Applying genome-wide approach to define the genetics of low-density lipoprotein oxidation in two large cohorts

Master's thesis
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Pro Gradu -tutkielma

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Otsikko: LDL:n hapettumisen genetiikan selvittäminen genomin laajuisella lähestymistavalla kahdessa suuressa kohortissa

Sivumäärä: 55 s.
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Tiivistelmä

Tausta: Sydän- ja verisuonitaudit ovat yleisin kuolinsyy ja suurin kansanterveysongelma nykymaailmassa. Hapettumalla muuntuneen LDL-liporoteiinin (oxLDL) katsotaan olevan keskeinen tekijä sydän- ja verisuonitauteihin johtavan ateroskleroosin synnyssä. LDL:n hapettumista koskevaa genetiikkaa tunnetaan huonosti.

Tavoitteet: Työssä jatkettiin ja laajennettiin aiemmin julkaistua oxLDL:n genomin laajuista tutkimustamme (GWAS). Tein nyt GWAS-meta-analyysin kahdelle suurelle kohortille aiemmassa työssä käytettyä (HapMap2, 2.2 miljoonaa varianttia) laajemman 1000 genomes -referenssigenomin avulla (n. 20 miljoonaa varianttia). Tavoitteena oli löytää uusia yhden nukleotidin polymorfismeja (SNP), jotka ovat yhteydessä LDL:n hapettumiseen.

Menetelmät ja aineistot: GWAS-analyyseissa käytettiin nuorta, tervettä populaatiota (A: N = 2080, 45 % miehiä, ikä 32 ± 5 vuotta) ja SNPTEST-ohjelmistoa sekä potilaspopulaatiota (B: N = 2912, 69 % miehiä, ikä 63 ± 11 vuotta) ja ProbABEL-ohjelmistoa. Aineistojen A ja B GWAS-meta-analyysi tehtiin GWAMA-ohjelmistolla. Lisäksi GWAS suoritettiin A-populaatiossa vakioimalla se aiemman tutkimuksen päälöydöksellä.

Tulokset: SNP rs676210 (Pro2739Leu) apolipoproteiini B:ssä oli ainoa itsenäisesti oxLDL:ään liittyvä SNP ($p = 8.7 \times 10^{-168}$) GWAS meta-analyysissä. Tämä tukee aiemman tutkimuksemme tuloksia, mutta uusia genomin laajuisesti merkitseviä ($p < 5 \times 10^{-8}$) tuloksia ei löytynyt.

Johtopäätökset: Tutkimuksessa ei löydetty uusia oxLDL-tasoihin liittyviä SNP:ja. Seuraava vaihe on tutkia rs676210:n vaikutuksia ja sen interaktioita ympäristötekijöiden kanssa tarkemmin suhteessa sydän- ja verisuonitauteihin.

Master's Thesis

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Abstract

Background: Cardiovascular diseases (CVD) are the number one killer and cause of disability in today's world. Oxidized low-density lipoprotein (oxLDL) is considered to be a key factor in the development of atherosclerosis which leads to CVD. The genetics of LDL oxidation are poorly known.

Aims: In this work, I continued and extended our previously published genome-wide association study (GWAS) on oxLDL. Previously, single-nucleotide polymorphisms (SNP) were imputed to HapMap2 reference with 2.2 million variants. The aim of the present study was to find novel SNPs associated with oxLDL in a meta-analysis of two GWASs with SNPs imputed now to the 1000 genomes reference (ca. 20 million variants).

Materials and Methods: GWASs were performed on a population of young healthy individuals (A: N=2,080, 45% male, aged 32±5 years, with the SNPTEST software), and in a patient sample (B: N=2,912, 69% male, 63±11 years, with the ProbABEL software), and a combined GWAS meta-analysis for cohort A and B data was carried out using the GWAMA software. Furthermore, the GWAS was run in the A population with top-SNP adjustment.

Results: The genetic variant rs676210 (Pro2739Leu) in apolipoprotein B (apoB) was the only SNP independently associated with oxLDL ($p=8.7 \times 10^{-168}$) in the GWAS meta-analysis. This is in accordance with our previously published study, and no new genome-wide significant hits were found.

Conclusions: No new oxLDL levels related SNPs were found in this study. The next step is to study the specific effects of rs676210 and its environmental factor interactions with cardiovascular endpoints more closely.

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Abbreviations

apoB	apolipoprotein B
BMI	body mass index
CAD	coronary artery disease
CVD	cardiovascular disease
ELISA	enzyme-linked immunosorbent assay
GWAS	genome-wide association study
ICAM-1	intercellular adhesion molecule 1
HDL	high-density lipoprotein
IDL	intermediate-density-lipoprotein
IL	interleukin
LDL	low-density lipoprotein
LURIC	the LUdwigshafen RIsk and Cardiovascular Health study
oxLDL	oxidized low-density lipoprotein
PCI	percutaneous coronary intervention
QQ	quantile quantile
SNP	single-nucleotide polymorphism
SSH	secure shell
VCAM-1	vascular cell adhesion molecule 1
VLDL	very-low-density-lipoprotein
YFS	the Young Finns Study

Abbreviations are defined at first mention in the abstract and in the review of the literature and used only for concepts that occur more than twice.

Introduction

The number one killer and cause of disability in the world is cardiovascular diseases (Nabel, Braunwald 2012) caused by atherosclerosis (Libby, Ridker et al. 2011). Atherosclerosis is an inflammatory disorder that progresses slowly in the artery walls. As the blood flow in the arteries is disturbed—i.e. plaques rupture—severe clinical symptoms are observed (Davis, Donnan 2012). Oxidized low-density lipoprotein (oxLDL) is known to be an important factor in the pathogenesis of atherosclerosis (Ishigaki, Oka et al. 2009).

The discovery of the human genome (Sachidanandam, Weissman et al. 2001) initiated an era of intense genetic studying and discoveries in the 21st century. In 2002, the discovery of correlation between nearby genetic variations (Gabriel, Schaffner et al. 2002) allowed the development of the HapMap database in 2005 (International HapMap Consortium 2005). High-throughput genotyping methods were developed (Hoheisel 2006), allowing the inexpensive and rapid genotyping of multiple single-nucleotide polymorphisms (SNP). Furthermore, the development of the statistical methods needed in bioinformatics (Marchini, Howie 2010) and the power of modern computers allowed the first genome-wide association study to be conducted (Klein, Zeiss et al. 2005).

Since then, hundreds of GWASs have been published (Manolio 2010)—not satisfying all the expectations (Manolio, Collins et al. 2009) but providing massive amounts of novel information on multiple common diseases (Hirschhorn, Gajdos 2011).

In this thesis, I continued our previous work (Mäkelä, Seppälä et al. 2012). In the previous study, a genome-wide association study (GWAS) on oxidized low-density lipoprotein (oxLDL) was conducted with a single cohort with SNPs imputed to HapMap2. In the present study, the work is expanded by performing a meta-analysis of GWASes with two cohorts and using 1000-genomes-imputed SNPs.

Review of the literature

The overall progress of the atherosclerotic process related to the genetics of oxidized LDL as based on the previous oxLDL GWAS (Mäkelä, Seppälä et al. 2012) is summarized in **Figure 1**. The concepts mentioned in the figure will be explained in the following sections.

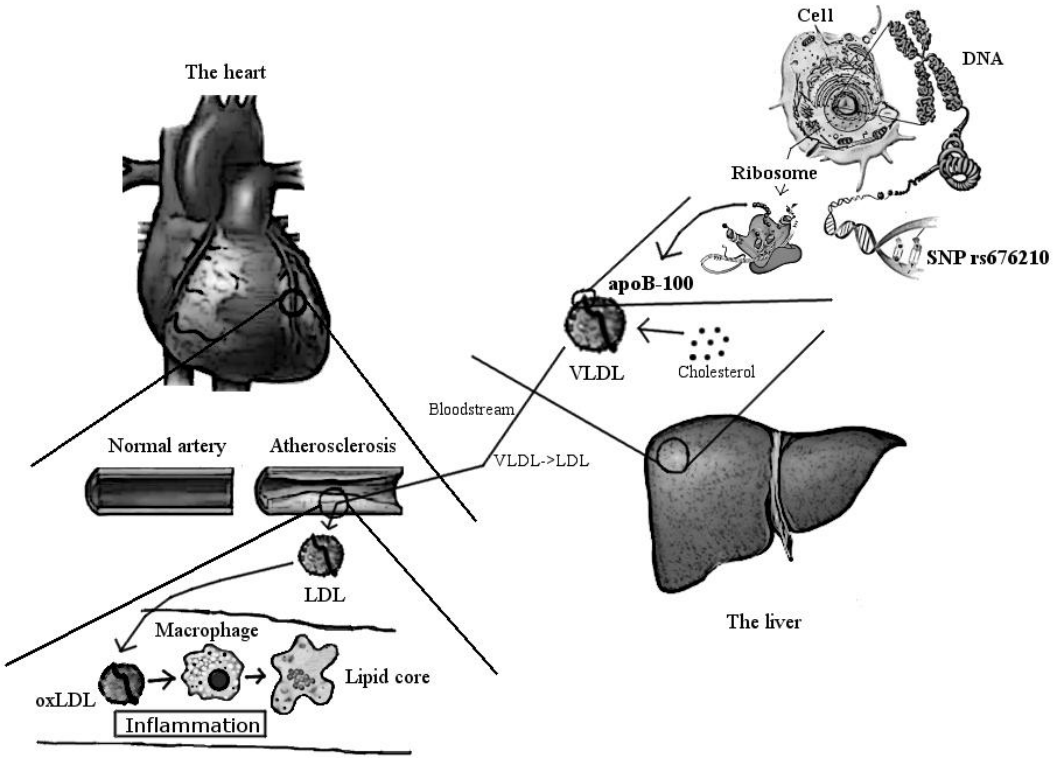


Figure 1. Oxidized LDL genetics in the intersection of inflammation and lipid metabolism of the pathogenesis of atherosclerosis

1. Oxidized low-density lipoprotein (oxLDL) and atherosclerosis

1.1 The journey of cholesterol and lipoproteins

Atherosclerosis is a slowly progressing inflammatory disorder of the artery walls that can lead to coronary artery disease (CAD) and stroke (Lusis 2012). The pathogenesis of atherosclerosis is an extremely complex process affected by multiple biological pathways (Weber, Noels 2011)**.**

In this thesis, I focus mainly on the intersection of the lipoprotein metabolism and inflammation regarding the genetics of LDL oxidation (**Figure 1**). The cholesterol, which is acquired from food (and transported by chylomicrons to the liver) or produced by the liver itself, is packed into very-low-density-lipoprotein (VLDL) particles formed in the liver (**Figure 2**). An apolipoprotein-B-particle (apoB) is also formed and incorporated in the VLDL particle. The best-known function of apoB is to act as a ligand for LDL receptors in various cells (Ooi, Russell et al. 2012).

