogenic FH to some degree. Thus, the overall prevalence of 1 in 836 in our sample can be viewed as

a lower bound for the true prevalence in Iceland. Similarly, due to the enrichment for high LDL cholesterol levels, the prevalence in the WGS subset (1 in 515) likely represents an upper bound for the true prevalence of monogenic FH in Iceland.

Our results suggest that the prevalence of monogenic FH in Iceland is considerably lower than estimates from recent genetic studies in large population samples from Denmark (1 in 217) (Benn et al., 2016), the United States (1 in 260 to 1 in 211) (Abul-Husn et al., 2016; Grzymski et al., 2020; Khera et al., 2016). and the United Kingdom (1 in 176) (Trinder et al., 2020) (see chapter 1.6.1 for details). Although methodological aspects relating to potential overestimation of monogenic FH prevalence in some of these studies (chapter 1.6.1) as well as the potential underestimation in our study may explain these differences to a degree, it is reasonable to conclude that it is likely that monogenic FH is less common in Iceland than in many European populations. The geographic isolation of Iceland and the resulting genetic homogeneity of its population offers a likely explanation (Helgason et al., 2003). For example, one of the most common FH mutations in Europe (Arg3527Gln in *APOB*) (Liyanage et al., 2011) is 10 times more common in populations outside of Iceland (allele frequency in Iceland is 3×10^{-5}, versus 3×10^{-4} in the Genome Aggregation Database (*Genome Aggregation Database (GnomAD)*, 2021)). In fact, in our study, Arg3527Gln was clustered within three unrelated families. In each family, the mutation could be traced to a likely foreign origin.

We observed evidence that the diversity of FH mutations in Iceland has increased during the last century. We found that 5 out of 20 FH mutations in our study (including Arg3527Gln) could be traced to foreign ancestors who likely settled in Iceland during the last century or so. Historically, Iceland has been a homogeneous and isolated society with little immigration of foreigners. In the last three decades, however, immigration to Iceland has grown considerably. For example, in 1996, 95% of the population did not have a recent foreign background whereas two decades later, in 2017, this number had fallen to 83% (Heleniak & Sigurjonsdottir, 2018). In 2020, immigrants (those with both parents foreign born and all grandparents foreign born) comprised over 15% of the Icelandic population (Statistics Iceland, 2020). Along with the projected increase in immigration over the next decades, the ethnical and genetic diversity of the Icelandic population will continue to grow (Heleniak & Sanchez Gaessen, 2016). It is thus logical to assume that FH mutations from other populations will be introduced to the Icelandic gene pool over time. With growing immigration from countries with higher prevalence of monogenic FH, the overall prevalence in Iceland could also be expected to rise over time.

5.4 Clinical FH is common but is rarely caused by a single mutation

In **paper III**, we screened for the high-risk clinical FH phenotype among 79,000 adult participants using the modified DLCN criteria. In this sample, we found that 2.2% could

be classified as having clinical FH (defined as probable or definite FH according to the criteria). This is considerably higher than previous estimates in large population samples (up to 1.2%) (Abul-Husn et al., 2016; Benn et al., 2012, 2016; Bucholz et al., 2018) (see chapter 1.6.1). One explanation that may account for these differences is the quality of the data we used to inform the DLCN criteria. When applying the DLCN criteria to large datasets, lack of information will inevitably lead to underestimation of the prevalence of clinical FH. Our study benefitted from nationwide coverage for diagnoses of premature CAD and longitudinal cholesterol measurements that account for nearly all cholesterol measurements taken in Iceland over the last three decades. In addition, we used comprehensive genealogical information in order to accurately define family history variables that were not subject to recall bias. This contrasts with some previous studies, where lack of available information may have led to underestimation of clinical FH (see chapter 1.6.1). Thus, the relatively higher prevalence of clinical FH in Iceland may be related to the quality of information used to inform the DLCN criteria, and does not necessarily indicate that the true prevalence of clinical FH is higher in Iceland than elsewhere.

We found that only 5% of individuals with the clinical FH phenotype had monogenic FH. In other words, 95% of these individuals did not carry a causative mutation. This is in line with results in the Geisinger Health System study (Abul-Husn et al., 2016) where, out of the 1.2% who had clinical FH, only 9% had monogenic FH. These findings strongly suggest that the clinical FH phenotype is much more common than monogenic FH. Consistent with previous observations (Mariano et al., 2020; Talmud et al., 2013; J. Wang et al., 2016), we found that polygenic influences are likely to account for a large fraction of cases of clinical FH where a mutation is not identified. Taken together, clinical FH represents a relatively common hypercholesterolemia syndrome that has a strong polygenic component, but is caused by monogenic FH in only a minority of cases.

5.5 Monogenic and clinical FH are severely undertreated

In **paper III**, we found that the majority of individuals with monogenic FH and clinical FH were severely undertreated with cholesterol-lowering medications in Iceland. Both conditions are characterized by extremely high cardiovascular risk, where aggressive LDL-lowering is recommended to reduce that risk (Mach et al., 2020). We found that a minority reached a LDL cholesterol goal of <2.6 mmol/l (11% and 25%, respectively) as recommended by the 2016 ESC/EAS cholesterol guidelines (Catapano et al., 2016) and even fewer reached <1.8 mmol/l (0% and 5%) as suggested by the more recent 2019 ESC/EAS guidelines for primary prevention in FH (Mach et al., 2020). The overall number of individuals that did not achieve guideline-directed goals is likely greater, however, as some of these individuals have manifest cardiovascular disease and the goals for secondary prevention in FH are considerably lower (Catapano et al., 2016; Mach et al., 2020). Our findings are consistent with large body of evidence that FH is

undertreated in most countries (Nordestgaard et al., 2013). Due to the fact that around 2% of Icelandic adults may have clinical FH, the severe undertreatment of this relatively large group of high-risk individuals represents a significant public health problem.

Likely explanations for undertreatment include clinical underdiagnosis and underuse of effective cholesterol-lowering medications such as high-intensity statins with the addition of ezetimibe (only about 40% of individuals with monogenic FH were prescribed high-intensity statins). Our analyses did not include the newer and more potent PCSK9 inhibitors as they had only recently been approved and were in limited use before the end of the study period in 2018.

Our findings mandate the need for improved recognition and diagnosis of both monogenic FH and clinical FH in Iceland. This could be aided by public health initiatives in order to: (i) increase awareness of FH among clinicians, (ii) improve clinical screening for hypercholesterolemia and FH, (iii) facilitate referrals for genetic testing and subsequent family cascade screening, (iv) increase the use of effective cholesterol-lowering therapies in order to achieve guideline-directed targets.

5.6 Implications for diagnosis of monogenic FH in Iceland

In **paper III**, we characterized 20 mutations that cause monogenic FH in Iceland. The founder mutation (splice donor mutation in *LDLR*, c.694+2T>C) that has been thought to account for the majority of monogenic FH in Iceland (Gudnason et al., 1997) accounted for about 40% of cases in our study. Prior to the publication of **paper III**, nine mutations were known to cause FH in Iceland (see chapter 1.6.1). Thus, over half of the FH mutations we identified were not previously known to cause monogenic FH in Iceland. It is reasonable to assume that the 20 mutations we identified likely account for the vast majority of cases of monogenic FH in Iceland.

5.6.1 Implications for clinical genetic testing in Iceland

Currently, genetic testing for FH at the only clinical genetics laboratory in Iceland (Landspítali – The National University Hospital) is limited to two *LDLR* mutations: c.694+2T>C (the Icelandic founder mutation) and Ala540Thr (personal communication, Eiríkur Briem, 2021). When neither of these mutations are found, the sample is often sent abroad for genotyping of a panel of FH mutations. Such panel genotyping has identified Icelandic carriers of seven additional FH mutations in *LDLR*: c.-152C>T, c.190+4A>T, Asp307Asn, Tyr375Cys, Tyr576Cys, Asp707Val, 2 kb deletion of exons 9 and 10 (personal communication, Eiríkur Briem 2021). All the mutations that have been detected upon clinical genetic testing in Iceland, with the exception of c.190+4A>T, were also identified in our study where they accounted for 72% of cases of monogenic FH. Thus, theoretically, if the currently available genotyping methods were used in the individuals with monogenic FH in our study, one would expect to detect a causative mutation in at least over 70% of cases.

Therefore, the primary reason for the apparent underdiagnosis of monogenic FH in Iceland is not due to inadequacy of the available genetic testing methods, but rather their underutilization. This is not a problem unique to Iceland, however, as genetic testing for FH is underused in most countries (Nordestgaard et al., 2013; Sturm et al., 2018). There are many possible reasons for the underuse of genetic testing for FH in Iceland. These may include: (i) lack of clinical recognition of hypercholesterolemic individuals who are at high risk of having monogenic FH (e.g. strong family history or the clinical FH phenotype), (ii) lack of clinical screening of family members of individuals at risk of having monogenic FH, (iii) clinical deferral of testing due to perceived high cost or lack of perceived benefit by the treating physician, and (iv) lack of Icelandic guidelines on when primary care providers and medical specialists should refer for genetic testing.

Our findings can be used to directly improve the yield and lower the cost of genetic testing of FH in Iceland. For example, the 20 identified mutations could form the basis for an Icelandic-specific mutation panel. Genetic testing of mutations on this panel could be carried out at the clinical genetics laboratory at Landspítali. For initial genetic testing for suspected FH, use of this panel would undoubtedly increase the overall yield at Landspítali and thus lower the costs and turn-around time associated with sending samples abroad for panel testing. An analysis of the cost-effectiveness of FH panel testing at Landspítali is needed, but we are confident that the current methods of genetic testing can be greatly improved based on our findings.

Taken together, the underdiagnosis of monogenic FH in Iceland is mainly due to the underuse of genetic testing. Panel testing for the identified FH mutations would improve the yield of genetic testing and could be carried out at Landspítali. There is an urgent need to implement strategies within the public healthcare system to increase clinical recognition of the severe hypercholesterolemia phenotype and increase the use of genetic testing in high-risk groups.

5.6.2 Potential usage of existing genetic information at deCODE genetics

Another clinical implication of our findings relates to the possibility of notifying participants that carry FH mutations of their genotype and the associated risk. In accordance with licenses under which deCODE genetics conducts research, participants are not approached and informed of their genotype. Recently, deCODE genetics undertook a project to offer genetic results for the *BRCA2* founder mutation (carrier frequency 0.7-0.8%), a major cause of hereditary breast cancer in Iceland (Stefansdottir et al., 2020). In May 2018, deCODE genetics launched a website (arfgerd.is) where participants, and other Icelanders willing to participate by giving a DNA sample, could sign an informed consent to voluntarily receive their *BRCA2* status. One year after its launch, over 46,000 Icelanders had received genetic results through the website and 352 (0.77%) been informed of a positive result and offered genetic

counselling (Stefansdottir et al., 2020). From the perspective of genetic counsellors at Landspítali, the web-based return of genetic results for *BRCA2* mutations was found to work well in the Icelandic society and the overall experience was positive (Stefansdottir et al., 2020).

Like *BRCA2* mutations that cause hereditary breast cancer, mutations that cause FH are clinically actionable (Centers for Disease Control and Prevention, 2019). Unlike in *BRCA2*, where surgery with bilateral mastectomy is the only definite method for primary prevention, preventive therapy in FH mutation carriers is non-invasive and is based on lifelong medical treatment, most often with pills taken once per day. Preventive therapy in monogenic FH reduces atherosclerotic events and is most effective when started early, preferentially in childhood (Luirink et al., 2019). In primary prevention for monogenic FH, an accurate and early diagnosis is paramount. It is reasonable to assume that most individuals with monogenic FH in our study are unaware of their inherited risk and that their obvious undertreatment is largely attributed to underdiagnosis. It is also reasonable to assume that if these individuals were aware of their inherited risk and were offered effective treatment to lower that risk, many would opt for treatment. Therefore, it is likely that a program to return genetic results for FH mutations to deCODE genetics' research participants could have a positive impact on health of many mutation carriers and potentially save lives.

The design and implementation of a program to return genetic results for FH mutations to participants could build on the experience gained by the *BRCA2* initiative and make use of existing infrastructure and expertise. In comparison with *BRCA2*, however, the rarity of FH mutations (affecting about 1:800 vs about 1:125 for *BRCA2*) may reduce the overall yield. In a theoretical example, if genetic results for FH were returned to an unselected sample of 46,000 individuals (same as received results for *BRCA2* over the course of one year), only about 60 individuals with monogenic FH would be identified based on the prevalence of about 1 in 800. Thus, it could be useful to focus especially on risk groups with higher prevalence of monogenic FH in our study, such as those with LDL cholesterol above 4.9 mmol/l (prevalence of 1 in 133) and premature CAD (1 in 69), and those with a known family history. Additionally, possibly implementing access for health professionals to order and receive genetic information on behalf of their patients, based on their informed consent, could also increase the yield for diagnosis of monogenic FH. As with *BRCA2*, all participants who are willing to receive information on FH mutations would receive ample information, provide informed consents and those with a positive result be provided with genetic counselling and means for clinical follow-up and further screening among family members. The details on the design and implementation of such a program is beyond the scope of this work.

5.7 Importance of accurate terminology in FH

In **paper III**, we demonstrated that there are important differences between monogenic FH and the clinical FH phenotype. The differences between these partially overlapping entities relate to several factors: (i) *Cause*. Monogenic FH is a genetic disease caused by a single FH mutation. Clinical FH is a phenotype that mimics the late clinical manifestations in monogenic FH, but is most commonly due polygenic influences, environmental factors, or a combination thereof. (ii) *Diagnosis*. Monogenic FH can be diagnosed with a genetic test. Clinical FH is by definition a phenotypic diagnosis and cannot be diagnosed with a genetic test. (iii) *Identification of asymptomatic individuals for primary prevention*. This is possible in monogenic FH, as genetic testing can identify mutation carriers at any age, even before the onset of significant atherosclerosis. Clinical FH, however, is most often diagnosed when cardiovascular disease is established and only rarely are these individuals asymptomatic. In addition, the DLCN criteria are not appropriate for use in children (Wiegman et al., 2015). (iv) *Co-morbidities*. Individuals with clinical FH are generally older and more often have manifestations of cardiovascular disease and greater burden of risk factors such as hypertension and smoking.

In clinical practice and in the scientific literature, the term ‘FH’ can mean several things. Usually, ‘FH’ is either a diagnosis based on genotype (i.e. monogenic FH) or phenotype (i.e. clinical FH). Sometimes, however, terms such as ‘heterozygous FH’ are confusingly used to refer to the clinical FH phenotype regardless of whether a causative mutation is found or not (Fahed et al., 2011). This use of ‘heterozygous FH’ dates back to the 1960s, when the phenotypes associated with heterozygous and homozygous FH were first described and differentiated (Khachadurian, 1964). In the following decades, diagnoses of ‘heterozygous FH’ or ‘homozygous FH’ were usually based on the clinical presentation alone, as the genetic architecture of FH was largely unknown until the 1980s and genetic testing limited. As genetic testing has become increasingly available and accurate, the use of the term ‘heterozygous FH’ to refer to a clinical phenotype is misleading. This is because ‘heterozygous’ implies the presence of a causative mutation and thus inappropriate in the absence of one. Monogenic FH and the clinical FH phenotype have even been combined in large meta-analyses to estimate the prevalence of ‘heterozygous FH’ (Beheshti et al., 2020). This practice can easily lead to erroneous conclusions, as monogenic FH and clinical FH are only partially overlapping entities and are not interchangeable.

Taken together, the umbrella term ‘FH’ represents several related but non-interchangeable entities. Several groups have recognized the need for refinement of the terminology used with respect to FH (Brandts et al., 2020; Fahed et al., 2011; Masana et al., 2019). For example, the use of the genetic terms ‘heterozygous FH’ and ‘monogenic FH’ should be restricted to cases where a mutation has been identified. In cases where FH is strongly suspected based on clinical presentation but a causative

mutation has not been identified, terms such as ‘clinical FH’ (if fulfilling one of the clinical criteria for FH, see chapter 1.6.1) or ‘suspected FH’ should be used instead. Our findings support that genetic testing should be carried in all individuals with a phenotype compatible with FH to identify those with monogenic FH, as the presence of a mutation entails specific implications for family cascade screening and management (Brandts et al., 2020).

5.8 Discovery of a gain-of-function mutation in *LDLR*

In **paper IV**, we describe the discovery of a gain-of-function mutation in *LDLR*. This is the first report of a gain-of-function mutation in *LDLR* with a large effect on levels of LDL cholesterol. Previously, several LDL-lowering variants had been described in *LDLR*, but their impact on LDL cholesterol was mild to moderate (Gretarsdottir et al., 2015; Natarajan et al., 2018; Van Zyl et al., 2014).

The mutation, a 2.5 kb deletion in the 3’ UTR, was found in seven heterozygous carriers from a single family in Iceland who all had very low levels of LDL cholesterol (mean 0.87 mmol/l, <1st percentile of the population distribution). Carriers of the deletion had 74% lower calculated LDL cholesterol and 54% lower levels of apoB, compared to non-carriers. Functional analyses showed that the deletion affects *LDLR* polyadenylation *in cis*, leading to the obligate use of a proximal alternative polyadenylation site. This, in turn, causes considerable shortening of the 3’ UTR on *LDLR* mRNA (**Figure 10**, chapter 4.3). This shorter 3’ UTR, that lacks negative regulatory elements, likely escapes various repressive elements such as microRNAs and thus results in almost two-fold higher expression of the LDL receptor in cells derived from carriers of the deletion. Higher expression of the LDL receptor, in turn, leads to increased hepatic LDL clearance and thus lower plasma LDL levels. The observed effects of the deletion are in line with what could be expected based on shortening of the *LDLR* mRNA 3’ UTR as indicated by previous studies (Goedeke et al., 2015; Jiang et al., 2015; Knouff et al., 2001; H. Li et al., 2009; Wagschal et al., 2015; Wilson et al., 1998; Yang et al., 2014), but our study represents the first demonstration in humans that shortening of the 3’ UTR of *LDLR* mRNA increases expression of LDL receptors and thus lowers plasma LDL.

These findings highlight the importance of the 3’ UTR in regulation of *LDLR* mRNA expression levels. Our discovery of a mutation that causes 3’ UTR shortening and extreme lowering of plasma LDL in humans offers compelling evidence that therapeutic alteration of the 3’ UTR length and its negative regulatory elements could be effective in lowering plasma LDL.

5.9 Are extremely low levels of LDL cholesterol harmful?

The cholesterol required for various biological processes within cells is generally derived from *de novo* synthesis or through uptake of circulating LDL via LDL receptors (Luo et al., 2020). For decades, it has been debated whether low levels of circulating

LDL cholesterol may impair cholesterol-reliant processes and thus have an adverse impact on health (Hartz et al., 2019; Olsson et al., 2017). This debate has been revitalized in recent years following the introduction of high-potency statins and PCSK9 inhibitors, which together may lower LDL cholesterol to levels below 0.5 mmol/l (Sabatine et al., 2018). Based on currently available evidence, extreme LDL-lowering with PCSK9 inhibitors on top of high-intensity statins appears to be safe and not associated with significant adverse effects (Cybulska et al., 2020; Giugliano et al., 2017; Koren et al., 2019; Robinson et al., 2017; Sabatine et al., 2017; Schwartz et al., 2018; Wang et al., 2020). However, as of 2021, the effects of long-term treatment with PCSK9 inhibitors beyond five years are still unknown.

Genetic mutations are present from birth and remain constant throughout life. Mutations that cause extreme LDL-lowering thus offer an opportunity to assess the potential health implications of having lifelong very low levels of LDL cholesterol. Monogenic conditions that cause extremely low LDL levels may be divided into two groups according to their primary mechanism by which they lower plasma LDL: (i) Mutations that impair apoB lipoprotein production (heterozygous *APOB* loss-of-function mutations causing FHBL, and ultrarare homozygous conditions such as abetalipoproteinemia and chylomicron retention disease), and (ii) Mutations that increase LDL clearance via LDL receptors (homozygous *PCSK9* loss-of-function and heterozygous *LDLR* gain-of-function [described in **paper IV**]). As the primary mechanism underlying the LDL-lowering effects of statins and PCSK9 inhibitors is up-regulation of LDL receptors (Horton et al., 2009), these effects are best reflected by mutations in the latter group. In comparison, mutations that impair apoB lipoprotein production and cause FHBL are not as suitable to assess the lifelong effects of LDL-lowering due to the different mechanism and known systemic adverse effects such as steatohepatitis (see chapter 1.6.2).

