Genetic Epidemiology and Lipids

A Pattern So Grand and Complex

Aaron Isaacs

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The belief that there is only one truth and that oneself is in possession of it seems to me the deepest root of all evil that is in the world.

– Max Born

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Chapter 1.3

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Chapter 2.1

Isaacs, A., Sayed-Tabatabaei, F.A., Hofman, A., Oostra, B.A., Klungel, O.H., Maitland-vander Zee, A.H., Stricker, B.H., Witteman, J.C. & van Duijn, C.M. The CETP I405V polymorphism is associated with increased HDL levels and decreased risk of myocardial infarction: the Rotterdam Study. *Eur J Cardiov Prev R.* In Press (2007).

Chapter 2.2

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Chapter 2.3

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Chapter 2.4

van der Vleuten, G.M., Isaacs, A., Zeng, W.W., ter Avest, E., Talmud, P.J., Dallinga-Thie, G.M., van Duijn, C.M., Stalenhoef, A.F. & de Graaf, J. Haplotype analyses of the APOA5 gene in patients with familial combined hyperlipidemia. *Biochim Biophys Acta.* 1772: 81-8 (2007).

Chapter 2.5

Isaacs, A., Jarvelin, M.R., Aulchenko, Y.S., Service, S.K., Ruokonen, A., DeYoung, J., Liu, F., Hartikainen, A.L., Sayed-Tabatabaei, F.A., Pouta, A., Witteman, J.C., Freimer, N.B., Oostra, B.A.,

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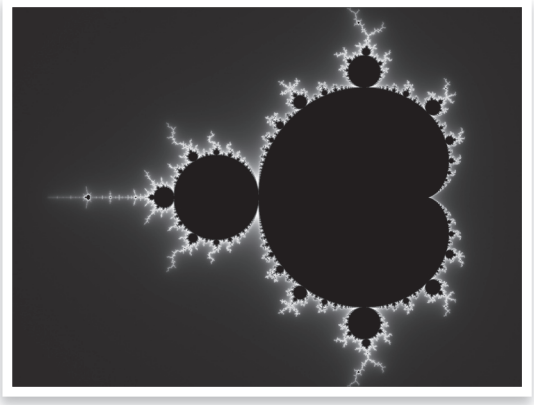
Chapter 3.1

Isaacs, A., Aulchenko, Y.S., Kayser, M., Liu, F., Sijbrands, E.J., Witteman, J.C., Oostra, B.A. & van Duijn, C.M. Genome-wide association analysis of plasma lipids determines novel loci affecting total cholesterol, triglycerides and triglyceride/HDL Ratio: the ERF Study. *In Progress.* (2007).

CONTENTS

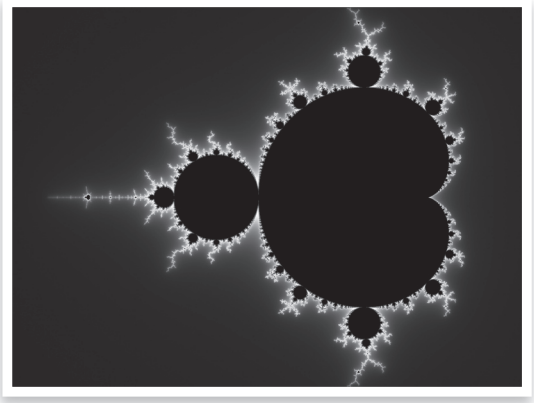
Chapter 1

Introduction



Chapter 1.1

General Introduction and Scope of the Thesis



LIPIDS AND CARDIOVASCULAR DISEASE

Intensive research over the course of the last several decades led to dramatic improvements in understanding, treating, and preventing cardiovascular disease. Despite this progress, cardiovascular disease remains the leading cause of mortality in both high- and low-income countries, and the leading source of morbidity in high-income countries.[1]

Lipids are well known determinants of cardiovascular disease risk; epidemiological evidence was published long ago.[2,3] Total cholesterol and low-density lipoprotein cholesterol (LDL), which tend to be strongly correlated, are associated with increased disease risk. LDL can be readily oxidized, which causes inflammation and atherosclerosis to occur,[4] and small, dense LDL particles are considered the most atherogenic lipid particles.[5]

High-density lipoprotein cholesterol (HDL), by contrast, is associated with beneficial effects, in terms of cardiovascular disease risk. Several mechanisms are likely to account for the protective consequences of increased HDL concentrations. HDL exhibits anti-inflammatory properties.[6] Additionally, it is thought to be an anti-oxidant,[7] helping to offset the deleterious effects of oxidized LDL. HDL is also involved in reverse cholesterol transport (i.e. the sequestration of cholesterol to the liver for metabolism),[8] which helps to reduce the amount of circulating lipid.

Currently, LDL and HDL are considered independent determinants of cardiovascular disease. The effects of triglycerides (TG), by contrast, are still being debated. TG is usually correlated with total cholesterol and LDL and inversely correlated with HDL. Cholesteryl ester transfer protein (CETP) is largely responsible for this inverse relationship. Activated CETP facilitates the exchange of HDL cholesteryl esters with TG from very low-density lipoproteins and LDL. Increased TG activates CETP, resulting in lower HDL levels and more small, dense LDL particles. It has, therefore, been difficult to distinguish the effects of TG independent of it's effect on other lipids.[9] Together with decreased HDL and increased small, dense LDL, raised TG forms the "TG-HDL axis", or atherogenic dyslipidemia, which is a noted determinant of cardiovascular disease risk, as well as a feature of diabetic and metabolic syndrome dyslipidemia.[10]

Pharmaceutical management of lipid levels is a major focus in cardiovascular disease prevention and management.[11] Indeed, the medication with the largest worldwide sales last year, Lipitor, generated over $13 billion in revenue. These treatments are effective in reducing primary and secondary cardiovascular events.[12] To date, lipid-modifying therapy focused largely on reducing total and LDL cholesterol; more recently, extensive efforts have gone into developing pharmaceutical interventions that will raise, or improve the quality of, HDL.[13-15] A recent study found that simultaneously decreasing LDL and increasing HDL through statin use might cause atherosclerotic regression.[16]

Lipid levels are ideal examples of complex traits, determined by large numbers of genes, each, typically, with a small effect. A 2005 review of genes and chromosomal regions suspected of involvement in determining HDL levels, for example, lists about 50 potential candidate

genes and 30 quantitative trait loci.[17] Numerous genes influencing various lipid parameters have been identified thus far. These include genes such as Apolipoproteins A-I, A-II, B, and E, CETP, a number of lipases (including lipoprotein, hepatic, endothelial, and pancreatic lipases), the adenosine binding cassettes (such as ABCA1), and lecithin:cholesteryl acyl transferase (LCAT), among many others. Despite this seeming wealth of information, there is wide acknowledgement that many genes (and proteins) remain unknown. Moreover, many of those that are known are not well characterized.

Although necessarily brief, this summary of the role lipids play in cardiovascular disease highlights some of the most salient points. An impressive number of journal articles and other publications on lipids have been published to date (a PubMed search of "lipids" turns up ~705,000 hits); many excellent reviews, Rader's overview of HDL for example,[13] can be obtained for more in-depth background.

GENETIC EPIDEMIOLOGY

In it's early years, the focus of genetic epidemiology was primarily on identifying the genes underlying monogenic, or, perhaps, oligogenic, disorders with clear patterns of Mendelian inheritance.[18,19] Although monogenic disorders are important for our knowledge of the mechanisms of disease, and genes that are involved, they tend to affect a limited number of individuals, while complex genetics plays a more important role in the majority of patients.[20,21] As more and more of these genes were discovered, the core function of the discipline began to shift towards the identification of genes involved in complex, polygenic phenotypes, such as lipid levels and distributions.[22]

Genetic epidemiologists use two broad classes of analyses to identify and characterize genetic involvement in complex phenotypes: candidate gene methods and genome-wide approaches. Studies in these two broad classes can be further categorized as either family- or population-based.

Candidate Gene Studies

Inquiries into possible candidate genes require a strong *a priori* hypothesis that a gene is involved in the phenotype being examined.[23] They typically involve genotyping a limited number of polymorphisms, which are then tested for association with the trait of interest, either as individual SNPs or as haplotypes. In family-based candidate gene studies, analysis options include transmission-disequilibrium tests (TDT),[24] and it's extensions and offshoots (such as FBAT),[25] as well as other methods, such as the measured genotype approach.[26] Advantages to studying families in candidate gene approaches are that it is often possible to directly infer haplotype phase from unphased genotypes due to the additional information afforded

through parental and sibling genotypic information[27] and that they are robust to population stratification.[28] A disadvantage of using families is that variants determining complex phenotypes, which may be common and with small effects, can be more difficult to identify.[29]

In general populations, the tools of classical epidemiology are used to explore gene-phenotype associations. These methods include linear and logistic regression, survival analysis, and analysis of variance applied to understanding genotype, or haplotype, associations with phenotypic information. Population-based samples possess some advantages compared to families, in that they are easier to ascertain, and in greater numbers, which affects statistical power. There are also disadvantages, such as greater susceptibility to population admixture and more complex procedures, and greater uncertainty, in haplotype estimation.[30]

Genome-Wide Studies

Genome-wide strategies, on the other hand, seek to localize novel genes using information spanning the entire genome. In family-based designs, the traditional method of choice is linkage analysis. This type of genome-wide screen incorporates pedigree and widely spaced (generally ~ 10 cM) microsatellite marker information to identify broad chromosomal regions that may harbor a gene involved in determining the phenotype under study. This type of approach was broadly successful in finding rare genetic variants with large effect sizes for a wide variety of phenotypes. The usefulness of linkage analysis in finding more common polymorphisms with smaller effects has, however, been more limited.[31,32]

Over the last few years, a new approach to genome-wide analysis became available, spurred by the development of micro-array genotyping assays. These micro-arrays allow for the rapid genotyping of many thousands, hundreds of thousands, or even millions, of SNPs.[33] This technological revolution, in turn, brought about the dawn of the age of genome-wide association analyses. This type of data can be considered in family studies, using software such as FBAT, but the real advantage genome-wide association provides is that large samples of unrelated individuals can also be analyzed.

Genome-wide association is an excellent method of identifying genetic variations that may be frequent and have small effect sizes.[30,34] Unlike candidate gene approaches, genome-wide analysis offers the potential to identify novel genes not previously identified with the phenotype of interest. The downside, however, is the multiple testing dilemma; establishing genome-wide significance can be difficult.[35] Several methods exist to attempt to correct for multiple testing, such as the Bonferroni correction, False Discovery Rate,[36] and permutation analysis. These are all quite punitive, although permutation is considered the proverbial "gold standard" in dealing with this issue, since it helps to account for the correlations between SNPS.[34] The problem with genome-wide permutation is that it is computationally intensive; however, the new R package for genome-wide analysis, GenABEL, easily and rapidly implements this procedure.[37]

Although these multiple testing corrections may help to control type I error rates, they are of marginal utility in identifying chance findings or excluding them. Several methods were introduced to further reduce false positives, including multi-stage and joint analysis designs.[38] In the end, however, the best way to ensure the accuracy of genome-wide findings is through replication or functional validation. Ideally, replications should utilize numerous independent samples; a number of journals, such as Nature Genetics, currently request numerous replication samples in addition to the discovery population. Validation, through functional studies in cells or animal models, is also an excellent method for confirming findings from genome-wide analyses, although it requires identification of the specific causal variant. These methods are especially important when newly identified loci were previously uncharacterized.

Other Considerations

A variety of other options also exists to help genetic epidemiologists to identify or characterize genes. One of these options is meta-analysis, which allows for the combination of results from multiple studies (either candidate gene or genome-wide). This technique may help to increase power and resolve inconsistent observations of association,[39] although they may be susceptible to population admixtures.

Other important considerations relate to the specific properties of the study population. The collection of genetically isolated populations, for example, offers several benefits. These include a reduction in genetic complexity for rare variants, a decrease in environmental variability, and an increased extent of linkage disequilibrium (LD).[40,41] The longer stretches of LD make it possible to conduct genome-wide association studies with fewer markers than would be necessary in a general population (and also leads to longer haplotypes),[42] while reduced genetic heterogeneity simplifies the task of identifying associated variants. There is a risk that findings in isolated populations may not generalize to the population at large, however, common variants that influence a phenotype, such as lipids, are unlikely to be greatly affected by genetic isolation.[43]

SCOPE OF THE THESIS

The goal of the work presented in this thesis was to use the tools of genetic epidemiology to help unravel the determinants of circulating lipid levels. These studies incorporate many different analytical methods and approaches, ranging from small families selected for affection status to large pedigrees in an isolated population ascertained irrespective of phenotype; from single SNPs in candidate genes to genome-wide analysis of hundreds of thousands of variants.

In Chapter 1.2, estimates of the proportion of variance in lipid traits attributable to additive, polygenic effects were estimated in a recently genetically isolated population. Additionally, the association of lipids with inbreeding was assessed, and the effect of the well-known Apolipoprotein E polymorphism was measured. In Chapter 1.3, meta-analysis was used to explore the association of a common hepatic lipase promoter polymorphism with lipid levels. In Chapters 2.1 and 2.2, the prospective follow-up cohort from the Rotterdam Study was used to examine the effects of previously characterized polymorphisms in the hepatic lipase and cholesteryl ester transfer protein genes on lipid levels, atherosclerosis proxies, and incidence of myocardial infarction. The interaction between these two SNPs was also evaluated. Chapters 2.3 and 2.4 used families affected by familial combined hyperlipidemia (FCH) to study the effects of two genes, upstream stimulatory factor 1 and Apolipoprotein A-V, on both FCH status and a wide range of lipid parameters. A region-wide analysis of the Apolipoprotein L gene cluster on chromosome 22 comprises Chapter 2.5. Lastly, in Chapter 3.1, a genome-wide association analysis of 250,000 SNPs was conducted.

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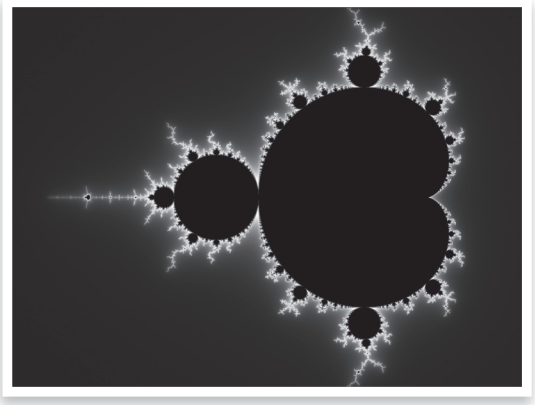
Chapter 1.2

Heritabilities, Apolipoprotein E, and Effects of Inbreeding on Plasma Lipids in a Genetically Isolated Population: The Erasmus Rucphen Family Study



ABSTRACT

Despite considerable progress in unravelling the genetic basis of dyslipidemias, most findings are based on families with extreme phenotypes. We studied lipid levels in an extended pedigree ascertained irrespective of phenotype from the population of a recent genetic isolate in the Netherlands. Heritabilities of plasma lipid measures were examined; this analysis also included estimates of the proportion of variance attributable to ApoE genotype. The association between inbreeding and lipids was also considered, as a substantial fraction of the population had known inbreeding. 868 individuals from this pedigree, containing more than 60,000 people over fifteen generations, were investigated in this study. Laboratory analysis of these subjects included the determination of fasting plasma lipids. ApoE ε2/3/4 status was ascertained using TaqMan assays. Heritabilities for plasma lipids were estimated with adjustments for multiple covariates using SOLAR. Heritabilities for total cholesterol (TC), high-density lipoprotein cholesterol (HDL), low-density lipoprotein cholesterol (LDL), triglycerides (TG), TC/HDL ratio, and TG/HDL ratio were found to be 0.35, 0.56, 0.30, 0.24, 0.49, and 0.39, respectively. The addition of ApoE genotype in the model significantly decreased these estimates (Δh^2 = -0.030, -0.004, -0.054, and -0.006 for TC, HDL, LDL, and TG). In a further analysis, TC and LDL were positively associated with the extent of inbreeding (P_{trend} = 0.02 and P_{trend} = 0.05, respectively). These data provide estimates of lipid heritability unbiased due to selection and suggest that this population represents a good opportunity to localize novel genes influencing plasma lipid levels.

Cardiovascular disease remains the leading cause of death in the United States, Europe, and portions of Asia.[1] High levels of total cholesterol (TC), triglycerides (TG) and low-density lipoprotein cholesterol (LDL) and low levels of high-density lipoprotein cholesterol (HDL) are well-established coronary heart disease (CHD) risk factors.[2] Plasma lipid levels themselves have emerged as important therapeutic targets, which can reduce the incidence of CHD.[3,4]

Detection of genetic variants leading to altered lipid profiles might prove useful in the diagnosis, prevention, and treatment of disease. Heritabilities of various plasma lipids in other family-based studies have been previously reported to range from 0.39 to 0.62 for TC, 0.39 to 0.83 for HDL, 0.24 to 0.50 for LDL, 0.20 to 0.55 for TG and 0.34 for TG/HDL ratio;[5-14] even more extreme values were noted in recent twin studies.[15,16] Several factors may explain the wide variation in these estimates. Until now, genetic studies have been performed in extended families selected on the basis of dyslipidemia, or in affected sib-pairs, usually with comparatively small sizes, which inflate heritability estimates. Furthermore, there is disagreement concerning whether common traits are caused by numerous common genetic variants (the common disease/common variant theory) or by rare, population specific mutations. If the common disease/rare variant theory holds, specifically ascertained populations might have higher heritabilities than found in less selected populations. By contrast, we studied the heritability of plasma lipids in an extended family, derived from a genetically isolated population. This family was not selected based on the presence of dyslipidemia.

The apolipoprotein E (ApoE) ε2/3/4 polymorphism, which results in three ApoE protein isoforms determined by missense mutations in the 112th and 158th amino acids, is one of the most well-described variations affecting plasma lipid values.[17] Differences in binding affinities between the three isoforms are dramatic, and lead to these observed differences in lipid levels.[18] In comparison to the referent ApoE ε3/3 group, the ApoE ε4 allele is strongly associated with increased TC, LDL, and TG, while the ApoE ε2 allele is associated with decreased TC and LDL and increased TG. [19,20] The effects on HDL are less clear, perhaps context dependent, although ε2 is generally associated with increases, and ε4 with decreases, in HDL.[21,22] ApoE was further associated with a variety of ailments, such as cardiovascular disease[23] and Alzheimer's Disease.[24]

The availability of a genealogical database detailing this population allowed for the construction of a single, large (greater than 60,000 individual) pedigree. This information also enabled us to examine the effects of inbreeding on plasma lipid levels. Despite the anticipation of inbreeding effects on plasma lipids, there was no evidence of this in a prior study,[25] although studies of other cardiovascular disease phenotypes have shown that inbreeding may play a role.[26,27]

The aim of this study was to estimate the heritability of plasma lipids in a recent genetic isolate in the Netherlands and to evaluate the degree to which ApoE contributes to these estimates. A further aim was to evaluate the effect of inbreeding on lipid levels.