VLDL is released to the bloodstream to transport cholesterol and various other substances into cells that require them. After interacting with HDL, or releasing some of the contents to tissues, VLDL becomes denser and turns into what is called intermediate-density-lipoprotein (IDL) (**Figure 2**). After more of the contents are released, the particle becomes even denser, turning into low-density-lipoprotein (LDL). The apoB moiety is present in each of the particles.

In normal lipoprotein metabolism, LDL is transported by the arteries to various tissues that require cholesterol and other contents of the particle for their function. After releasing the contents, the particle travels back to the liver where it is incorporated into hepatocytes by the LDL receptor and degraded (Yazdanyar, Jiang 2012). The liver creates new VLDL particles, and the journey starts again from the beginning.

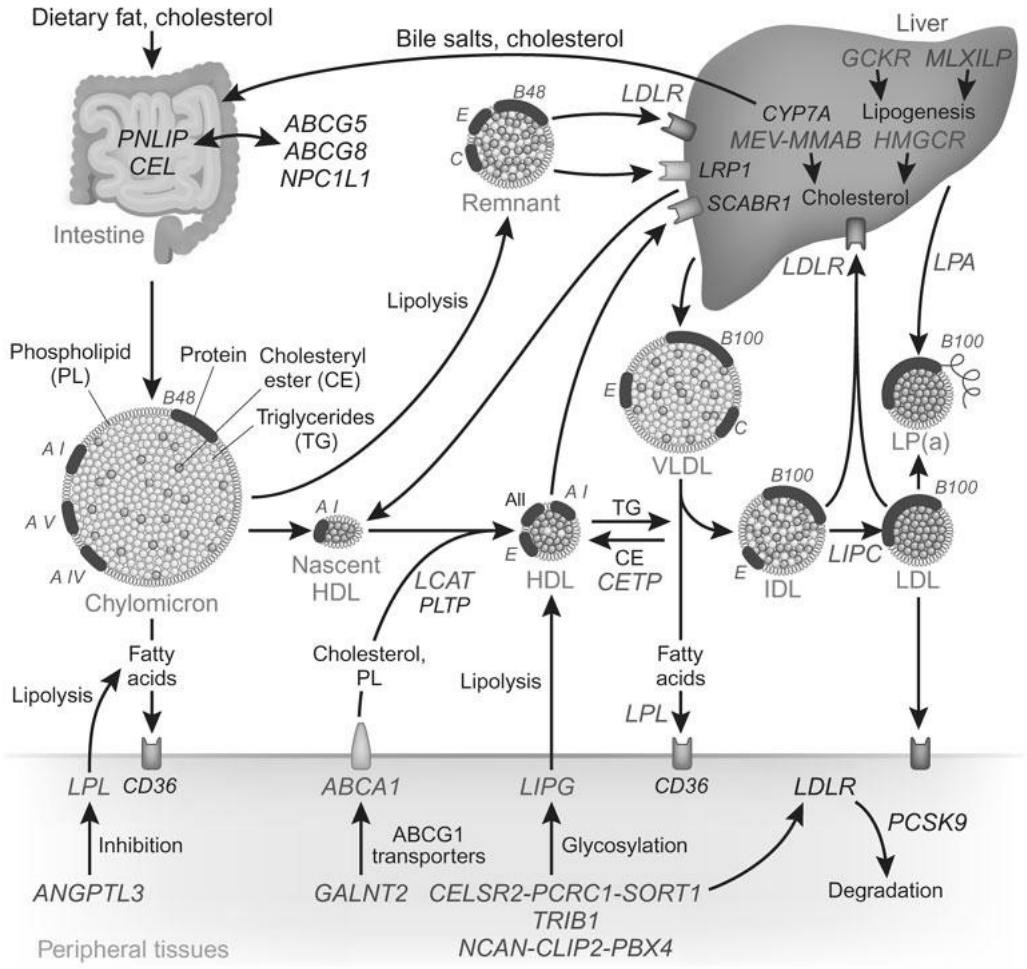


Figure 2. The highly complex nature of lipoprotein metabolism (Lusis, Pajukanta 2008)

1.2 LDL oxidation and atherosclerotic plaque formation

If certain conditions are met when LDL is travelling in the arteries, it can get lost on its way to the tissues and become stuck in the intima part of the arteries (**Figure 1**). These conditions include, for example, high shear stress, high blood pressure, diabetes, hypercholesterolemia, certain immunological signaling (Huo, Schober et al. 2003) by the endothelial cells covering the artery, etc. (Nabel, Braunwald 2012).

LDL cannot release itself from the intima (Kwon, Schroeder et al. 2008) and begins to be modified by its environment. Especially the apoB moiety is bound to the negatively charged extracellular matrix proteoglycans (Weber, Noels 2011). Reactive oxygen species and enzymes such as myeloperoxidase and lipoxygenases from inflammatory cells start

the oxidative modification of the LDL particle. When this foreign particle is noticed, the endothelial cells begin signaling that something is amiss. They express adhesion molecules and secrete chemokines to attract immune cells to the crime scene (Hansson, Hermansson 2011). Moreover, the displacement of platelet-derived chemokines drive the infiltration of the immune cells.

As monocytes traveling in the bloodstream hear the call of the endothelial cells, they adhere to the adhesion molecules on top of the endothelial cells and start their signature movement called tethering and rolling (Woollard, Geissmann 2010). After this, the monocyte is firmly adhered to the endothelial surface by intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) proteins. The monocyte then starts to move inside the artery wall by means of lateral transmigration and diapedesis between the endothelial cells.

As the monocyte finds itself in a tissue (in this case, the intima of the artery) certain immunological signaling causes it to specialize into a macrophage. Normally, LDL is internalized to cells by so-called Brown-Goldstein LDL-receptor (Brown, Goldstein 1983). Within this process is a mechanism that controls the internalization so that cells cannot get overfilled with LDL. However, as LDL is modified in the intima, it loses its typical form and turns into what is called oxidized LDL (oxLDL) (Ishigaki, Oka et al. 2009). As macrophages specialize in cleaning up unfamiliar material from the body, they have a mechanism for recognizing the oxLDL particle, also called the scavenger receptor (Woollard, Geissmann 2010). The problem with this receptor is that it does not have a feedback mechanism that controls how much oxLDL has been incorporated into the macrophage (**Figure 3**).

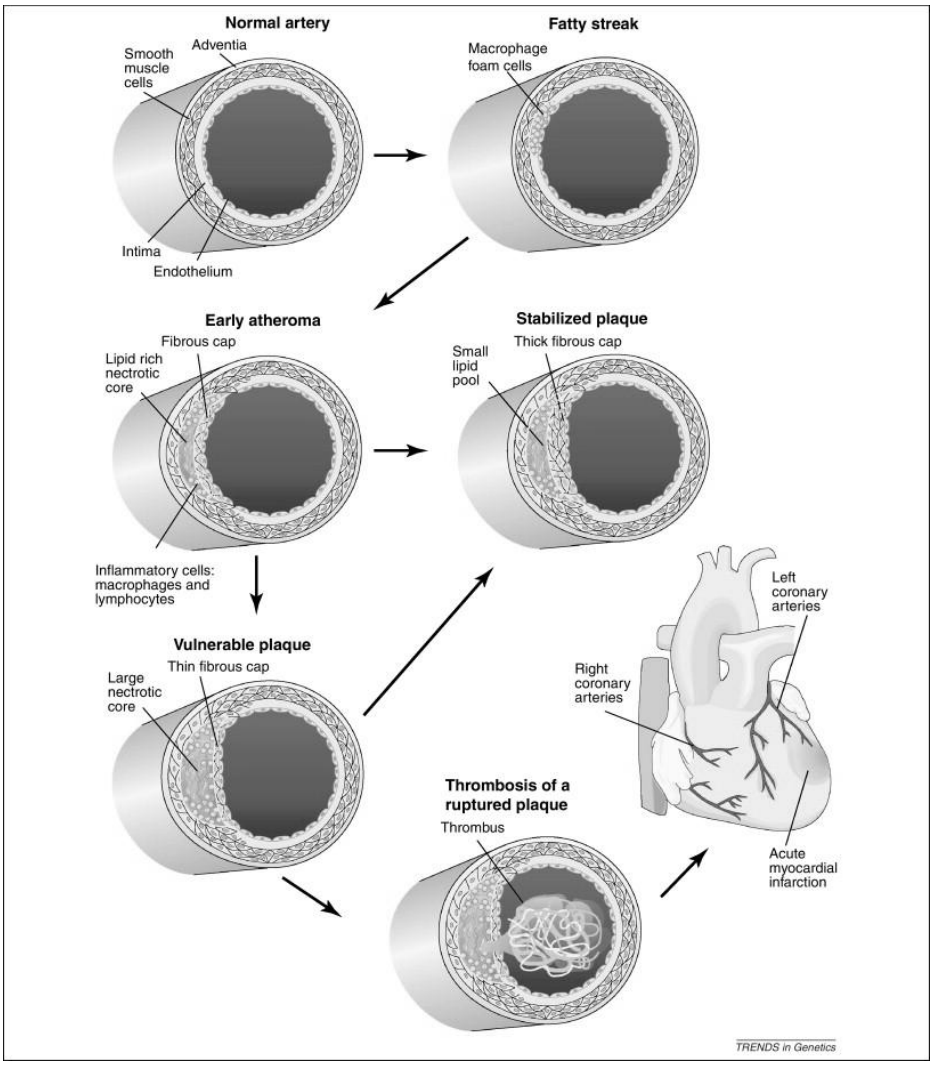


Figure 3. The process of atherosclerosis that can take decades (Lusis 2012)

As years go by and more and more LDL gets stuck in the intima, the macrophages become filled with the cholesterol delivered by the particles (Moore, Tabas 2011). A macrophage full of cholesterol is called a foam cell. Foam cells form the classical formation seen in histological staining of the arteries. As more and more foam cells are formed, they can be seen even with the naked eye on the artery wall (Nakashima, Fujii et al. 2007) and are called fatty streaks.