In **paper IV**, we carried out a comprehensive analysis of the carriers of *LDLR* gain-of-function deletion in order to assess whether lifelong very low levels of LDL cholesterol are potentially deleterious to human health. Through review of medical records of all seven carriers and an in-depth phenotyping study of the four that accepted to participate in the study, we did not find compelling evidence that the deletion may have an adverse impact on their health. An important limitation, however, is that due to the extreme rarity of the deletion, our analyses were not powered to detect small or moderate differences. Thus, although we did not find evidence of highly penetrant adverse effects, we cannot exclude milder effects. Previously, complete genetic deficiency *PCSK9* causing LDL cholesterol levels around 0.40 mmol/l was demonstrated to be viable and possibly benign, but limited to only two individuals (Hooper et al., 2007; Zhao et al., 2006). Despite having only seven carriers of the *LDLR* deletion, our study represents the largest and most comprehensive characterization of individuals with isolated extremely low LDL levels due to a monogenic condition.

We noted a non-significant trend toward mildly impaired performance on several neurocognitive tests in the four carriers tested. Although these analyses were underpowered to conclude on any potential relationship, a potential link between very low LDL concentrations and impaired neurocognition has been hypothesized (Banach et al., 2017). The evidence, however, has been largely inconsistent and inconclusive (Banach et al., 2017). Notably, data from large clinical trials of statins and PCSK9 inhibitors have not supported that intense LDL-lowering may contribute to cognitive impairment (Gencer et al., 2020; Giugliano et al., 2017; Swiger et al., 2013). One limitation, however, is that data on the effects of PCSK9 inhibitors is limited to a relative short median follow-up period of about two years (Gencer et al., 2020; Giugliano et al., 2017). Thus, the potential adverse effects of intense LDL-lowering with PCSK9 inhibitors over many years and even decades remain unknown. Taken together, our findings are inconclusive and neither support nor refute that lifelong very low plasma LDL may influence neurocognition. If given that very low LDL levels truly contribute to impaired cognition, however, our findings may suggest that the effect is likely to be mild and thus outweighed by the atheroprotective effects.

The causal and log-linear relationship between levels of LDL cholesterol and risk of CAD is well established (Borén et al., 2020). As a result, the observed 74% lowering of calculated LDL cholesterol due to the *LDLR* gain-of-function deletion would be expected to result in considerable atheroprotection. Mendelian randomization studies have estimated that genetically lower LDL cholesterol by 0.35 mmol/l reduces the risk of CAD by 21% (odds ratio, 0.79) (Ference et al., 2017). If these results are extrapolated to the *LDLR* deletion, the observed lowering of LDL cholesterol by 2.5 mmol/l (adjusted absolute difference between carriers and non-carriers) could be expected to lower CAD risk by about 80%.[a] In our study, upon review of medical records, none of the carriers had established atherosclerotic disease. One carrier had undergone coronary angiography with a finding of angiographically normal coronary arteries, but none had undergone imaging for subclinical atherosclerosis. Therefore, due to the small sample size and lack of information on extent of coronary atherosclerosis in the carriers, we were unable to directly assess the likely atheroprotective effects of the *LDLR* deletion.

Taken together, our in-depth phenotypic analyses of carriers of the *LDLR* deletion do not indicate that having lifelong very low levels of LDL cholesterol may be significantly detrimental to human health. However, we cannot exclude potential adverse effects with a mild-to-moderate effect size or those that were not directly assessed in our in-depth phenotypic study.

[a] If a decrease in LDL cholesterol by 0.35 mmol/l corresponds to odds ratio (OR) of 0.79, then the OR for decrease in LDL cholesterol by 2.48 mmol/l can be calculated as follows (assuming a log-linear relationship): log(OR) = log(0.79) × (2.48/0.35) = -1.67, which gives OR = exp(-1.67) = 0.19.

6 Strengths and limitations

The studies presented in this thesis have several important strengths in common. First, the studies were made possible by the unique deCODE genetics database which holds extensive information on the genotypes and phenotypes of over 160,000 genotyped Icelanders. The large fraction of participants who have undergone WGS (almost 50,000 as of 2020) and accurate imputation methods, facilitated by genetic homogeneity of the Icelandic population and known genealogy, enable accurate study of millions of common and rare sequence variants identified in this population. Second, the WGS subsample is enriched for individuals with extreme phenotypes (e.g. very high or very low levels of LDL cholesterol) due to their intentional selection for WGS. This enrichment has facilitated the identification of rare sequence variants with a large impact on LDL cholesterol, such as those presented in papers III and IV. Third, most phenotypes were obtained from large healthcare institutions that serve the majority of the Icelandic population, including the only tertiary care hospital and the only cardiac catheterization lab in Iceland, as well as several nationwide clinical registries. Thus, the availability of most phenotypes is relatively unbiased and only limited by their acquisition in the clinical setting (i.e. decision of the treating physician to order a certain blood test). Of particular relevance, cholesterol measurements spanning almost three decades were available for over 100,000 participants. In addition, a complete nationwide registry of all drug prescriptions in Iceland covering a period of over 15 years was used to infer statin use among the participants.

Several limitations of these studies deserve mention. First, although our genotyped sample represents a large fraction of the Icelandic population, it is not a random sample and may thus not be representative of the Icelandic population in some respects. Second, the availability of most phenotypes was limited by their acquisition in clinical practice. For example, disease diagnoses, clinical registry data and blood samples are dependent on various individual-related factors (e.g. nature of symptoms, willingness and ability to seek medical help, socioeconomic status and place of residence) as well as physician-related factors (e.g. assessment of clinical symptoms, availability of diagnostic tests, choice of diagnostic tests, registration of diagnoses). In some cases, this non-uniform coverage of phenotypes in our sample may introduce bias, especially if the availability of a certain phenotype (e.g. probability of having a certain blood measurement) is influenced by the genotype of interest. Third, approximately a third of the overall genotyped sample has undergone WGS in addition to chip genotyping. Therefore, ultrarare variants that are only found in those who have not undergone WGS (i.e. chip-genotyped only, about 110,000 individuals) will inevitably go undetected in our data. This has particular relevance to identification of FH mutations, where potential mutations present only in those who have not undergone WGS will be missed, leading to underestimation of monogenic FH prevalence.

7 Concluding remarks and future directions

Cardiovascular disease remains one of the most important causes of morbidity and mortality worldwide (Murray et al., 2012). Over the last two decades, the study of human genetics has improved our understanding of the complex pathology of cardiovascular disease and contribution of its risk factors (Musunuru & Kathiresan, 2019). Insights gained from genetic studies have led to the development of new therapeutics to lower cardiovascular risk, as exemplified by the success of PCSK9 inhibitors (Horton et al., 2009). The continuing study of rare and common genetic variation is an important endeavor that will likely lead to further advances in prevention and management of cardiovascular disease.

This thesis focused on common and rare sequence variants that influence the risk of CAD and plasma levels of atherogenic lipoproteins. **Paper I** highlighted the contribution of common genetic risk factors of CAD to the coronary atherosclerotic process. **Paper II** studied common genetic determinants of atherogenic lipoproteins and how they relate to coronary atherosclerosis. **Paper III** provided a comprehensive analysis of monogenic FH and clinical FH in a large fraction from the Icelandic population, underscoring their underdiagnosis and undertreatment. **Paper IV** described the discovery of a novel *LDLR* gain-of-function mutation that causes extreme lowering of plasma LDL through increased LDL receptor expression.

My sincere hope is that the incremental gain in knowledge provided by these studies will be an inspiration for further studies to advance our understanding of the genetics of cardiovascular disease. Future research in this field has the potential to improve prevention and management of cardiovascular disease. With respect to FH, there is a great need to improve its diagnosis and management in Iceland and worldwide. With improved diagnosis of FH and early institution of effective LDL-lowering treatment, we clearly have the possibility to save lives. My hope is that the work on FH presented within this thesis will eventually lead to increased recognition and improvement in the management of individuals with FH in Iceland. The discovery of the gain-of-function mutation in *LDLR* (3' UTR deletion) deserves special mention, as this finding highlights the profound implication of the 3' UTR in regulation of *LDLR* mRNA expression. Further studies are needed to study the factors that influence alternative polyadenylation in *LDLR* (and thus the mRNA 3' UTR length) and the mechanism of 3' UTR-associated negative regulatory elements, in order to assess the possibility of molecular intervention to influence these processes. Such studies could potentially unveil novel methods to lower plasma LDL and thus reduce cardiovascular risk.

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Appendix

Paper I

Common Sequence Variants Associated With Coronary Artery Disease Correlate With the Extent of Coronary Atherosclerosis

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Objective—Single-nucleotide polymorphisms predisposing to coronary artery disease (CAD) have been shown to predict cardiovascular risk in healthy individuals when combined into a genetic risk score (GRS). We examined whether the cumulative burden of known genetic risk variants associated with risk of CAD influences the development and progression of coronary atherosclerosis.

Approach and Results—We investigated the combined effects of all known CAD variants in a cross-sectional study of 8622 Icelandic patients with angiographically significant CAD (≥50% diameter stenosis). We constructed a GRS based on 50 CAD variants and tested for association with the number of diseased coronary arteries on angiography. In models adjusted for traditional cardiovascular risk factors, the GRS associated significantly with CAD extent (difference per SD increase in GRS, 0.076; $P=7.3\times10^{-17}$). When compared with the bottom GRS quintile, patients in the top GRS quintile were roughly 1.67× more likely to have multivessel disease (odds ratio, 1.67; 95% confidence interval, 1.45–1.94). The GRS significantly improved prediction of multivessel disease over traditional cardiovascular risk factors (χ^2 likelihood ratio 48.1; $P<0.0001$) and modestly improved discrimination, as estimated by the *C*-statistic (without GRS versus with GRS, 64.0% versus 64.8%) and the integrated discrimination improvement (0.52%). Furthermore, the GRS associated with an earlier age at diagnosis of angiographic CAD. These findings were replicated in an independent sample from the Emory Biobank study (n=1853).

Conclusions—When combined into a single GRS, known genetic risk variants for CAD contribute significantly to the extent of coronary atherosclerosis in patients with significant angiographic disease. **(*Arterioscler Thromb Vasc Biol*. 2015;35:1526-1531. DOI: 10.1161/ATVBAHA.114.304985.)**

Key Words: atherosclerosis ■ coronary disease ■ genetics

Coronary artery disease (CAD) is a complex disease with both environmental and heritable contributions.[1] To date, genome-wide association studies have yielded common single-nucleotide polymorphisms (SNPs) at 50 chromosomal loci associated with risk of CAD.[2] Multilocus genetic risk scores (GRSs) combining multiple SNPs with modest effects on cardiovascular risk have been shown to predict incident cardiovascular events in several prospective cohorts of European ancestry.[3–10] GRSs based on common CAD risk variants have been associated with atherosclerotic phenotypes such as peripheral artery disease,[11] carotid intima-media thickness,[12] and coronary artery calcium,[6] which is an indirect measure of atherosclerotic burden.

Coronary angiography remains the "gold standard" in quantifying the extent and severity of CAD and thus atherosclerotic burden. Previous studies have shown that genetic sequence variants at chromosome 9p21 and in the apolipoprotein(a) gene (*LPA*) not only associate with risk of CAD but also predict the extent of angiographic CAD, suggesting a role for these loci in influencing the development and progression of coronary atherosclerosis.[13–15] In this study, we evaluated the effects of all known common genetic variants associated

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Nonstandard Abbreviations and Acronyms	
CAD	coronary artery disease
GRS	genetic risk score
SNP	single-nucleotide polymorphism

with risk of CAD on the extent of coronary atherosclerosis in patients with significant CAD on coronary angiography, both individually and combined in a GRS.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

Characteristics of the Patients

A total of 8622 Icelandic patients with significant angiographic CAD (≥50% diameter stenosis) were included in the main analysis. Replication was sought in 1853 patients from the Emory Biobank. All participants were of European ancestry. Characteristics of the study patients are shown in Table 1. Diabetes mellitus, hypertension, and hyperlipidemia were more common in patients from the Emory Biobank, whereas Icelandic patients tended to be younger and were more likely to be current smokers. On average, patients from the Emory Biobank had more extensive coronary disease and were more likely to have history of myocardial infarction and coronary revascularization.

Association With CAD Extent

Among the 50 SNPs tested, rs1333049 at chromosome 9p21 and rs10455872 in *LPA* associated significantly ($P<0.001$) with the number of coronary arteries with at least 50% diameter stenosis in a combined analysis of the samples, adjusting for traditional cardiovascular risk factors: age, sex, hyperlipidemia, diabetes mellitus, hypertension, current smoking, and former smoking (Table I in the online-only Data Supplement). Figure 1 illustrates the linear relationship between the magnitude of the effects of individual SNPs on CAD extent and their respective odds ratio for the risk of CAD, previously reported in meta-analyses of genome-wide association studies (Table I in the online-only Data Supplement).

The combined GRS was strongly associated with CAD extent when adjusting for traditional cardiovascular risk factors (difference per SD increase in GRS, 0.076; $P=7.3\times10^{-17}$). Estimates in models adjusting for traditional cardiovascular risk factors did not differ substantially from those in models adjusted for age and sex only (Table II in the online-only Data Supplement), and the association remained significant after further consecutive adjustment for family history of premature CAD (difference per SD increase in GRS, 0.072; $P=3.0\times10^{-15}$; Table III in the online-only Data Supplement). To further illustrate this relationship, we divided patients into quintiles based on the GRS and compared the proportion of patients with multivessel disease (≥2 coronary arteries with at least 50% diameter stenosis) between the top and the bottom quintiles (Figure 2). Roughly 65% of patients in the top quintile had multivessel disease compared with 56% of patients in the bottom quintile (Table IV in the online-only Data Supplement). Thus, patients who were in the top quintile of the GRS were 1.67× more likely to have multivessel disease compared with patients in the bottom quintile (adjusted odds ratio, 1.67; 95% confidence interval, 1.45–1.94; Table 2).

Table 1. Characteristics of the Patients

Characteristics	Iceland (n=8622)	Emory Biobank (n=1853)	*P* Value*
Age, y	64.4 (10.7)	65.6 (10.6)	<0.001
Male sex, %	75.1	73.8	0.24
Diabetes mellitus, %	11.4	31.5	<0.001
Hypertension, %	54.3	70.7	<0.001
Hyperlipidemia, %	50.3	74.6	<0.001
Current smoker, %	27.4	14.7	<0.001
Former smoker, %	47.6	47.9	0.83
Previous MI, %	29.7	49.2	<0.001
Previous PCI, %	4.2	57.5	<0.001
Previous CABG, %	7.6	31.3	<0.001
Family history, %	43.1	44.7	0.21
No. of diseased vessels†	1.94 (0.88)	2.10 (0.91)	<0.001
1-vessel disease	39.1	32.0	<0.001
2-vessel disease	30.3	31.6	0.30
3-vessel disease	28.1	31.3	0.007
4-vessel disease	2.5	5.1	<0.001

Data are presented as percentages or means (SD). CABG indicates coronary artery bypass grafting; MI, myocardial infarction; and PCI, percutaneous coronary intervention.

*P values for continuous variables were calculated using Student t test. P values for categorical variables were calculated using the χ^2 test.

†Total number of coronary arteries with at least 50% stenosis on coronary angiography (left anterior descending, circumflex, the right main coronary artery, and the left main coronary artery).

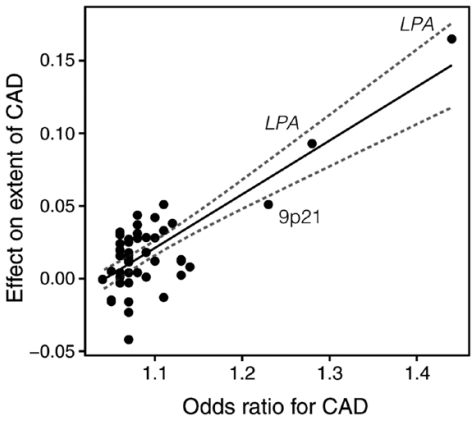

Figure 1. The effects of 50 single-nucleotide polymorphisms (SNPs) on the extent of coronary artery disease (CAD), expressed as the increase in number of diseased coronary arteries (with at least 50% stenosis) per SNP risk allele, plotted against their respective effect on CAD risk (odds ratio), previously reported in meta-analyses of genome-wide association studies (Table I in the online-only Data Supplement for references). Combined effect sizes in the Icelandic and Emory Biobank samples are presented where available. The solid line denotes best linear fit, the dashed lines indicate 95% confidence limits.

Because variants at chromosome 9p21 and *LPA* have previously been reported to associate with the extent of angiographic CAD,[13–15] we investigated whether the effect of the GRS was dominated by these variants. After excluding variants at chromosome 9p21 (rs1333049) and *LPA* (rs10455872 and rs3798220) from the GRS, the association remained significant (P=8.1×10^{-8}; Table 2). Similar results were obtained in models additionally adjusted for family history of premature CAD and in models adjusted for age and sex only (Table II and Table III in the online-only Data Supplement).

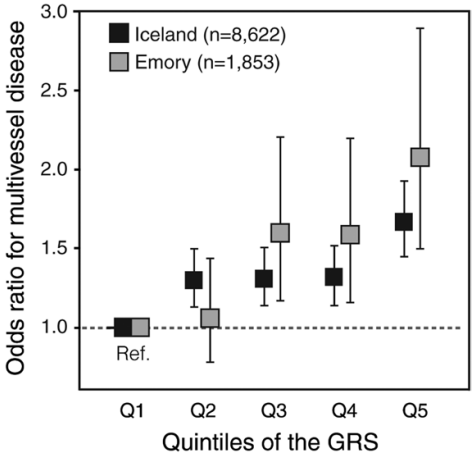

Figure 2. Adjusted odds ratios for multivessel disease by quintiles of the genetic risk score (GRS) in the Icelandic (black) and Emory Biobank (gray) samples. Odds ratios are referenced to the bottom GRS quintile and presented with 95% confidence intervals.

Model Performance

As shown in Table 3, the GRS significantly improved prediction of multivessel disease over cardiovascular risk factors in models including age and sex only (model 1), traditional cardiovascular risk factors (model 2), and family history of premature CAD (model 3), as evaluated by likelihood ratio tests (P<0.0001). To estimate the improvement in discrimination, we compared the *C*-statistics (area under the receiver-operating-characteristic curve) for models with and without the GRS. The *C*-statistics for the models without the GRS ranged from 62.9% to 64.8% (Table 3). Addition of the GRS to the models resulted in modest increases in the *C*-statistic, ranging from 0.6% to 0.8% (Table 3). Similarly, the integrated discrimination improvement ranged from 0.46% to 0.53%, indicating a marginal improvement in discrimination for multivessel disease with the addition of the GRS (Table 3).

Association With Age at Angiography

The GRS associated significantly with age at angiography when adjusting for sex, hyperlipidemia, diabetes mellitus, hypertension, current smoking, and former smoking (difference per SD increase in GRS, −0.90 years; P=7.2×10^{-17}). This association persisted when variants at chromosome 9p21 and *LPA* were excluded from the GRS (difference per SD increase in GRS, −0.64 years; P=2.5×10^{-9}). Patients in the top quintile of the GRS were on average 2.4 years younger than patients in the bottom quintile (63.6 years compared with 66.0 years), as shown in Table IV in the online-only Data Supplement.

Replication

In the Emory Biobank sample, the GRS based on 32 SNPs was significantly associated with CAD extent (difference per SD increase in GRS, 0.115; P=2.6×10^{-8}; Table 2). In the Emory Biobank sample, 77% of patients in the top quintile of the GRS had multivessel disease compared with 62% of patients in the bottom quintile (Table V in the online-only Data Supplement), corresponding to an adjusted odds ratio of 2.08 (95% confidence interval, 1.50–2.90; Table 2). As shown in Table 3, The GRS significantly improved prediction of multivessel disease over cardiovascular risk factors and modestly improved discrimination, as estimated by the increase in the *C*-statistics for the models with the addition of the GRS (ranging from 1.7% to 1.9%) and the integrated discrimination improvement (1.5% for all models). The GRS associated with age at angiography when adjusting for sex, hyperlipidemia, diabetes mellitus, hypertension, current smoking, and former smoking (difference per SD increase in GRS, −0.60 years; P=0.011), but the association was not significant when variants at 9p21 and *LPA* were excluded from the GRS (difference per SD increase in GRS, −0.44 years; P=0.062).