MATERIALS AND METHODS

Study Population

The present study was conducted in a large family-based cohort study (the Erasmus Rucphen Family [ERF] Study). The cohort was derived from a recent genetic isolate in the southwest Netherlands for which a comprehensive genealogical database exists. This population was founded in the middle of the 18th century by approximately 150 individuals and was isolated until the last few decades. Characterized by rapid growth and minimal immigration, the isolate now includes approximately 20,000 inhabitants. Twenty couples living in the region in the 19th century were chosen. These couples parented a minimum of 6 children, each of whom was baptized between 1880 and 1900 in the community church. All living descendants of these pairs (as well as their spouses), ascertained on the basis of municipal and baptismal records, were traced and invited to participate ($n \approx 3{,}000$).

Subjects completed an interview with a physician and a thorough medical examination during a visit to the research center. The interview included questions concerning smoking (number of cigarettes per day) and alcohol consumption (grams per day) habits, lipid lowering and hormone replacement therapy, and diabetic medications. Participants were requested to bring the medications they use to the research center. All details pertaining to their prescriptions were discussed with a research physician. Height and weight data were collected and used to calculate body mass index. Blood pressure was measured twice on the right arm in a sitting position after at least five minutes rest, using an automated device (OMRON 711); the average of the two values was used for analysis.

All participants gave informed consent, and the Medical Ethics Committee of the Erasmus Medical Center approved the study protocol. The present study is based on 868 individuals, from an extended pedigree of almost 60,000 over fifteen generations, for whom plasma lipid data and ApoE ε2/3/4 genotype was available at the time of analysis.

Laboratory Analysis

Fasting blood samples were collected during the participant's visit to the research center. A Synchron LX20 (Beckman Coulter Inc., Fullerton, CA. U.S.A.) spectrophotometric chemical analyzer was utilized for the determination of plasma lipid values, as well as fasting plasma glucose levels. Diabetes mellitus was defined by one of two common criteria: use of glucose lowering medication or a plasma glucose level greater than 7.0 mmol/L.[28]

Genomic DNA was extracted from whole blood samples drawn at the baseline examination, utilizing the salting out method.[29] Subjects were genotyped for the ApoE ε2/3/4 polymorphism with two TaqMan allelic discrimination Assays-By-Design (Applied Biosystems, Foster City, CA), targeting SNPs in the 112th (rs429358) and 158th (rs7412) amino acids of the

ApoE gene.[30] Forward and reverse primer sequences for the position 112 polymorphism were 5′- GGGCGCGGACATGGA-3′ and 5′- CCTCGCCGCGGTACTG-3′, respectively. The minor groove binding probes were 5′- CGGCCGCGCACGT-VIC-3′ and 5′- CGGCCGCACACGT-FAM-3′. For the SNP at position 158, forward and reverse primer sequences were 5′- TCCGCGATGCCGATGAC-3′ and 5′- CCCCGGCCTGGTACAC-3′, respectively. The minor groove binding probes were 5′- CAGGCGCTTCTGC-VIC-3′ and 5′- CAGGCACTTCTGC-FAM-3′. Both assays were designed using the reverse strand.

The assays utilized 5 nanograms of genomic DNA and 5 microliter reaction volumes. The amplification and extension protocol was as follows: an initial activation step of 10 min at 95°C preceded 40 cycles of denaturation at 95°C for 15 seconds and annealing and extension at 50°C for 60 seconds. Allele-specific fluorescence was then analyzed on an ABI Prism 7900HT Sequence Detection System with SDS v 2.1 (Applied Biosystems, Foster City, CA).

Statistical Analysis

Multiple linear regression models were fitted in SPSS 11.0 to examine the association of covariates with plasma lipids and to assess the distributional assumption of normality. For heritability estimation, covariates were chosen on the basis of statistical significance ($P < 0.10$). For these analyses, HDL, TG, TG/HDL, and TC/HDL were natural log transformed to yield normally distributed residuals. The normality of residuals was tested using a one-sample Kolmogorov-Smirnov test.

Estimates of heritability, utilizing a variance component approach based on maximum likelihood procedures, were calculated in SOLAR version 2.1.4.[31] Heritability (h^2) is defined as the ratio of additive genetic variance to total phenotypic variance unexplained by covariates, and does not take into account epistasis or dominance. To determine the proportion of variance attributable to ApoE genotype, heritabilities were computed with and without genotypic data. ApoE ε2/3/4 status was coded using the 3/3 genotype as a baseline, with a separate variable coding for each of the other possible genotypes (2/2, 2/3, 2/4, 3/4, and 4/4). Comparison of the log likelihoods of these models allowed us to assess the significance of the differences. To evaluate the effect of including individuals receiving lipid-lowering therapy, heritabilities were also estimated excluding those individuals.

Inbreeding coefficients were calculated according to Meuwissen's method, as implemented in PEDIG.[32] Spearman's correlation coefficients were calculated for inbreeding versus the lipid outcomes. To predict the impact of these associations on lipid levels, a linear regression model assessing inbreeding coefficient quartile versus plasma lipid levels was realized. The quartiles were used due to the large skew in the distribution resulting from the many individuals who did not possess measurable inbreeding (n = 200). These analyses were executed utilizing SPSS 11.0.

RESULTS

Approximately 3,000 individuals were invited to participate in the ERF study. At the time of genotyping and analysis, about 1,000 of these samples were ready for use; after exclusion of those with missing information and/or genotype, 868 were included in the described study. Table one shows descriptive statistics for classical cardiovascular risk factors stratified by gender. Men had higher mean BMI and blood pressure (both systolic and diastolic). In this population, women smoked more often than men, while significantly more men consumed alcohol. HDL and TG levels were substantially different (as, consequently, were the TG/HDL and TC/HDL ratios). There were no significant differences, however, between the sexes with respect to TC or LDL. Men also received lipid-lowering therapy more frequently than women.

The mean levels of TC in this population were relatively high given the age distribution. Considering a clinical cut-off of 6.5 mmol/L to determine hyperlipidemia, the mean TC level was less than one standard deviation lower than this value. Approximately 20% of the population had TC levels greater than, or equal to, 6.5 mmol/L. This pattern was observed in both men and women.

Table 1: Summary statistics in the ERF population

	Women (n = 517)	Men (n = 351)
Age (years)	51.00 ± 14.51	53.80 ± 13.52*
BMI (kg/m^2)	26.68 ± 4.82	27.59 ± 4.29*
Smoking % (n)	47.2 (244)	32.5 (114)*
Alcohol Use % (n)	33.7 (174)	69.8 (245)*
SBP (mm Hg)	138.82 ± 22.76	144.30 ± 19.44*
DBP (mm Hg)	78.98 ± 9.62	82.09 ± 10.21*
TC (mmol/L)	5.63 ± 1.17	5.52 ± 1.11
HDL (mmol/L)	1.37 ± 0.36	1.13 ± 0.31*
LDL (mmol/L)	3.72 ± 1.04	3.73 ± 0.98
TG (mmol/L)	1.28 ± 0.63	1.58 ± 1.00*
TC/HDL Ratio	4.32 ± 1.19	5.16 ± 1.41*
TG/HDL Ratio	1.03 ± 0.67	1.58 ± 1.27*
Diabetes mellitus % (n)	6.2 (32)	7.1 (25)
LLT % (n)	16.8 (87)	22.2 (78)*
HRT % (n)	3.3 (17)	NA
ApoE ε2 Frequency	0.05	0.05
ApoE ε3 Frequency	0.75	0.74
ApoE ε4 Frequency	0.20	0.20

BMI = Body Mass Index, SBP = Systolic Blood Pressure, DBP = Diastolic Blood Pressure, TC = Total Cholesterol, HDL = High-Density Lipoprotein Cholesterol, LDL = Low-Density Lipoprotein Cholesterol, TG = Triglycerides, LLT = Lipid Lowering Therapy, HRT = Hormone Replacement Therapy.

* Significantly different from women.

Values presented are mean ± standard deviation for continuous traits and percent (number) for non-continuous traits.

Table 2: Coefficients and *P*-values for covariates by outcome measure

	TC		HDL		LDL		TG		TG/HDL Ratio		TC/HDL Ratio	
Covariate	β	P-value	β	P-value	β	P-value	β	P-value	β	P-value	β	P-value
Age	0.127	<0.001	0.032	0.026	0.106	<0.001	*0.0499*	*0.101*	*0.016*	*0.665*	0.061	<0.001
Sex	-1.140	<0.001	*0.338*	*0.178*	-1.108	<0.001	*-0.566*	*0.267*	*-0.890*	*0.147*	-1.151	<0.001
Smoking	0.007	0.049	-0.015	<0.001	0.010	0.002	0.016	0.021	0.030	<0.001	0.020	<0.001
Alcohol	*-0.006*	*0.165*	0.010	0.006	-0.015	<0.001	0.025	0.001	*0.015*	*0.104*	-0.014	<0.001
Age*sex	-0.023	<0.001	-0.008	0.079	-0.019	<0.001	*-0.005*	*0.558*	*0.003*	*0.798*	-0.008	0.081
Age^2	-0.001	<0.001	*-0.000*	*0.206*	-0.001	<0.001	*-0.000*	*0.274*	*-0.000*	*0.713*	-0.000	0.006
DM	-0.248	0.083	-0.270	0.045	-0.338	0.008	0.615	0.022	0.889	0.006	*0.109*	*0.442*
Inbreeding	19.113	0.003	11.082	0.079	13.945	0.011	*13.978*	*0.215*	*4.549*	*0.751*	*2.706*	*0.676*
BMI	*-0.003*	*0.741*	-0.063	<0.001	*0.004*	*0.594*	0.105	<0.001	0.170	<0.001	0.060	<0.001
HRT	*-0.276*	*0.297*	*0.134*	*0.593*	*-0.328*	*0.162*	*-0.439*	*0.381*	*-0.576*	*0.341*	*-0.378*	*0.152*
LLT	-0.908	<0.001	-0.267	0.002	-0.914	<0.001	0.737	<0.001	0.973	<0.001	-0.425	<0.001

DM = Diabetes Mellitus, BMI = Body Mass Index, HRT = Hormone Replacement Therapy, LLT = Lipid Lowering Therapy.

Italicised covariates were excluded from the full model.

The relationship between lipid levels and classical risk factors revealed that regression coefficients were of anticipated magnitudes and directions (Table Two). Heritability estimates were computed for all plasma lipid outcomes using two adjusted models (Table Three). The full models included covariates (age, sex, age*sex, age^2, smoking status, alcohol consumption, lipid-lowering therapy, diabetes status, inbreeding coefficient, body mass index, and hormone replacement therapy) significant at a level of $P < 0.10$. The highest heritability estimates were found for HDL and TC, and, consequently, TC/HDL ratio. The lowest estimates were found for LDL and TG (Table Three). All of these estimates were highly significant; the *P*-values for the full model estimates for each outcome were found to be < 0.0002.

Table 3: Plasma lipid heritability estimates in ERF

Outcome	Heritability (SEM)		Proportion of variance due to covariates		ApoE Genotype		
	Age & Sex adjusted	Full Model adjusted	Age & Sex adjusted	Full Model adjusted	Δh^2	Prop. of variance	*P*-Value
TC	0.19 (0.07)	0.35 (0.09)	0.02	0.16	-0.030	0.031	0.006
HDL	0.51 (0.08)	0.56 (0.08)	0.11	0.21	-0.004	0.011	0.014
LDL	0.17 (0.07)	0.30 (0.09)	0.02	0.19	-0.054	0.053	< 0.001
TG	0.28 (0.07)	0.24 (0.07)	0.04	0.14	-0.006	0.008	0.021
TC/HDL Ratio	0.38 (0.07)	0.49 (0.08)	0.10	0.20	-	-	-
TG/HDL Ratio	0.37 (0.07)	0.39 (0.08)	0.08	0.17	-	-	-

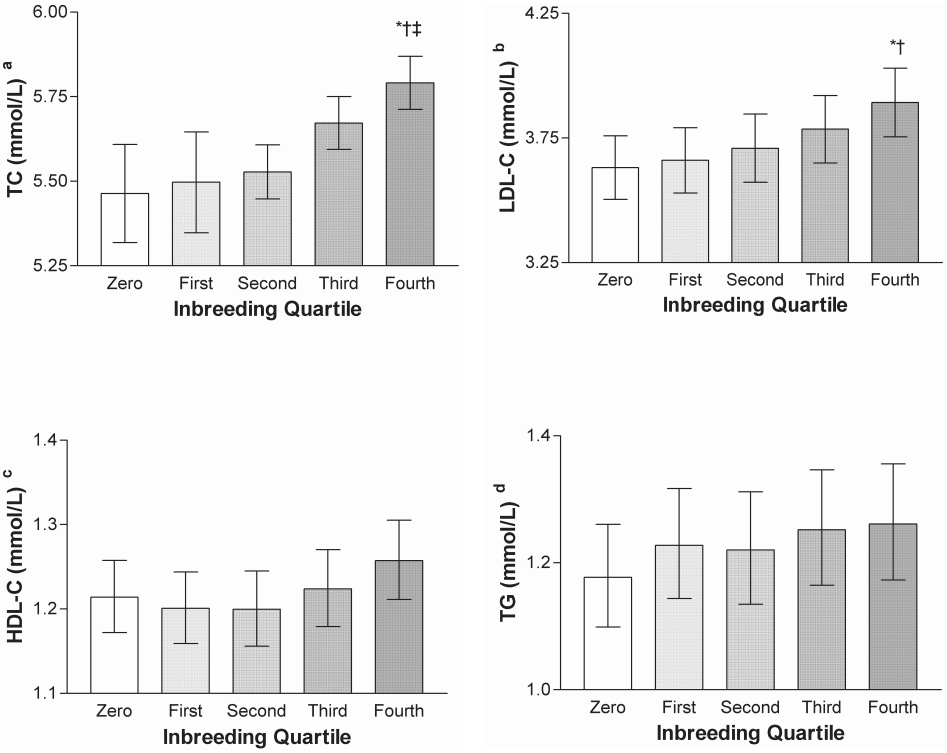
TC = Total Cholesterol, HDL = High-Density Lipoprotein Cholesterol, LDL = Low-Density Lipoprotein Cholesterol, TG = Triglycerides, Δh^2 = Change in Heritability, Prop. of variance = Proportion of Variance Attributable to ApoE Genotype.

Full model controlled for age, sex, smoking, alcohol use, age*sex, age2, diabetes mellitus, inbreeding, BMI, hormone replacement therapy and lipid lowering therapy.

Excluding individuals on lipid-lowering therapy did not dramatically affect these findings. Heritabilities increased somewhat for each outcome when these individuals were not included; these differences ranged from 0.0005 (for TC/HDL ratio) to 0.039 (for TC and HDL). These increases were of the anticipated magnitude and direction, given the random variance introduced by differing levels of efficacy, compliance, and dosage among treated individuals.

Inbreeding was a significant covariate in the heritabilities estimated for both TC and LDL (Table Two). In light of this result, the relationship between inbreeding and lipids was assessed. Spearman's correlation coefficients for TC, HDL, LDL, and TG (*P*-value) were 0.18 ($P < 0.001$), 0.04 ($P = 0.185$), 0.15 ($P < 0.001$), and 0.09 ($P = 0.006$), respectively. These associations, adjusted for numerous covariates, persisted for both TC and LDL when inbreeding quartiles were considered (Figure One). TC ($P_{trend} = 0.02$) and LDL ($P_{trend} = 0.05$) increased significantly with level of inbreeding. HDL, while not significant, did indicate a tendency for more inbred individuals to have higher levels. In addition, inbred individuals received more frequent prescription of lipid-lowering therapy; 19.9% of the inbred group received such therapy, compared to 13.1% in the non-inbred group ($P = 0.03$).

Figure 1: Plasma lipid means (95% CI) by inbreeding quartiles



All outcomes adjusted for age, sex, smoking, alcohol use, age*sex, age^2, diabetes mellitus status, hormone replacement therapy, and lipid lowering therapy.

* Significantly different from zero inbreeding. † Significantly different from first quartile. ‡ Significantly different from second quartile.

[a] *P* for trend = 0.02, [b] *P* for trend = 0.05, [c] *P* for trend = 0.41, [d] *P* for trend = 0.69.

ApoE genotype was successfully assessed in over 95% of subjects. Mendelian inconsistencies forced the removal of a few individuals/trios (n = 7). ApoE genotype accounted for significant proportions of the heritabilities of all analysed outcomes (the ratios were not further analysed, as they did not produce informative results) (Table Three). Reductions of 0.030, 0.004, 0.054, and 0.006 were observed for TC, HDL, LDL, and TG, respectively. These values correspond to differences of 3.1%, 1.1%, 5.3%, and 0.8% of the total trait variance.

DISCUSSION

In this large, family-based study in a non-phenotypically selected pedigree, all heritability estimates of fasting plasma lipids (TC, HDL, LDL, TG, TG/HDL ratio, and TC/HDL ratio) were highly significant. These estimates ranged from 0.24 for TG to 0.56 for HDL. The inclusion of ApoE genotype caused significant decreases in these estimates, ranging from 0.70% (HDL) to

17.72% (LDL). A further analysis of the association of inbreeding with plasma lipids revealed significant, if modest, correlations between inbreeding and TC, LDL, and TG. Significant trends of TC and LDL increasing with inbreeding quartile were observed. HDL levels also tended to increase with the extent of inbreeding, although this trend fell well short of the conventional significance level. To our knowledge, this is the first study to document an association between inbreeding and plasma lipids.

These estimates of heritability are in the range of previous estimates, although some publications reported heritabilities higher than those obtained in this study. Several factors may explain this. The simplest revolves around the use of different populations, which may have differing genetic contributions to lipid levels. Another is that prior estimates tended to be obtained from selected pedigrees (i.e. selected on the basis of disease status),[8] whereas this analysis features a "randomly" (i.e. non-phenotypically) selected pedigree. Furthermore, many previous reports have utilized multiple small pedigrees in their analysis.[10] The use of small pedigrees, with closely related individuals, tends to inflate heritability estimates compared to larger pedigrees containing distant relations.[33] This study, which used only one large pedigree, minimizes this problem by virtue of its design.

Although a shared household environment may influence plasma lipids through shared dietary and exercise habits, the homogeneity of the isolated population suggests that this is unlikely to appreciably alter these results. Furthermore, inclusion of BMI as a covariate served as a proxy for these two traits. BMI was significantly associated with all outcomes except TC and LDL; its presence in the model did not alter the significance levels of our estimates of heritability.

Dominance variance, which, in conjunction with additive and environmental variance, comprises broad sense heritability, was also not estimated in this study due to the computational problems involved. Dominance variance has been previously demonstrated to have very little influence on heritability estimates for HDL and TG levels, although it exerted a greater effect on LDL.[34] This would suggest that estimates for HDL and TG are likely accurate, and LDL might be somewhat underestimated in this study.