When macrophages cannot fulfill their normal duties as they are filled with cholesterol, they start to die apoptotically (Seimon, Nadolski et al. 2010). The cholesterol (and other substances) begin to form the so-called necrotic core in the intima. As this material is foreign to the vessel wall, protective measures are launched. Smooth muscle cells from

the outer parts of the artery (adventitia) travel to the scene and start to form a so-called fibrous cap around the foreign material to sequestrate it from the environment (Lusis 2012).

The composition of the fibrous cap is known to be essential in determining how dangerous atherosclerosis is to an individual (Lusis 2012). A strong and thick fibrous cap is known to protect the foreign material securely and will not rupture easily. For some reason, some individuals form a thin fibrous cap. In these caps, the SMCs can be replaced with macrophages (Weber, Noels 2011).

The process of atherosclerosis can already start in childhood. The formation of the atheromatous plaques with the fibrous cap can take 50 years (Nabel, Braunwald 2012). Atherosclerosis is a devious disease as it can go undetected all this time.

The first clinical symptoms observed are usually severe chest pain that occurs if the atheromatous plaque interrupts the blood flow to the heart muscle (Nabel, Braunwald 2012). Fortunately, modern medicine can usually treat these conditions easily with percutaneous coronary intervention (PCI).

The most severe case is the rupture of a thinly capped atheromatous plaque (**Figure 3**). This kind of plaque can go undetected as it can be formed without disturbing the blood flow in the artery (Lusis 2012). Acute myocardial infarction caused by such plaques can be too severe to treat and a life is taken by sudden cardiac death.

1.3 Methods for the measurement of LDL oxidation

The lipoproteins are multi-molecular by nature (Lusis, Pajukanta 2008). They are formed by (i) a lipid core which contains fat-soluble substances such as cholesterol esters, triglycerides, vitamins, etc.; by (ii) a phospholipid bi-layer which forms the amphipathic surface of the particle separating the hydrophobic core from the hydrophilic environment; and by (iii) apo-lipoproteins which, e.g., guide the particles to the right places by acting as ligands for cell surface receptors (**Figure 4**).

Due to the multi-molecular nature of LDL, oxLDL can be defined in multiple ways (Brinkley, Nicklas et al. 2009), and, therefore, multiple methods exist for its measurement. The different compartments can oxidize together or separately, and many metabolites that indicate LDL oxidation are also formed.

One of the most widely used method is the Mercodia oxLDL assay (Holvoet, Stassen et al. 1998). **Figure 4** illustrates the principle of the assay. It measures the modification of the apoB moiety (Segrest, Jones et al. 2001) of LDL. When the LDL particle lies in the hostile environment of the artery intima, the lysine residues of apoB are substituted by aldehydes, which is recognized by the monoclonal 4E6 antibody of this enzyme-linked immunosorbent assay (ELISA).

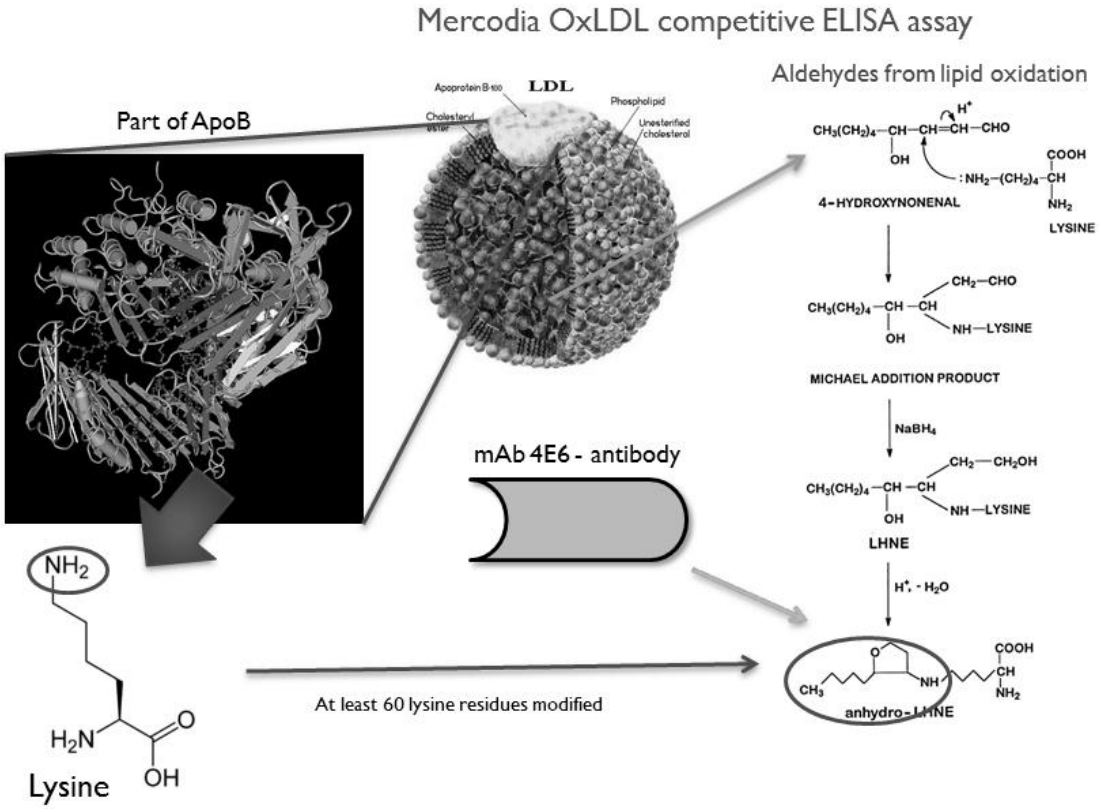


Figure 4. The principle of the Mercodia oxLDL assay.

Of the other methods, LDL diene conjugation, for example, measures the oxidation of the lipid compartment of the LDL particle (Ahotupa, Marniemi et al. 1998). LDL baseline diene conjugation can be measured by determining the level of baseline diene conjugation in lipids extracted from LDL. First, serum LDL is isolated by means of precipitation with buffered heparin. Lipids are then extracted from LDL samples with chloroform-methanol,

dried under nitrogen, then redissolved in cyclohexane and analyzed spectrophotometrically at 234 nm.

It is extremely important to consider the limitations of the measurement method when interpreting the results.

The Mercodia assay has been used in multiple studies; however, the results are inconclusive. Some studies show that oxLDL predicts cardiac syndromes (Meisinger, Baumert et al. 2005, Shimada, Mokuno et al. 2004, Tsimikas 2006), coronary artery disease (CAD) severity (Uzun, Zengin et al. 2004), plaque instability (Nishi, Itabe et al. 2002), cerebral infarction (Uno, Kitazato et al. 2003), and restenosis (Naruko, Ueda et al. 2006). However, some larger studies have been unable to replicate these results (Ishigaki, Oka et al. 2009).

Interestingly, the removal of oxLDL has been shown to prevent atherosclerosis in mice (Ishigaki, Katagiri et al. 2008).

2. Genome-wide association study (GWAS)

Since the discovery of the human genome (Sachidanandam, Weissman et al. 2001), the understanding of human genetics has taken huge leaps. In 2002, it was discovered that there is correlation between nearby genetic variations (Gabriel, Schaffner et al. 2002), and the HapMap database was developed in 2005 (International HapMap Consortium 2005). The idea of a genotyping microarray first came about in 1986 (Poustka, Pohl et al. 1986). Since then, microarray technology has seen huge advances (Hoheisel 2006), and inexpensive and rapid genotyping of multiple single-nucleotide polymorphisms (SNP) has become available to a wide audience. Moreover, excellent statistical methods in bioinformatics have been developed (Marchini, Howie 2010), allowing the first genome-wide association study to be performed (Klein, Zeiss et al. 2005).

To date, hundreds of GWASs have been published (Manolio 2010). The GWAS method has its problems (Manolio, Collins et al. 2009), but the approach has facilitated the location of hundreds of novel complex disease-associated loci in the human genome (Hirschhorn, Gajdos 2011)—the most recent being the extensive GWAS on CAD (CARDIoGRAMplusC4D Consortium, Deloukas et al. 2012) (**Figure 5**).

The theory behind a GWAS is the so-called common disease–common variant hypothesis (Manolio, Brooks et al. 2008). It postulates that individuals are predisposed to complex diseases by carrying many varied alleles with small independent effect but with a large combined effect, ultimately manifesting as, e.g., coronary artery disease. In a GWAS, the idea is to harvest these areas in studies with thousands of individuals to find all the small variations in the genome that impose a higher risk of the studied disease.

The main critique towards GWASs (McClellan, King 2010) comes from the evolutionary perspective: the rare and harmful mutations have been removed during many generations (Barreiro, Laval et al. 2008). The majority of the variation in the human genome is quite recent. Moreover, the majority of GWAS findings are on so-called gene deserts where there is no known mechanism of function. Furthermore, the inability to find a plausible biological explanation to the associations compels McClellan to infer that the

majority of the findings could be spurious mostly due to unaccounted population stratification (McClellan, King 2010).

However, GWASs are planned so that they tag the most probable areas with an association to the studied phenotype (Klein, Xu et al. 2010, Wang, Bucan et al. 2010). This is a built-in feature of the technique, because it takes advantage of the linkage disequilibrium (LD) of the human genome. It was discovered in the early 21st century that the genome is most likely structured so that large LD blocks are passed down through the generations (Gabriel, Schaffner et al. 2002). This means that nearby single-nucleotide polymorphisms (SNPs) are highly correlated with each other. The genotyping arrays were therefore designed to capture the variation in the genome by taking one tag from each of these blocks. It is thus not possible to find the exact spot behind the association, merely the most probable area. Hence, further studies are required to pinpoint the mechanism behind the associations.