Association With CAD Extent When Including Individuals With Nonsignificant CAD

As expected, the association between the GRS and CAD extent was even more pronounced when individuals with

Table 2. Association of the GRS With the Number of Diseased Coronary Arteries on Coronary Angiography

	No. of SNPs	Difference Per SD Increase (95% CI)	SE	*P* Value	Contrast Top vs Bottom GRS Quintile OR for Multivessel Disease* (95% CI)
Iceland (n=8622)					
Full GRS	50	0.076 (0.058–0.094)	0.0091	7.3×10^{-17}	1.67 (1.45–1.94)
Full GRS excluding 9p21 and *LPA*	47	0.049 (0.031–0.066)	0.0091	8.1×10^{-8}	1.32 (1.14–1.52)
Restricted GRS	32	0.072 (0.054–0.089)	0.0091	3.2×10^{-15}	1.55 (1.34–1.78)
Emory Biobank (n=1853)					
GRS	32	0.115 (0.075–0.155)	0.021	2.6×10^{-8}	2.08 (1.50–2.90)
GRS excluding 9p21 and *LPA*	29	0.070 (0.029–0.110)	0.021	7.3×10^{-4}	1.50 (1.09–2.08)

Associations were tested using linear and logistic regression models adjusted for traditional cardiovascular risk factors (age, sex, hyperlipidemia, diabetes mellitus, hypertension, current smoking, and former smoking). CI indicates confidence interval; GRS indicates genetic risk score; OR, odds ratio; and SNP, single-nucleotide polymorphisms.

*Multivessel disease was defined as having at least 2 coronary arteries with ≥50% stenosis on coronary angiography.

nonsignificant CAD (<50% stenosis) were also included (Table VI and Figure I in the online-only Data Supplement).

Discussion

In this study, we demonstrate that a genetic score based on known common CAD risk variants is strongly associated with the extent of coronary atherosclerosis in patients with established angiographic CAD. This association is independent of traditional cardiovascular risk factors and family history of CAD. We found that the GRS significantly improved prediction of multivessel disease over established cardiovascular risk factors although the improvement in discrimination was modest. Compared with patients in the bottom quintile of the GRS, patients in the top quintile were roughly 1.67× (Iceland) and 2.08× (Emory Biobank) more likely to have multivessel disease. Furthermore, we found that the GRS associated with younger age at angiography, consistent with an earlier disease onset for individuals with a high burden of common genetic risk variants for CAD.

Previously, a genetic variant at chromosome 9p21 and 2 variants at *LPA* were shown to influence the extent of coronary atherosclerosis as determined by coronary angiography.[13–15] In keeping with these findings, these variants showed the strongest association with CAD extent in the present study. Because of their large effect sizes on the risk of CAD, they were assigned the greatest weights in the GRS. To evaluate whether the GRS was dominated by these loci, we excluded them from the GRS in a separate analysis. We found that the GRS restricted to variants outside chromosome 9p21 and *LPA* was also significantly associated with CAD extent, despite showing a somewhat weaker effect than that of the unrestricted GRS. These results show that currently known genetic risk variants for CAD, not previously associated with CAD extent, collectively associate with extent of coronary atherosclerosis. This suggests that many of these genetic variants influence the development of coronary atherosclerosis although the effect of a single variant is likely to be small.

Previous studies have suggested that some genetic variants associated with CAD may primarily promote coronary atherosclerosis, whereas other variants may predispose to myocardial infarction in the presence of coronary atheroma. For example,

Table 3. Model Prediction for Multivessel Disease With and Without the GRS

	C-Statistic					
Model Covariates	Without GRS, %	With GRS, %	Increase, %	IDI, %	LR χ^2	*P* Value*
Iceland (n=8622)†						
Model 1: age and sex only	62.9	63.7	0.8	0.53	48.7	<0.0001
Model 2: Traditional cardiovascular risk factors‡	64.0	64.8	0.8	0.52	48.1	<0.0001
Model 3: Traditional cardiovascular risk factors‡ and family history of premature CAD	64.8	65.4	0.6	0.46	42.7	<0.0001
Emory Biobank (n=1853)§						
Model 1: age and sex only	60.9	62.8	1.9	1.5	28.1	<0.0001
Model 2: Traditional cardiovascular risk factors‡	62.1	63.8	1.7	1.5	28.4	<0.0001
Model 3: Traditional cardiovascular risk factors‡ and family history of CAD	62.2	63.9	1.7	1.5	28.4	<0.0001

C-statistics and the IDI are reported as percentages. CAD indicates coronary artery disease; GRS, genetic risk score; IDI, integrated discrimination improvement; LR, likelihood ratio; and SNP, single-nucleotide polymorphisms.

*All *P* values reported are from likelihood ratio χ^2 tests for nested models.

†GRS based on 50 SNPs.

‡Traditional cardiovascular risk factors were defined as age, sex, hyperlipidemia, diabetes mellitus, hypertension, current smoking, and former smoking.

§GRS based on 32 SNPs.

chromosome 9p21 has been shown to associate primarily with coronary atherosclerosis but not myocardial infarction per se.[16,17] Reilly et al[18] showed that 12 genome-wide significant CAD variants did not associate individually with myocardial infarction among patients with angiographic CAD. Extending these observations, Patel et al[19] showed that a GRS based on 11 CAD risk variants associated with prevalent myocardial infarction in individuals undergoing coronary angiography but not when the analysis was restricted to patients with established angiographic CAD. These studies suggest that genetic risk variants for CAD, identified in early large-scale genome-wide association studies, relate primarily to coronary atherosclerosis and may have a minimal role in plaque rupture or thrombosis leading to acute coronary events. Our findings support the hypothesis that most common CAD variants identified to date influence the development of coronary atherosclerosis. Although the extent and overall burden of angiographic CAD unequivocally increase the risk of adverse cardiovascular events,[20,21] it remains to be established whether genotype scores based on common CAD variants are predictive of cardiovascular events in patients with established disease. Large prospective studies are warranted to evaluate the potential clinical use of genomic data as prognostic factors in patients with established CAD.

Our study should be interpreted in the context of several important limitations. First, we used standard coronary angiography to assess and quantify the extent of coronary atherosclerosis as the number of coronary arteries with at least 50% diameter stenosis. Although angiography is the most widely used and validated method for CAD assessment, it does not provide information on the volume or composition of the atherosclerotic plaque.[22] In the Icelandic sample, angiographic data for calculation of more sophisticated angiographic scoring systems such as the Gensini score or Duke CAD Severity Index were not available. Second, the GRS used for replication analyses in the Emory Biobank was constructed from an available 32 SNP subset of the 50 SNPs and was therefore not directly comparable with the GRS used for the main analyses. The main strengths of our study include large sample sizes and an unbiased nationwide coverage for the selection of the larger sample of Icelandic patients.

In summary, we have demonstrated that a combined GRS based on known common genetic risk variants for CAD is associated with the extent of coronary atherosclerosis in 2 independent populations of patients with established angiographic CAD. These findings show that patients with CAD with a high burden of common genetic variants associated with CAD risk are more likely to have extensive coronary disease than those who carry a low burden of such risk variants.

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Disclosures

E. Bjornsson, A. Helgadottir, D.F. Gudbjartsson, G. Thorleifsson, U. Thorsteinsdottir, and K. Stefansson are employees of deCODE Genetics/Amgen Inc. The other authors report no conflicts.

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Significance

Previous studies have shown that common genetic risk variants for coronary artery disease at chromosome 9p21 and in the lipoprotein(a) gene associate with angiographic extent of the disease, suggesting a role for these loci in the development of coronary atherosclerosis. In this study, we show that the cumulative burden of currently known genetic risk variants for coronary artery disease associates significantly with the extent of coronary atherosclerosis in 2 independent populations of patients with established angiographic coronary artery disease. Compared with patients in the bottom quintile of the genetic score, patients in the top quintile were significantly more likely to have multivessel disease.

Paper II

JAMA Cardiology | Original Investigation

Association of Genetically Predicted Lipid Levels With the Extent of Coronary Atherosclerosis in Icelandic Adults

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IMPORTANCE Genetic studies have evaluated the influence of blood lipid levels on the risk of coronary artery disease (CAD), but less is known about how they are associated with the extent of coronary atherosclerosis.

OBJECTIVE To estimate the contributions of genetically predicted blood lipid levels on the extent of coronary atherosclerosis.

DESIGN, SETTING, AND PARTICIPANTS This genetic study included Icelandic adults who had undergone coronary angiography or assessment of coronary artery calcium using cardiac computed tomography. The study incorporates data collected from January 1987 to December 2017 in Iceland in the Swedish Coronary Angiography and Angioplasty Registry and 2 registries of individuals who had undergone percutaneous coronary interventions and coronary artery bypass grafting. For each participant, genetic scores were calculated for levels of non–high-density lipoprotein cholesterol (non–HDL-C), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), and triglycerides, based on reported effect sizes of 345 independent, lipid-associated variants. The genetic scores' predictive ability for lipid levels was assessed in more than 87 000 Icelandic adults. A mendelian randomization approach was used to estimate the contribution of each lipid trait.

EXPOSURES Genetic scores for levels of non–HDL-C, LDL-C, HDL-C, and triglycerides.

MAIN OUTCOMES AND MEASURES The extent of angiographic CAD and coronary artery calcium quantity.

RESULTS A total of 12 460 adults (mean [SD] age, 65.1 [10.7] years; 8383 men [67.3%]) underwent coronary angiography, and 4837 had coronary artery calcium assessed by computed tomography. A genetically predicted increase in non–HDL-C levels by 1 SD (38 mg/dL [to convert to millimoles per liter, multiply by 0.0259]) was associated with greater odds of obstructive CAD (odds ratio [OR], 1.83 [95% CI, 1.63-2.07]; $P = 2.8 \times 10^{-23}$). Among patients with obstructive CAD, there were significant associations with multivessel disease (OR, 1.26 [95% CI, 1.11-1.44]; $P = 4.1 \times 10^{-4}$) and 3-vessel disease (OR, 1.47 [95% CI, 1.26-1.72]; $P = 9.2 \times 10^{-7}$). There were also significant associations with the presence of coronary artery calcium (OR, 2.04 [95% CI, 1.70-2.44]; $P = 5.3 \times 10^{-15}$) and $\log_e$-transformed coronary artery calcium (effect, 0.70 [95% CI, 0.53-0.87]; $P = 1.0 \times 10^{-15}$). Genetically predicted levels of non–HDL-C remained associated with obstructive CAD and coronary artery calcium extent even after accounting for the association with LDL-C. Genetically predicted levels of HDL-C and triglycerides were associated individually with the extent of coronary atherosclerosis, but not after accounting for the association with non–HDL cholesterol.

CONCLUSIONS AND RELEVANCE In this study, genetically predicted levels of non–HDL-C were associated with the extent of coronary atherosclerosis as estimated by 2 different methods. The association was stronger than for genetically predicted levels of LDL-C. These findings further support the notion that non–HDL-C may be a better marker of the overall burden of atherogenic lipoproteins than LDL-C.

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Supplemental content

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Low-density lipoprotein cholesterol (LDL-C) is an established risk factor for coronary artery disease (CAD).[1] Traditionally, LDL-C has been regarded as the primary marker of atherogenic lipoproteins and a treatment target for lipid-lowering therapies.[1] However, there is growing evidence that LDL-C may not be the best marker of the cardiovascular risk conferred by atherogenic lipoproteins. Epidemiological studies[2-6] have shown that non–high-density lipoprotein cholesterol (non-HDL-C) and apolipoprotein B, which are highly correlated, are superior to LDL-C for cardiovascular risk prediction in healthy individuals and patients with coronary disease. Experimental evidence from clinical trials[7-9] shows that aggressive lowering of LDL-C can slow progression and even induce regression of coronary atherosclerosis, as assessed by intravascular ultrasonography. A recent analysis of clinical trial data,[10] however, showed that changes in coronary atheroma volume may be more closely associated with levels of non-HDL-C than LDL-C.

Mendelian randomization is a method in which genetic information is used to infer whether an exposure is causally associated with an outcome (eg, a disease).[11] Using this method, genetic studies[1] have supported a potential causal role of LDL-C in coronary disease, but few have assessed non-HDL-C directly.[12] In a previous study using mendelian randomization,[13] we provided evidence to support a potential causal role of non-HDL-C in coronary diseases and showed that genetically predicted non-HDL-C levels were more significantly associated than LDL-C was with risk of CAD. Although genetic scores for lipid levels have been widely studied in the context of cardiovascular risk, less is known about their association with measures of the extent of coronary atherosclerosis. To our knowledge, 2 studies[14,15] have associated genetic scores for lipid levels with the extent of coronary atherosclerosis. Both studies evaluated associations with coronary artery calcium (CAC), a noninvasive marker of the overall coronary atherosclerotic burden.[16] A genetic score for LDL-C was associated with higher CAC in one study[14] but not the other.[15] In addition, one of the studies[14] evaluated genetic scores for HDL-C and triglyceride levels and did not find significant associations with CAC. In the present study, we used mendelian randomization to evaluate the contributions of individual lipid traits on the extent of coronary atherosclerosis in a data set of Icelandic adults undergoing coronary angiography and evaluation of CAC.

Key Points

Question Are genetically predicted lipid levels associated with the extent of coronary atherosclerosis?

Findings This study found that a genetic score for non–high-density lipoprotein cholesterol was significantly associated with the extent of coronary atherosclerosis, as estimated by coronary angiography or coronary calcium scanning in Icelandic adults. The association persists after accounting for low-density lipoprotein cholesterol.

Meaning Elevated non–high-density lipoprotein cholesterol is associated with the development of coronary atherosclerosis and may be a better marker for atherogenic lipoproteins than low-density lipoprotein cholesterol.

Methods

Study Participants

We identified Icelandic adults who had undergone coronary angiography for any indication at Landspítali–The National University Hospital in Reykjavík, the only interventional cardiology center in Iceland. The data were obtained from 3 clinical registries, as described previously.[17] First was the Swedish Coronary Angiography and Angioplasty Registry (SCAAR), which holds data on all consecutive individuals undergoing coronary angiography and percutaneous coronary intervention in Iceland since January 1, 2007.[18,19] From SCAAR, we obtained data collected prospectively between January 1, 2007, and December 31, 2017 (including 13 437 procedures for 9885 adults who had been genotyped). Second, we used a registry of all percutaneous coronary intervention procedures performed in Iceland between January 1, 1987, and December 31, 2006 (including 5386 procedures for 3743 patients who had been genotyped). Finally, we used a registry of coronary-artery bypass grafting procedures performed in Iceland, which holds data on patients who underwent preprocedural coronary angiography between January 1, 2001, and December 31, 2013 (1309 procedures for 1309 patients who had been genotyped).[20] For the main analyses, the 3 data sources were combined into a single data set (eFigure 1 in the Supplement); for individuals with multiple procedures, we only used the earliest record (n = 12 728 unique individuals). Information on cardiovascular risk factors was obtained from these registries. In the combined data set, hypertension, diabetes, and hyperlipidemia were defined by previous diagnosis of the respective condition or medical treatment at the time of angiography (with antihypertensive, antidiabetic, or lipid-lowering medication, respectively). Individuals with missing data were removed prior to analyses (n = 268), resulting in a total sample size of 12 460.

We identified Icelandic adults who underwent cardiac computed tomography for any indication at Röntgen Domus, the largest privately operated medical imaging clinic in the country. Imaging was performed between January 4, 2009, and October 31, 2017. A CAC score (Agatston score)[21] was available for 4837 individuals who had been genotyped. For each individual, we used the earliest record only. Information on cardiovascular risk factors, other than age at the time of procedure and sex, was not available. All participants donated samples for genotyping and provided informed consent as part of various genetic programs at deCODE genetics. The study was approved by the Data Protection Authority of Iceland and the National Bioethics Committee of Iceland. Personal identities of the participants were encrypted with a third-party system provided by the Data Protection Authority of Iceland.

Coronary Angiography

Coronary angiograms were evaluated by the interventional cardiologists performing the procedures. Angiographic extent of

CAD was quantified as the number of major epicardial coronary arteries (the left anterior descending artery, the circumflex artery, or the right coronary artery) with at least 50% luminal diameter stenosis (significant stenosis), ranging from 0 to 3 diseased coronary arteries. Obstructive CAD was defined as having 1 to 3 coronary arteries with significant stenosis or significant stenosis in the left main coronary artery. No or nonobstructive CAD was defined as having less than 50% stenosis in all 3 major coronary arteries and the left main coronary artery. Patients with obstructive CAD and without left main disease were categorized as having 1-vessel, 2-vessel, or 3-vessel disease, based on the number of coronary arteries with significant stenosis. Those with left main disease were categorized separately. Multivessel disease was defined as having 2-vessel or 3-vessel disease or left main disease.

Quantification of Coronary Artery Calcium

Coronary artery calcium was assessed using cardiac-gated multidetector computed tomography scanners (Aquilion [Toshiba Medical Systems]) with a slice thickness of 0.5 to 3 mm. Scans were read by radiologists, and CAC was quantified using a CAC score (Agatston score).[21]

Genotyping and Imputation

Genotyping and imputation methods were as previously described.[22,23] Briefly, DNA sequence variants identified in the genomes of 28 075 Icelandic adults whose whole genomes have been sequenced were imputed into 155 250 Icelanders who had been genotyped using various Illumina single-nucleotide polymorphism chips and their genotypes phased using long-range phasing.[22-24]

Genetic Scores

We constructed individual-level genetic scores for levels of non-HDL-C, LDL-C, HDL-C, and triglycerides based on variants identified in a recent large-scale, exome-wide association study of lipid levels.[25] That study reported 444 single-nucleotide polymorphisms in 250 loci with minor allele frequency ranging from 6.7×10^{-6} to 0.49. Each variant was reported to associate independently with at least 1 lipid level (total cholesterol, LDL-C, HDL-C, or triglycerides) at $P < 2.1 \times 10^{-7}$, a Bonferroni correction for the testing of 242 289 variants. In our study, a total of 414 variants were observed in the population that had been genotyped (n = 155 250), of which 412 had good imputation quality (imputation information of at least 0.90); 2 variants with imputation information less than 0.90 were excluded (eTable 1 in the Supplement). For the calculation of the genetic scores, to minimize potential bias associated with including correlated variants, we used a subset of 345 variants with pairwise r^2 less than 0.20 (eTable 1 in the Supplement). Based on this set, we calculated the genetic scores by summing the product of the allele count and the corresponding effect size for each variant. A flowchart summarizing the selection of variants and calculation of the genetic scores is presented in eFigure 2 in the Supplement.

For the genetic scores for LDL-C, HDL-C, and triglycerides, we used the effect sizes (with SDs) as previously reported[25]; these were estimated by using data from more than 300 000 Europeans, of whom less than 1% were Icelandic. Because association results for non-HDL-C were not available from this resource, we used the reported effect sizes for total cholesterol and HDL-C to derive effect sizes for non-HDL-C. To generate an equation to estimate the association with non-HDL-C, we analyzed the lipid effect sizes of 48 463 variants with minor allele frequency greater than 1.0% that were associated with each lipid trait at P values less than .05 in the Icelandic population (sample sizes: 93 556, 103 599, and 93 746 individuals for non-HDL-C, total cholesterol, and HDL-C, respectively). Ordinary least-squares regression estimated the non-HDL-C effect size as 0.979 × total cholesterol effect size - 0.354 × HDL-C effect size (with all effect sizes in SDs, estimated on inverse normal-transformed values). There was a high correlation between estimated and observed effect sizes on non-HDL-C (R^2 = 0.99). This equation had high predictive accuracy (R^2 = 0.93) in an external validation sample from the UK Biobank[26] (eMethods in the Supplement), using a set of 50 000 randomly selected variants with minor allele frequency greater than 1.0% and effect sizes based on cholesterol measurements for more than 358 000 individuals. Thus, the effect sizes of variants on non-HDL-C can be reliably predicted from their associations with total cholesterol and HDL-C, consistent with the fact that non-HDL-C is calculated directly from these lipid measurements (total cholesterol level − HDL-C level). We used this equation to estimate effect sizes for non-HDL-C from the effect sizes for total cholesterol and HDL-C levels, as previously reported,[25] and used the estimated effect sizes for calculation of the genetic score for non-HDL-C. Effect sizes used for the calculation of the genetic scores are shown in eTable 1 in the Supplement.

Each genetic score was associated with its respective lipid level in samples of more than 87 000 Icelandic adults for whom genotyping was performed (eMethods and eTable 2 in the Supplement). The genetic score for non-HDL-C explained 12.8% of the variance in non-HDL-C levels. For LDL-C, HDL-C, and triglycerides, the variance explained by the corresponding genetic score was 13.1%, 11.3%, and 8.9%, respectively (eTable 2 in the Supplement). The genetic scores showed weak to moderate pairwise correlation, with the exception of the genetic scores for non-HDL-C and LDL-C, which were highly correlated (r = 0.95; eTable 3 in the Supplement).