The ApoE analysis provides a reliable estimate of the proportion of trait variance due to ApoE ε2/3/4 status and demonstrates the power of the ERF study design to detect variations that cause comparatively small changes in a given lipid outcome. The amount of LDL variation attributable to this very well-described variant corresponds well with a previous estimate,[19] while the results for the other studied outcomes are also in line with expectations. The large amounts of residual heritability for all traits indicate that other genetic variations also play important roles in the determination of lipid levels in this population. These may include numerous polymorphisms with small effects, or gene-gene interactions, as well as genes with major effects (although these are likely to have been previously detected).

The increase of TC and LDL, and, to a lesser extent HDL, associated with inbreeding suggests that genetic factors, possibly recessive, have a substantial effect on plasma lipids in this

population. The lack of clear statistical association between inbreeding and HDL levels may be a result of insufficient statistical power, since the variation in HDL was considerably smaller than for either TC or LDL. Inbreeding has been previously associated with cardiovascular disease, particularly hypertension,[26] but not to lipid levels. Estimates that as many as 23% of amino acid mutations are somewhat deleterious, and present mostly in heterozygous individuals,[35] suggests that consanguinity should increase homozygosity at these sites and lead to more pronounced effects in inbred populations.

Taken together, the heritability estimates (with and without ApoE) and the association of inbreeding with plasma lipids, offer strong evidence for the presence of genetic variants influencing lipid levels. It is likely that some deleterious recessive alleles occur with increased rates of homozygosity in this population, which ought to feature a smaller number of variants to begin with (due to the founder effect). ApoE does not account for most of the estimated additive genetic variance of these traits. Especially for HDL, with a high heritability estimate, and TC and LDL, with reasonably high heritabilities and an association with inbreeding, this population offers a good opportunity to discover genetic variants associated with these traits.

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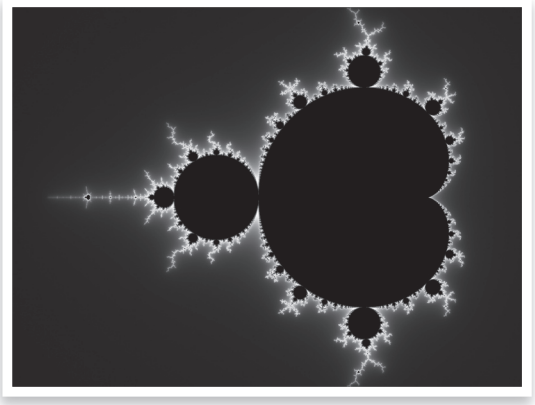
Chapter 1.3

The –514 C>T Hepatic Lipase Promoter Region (LIPC) Polymorphism and Plasma Lipids, A Meta-analysis



ABSTRACT

Investigations of the –514 C>T single nucleotide polymorphism (SNP) in the hepatic lipase (HL) gene promoter region (LIPC) have yielded contradictory results regarding its association with changes in plasma lipids. The current study is a meta-analysis of 25 publications on this SNP, comprising over 24,000 individuals, and its relationship with total cholesterol (TC), low-density lipoprotein cholesterol (LDL), high-density lipoprotein cholesterol (HDL), triglycerides (TG) and HL activity. Significant decreases were observed in HL activity for both the CT and TT genotypes compared to the CC genotype [Weighted Mean Difference (WMD) = -5.83 mmol/L/hr [95% Confidence Interval (95% CI): -8.48, -3.17] and –11.05 mmol/L/hr [95% CI: -14.74, -7.36], respectively]. Moreover, significant increases in HDL were found; the CT to CC comparison showed an increase in WMD of 0.04 mmol/L [95% CI: 0.02, 0.05] and the increase in the TT *vs.* CC difference was WMD of 0.09 mmol/L [95% CI: 0.07, 0.12]. These changes appear to be stepwise, implying an allele dosage effect. All *P*-values for these associations were less than 0.001. This meta-analysis demonstrates the importance of the –514C>T SNP in determining HL activity and plasma HDL concentration and helps quantify the role that hepatic lipase plays in the metabolism of HDL.

Hepatic lipase (HL), a glycoprotein member of the lipase superfamily, plays an important role in the metabolism and modeling of both pro- and antiatherogenic lipoproteins. Synthesized and secreted by the liver, HL carries out several metabolic functions, including the hydrolysis of triglycerides, the lypolysis of phospholipids, the modeling of small, dense atherogenic low-density lipoprotein cholesterol (LDL) particles, and the catabolism of high-density lipoprotein cholesterol (HDL).[1]

Numerous polymorphisms in the HL gene, located at 15q21 and spanning nine exons, are under investigation. Four common single nucleotide polymorphisms (SNPs) are located in the promoter region (an A>G at –763 bp, a T>C at –710 bp, a C>T -514 bp upstream of the promoter, and a G>A SNP at –250 bp); these have been reported to be in complete linkage disequilibrium.[2,3] One in particular, designated alternately as the –480 C>T or the –514 C>T single nucleotide polymorphism (SNP),[4,5] has received considerable attention. This polymorphism, located in the promoter region, has been demonstrated to influence HL activity levels.[6-13] HL activity is substantially decreased in carriers of the T allele. In addition, in some populations, the –514 SNP (as the polymorphism will be designated in this article) demonstrated a significant positive effect on plasma HDL levels.[11,14-24] This finding, however, has been attenuated by other studies that fail to demonstrate an association.[7,8,12,25-30] With respect to other plasma lipids, one study documents a marginally significant association between the SNP and increased total cholesterol (TC) in male control subjects.[29]

The use of widely varying, sometimes heterogeneous, populations as well as the small sample sizes used in many of these studies may explain these discrepancies, especially considering the comparatively small measurable association of the mutation, with respect to plasma lipids. In an effort to resolve these differences and gain some indication of the overall clinical significance of this polymorphism, the current study examines the literature available through January 2004 in a meta-analysis, a method that may help to alleviate the inconsistencies traditionally associated with genetic association studies.[31]

MATERIALS AND METHODS

PubMed, the on-line bibliographic resource, provided the means for identifying association studies pertaining to the –514 C>T polymorphism and HL activity and plasma lipids. These articles, supplemented by others extracted from their references and by additional information obtained via contact with the authors, totalled 47. Studies presenting either HL activities, plasma lipid levels, or both were considered for inclusion. Twelve studies were excluded due to the fact that relevant data (such as plasma lipid concentrations or number sampled for each genotype) were not presented.[32-43] Nine others failed to distinguish between the CT and TT genotypes and did not enter into the meta-analysis for this reason.[16,25,44-50] Another study appeared to present a subset of data published in a separate article.[51]

Altogether, 25 studies contributed to this meta-analysis. Sample sizes varied widely, ranging from 21 - 9,117. Three studies utilized a case-control design in which the cases were comprised of coronary artery disease (CAD) patients,[9,11,29], one used hemodialysis patients as cases,[52] and five included only high-risk individuals.[8,9,13,17,52] One study, by Shohet *et al.*,[9] described a case-control study and also presented data for a group of CAD patients. Two reports examined the effect of the –514C>T polymorphism on patients receiving lipid-lowering therapy;[12,13] only baseline data entered into this analysis. One article documented cross-sectional analysis of a large cohort.[53] The remaining publications studied either random samples from the general population or relatively healthy individuals. Included are several cohort-based analyses; in these instances, however, the design was that of a nested case-control study.

Ten of the studies included only male subjects;[6,8-13,17,28,30] similarly, two analysed only females.[20,54] The remainder sampled both genders; several studies reported significant differences between men and women. Because of this, both overall and gender-stratified analyses were performed to determine if this gender difference might affect results.

Measurements of plasma lipids took place via colorimetric enzymatic reaction in articles included in the meta-analysis. The HDL fractions, in most instances, were determined by measurement of the cholesterol levels in the supernatant after precipitation of apolipoprotein B-containing particles. LDL levels were usually estimated via Friedewald's equation.

Genotype and allele proportions in these samples did not, for the most part, differ from Hardy-Weinberg equilibrium (HWE). One population that failed to meet HWE reported on a high-risk population,[11] in which deviations from Hardy-Weinberg proportions are expected if the gene is associated with the risk of disease. Another study, by Fang *et al.*,[26] was in accordance with HWE overall and in men, but not women.

Statistical Analysis

Entry of the available data into Cochrane's Review Manager (RevMan version 4.2, the Cochrane Collaboration, Oxford, UK) allowed for extensive analysis. Before pooling the studies, tests for homogeneity were performed. Sensitivity analysis was conducted by reviewing the included studies a second time to detect sources of heterogeneity and, consequently, to perform additional analysis with and without those studies. In those cases where standard errors were originally reported, standard deviations were calculated. All data in this analysis are presented as the mean ± standard deviation. The method of moments technique, proposed by DerSimonian and Laird,[55] provided a way to calculate weighted mean differences (WMD) in a random effects model for pooled data.

Published findings showing differences on the basis of gender necessitated stratified analysis by sex. Differences in allele frequency between ethnic groups further necessitated a breakdown by ethnicity (Caucasian, African-American, and Asian). Finally, stratification by population (high or low risk) was performed to determine whether risk factor profiles played a role in

this association. Risk categories were determined on the basis of the presence (or absence) of disease (such as CAD or diabetes) or other risk factors (such as children of CAD patients).

Finally, utilization of funnel plots allowed us to ascertain the possible influence of publication bias. These plots were conducted with RevMan software.

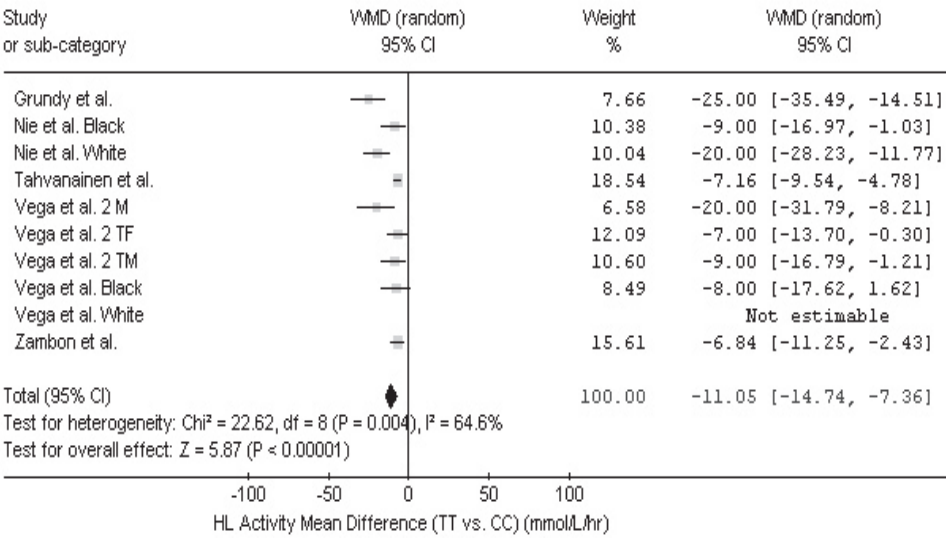
RESULTS

Twenty-five studies included in this analysis comprised 24,252 subjects, although this total fluctuates according to the data available for a given outcome. Six articles (791 persons) provided the data analysed for HL activity; 23 included data analysed for plasma HDL levels (23,792 individuals). Anderson *et al.*[53] (n = 9,117) and Couture *et al.*[14] (n = 2,667) used the largest samples, both in Caucasian populations. The majority of the studies sampled middle-aged subjects, although several used younger groups and others consisted largely of more elderly people.

Overall, the frequency of the T allele was 25.3%. This estimate varied widely in the studies, ranging from just over 55% in an Oji-Cree (indigenous Canadian) ethnic population[27] to 17% in one of the Caucasian samples.[15] Table 1 details the number of subjects and genotype distributions of the included studies.

HL activity decreased significantly for both the CT and the TT genotypes compared with the CC genotype (WMD = -5.83 mmol/L/hr [95% CI: -8.48, -3.17], $P < 0.001$, and –11.05 mmol/L/hr [95% CI: -14.74, -7.36], $P < 0.001$, respectively). Figure 1 shows the negative WMDs in HL

Figure 1: Hepatic lipase activity for TT versus CC genotypes



T: Turkish population; M: Male; F: Female

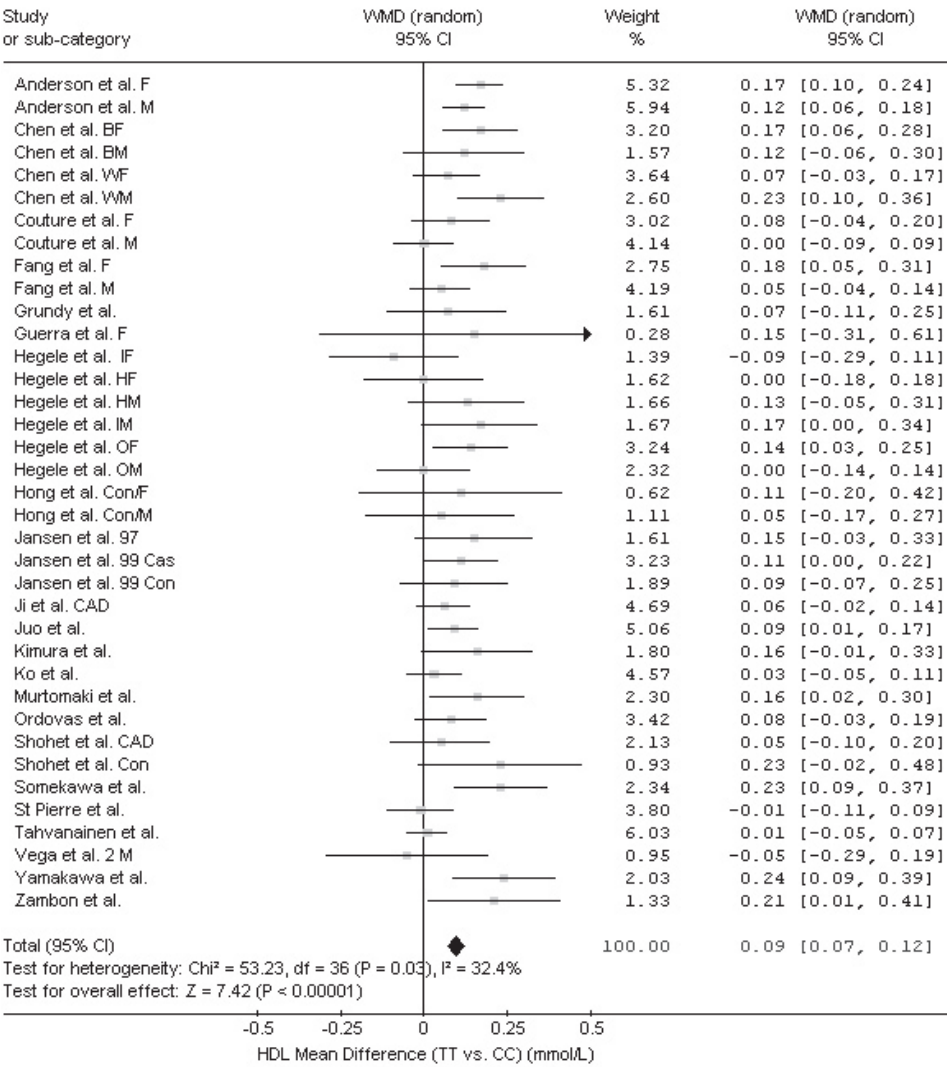
Table 1: Characteristics of included studies

First Author	Year	Country/ Region	Sample Size	Genotype Frequencies (%)			HWE *P*-value
				CC	CT	TT	
Anderson *et al.* [53]	2003	Denmark	9117	5662 (62.1)	3048 (33.4)	407 (4.5)	0.90
Chen *et al.*[22]	2003	United States	2462	1159 (47.1)	970 (39.4)	333 (13.5)	0.00
Couture *et al.*[14]	2000	United States	2667	1690 (63.4)	872 (32.7)	105 (3.9)	0.57
Fang *et al.*[26]	2002	China	823	336 (40.8)	380 (46.2)	107 (13.0)	0.98
Grundy *et al.*[12]	1999	United States	21	11 (52.4)	4 (19.0)	6 (28.6)	0.01
Guerra *et al.*[15]	1997	United States	139	97 (69.8)	37 (26.6)	5 (3.6)	0.54
Hegele *et al.*[27]	1999	Canada	1195	559 (46.8)	484 (40.5)	152 (12.7)	0.00
Hong *et al.*[29]	2000	Korea	124	39 (31.5)	61 (49.2)	24 (19.4)	0.99
Jansen *et al.*[11]	1997	Netherlands	612	367 (60.0)	234 (38.2)	11 (1.8)	0.00
Jansen *et al.*[17]	1999	Netherlands	790	468 (59.2)	288 (36.5)	34 (4.3)	0.21
Ji *et al.*[23]	2002	Australia	820	514 (62.7)	270 (32.9)	36 (4.4)	0.94
Juo *et al.*[28]	2001	United States	578	137 (23.7)	283 (49.0)	158 (27.3)	0.64
Kimura *et al.*[52]	2003	Japan	183	54 (29.5)	79 (43.2)	50 (27.3)	0.07
Ko *et al.*[24]	2004	Taiwan	716	295 (41.2)	331 (46.2)	90 (12.6)	0.85
Murtomaki *et al.*[18]	1997	Finland	270	144 (53.3)	110 (40.7)	16 (5.9)	0.40
Nie *et al.*[10]	1998	United States	169	80 (47.3)	68 (40.2)	21 (12.4)	0.28
Ordovas *et al.*[19]	2002	United States	2130	1359 (63.8)	698 (32.8)	73 (3.4)	0.15
Shohet *et al.*[42]	1999	United States	399	266 (66.7)	117 (29.3)	16 (4.0)	0.49
Somekawa *et al.* [54]	2002	Japan	209	67 (32.1)	85 (40.7)	57 (27.3)	0.01
St. Pierre *et al.*[30]	2003	Canada	235	149 (63.4)	75 (31.9)	11 (4.7)	0.69
Tahvanainen *et al.*[8]	1998	Finland	376	212 (56.4)	138 (36.7)	26 (6.9)	0.59
Vega *et al.*[6]	1998	United States	83	38 (45.8)	32 (38.6)	13 (15.7)	0.17
Vega *et al.*[7]	1998	Europe	94	56 (59.6)	-	38 (40.4)	-
Yamakawa-Kobayashi *et al.*[20]	2002	Japan	174	57 (32.8)	69 (39.7)	48 (27.6)	0.01
Zambon *et al.*[13]	2001	United States	49	25 (51.0)	20 (40.8)	4 (8.2)	1.00

activity for carriers of the TT and CC genotypes. The difference in HL activity between the TT and CT carriers also reached statistical significance (-3.98 mmol/L/hr [95% CI: -5.90, -2.05], $P < 0.001$).