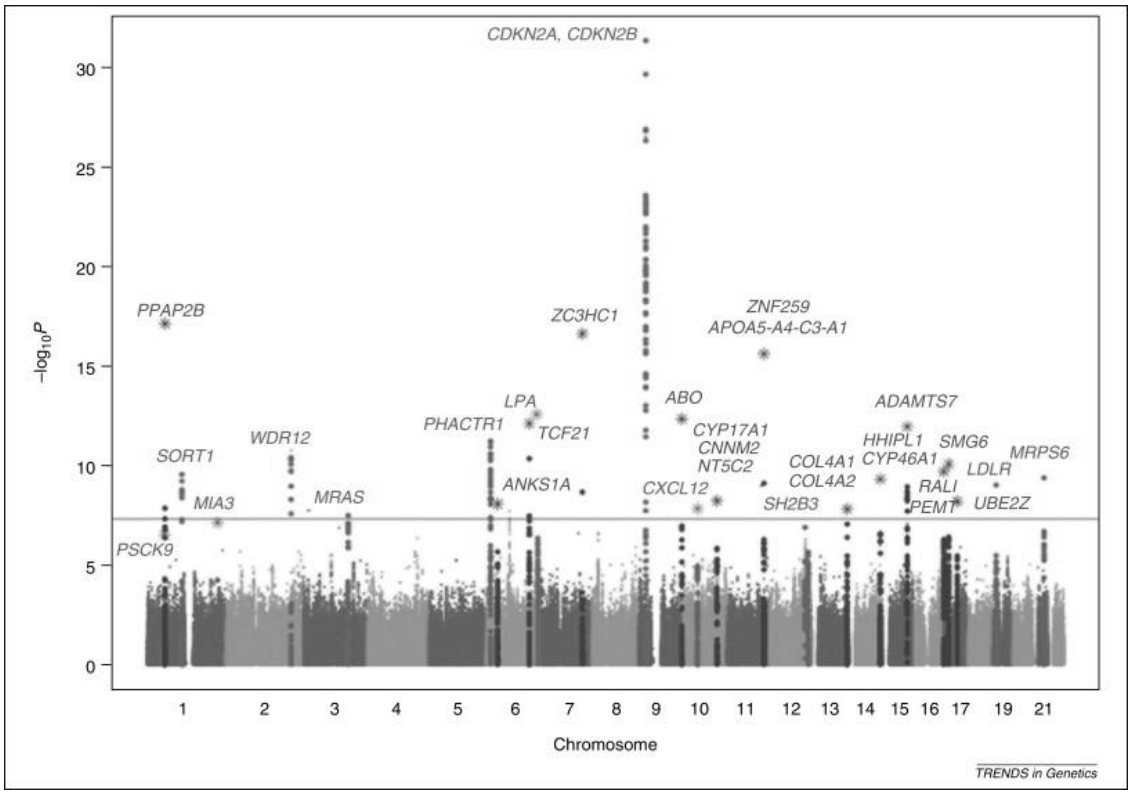


Figure 5. Findings of recent coronary artery disease (CAD) GWASs (CARDIoGRAMplusC4D Consortium, Deloukas et al. 2012, Lusis 2012)

To explain why most associations are found in gene deserts, there could be a yet unknown mechanism underlying such associations (Klein, Xu et al. 2010, Wang, Bucan et al. 2010). Moreover, more gene-gene and gene-environment interaction studies will be needed to determine where the elusive heritability lies (Zuk, Hechter et al. 2012).

So far, GWASs have been a success story, bringing about vast collaborative projects worldwide to illuminate the largely unknown mechanisms behind complex diseases. The major challenge is translating this knowledge into clinical practice (Fugger, McVean et al. 2012).

Hundreds of loci are known to be involved in lipoprotein metabolism (Lusis, Pajukanta 2008) and the pathogenesis of atherosclerosis (Lusis, Fogelman et al. 2004, Lusis, Fogelman et al. 2004). Large GWASs have been conducted to detect multiple new loci associated with CAD (Schunkert, Konig et al. 2011, CARDIoGRAMplusC4D Consortium, Deloukas et al. 2012) and lipid metabolism (Teslovich, Musunuru et al.

2010, Willer, Sanna et al. 2008). Moreover, the availability of next-generation sequencing in the near future will bring about immense amounts of additional information (Shendure, Ji 2008).

One possibility would be to utilize GWAS results in genetics testing (Grosse, Khoury 2006). Attempts have been made to use GWAS results in predicting disease susceptibility, but this objective has been elusive in practice (Ripatti, Tikkanen et al. 2010). A second avenue is to find out whether the GWAS-tagged regions could be utilized as intervention targets (Fugger, McVean et al. 2012).

2.1 Genetics of LDL oxidation

Even though more and more genetic studies are published every day, the genetic variation affecting LDL oxidation is mostly unknown. We published the first GWAS on oxLDL, discovering one SNP highly associated with serum oxLDL levels (Mäkelä, Seppälä et al. 2012). We found one SNP in the apoB locus that causes a missense mutation in the apoB particle (**Figure 1**). The goal of the present thesis is to determine whether further loci could be defined with more advanced bioinformatic methods.

Objectives

The specific aims of this study were

1. To expand a previously conducted GWAS (Mäkelä, Seppälä et al. 2012) by means of a GWAS meta-analysis of two large cohorts imputed to the broader 1000 genomes reference genome to find additional genetic variation affecting to the oxidation of serum LDL

2. To verify whether any new information was acquired by running the GWAS again by adjusting for the top SNP (rs676210) found in the previous study by using conditional analysis method (Mäkelä, Seppälä et al. 2012)

Materials and methods

1. Study populations and ethical statements

The Cardiovascular Risk in Young Finns Study (YFS)

YFS is a longitudinal Finnish population sample for studying cardiovascular risk factors and the evolution cardiovascular diseases from childhood to adulthood (Raitakari, Juonala et al. 2008). The first cross-sectional study was conducted in 1980 at five centers (Tampere, Helsinki, Turku, Oulu, and Kuopio). A total of 3,596 subjects in the age groups of 3, 6, 9, 12, 15, and 18 were selected randomly from the national population register.

The subjects have been re-examined as adolescents in 1983 and 1986 and as adults in 2001, 2007, and 2012. In the present study, the data from 2001 was used.

In 2001, a total of 2,283 participants aged from 24 to 39 years were examined for numerous study variables (Raitakari, Juonala et al. 2008). Mainly the measurement of oxLDL was used in the current study. Genotype and phenotype data were available for 2,080 subjects, and they formed the present study population.

The Ludwigshafen Risk and Cardiovascular Health (LURIC) study

The LURIC study consists of 3,316 Caucasian patients who were referred to coronary angiography due to chest pain at a tertiary care center in Southwest Germany between 1997 and 2000 (Winkelmann, März et al. 2001).

All the necessary covariate and endpoint data (genotyping and oxLDL-measurement) were available for 2,912 LURIC patients. They formed the present study population.

Ethical statements

Both studies (YFS and LURIC) were conducted according to the guidelines of the Declaration of Helsinki. The study protocols were approved by local ethics committees. All participants gave an informed consent.

2. Clinical and biochemical characteristics of the study cohorts

In both studies, the circulating oxLDL levels were measured with the same competitive enzyme-linked immunosorbent assay (ELISA) utilizing a specific murine monoclonal antibody, mAB 4E6 (Holvoet, Stassen et al. 1998) (Mercodia, Uppsala, Sweden; detection limit < 0.3 U/l).

The assay utilizes the phenomenon where a conformational epitope is formed at the apoB moiety of LDL when at least 60 lysine residues are substituted with aldehydes while the LDL is oxidized (**Figure 4**). Moreover, aldehydes released from activated platelets or the endothelial cells under oxidative stress may also induce the modification of apoB (Paniagua, Lopez-Miranda et al. 2005).

In both studies, venous blood samples were drawn after an overnight fast. In YFS, standard methods were used for the determination of serum total cholesterol, triglycerides, and high-density lipoprotein (HDL) cholesterol. LDL cholesterol was calculated in YFS by the Friedewald formula: LDL cholesterol = total cholesterol – HDL cholesterol – triglyceride-concentration / 2.2 (Friedewald, Levy et al. 1972). In LURIC, lipoproteins were separated by means of a combined ultracentrifugation–precipitation method (ß-quantification) (Winkelmann, März et al. 2001).

In YFS, for ApoB analysis a immunoturbidometrical assay was used (Orion Diagnostica, Espoo, Finland); the interassay CV was 2.8% for apoB (Juonala, Viikari et al. 2008). *In LURIC*, apoB was analyzed by means of a photometric assay which uses antihuman apoB antibody (apoB Test, Rolf Greiner Bio-chemica, Flacht, Germany).

In both studies, the formula body-mass index (BMI) = weight (kg) / height (m) ^{2} was used for BMI calculation.

3. Genotyping and quality control

The YFS employed a custom-built Illumina Human 670k BeadChip for genotyping at the Welcome Trust Sanger Institute. Peripheral blood leukocytes were used for the extraction of genomic DNA. A commercially available kit and Qiagen BioRobot M48 Workstation were used according to the manufacturer's instructions (Qiagen, Hilden, Germany). Genotype calling was carried out with the Illuminus clustering algorithm. Following the Sanger genotyping pipeline quality control (QC) criteria (duplicated samples, heterozygosity, Sequenom fingerprint discrepancy, or low call rate), 56 samples failed to meet the criteria. From the remaining 2,500 samples, 3 were removed due to low call rate (<0.95), 54 were excluded because of possible relatedness (pi-hat > 0.2), and one subject failed gender check. The Hardy–Weinberg equilibrium (HWE) test excluded 11,766 SNPs ($p \leq 10^{-6}$), and 7,746 SNPs failed the missingness test (call rate < 0.95). A further 34,596 SNPs failed the frequency test (MAF < 0.01). After QC, 2,442 samples with 546,677 genotyped SNPs were available for the GWAS analysis.

The imputation of SNPs refers to using the LD structure of the genome in the prediction of the SNPs that have not been genotyped (Marchini, Howie 2010). Genotype imputation was performed in the earlier study (Mäkelä, Seppälä et al. 2012) using MACH 1.0 (Li, Willer et al. 2010) and HapMap II CEU (release 22, NCBI build 36, dbSNP 126) samples as a reference. After imputation, 2,543,887 SNPs were available. SNPs with a squared correlation (r^2) of < 0.30 between imputed and true genotypes were eliminated from the analysis. Palindromic A/T and C/G SNPs were removed before imputation. After imputation, 2,543,887 SNPs were available.

In this study, to get more SNPs for analysis, genotype imputation was performed using IMPUTE2 and 1000 Genomes Interim Phase I EUR July 2011 haplotypes as a reference. SNPs with info ≥ 0.4 were considered well imputed. After imputation, more than 10 million SNPs were available for analysis.