Statistical Analysis

Mendelian Randomization

We used a mendelian randomization approach involving genetic scores as instrumental variables to infer potential causal contributions of individual lipid traits. Because of the pleiotropy of lipid-associated variants (ie, each variant being commonly associated with more than 1 lipid level[25]), the association of a genetic score for a given lipid level may be confounded by its correlation with other lipid levels. To account for these pleiotropic effects, we conducted joint analyses in which the association of a given genetic score (eg, for LDL-C) was adjusted for genetic scores for other lipid levels (eg, for HDL-C and triglycerides) by including them as covariates in the model. Recently, we applied this approach in another mendelian ran-

Table 1. Characteristics of the Coronary Angiography Sample

Characteristic	No. (%)
Patients, No.	12 460
Age, mean (SD), y	65.1 (10.7)
Male	8383 (67.3)
Diabetes mellitus[a]	1467 (11.8)
Hypertension[b]	7184 (57.7)
Hyperlipidemia[c]	6599 (53.2)
Current smoking	2693 (21.6)
Former smoking	6191 (49.7)
Medical history	
Myocardial infarction	2221 (18.2)
Percutaneous coronary intervention	330 (2.7)
Coronary artery bypass grafting	659 (5.3)
Angiographic findings	
No or nonobstructive coronary artery disease	3476 (27.9)
Obstructive coronary artery disease	8984 (72.1)
1-Vessel disease[d]	3695 (31.6)
2-Vessel disease[d]	2462 (21.0)
3-Vessel disease[d]	2072 (17.7)
Left main disease	755 (6.1)

[a] Previous diagnosis of diabetes mellitus or the use of antidiabetic medication.

[b] Previous diagnosis of hypertension or the use of antihypertensive medication.

[c] Previous diagnosis of hypercholesterolemia or the use of lipid-lowering medication.

[d] Without left main disease.

domization study to infer the potential causal role of lipid levels in the context of risk of coronary disease.[13]

Association Analyses

We used logistic regression models to test for associations with dichotomized measures of angiographic extent of CAD and CAC. Linear regression models were used to test for associations with CAC as a continuous variable as the natural logarithm of the CAC score plus 1 ($\log_e$[CAC score +1]). Association analyses involving angiographic extent of CAD were adjusted for age, age^2, sex, diabetes, hypertension, and current and former smoking status. Results did not differ materially when adjusted only for age, age^2, and sex (eTable 4 in the Supplement). Association analyses of CAC were adjusted for age, age^2, and sex. Unless otherwise noted, effect size estimates for the genetic scores were scaled to correspond to a 1-SD increase in multivariate-adjusted residuals of the respective lipid level (for non-HDL-C, LDL-C, and HDL-C) or doubling of triglyceride levels, as estimated in more than 87 000 samples from Icelandic adults (eTable 5 in the Supplement).

For all tests, a 2-tailed $P < .05$ was considered statistically significant. Analyses were conducted using R version 3.3.2 (R Project for Statistical Computing).

Results

A total of 12 460 Icelandic adults who had been genotyped and had angiographic data available were identified (**Table 1**). The mean (SD) age was 65.1 (10.7) years; 8383 (67.3%) were men, and 8984 (72.1%) had obstructive CAD (at least 50% diameter stenosis in at least 1 coronary artery). Among patients with obstructive CAD, 5289 (58.9%) had multivessel disease (at least 2-vessel disease or left main disease).

Genetic Scores for Lipid Levels and Obstructive CAD

We assessed whether the genetic scores for levels of non-HDL-C, LDL-C, HDL-C, and triglycerides were associated with the presence of obstructive CAD vs no or nonobstructive CAD (**Table 2**). The genetic scores for non-HDL-C, HDL-C, LDL-C, and triglycerides were all associated individually with obstructive CAD. A genetically predicted 1-SD increase in non-HDL-C levels (38 mg/dL [to convert to millimoles per liter, multiply by 0.0259]) was associated with an 83% higher risk of having obstructive CAD (OR, 1.83 [95% CI, 1.63-2.07]; $P = 2.8 \times 10^{-23}$; Table 2). Similarly, a genetically predicted 1-SD increase in LDL-C level (34 mg/dL [to convert to millimoles per liter, multiply by 0.0259]) was associated with a 73% higher risk of obstructive CAD (OR, 1.73 [95% CI, 1.54-1.95]; $P = 6.4 \times 10^{-20}$; Table 2).

The association of the genetic score for non-HDL-C remained significant after accounting for the genetic scores for HDL-C and triglycerides (OR, 1.75 [95% CI, 1.52-2.01]; $P = 3.2 \times 10^{-15}$), as were the association of the genetic score of LDL-C after accounting for the genetic scores for HDL-C and triglycerides (OR, 1.63 [95% CI, 1.44-1.84]; $P = 3.0 \times 10^{-15}$). However, the genetic score for non-HDL-C conferred additional risk of obstructive CAD after accounting for the LDL-C genetic score (OR, 2.13 [95% CI, 1.47-3.10]; $P = 6.4 \times 10^{-5}$) while the association of the LDL-C genetic score was fully explained by the non-HDL-C genetic score (OR, 0.85 [95% CI, 0.59-1.23]; $P = .40$ after adjustment for the non-HDL-C genetic score; Table 2).

The genetic score for HDL-C showed a nominal association with obstructive CAD when adjusting for the genetic scores for non-HDL-C and triglycerides (OR, 0.83 [95% CI, 0.72-0.96]; $P = .01$; Table 2). The genetic score for triglycerides was nominally associated with obstructive CAD when adjusting for the genetic scores for LDL-C and HDL-C (OR, 1.35 [95% CI, 1.06-1.71]; $P = .01$) but not after adjustment for the non-HDL-C genetic score (OR, 0.99 [95% CI, 0.76-1.29]; $P = .94$; Table 2).

Genetic Scores for Lipid Levels and CAD Extent in Patients With Obstructive CAD

We tested associations with multivessel disease and 3-vessel disease among patients with obstructive CAD (n = 8984) (Table 2). A genetically predicted 1-SD increase in non-HDL-C was associated with a 26% higher risk of multivessel disease (OR, 1.26 [95% CI, 1.11-1.44]; $P = 4.1 \times 10^{-4}$) and a 47% higher risk of 3-vessel disease (OR, 1.47 [95% CI, 1.26-1.72]; $P = 9.2 \times 10^{-7}$). The association persisted after adjusting for the genetic scores for HDL-C and triglycerides (OR, 1.44 [95% CI, 1.21-1.73]; $P = 5.8 \times 10^{-5}$; Table 2). The genetic score for LDL-C showed similar associations (for multivessel disease: OR, 1.28 [95% CI, 1.12-1.45]; $P = 1.9 \times 10^{-4}$; after adjustment for the genetic scores for HDL-C and triglycerides: OR, 1.27 [95% CI, 1.11-1.45]; $P = 3.5 \times 10^{-4}$; for 3-vessel disease: OR, 1.43 [95% CI, 1.23-1.67]; $P = 3.8 \times 10^{-6}$; after adjustment: OR, 1.39 [95% CI, 1.19-1.63]; $P = 4.1 \times 10^{-5}$) (Table 2). Neither non-HDL-C nor

Table 2. Genetic Scores for Lipid Levels and Angiographic Extent of Coronary Artery Disease

	Obstructive Coronary Artery Disease in Overall Sample (N = 12 460)		Patients With Obstructive Coronary Artery Disease (n = 8984)			
			Multivessel Disease		3-Vessel Disease	
Covariates (Genetic Scores)[a]	Odds Ratio (95% CI)	*P* Value	Odds Ratio[b] (95% CI)	*P* Value	Odds Ratio[b] (95% CI)	*P* Value
Non-HDL-C	1.83 (1.63-2.07)	2.8×10^{-23}	1.26 (1.11-1.44)	4.1×10^{-4}	1.47 (1.26-1.72)	9.2×10^{-7}
HDL-C	1.74 (1.54-1.98)	3.7×10^{-18}	1.26 (1.10-1.45)	6.5×10^{-4}	1.46 (1.24-1.72)	4.1×10^{-6}
HDL-C and triglycerides	1.75 (1.52-2.01)	3.2×10^{-15}	1.31 (1.13-1.52)	4.6×10^{-4}	1.44 (1.21-1.73)	5.8×10^{-5}
LDL-C	2.13 (1.47-3.10)	6.4×10^{-5}	1.01 (0.68-1.50)	.97	1.49 (0.93-2.38)	.10
LDL-C	1.73 (1.54-1.95)	6.4×10^{-20}	1.28 (1.12-1.45)	1.9×10^{-4}	1.43 (1.23-1.67)	3.8×10^{-6}
HDL-C and triglycerides	1.63 (1.44-1.84)	3.0×10^{-15}	1.27 (1.11-1.45)	3.5×10^{-4}	1.39 (1.19-1.63)	4.1×10^{-5}
Non-HDL-C	0.85 (0.59-1.23)	.40	1.27 (0.86-1.87)	.24	0.99 (0.62-1.58)	.96
HDL-C	0.71 (0.62-0.80)	3.0×10^{-8}	0.94 (0.82-1.07)	.35	0.87 (0.75-1.02)	.09
LDL-C and triglycerides	0.83 (0.72-0.96)	.01	0.98 (0.85-1.13)	.77	0.99 (0.83-1.17)	.89
Non-HDL-C and triglycerides	0.83 (0.72-0.96)	.011	0.98 (0.85-1.13)	.78	0.99 (0.83-1.18)	.90
Triglycerides	1.86 (1.51-2.29)	6.4×10^{-9}	1.12 (0.89-1.39)	.34	1.45 (1.12-1.89)	.005
LDL-C and HDL-C	1.35 (1.06-1.71)	.014	1.01 (0.78-1.29)	.96	1.28 (0.95-1.73)	.10
Non-HDL-C and HDL-C	0.99 (0.76-1.29)	.94	0.87 (0.66-1.15)	.32	1.05 (0.75-1.46)	.78

Abbreviations: HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; non–HDL-C, non–high-density lipoprotein cholesterol.

SI conversion factor: To convert HDL-C, non–HDL-C, and LDL-C to mmol/L, multiply by 0.0259.

[a] In all models, age, age^2, sex, diabetes, hypertension, and current smoking and former smoking status were included as covariates in addition to the genetic scores. Sample sizes: obstructive coronary artery disease (8984 affected individuals and 3476 control individuals), multivessel disease (5289 affected individuals and 3695 control individuals), and 3-vessel disease (2072 affected individuals and 6157 control participants).

[b] Odds ratios are scaled to correspond to a 1-SD increase in the respective cholesterol trait or doubling of triglyceride levels. For non–HDL-C, LDL-C, and HDL-C, this corresponds to 38 mg/dL (0.97 mmol/L), 34 mg/dL (0.87 mmol/L), and 15 mg/dL (0.38 mmol/L), respectively.

Table 3. Genetic Scores for Lipid Levels and Coronary Artery Calcium

	Coronary Artery Calcium Score Greater Than 0 (n = 4837)		$\log_e$-Transformed Coronary Artery Calcium Score[b] (n = 4837)	
Covariates (Genetic Scores)[a]	Odds Ratio[c] (95% CI)	*P* Value	Effect Size (95% CI)	*P* Value
non–HDL-C	2.04 (1.70-2.44)	5.3×10^{-15}	0.70 (0.53-0.87)	1.0×10^{-15}
HDL-C	2.05 (1.70-2.48)	4.2×10^{-14}	0.67 (0.50-0.85)	1.4×10^{-13}
HDL-C and triglycerides	2.07 (1.69-2.55)	3.9×10^{-12}	0.70 (0.50-0.90)	3.5×10^{-12}
LDL-C	2.06 (1.18-3.60)	.01	0.80 (0.27-1.33)	.003
LDL-C	1.91 (1.60-2.27)	1.4×10^{-13}	0.62 (0.46-0.79)	7.6×10^{-14}
HDL-C and triglycerides	1.84 (1.54-2.19)	9.8×10^{-12}	0.58 (0.41-0.74)	1.4×10^{-11}
Non–HDL-C	0.99 (0.58-1.69)	.97	−0.10 (−0.61 to 0.41)	.70
HDL-C	0.87 (0.76-0.99)	.04	−0.22 (−0.35 to −0.09)	.001
LDL-C and triglycerides	1.01 (0.86-1.18)	.91	−0.09 (−0.23 to 0.06)	.26
Non–HDL-C and triglycerides	1.01 (0.87-1.18)	.88	−0.07 (−0.20 to 0.07)	.34
Triglycerides	1.43 (1.18-1.74)	3.2×10^{-4}	0.37 (0.18-0.56)	1.4×10^{-4}
LDL-C and HDL-C	1.26 (1.00-1.57)	.047	0.18 (−0.04 to 0.39)	.10
Non–HDL-C and HDL-C	0.97 (0.76-1.24)	.83	−0.07 (−0.3 to 0.17)	.57

Abbreviations: HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol.

SI conversion factor: To convert HDL-C, non–HDL-C, and LDL-C to mmol/L, multiply by 0.0259.

[a] In all models, age, age^2, and sex were included as covariates, in addition to the genetic scores.

[b] $\log_e$(CAC score + 1).

[c] Odds ratios and linear regression coefficients are scaled to correspond to a 1-SD increase in the respective cholesterol trait or doubling of triglyceride levels. For non–HDL-C, LDL-C, and HDL-C, this corresponds to 37 mg/dL (0.97 mmol/L), 37 mg/dL (0.87 mmol/L), and 15 mg/dL (0.38 mmol/L), respectively.

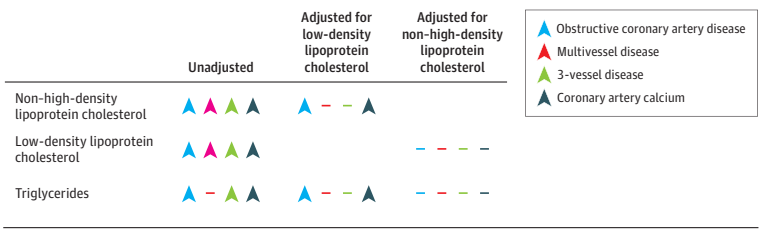
LDL-C remained significant after adjusting for of the other. The genetic scores for HDL-C and triglycerides were not associated with multivessel disease or 3-vessel disease in adjusted models.

Genetic Scores for Lipid Levels and Coronary Artery Calcium

In addition to angiographic measures of CAD extent, we tested whether the genetic scores were associated with the presence and extent of CAC as assessed by cardiac computed tomography. A CAC score was available for 4837 individuals on whom genotype data were available. The mean (SD) age was 58.4 (9.7) years, and 2377 (49.1%) were men. The median CAC score was 3.8 (range, 0-5223; mean [SD], 134 [353]). Coronary artery calcium was present in 2598 (53.7%) (CAC score >0), indicating the presence of coronary atherosclerosis, and 1211 (25.0%) had moderate to extensive CAC, defined[16] as a CAC score greater than 100.

Association results for the presence and extent of CAC were similar to those for angiographic extent of CAD (**Table 3**). The genetic score for non–HDL-C was associated with the presence of CAC (OR, 2.04 [95% CI, 1.70-2.44]; $P = 5.3 \times 10^{-15}$) and $\log_e$-transformed CAC score (0.70 [95% CI, 0.53-0.87]; $P = 1.0 \times 10^{-15}$). The genetic score for non–HDL-C was associated with the presence and extent of CAC after accounting for the LDL-C genetic score (OR, 2.06 [95% CI, 1.18-3.60]; $P = .01$; $\log_e$-transformed CAC score, 0.80 [95% CI, 0.27-1.33]; $P = .003$,

Figure. Summary of Main Findings



Color-coded arrowheads denote the direction of the association of the respective genetic score with phenotypes of coronary artery disease extent. Dashes indicate nonsignificant associations.

respectively), but no association remained for the genetic score for LDL-C after adjusting for the non-HDL-C genetic score (Table 3). The genetic scores for HDL-C and triglycerides were not associated with CAC in adjusted models (Table 3).

Non–HDL-C Genetic Score and Risk of CAD

Previously, we demonstrated a robust association between genetically predicted levels of non-HDL-C and risk of CAD.[13] We sought to validate the association of the current non-HDL-C genetic score with CAD among Icelandic adults with replication in the UK Biobank (eMethods and eTable 6 in the Supplement). The non-HDL-C genetic score was associated with increased risk of CAD in the Icelandic population (OR, 1.61 [95% CI, 1.51-1.71]; $P = 1.2 \times 10^{-49}$; 19 123 affected individuals and 124 461 control individuals) and the UK Biobank (OR, 1.52 [95% CI, 1.47-1.57]; $P = 4.4 \times 10^{-138}$; 28 110 affected individuals and 380 455 control individuals). Furthermore, the association remained significant after adjustment for the LDL-C genetic score in both samples (OR, 1.79 [95% CI, 1.47-2.18]; $P = 7.8 \times 10^{-9}$ in Icelandic adults and OR, 1.81 [95% CI, 1.63-2.01]; $P = 5.9 \times 10^{-28}$ in the UK Biobank), in line with our previous findings.[13]

Discussion

In this study, we used genetic scores to evaluate the associations of commonly measured lipid levels with the extent of coronary atherosclerosis, as assessed by 2 different methods. The main findings of this study were that (1) genetically predicted levels of non-HDL-C and LDL-C were consistently associated with greater extents of coronary atherosclerosis, (2) the genetic score for non-HDL-C was most significantly associated with the extent of CAD and provides additional predictive value beyond the LDL-C genetic score, and (3) genetically predicted levels of HDL-C and triglycerides were not significantly associated with the extent of coronary atherosclerosis after accounting for the contribution of non-HDL-C.

The non-HDL-C fraction represents the sum of cholesterol carried by all atherogenic, apolipoprotein B–containing lipoproteins. Most non-HDL-C is found within LDL particles (as LDL-C), while the remainder is carried by triglyceride-rich lipoproteins (intermediate-density lipoproteins, very-low-density lipoproteins, and chylomicron remnants) and, to a lesser degree, lipoprotein(a).[27] Recently, we undertook a mendelian randomization analysis that supported a direct involvement of non-HDL-C, but not triglycerides or HDL-C, in the development of CAD.[13] In that analysis, non-HDL-C was associated with CAD risk and provided predictive power beyond that of a genetic score for LDL-C.

In contrast with the previous study, which compared individuals with CAD to population controls, in the present study, we studied measures of CAD extent in a population undergoing invasive or noninvasive assessment of CAD. We found that a genetic score for non-HDL-C was significantly associated with multiple measures of coronary atherosclerotic burden. In line with our previous findings, the genetic score for non-HDL-C was associated significantly with the risk of obstructive CAD and presence of coronary calcium, even after accounting for the contribution of LDL-C. This residual association may reflect the influence of the cholesterol carried within triglyceride-rich lipoproteins, also known as remnant cholesterol, which is a subfraction of non-HDL-C. Accumulating evidence suggests that triglyceride-rich lipoproteins are associated with the risk of CAD,[28-37] most likely because of their content of cholesteryl esters.[13,38,39] In line with this, the association of the genetic score for triglycerides with the extent of angiographic CAD is fully explained by the genetic score for non-HDL-C. These findings, summarized in the **Figure**, together with our previous results,[13] suggest that among the commonly measured lipid fractions in clinical practice, non-HDL-C may be the best overall marker of atherogenic lipoproteins and cardiovascular risk.

Mendelian randomization studies have consistently shown that HDL-C levels are not likely to contribute to the pathogenesis of CAD.[13,29,40,41] We observed a nominal association between the HDL-C genetic score and obstructive CAD after accounting for the genetic scores for non-HDL-C and triglycerides. However, there was no association with other measures of angiographic extent of CAD or the extent of coronary calcium. A possible explanation for these results is residual confounding due to the pleiotropic effects of multiple HDL-C variants on other lipid fractions that may not be accounted for in the adjusted models. Taken together, these results do not support the hypothesis that HDL-C contributes to the extent of coronary atherosclerosis.

These findings have implications for predicting potential cardiovascular benefit from lipid-lowering therapies, especially with respect to non-HDL-C and triglycerides. Consistent with a potential causal role of non-HDL-C and its major component LDL-C, reduction in these lipid fractions lowers cardiovascular risk in a dose-dependent manner.[42,43] On the other hand, trials of triglyceride-lowering therapies have produced

variable results.[44] Thus, it is unlikely that lowering triglycerides per se reduces cardiovascular risk, consistent with a likely noncausal role in atherogenesis. However, triglyceride-lowering therapies that also lower non–HDL-C and/or have nonlipid-associated vascular benefits would be expected to reduce cardiovascular risk.

The main strengths of this study include the large sample size of individuals with genotype, angiographic, and coronary calcium data available and consistent effect sizes of the genetic scores on different measures of the extent of coronary atherosclerosis. In addition, angiographic data were obtained from large nationwide angiography registries, reducing the risk of selection bias.