Plasma HDL levels also differed significantly among genotypes, although previous findings were inconsistent. Mean plasma HDL levels in the CT group were increased, on average, 0.04 mmol/L [95% CI: 0.02, 0.05] ($P < 0.001$), whereas mean plasma HDL levels for TT carriers increased 0.09 mmol/L [95% CI: 0.07, 0.12] ($P < 0.001$) compared with the CC group, as depicted in Figure 2. A significant difference also existed between the CT and TT subjects (0.06 mmol/L [95% CI: 0.04, 0.08], $P < 0.001$).

Figure 2: HDL concentrations for TT versus CC genotypes



I: Inuit population; H: Hutterite population; O: Oji-Cree population; M: Male; F: Female; CAD: Coronary artery disease group; Con: Control group; Cas: Designated cases

A significant difference also existed between the CC and TT genotypes for TC (0.09 mmol/L [95% CI: 0.02, 0.17], $P = 0.02$). Analysis of the other plasma lipids, including LDL and triglycerides, failed to reveal any significant differences (Table 2).

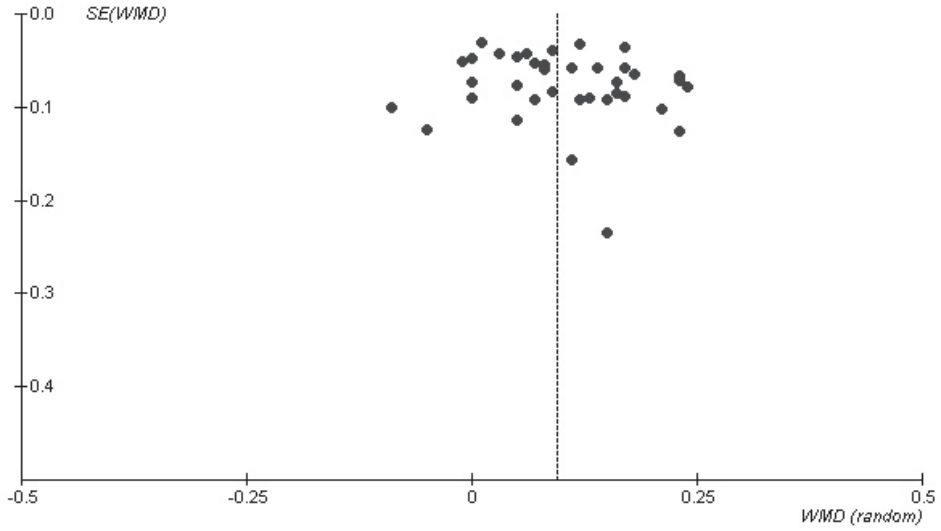
Stratification by gender, ethnicity (Caucasian, African-American, or Asian) and risk category (high or low) did not appreciably alter these associations (data not shown). The lone exception appeared to be a small, but significant, difference between WMDs in TC concentration when stratifying by ethnicity. Examining the CC and CT genotypes, the WMD of TC concentrations

Table 2: Plasma lipids and HL activity by genotype

Measurement	WMD [95% CI]	
	CT versus CC	TT versus CC
TC (mmol/L)	0.01 [-0.05, 0.07]	0.09 [0.02, 0.17]
HDL (mmol/L)	0.04 [0.02, 0.05]	0.09 [0.07, 0.12]
LDL (mmol/L)	0.02 [-0.02, 0.06]	-0.02 [-0.09, 0.06]
TG (mmol/L)	0.03 [0.00, 0.06]	0.04 [-0.05, 0.13]
HL Activity (mmol/L/hr)	-5.83 [-8.48, -3.17]	-11.05 [-14.74, -7.36]

WMD: Weighted mean difference; TC: Total cholesterol; HDL: High-density lipoprotein cholesterol; LDL: Low-density lipoprotein cholesterol; TG: Triglycerides; HL: Hepatic lipase

Figure 3: Funnel plot of studies analyzed for HDL



was significantly lower in Asians (WMD = -0.09 mmol/L [95% CI: -0.19, 0.01]), compared with that of Caucasians (WMD = 0.06 mmol/L [95% CI: 0.00, 0.12]).

Regarding HDL levels, stratification by ethnicity revealed that the direction of the association was consistent across ethnic groups, although the point estimates differed slightly. Funnel plots, performed to assess the possibility of publication bias, were quite symmetrical in all of the studied associations. Figure 3 shows this plot for the studies analysed for HDL.

DISCUSSION

This meta-analysis used data from more than 24,000 individuals, available from 25 published studies, to re-evaluate the association between the –514C>T promoter region SNP in the HL gene and plasma lipids and HL activity. The stepwise decrease in the point estimates for HL

(and stepwise increase in HDL levels) suggests that an allele dosage effect occurs in this association.

In several studies, the genotype frequencies did not conform to Hardy-Weinberg equilibrium proportions. In one of these, the population came from a high-risk category, in which deviations from HWE are expected. In several others, however, the sample came from general population groups. The reasons behind this are unclear, although genotyping errors remain a possibility. These studies introduced significant heterogeneity to the HDL comparisons. Their exclusion eliminated the heterogeneity, but did not appreciably alter the point estimates or CIs.

Any measurement errors in the laboratory analysis of plasma lipids would be equally distributed among the three genotypes, and, because weighted mean differences were calculated, this is unlikely to introduce an error in these results. The study by Grundy *et al.*[12] contained the smallest sample size (representing < 0.09% of the total). It also featured the third smallest standard deviation, according it a relatively large weight in the meta-analysis. The articles by Tahvanainen *et al.*[8] and Zambon[13] presented the smallest standard deviations, which, likewise, imparted high weights. Excluding these studies did not noticeably change the results, excluding the possibility of an over-weighting effect on the results obtained here.

The analysis of HL activity revealed evidence of significant heterogeneity. Although this heterogeneity might lead to an overestimation of the total estimate, sensitivity analysis demonstrated that heterogeneity did not alter the significant association between the genotype and HL activity, because all of the included studies show significant negative association between the presence of the T allele and HL activity. The Caucasian sample in the study by Vega *et al.*[6] included only one individual with the TT genotype; therefore, estimation of a WMD for HL activity was not possible.

An important potential source of bias in a meta-analysis is publication bias, because the likelihood of publishing a study is frequently related to the results it contains. The articles analysed in this meta-analysis, however, include many that reported negative findings. Funnel plots, used to determine the presence of publication bias, were highly symmetrical, suggesting that this form of bias is not an issue.

A previous study noted substantially reduced transcription of HL in murine hepatocyte cells (AML12).[3] The significant decrease in HL activity for both the CT and TT genotypes (with respect to the CC group) support the association between the presence of the T allele and decreased HL transcription, and, in turn, activity. Likewise, the significant increase observed in HDL levels when comparing CT and TT groups to CC support the association between this SNP and HDL levels, presumably as a result of decreased HL activity.

This analysis demonstrates the effect of the –514 C>T polymorphism on both HL activity and plasma HDL levels. The crucial question to ask concerns the effect of this SNP on cardiovascular morbidity and mortality. To date, few studies have addressed this issue. Based on the findings of the present study, we predict that TT carriers face a reduced risk of CAD, perhaps as

a consequence of increased HDL levels or due to previously noted associations with increased LDL buoyancy.[13,50] Surprisingly, some studies suggest an increased risk, which requires further research.

Briefly, this meta-analysis of 25 articles shows a positive association between the −514 C>T polymorphism and HDL levels and a negative association between genotype and HL activity. These associations are largely unaffected by gender, ethnicity, and risk category.

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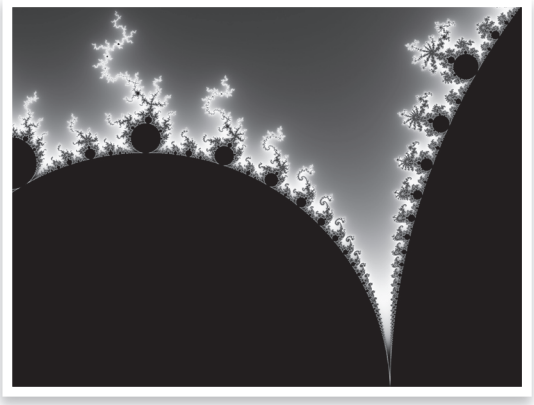
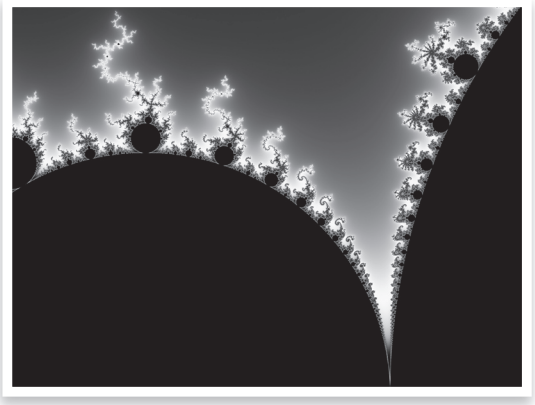
Chapter 2

Candidate Gene Studies



Chapter 2.1

The CETP I405V Polymorphism Is Associated with Increased HDL Levels and Decreased Risk of Myocardial Infarction: The Rotterdam Study



ABSTRACT

Background and Design: The CETP I405V polymorphism's effect on lipid levels, atherosclerosis, and myocardial infarction (MI) was examined in 6,421 participants from the Rotterdam Study.

Methods: Quantitative outcomes were studied with linear models; Cox models were used to assess MI risk.

Results: HDL increased by 0.06[0.03,0.09] mmol/L in VV carriers. The V allele was further associated with decreased MI risk in men (hazard ratio [95% CI] = 0.51 [0.39,0.67], VV vs. II) (P_{trend}=0.01).

Conclusions: This study provides additional evidence for the association of CETP with HDL levels and suggests that CETP is an atherogenic protein increasing the risk of MI.

INTRODUCTION

Cholesteryl ester transfer protein (CETP) facilitates the transfer of cholesteryl esters from HDL to triglyceride-rich lipoproteins in exchange for triglycerides. This function fostered considerable interest in CETP's role in cardiovascular disease; recent reviews suggested that CETP might be a target for reducing disease incidence.[1]

The CETP I405V (rs5882) polymorphism, located in a known binding domain,[2] causes an isoleucine/valine shift, leading to decreased protein levels.[3] This polymorphism's impact on HDL levels was clearly established in a recent meta-analysis.[4] It was additionally associated with decreased low-density lipoprotein (LDL) particle size.[5]

The relationship with coronary heart disease (CHD) is less well understood; previous studies reported discordant results.[3,6] To further elucidate the role of the I405V polymorphism in CHD, this study examined its association with lipids, common carotid intima media thickness (IMT) and plaques, and incidence of myocardial infarction (MI) in a population-based cohort in Rotterdam, the Netherlands.

MATERIALS AND METHODS

Study population

This study was embedded in the Rotterdam Study,[7] a single-center prospective cohort of 7,983 individuals aged 55 and older. Baseline medical examinations, including details of alcohol consumption, smoking, lipid-lowering therapy (LLT), and diabetes, took place between 1990-1993. Serum lipid measurements of total cholesterol (TC) were determined enzymatically, as were HDL levels after precipitation of apolipoproteinB containing particles. IMT was assessed by ultrasonography.[8] Carotid plaque score was determined by the number of sites (common, internal, and bifurcation; left and right) that showed visible focal widening protruding into the luminal space (scored 0-6).

Follow-up data between baseline and January 1, 2003 included incidence of MI. Information on MIs was obtained through a computerized reporting system. Two physicians examined patients' medical records and verified the events; an expert in the field resolved discordant diagnoses. In the case of multiple events, the first was used for analysis; prevalent MIs were excluded.

Genotyping

DNA was extracted from blood drawn at baseline, utilizing the salting-out method.[9] Subjects were genotyped with a TaqMan Assay-By-Design and analyzed on an ABI Prism 7900HT with SDSv2.1 (Applied Biosystems, Foster City, CA).

Statistical analysis

Hardy-Weinberg proportions were assessed by exact test. General characteristics were compared by analysis of variance (ANOVA) for continuous and χ^2 for dichotomous variables. After gender stratification, TC, HDL, TC/HDL ratio, IMT, and plaques were examined using a general linear model adjusted for age, smoking, alcohol consumption, body-mass index (BMI), LLT, and diabetes. Log-transformed lipid and IMT values were utilized to improve normality.

Cox models assessed the relationship between the I405V polymorphism and MI. Two models were implemented, following stratification by gender. The first included I405V genotype, age, smoking, BMI, systole, diastole, diabetes, and TC. The second additionally included HDL.

RESULTS

DNA was available for 6,571 participants; 6,421 (2,607 males and 3,814 females) were typed for the I405V polymorphism (97.7%). Genotype frequencies did not differ from Hardy-Weinberg expectations ($P = 0.41$). No differences existed between genotypes for baseline characteristics. Serum lipids, however, differed significantly. VV subjects possessed higher HDL levels in males ($P = 0.01$) and females ($P < 0.01$). This led to lower TC/HDL ratios. In males, the TC/HDL ratio in II carriers differed from VV carriers ($P = 0.05$). In females, there was a significant trend, with TC/HDL ratio decreasing with the number of V alleles ($P = 0.04$). There were no differences between genotypes with respect to TC, IMT or plaques (table 1).

Mean follow-up was 8.80 years for men, during which 140 events occurred. For women, 89 events occurred, with a mean follow-up of 9.35 years. In Cox analysis of incident MI, women showed no risk difference between genotypes ($P_{trend} = 0.89$). In men, the V allele was associated with decreased MI risk ($P_{trend} = 0.01$). IV and VV carriers had hazard ratios of 0.71[95% Confidence Interval (95% CI): 0.54, 0.93] and 0.51[95% CI: 0.39, 0.67], respectively, compared to the II reference group. Including HDL as a covariate did not appreciably alter this association (IV and VV hazard ratios of 0.72 [95% CI: 0.55, 0.95] and 0.52 [95% CI: 0.40, 0.68], respectively).

Table 1: Lipid levels and atherosclerosis by genotype and gender

		Genotype						P_{trend}
		II		IV		VV		
TC (mmol/L)	Male	6.23	6.17,6.31	6.30	6.23,6.37	6.19	6.04,6.34	0.36
	Female	6.79	6.73,6.85	6.79	6.73,6.86	6.83	6.70,6.97	0.84
HDL-C (mmol/L)	Male	1.16	1.14,1.18	1.20	1.18,1.21*	1.21	1.17,1.24*	0.01
	Female	1.40	1.38,1.41	1.41	1.39,1.43	1.47	1.43,1.51* **	<0.01
TC/HDL Ratio	Male	5.37	5.28,5.46	5.26	5.17,5.36	5.12	4.93,5.32*	0.09
	Female	4.85	4.78,4.93	4.82	4.74,4.90	4.64	4.49,4.79* **	0.04
Common Carotid IMT (mm)	Male	0.80	0.79,0.81	0.82	0.81,0.83	0.80	0.78,0.82	0.07
	Female	0.74	0.74,0.75	0.75	0.74,0.76	0.75	0.74,0.77	0.54
No. of Carotid Plaques ≥ 3 (%)	Male	28.0	24.9,31.1	27.4	24.3,30.5	27.7	21.0,34.3	0.96
	Female	17.3	15.2,19.4	16.1	13.9,18.3	17.2	12.8,21.7	0.72

TC, Total Cholesterol; HDL, High Density Lipoprotein Cholesterol; IMT, Intima Media Thickness.

* Significantly different from II ($P < 0.05$). ** Significantly different from IV ($P < 0.05$).

Values are geometric means and 95% CIs, except for plaques, which are % and 95% CI.

Model controlled for age, body mass index, alcohol consumption, smoking, lipid-lowering therapy, and diabetes.

DISCUSSION

The higher HDL levels in V carriers is consistent with previously noted decreases in CETP activity[6] and a meta-analysis that observed a 0.05 mmol/L difference in HDL between homozygous genotypes.[4] Here, similar results were observed, both in magnitude and direction.

CETP's role in metabolizing HDL while re-modeling LDL suggests that CETP promotes atherosclerosis, whereas CETP's reverse cholesterol transport function suggests an anti-atherogenic role.[1] In the Rotterdam cohort, the V allele was associated with lower MI risk in males, supporting the atherogenic view. The inclusion of HDL in the Cox models does not affect the results; this implies that the polymorphism's influence may be due to factors other than increasing HDL levels. Perturbing CETP's role in modeling LDL particles, for example, may give rise to the observed protective effect independent of incremental increases in HDL.

Although increased HDL was observed in both genders, the effect on MI risk was demonstrated only in men. Differences in sex steroid hormones are one possible explanation and a recent publication suggests that, independent of hormonal levels, the presence of a second X chromosome leads to differences in lipid profiles.[10]

In total, these findings provide circumstantial evidence for the atherogenicity of CETP. They also suggest that CETP inhibition might be a therapeutically beneficial target in the prevention of MI, although the data only support this inference for men. Given male's higher incidence of MI, this is a promising subject for further research.

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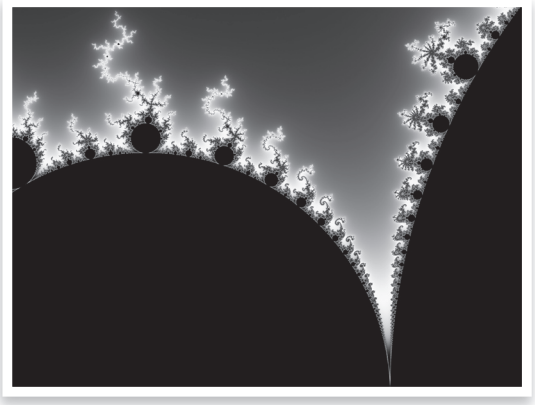
Chapter 2.2

Epistatic Effect of Cholesteryl Ester Transfer Protein and Hepatic Lipase on Serum HDL Levels: The Rotterdam Study



ABSTRACT

Objectives: Polymorphisms in the hepatic lipase (LIPC -514C>T) and cholesteryl ester transfer protein (CETP I405V) genes affect HDL levels but their relationship with cardiovascular disease, and their combined effect, is unclear. The objectives of the current study were to characterize the effect of the hepatic lipase variant, and it's interaction with the CETP variant, in terms of cholesterol levels, atherosclerosis, and risk of myocardial infarction (MI).

Design: The study was conducted in the Rotterdam Study, a large single-center prospective cohort study in people aged 55 years and older. Lipid levels were analyzed using linear regression models and risk of MI was assessed with Cox' proportional hazards models.

Results: The hepatic lipase variant was associated with an increase in serum HDL levels of 0.11 mmol/L in both genders, while an increased risk of MI was observed only in men (hazard ratio =1.32 [95% Confidence Interval (CI): 1.05 - 1.66] for CT versus CC and 1.75 [95% CI: 1.39 - 2.20] for TT versus CC). This effect was independent of serum HDL. LIPC –514C>T interacted with CETP I405V with respect to serum HDL levels. Those homozygous for both mutations saw a marked elevation in HDL (0.29 mmol/L, $P_{interaction} = 0.05$). These increased HDL levels, however, were not inversely associated with atherosclerosis or MI risk.