Genomic DNA was prepared from EDTA anticoagulated peripheral blood by means of a common salting-out procedure in LURIC. The Affymetrix Genome-Wide Human SNP Array 6.0, and the Affymetrix Mapping 500K Array were used for genotyping. The following QC criteria were applied: individual call rate < 0.95, SNP call rate < 0.98, HWE p value < 10^{-4}, and MAF < 0.01. Genotype imputation was completed with MACH 1.0 (Li, Willer et al. 2010) and 1000 Genomes Pilot + HapMap 3 release 2 samples as a reference. SNPs with a squared correlation of ≥ 0.30 between imputed and true genotypes were considered well imputed. Both genotype and clinical data were available for 2,912 samples in the present study.

4. Performing the GWAS and the meta-analysis

4.1 Data preparation for statistical analysis

I had the data from both cohorts available on my standard desktop computer (Dell Optiplex 780). The data consists of three parts: (i) the phenotypic data, (ii) the genotypic SNP data, and (iii) the imputed SNP data. I used R Statistical package v. 2.11.1 (http://www.r-project.org) for reading in the phenotypic data of both cohorts in separate files (**Code 1**).

```
a <-read.table("YFS_data.dat"  ,header=T,sep="\t",comment="",quote="")
b <-read.table("LURIC_data.dat",header=T,sep="\t",comment="",quote="")
```

Code 1 – R-code for reading the data files

The required variables were selected from the data files (oxLDL, age, sex, BMI, and apoB). Principal components were also included and used to control for population stratification (Price, Patterson et al. 2006). The oxLDL measurement was transformed close to normal with inverse normal transformation. To get one variable for the GWAS analysis, a linear regression model was used with Y=oxLDL as well as age, sex, BMI, and principal components as covariates. The standardized residuals (mean 0, s.d. 1) obtained from this linear regression model were applied as the endpoint in the GWAS calculations in both cohorts. (**Code 2**)

```
# select the needed data
d <- a[c("study_ID", "oxldl", "age", "sex", "apoB")]
d <- na.omit(d)

# load the precalculated principal components
principal_components <- read.table("mds.dat", header=T)

d <- merge(d, principal_components, by=1, all=F, sort=F)

# normal transform the oxLDL variable
d["oxldltrC"]  <- qqnorm(d["oxldl"][,], plot.it = F)$x

# with the linear regression model and obtain residuals for the GWAS
d_lm <- lm(d$oxldltrC ~ d$sex + d$age+ d$bmi+ d$C1 + d$C11+ d$C15 )
d$oxldltrC_res <- residuals(d_lm)
```

Code 2 – R-code for preparing the data for GWAS

The data was saved and the standard GWAS software used for the calculations during next steps. The GWAS with top-SNP adjustment in YFS was performed identically, with the exception of adding rs676210 as a covariate before extracting the residuals.

4.2 Individual GWAS analyses

Because of the differences in the format of the genotypic and imputed data, the SNPTEST software was used for the YFS data and the ProbABEL software for the LURIC data. Both pieces of software fit a linear regression model (**Figure 6)** for each genotyped and imputed SNP and print out the result in the result file for each SNP

separately. Genotypes were coded as 0, 1, or 2 when the SNP was genotyped, and by dosage (scale 0–2) when imputed. SNPTEST and ProbABEL take in to account the uncertainty of imputation with the imputed SNPs.

$$E[\mathbf{Y}] = \mathbf{X}\boldsymbol{\beta} = \mathbf{X}_x\boldsymbol{\beta}_x + \mathbf{X}_g\boldsymbol{\beta}_g$$

Figure 6. Linear regression in ProbABEL (Aulchenko, Struchalin et al. 2010).

An SSH connection was created to a separate UNIX environment that utilizes a high-performance computer cluster for the calculations. The phenotypic, genotypic SNPs, and imputed SNPs data were uploaded to the server.

For YFS, a separate process for each chromosome was initiated for the SNPTEST software. For LURIC, similarly, a separate process was initiated for each chromosome utilizing the ProbABEL software. (**Code 3**)

```
grun -n oxldlC_1 snptest -data yfs_1000g_data.bgen oxldlC.sample -
frequentist 1 -method expected -pheno oxldlC -nowarn -o
oxldlC_chr1.txt

grun -n ab_oxldl_1_chr1 -mem 8G palinear --info
luric_1000g_data_chr1.mlinfo --dose luric_1000g_data_chr1.mldose --
pheno abel_oxldl_luric.phe --out oxldl_chr1
```

Code 3 – UNIX commands for creating processes for the two programs on chromosome 1. Similarly, the commands are run for chromosomes 2–22.

4.3 Extracting and combining the results for meta-analysis

Both ProbABEL and SNPTEST output result in separate result files for each chromosome. The next step is to combine the results into one file.

For ProbABEL, an R script was used (**Code 4**). It loops through each chromosome result file and picks the variables needed for the figure drawing and meta-analysis. Similarly, the results of SNPTEST were incorporated into one file.

Once the results were in a single file, QQ and Manhattan plots were drawn of the results for both cohorts separately. R script was used for the drawing. For the QQ plot, the p value for each SNP is plotted together with the expected distribution to see if there is any deviation from normal distribution. In the Manhattan plot, the p value of each SNP is plotted on a chromosome map to see where the associations come from. SNPs that associated with oxLDL with an a priori set Bonferroni-corrected p value $< 5 \times 10^{-8}$ were marked on the figures to see whether there were statistically significant associations (**Figure 7**).

```
  #read and process ProbABEL results for each chromosome 1-22
  for (i in 1:22) {

      filename <- paste(out_path, trait, "_chr", i, "_add.out.txt",
sep="")
      if (file.access(filename, mode = 0) == -1) {
        msg(paste("ProbABEL: No such file", filename))
        next
      }

      msg(paste("ProbABEL: Reading file", filename))
      x <- read.table(filename, header=T, na.strings="NA", as.is=T)
      names(x)[match("name", names(x))] <- "SNP"

      # Base position for the imputed data
        filename <- paste(luric_path,
"1000g_pilot/1000g_pilot_liftover_chr",i,".txt", sep="")
        msg(paste("Reading file", filename))
        hg19 <- read.table(filename, header=T, as.is=T, quote="",
comment="", sep="\t")
        names(hg19) =
c("CHR","HG18_POS","HG19_POS","HG18_NAME","HG19_NAME","HG19_SNPID","GEN
E")

        x <- cbind(x["HG19_NAME"],x["HG19_SNPID"], x["CHR"],
x["HG19_POS"], x["AA1"], x["AA2"], "STRAND" = "+", x["MAF"], x["n"],
x["beta2"], x["sebeta_SNP_add"], x["p"], x["Rsq"], x["Quality"],
"IMPUTED" = 1)



      # Sort by CHR and BP for Manhattan plot
      xy <- xy[order(xy$CHR, xy$BP, na.last=NA, decreasing=F), ]

      msg(paste("Writing file", out_filename))
      write.table(meta, out_filename, append=app_flag,
col.names=!app_flag, row.names=F, sep ="\t",  quote=F, na=out_na,
dec=out_dec)

  }
```

Code 4 – R script for processing the output of ProbABEL. Similar code was used for SNPTEST output.

4.4 Meta-analysis

In the meta-analysis, the results of the two GWASs are summarized. This was accomplished with GWAMA software (Magi, Morris 2010), which accepts the result files of ProbABEL and SNPTEST. GWAMA calculates the summary of the association for each SNP that is found in both cohorts with fixed-effects meta-analysis.

```
  pheno=$1

  echo "Step 1/3: Writing $pheno".in" file"
  echo $pheno"_yfs.txt" > $pheno".in"
  echo $pheno"_luric.txt" >> $pheno".in"

  echo "Step 2/3: Running fixed effects meta-analysis for $pheno"
  GWAMA -i $pheno".in" -o $pheno"_fixed" -gc -qt

  echo "Step 3/3: Sorting results and picking top hits from fixed effects
results"
  head -1 $pheno"_fixed.out" > $pheno"_fixed_sort.out"
  tail +2 $pheno"_fixed.out" | sort -nk 10 >> $pheno"_fixed_sort.out"
  head -1000 $pheno"_fixed_sort.out" > $pheno"_fixed_top.txt"
  rm $pheno"_fixed_sort.out"
```

Code 5 – The GWAMA meta-analysis shell script for UNIX

Similarly to the individual GWASs, QQ and Manhattan plots were drawn. Moreover, regions harboring association signals were visualized using LocusZoom (Pruim, Welch et al. 2010).

Results

1. GWASs in YFS and LURIC

The YFS and LURIC cohorts are quite different, since YFS is a population sample and LURIC a patient sample. Moreover, there was a female predominance in YFS, while the patients in LURIC were predominantly male (**Table 1**). Furthermore, there were age differences between the cohorts, with YFS as the younger and LURIC as the older population. The mean oxLDL levels also varied, being higher in YFS and lower in LURIC (**Table 1**).

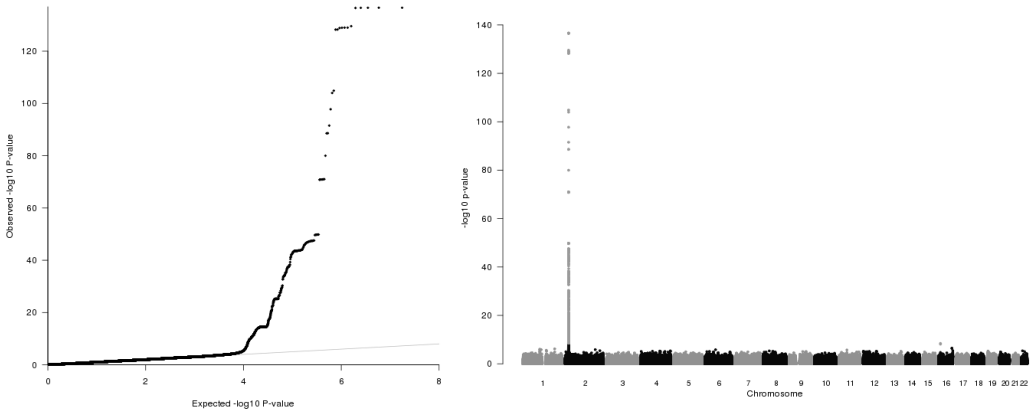
Table 1. General characteristics of the study populations.

Study	Acronym	n*	Age, years†	% Male	OxLDL (U/l) †
The Cardiovascular Risk in Young Finns Study	**YFS**	2080	31.7 (5.0)	45.2%	83.4 (25.1)
The Ludwigshafen Risk and Cardiovascular Health Study	**LURIC**	2912	62.6 (10.7)	69.3%	75.0 (28.3)

Abbreviations: n, number. *n for subjects having data required for the present study; †values expressed as means (standard deviations).