Limitations

This study has several limitations. We attempted to disentangle the contributions of each lipid level by evaluating the respective genetic score while adjusting for genetic scores for other lipid levels. However, unmeasured pleiotropy of the genetic scores may not be accounted for in the adjusted models. In turn, this may limit the interpretation of causality in the mendelian randomization analyses. Another limitation is the use of estimated variant effect sizes on the calculation of the non–HDL-C genetic score. However, as evident by the high agreement between calculated and observed effect sizes on non–HDL-C in Iceland and the UK Biobank, this approach provides a reliable estimate of non–HDL-C effect sizes.

Conclusions

In this study, we have demonstrated that genetically predicted levels of non–HDL-C were associated with the extent of coronary atherosclerosis and provide predictive power beyond genetically predicted LDL-C levels. These results support the notion that non–HDL-C may be a better measure of the overall burden of atherogenic lipoproteins and cardiovascular risk than LDL-C.

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Paper III

Arteriosclerosis, Thrombosis, and Vascular Biology

CLINICAL AND POPULATION STUDIES

Large-Scale Screening for Monogenic and Clinically Defined Familial Hypercholesterolemia in Iceland

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OBJECTIVE: Familial hypercholesterolemia (FH) is traditionally defined as a monogenic disease characterized by severely elevated LDL-C (low-density lipoprotein cholesterol) levels. In practice, FH is commonly a clinical diagnosis without confirmation of a causative mutation. In this study, we sought to characterize and compare monogenic and clinically defined FH in a large sample of Icelanders.

APPROACH AND RESULTS: We whole-genome sequenced 49 962 Icelanders and imputed the identified variants into an overall sample of 166 281 chip-genotyped Icelanders. We identified 20 FH mutations in *LDLR*, *APOB*, and *PCSK9* with combined prevalence of 1 in 836. Monogenic FH was associated with severely elevated LDL-C levels and increased risk of premature coronary disease, aortic valve stenosis, and high burden of coronary atherosclerosis. We used a modified version of the Dutch Lipid Clinic Network criteria to screen for the clinical FH phenotype among living adult participants (N=79 058). Clinical FH was found in 2.2% of participants, of whom only 5.2% had monogenic FH. Mutation-negative clinical FH has a strong polygenic basis. Both individuals with monogenic FH and individuals with mutation-negative clinical FH were markedly undertreated with cholesterol-lowering medications and only a minority attained an LDL-C target of <2.6 mmol/L (<100 mg/dL; 11.0% and 24.9%, respectively) or <1.8 mmol/L (<70 mg/dL; 0.0% and 5.2%, respectively), as recommended for primary prevention by European Society of Cardiology/European Atherosclerosis Society cholesterol guidelines.

CONCLUSIONS: Clinically defined FH is a relatively common phenotype that is explained by monogenic FH in only a minority of cases. Both monogenic and clinical FH confer high cardiovascular risk but are markedly undertreated.

GRAPHIC ABSTRACT: A graphic abstract is available for this article.

Key Words: genetic screening ■ genetics ■ hypercholesterolemia ■ lipids ■ mutation

Familial hypercholesterolemia (FH) is a genetic disorder characterized by markedly elevated levels of LDL-C (low-density lipoprotein cholesterol), leading to premature cardiovascular disease and death.[1] Despite advances in genetic diagnostics and the availability of effective cholesterol-lowering treatment, FH remains underdiagnosed and undertreated in most countries.[2]

See accompanying editorial on page 2629

FH is classically defined as an autosomal dominant, monogenic disease caused by highly penetrant mutations in the genes encoding the LDL receptor (*LDLR*), apolipoprotein B (*APOB*), or proprotein convertase

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For Sources of Funding and Disclosures, see page 2626.

Nonstandard Abbreviations and Acronyms

CAD	coronary artery disease
DLCN	Dutch Lipid Clinic Network
FH	familial hypercholesterolemia
HDL-C	high-density lipoprotein cholesterol
ICD	*International Classification of Diseases*
LDL-C	low-density lipoprotein cholesterol
PCSK9	proprotein convertase subtilisin/kexin type 9
WGS	whole-genome sequencing

Highlights

- Monogenic familial hypercholesterolemia (FH) was found in 199 of 166 281 genotyped Icelanders, a prevalence of 1 in 836.
- Monogenic FH associated with high lifetime cumulative exposure to LDL-C (low-density lipoprotein cholesterol), increased risk of coronary disease and aortic valve stenosis, but not ischemic stroke.
- Clinically defined FH (using the Dutch Lipid Clinic Network criteria) was observed in 2.2% of adults with available cholesterol measurements. Only small minority (5.2%) had monogenic FH.
- Both monogenic FH and clinically defined FH were severely undertreated with cholesterol-lowering medications.

subtilisin/kexin type 9 (*PCSK9*).[3] The prevalence of monogenic FH has been traditionally estimated to be 1 in 500 but recent genetic studies in European and North American populations indicate that the prevalence may be >1 in 250.[4–9] Such estimates, however, depend on the criteria used for defining FH mutations and may differ between populations.

In practice, FH is most commonly diagnosed on the basis of clinical presentation and genetic testing is rarely performed to confirm the diagnosis.[10] Among individuals who undergo genetic testing for FH in tertiary lipid clinics, only 40%–50% are found to have a monogenic cause.[11–13] A substantial fraction of those with a clinical diagnosis of FH but no demonstrable FH mutation may have a polygenic basis for hypercholesterolemia, but environmental and lifestyle factors also play a role.[11–13] Thus, in general, the term FH encompasses 2 partially overlapping entities: classical monogenic FH and the more complex FH clinical phenotype. The use of genetic testing to identify individuals with monogenic FH has important implications for clinical decisions involving family screening, genetic counseling, risk stratification, and therapeutic choices.[10]

In this study, we investigated the prevalence and characteristics of monogenic FH and clinically defined FH in Iceland. First, we examined the prevalence and impact of monogenic FH in over 160 000 genotyped Icelanders. We then determined the prevalence of clinical FH and estimated the contribution of monogenic FH and polygenic burden toward clinical FH, using a subsample of over 79 000 participants. Finally, we assessed the contemporary use and effectiveness of cholesterol-lowering treatment in individuals with monogenic FH and clinical FH.

METHODS

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Study Population

This study is based on a genotyped sample of 166 281 Icelandic participants. This sample comprises voluntary participants of various genetic research projects at deCODE genetics, Reykjavík, Iceland, and this study population has been described in detail previously.[14] All analyses presented in this study were conducted in the entire sample or relevant subsamples. All participants donated samples for genotyping and provided informed consents. The study was approved by the National Bioethics Committee of Iceland (VSNb2015080003-03.01 and VSNb2015010033-03.12 with amendments). Personal identities of the participants were encrypted with a third-party system, provided by the Data Protection Authority of Iceland. Genotype information was not disclosed to the study participants.

Laboratory Measurements

Measurements of total cholesterol, HDL-C (high-density lipoprotein cholesterol) and triglycerides, taken between 1990 and 2019, were obtained from Landspítali - The National University Hospital (LUH) in Reykjavík, the largest and only tertiary referral hospital in Iceland; the Laboratory in Mjódd, Reykjavík; Akureyri Hospital, a Regional Hospital in North Iceland, and from the deCODE genetics laboratory. Measurements were taken either in a fasting or nonfasting state. Levels of LDL-C were calculated using the Friedewald[15] equation for triglyceride levels <4.00 mmol/L. Lipoprotein(a) was measured at the laboratory at deCODE genetics using a Tina-quant Lipoprotein(a) Gen.2 (Roche Diagnostics) immunoturbidimetric assay.

Atherosclerotic Diseases

Cases were defined as described below. Unless otherwise noted, diagnostic codes and information obtained from clinical registries were not validated.

Coronary Artery Disease

Coronary artery disease (CAD) was defined as previously described,[16] primarily on the basis of *International Classification of Diseases* (*ICD*) codes indicative of CAD (including myocardial infarction). Cases with CAD were identified based on discharge diagnoses from LUH (*ICD-9* codes 410.*, 411.*, 412.*, and 414.* or *ICD-10* codes I20.0, I21.*, I22.*, I23.*, I24.*, and I25.*), documentation of obstructive CAD in nationwide coronary angiography registries at LUH[17] and relevant surgical procedure codes

from LUH. CAD case status was also assigned based on the same *ICD* codes for CAD listed as the cause or contributing cause of death, in the Icelandic death registry. Early-onset CAD was defined as CAD occurring before age 50 years for men and 60 years for females.

Coronary Revascularization

All procedures were performed at LUH, the only center for interventional cardiology and cardiothoracic surgery in Iceland. Individuals who underwent percutaneous coronary intervention (years 1985–2017) were identified using nationwide coronary angiography registries[17] and relevant procedure codes, and those who underwent coronary artery bypass surgery (years 1987–2017) were identified through relevant surgical procedure codes.

Peripheral Artery Disease

Cases were identified based on discharge diagnoses (*ICD-10*: I70.2, I70.9, and I73.9) and relevant surgical procedure codes at LUH between years 1998 and 2016. A subset of cases (ascertained during years 1998–2006) was clinically validated by a vascular surgeon, as previously described.[18]

Ischemic Stroke

Cases were identified from either a registry of individuals with a validated diagnosis of ischemic stroke or transient ischemic attack at LUH during the years 1993 to 2013, as described previously,[19] or relevant discharge diagnoses at LUH between years 2014 and 2016 (*ICD-10* codes: I63 and G45).

Aortic Valve Stenosis

Cases were identified based on relevant discharge diagnoses (*ICD-10* codes I35.0 or I35.2) or the relevant NOMESCO classification of surgical procedure codes (FMA, FMD, and subcodes) at LUH, between years 1983 and 2016, as previously described.[20]

Extent of Coronary Atherosclerosis

Coronary Angiography

Individuals were identified in the Swedish Coronary Angiography and Angioplasty Registry, which holds data on all consecutive individuals undergoing coronary angiography and percutaneous coronary intervention in Iceland from January 1, 2007.[17,21] Here, we used data through December 31, 2017. Obstructive CAD was defined as having ≥50% diameter stenosis in one or more epicardial coronary artery, including the left main stem. Multivessel disease was defined as having ≥50% diameter stenosis in at least 2 epicardial coronary arteries or left main disease.

Coronary Artery Calcium

Individuals underwent coronary artery calcium (CAC) scanning for any indication at Röntgen Domus, the largest privately operated medical imaging clinic in Iceland. Imaging was performed between January 4, 2009, and October 31, 2017.[17] CAC was assessed using cardiac-gated multidetector computed tomography scanners (Aquilion, Toshiba Medical Systems) with a slice thickness of 0.5 to 3 mm. Scans were read by radiologists and CAC was quantified using a CAC score (Agatston score[22]).

Genotyping and Whole-Genome Sequencing

The methods used for whole-genome sequencing (WGS), calling of single-nucleotide polymorphisms and small insertions/deletions (up to a length of 60 bp), long-range phasing and imputation were as described previously.[14,23,24] Briefly, a total of 166 281 Icelanders were genotyped using various Illumina single-nucleotide polymorphism chips and their genotypes phased using long-range phasing. A subsample of 49 962 underwent WGS (median depth, 39×), and the identified DNA sequence variants were imputed into the overall sample. Individuals were chosen for WGS based on various conditions, including extremes of cholesterol levels.[25] Consequently, the WGS subsample is enriched for individuals with high LDL-C as well as various cardiovascular phenotypes (Table I in the Data Supplement). We searched for copy-number variants (eg, deletions) in *LDLR* using several methods based on WGS data (PopDel,[26] DELLY,[27] Graphtyper,[28] and Manta[29]), single-nucleotide polymorphism genotypes (PennCNV[30]) and long-read sequences of 3622 Icelanders.[31]

Genotype imputation was performed as previously described,[14,23] as outlined in the Data Supplement. We used Sanger sequencing to validate the genotypes of all predicted carriers based on imputation, in addition to confirming the genotypes of carriers who had undergone WGS. Furthermore, we used the comprehensive Icelandic genealogical database[32] to direct extensive Sanger sequencing among relatives of carriers, to validate their imputed genotypes and search for additional carriers. The directly assessed genotypes were then used as a training set for reimputation of the variants. The majority of FH mutations (13/20) had imputation information of at least 0.89, reflecting accurate imputation (Table II in the Data Supplement). We were unable to impute 5 mutations (4 singletons and 1 with 2 carriers) as the genotypes could not be placed onto haplotypes with high confidence and thus were imputed to a 0% frequency.

Definition of FH Mutations

Mutations were considered to potentially cause FH if they met one of the following criteria:

1. Predicted loss-of-function mutations in *LDLR*. All predicted loss-of-function mutations in *LDLR* were considered to be FH mutations, that is, nonsense mutations (premature stop-codon), essential splice variants (donor or acceptor), insertion/deletion (indels) that cause frameshift or larger copy-number variants (eg, deletions) involving exons.
2. Reported FH mutations in ClinVar. We retrieved data from ClinVar for variants in *LDLR*, *APOB,* and *PCSK9* (http: https://www.ncbi.nlm.nih.gov/clinvar/, accessed November 11, 2019). Variants were considered if they were annotated as either Pathogenic or Likely pathogenic. Variants with Conflicting interpretations of pathogenicity were considered if at least half of submissions annotated the variant as Pathogenic or Likely pathogenic.
3. *LDLR* missense mutations at the same position as pathogenic mutations. We considered rare *LDLR* missense variants that cause an amino acid change at the same position as a mutation designated as Pathogenic or Likely pathogenic in ClinVar.

Mutations meeting the above criteria were manually curated and excluded if the allele frequency in our data was inconsistent with FH (eg, >0.1%) or if the phenotypes of the carriers were grossly inconsistent with FH (eg, low or normal levels of LDL-C if not on lipid-lowering medications). The selection process is outlined in Figure I in the Data Supplement.

Search for Additional FH Mutations

We searched for other, potential FH mutations by assessing rare sequence variants (allele frequency below 0.1%) in *LDLR*, *APOB*, *PCSK9*, *APOE*, *LDLRAP1*, *ABCG5*, and *ABCG8*. Of the identified variants, none associated with a large increase in LDL-C levels (ie, at least 1 mmol/L at $P<0.05$, under additive and recessive models) among 104 828 genotyped Icelanders. In brief, we did not identify additional mutations in these genes that are likely to cause FH in Iceland.

Drug Prescription Data

Prescriptions of cholesterol-lowering medications (ATC code C10) were obtained from a nationwide registry maintained by the Directorate of Health that contains all issued drug prescriptions in Iceland between January 1, 2003, and December 31, 2018. Statin potency was assigned as described in the 2013 American College of Cardiology/American Heart Association cholesterol guidelines[33] (Table III in the Data Supplement).

Definition of Clinical FH

We used a modified version of the Dutch Lipid Clinic Network (DLCN) criteria that exclude physical examination findings and genetic information. In brief, each individual is assigned a score based on family history of hypercholesterolemia or premature cardiovascular disease (maximum 2 points), personal history of premature cardiovascular disease (maximum 2 points), and the maximum documented LDL-C (maxLDL-C) levels for the individual (maximum 8 points; see Table IV in the Data Supplement for details). Family history variables were created using the Icelandic genealogical database[32] (to identify first-degree relatives) coupled with relevant clinical data. Clinical FH was defined as probable FH (score 6–8) or definite FH (score >8). These criteria were applied to genotyped participants that were alive and between the ages of 20 and 80 years, with at least one available LDL-C measurement. Participants with no available LDL-C measurement were excluded.

Polygenic Contribution in Mutation-Negative Clinical FH

We estimated the polygenic contribution in mutation-negative clinical FH using a genetic score for LDL-C. We used a weighted genetic score based on the effects of 345 lipid-associated variants on LDL-C levels in an exome-wide association study of >300 000 individuals,[34] as previously described.[17] In a sample of 98 497 genotyped Icelanders with available information, the genetic score explained 12.3% of the variance (R^2) in maxLDL-C and associated with an increase by 1.04 mmol/L per 1-unit increase in the genetic score ($P<10^{-300}$). A 1-unit increase in the genetic score approximates an increase by one SD in LDL-C levels, based on the aggregate effects of the individual variants.

Statistical Analyses

A generalized form of linear or logistic regression that accounts for the relatedness between individuals and potential population stratification was used to test for associations with quantitative traits and diseases. For association analyses, levels of LDL-C were adjusted for statin use: for individuals who were prescribed statins within one year before measurement, total cholesterol was divided by 0.8 (Liu et al[34]), and the modified value was used for calculation of LDL-C. Measurements taken before January 1, 2003, and after December 31, 2018 (24% of all measurements) were not adjusted for statin use due to unavailable prescription data. Unadjusted values were used in analyses involving cumulative LDL-C exposure and LDL-C target attainment. For lipid traits, residuals were obtained after adjustment for age, age^2, year of birth, sex, measurement site, and county of birth. The adjusted residuals were used as outcome variables in association analyses for lipid traits. For associations with maxLDL-C, raw, non-normalized adjusted residuals were used to better retain information from outliers (ie, individuals with very high maxLDL-C). For lipid traits other than maxLDL-C, the mean values of adjusted residuals (for each individual) were transformed to a normal distribution with a mean of 0 and a SD of 1. Unless otherwise specified, controls in logistic regression analyses comprise noncases for a given phenotype in the overall genotyped population. Data were analyzed using R software (The R Foundation for Statistical Computing), and $P<0.05$ was considered to be statistically significant.

RESULTS

Prevalence of Monogenic FH

We identified 20 FH mutations in 49 962 Icelanders whose genomes had been sequenced. Most of the mutations are located in *LDLR* (3 loss-of-function mutations, 12 missense mutations, and 1 promoter variant), 3 in *PCSK9* (missense mutations) and 1 in *APOB* (missense mutation; Table 1 and Table II in the Data Supplement). These variants were imputed into an additional 116 319 chip-genotyped individuals to identify additional carriers. The genotypes of all identified carriers were confirmed with Sanger sequencing. A diagram showing the structure of the overall genotyped sample and subsamples are shown in Figure II in the Data Supplement.

In the overall sample (N=166 281), we identified 199 heterozygous FH mutation carriers. This corresponds to a monogenic FH prevalence of 1 in 836 (0.12%). Of the 199 identified FH mutation carriers, 98 (49%) had undergone WGS. The prevalence of monogenic FH was ≈2-fold higher among those who underwent WGS (1 in 515 [0.19%]), compared with those who did not (1 in 1149 [0.087%]; Table I in the Data Supplement). This is likely due to the intentional enrichment for individuals with severe hypercholesterolemia (eg, 1.9-fold enrichment for LDL-C >99th percentile) and various cardiovascular phenotypes in the WGS subsample (Table I in the Data Supplement).

Table 1. FH Mutations Found in the Overall Genotyped Sample of 166 281 Icelanders

Gene	Position (hg38)	Alleles*	Mutation	Type	Carriers (N)	Allele frequency† (%)	Previously identified in Iceland
PCSK9	chr1:55043921	C/T	Arg96Cys	Missense	3	9.0×10^{-4}	
	chr1:55044020	G/A	Asp129Asn	Missense	1	3.0×10^{-4}	
	chr1:55052398	G/A	Arg215His	Missense	1	3.0×10^{-4}	
APOB	chr2:21006288	G/A	Arg3527Gln	Missense	10	3.0×10^{-3}	
LDLR	chr19:11089397	C/T	c.-152C>T	Promoter	21	6.3×10^{-3}	
	chr19:11102772	A/T	Asp100Val	Missense	1	3.0×10^{-4}	
	chr19:11105315	G/A	Gly137Ser	Missense	2	6.0×10^{-4}	
	chr19:11105599	C/A	Cys231Ter	Stop gained (LoF)	5	1.5×10^{-3}	Yes[36]
	chr19:11105602	T/C	c.694+2T>C	Splice donor (LoF)	80	0.024	Yes[35]
	chr19:11106640	G/A	Arg257Gln	Missense	2	6.0×10^{-4}	
	chr19:11107493	G/A	Asp307Asn	Missense	20	6.0×10^{-3}	Yes[36]
	chr19:11111577	A/G	Tyr375Cys	Missense	3	9.0×10^{-4}	Yes[36]
	chr19:11113337	C/T	Arg416Trp	Missense	1	3.0×10^{-4}	
	chr19:11113398	T/C	Val436Ala	Missense	5	1.5×10^{-3}	
	chr19:11116125	G/A	Ala540Thr	Missense	20	6.0×10^{-3}	Yes[36]
	chr19:11116198	A/G	Asn564Ser	Missense	1	3.0×10^{-4}	
	chr19:11116880	A/C	Tyr576Ser	Missense	11	3.3×10^{-3}	Yes[36]
	chr19:11120502	A/T	Asp707Val	Missense	5	1.5×10^{-3}	Yes[36]
	chr19:11112202-11114606	Deletion/no deletion	Ex9-10DEL	Deletion (LoF)	5	1.5×10^{-3}	Yes[37]
	chr19:11129598	C/G	Asn825Lys	Missense	2	6.0×10^{-4}	

FH indicates familial hypercholesterolemia; and LoF, loss-of-function.