Conclusions: LIPC genotype affects HDL levels, and risk of MI in males. The interaction of this variant with CETP on HDL levels helps elucidate the underlying mechanisms and suggests that the beneficial effects of CETP inhibition may vary in particular subgroups.

Despite considerable progress in the prevention and treatment of myocardial infarction (MI) in recent decades, MI remains a leading cause of morbidity and mortality in the Western world, and, increasingly, in other regions. Low levels of circulating high-density lipoprotein cholesterol (HDL) are one of the well-known risk factors for MI. HDL lowers risk through a combination of anti-inflammatory and anti-oxidant properties[1] and is the most frequently perturbed lipid measurement in families with coronary heart disease.[2] Increasing the concentration of HDL is a promising approach to reduce cardiovascular risk.[3]

The genes encoding hepatic lipase (LIPC) and cholesteryl ester transfer protein (CETP) are consistently associated with circulating HDL. The T allele of the -514 C>T single nucleotide polymorphism (SNP) in the promoter region of the LIPC gene is associated with a substantial decrease in hepatic lipase activity and a modest, but significant, elevation of HDL levels.[4] The V allele of the CETP I405V polymorphism, which leads to an isoleucine to valine substitution in the 405th residue of the CETP protein, is strongly associated with decreased CETP activity and mass. Through this mechanism, the CETP SNP causes significant increases in HDL levels.[5]

Both hepatic lipase and CETP are involved in modeling and re-modeling HDL sub-species, particularly intermediate sized α-1 particles.[2] CETP-mediated TG enrichment of HDL notably increases the ability of hepatic lipase to model HDL.[6] As such, the potential interaction between LIPC and CETP variants on lipid levels is of interest. In interaction studies published to date, the joint effects of the LIPC –514C>T genotype with the CETP Taq1B and –1337C>T polymorphisms revealed no significant evidence for epistasis.[7-9]

Although the relationships between LIPC and CETP and HDL are well established, their roles in modulating cardiovascular disease risk are less clear. Previous findings on the effect of the LIPC –514 C>T polymorphism on MI risk were inconsistent. Two studies found no association,[10,11] although the latter study showed a borderline deleterious effect of the T allele. In two other reports, the T allele was variously associated with a protective effect on MI risk in coronary heart disease patients[12] and an increased risk of acute MI in males.[13] This second finding is counter-intuitive, as the T allele is associated with increased HDL levels.

Most previous studies examining the relationship between CETP variants and MI risk have focused on the intronic Taq1B variant. One of these found no effect on MI risk,[14] while others found effects in the anticipated direction (increased HDL and decreased MI risk with the CETP Taq1B B2 allele).[15,16] The role of the CETP I405V variant is similarly unclear. Several studies evaluated the impact of the CETP I405V SNP on MI risk. Of these, some found no effect[17,18] and another observed no differences in allele frequencies between children of early MI patients and controls.[19] The CETP I405V polymorphism was previously studied in the Rotterdam Study, and associated with an 0.06 mmol/L increase in HDL levels in both men and women, and a significant decrease in MI risk in males.[20]

Epistatic effects that predispose one to incidence of coronary disease, such as MI, are increasingly being explored.[21] Prior studies suggested a possible interaction between CETP and LIPC variants with respect to coronary artery disease markers (angiography and coronary ste-

nosis index).[8,9] To date, no research addressing this potential interaction in terms of modifying MI risk has been published and population-based data with respect to MI are also lacking. An interaction between these LIPC and CETP variants, which decrease protein activity and increase HDL cholesterol levels, should, in theory, decrease atherosclerosis and reduce the incidence of MI in our prospective cohort study if the function of the resulting HDL particles remains unchanged.

The present study was conducted with two aims. The first was to asses the impact of the LIPC –514 C>T polymorphism with respect to serum lipid levels, atherosclerosis proxies, and incidence of MI. The second objective was to determine whether there was evidence for an interaction between this polymorphism and the CETP I405V polymorphism.

MATERIALS AND METHODS

Study population

This study was embedded in the Rotterdam Study. Previous descriptions of this population have been published.[22] Briefly, the Rotterdam Study is a single-center prospective cohort comprised of 7,983 individuals aged 55 and older at the study's inception. Baseline examinations took place between 1990 and 1993. All participants completed written informed consents and the Medical Ethics Committee at Erasmus University approved the protocols for the ascertainment and examination of human subjects. At their baseline examination, these individuals completed interviews with a trained research assistant including details of alcohol consumption, smoking, hormone replacement therapy, lipid lowering therapy, and diabetes status, and underwent medical examinations at the research center. Serum lipid measurements of total cholesterol and HDL were determined enzymatically, using an automated procedure.[23] Common carotid intima media thickness was assessed by ultrasonography, as previously described.[24] Carotid plaque score was determined by the number of sites (common carotid, internal carotid, and bifurcation on both the left and right sides) that showed visible focal widening protruding into the luminal space, and scored from zero to six.

Follow-up data collection between baseline (1990 - 1993) and January 1, 2005 included data on the incidence of MI. Information on fatal and non-fatal MIs was obtained from general practitioners through a computerized reporting system. Two research physicians examined the patients' medical records and verified the events. When these physicians disagreed, a medical expert in the field determined the diagnosis. In the case of multiple events, the first event was used for this analysis; prevalent MIs were excluded.

Genotyping

Genomic DNA was extracted from whole blood samples drawn at the baseline examination, utilizing the salting out method. Subjects (n = 6,571) were genotyped for the both polymorphisms with a TaqMan allelic discrimination Assay-By-Design (Applied Biosystems, Foster City, CA). Forward and reverse primer sequences for the I405V SNP were 5′- CTCACCATGGGCATTTGATTGG-3′ and 5′- CGGTGATCATTGACTGCAGGAA-3′, respectively. The minor groove binding probes were 5′- CTCCGAGTCCGTCCAGA-VIC-3′ and 5′- TCCGAGTCCATCCAGA-FAM-3′. For the LIPC SNP, forward and reverse primers were 5′- TTTGCTTCTTCGTCAGCTCCTT – 3′ and 5′- GTCAAAGTGTGGTGCAGAAAACC-3′; probes were 5′- CTTCACCCCCATGTCAA-VIC-3′ and 5′- TTCACCCCCGTGTCAA- FAM -3′.

The assays utilized 5 nanograms of genomic DNA and 5 microliter reaction volumes. The amplification and extension protocol was as follows: an initial activation step of 10 min at 95ºC preceded 40 cycles of denaturation at 95ºC for 15 seconds and annealing and extension at 50ºC for 60 seconds. Allele-specific fluorescence was then analyzed on an ABI Prism 7900HT Sequence Detection System with SDS v 2.1 (Applied Biosystems, Foster City, CA).

Statistical analysis

Deviations from Hardy-Weinberg proportions were evaluated using an exact test.[25] General characteristics were compared using univariate analysis of variance (ANOVA) for continuous variables and χ^2 for dichotomous variables. After stratification by gender, serum lipid outcomes (total cholesterol, HDL, and total cholesterol /HDL ratio) and carotid IMT and plaques were examined with respect to the two polymorphisms. These analyses were conducted with a general linear model adjusting for age, smoking, alcohol consumption, body-mass index, lipid lowering therapy, and diabetes mellitus. As the residuals for the lipid measures and IMT were not normally distributed, these values were log transformed to improve their normality.

Cox Proportional Hazards models were implemented to assess the relationship between the polymorphisms and incidence of MI, using follow-up time on the independent axis. The data were stratified by gender and three models were fitted. The first was adjusted only for age. In the second model, the effect of the genotype was adjusted for classical MI risk factors including age, smoking, BMI, systolic and diastolic blood pressure, diabetes mellitus, and total serum cholesterol. In the third model, adjustments were made for these classical risk factors and, additionally, serum HDL levels to assess effects of the gene independent of HDL.

To test for interaction between the genes, all possible combinations of genotypes of the two SNPs were analyzed. Models with/without the combined effect were then tested for differences using a likelihood ratio test.

RESULTS

The CETP SNP was successfully typed in 6,421 individuals (97.7%) and the LIPC SNP in 6,239 subjects (94.9%). A total of 6,148 participants (93.6%) were genotyped for both polymorphisms. Genotype and allele distributions for both SNPs were in Hardy-Weinberg proportions ($P_{LIPC} = 0.13$ and $P_{CETP} = 0.42$).

As previously published, baseline characteristics did not differ between genotypes with respect to the CETP I405V polymorphism.[20] Similarly, no differences for these traits were observed by LIPC genotype, with the exception of systolic blood pressure. Systolic blood pressure was highest in individuals heterozygous for the T allele and lowest in TT carriers (Table 1).

The LIPC genotype was significantly associated with increased serum HDL levels (Table 2). The difference between homozygous groups was 0.11 mmol/L in both genders ($P < 0.001$), while heterozygotes saw more modest increases (0.04 mmol/L in males and 0.03 mmol/L in females). Subsequent adjustment for factors known to influence serum HDL levels did not change these findings. No statistically significant differences were noted for either genotype for total cholesterol (TC). As a consequence of the association to serum HDL, the serum TC/HDL ratio was decreased in LIPC-T carriers in both genders ($P_{male} < 0.05$ and $P_{female} = 0.01$). Despite these changes in HDL and TC/HDL ratio, no differences were observed for the common carotid IMT and plaque score (Table 2). As previously described, the CETP I405V SNP was associated with an increase of 0.06 mmol/L in serum HDL in both genders and a decrease in serum TC/HDL ratio of 0.18 in males and 0.24 in females.[20]

Serum HDL was strongly associated with a decreased risk of MI ($P < 0.001$ overall, $P = 0.001$ in males, and $P = 0.002$ in females). The LIPC T allele, however, which raised HDL levels, did not

Table 1: General characteristics of the study population by LIPC/CETP Genotype

	LIPC Genotype				CETP Genotype			
	CC (n = 3901)	CT (n = 2039)	TT (n = 299)	*P*-value	II (n = 2949)	IV (n = 2826)	VV (n = 646)	*P*-value
Male Gender (%)	1577 (40.4)	821 (40.3)	134 (44.8)	0.31	1182 (40.1)	1157 (40.9)	268 (41.5)	0.71
Age (Years)	69.37 (0.15)	69.72 (0.20)	68.85 (0.54)	0.19	69.45 (0.17)	69.61 (0.17)	69.21 (0.35)	0.56
BMI (kg/m^2)	26.26 (0.06)	26.34 (0.08)	26.16 (0.22)	0.62	26.33 (0.07)	26.20 (0.07)	26.36 (0.15)	0.37
SBP (mm Hg)	138.74 (0.36)	140.63 (0.50)	137.94 (1.34)	0.01	139.74 (0.42)	139.13 (0.43)	138.42 (0.89)	0.33
DBP (mm Hg)	73.67 (0.19)	73.89 (0.26)	73.11 (0.69)	0.52	73.92 (0.21)	73.68 (0.22)	73.11 (0.47)	0.27
Diabetes (%)	381 (9.8)	224 (11.0)	38 (12.8)	0.12	297 (10.1)	288 (10.2)	62 (9.6)	0.91
Prevalent MI (%)	465 (12.9)	257 (13.5)	35 (12.7)	0.80	364 (13.4)	342 (13.1)	78 (13.2)	0.96
LLT (%)	88 (3.1)	53 (3.6)	6 (2.8)	0.73	77 (3.7)	65 (3.1)	15 (3.2)	0.88
Current Smoker (%)	878 (22.5)	422 (20.7)	77 (25.8)	0.08	658 (22.3)	617 (21.8)	140 (21.7)	0.88
Alcohol (%)	2359 (78.9)	1252 (79.2)	193 (85.0)	0.09	1827 (80.0)	1709 (79.2)	390 (78.9)	0.77

Continuous traits: mean (SEM), ANOVA *P*-value. Dichotomous traits: n (%), χ^2 *P*-value.

BMI: Body Mass Index; SBP: Systolic Blood Pressure; DBP: Diastolic Blood Pressure; MI: Myocardial Infarction; LLT: Lipid-lowering Therapy

Table 2: Mean lipid levels and atherosclerosis measures by LIPC and CETP genotypes

		LIPC Genotype					CETP Genotype				
Outcome		CC	CT	TT	P_{crude}	$P_{adjusted}$	II	IV	VV	P_{crude}	$P_{adjusted}$
TC (mmol/L)	Male	6.28 (0.029)	6.33 (0.041)	6.42 (0.112)	0.282	0.140	6.28 (0.034)	6.34 (0.034)	6.29 (0.077)	0.497	0.280
	Female	6.80 (0.026)	6.86 (0.036)	6.77 (0.085)	0.283	0.259	6.83 (0.029)	6.79 (0.031)	6.83 (0.061)	0.588	0.712
HDL (mmol/L)	Male	1.19 (0.008)	1.23 (0.013)	1.30 (0.026)	< 0.001	< 0.001	1.19 (0.009)	1.23 (0.010)	1.25 (0.022)	0.005	0.002
	Female	1.42 (0.008)	1.45 (0.010)	1.53 (0.032)	< 0.001	< 0.001	1.43 (0.009)	1.43 (0.009)	1.49 (0.020)	0.007	0.007
TC/HDL Ratio	Male	5.55 (0.040)	5.46 (0.057)	5.22 (0.146)	0.047	0.056	5.55 (0.047)	5.46 (0.047)	5.37 (0.103)	0.159	0.030
	Female	5.10 (0.033)	5.02 (0.047)	4.72 (0.115)	0.010	0.001	5.11 (0.038)	5.05 (0.040)	4.87 (0.079)	0.035	0.018
Common Carotid IMT (mm)	Male	0.83 (0.005)	0.83 (0.007)	0.82 (0.021)	0.644	0.695	0.82 (0.006)	0.83 (0.006)	0.82 (0.012)	0.364	0.267
	Female	0.77 (0.004)	0.78 (0.005)	0.75 (0.014)	0.134	0.550	0.77 (0.004)	0.78 (0.004)	0.78 (0.011)	0.559	0.574
No. of Carotid Plaques ≥ 3 (%)	Male	336 (27.3)	204 (31.1)	34 (32.7)	0.153	0.200	271 (29.5)	259 (28.1)	61 (29.8)	0.768	0.884
	Female	358 (19.8)	168 (17.8)	18 (15.1)	0.234	0.092	273 (20.0)	241 (18.7)	61 (20.6)	0.615	0.871

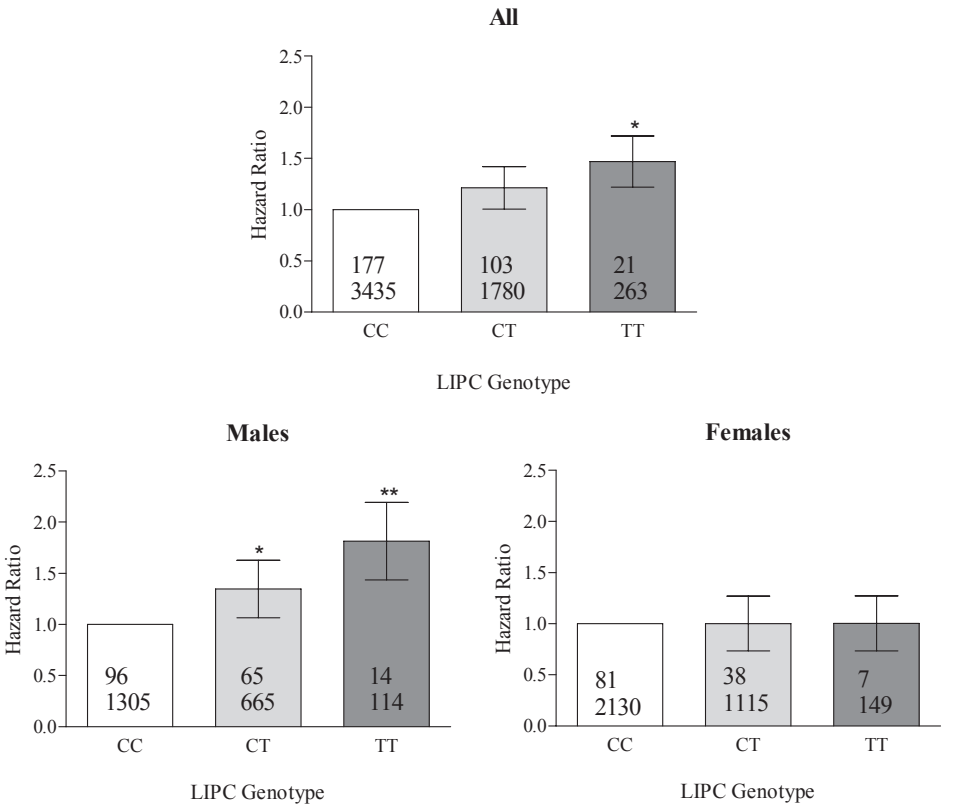
Values presented are mean (SEM), except for plaques (n (%)).

P_{crude} = analysis of variance (ANOVA) *P*-value (χ^2 for plaques).

$P_{adjusted}$ is from regression model containing age, body mass index, alcohol, smoking, lipid-lowering therapy, and diabetes. IMT and plaques are additionally adjusted for systolic and diastolic blood pressure.

TC: Total Cholesterol; HDL: High-Density Lipoprotein Cholesterol; IMT: Intima Media Thickness

Figure 1: MI hazard by LIPC genotype



Model adjusted for gender, age, body mass index, smoking, diabetes, systolic and diastolic blood pressure, total cholesterol and HDL.