The GWAS results from YFS and LURIC are presented in **Figure 7**.

YFS



LURIC

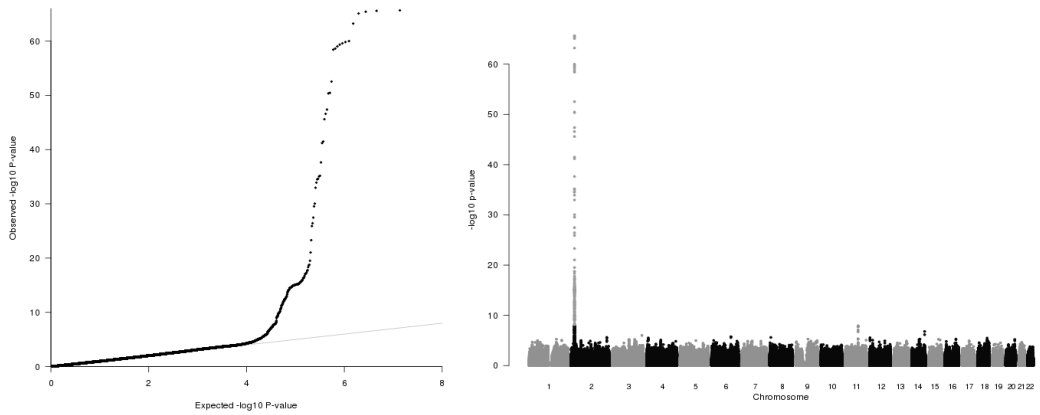


Figure 7. QQ and manhattan plots for the Young Finns Study (YFS) and the Ludwigshafen Risk and Cardiovascular Health (LURIC) study

In the quartile-quartile (QQ) plot, the gray line represents normal distribution and the p values of the GWAS SNPs are plotted on the y-axis. It shows a clear deviance from normal distribution with the GWAS results. This means that the results are most probably not due to chance.

The Manhattan plot maps the SNP p values on chromosomes. On the x-axis is the chromosome number (1-22), and the y-axis shows the –log of the association p value. On

the Manhattan plots, it is clearly shown that the results are similar in YFS and LURIC. The significant associations are found on chromosome two.

2. Meta-analysis of the GWAS results

In the meta-analysis, we confirmed the strong association in the chromosome two region (**Figure 8, Supplementary Table 1**). A total of 635 SNPs were associated with oxLDL with genome-wide statistical significance ($P < 5 \times 10^{-8}$).

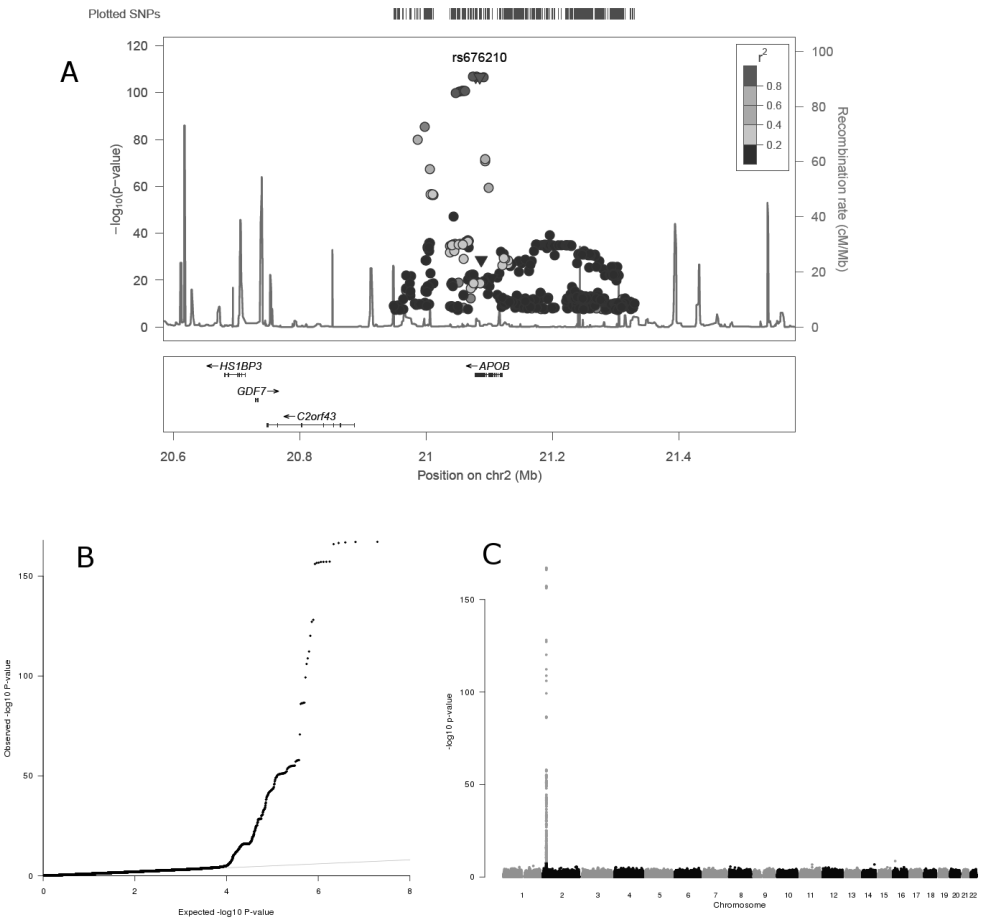


Figure 8. The meta-analysis results.

The panels B and C in **Figure 8** show similar results as those found in the individual GWASs. The A panel shows a zoom-in in the main association region in chromosome 2. On the y-axis is the –log p value of the SNPs. The gray line represents the recombination rate on the chromosome (the approximated LD blocks). The panel shows genes in the region.

All of the SNPs were on the apolipoprotein B-100 precursor coding region (OMIM 107730) on chromosome 2.

3. Top SNP adjustment

I also ran the GWAS for YFS with top SNP adjustment (**Figure 9**). There were no new associations in addition to the previously published one. This probably means that all of the 635 SNPs are correlated with rs676210 (due to linkage disequilibrium) so that the strong association of rs676210 with oxLDL also shows a false association with the other SNPs.

These results confirm that the strongest association with oxLDL is most probably due to rs676210. A larger population could be needed to find possible further associations.

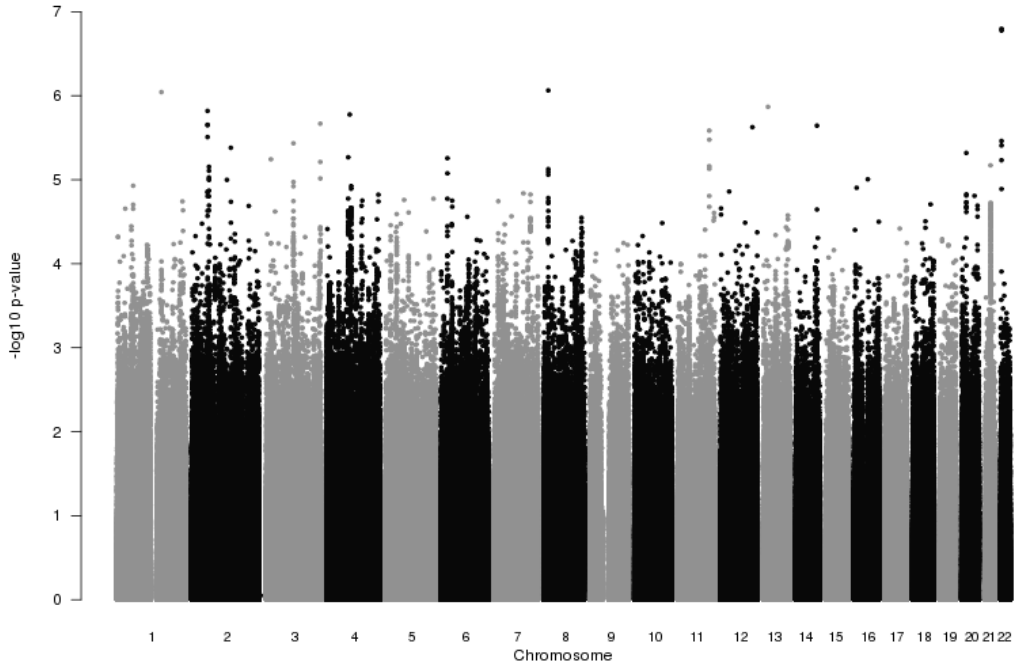


Figure 9. No further associations at $p < 5x10^{-8}$ after running the GWAS with top SNP adjustment in the YFS

Discussion

I performed a GWAS meta-analysis on oxLDL in two large cohorts and confirmed a previously found APOB locus on chromosome two to be associated with oxLDL. Of the hundreds of SNPs associated with circulating oxLDL levels in a healthy Caucasian adult population (YFS) and a patient population (LURIC), only one missense mutation leading to a proline-to-leucine interchange in ApoB (rs676210) on chromosome two remained significant in further analysis. This result is in accordance with our previously published study (Mäkelä, Seppälä et al. 2012) and, unfortunately, no new genome-wide significant loci were found.

1. The GWAS results

The differences in the measurement of oxLDL could affect the results. There were differences in the mean oxLDL levels of the respective study cohorts even though the same assay was used. This could be due to levels of oxidants or anti-oxidants, different storage conditions, or differences in the use of statin medication (Ky, Burke et al. 2008). In LURIC, there was a higher proportion of statin users in comparison to the healthier YFS population. However, the GWAS results were parallel in spite of the differences.

The meta-analysis results and the additional rs676210-conditioned analysis confirmed the association of rs676210 with oxLDL in these two cohorts.

ApoB is coded by the gene apolipoprotein B-100 precursor coding region (OMIM 107730) on chromosome two. The top SNP, rs676210, causes a Proline -> Leucine missense mutation in the apoB protein.

2. Genetics of LDL oxidation

There are few previous reports about the genetics of LDL oxidation. The interleukin-1b (IL-1B) gene has been associated with oxLDL levels (Manica-Cattani, Medeiros Frescura Duarte et al. 2012), supporting the role of genetic variation in LDL oxidation. To our knowledge, ours was the first GWAS on circulating oxLDL (Mäkelä, Seppälä et al. 2012). In our study, rs676210 association was confirmed by employing a forward selection algorithm, performing haplotype analyses, assessing the damage-producing probability of the SNPs, and running the GWAS with top-SNP adjustment.