*Reference allele/alternative allele.

†Allele frequency in the combined, overall sample of 166 281 genotyped individuals. Mutations were identified in the subsample of 49 962 individuals who underwent both whole-genome sequencing and chip genotyping. The genotypes of the additional 116 319 individuals who were only chip genotyped were imputed. Sanger sequencing followed by reimputation was used to confirm genotypes and improve imputation accuracy (Methods).

The most common single FH mutation was a known founder mutation in Iceland,[35,38] a splice donor mutation in *LDLR* (c.694+2T>C) carried by 80 individuals and thus explaining 40.2% of monogenic FH in the overall sample. Five mutations are likely of a recent foreign origin and appear to have been introduced to the Icelandic gene pool during the last century (Data Supplement). Of the 20 mutations, 12 have not been described previously in Iceland (Table 1).

Lipid Levels in Monogenic FH

The maximum documented LDL-C level (maxLDL-C) in individuals with monogenic FH (N=175) was 7.15 mmol/L on average, compared with 3.94 mmol/L in noncarriers (N=104 653; Figure 1A). Levels of maxLDL-C were adjusted for statin use to approximate untreated levels (Methods). Monogenic FH associated with higher maxLDL-C by 3.37 mmol/L (95% CI, 3.16–3.58, $P<10^{-300}$; Table 2). Influence on maxLDL-C by mutation class are shown in Figure 1B and Table V in the Data Supplement. In addition, monogenic FH associated nominally with higher levels of lipoprotein(a) (β=0.35 SD [95% CI, 0.027–0.68]; P=0.034) but lower levels of triglycerides (β=–0.27 SD [95% CI, –0.41 to –0.16]; $P=2.4\times10^{-4}$) and HDL-C (β=–0.22 SD [95% CI, –0.07 to –0.37]; P=0.0032; Table 2), consistent with previous observations.[39,40]

Cumulative Lifetime Exposure to LDL-C

Figure 2 shows the relationship between monogenic FH and estimated cumulative exposure to LDL-C in adults aged 20 to 80 years in our data, expressed in units of mmol/L years. Cumulative exposure to LDL-C is the cumulative sum of mean LDL-C in mmol/L × years across age groups, based on LDL-C measurements taken over a period of nearly 3 decades (years 1990–2019; Figure III in the Data Supplement). As shown in Figure 2, individuals with monogenic FH have high cumulative LDL-C exposure throughout adult life. For example, the estimated cumulative LDL-C exposure of a 40-year-old individual with monogenic FH is similar to that of a 70-year-old noncarrier.

Atherosclerotic Diseases and Aortic Valve Stenosis

Monogenic FH associated with 3.4-fold greater risk of CAD (OR, 3.43 [95% CI, 2.25–5.22]; $P=9.8\times10^{-9}$)

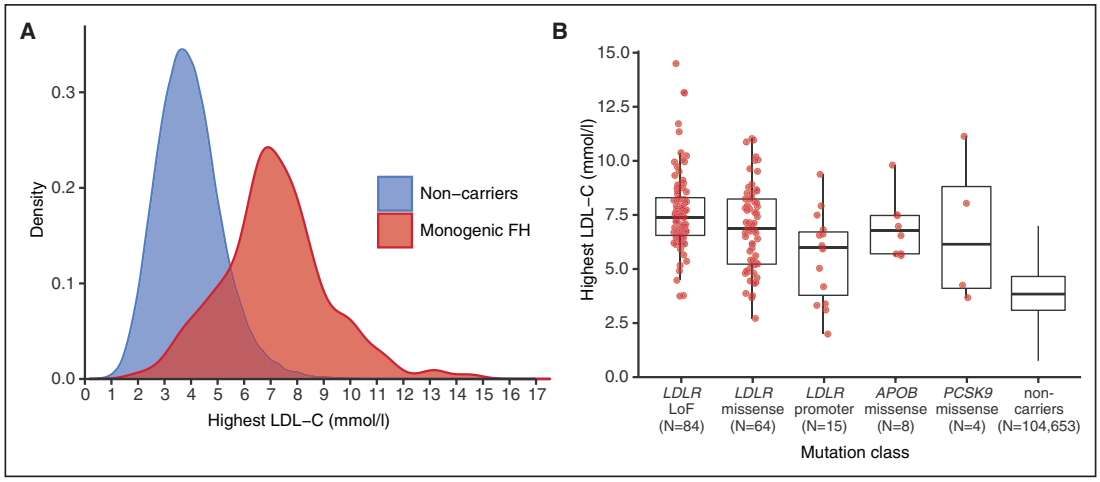

Figure 1. Monogenic familial hypercholesterolemia (FH) and LDL-C (low-density lipoprotein cholesterol) levels. **A** shows the distribution of the maximum documented LDL-C levels (maxLDL-C) in the subsample of 104 828 participants who had available LDL-C measurements. Individuals with monogenic FH are indicated with red (N=175) and FH mutation noncarriers with blue (N=104 653). **B** shows the distribution of maxLDL-C levels by FH mutation class. To convert LDL-C levels from mmol/L to mg/dL, multiply by 38.6.

and 5.1-fold higher risk of early-onset CAD (before age 50 years for men and 60 years for women; OR, 5.14 [95% CI, 2.84–9.28]; P=5.9×10^{-8}; Table 3). Associations stratified by mutation class are shown in Table VI in the Data Supplement. Individuals with monogenic FH were diagnosed with CAD (N=46) at a mean age of 57.7 years (SD 11.4 years), that is 8.4 years earlier than noncarriers (N=19 628, mean 66.1 years [SD 12.9 years]; P=3.7×10^{-7}). In addition, individuals with monogenic FH were more likely to have undergone coronary revascularization with percutaneous coronary intervention or coronary artery bypass surgery (Table 3). We did not observe associations with other atherosclerotic diseases such as peripheral artery disease (OR, 1.05 [95% CI, 0.33–3.38]; P=0.93) or ischemic stroke (OR, 0.88 [95% CI, 0.36–2.15]; P=0.78).

We evaluated the association between monogenic FH and measures of the extent of coronary atherosclerosis, as assessed by conventional coronary angiography (34 individuals with monogenic FH and 11 212 noncarriers) or noninvasive CAC scanning (18 individuals with monogenic FH, 5844 noncarriers). Characteristics of the samples are shown in Table VII in the Data Supplement. We observed an association with higher risk of having obstructive angiographic CAD (OR, 2.44 [95% CI, 1.11–5.36]; P=0.026) and left main disease (OR, 4.81 [95% CI, 2.02–11.44]; P=0.00038), adjusting for age and sex (Table VIII in the Data Supplement). Monogenic FH associated with the presence of coronary calcium (CAC score >0; OR, 5.68 [95% CI, 1.67–19.30]; P=0.0053) and CAC score >400 (OR, 11.48 [95% CI, 3.64–36.18]; P=3.1×10^{-5}), adjusting for age and sex (Table VIII in the Data Supplement). Thus, monogenic FH associated with greater burden of coronary atherosclerosis as assessed by either coronary angiography or CAC scanning.

An association between monogenic FH and increased risk of aortic valve stenosis was recently reported in Norway.[41] We tested for association with aortic valve stenosis and found that individuals with monogenic FH had 3.4-fold higher risk of aortic valve stenosis than noncarriers (OR, 3.41 [95% CI, 1.16–10.05]; P=0.026; Table 3).

Table 2. Association of Monogenic FH With Blood Lipid Levels

		Monogenic FH		Noncarriers		Adjusted difference	
Lipid trait	N total*	N	Mean (SD)	N	Mean (SD)	β (95% CI)	*P* value
LDL-C (maximum), mmol/L†	104 828	175	7.15 (2.02)	104 653	3.94 (1.23)	+3.37 mmol/L (3.16 to 3.58)	<10^{-300}
Triglycerides, mmol/L	109 550	178	1.24 (0.64)	109 372	1.44 (0.80)	−0.27 SD (−0.41 to −0.16)	2.4×10^{-4}
HDL-C, mmol/L	110 076	177	1.35 (0.38)	109 899	1.45 (0.42)	−0.22 SD (−0.07 to −0.37)	0.0032
Lipoprotein(a), nmol/L	24 257	36	61.1 (102)	24 221	41.5 (63.2)	+0.35 SD (0.027 to 0.68)	0.034

Values for LDL-C, HDL-C, and triglycerides are given in mmol/L. To convert to mg/dL, multiply by 38.6 for LDL-C and HDL-C, and by 88.6 for triglycerides. FH indicates familial hypercholesterolemia; HDL-C, high-density lipoprotein cholesterol; and LDL-C, low-density lipoprotein cholesterol.

*No. of genotyped participants with at least one available measurement of the relevant lipid trait.

†Adjusted for statin use (Methods).

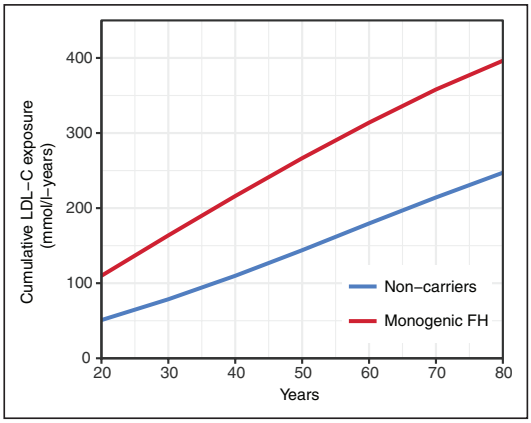

Figure 2. Cumulative lifetime exposure to LDL-C (low-density lipoprotein cholesterol).
Shown is the estimated average lifetime cumulative exposure to LDL-C, in units of mmol/L years. Individuals with monogenic familial hypercholesterolemia (FH; N=175) are shown in red, and non-carriers (N=104 653) in blue. Here, LDL-C measurements were not adjusted for statin use and thus reflect actual exposure to LDL-C. To convert mmol/L years to mg/dL years, multiply by 38.6.

Lifespan

Among individuals who lived to be at least 50 years old and were born after 1880 (N=48 628), individuals with monogenic FH had shorter lifespan by an average of 3.6 years (95% CI, 1.0–6.2; *P*=0.0066).

Prevalence by Maximum LDL-C Level and Diagnosis of Early-Onset CAD

We assessed the prevalence of monogenic FH by different strata of maxLDL-C levels and by diagnosis of early-onset CAD (Table IX in the Data Supplement). The prevalence was 1 in 599 (0.17%) among genotyped individuals with at least one LDL-C measurement available (N=104 828). Among those with maxLDL-C≥4.9 mmol/L (N=20,507), the prevalence was 1 in 134 (0.75%) and among those with both maxLDL-C≥4.9 mmol/L and early-onset CAD (N=1247), the prevalence was almost 2-fold higher (1 in 69 or 1.44%). The highest prevalence (11.4%) was observed in individuals with maxLDL-C over 8.5 mmol/L (N=325).

Clinically Defined FH and the Contribution of Monogenic FH

We screened for the clinical FH phenotype using a modified version of the DLCN criteria that exclude genotype data and physical examination findings. We screened a subsample of the overall genotyped sample, consisting of 79 058 living participants between the ages of 20 and 80 years that had at least one LDL-C measurement (summarized in Figure IV in the Data Supplement). Their mean age was 57.7 years and 45.0% were male (Table X in the Data Supplement). The prevalence of monogenic FH in this sample was 0.18% (Table XI in the Data Supplement).

A total of 1736 (2.2%) individuals fulfilled the criteria for clinical FH (probable or definite FH). The prevalence of clinical FH increased with age and was highest in those between the ages of 70 and 80 years (3.8%; Table XII in the Data Supplement). Overall, only 5.2% (N=90) of individuals with clinical FH were found to have monogenic FH (20.3% [N=29] of individuals with definite FH and 3.8% [N=61] with probable FH, Table XI in the Data Supplement).

Comparing Monogenic FH and Mutation-Negative Clinical FH

We explored the differences between individuals with a purely genetic diagnosis of FH (ie, monogenic FH) and those with clinical diagnosis of FH where no causative mutation is found. For this analysis, we compared the characteristics of individuals with monogenic FH (irrespective of DLCN classification) and those with mutation-negative clinical FH, defined as the subsample of individuals with clinical FH who did not carry an FH mutation (N=1736–90=1646). As individuals with

Table 3. Association of Monogenic FH With Atherosclerotic Diseases and Aortic Valve Stenosis

Disease	Cases*	Controls†	OR (95% CI)	*P* value
Coronary artery disease	19 674 (46)	129 508	3.43 (2.25–5.22)	9.8×10^{-9}
Coronary artery disease, early onset‡	3473 (19)	145 415	5.14 (2.84–9.28)	5.9×10^{-8}
Percutaneous coronary intervention	4067 (15)	139 646	4.14 (2.14–8.04)	2.6×10^{-5}
Coronary artery bypass surgery	3747 (15)	144 764	5.05 (2.55–10.03)	3.6×10^{-6}
Peripheral artery disease	2601 (3)	144 735	1.05 (0.33–3.38)	0.93
Ischemic stroke	5156 (5)	144 400	0.88 (0.36–2.15)	0.78
Aortic valve stenosis	1662 (5)	144 941	3.41 (1.16–10.05)	0.026

FH, familial hypercholesterolemia; and OR, odds ratio.
*No. of cases that have monogenic FH are given within parentheses.
†Controls are noncases for a given phenotype from the overall genotyped sample.
‡Age at diagnosis <50 y for men and <60 y for women.

mutation-negative clinical FH were alive by definition, we included only living individuals with monogenic FH (N=166) for this comparison.

Individuals with monogenic FH were younger than individuals with mutation-negative clinical FH (mean age, 53.9 versus 66.4 years, $P<0.0001$). Individuals with monogenic FH were more likely to have extreme hypercholesterolemia (maxLDL-C≥8.5 mmol/L; 19.3% versus 10.9%, $P=0.00064$) and family history of either hypercholesterolemia (maxLDL-C above 95th percentile; 71.1% versus 62.3%, $P<0.0001$) or clinical FH (68.1% versus 52.2%, $P<0.0001$), but lower prevalence of early-onset CAD (10.2% versus 33.2%, $P<0.0001$), hypertension (21.7% versus 51.3%, $P<0.0001$), and ever smoking (28.3% versus 47.1%, $P=0.0091$), with P values adjusted for age and sex (Table XIII in the Data Supplement).

We evaluated prescribing patterns of cholesterol-lowering drugs using nationwide drug prescription data for lipid-lowering medications prescribed from 2003 to 2018. During this period, individuals with monogenic FH were less likely than individuals with mutation-negative clinical FH to have received a prescription of any statin (75.9% versus 96.9%, $P<0.0001$) but were more likely to have received a high-potency statin (55.4% versus 46.2%, $P=0.00015$), ezetimibe (28.9% versus 11.1%, $P<0.0001$), and PCSK9 (proprotein convertase subtilisin/kexin type 9) inhibitors (3.0% versus 0.73%, $P=0.048$), with P values adjusted for age and sex (Table XIII in the Data Supplement). At the time of first prescription of a lipid-lowering medication, individuals with monogenic FH were on average 9.9 years younger than those with mutation-negative clinical FH (mean age, 44.8 versus 54.4 years; adjusted difference, −9.9 years; $P<0.0001$), after accounting for sex.

Prescription Patterns and Effectiveness of Cholesterol-Lowering Treatment

Figure 3 shows the latest unadjusted LDL-C measurement (years 2004–2018) by prescription of statins (highest potency class) and ezetimibe in the preceding year for living individuals with monogenic FH (N=135, mean age 56.0 years) and mutation-negative clinical FH (N=1508, mean age 66.4 years). Individuals that did not have an LDL-C measurement during this time period were not included. During the year preceding the measurement, high-potency statins were prescribed to 40.0% and 21.9% of individuals with monogenic FH and mutation-negative clinical FH, respectively. The fraction of those who received neither statins nor ezetimibe was 28.1% and 17.9%, respectively. Only 11.0% of individuals with monogenic FH and 24.9% with mutation-negative clinical FH attained an LDL-C level <2.6 mmol/L, the target endorsed by the 2016 European Society of

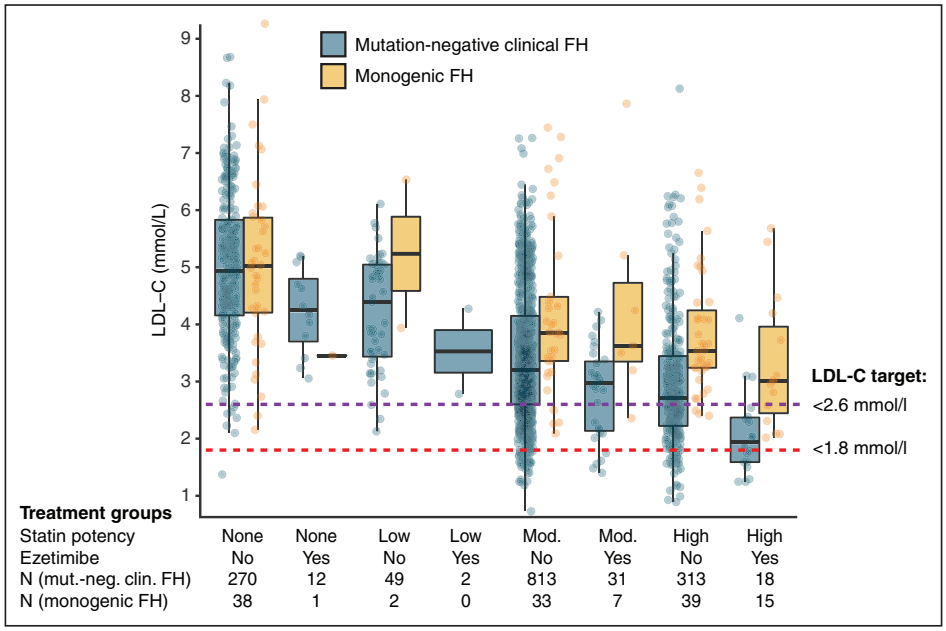

Figure 3. Prescription patterns and effectiveness of cholesterol-lowering therapy in living individuals with monogenic familial hypercholesterolemia (FH; yellow, N=135) and mutation-negative clinical FH (blue, N=1508).
Shown is the latest available LDL-C (low-density lipoprotein cholesterol) measurement (years 2004–2018) as a function of potency of the prescribed cholesterol-lowering therapy (ie, prescriptions of statins and ezetimibe) during the preceding year. Here, LDL-C values were not adjusted for statin use. Horizontal lines indicate the recommended target levels for primary prevention in FH according to the European Society of Cardiology (ESC) and European Atherosclerosis Society (EAS) guidelines from 2016 (purple, <2.6 mmol/L)[42] and 2019 (red, <1.8 mmol/L).[43]

Cardiology/European Atherosclerosis Society Guidelines for the management of dyslipidaemias[42] for primary prevention in FH (Figure 3). No individual with monogenic FH and only 5.2% with mutation-negative clinical FH attained an LDL-C level <1.8 mmol/L, the target recommended by the 2019 European Society of Cardiology/European Atherosclerosis Society guidelines[43] for primary prevention in FH, in the absence of atherosclerotic disease and other major cardiovascular risk factors. These data demonstrate that both individuals with monogenic FH and individuals with mutation-negative clinical FH are markedly undertreated.

Polygenic Contribution in Mutation-Negative Clinical FH

We estimated the polygenic contribution in mutation-negative clinical FH using an LDL-C genetic score based on 345 lipid-associated variants (Methods). These analyses were performed in a subsample of 72 926 individuals from the overall genotyped sample who (1) were classified using the DLCN criteria, (2) had an available genetic score, and (3) did not have monogenic FH. This sample consists of 1564 individuals with mutation-negative clinical FH and 71 362 controls (ie, unlikely or possible FH according to the DLCN criteria).

An increase in the genetic score corresponding to 1-SD increase in LDL-C (≈1.04 mmol/L increase in maxLDL-C) was associated with about 9-fold higher risk of mutation-negative clinical FH (OR, 9.25, P=3.5×10^{-138}) and 2-fold higher risk of early-onset CAD (OR, 1.94, P=7.6×10^{-31}) but was not associated with risk of ischemic stroke (Table XIV in the Data Supplement). A total of 78.7% of mutation-negative clinical FH cases had values above the 50th percentile in the overall distribution, 58.7% above the 70th percentile, 26.2% above the 90th percentile, and 15.0% above the 95th percentile (Figure 4A). These results show that a large fraction of mutation-negative clinical FH individuals has a high polygenic burden of LDL-C-raising sequence variants.