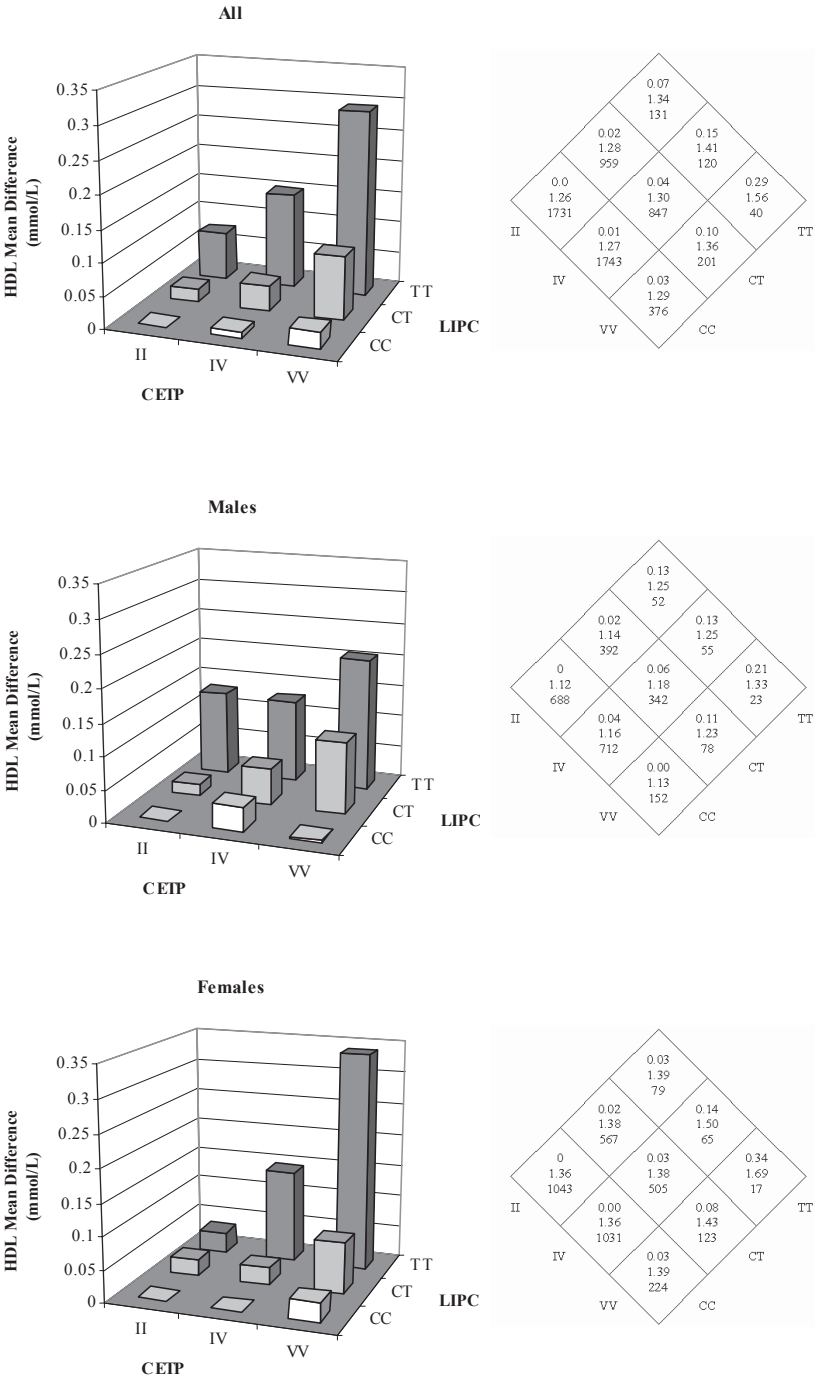
* Significantly different from CC ($P < 0.05$). ** Significantly different from CT ($P < 0.05$).

Numbers in each bar represent number of cases (n) and number of subjects (N).

result in an inverse association between number of T alleles and MI risk (Figure 1). In females, no association was found. In males, the T allele of the LIPC genotype was associated with an increase in MI hazard ($P_{trend} = 0.02$) despite the finding that this allele was associated with high serum HDL levels (Table 2). The hazard for CT carriers was 1.32 [95% Confidence Interval (CI): 1.05 - 1.66] and for TT carriers was 1.75 [95% CI: 1.39 - 2.20]. Inclusion of classical risk factors in the model did not alter these results ($HR_{CT} = 1.30$ [95% CI: 1.03 - 1.65] and $HR_{TT} = 1.70$ [95% CI: 1.35 - 2.15]. These hazards remained significantly increased after inclusion of HDL in the model ($HR_{CT} = 1.35$ [95% CI: 1.07 - 1.70] and $HR_{TT} = 1.82$ [95% CI: 1.44 - 2.30] ($P_{trend} = 0.01$) (Figure 1). As described earlier, the V allele of the CETP I405V polymorphism significantly decreased MI risk in males ($HR_{IV} = 0.75$ [95% CI: 0.59 - 0.95] and $HR_{VV} = 0.56$ [95% CI: 0.44 - 0.71]).

Analysis of the combined effects of the two genotypes suggests interaction at the serum HDL level (Figure 2). The mean difference in serum HDL between LIPC-TT/CETP-VV carriers, compared to the LIPC-CC/CETP-II genotype group, was 0.29 mmol/L. This value is considerably

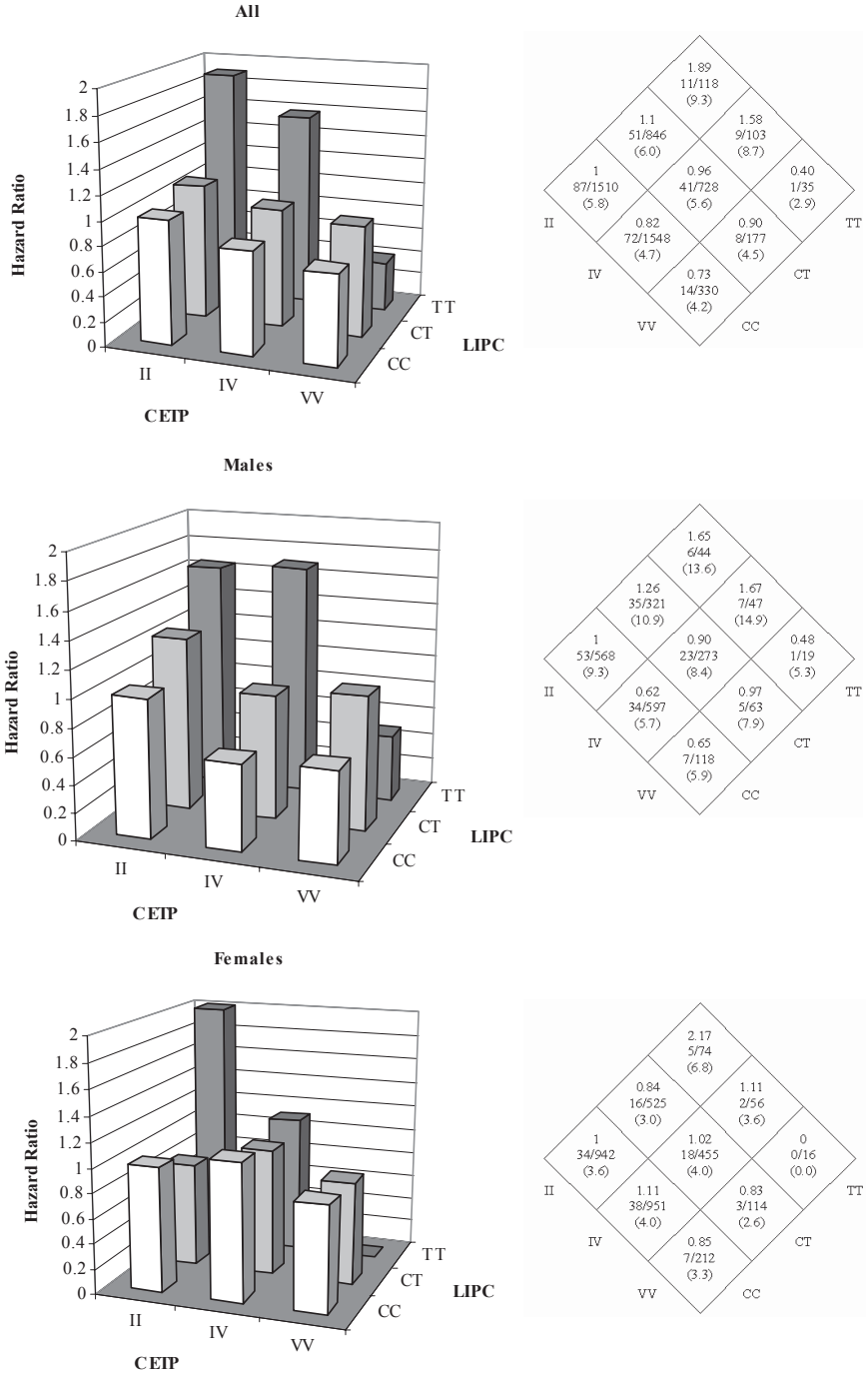
Figure 2: HDL mean differences by combined CETP/LIPC genotype



Model adjusted for gender, age, body mass index, alcohol, smoking, lipid-lowering therapy, and diabetes.

The numbers in the inset represent mean difference, mean and number of individuals for the corresponding bar.

Figure 3: MI hazard ratio by combined CETP/LIPC genotype



Model adjusted for gender, age, body mass index, smoking, diabetes, systolic and diastolic blood pressure, total cholesterol and HDL.

The numbers in the inset represent hazard ratio, number of cases/total number, (%) for the corresponding bar.

greater than expected from the sum of the mean differences in serum HDL due to the individual genes (CETP-VV versus CETP-II = 0.02 mmol/L and LIPC-TT versus LIPC-CC 0.06 mmol/L) ($P_{interaction}$ = 0.05). Intermediate combinations (i.e. heterozygous for one or both variants) also showed increases larger than expected for the two SNPs individually. These patterns were similar after stratification by gender (Figure 2), although the observed combined effect of the genes was stronger in women than in men (0.34 mmol/L, P = 0.03 and 0.21 mmol/L, P = 0.13, respectively). No significant differences were observed for IMT (data not shown).

The interaction observed for serum HDL levels did not translate into MI hazards (Figure 3). Although none of the hazards were significantly increased in either the overall analysis or the analysis stratified by gender, the patterns in MI hazard diverged from the patterns predicted from the effect of the two genes on serum HDL (Figure 2). The point-estimate for MI risk was highest in those who carried the combination of the CETP-II and LIPC-TT genotypes, while this genotype combination was associated with higher serum HDL levels compared to the CETP-II and LIPC-CC combination. When studying the combined effects of CETP and LIPC, the MI hazard decreased with the number of CETP V alleles, as predicted by the effect of this SNP on serum HDL levels. In contrast, the hazard of MI increased with the number of LIPC T alleles, although none of these hazard ratios were significantly increased, overall or in men. The only exception to these trends was in the CETP-VV/LIPC-TT group, which showed the lowest risk. This group is very small, however, making the risk estimate unreliable.

DISCUSSION

In this large, population-based cohort of elderly Dutch individuals, the LIPC –514C>T polymorphism was associated with increased HDL levels in both genders, and a significantly enhanced risk of MI in males. The increase in serum lipid levels attributable to the –514C>T SNP observed in this study, 0.11 mmol/L, is in line with results from a recent meta-analysis, which estimated a 0.09 [95% CI: 0.07 - 0.12] mmol/L increase in HDL levels.[4] The LIPC variant resulted in increased serum HDL while increasing MI risk in males.

When studying the interaction between the LIPC–514C>T polymorphism and the CETP I405V polymorphism, there was significant evidence for interaction between the two SNPs, leading to marked increases in serum HDL in those individuals homozygous for the minor allele of both variants. This large departure from additivity was statistically significant, and suggests epistasis between the two genes. A plausible mechanism for this interaction may result from the decreased catabolic efficacy of hepatic lipase with respect to triglyceride-poor HDL.[3] Decreases in CETP activity due to I405V genotype would lead to diminished CETP-mediated TG enrichment of HDL, and a subsequent reduction in hepatic lipase efficiency, in addition to the decrease in lipase activity due solely to –514C>T genotype.

This interaction may have consequences in terms of therapeutic CETP inhibition, which is currently the subject of intense interest.[26,27] Since the LIPC variant seems to modify the effects of the CETP variant, it is likely that the LIPC genotype may also be involved in the response to pharmaceutical CETP inhibition. Recently, however, the clinical trial of a CETP inhibitor, torcetrapib, ceased because the drug caused excess mortality.[28] Slight increases in systolic blood pressure were noted in early trials and may explain these adverse effects. Alternatively, HDL may become dysfunctional during CETP inhibition. At the least, these results suggest this topic warrants further study.

The interaction between CETP and LIPC, however, did not translate into the expected reduction in risk of MI. While studying the joint effect of the two genes, no significant associations with the hazard of MI could be observed in either the overall or gender specific analysis. The lack of association in the interaction model may be explained in large part by the lack of statistical power to study interactions,[29] even for a large study, such as the Rotterdam Study. Specifically, the number of dual rare homozygotes, and the number of events observed in this subgroup, was insufficient to analyze this interaction in terms of MI hazard.

Several hypotheses may help to explain the seemingly paradoxical associations between the effects of LIPC on circulating HDL and MI. One potential explanation revolves around the fact that different HDL sub-species may result in different risk levels[30] and that some subfractions may be atherogenic under some conditions.[31] Unfortunately, HDL subfractions were not determined in the Rotterdam Study. Another explanation for the apparently contradictory results is that the increase in MI risk by LIPC genotype is mediated by the non-lipolytic function of hepatic lipase, which may enhance atherosclerotic development. LIPC can reduce remnant and LDL cholesterol through this non-catalytic pathway. This reduction, demonstrated in mouse models,[32,33] implies that benefits derived from increased serum HDL may be outweighed by decreased clearance of ApoB containing particles. A third plausible explanation might relate to the effects of hepatic lipase in terms of the oxidation status of LDL particles, since oxidized LDL particles are thought to play an important role in atherogenesis.[34,35] A study of LIPC –514C>T in familial combined hyperlipidemia observed that the T allele led to increases in malondialdehyde-modified LDL, [36] a species of oxidized LDL that may be a useful biomarker for atherosclerosis.[37] Assuming that this process also occurs in non-affected individuals might explain the results obtained in the current study.

In this study, increased HDL was observed in both genders, but increased MI risk was demonstrated only in men. Previous studies on CETP and LIPC genetic variation also noted gender differences.[38,39] Differences in sex steroid hormone levels and activity are one possible explanation, although these women were post-menopausal. Additionally, women tend to experience MI later in life than men,[40] and are, therefore, less likely to become cases during the follow-up period; the noted differences might be due to this factor. Lastly, the possibility of a chance finding cannot be excluded.

The apparent discrepancy between the effect of LIPC genotype on HDL levels and risk of incidence of MI observed in men suggests that attempts to identify groups at high risk for MI based on genotypes associated with a favourable lipid profile may be hindered by unexpected associations and emphasizes the importance of studying clinically more relevant outcomes, such as MI. These efforts will be further complicated by the large number of, as yet, unidentified genes and interactions which are likely to affect both lipid levels and MI risk.

In summation, the T allele of the LIPC –514 C>T polymorphism was significantly associated with HDL levels in both genders, and an increased risk of MI in males. The interaction between this SNP and the CETP I405V variant was also examined. Those individuals homozygous for both mutations (LIPC-TT/CETP-VV) possessed markedly increased serum HDL levels, but this did not appear to affect MI risk in the present study.

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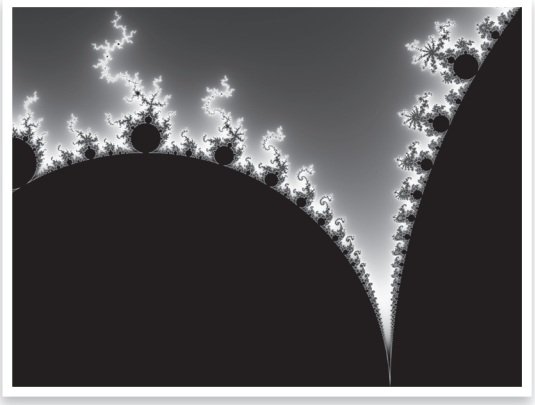
Chapter 2.3

The Involvement of Upstream Stimulatory Factor 1 in Dutch Patients with Familial Combined Hyperlipidemia



ABSTRACT

Recently, the upstream stimulatory factor 1 gene (*USF1*) was proposed as a candidate gene for familial combined hyperlipidemia (FCH). In this study, we examined the previously identified risk haplotype of *USF1* with respect to FCH and its related phenotypes in 36 Dutch FCH families. The diagnosis of FCH was based on both the traditional diagnostic criteria and a nomogram. The two polymorphisms, USF1s1 and USF1s2, were in complete linkage disequilibrium. No association was found for the individual single nucleotide polymorphisms (SNPs) with FCH defined by the nomogram (USF1s1, $P = 0.53$; USF1s2, $P = 0.53$), whereas suggestive associations were found when using the traditional diagnostic criteria for FCH (USF1s1, $P = 0.08$; USF1s2, $P = 0.07$). *USF1* was associated with total cholesterol (USF1s1, $P = 0.05$; USF1s2, $P = 0.04$) and apolipoprotein B (USF1s1, $P = 0.06$; USF1s2, $P = 0.04$). Small dense LDL showed a suggestive association (USF1s1, $P = 0.10$; USF1s2, $P = 0.09$). The results from the haplotype analyses supported the results obtained for the individual SNPs. In conclusion, the previously identified risk haplotype of *USF1* showed a suggestive association with FCH and contributed to the related lipid traits in our Dutch FCH families.

Familial combined hyperlipidemia (FCH; Online Mendelian Inheritance in Man 144250) is the most common genetic lipid disorder of unknown cause in humans, affecting up to 5% of the general population.[1] Major characteristics of FCH include increased levels of apolipoprotein B (apoB), triglycerides (TGs), and/or plasma total cholesterol (TC). Other FCH phenotypes are decreased levels of high density lipoprotein cholesterol and the presence of small, dense low density lipoprotein (sdLDL). In addition, patients with FCH have a 2-fold increased risk of cardiovascular disease (CVD) and are often obese and insulin-resistant.[2]

During the past few years, several groups have performed linkage analyses in an attempt to determine the genetic defects causing FCH.[3-8] By doing this, a locus on chromosome 1q21-23 was identified.[4,7-9] The first candidate gene proposed in this region was the thioredoxin-interacting protein gene (*TXNIP*).[10] It was demonstrated, however, that *TXNIP* was not a major contributor to the FCH phenotype.[11-13] More recently, a second candidate gene, the upstream stimulatory factor 1 gene (*USF1*), was suggested as the prime candidate gene for FCH in this linkage region in 60 Finnish FCH families.[12] Twenty-three single nucleotide polymorphisms (SNPs) were reported in the *USF1* gene. Two of these SNPs, USF1s1 (3' untranslated region; rs3737787) and USF1s2 (intron 7; rs2073658), individually and combined into a haplotype, showed linkage and association with FCH and multiple phenotypes of FCH, including TG, apoB, TC, and sdLDL, implying that *USF1* contributes to the complex pathophysiology of FCH in these Finnish FCH families.[12] The association of *USF1* with FCH, however, was strongest in males with increased levels of TGs.[12]

Huertas-Vazquez *et al.*[5] reported that *USF1* was associated with FCH and increased TG levels in 24 Mexican FCH families, whereas other phenotypes were not investigated. No sex-specific effect of *USF1* was found in these families. Coon *et al.*[14] investigated the association of *USF1* with FCH in Utah pedigrees ascertained for early death attributable to coronary heart disease (CHD), early stroke, or early-onset hypertension. They reported suggestive associations for FCH, TG, and low density lipoprotein cholesterol (LDL) levels in the pedigrees ascertained for early stroke and early onset of hypertension. However, in the families ascertained for early death attributable to CHD, in which 60% of the FCH patients were present, no association of *USF1* with FCH was found.[14]

The USF1 protein regulates the transcriptional activation of a variety of genes involved in glucose and lipid metabolism and in the development of atherosclerosis.[15,16] USF1 plays a pivotal role in adipose tissue, where it influences de novo lipogenesis by mediating the glucose-regulated expression of hormone-sensitive lipase (HSL), a key enzyme in the regulation of lipid storage in adipose tissue.[17] An interaction between SNPs in the *HSL* and *USF1* genes in postprandial TG levels has been reported previously.[18] Despite this, HSL activity in adipose tissue was unchanged in Finnish patients with FCH.[19] USF1 also influences the transcription of fatty acid synthase (FAS), which is involved in the synthesis of fatty acids.[15,20] FAS might play a role in FCH, as patients with FCH have decreased circulating levels of FAS ligand.[21] USF1 also plays a role in the transcription of several apolipoproteins (APOCIII, APOAII, and APOE),[16,22]

glucokinase,[23] hepatic lipase,[24] angiotensinogen,[25] and ABCA1.[26] Genetic variations in *APOCIII*, *APOAII*, and hepatic lipase have been associated with FCH;[27-30], the role of glucokinase, angiotensinogen, and *ABCA1* in FCH has not been explored yet.