There are a few other studies related to the genetic variant rs676210. In a large lipid GWAS, the rs676210 minor allele (A) was associated with lower triglyceride, total cholesterol, and LDL cholesterol levels, and with higher HDL cholesterol levels (Teslovich, Musunuru et al. 2010). Furthermore, rs676210 has been found to associate with triglycerides, VLDL-related fractions, and mean VLDL/LDL size (Chasman, Pare et al. 2009). In addition, rs676210 has been linked to an improved response to fenofibrate treatment (Wojczynski, Gao et al. 2010). The treatment was reported to lower triglyceride levels by 24.7%, 28.3%, and 34.5% according to the rs676210 genotypes GG, GA, and AA, respectively. Fenofibrates have been shown to have effect on oxLDL (Hogue, Lamarche et al. 2008).

The reason why rs676210 associates with oxLDL could the that the missense mutation in apoB (Kumar, Butcher et al. 2011) makes LDL more prone to oxidation in the intima of the arteries. The atherosclerotic process involves hundreds of genes with which LDL interacts (**Figure 10**). For example, the mutation could cause the apoB moiety to change the 3D structure so that LDL is more easily stuck in the proteoglycans in the matrix of the intima (Weber, Noels 2011). Therefore, the LDL particle could be exposed to the oxidative processes longer.

We have now tagged one locus with LDL oxidation. This association requires extensive further study to determine the more specific effects of rs676210 (Fugger, McVean et al. 2012).

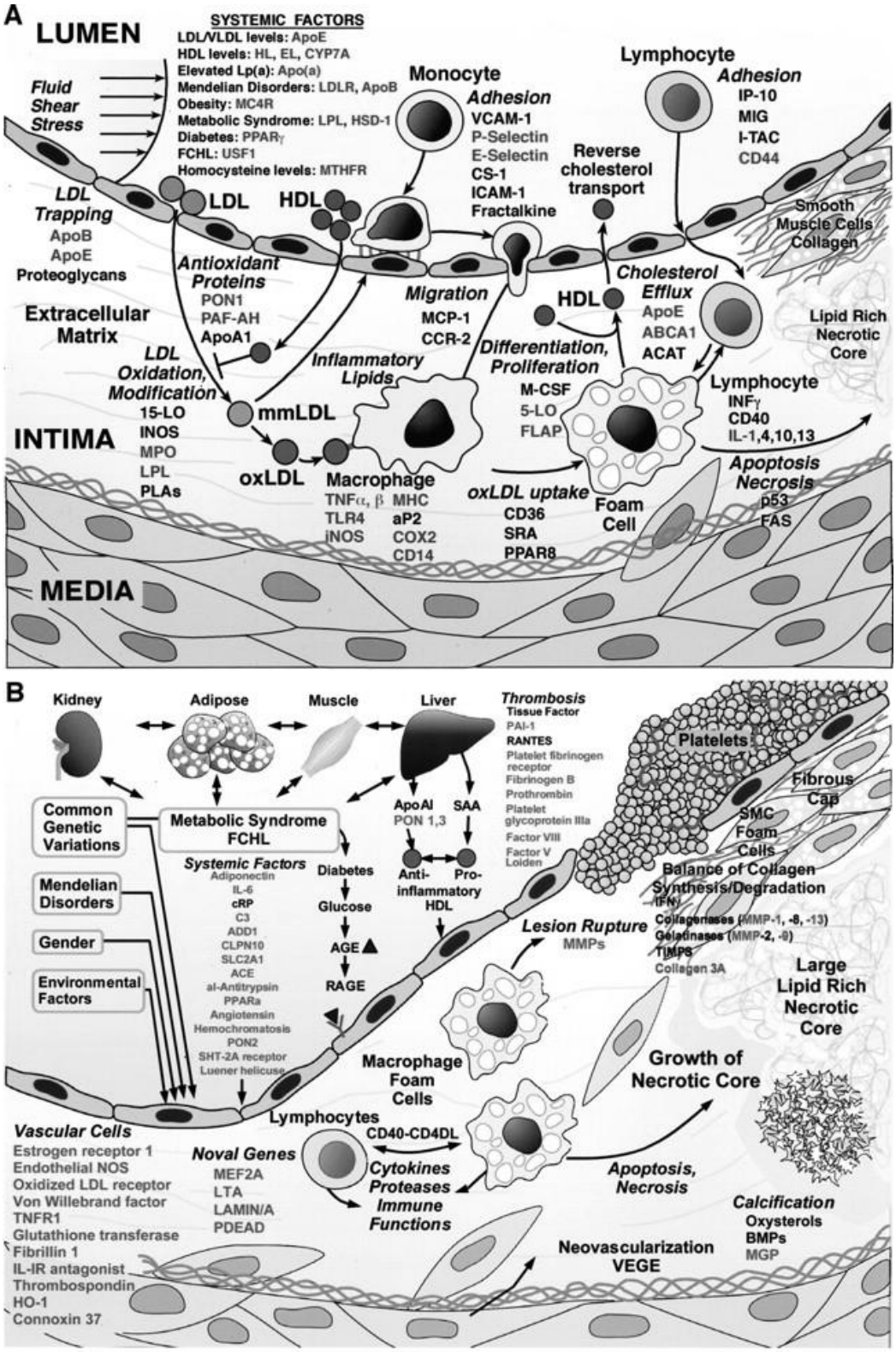


Figure 10. The genes involved in atherosclerosis (Lusis, Fogelman et al. 2004)

3. Limitations and strengths of the study

The main reason for not finding additional signals could be the small number of study subjects. In the largest GWASs, hundreds of thousands of individuals are required to significantly find the weakest signals.

The two cohorts used are also quite heterogenic, which could dampen the weaker signals (Han, Eskin 2012). Moreover, the analysis was performed only with Caucasian subjects and cannot be generalized to other populations.

Furthermore, a meta-analysis of the top-SNP-adjusted GWAS was not done. However, there most probably would not have been additional signals.

The oxLDL was measured with a method that only takes in to account the changes in the apoB protein part of the LDL particle. This does not provide a comprehensive view of LDL oxidation, and further studies with different measurements of LDL oxidation should be conducted.

The greatest strength of the study is the replication of the major results in two independent cohorts. Furthermore, the quality control before the GWAS was carried out rigorously.

There were significant differences in sex distributions and mean ages between the cohorts. However, the association of rs676210 with oxLDL concentration remained significant in spite of these and other differences in the background populations.

4. Future directions

It is hypothesized that the elusive heritability could be hidden in gene–environment interactions (Manolio, Collins et al. 2009). It is also known that multiple modifiable risk factors increase the risk of cardiovascular events. Major of such risk factors are, e.g., smoking (Pipe, Papadakis et al. 2010), diet, exercise, hypertension, diabetes, and obesity (Nabel, Braunwald 2012). The next step could be to study whether rs676210 acts differently in smokers and non-smokers (Kassi, Dalamaga et al. 2009) or with other oxidation related environmental factors.

Summary and conclusions

The main findings and conclusions were

1. I successfully expanded the previously conducted GWAS (Mäkelä, Seppälä et al. 2012) by GWAS meta-analysis in two large cohorts imputed to 1000 genomes reference genome. The previously significantly associated locus on chromosome two was replicated in this study.

2. The GWAS was run again by adjusting for the top-SNP in the previous GWAS (rs676210) to verify whether any new SNPs were found. Unfortunately rs676210 remained the only SNP independently associated with serum oxLDL.

The present findings support our previous findings, and no novel associations were found. The missense mutation (Pro2739Leu, rs676210) causing a proline-to-leucine interchange in apoB seems to influence LDL oxidation in a stepwise manner. The next step is to further clarify the mechanism behind the association by studying, for example, whether the SNP acts differently in smokers and non-smokers with cardiovascular endpoints.

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Supplementary Data

Table 2. The single-nucleotide polymorphisms (SNP) found in the meta-analysis. All of the SNPs were on and near the apolipoprotein B-100 precursor coding region (OMIM 107730) on chromosome 2 (except for rs4781086 on chromosome 16; it did not, however, remain significant in further analyses).

rs-number	chromosome	position	p value	rs-number	chromosome	position	p value
rs673548	2	21237544	7.25E-168	rs538928	2	21389019	1.10E-16
rs676210	2	21231524	8.67E-168	rs559967	2	21395598	1.10E-16
rs2678379	2	21226560	1.40E-167	rs477146	2	21396334	1.10E-16
rs1042034	2	21225281	2.76E-167	rs312983	2	21378580	1.11E-16
rs4665710	2	21221035	1.03E-166	rs522250	2	21387113	1.11E-16
rs7557067	2	21208211	5.72E-158	rs563696	2	21389430	1.11E-16
rs6544366	2	21204025	6.68E-158	rs312985	2	21378805	1.12E-16
rs10184054	2	21203877	7.43E-158	rs529396	2	21381268	1.13E-16
rs4564803	2	21205502	8.38E-158	rs530474	2	21381435	1.13E-16
rs6754295	2	21206183	1.93E-157	rs554414	2	21394892	1.13E-16
rs11902417	2	21198900	2.55E-157	rs556504	2	21395261	1.13E-16
rs6728178	2	21193946	8.06E-157	rs559318	2	21381490	1.14E-16
rs6711016	2	21123352	7.73E-129	rs532300	2	21381608	1.14E-16
rs56296027	2	21134011	7.86E-128	rs533211	2	21381702	1.14E-16
rs78980904	2	21121464	7.51E-121	rs560522	2	21382363	1.14E-16
rs11680233	2	21240031	5.58E-113	rs527034	2	21382786	1.14E-16
rs11126598	2	21240364	1.53E-109	rs553523	2	21394798	1.14E-16
rs3791980	2	21245329	9.52E-107	rs479545	2	21389897	1.16E-16
rs4296389	2	21142994	5.27E-100	rs493404	2	21394340	1.16E-16
rs3923037	2	21148274	2.37E-87	rs549959	2	21394458	1.16E-16
rs6732011	2	21146521	2.91E-87	rs496100	2	21394614	1.16E-16
rs10180633	2	21144829	3.94E-87	rs492494	2	21394248	1.17E-16
rs4426495	2	21143982	4.54E-87	rs490757	2	21388224	1.18E-16
rs10183548	2	21145499	9.16E-87	rs578864	2	21393937	1.18E-16
rs1318005	2	21214208	1.23E-58	rs492365	2	21394201	1.18E-16
rs1318006	2	21214152	1.29E-58	rs488507	2	21393689	1.19E-16
rs4665642	2	21213185	1.89E-58	rs492364	2	21394199	1.21E-16
rs6722374	2	21213062	1.99E-58	rs538862	2	21404980	1.23E-16
rs1320143	2	21212348	4.29E-58	rs12997242	2	21381177	1.25E-16
rs1318004	2	21214209	7.51E-58	rs484268	2	21406471	1.26E-16
rs10495713	2	21200519	6.87E-56	rs499560	2	21404398	1.27E-16
rs34335269	2	21196250	8.30E-56	rs312984	2	21378778	1.28E-16