We compared the risk of mutation-negative clinical FH by percentiles of the genetic score, relative to individuals in the middle quintile (40–59th percentile), adjusting for age and sex. There was a trend toward higher maxLDL-C and higher risk of mutation-negative clinical FH with increasing percentiles of the genetic score (Figure V in the Data Supplement and Figure 4B). Individuals with a genetic score at or above the 99.9th percentile (N=58) had a mean maxLDL-C of 5.0 mmol/L and the prevalence of mutation-negative clinical FH in this percentile was 9.6% (OR, 5.74, P=1.6×10^{-5}). Compared with monogenic FH, individuals with a genetic score at or above the 99.9th percentile had lower estimated cumulative lifetime exposure to LDL-C (Figure VI in the Data Supplement).

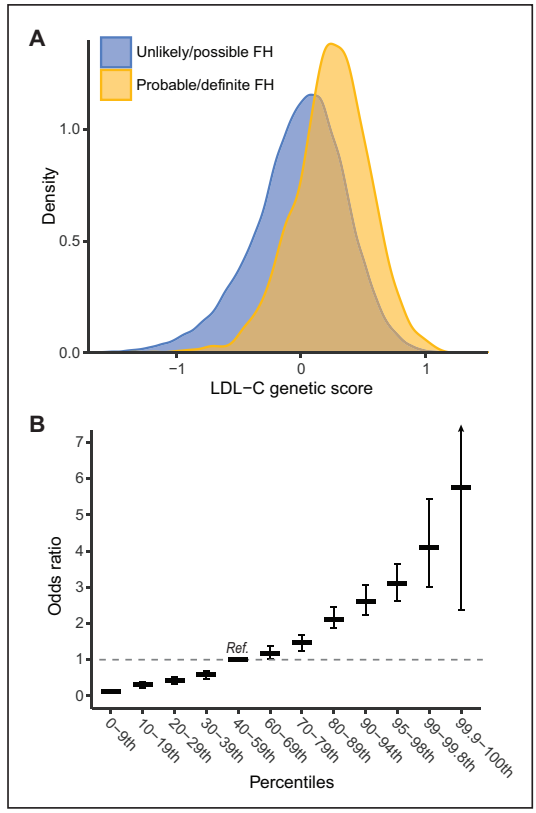

Figure 4. Polygenic contribution to mutation-negative clinical familial hypercholesterolemia (FH).
A shows the distribution of the LDL-C (low-density lipoprotein cholesterol) genetic score by clinical FH status according to a modified version of the Dutch Lipid Clinic Network criteria, excluding individuals with monogenic FH. Yellow indicates clinical FH (probable or definite FH, N=1564) and blue indicates controls (unlikely or possible FH, N=71 362). **B** shows odds ratios for clinical FH by percentiles of the LDL-C genetic score, given relative to the middle quintile (40–59th percentile). 95% CIs are presented.

DISCUSSION

We found that the prevalence of monogenic FH was 1 in 836 in the overall sample of 166 281 genotyped individuals, representing a large fraction of the Icelandic population (364.134 inhabitants on January 1, 2020, Statistics Iceland). We observed a higher prevalence in the nonrandom subsample of 49 962 individuals who had been selected for WGS (1 in 515). This is expected due to the intentional enrichment for individuals with high LDL-C and various cardiovascular phenotypes in this subsample. Thus, the prevalence of monogenic FH in the WGS subsample likely overestimates the true prevalence in the Icelandic population. Because WGS was not performed on all study participants, we may have missed ultra-rare and private FH mutations that are only present in the 116 319 individuals who did not undergo WGS, resulting

in underestimation of the prevalence in the overall genotyped sample. Nevertheless, our data suggest that the prevalence of monogenic FH in Iceland is considerably lower than recent estimates from large genetic studies in Denmark[6] (1 in 217), the United States[5,7,9] (from 1 in 260 to 1 in 211) and the UK Biobank[8] (1 in 176). The comparatively low prevalence of monogenic FH in our study may be related the geographic isolation and genetic homogeneity of the Icelandic population.[44] In addition, because we applied a conservative approach in the selection of mutations assumed to be causative of FH (eg, in-silico predictions were not considered) we may have missed some true FH mutations. Taken together, our findings suggest that the prevalence of monogenic FH in the Icelandic population is likely lower than recent estimates in several European populations.

Individuals with monogenic FH are exposed to high plasma LDL from early life and throughout adulthood.[45] Using LDL-C measurements spanning 3 decades for over 100 000 individuals, we demonstrated a high cumulative lifetime exposure to LDL in Icelanders with monogenic FH, consistent with previous studies.[7,46] Monogenic FH was strongly associated with increased risk of premature coronary disease and greater burden of coronary atherosclerosis, as previously described.[5–7,47–49] We did not observe increased risk of ischemic stroke in monogenic FH, in keeping with previous studies,[40,50] indicating that high LDL levels may not influence the development of atherosclerotic lesions to the same extent in all arteries. Our results corroborate recent findings of an increased risk of aortic valve stenosis in monogenic FH,[41] consistent with a causal role of LDL in the development of aortic valve stenosis.[20,51,52]

We observed that 2.2% of 79 000 living adult participants with at least one LDL-C measurement could be classified as having clinical FH, defined as probable or definite FH according to a modified version of the DLCN criteria. Clinical FH was over 10-times more common than monogenic FH in this sample (2.2% versus 0.18%). Of note, individuals with clinical FH were more likely than individuals with monogenic FH to have early-onset CAD (33% versus 10%). Although not entirely clear, this may reflect enrichment for cases of early-onset CAD due to its weight in the DLCN criteria (giving 2 points), older age (mean age, 66 versus 54 years), or both. Previous estimates of the prevalence of clinical FH in large population-based studies, using DLCN criteria, have ranged between 0.35% and 1.2%.[5,6,53,54] The comparatively high prevalence observed in our study may be explained, at least in part, by the use of comprehensive genealogical information providing an accurate family history that is not subject to recall bias. We found that only about 5% of individuals with clinical FH had monogenic FH. This observation is consistent with a study among 46 285 participants in an electronic health records-linked biobank where only about 9% of individuals with clinical FH carried an FH mutation.[5] By contrast, in tertiary lipid clinics, a monogenic cause is commonly found in 40% to 50% of cases.[11–13] This is not surprising, however, as individuals who are referred to lipid clinics represent a highly selected population with high a priori probability of having a causative mutation. Thus, our findings indicate that on the population scale, the clinical FH phenotype is likely caused by monogenic FH in only a small minority of cases.

Our results demonstrate that polygenic susceptibility to elevated plasma LDL-C is an important contributor to development of mutation-negative clinical FH, consistent with previous studies.[11–13] In contrast to one previous study[55] but consistent with a recent report,[56] our study shows that having an extreme value of a LDL-C genetic score is not comparable to having monogenic FH. Compared with monogenic FH, individuals with a genetic score at or above the 99.9th percentile had lower maxLDL-C levels (mean, 5.0 versus 7.15 mmol/L), lower estimated cumulative lifetime exposure to LDL-C and substantially lower prevalence of clinical FH (9.6% versus 64%). These results are also consistent with previous findings showing a greater risk of atherosclerotic cardiovascular disease[8] and higher severity of preclinical atherosclerosis[57] in individuals with monogenic FH, compared with those considered to have polygenic hypercholesterolemia on the basis of a high LDL-C genetic score. Thus, a high LDL-C genetic score is a marker of polygenic predisposition to hypercholesterolemia and the clinical FH phenotype, but it does not have a penetrance comparable to that of monogenic FH.

The present findings have clinical implications. First, our results show that the majority of Icelanders with monogenic and clinically defined FH are markedly undertreated with cholesterol-lowering medications, as is the case in most countries.[2] Here, only a small minority reached a target of LDL-C<2.6 mmol/L (11% and 25%, respectively) as suggested by the 2016 European Society of Cardiology/European Atherosclerosis Society guidelines,[42] and even fewer reached <1.8 mmol/L (0% and 5%) as suggested by the recent 2019 European Society of Cardiology/European Atherosclerosis Society guidelines.[43] Note that these targets are only appropriate for primary prevention in individuals with FH without other major cardiovascular risk factors. Thus, the degree of undertreatment in our data is underestimated by these numbers, as lower targets would apply for those with manifest atherosclerotic disease or otherwise classified at very high risk. The most likely explanation for undertreatment is clinical underdiagnosis due to several factors, including inadequate awareness of FH among clinicians and underuse of genetic testing and family cascade screening. In addition, among individuals with a known diagnosis of FH, lack of appropriate escalation of therapy as well as lack of patient

education and motivation are likely contributing factors. Second, the yield of clinical genetic testing for FH and subsequent family cascade screening in Iceland can be improved by incorporating the panel of FH mutations identified in this study. Third, the obvious underdiagnosis and undertreatment of FH in Iceland calls for public health care initiatives to improve diagnosis and appropriate treatment of FH, including clinician awareness and facilitation of referrals for genetic testing and subsequent family cascade screening.

Limitations

Several limitations to this study deserve mention. We chose a conservative approach in defining FH mutations which limits false-positives but comes at the expense that some very rare mutations that truly cause FH may have been missed. Identification of FH mutations was based on WGS in approximately a third of the overall sample and thus we may have missed FH mutations only present in those that were not sequenced. However, these mutations would likely be extremely rare and thus not have significant impact on the estimated prevalence of monogenic FH. Similarly, we cannot exclude the presence of undetected, potentially pathogenic copy-number variants in *LDLR* in our data. Although widely used in registry studies,[5,6,58] the DLCN criteria were not designed for screening at a population level and may thus not be ideal for this purpose. Analyses were based on LDL-C measurements taken for various clinical indications and thus this sample may be enriched for individuals with high LDL-C levels. Prevalence estimate of clinical FH is subject to an inherent selection bias related to genotyping status and the availability of LDL-C measurements and thus our estimate may be biased upwards. The use of cholesterol-lowering drugs was inferred from drug prescription data and may not accurately reflect the actual use in some cases.

Conclusions

Our findings indicate that the prevalence of monogenic FH in Iceland is lower than many contemporary estimates in European and North American populations. Clinical FH is a relatively common high-risk cardiovascular phenotype that has a strong polygenic basis but is rarely caused by an FH mutation. Both individuals with monogenic FH and individuals with mutation-negative clinical FH are markedly undertreated with cholesterol-lowering agents in Iceland. These results emphasize an urgent need for improved diagnosis and appropriate treatment of monogenic and clinically defined FH.

ARTICLE INFORMATION

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Supplemental Materials

Note
Supplementary Methods
Data Supplement Tables I–XV
Data Supplement Figures I–VI
References [59,60]

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Paper IV

Circulation: Genomic and Precision Medicine

ORIGINAL ARTICLE

Lifelong Reduction in LDL (Low-Density Lipoprotein) Cholesterol due to a Gain-of-Function Mutation in *LDLR*

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BACKGROUND: Loss-of-function mutations in the LDL (low-density lipoprotein) receptor gene (*LDLR*) cause elevated levels of LDL cholesterol and premature cardiovascular disease. To date, a gain-of-function mutation in *LDLR* with a large effect on LDL cholesterol levels has not been described. Here, we searched for sequence variants in *LDLR* that have a large effect on LDL cholesterol levels.

METHODS: We analyzed whole-genome sequencing data from 43 202 Icelanders. Single-nucleotide polymorphisms and structural variants including deletions, insertions, and duplications were genotyped using whole-genome sequencing-based data. LDL cholesterol associations were carried out in a sample of >100 000 Icelanders with genetic information (imputed or whole-genome sequencing). Molecular analyses were performed using RNA sequencing and protein expression assays in Epstein-Barr virus-transformed lymphocytes.

RESULTS: We discovered a 2.5-kb deletion (del2.5) overlapping the 3′ untranslated region of *LDLR* in 7 heterozygous carriers from a single family. Mean level of LDL cholesterol was 74% lower in del2.5 carriers than in 101 851 noncarriers, a difference of 2.48 mmol/L (96 mg/dL; $P=8.4\times10^{-8}$). Del2.5 results in production of an alternative mRNA isoform with a truncated 3′ untranslated region. The truncation leads to a loss of target sites for microRNAs known to repress translation of *LDLR*. In Epstein-Barr virus-transformed lymphocytes derived from del2.5 carriers, expression of alternative mRNA isoform was 1.84-fold higher than the wild-type isoform ($P=0.0013$), and there was 1.79-fold higher surface expression of the LDL receptor than in noncarriers ($P=0.0086$). We did not find a highly penetrant detrimental impact of lifelong very low levels of LDL cholesterol due to del2.5 on health of the carriers.

CONCLUSIONS: Del2.5 is the first reported gain-of-function mutation in *LDLR* causing a large reduction in LDL cholesterol. These data point to a role for alternative polyadenylation of *LDLR* mRNA as a potent regulator of LDL receptor expression in humans.

Key Words: cardiovascular disease ■ genetics ■ lipids ■ microRNA ■ polyadenylation

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Nonstandard Abbreviations and Acronyms

3′ UTR	3′ untranslated region
LDL	low-density lipoprotein
PAS	polyadenylation site
PCSK9	proprotein convertase subtilisin/kexin type 9
SREBP	sterol regulatory element-binding protein
WGS	whole-genome sequencing

Elevated levels of LDL cholesterol play a central role in the pathogenesis of coronary heart disease.[1] Drugs that lower LDL cholesterol remain the cornerstone of medical therapy; these include statins and inhibitors of the enzyme PCSK9 (proprotein convertase subtilisin/kexin type 9).[2] Both mediate their effect through enhanced function of the LDL receptor pathway, the key regulator of plasma LDL levels.[3] Defects in the LDL receptor or its regulation result in high levels of LDL and premature cardiovascular disease, as demonstrated by high impact loss-of-function mutations in the *LDLR* gene that cause familial hypercholesterolemia.[4] In contrast, gain-of-function mutations in *LDLR* would be expected to cause reduction in LDL cholesterol levels and protect against cardiovascular disease. Sequence variants in *LDLR* that result in a modest gain-of-function and thus moderate reduction in LDL cholesterol have been described.[5–7] However, a gain-of-function mutation with a large effect on LDL cholesterol has hitherto not been described in *LDLR*.

Here, we searched for sequence variants in *LDLR* with a large effect on LDL cholesterol levels in a large sample of Icelanders. We discovered a rare 2.5 kilobase (kb) deletion (del2.5) in the 3′ untranslated region (UTR) of *LDLR* that causes enhanced expression of the LDL receptor and a large reduction in circulating LDL cholesterol, consistent with a gain-of-function effect.

METHODS

This study was based on a large database of DNA sequence variation in the Icelandic population combined with extensive phenotypic data, which has been described previously.[8–10] A full description of the methods and materials used in this study is available in the Data Supplement. Additional data that support the findings of this study are available from the corresponding authors upon reasonable request. The study was approved by the Data Protection Authority of Iceland and The National Bioethics Committee of Iceland and complies with the Declaration of Helsinki.

RESULTS

A Gain-of-Function Deletion in *LDLR*

We searched for rare sequence variants in *LDLR* with a large effect on LDL cholesterol using whole-genome sequencing (WGS) data from 43 202 Icelanders. We searched for deletions and other structural variants in *LDLR* using several WGS-based methods, as described in the Data Supplement. The WGS dataset was enriched for individuals at the extremes of the distribution of LDL cholesterol levels. The proportion that had undergone WGS was 30.3% in the overall set of chip-typed individuals with available LDL cholesterol levels (n=101 857), compared with 49.0% and 54.0% for LDL cholesterol levels less than first or >99th percentile, respectively. This corresponds to enrichment by 1.62- and 1.79-fold, respectively (Data Supplement).

We detected 4 mutations previously reported to cause familial hypercholesterolemia in Iceland[11–13] (Table I in the Data Supplement). In addition, using the PopDel software that is able to detect deletions of 500 to 10 000 bp in WGS-based data,[14] we identified a 2.5 kb (2485 bp) deletion (del2.5) overlapping the distal portion of the 3′ untranslated region (3′ UTR; genomic coordinates [hg38], chr19:11, 133, 108-11, 135, 592, Figure 1A) in 3 closely related individuals (family members I.2, II.4, and III.4, Figure 1B). This deletion was not found in any of the other 43 199 individuals in the WGS dataset and was not detected with the other variant calling tools used. Following WGS of DNA samples from other family members, we identified 4 additional carriers of del2.5 (family members II.1, II.7, III.2, and III.3). All 7 carriers are heterozygous for del2.5 and cluster within 3 generations (Figure 1B). Haplotype analyses indicate that del2.5 arose as a de novo mutation in family member I.2 (Figure II in the Data Supplement) which, together with the absence of del2.5 from other individuals in the WGS dataset, suggests that del2.5 is unique to this family.

Carriers of del2.5 with available blood lipid measurements (n=6) had levels of calculated LDL cholesterol within the first percentile (adjusted for age and sex), with a mean of 0.87 mmol/L (Figure 1B and 1C). Levels of calculated LDL cholesterol of carriers were lower than those of 101 851 population-based noncarriers (mean, 3.34 mmol/L; SD, 0.90 mmol/L), with a difference of 2.48 mmol/L (P=8.4×10^{-8}; Table). This corresponds to 74% lower levels of calculated LDL cholesterol. Comparable results were observed for directly measured LDL cholesterol (Table). LDL cholesterol levels of the carriers were consistently low in multiple measurements over several decades of life (youngest at 20 years, oldest at 85 years; data not shown). Del2.5 carriers also had lower levels of total cholesterol (difference, 47% lower; P=1.6×10^{-6}), non-high-density lipoprotein cholesterol (difference, 63% lower; P=1.2×10^{-7}), and apolipoprotein B (difference, 54% lower; P=1.4×10^{-6}; Table). Levels of high-density lipoprotein cholesterol, triglycerides, and lipoprotein(a) did not differ significantly between carriers and noncarriers. Noncarriers within the family did not exhibit hypocholesterolemia (Figure 1B). Pathogenic mutations in genes linked to monogenic disorders involving low LDL cholesterol levels (eg, *APOB*, [Apolipoprotein B] *PCSK9*, *MTTP* [microsomal triglyceride transfer protein], *APOC3* [apolipoprotein C-III], *ANGPTLs*

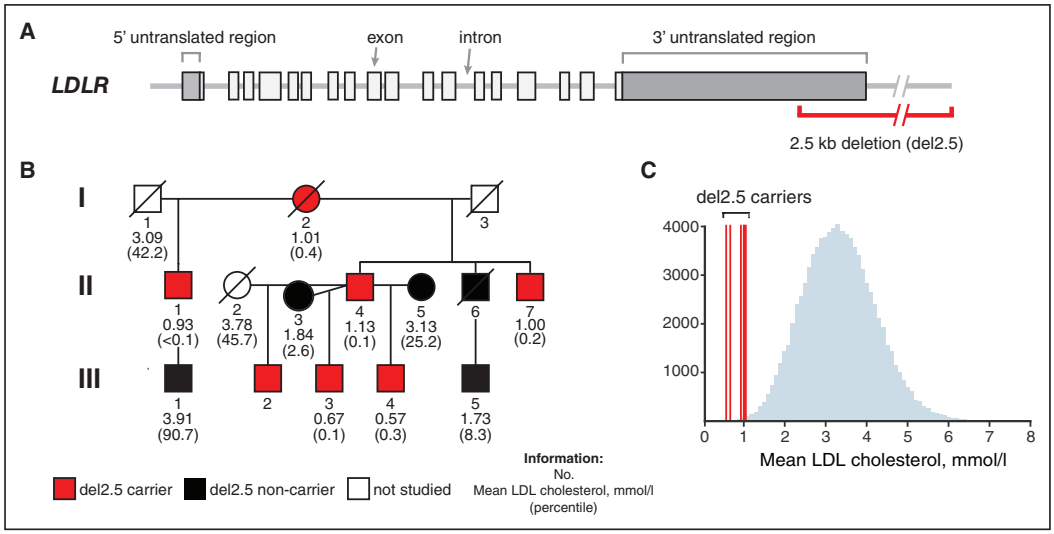

Figure 1. *LDLR* del2.5 deletion reduces circulating LDL cholesterol in carriers.
A, Shows the location of the 2.5 kilobase deletion (del2.5) in the LDL (low-density lipoprotein) receptor gene (*LDLR*). Del2.5 removes the distal part of the 3′ untranslated region and extends beyond the gene (Figure I in the Data Supplement). **B**, Shows the pedigree of the family. Red denotes carriers of del2.5 and black denotes genotyped noncarriers. The numbers presented below each individual are the individual's number (within each generation), mean level of LDL cholesterol in units of mmol/L, and age- and sex-adjusted percentile in the Icelandic population. None of the carriers of del2.5 were known to take statins. To maintain anonymity, some nonparticipants and genotyped noncarriers are omitted from the pedigree. **C**, Shows the distribution of mean levels of calculated LDL cholesterol in 101 857 genotyped Icelanders. Values for the 6 carriers with available measurements are indicated with red lines. To convert the values for LDL cholesterol from mmol/L to mg/dL, multiply by 38.6.