In this study, we evaluated the putative role of the *USF1* gene in Dutch FCH families. We investigated the effect of two individual SNPs, and the haplotypes formed by these two SNPs, on FCH and its associated phenotypes, including not only lipids and lipoproteins but also parameters of obesity and insulin resistance.

MATERIALS AND METHODS

Study population

The FCH population was ascertained in 1994 and followed up and expanded in 1999. The families were ascertained through probands recruited from the outpatient lipid clinic of the Radboud University Nijmegen Medical Center. In 1994 and 1999, we ascertained families in which the proband exhibited a combined hyperlipidemia, with both plasma TC and TG levels above the age- and gender-related 90th percentile,[31] during several periods in which they were not treated with lipid-lowering drugs and despite dietary advice. Additionally, a first-degree relative had increased levels of TC and/or TG above the 90th percentile, and the proband, or a first-degree relative, suffered from premature (before the age of 60 years) CVD. Families were excluded when probands were diagnosed with underlying diseases causing hyperlipidemia (i.e., diabetes mellitus types 1 and 2, hypothyroidism, and hepatic or renal impairment), a first-degree relative had tendon xanthomata, or probands were homozygous for the *APOE2* allele.

In this study, we used the data of all participating subjects in 1999, including the spouses. The total population consists of 36 families from multiple (two to four) generations and comprises 611 subjects with known genealogic and phenotypic data. The diagnosis of FCH was based on *1*) the traditional diagnostic criteria, including TC and/or TG levels above the 90th percentile,[32] and *2*) the nomogram, as recently described by our group.[33] Plasma TG and TC levels, adjusted for age and gender, and absolute apoB levels were applied to the nomogram to calculate the probability of being affected with FCH. When this probability is >60%, the diagnostic phenotype is present in at least one first-degree relative, and premature CVD (before the age of 60 years) is present in at least one individual in the family, the individual is defined as affected by FCH. In 26 of the 36 FCH families, ascertained through the traditional criteria,[32] the proband also fulfilled the criterion of the nomogram for the FCH diagnosis; for the other 10 probands, the nomogram could not be applied because of missing apoB data. The normolipidemic relatives (n = 390), unaffected spouses of both the FCH patients and the normolipidemic relatives (n = 64), and subjects without known phenotypic and/or genotypic

data (n = 230) were included in the family-based analyses. After withdrawal of lipid-lowering medication for 4 weeks and an overnight fast, blood was drawn by venipuncture. The maximum waist circumference (cm) at the umbilical level was measured in the late exhalation phase while standing. All included subjects were Caucasian and older than 10 years. The ethical committee of the Radboud University Nijmegen Medical Center approved the study protocol, and the procedures followed were in accordance with institutional guidelines. All subjects gave informed consent.

Biochemical analyses

Plasma TC and TG were determined by commercially available enzymatic reagents (Boehringer-Mannheim, Mannheim, Germany, catalog No. 237574; and Sera Pak, Miles, Belgium, catalog No. 6639, respectively). Total plasma apoB concentrations were measured by immunonephelometry.[34] LDL subfractions were separated by single-spin density gradient ultracentrifugation, according to a previously described method.[35] A continuous variable, K, represents the LDL subfraction profile of each individual. A negative K value ($K \leq -0.1$) reflects a more dense LDL subfraction profile, and a positive K value ($K > -0.1$) reflects a more buoyant profile. Glucose concentrations were analyzed in duplicate using the oxidation technique (Beckman® Glucose Analyser2; Beckman Instruments, Inc.). Plasma insulin concentrations were ascertained using a double antibody method.[36] Insulin resistance was assessed by the homeostasis model assessment (HOMA) method.[37]

DNA was obtained from peripheral blood lymphocytes using a standard technique. Microsatellite markers, D1s104 and D1s1677, were amplified by PCR and analyzed on polyacrylamide gels, as described by Hughes.[38] D1s104 and D1s1677 were successfully genotyped in 611 and 545 individuals, respectively. The genotyping of D1s1677 failed in 38 FCH patients. For the *USF1* variants, PCRs were performed in a final volume of 50 µl at an annealing temperature of 53.7°C for both polymorphisms. Primer sequences for USF1s1 were 5′-GGTGTGTCCTTGAACTGAG-3′ (forward) and 5′-CAAGCCAGAGCATCACCTG-3′ (reverse), and those for USF1s2 were 5′-CTTTAGTAGAGACAGGGTTTCAC-3′ (forward) and 5′-GATTTAGCAGGTATTAGGAGCA-3′ (reverse). The mismatch (underlined) for USF1s2 was introduced to create a restriction site for BsiHKA I. The PCR products were digested with either 10 units of BstF5 I (USF1s1) or 10 units of BsiHKA I (USF1s2) at 65°C (New England Biolabs); subsequently, the resulting fragments (242, 172, and 70 bp in heterozygotes for USF1s1 and 154, 136, and 18 bp in heterozygotes for USF1s2) were separated on agarose gels. USF1s1 and USF1s2 were genotyped in all 611 individuals, including 157 FCH patients. The genotyping of USF1s2, however, failed for six individuals, including three FCH patients.

Quantitative real-time PCR analysis of USF1 expression in peripheral blood mononuclear cells

USF1 mRNA expression levels were quantified in peripheral blood mononuclear cells (PBMCs) of 30 FCH patients and 30 sex-matched normolipidemic relatives randomly selected from our study population, as described previously.[39] RNA was isolated from PBMCs, including both lymphocytes and monocytes, and reversed-transcribed to cDNA. Quantitative real-time PCR was carried out in a total of 25 µl containing 2 µl of cDNA, 0.25x SYBR® green solution (Invitrogen), 1x fluorescein (Bio-Rad), 2 mM $MgCl_2$, 50 ng of forward (5′-ATGACCCAGGCGGTGATCCA-3′) and reverse (5′-GACGCTCCACTTCATTATGC-3′) primers, 100 µM deoxynucleoside triphosphates, 1x Ampli*Taq* Gold amplification buffer, and 1.5 unit of Ampli*Taq* Gold DNA polymerase (Applied Biosystems). PCR conditions were as follows: a hot start at 95°C for 10 min, followed by 40 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. Samples were run in duplicate on the Icycler iQ real-time PCR detection system (Bio-Rad) to determine the threshold cycle.[40] Expression levels were normalized to beta-2-microglobulin by comparative quantification using the $\Delta\Delta C_t$ method.[41]

Statistical analyses

The characteristics of the study population are expressed as means ± SD. Before further statistical analyses, extended Mendelian error-checking was performed for 36 families, including 219 nuclear families (n = 611), with PedCheck.[42] Data were available for only 25 of the 36 probands, because 11 probands were already recruited in 1994 and did not participate in 1999. For families with Mendelian inconsistencies, which can be attributable to paternity problems and/or misgenotyping, problematic genotypes were set to missing for the complete nuclear families or the isolated problematic individuals [n = 10 (1.6%)]. The parental data for each polymorphism were tested for Hardy-Weinberg equilibrium by use of an exact test. Variables with skewed distribution, including TGs and the HOMA index, were logarithmically transformed. Multipoint parametric linkage analysis of the two microsatellite markers on chromosome 1 and the two *USF1* polymorphisms was performed for three traits, the FCH trait, defined by both the traditional diagnostic criteria and the nomogram, and the TG trait, defined by TG levels above the 90th percentile,[31] using SIMWALK2.[43] Data were prepared using MEGA2.[44] We assumed a disease allele frequency of 0.01 and 0.10 under the dominant and recessive models of inheritance, respectively. The assumed penetrance in the dominant model was 0.99. In the recessive model, the assumed penetrance values with risk and nonrisk genotypes were 0.99 and 0.50, respectively. A phenocopy rate of 0.01 was assumed. These conditions are comparable to those used by Pajukanta *et al.*[7] Two-point nonparametric linkage analyses of the two microsatellite markers and the *USF1* polymorphisms were carried out using SOLAR 2.1.4 software.[45] The presence of nonparametric linkage was tested for FCH and the related quanti-

tative traits, including apoB, TC, and TG levels, and the presence of sdLDL, represented by the K value. The HAPLOVIEW program[46] was used to calculate allele and haplotype frequencies and to calculate the linkage disequilibrium between the two SNPs. HAPLOVIEW was also used to calculate transmission disequilibrium association with permutation analysis to correct for multiple testing.

Associations between the individual polymorphisms or haplotypes and FCH and its related phenotypes in our extended families were determined using two programs: the family-based association test (FBAT)[47] and the pedigree-based association test (PBAT)[48] software. An additive model of inheritance was used, as the mode of inheritance of FCH is unknown and this model is particularly robust.[47] FBAT handles pedigrees by breaking the pedigrees into all possible nuclear families and evaluating their contributions to the test statistics. According to FBAT, our 36 FCH families included 219 nuclear families. The –e option of FBAT, which computes the test statistics using an empirical variance estimator,[49] was implemented, because the nuclear families were not independent. The –p option, which performs the Monte Carlo permutation procedure, was used to correct for multiple testing. As Pajukanta *et al.*[12] reported a sex-specific effect for USF1, adjustments were made for age and gender by calculating the unstandardized residuals. No adjustment for age and gender was made for FCH, as the FCH diagnosis is already corrected for age and gender. For the haplotype analysis, the haplotype-based association test (HBAT) command of the FBAT program was used, using the –e and –p options.

PBAT is capable of handling extended pedigrees without breaking them into nuclear families. Despite this, our extended pedigrees had to be divided into smaller units, because of computational limitations. This resulted in a total of 92 pedigrees, with a maximum of 14 nonfounders per pedigree. Age and gender were used as covariates in the models. All tested variables, except for the dichotomous variables, were transformed to a Z score, as recommended by PBAT.[48]

The proportions of trait variations for TC, apoB, and sdLDL explicable by *USF1* polymorphisms were tested by means of generalized estimating equations because of possibly correlated values within families.

Differences were considered statistically significant at $P \leq 0.05$. These analyses were conducted using SPSS 12.0.1, PedCheck 1.1, MEGA2, SIMWALK2, SOLAR 2.1.4, HAPLOVIEW 3.2, FBAT 3.2, and PBAT 3.0.

RESULTS

Subject characteristics

According to both the traditional diagnostic criteria and the nomogram, 135 subjects were affected with FCH. In addition, 38 patients were affected with FCH according to either the traditional diagnostic criteria (n = 16) or the nomogram (n = 22) as the diagnostic criterion. Therefore, in total, 157 subjects were diagnosed with FCH according to the nomogram. Descriptive statistics of anthropometric and metabolic characteristics of the study population, presented below and in Table 1, were based on the nomogram as the diagnostic criterion for FCH.

FCH patients were older than normolipidemic relatives but younger than spouses. The higher prevalence of CVD in patients with FCH was evident, compared with normolipidemic relatives and spouses. The mean waist circumference of patients with FCH was significantly higher compared with normolipidemic relatives. By definition, FCH patients were characterized by increased plasma TC, TG, and apoB levels. Furthermore, FCH patients exhibited higher levels of sdLDL, as reflected by a lower K value. Finally, FCH patients were more insulin-resistant, as reflected by a higher HOMA index. In the total population, 126 subjects showed the TG trait, as defined by TG levels > 90th percentile, including 52 men. In Table 1, the distribution of the TG trait among FCH family members is presented.

Table 1: Characteristics of patients with familial combined hyperlipidemia, normolipidemic relatives and spouses

Characteristics	FCH patients (n = 157)	NL relatives (n = 390)	Spouses (n = 64)
Allele frequency (Wt) (USF1s1/USF1s2)	0.75 / 0.75	0.69 / 0.69	0.72 / 0.72
Gender (males)	75 (47.8%)	177 (45.4%)	31 (48.4%)
Age (years)	47.0 (15.6) *†	37.9 (15.7)‡	56.0 (10.5)
CVD	32 (20.4%) *†	20 (5.1%)	4 (6.3%)
Waist (cm)	87.2 (12.2) *	77.7 (10.9)‡	84.3 (12.5)
TC (mmol/L)	6.5 (1.1) *†	4.9 (0.9)‡	5.2 (0.8)
TG (mmol/L)	3.1 (1.5) *†	1.2 (0.5)	1.3 (0.6)
Apo B (mg/L)	1370 (264) *†	960 (220)	996 (175)
LDL (mmol/L)	4.1 (1.2) *†	3.2 (0.9) ‡	3.4 (0.7)
K-value	-0.26 (0.26) *†	0.05 (0.19)	0.05 (0.25)
HOMA	3.4 (1.8) *†	2.3 (1.3)‡	2.7 (1.3)

apoB, apolipoprotein B; CVD, cardiovascular disease; FCH, familial combined hyperlipidemia; HOMA, homeostasis model assessment; LDL, low density lipoprotein cholesterol; TC, total cholesterol; TG, triglyceride; USF1, upstream stimulatory factor 1.

FCH diagnosis was based on the nomogram. Bivariate variables are presented as number (%); continuous variables are presented as mean (SD). Allele frequencies are given for the wild-type alleles of USF1s1 and USF1s2; for K value, a value <–0.1 represents the presence of small, dense LDLs.

* $P < 0.05$, compared to NL relatives; † $P < 0.05$, compared to spouses; ‡ $P < 0.05$, compared to spouses.

For both USF1s1 and USF1s2, the wild-type allele (C and A, respectively) had a frequency of 0.81 in probands. The wild-type allele frequency of 0.72 in spouses was comparable to the frequency found in probands ($P = 0.30$). The distributions of the parental alleles and genotypes for both polymorphisms were in Hardy-Weinberg proportions. Analysis performed with HAPLOVIEW showed that the two polymorphisms were in complete linkage disequilibrium ($D' = 1.0$, $R^2 = 1.0$), resulting in the presence of only two haplotypes in our study population. The C-A haplotype had an overall frequency of 71% (n = 853), and the T-G haplotype frequency was 29% (n = 357).

In total, 302 individuals (85 FCH patients, 184 normolipidemic relatives, and 33 spouses) carried two common alleles for both SNPs. Two hundred fifty subjects (59 FCH patients, 165 normolipidemic relatives, and 26 spouses) were heterozygous for both SNPs, and 53 subjects (9 FCH patients, 39 normolipidemic relatives, and 5 spouses) carried two rare alleles for both SNPs.

With a transmission disequilibrium association test, we observed no overtransmission of one of the alleles (USF1s1, $P = 0.62$; USF1s2, $P = 0.62$) or haplotypes ($P = 0.32$) to the FCH patients. Correction for multiple testing by permutation analysis did not alter these results.

Linkage analyses

Parametric and nonparametric linkage analyses for the 1q21-23 region were performed using the two SNPs in the *USF1* gene and two microsatellite markers on chromosome 1, D1s104 and D1s1677, previously identified as the markers underlying the linkage signal on 1q21-23 in the Finnish population. Two-point nonparametric linkage analyses of these two markers and the two SNPs in the *USF1* gene did not show evidence of linkage with the FCH trait [log of the odds (lod) scores = 0.0 for the nomogram and 0.0–0.3 for the traditional diagnostic criteria], the TG trait (lod score = 0.0–0.2), or any of the related quantitative traits, including apoB, TC, and LDL levels and the presence of sdLDL (all lod scores < 0.5). As parametric linkage analyses can be more powerful than nonparametric linkage analyses in our extended families, we also performed parametric linkage analyses, which also did not show any evidence of linkage with the FCH trait (lod score = 0.0 for the nomogram and 0.6 for the traditional diagnostic criteria) and the TG trait, defined by TG levels above the 90th percentile (lod score = 0.5).

Association and haplotype analyses

In Table 2, the associations of the polymorphisms of the *USF1* gene, individually and combined into haplotypes, with FCH and its related phenotypes in the total population are presented. No statistical association of the *USF1* gene with FCH, defined by the nomogram, was found. Applying the traditional diagnostic criteria of FCH revealed a suggestive association of the *USF1* gene with FCH. No association of the *USF1* gene with the TG trait was found. How-

Table 2: Association of the individual polymorphisms and haplotypes of the *USF1* gene with FCH and its related phenotypes after correction for age and gender in the total population

	FBAT -e		FBAT -p		HBAT -e	HBAT -p	PBAT -e
	USF1s1	USF1s2	USF1s1	USF1s2	Overall Haplotype	Overall Haplotype	Risk Haplotype
FCH	0.45	0.52	0.53	0.53	0.52	0.55	0.95
TC	0.08	0.06	0.05	0.04	0.06	0.05	0.11
TG	0.31	0.27	0.39	0.33	0.27	0.38	0.61
Apo B	0.05	0.05	0.06	0.04	0.04	0.05	0.12
LDL	0.31	0.24	0.24	0.20	0.27	0.22	0.27
K-value	0.09	0.09	0.10	0.09	0.09	0.11	0.46
Waist	0.66	0.71	0.68	0.69	0.65	0.67	0.55
HOMA	0.43	0.57	0.53	0.69	0.60	0.73	0.43

FBAT, family-based association test; HBAT, haplotype-based association test; PBAT, pedigree-based association test.

Presented are the obtained *P* values for the individual single nucleotide polymorphisms and haplotypes in the total population.

For K value, a value < –0.1 represents the presence of small dense LDLs. –e indicates correction for the nonindependence of the nuclear families; –p indicates correction for multiple testing by permutation tests.

ever, after correction for age and gender, suggestive associations for the wild-type haplotype, previously identified as the risk haplotype by Pajukanta *et al.*,[12] were obtained for TC, apoB, and sdLDL when the structures of the pedigrees were taken into account (Table 2). Correcting for multiple testing, with the –p option, resulted in an association of USF1s1 and USF1s2 with TC ($P = 0.05$ and $P = 0.04$, respectively) and apoB ($P = 0.06$ and $P = 0.04$, respectively). sdLDL showed suggestive associations with USF1s1 ($P = 0.10$) and USF1s2 ($P = 0.09$). The *USF1* gene, however, could only explain a small proportion of the variation in plasma TC (1.7%), apoB (1.5%), and sdLDL (0.5%) levels. LDL and the obesity- and insulin resistance-related phenotypes of FCH were not associated with *USF1* (Table 2).