rs56213756	2	21206995	8.45E-56	rs473770	2	21403965	1.28E-16
rs6733447	2	21198136	9.09E-56	rs507627	2	21403829	1.29E-16
rs56403064	2	21206906	9.17E-56	rs563752	2	21396013	1.30E-16
rs7571469	2	21204481	1.06E-55	rs477964	2	21402916	1.30E-16
rs17041679	2	21195412	1.28E-55	rs578095	2	21393866	1.31E-16
rs35893087	2	21192523	1.39E-55	rs563719	2	21396001	1.31E-16
rs9789416	2	21206052	1.71E-55	rs505813	2	21403639	1.31E-16
rs1320145	2	21192362	1.94E-55	rs537810	2	21404816	1.31E-16
rs17041662	2	21189068	4.07E-55	rs12994068	2	21118983	1.32E-16
rs4665790	2	21188570	4.18E-55	rs567071	2	21405790	1.32E-16
rs1320144	2	21192112	4.98E-55	rs521685	2	21401631	1.37E-16
rs6721844	2	21212571	1.31E-54	rs521755	2	21401700	1.37E-16
rs4665639	2	21186891	1.53E-54	rs522661	2	21401801	1.37E-16
rs17041663	2	21190238	4.79E-54	rs473448	2	21402456	1.37E-16
rs34059329	2	21209766	5.61E-53	rs476124	2	21402716	1.37E-16
rs1344063	2	21184545	8.35E-53	rs522761	2	21401789	1.38E-16
rs645456	2	21317485	9.66E-53	rs488922	2	21406942	1.39E-16
rs581998	2	21320481	2.14E-52	rs312936	2	21408647	1.41E-16
rs534100	2	21321158	3.08E-52	rs1712249	2	21385974	1.42E-16
rs312942	2	21321866	3.38E-52	rs561850	2	21395805	1.42E-16
rs56327713	2	21206836	3.39E-52	rs489022	2	21406981	1.42E-16
rs35131127	2	21205914	5.12E-52	rs547179	2	21384662	1.44E-16
rs312957	2	21342312	5.69E-52	rs1652422	2	21383841	1.46E-16
rs312967	2	21367516	5.97E-52	rs572246	2	21385149	1.46E-16
rs312961	2	21349199	6.31E-52	rs522963	2	21386957	1.46E-16
rs312968	2	21368451	6.56E-52	rs573314	2	21385161	1.47E-16
rs312964	2	21355430	6.90E-52	rs544450	2	21384358	1.48E-16
rs312969	2	21369568	7.06E-52	rs544655	2	21384441	1.49E-16
rs312966	2	21360583	7.66E-52	rs557316	2	21395337	1.55E-16
rs312959	2	21346577	7.90E-52	rs475887	2	21389485	1.56E-16
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rs312956	2	21342092	9.86E-52	rs558342	2	21395469	1.65E-16
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rs312946	2	21328999	1.30E-51	rs312937	2	21408841	1.82E-16
rs312947	2	21330769	1.32E-51	rs547239	2	21384686	1.97E-16
rs312955	2	21341893	1.61E-51	rs520861	2	21387239	2.02E-16
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rs6706783	2	21191409	1.88E-51	rs312938	2	21409165	2.08E-16
rs312978	2	21376584	2.20E-51	rs312940	2	21409717	2.16E-16
rs312949	2	21334283	2.28E-51	rs938848	2	21196847	2.28E-16
rs312977	2	21376312	3.29E-51	rs484399	2	21406568	2.28E-16
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rs312950	2	21336222	5.83E-51	rs10495712	2	21196112	2.40E-16
rs6705668	2	21142546	1.24E-50	rs62120842	2	21196447	2.43E-16
rs4274565	2	21141909	3.75E-50	rs544039	2	21398985	2.44E-16

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rs11693076	2	21140033	1.24E-49	rs524455	2	21415728	3.79E-16
rs4606879	2	21138868	1.03E-48	rs2337381	2	21186121	4.35E-16
rs11903024	2	21138740	1.06E-48	rs68093099	2	21190107	4.52E-16
rs7604788	2	21190024	3.47E-45	rs62120837	2	21187371	4.74E-16
rs557624	2	21398080	4.63E-45	rs62120838	2	21187513	4.80E-16
rs588245	2	21270057	4.52E-44	rs4665788	2	21188488	5.05E-16
rs523095	2	21387193	8.55E-44	rs1801695	2	21224853	5.11E-16
rs552658	2	21388146	9.32E-44	rs62120847	2	21205920	5.33E-16
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rs564539	2	21389505	1.08E-43	rs535713	2	21414072	5.55E-16
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rs666126	2	21282435	1.48E-40	rs17042000	2	21422928	3.39E-15
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rs447986	2	21439498	3.93E-34	rs4665176	2	21305853	2.83E-14
rs385777	2	21439897	4.16E-34	rs6708168	2	21301641	2.85E-14
rs423836	2	21449098	4.89E-34	rs61539677	2	21305195	2.93E-14
rs384302	2	21443231	5.14E-34	rs754523	2	21311691	2.99E-14
rs679899	2	21250914	5.27E-34	rs62122517	2	21298811	3.08E-14
rs520354	2	21259612	8.75E-34	rs62122520	2	21313430	3.30E-14
rs387752	2	21444678	1.09E-33	rs6761300	2	21306974	3.41E-14
rs76423192	2	21185419	1.17E-33	rs56079819	2	21314851	3.58E-14
rs1429972	2	21309758	1.42E-33	rs12477249	2	21400661	3.71E-14
rs1429973	2	21309281	1.74E-33	rs78120576	2	21318633	3.98E-14
rs11694766	2	21426779	4.82E-33	rs62120852	2	21213544	4.65E-14
rs693	2	21232195	6.78E-33	rs55938327	2	21209542	5.37E-14
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rs6756501	2	21218805	3.15E-31	rs34916112	2	21339285	8.26E-14
rs34776010	2	21216247	3.20E-31	rs11693870	2	21320250	8.41E-14
rs952274	2	21221357	3.21E-31	rs754524	2	21311541	9.11E-14
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rs4503953	2	21105335	5.18E-31	rs4665178	2	21321721	1.13E-13
rs12710745	2	21112689	6.41E-31	rs56090741	2	21323651	1.13E-13
chr2:21290063	2	21290063	9.83E-31	rs4665179	2	21321724	1.14E-13
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rs312050	2	21429198	3.42E-29	rs481069	2	21281856	1.31E-13
rs364390	2	21449787	3.42E-29	rs668948	2	21291529	1.32E-13
rs427642	2	21449940	3.45E-29	rs541041	2	21294975	1.35E-13
rs431802	2	21449791	3.48E-29	rs7567217	2	21303470	1.37E-13
rs437775	2	21438794	3.61E-29	rs13011615	2	21274167	1.39E-13
rs436941	2	21438863	3.63E-29	rs581411	2	21289432	1.53E-13
rs403772	2	21448480	3.70E-29	rs563290	2	21288226	1.64E-13
rs398532	2	21438480	3.72E-29	rs562338	2	21288321	1.64E-13
rs364381	2	21439753	3.72E-29	rs580889	2	21290067	2.70E-13
rs437624	2	21440004	3.75E-29	rs10221768	2	21417431	2.97E-13
rs384429	2	21447727	3.75E-29	rs6756284	2	21309856	3.30E-13
rs435647	2	21438180	3.78E-29	rs312980	2	21377938	3.79E-13

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rs414611	2	21446994	3.85E-29	rs11687710	2	21371068	3.96E-13
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rs430872	2	21443112	3.95E-29	rs585967	2	21270554	5.83E-13
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rs405983	2	21438129	4.34E-29	rs71435593	2	21302020	6.58E-13
rs393495	2	21450207	4.76E-29	rs62135035	2	21346521	6.77E-13
rs368523	2	21447687	8.59E-29	rs2195597	2	21331185	7.14E-13
rs427095	2	21440918	1.29E-28	rs2163203	2	21331137	7.18E-13
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rs56984418	2	21217230	9.81E-28	rs56201838	2	21328501	7.40E-13
rs57947200	2	21217229	1.00E-27	rs5013879	2	21136113	7.57E-13
rs80219287	2	21080701	7.92E-26	rs2115838	2	21350324	7.64E-13
rs3749054	2	21237238	8.24E-26	rs403880	2	21449408	7.66E-13
rs75319532	2	21056947	8.58E-26	rs34722314	2	21271707	8.12E-13
rs1800479	2	21227383	8.82E-26	rs1713222	2	21271323	8.24E-13
rs62122484	2	21223572	9.84E-26	rs12621605	2	21454659	9.24E-13
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rs13014683	2	21213126	1.77E-25	rs12052779	2	21333932	9.86E-13
rs1042031	2	21225753	2.19E-25	rs950662	2	21455548	9.86E-13
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rs80169634	2	21130491	1.49E-24	rs950661	2	21455941	9.93E-13
rs11673889	2	21104631	1.91E-24	rs13414558	2	21457147	9.99E-13
rs67212568	2	21208484	3.71E-24	rs13414740	2	21457327	1.00E-12
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rs13020417	2	21144834	5.24E-23	rs531819	2	21263639	1.20E-12
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rs77856692	2	21385318	7.43E-23	rs432483	2	21450349	1.55E-12
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rs3935557	2	21141725	1.14E-21	rs428696	2	21448607	1.67E-12
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rs78357953	2	21422826	1.83E-21	rs380240	2	21446653	1.87E-12
rs4502373	2	21139470	6.10E-21	rs367162	2	21444779	1.89E-12
rs4300790	2	21139417	6.54E-21	rs515135	2	21286057	1.93E-12

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rs4368289	2	21135723	2.41E-20	rs11685356	2	21197159	2.42E-12
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rs489010	2	21390992	1.01E-16	chr2:21401901	2	21401901	3.94E-10
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