[angiopoietin-like genes] and *SAR1B* [secretion-associated ras-related GTPase 1B])[15] were not detected in WGS data from del2.5 carriers. Taken together, del2.5 in *LDLR* causes primarily a large reduction in LDL cholesterol, consistent with a gain-of-function effect.

As PCSK9 is a major regulator of LDL metabolism, we compared circulating levels of PCSK9 in peripheral blood from del2.5 carriers (n=4) to those from noncarriers (n=74). Levels of PCSK9 were lower by 34% in the carriers (*P*=0.021; Figure III in the Data Supplement), consistent with positive correlation between levels of PCSK9 and LDL cholesterol in individuals not treated with statins.[16,17]

Del2.5 Causes Shortening of the 3′ UTR of *LDLR* mRNA

Del2.5 removes ≈700 bp of the distal end of the 3′ UTR of *LDLR*, which includes the distal polyadenylation site (PAS; approximately at chr19:11,133,820) that gives rise to the canonical *LDLR* mRNA isoform

Table. Associations of del2.5 With Lipid Traits

	Carriers			Noncarriers			Difference	
Lipid traits	n	Mean (SD)	Range	n	Mean (SD)	Median (IQR)	Adjusted (units in SD)	*P* value
Total cholesterol, mmol/L	6	2.88 (0.45)	2.25–3.60	113 349	5.40 (0.98)	5.38 (4.71–6.04)	−2.75	1.6×10^{-6}
Non-HDL cholesterol, mmol/L	6	1.48 (0.38)	0.93–1.96	106 858	3.98 (0.98)	3.94 (3.30–4.61)	−2.96	1.2×10^{-7}
LDL cholesterol, mmol/L								
Calculated	6	0.86 (0.19)	0.57–1.01	101 851	3.34 (0.90)	3.31 (2.71–3.92)	−2.95	8.4×10^{-8}
Measured	4	1.01 (0.63)	0.35–1.87	12 569	3.43 (1.03)	3.36 (2.68–4.07)	−2.96	2.7×10^{-7}
HDL cholesterol, mmol/L	6	1.41 (0.38)	1.14–2.00	107 030	1.45 (0.42)	1.40 (1.15–1.69)	0.19	0.79
Triglycerides, mmol/L	6	1.33 (0.59)	0.57–2.38	94 624	1.44 (0.80)	1.25 (0.92–1.74)	0.18	0.80
Apolipoprotein A, g/L	4	1.46 (0.16)	1.29–1.67	23 871	1.62 (0.31)	1.58 (1.40–1.80)	−0.25	0.69
Apolipoprotein B, g/L	4	0.47 (0.17)	0.32–0.70	23 871	1.03 (0.27)	1.01 (0.84–1.20)	−2.79	1.4×10^{-6}
Lipoprotein (a), nmol/L	4	8.30 (5.07)	4.37–15.65	23 819	41.50 (63.14)	13.89 (6.12–41.08)	−0.93	0.16

To convert the values for cholesterol from mmol/L to mg/dL, multiply by 38.67. To convert the values for triglycerides from mmol/L to mg/dL, multiply by 88.57. Difference is shown in units of SDs (for inverse-rank normalized residuals). *P* values were derived from linear regression models. HDL indicates high-density lipoprotein; IQR, interquartile range; and LDL, low-density lipoprotein.

(RefSeq transcript NM_000527.4). As polyadenylation is essential for the translation of mRNA into protein, we hypothesized that an alternative PAS would be used in the presence of del2.5, giving rise to an alternative mRNA isoform. Consistent with this, using 3′ rapid amplification of cDNA, we observed 2 primary mRNA isoforms differing in the length of their 3′ UTRs in peripheral blood from a heterozygous del2.5 carrier (family member II.4; Figure 2A and 2B). In addition to isoforms with the full-length 3′ UTR (2500 nucleotides), we observed high abundance of isoforms with a shorter 3′ UTR (844 nucleotides). The shorter 3′ UTR results from the use of an alternative, proximal PAS, ≈1 kb upstream of del2.5. The use of this alternative PAS is supported by databases of RNA sequences[18] and expressed sequence tags[19] in human cell lines (Figures IV and V in the Data Supplement).

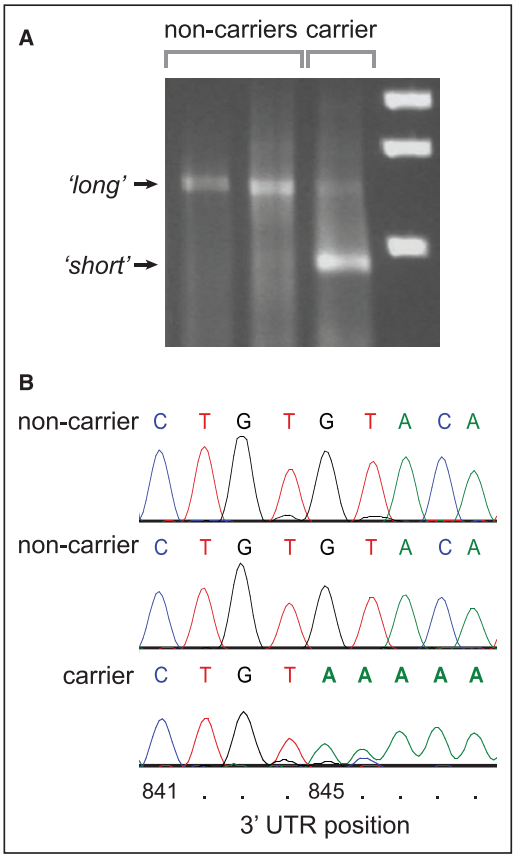

Figure 2. *LDLR* del2.5 removes the canonical polyadenylation site generating shorter LDLR mRNA.
A, Depicts the results of 3′rapid amplification of cDNA of *LDLR* mRNA isolated from peripheral blood of a del2.5 carrier (family member II.4) and 2= noncarriers, demonstrating the presence of a shortened 3′untranslated region (3′UTR) in the del2.5 carrier. **B**, Shows a Sanger sequencing chromatogram of *LDLR* cDNA demonstrating polyadenylation at a proximal site in the 3′ UTR (position 845; genomic coordinate, chr19:11,132,161 [hg38]).

The Short 3′ UTR Leads to Higher Expression of the LDL Receptor

Shortening of 3′ UTRs may increase stability and translational efficiency of mRNA through the loss of negative regulatory elements such as microRNA target sites.[20] Several microRNAs have been shown to repress the translation of *LDLR* mRNA and alter plasma LDL cholesterol in vivo.[21–24] The majority of target sites for these microRNAs are located distally to the alternative PAS and thus are not found in the shorter 3′ UTR of the *LDLR* mRNA generated by the del2.5 allele (Table II in the Data Supplement). Thus, the shorter 3′ UTR would be expected to confer greater mRNA stability and a subsequent greater protein production than the full-length 3′ UTR. To investigate this, we studied *LDLR* mRNA and protein expression in Epstein-Barr virus-transformed lymphocytes derived from 4 carriers of del2.5 and up to 20 noncarrier controls (8 for mRNA expression, 20 for protein expression). We used Epstein-Barr virus-transformed lymphocytes for their relative ease of access and because they express both *LDLR* and the microRNAs know to regulate *LDLR* expression[21–24] (Data Supplement).

Using RNA sequencing, we observed lower coverage in the region distal to the alternative PAS in del2.5 carriers than in 8 matched controls, consistent with a more frequent use of this alternative PAS (Figure 3A). In an allele-specific analysis, we observed that *LDLR* mRNA originating from the del2.5 allele was more abundant than mRNA arising from the unaffected allele (wild-type) in the del2.5 carriers (fold-difference, 1.84; P=0.0013). Furthermore, the del2.5 allele was more highly expressed than the wild-type allele in noncarriers (fold-difference, 1.55; P=0.0063; Figure 3B). Expression of the wild-type allele did not differ significantly between carriers and noncarriers (P=0.18). Surface expression of the LDL receptor was quantified with the use of flow cytometry as described in the Data Supplement. Surface expression of the LDL receptor was on average 1.79-fold higher in cells derived from del2.5 carriers compared with those derived from 20 noncarriers (P=0.0086; Figure 3C). Thus, cells derived from del2.5 carriers demonstrated higher expression of the LDL receptor than in those from noncarriers, both at the mRNA and protein level.

Health of the del2.5 Carriers

Concerns have been raised that extreme lowering of LDL cholesterol with potent lipid-lowering therapy may have an adverse impact on health; including cognitive impairment, type 2 diabetes, intracerebral hemorrhage, and impaired steroid hormone synthesis.[25,26] To explore whether lifelong extremely low LDL cholesterol may have

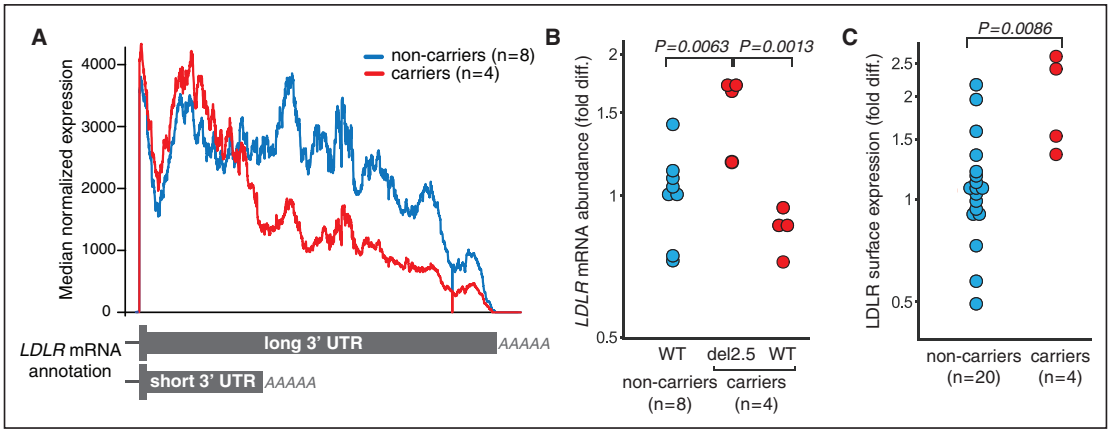

Figure 3. Increase in *LDRR* mRNA and surface expression of the LDLR in carriers of del2.5.
A and **B**, Show results of RNA sequencing in Epstein-Barr virus-transformed lymphocytes derived from 4 del2.5 carriers and 8 matched noncarriers. **A**, Shows the RNA sequencing coverage over exon 18 and the 3′ untranslated region (3′ UTR) of *LDLR*, expressed as median normalized expression levels. **B**, Shows the relative abundance of *LDLR* mRNA by phased alleles; wild-type (WT) for noncarriers (mean expression level), and del2.5 and WT for del2.5 carriers. **C**, Demonstrates relative surface expression levels of the LDL (low-density lipoprotein) receptor (LDLR) in Epstein-Barr virus-transformed lymphocytes from 4 del2.5 carriers and 20 noncarriers. For each individual, expression levels are expressed as fold-difference relative to a 1:1 matched control.

a deleterious effect on health, we performed a comprehensive phenotypic analysis of the del2.5 carriers. All identified carriers of del2.5 were invited to participate in the deCODE health study (described in the Data Supplement), of which 4 agreed to participate. In addition, we reviewed the medical records of all del2.5 carriers.

The oldest carrier died at the age of 85 years, and the remaining 6 are between ages of 35 and 65 years (rounded to nearest multiple of 5). Known diseases and conditions affecting the carriers are shown in Table III in the Data Supplement. Conditions affecting >1 carrier were hypertension (n=3) and osteoarthritis (n=3), which are common diseases in the Icelandic population (prevalence of 28% and 12% in the genotyped sample, respectively). No carrier had evidence of impaired steroid hormone synthesis or altered liver function (Tables IV and V in the Data Supplement). One carrier had adult-onset type 2 diabetes but other carriers (n=3) had values of glycated hemoglobin A1c and fasting glucose within the normal range. The results of cognitive testing in the 4 carriers who participated in the deCODE health study did not show significantly impaired cognitive function (P>0.05 for differences in mean standardized scores for cognitive tests including Rapid Visual Information Processing, Spatial Working Memory, Trail-Making Tests) as shown in Table VI in the Data Supplement. Taken together, we did not observe a highly penetrant adverse effect of extreme lifelong reduction in LDL cholesterol because of del2.5.

DISCUSSION

We have described a novel deletion in the 3′ UTR of *LDLR* that leads to increased expression of the LDL receptor and reduction of LDL cholesterol levels by 74%. Del2.5 is very rare in the Icelandic population, restricted to a 3 generation family and arose as a de novo mutation about a century ago. We detected del2.5 in large-scale WGS data enriched for individuals at the extremes of LDL cholesterol levels, using a newly developed algorithm, PopDel,[14] designed to detect 500 to 10 000 bp deletions in short read sequence data. To our knowledge, a gain-of-function mutation in *LDLR* leading to a large reduction in LDL cholesterol has not been described previously.

Our analyses indicate that the effect of del2.5 is mediated in cis, through removal of the canonical PAS on the distal end of the 3′ UTR, leading to use of a proximal, alternative PAS. This results in shortening of the *LDLR* mRNA 3′ UTR by two-thirds. The short 3′ UTR lacks negative regulatory elements that are located distally on the *LDLR* mRNA 3′ UTR, such as microRNA target sites[21,22] and adenylate-uridylate-rich elements.[27,28] The short 3′ UTR lacks the majority of target sites for microRNAs (miR-128-1, miR-148a, and miR-185) that have been shown to destabilize *LDLR* mRNA and repress its translation and significantly alter plasma LDL cholesterol in vivo.[21–24] (Figure 4). In addition, a loss of distally located adenylate-uridylate-rich elements may confer resistance to mRNA destabilization mediated by RNA-binding proteins.[27,28] Consistent with the observed effects of del2.5, in a transgenic mouse model, truncation of the human *LDLR* mRNA 3′ UTR has been shown to lead to increased mRNA stability and increased expression of the LDL receptor.[29] Since del2.5 carriers are all heterozygous, a reduction in LDL cholesterol by 74% is mediated by primary use of the alternative PAS in only

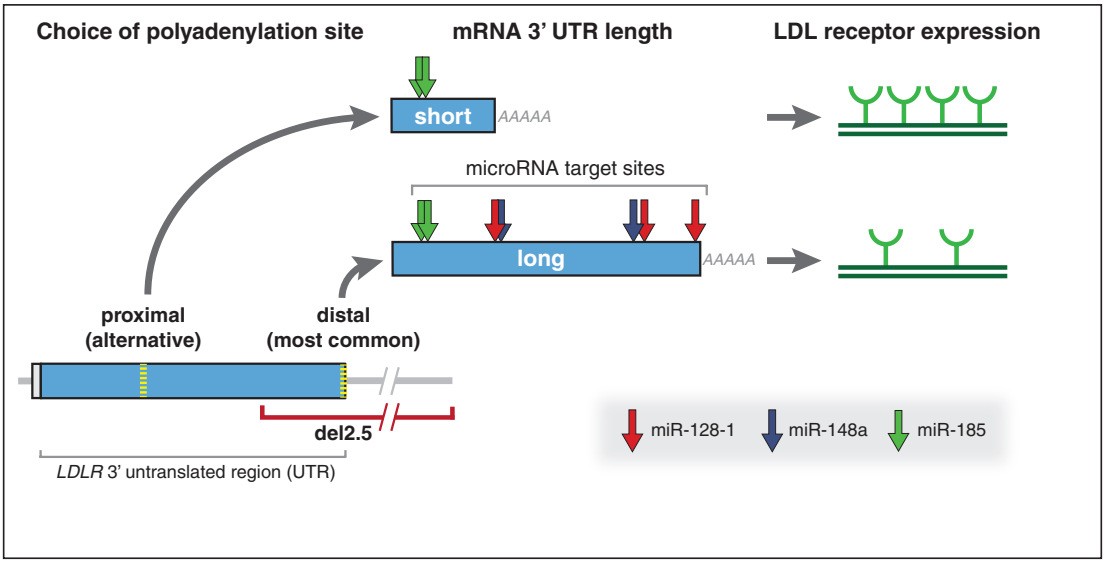

Figure 4. Schematic view of how differential usage of polyadenylation sites (PAS) in *LDLR* mRNA precursors may regulate LDL (low-density lipoprotein) receptor expression.
Through removing a region containing the distal PAS (canonical), del2.5 causes the alternative, proximal PAS to be primarily used. This leads to production of mRNA with a short 3′ untranslated region (3′ UTR) that lacks the majority of target sites for microRNAs that have been shown to negatively regulate *LDLR* mRNA stability and alter plasma LDL cholesterol in vivo. Loss of other negative regulatory sequences such as adenylate-uridylate-rich elements (not shown) may also play a role. The short 3′ UTR thus likely confers resistance to negative regulation, leading to increased *LDLR* mRNA stability and increased protein output.

one of the 2 copies of the *LDLR* gene. These findings highlight the important role of alternative polyadenylation and thus *LDLR* mRNA 3′ UTR length in the regulation of LDL receptor expression and plasma levels of LDL cholesterol.

PCSK9 is a major negative regulator of the LDL receptor and is co-upregulated with the LDL receptor in the setting of low intracellular concentrations of cholesterol, by the action of transcription factors called SREBPs (sterol regulatory element-binding proteins).[30,31] We observed modestly lower concentrations of circulating PCSK9 in del2.5 carriers. This suggests that del2.5 may lead to reduced activity of the SREBP pathway, possibly because of high levels of intracellular cholesterol resulting from high LDL receptor activity. Whereas statins lead to increased production of PCSK9, which may limit their efficacy,[30] del2.5 seems to associate with downregulation of PCSK9, which may contribute to the observed very low plasma levels of LDL cholesterol in carriers.

Safety analyses in pooled data from clinical trials of PCSK9 inhibitors indicate that very low levels of LDL cholesterol, even <0.65 mmol/L, are not associated with serious adverse outcomes.[25,32–36] However, the effects of long-term treatment beyond 5 years are unknown. As del2.5 is present from birth, the phenotypes of the carriers provide a unique insight into potential consequences of having very low levels of LDL cholesterol for many decades. Previously, complete genetic deficiency of PCSK9 (because of biallelic loss-of-function mutations) in only 2 individuals showed that extremely low LDL levels are viable, but other phenotypic data were limited.[37,38] We performed an in-depth phenotype analysis of the 7 del2.5 carriers and did not find a highly penetrant increase in risk of conditions that have been proposed to associate with extremely low LDL levels.[25] Familial hypobetalipoproteinemia because of loss-of-function mutations in *APOB* leads to similarly low levels of LDL cholesterol (generally 0.5–1.3 mmol/L) but predisposes to nonalcoholic fatty liver disease, likely due to triglyceride accumulation in hepatocytes.[39] However, unlike *APOB* loss-of-function mutations, LDL-lowering by del2.5 is not mediated through impaired lipoprotein synthesis and secretion but rather enhanced clearance of LDL from the circulation because of increased overall activity of the LDL receptor pathway. Thus, del2.5 would not be expected to predispose to nonalcoholic fatty liver disease, consistent with the absence of elevated liver transaminases in del2.5 carriers. Taken together, these results suggest that having LDL cholesterol levels that are comparable to those achieved with pharmacological inhibition of PCSK9, over many decades of life, is not likely associated with a major adverse impact on health. However, potential adverse effects that are less penetrant cannot be excluded because of the extreme rarity of del2.5.

Limitations

The small number of *LDLR* del2.5 carriers inherently leads to inadequate statistical power to detect modest differences. Although this does not affect the association with LDL cholesterol levels or LDL receptor expression due to the large effect, this is a limitation when assessing the effect of del2.5 on health of the carriers as modest effects cannot be detected.

Conclusions

We have described a novel deletion in the 3′ UTR of *LDLR* that leads to a large reduction in LDL cholesterol. To our knowledge, this is the first report of a naturally occurring, highly penetrant gain-of-function mutation in *LDLR*. These findings indicate that alternative polyadenylation of *LDLR* mRNA may be a potent regulator of LDL receptor expression in humans.

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