PBAT was also used to analyze the association between the haplotypes of the *USF1* gene and FCH and its associated phenotypes. PBAT does not implement an overall haplotype test but generates a *P* value for each haplotype. As shown in Table 2, the risk haplotype was not associated with FCH when the nomogram was used as the diagnostic criterion, but a suggestive association was found when using the traditional diagnostic criteria for FCH. The analysis of the association between *USF1* with other characteristics of FCH did not reveal any statistically significant association. The associations found for TC and apoB, however, pointed in the same direction as the associations found with the FBAT and HBAT statistics (Table 2).

In view of the fact that Pajukanta *et al.*[12] demonstrated a gender-specific effect of the *USF1* gene on FCH and TG levels in males, analyses of both the individual SNPs and the haplotypes of *USF1* were also performed in males only (Table 3). The male population (n = 283) included 65 FCH patients based on the traditional diagnostic criteria and 75 FCH patients based on the nomogram. In total, 52 men (18.5%) had TG levels > 90th percentile. No significant association of the *USF1* gene with the FCH or the TG trait was found, and only suggestive evidence for an

Table 3: Association of the individual polymorphisms and haplotypes of the *USF1* gene with FCH and its related phenotypes after correction for age and gender in males

Characteristics	FBAT -e		FBAT -p		HBAT -e Overall Haplotype	HBAT -p Overall Haplotype	PBAT -e Risk Haplotype
	USF1s1	USF1s2	USF1s1	USF1s2			
FCH	0.45	0.52	0.48	0.56	0.52	0.55	0.70
TC	0.33	0.30	0.32	0.29	0.30	0.34	0.11
TG	0.53	0.44	0.59	0.54	0.44	0.52	0.75
Apo B	0.12	0.10	0.13	0.12	0.10	0.13	0.05
LDL	0.63	0.64	0.57	0.62	0.64	0.61	0.26
K-value	0.15	0.12	0.23	0.19	0.12	0.22	0.65
Waist	0.11	0.10	0.06	0.06	0.10	0.06	0.15
HOMA	0.49	0.47	0.64	0.58	0.47	0.59	0.59

Presented are the obtained *P* values for the individual single nucleotide polymorphisms and haplotypes in males only.

For K value, a value <–0.1 represents the presence of small dense LDLs. –e, correction for the nonindependence of the nuclear families; –p, correction for multiple testing by permutation tests.

association in males was present for apoB levels and sdLDL, in accordance with the analysis in the complete families. Therefore, no apparent gender-specific effect was present for these parameters (Table 3). For the obesity parameter (waist circumference), we found suggestive evidence for an association with *USF1* in males only (Table 3).

Expression analyses of USF1 in PBMCs

To further explore the role of USF1 in FCH, expression levels of *USF1* in PBMCs were measured in 30 FCH patients (defined by the nomogram) and 30 controls. Anthropometric and metabolic characteristics of these subjects are presented in supplementary Table 1. The FCH

Supplementary table 1: Association of the individual polymorphisms and haplotypes of the *USF1* gene with two traits including (1) familial combined hyperlipidemia based on the traditional diagnostic criteria (TC and/or TG levels above the 90th percentile) and (2) TG levels above the 90th percentile

	FBAT -e		FBAT -p		HBAT -e Overall Haplotype	HBAT -p Overall Haplotype	PBAT -e Risk Haplotype
	USF1s1	USF1s2	USF1s1	USF1s2			
Total Population							
FCH	0.071	0.057	0.084	0.069	0.057	0.057	0.503
TG > 90th	0.658	0.658	0.671	0.694	0.658	0.690	0.956
Males only							
FCH	0.411	0.305	0.473	0.293	0.305	0.315	0.831
TG > 90th	0.976	0.976	0.938	0.936	0.976	0.943	0.997

For K value, a value <–0.1 represents the presence of small dense LDLs. –e, correction for the nonindependence of the nuclear families; –p, correction for multiple testing by permutation tests.

patients demonstrated a relative expression of 0.9 (0.5–2.2) for the *USF1* gene, compared with the control value of 1.0 (0.3–4.0), which was not significantly different ($P = 0.27$). Of the 30 FCH patients, 14 carried the risk haplotype, 12 were heterozygous for the USF1 mutations, and 4 were homozygous for the rare haplotype. In the control group, 12 subjects carried the risk haplotype, 15 were heterozygous, and 3 were homozygous for the rare haplotype. As the FCH patients carrying the *USF1* risk haplotype had a relative expression of 1.3 (0.5–1.9) and the FCH patients carrying the rare haplotype had a relative expression of 1.0 (0.6–1.6), no differences based on the haplotype were observed within the FCH patients. The results in the controls were comparable.

DISCUSSION

In this study, we investigated the putative role of *USF1* in Dutch FCH families. We show that the wild-type haplotype of *USF1*, previously identified as a risk haplotype,[12] was not associated with FCH when using the nomogram to diagnose FCH, which is based on plasma levels of TC, TG, and apoB; however, a suggestive association of *USF1* with FCH in our Dutch families was found when using the traditional lipid criteria to diagnose FCH. Furthermore, we report no association of *USF1* with the TG trait. The risk haplotype of *USF1*, however, was associated with other important phenotypes of FCH, including plasma TC levels, apoB levels, and the presence of sdLDL, accounting for ~1% of the variation in these phenotypes. We conclude that *USF1* shows a suggestive association with FCH and can be considered a modifier gene, contributing to related lipid traits in our Dutch FCH families.

Several groups have tried to replicate the results with regard to *USF1* found in the Finnish FCH families[12] (see supplementary Table 2). Huertas-Vazquez *et al.*[5] reported an association of *USF1* with FCH and TG levels in a Mexican population, although their results were less conclusive and no gender-specific effect was found. Coon *et al.*[14] also reported an association of *USF1* with FCH in Utah pedigrees ascertained for early death attributable to CHD, early stroke, or early-onset hypertension. When restricting the analyses to the families ascertained for early death attributable to CHD, in which 60% of the FCH patients were present, no evidence for the involvement of *USF1* in FCH, TG, or LDL levels was found, although the strongest association would be expected in these families. This lack of association in the families ascertained for early death attributable to CHD could be the result of limited power, as only a few families were ascertained for CHD (n = 17).[14] Another explanation could be the use of different diagnostic criteria for FCH in the different populations. To diagnose FCH, Pajukanta *et al.*[12] and Huertas-Vazquez *et al.*[5] used the traditional criteria, based on increased plasma TC and/or TG levels above the 90th percentile, adjusted for age and gender, whereas Coon *et al.*[14] defined FCH based on LDL and/or TG levels above the 90th percentile, adjusted for age and gender. In this study, we applied two diagnostic criteria for FCH: the recently described nomogram[33] and

Supplementary table 2: Summary of the associations of the *USF1* gene previously reported

			Total population			Males only		
			HHRR	GAMETE	FBAT	HHRR	GAMETE	FBAT
FCH	USF1s1	Pajukanta et al.	NS	~	~	0.04	0.05	~
		Huertas-Vazquez et al.	0.05	0.01	0.02	~	~	~
		Coon et al. (total group)	~	~	0.02	~	~	NS
		Coon et al. (CHD only)	~	~	NS	~	~	NS
	USF1s2	Pajukanta et al.	NS	~	~	0.04	NS	~
		Huertas-Vazquez et al.	NS	0.05	NS	~	~	~
		Coon et al. (total group)	~	~	NS	~	~	NS
		Coon et al. (CHD only)	~	~	NS	~	~	NS
	Haplotype	Pajukanta et al.	NS	0.00004*	0.02	0.009	0.0004*	~
		Huertas-Vazquez et al.	~	~	0.03	~	~	~
		Coon et al. (total group)	~	~	NS	~	~	NS
		Coon et al. (CHD only)	~	~	NS	~	~	NS
TG	Haplotype	Pajukanta et al.	0.05	0.00006*	~	0.00003	0.00001	~
		Huertas-Vazquez et al.	~	~	0.006	~	~	~
		Coon et al. (total group)	~	~	NS	~	~	0.01
		Coon et al. (CHD only)	~	~	NS	~	~	NS
TC	Haplotype	Pajukanta et al.	~	0.0001	~	~	0.007	~
apoB	Haplotype	Pajukanta et al.	~	0.00003	~	~	0.0007	~
sdLDL	Haplotype	Pajukanta et al.	~	0.002	~	~	0.01	~
LDL	Haplotype	Coon et al. (total group)	~	~	NS	~	~	0.01
		Coon et al. (CHD only)	~	~	NS	~	~	NS

the traditional FCH criteria.[32] We show that different diagnostic criteria affect the results of the association analysis. As shown in Table 2, a suggestive *P* value for the association of *USF1* with FCH was found when using the traditional FCH criteria, whereas no association was found when using the nomogram.

In sharp contrast to the results obtained by Pajukanta *et al.*,[12] who reported the strongest association of *USF1* in males with high TG levels, we report here no gender-specific effect and no association of *USF1* with high TG levels. The absence of this association in our population might be explained by the relatively mildly increased levels of TG in our study population compared with the Finnish population (2.8 vs. ~3.3 mmol/l, respectively).[50,51] FCH remains a multifactorial disease, with extensive genetic heterogeneity, in which the combination of many genes and the environment determine the expression of the phenotype, contributing to the apparently conflicting results in different FCH populations. Our results, however, are supported by a study in Mexican FCH families,[5] which also found no gender-specific effect.

Although we did not find an association between *USF1* and TG levels in this study, we were able to replicate some associations for other important phenotypes of FCH, including TC, apoB, and sdLDL. A study performed in 196 healthy obese Scandinavian women[52] and one

involving diabetes mellitus type 2 patients,[53] however, failed to demonstrate an association between *USF1* and lipid/lipoprotein levels. This suggests that the influence of *USF1*, as a modifier gene, on these lipid levels is specific for patients with FCH.

Recently, a close relationship between *USF1* and catecholamine-stimulated lipolysis in human fat cells was found,[52] and based on this, it was suggested that the adipocyte lipolysis-related effects of genetic variation in *USF1* can have clinical consequences against certain backgrounds such as FCH. In healthy young males, it was demonstrated that *USF1* is associated with glucose levels and has a modulating effect on the correlation between body mass index and TC levels.[18] In this study, however, we did not find an association between *USF1* and glucose, insulin levels, or obesity parameters. In males, however, a suggestive association was found between *USF1* and the obesity parameter, waist circumference.

In the search for candidate genes underlying complex genetic disorders, haplotype analyses have become a valuable tool. The most convincing results for the association of *USF1* with FCH, from Pajukanta *et al.*[12] and Huertas-Vazquez *et al.*,[5] were obtained when analyzing the *USF1* SNPs as haplotypes (see supplementary Table 2). In our study, however, the two SNPs resulted in only two haplotypes, so no increased power was obtained from haplotype analyses. The complete linkage disequilibrium between these two SNPs has been reported previously in 196 healthy obese women.[52] In this study, we used two programs for haplotype analyses, FBAT and PBAT. In FBAT, the presence of linkage is not mandatory for analyzing haplotypes. FBAT, however, splits up the pedigrees into nuclear families; thereby, important information about the family structure is lost. The second program, PBAT, does not require the presence of linkage and is said to be capable of handling extended pedigrees. PBAT, however, could not handle our extended pedigrees, necessitating their division into smaller units. Haplotype analyses in extended pedigrees, therefore, remain a challenge.

For the association analyses of *USF1* with FCH, Pajukanta *et al.*[12] and Huertas-Vazquez *et al.*[5] used several programs (see supplementary Table 2). In the haplotype-based relative risk and the genotype pedigree disequilibrium test programs, linkage is assumed to be present in the region under investigation, making these programs unsuitable in our situation of no linkage. The association of *USF1* with FCH in Mexican families was found using the gamete competition test program, which analyzes the presence of both linkage and association, so the significant result can represent the presence of linkage only, as reported for this region in this population.[5] Pajukanta *et al.*[12] resolved this problem in the Finnish FCH families by gene dropping.

In this study, we genotyped two of the SNPs present in the *USF1* gene. By genotyping these two SNPs, we did not capture the complete genetic variation in this region. It is possible, therefore, that in our FCH Dutch population, another haplotype is the major risk haplotype. This is unlikely, however, because not only in the Finnish families but also in the Mexican and American families, this particular haplotype was identified as the risk haplotype. Furthermore, associations of this haplotype with underlying phenotypes of FCH were present in these

Dutch FCH families. The identification of *USF1* as a modifier gene in our population, however, may be of great importance, as genome-wide linkage analyses in our population revealed no loci with lod scores reaching the level of suggestive linkage (data not shown), making it unlikely to find major genes for FCH.

Although *USF1* was thought to be a good candidate gene for FCH, our results do not support the role of *USF1* as a major gene in our population. This is reinforced by the lack of functionality of the genetic variations in *USF1*, as the two polymorphisms do not result in amino acid changes. The identification of a site for DNA binding proteins in the region of USF1s2 has been reported; however, no evidence was found for the presence of the suggested internal promoter causing the transcription of truncated mRNA.[54] Pajukanta and colleagues[54] reported differential expression of three of the USF1-regulated genes in adipose tissue based on the USF1s2 allele. The expression of *USF1* itself, however, was not different in adipose tissue. In this study, we have measured the expression of *USF1* in PBMCs and, consistent with the results of Pajukanta *et al.*,[12] we did not find any haplotype-dependent differences. Our decision to use PBMCs, in the absence of adipose tissue samples from our study population, was justified by a study by Morello *et al.*,[55] showing that the FCH-specific transcription profile was detectable in peripheral blood cells.

In conclusion, in our Dutch FCH patients, *USF1* shows a suggestive association with FCH and can be considered a modifier gene, contributing to related lipid traits in our Dutch FCH families.

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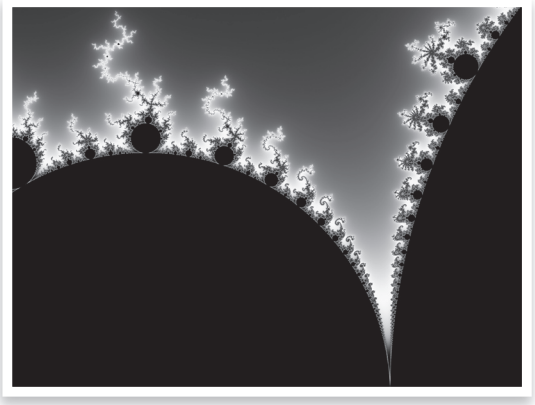
Chapter 2.4

Haplotype Analyses of the *APOA5* Gene in Patients with Familial Combined Hyperlipidemia



ABSTRACT

Background: Familial combined hyperlipidemia (FCH) is the most common genetic lipid disorder with an undefined genetic etiology. Apolipoprotein A5 gene (*APOA5*) variants were previously shown to contribute to FCH. The aim of the present study was to evaluate the association of *APOA5* variants with FCH and its related phenotypes in Dutch FCH patients. Furthermore, the effects of variants in the *APOA5* gene on carotid intima-media thickness (IMT) and cardiovascular disease (CVD) were examined.

Materials and methods: The study population consisted of 36 Dutch families, including 157 FCH patients. Two polymorphisms in the *APOA5* gene (– 1131T > C and S19W) were genotyped.

Results: Haplotype analysis of *APOA5* showed an association with FCH ($P = 0.029$), total cholesterol ($P = 0.031$), triglycerides ($P < 0.001$), apolipoprotein B ($P = 0.011$), HDL-cholesterol ($P = 0.013$), small dense LDL ($P = 0.010$) and remnant-like particle cholesterol ($P = 0.001$). Compared to S19 homozygotes, 19W carriers had an increased risk of FCH (OR = 1.6 [1.0–2.6]; $P = 0.026$) and a more atherogenic lipid profile, reflected by higher triglyceride (+ 22%) and apolipoprotein B levels (+ 5%), decreased HDL-cholesterol levels (– 7%) and an increased prevalence of small dense LDL (16% vs. 26%). In carriers of the – 1131C allele, small dense LDL was more prevalent than in – 1131T homozygotes (29% vs. 16%). No association of the *APOA5* gene with IMT and CVD was evident.

Conclusion: In Dutch FCH families, variants in the APOA5 gene are associated with FCH and an atherogenic lipid profile.

Familial combined hyperlipidemia (FCH) is the most common lipid disorder of unknown genetic etiology, affecting 2–5% of the general population.[1,2] Major characteristics of FCH include elevated plasma levels of apolipoprotein B (apoB), triglycerides (TG) and/or total cholesterol (TC). Other phenotypes include decreased levels of high-density lipoprotein cholesterol (HDL) and the presence of small, dense low-density lipoproteins (sdLDL). In addition, FCH patients have an increased risk of cardiovascular disease (CVD) and are often obese and insulin resistant.[3]

Despite decades of research, the complex genetics of FCH are still not fully understood. Several linkage analyses were performed, leading to the identification of multiple loci for FCH.[4-9] One repeatedly identified locus is located on chromosome 11q, a site involved in modulating the expression of FCH.[10-12] This region includes the apolipoprotein *A1–C3–A4–A5* gene cluster.[13] The apolipoprotein A5 (*APOA5)* gene encodes the apolipoprotein AV protein (APOAV), which is exclusively expressed in the liver. APOAV is found on very low-density lipoprotein (VLDL), HDL and chylomicrons and is, compared to other apolipoproteins, present in very low plasma concentrations.[14,15] Variations in the *APOA5* gene are related to TG levels, however, the underlying mechanism is not yet fully understood.[16] One hypothesis, based on mouse studies, suggests that APOAV modulates TG levels by guiding VLDL and chylomicrons to proteoglycan-bound lipoprotein lipase for lipolysis and by increasing VLDL clearance.[17,18]

In the human *APOA5* gene, several single-nucleotide polymorphisms (SNPs) have been identified (– 1131T > C, – 3A > G, S19W (56C > G), IVS3 + 476G > A and 1259T > C).[19,20] The three major haplotypes, representing approximately 98% of all haplotypes in these populations, were defined by the – 1131T > C and the S19W SNPs.[19,20] In Caucasians, the rare alleles of these two SNPs were associated with elevated plasma levels of TC, TG, remnant-like particle cholesterol (RLP), LDL cholesterol (LDL) and apoB, decreased HDL levels and the presence of sdLDL.[13,20,21] In patients with FCH, *APOA5* polymorphisms were associated with an increased risk of FCH and related lipid phenotypes, including TG, apoB and HDL levels and LDL particle size.[11,22,23] The relationship between variants in the *APOA5* gene and RLP levels was not previously studied in FCH patients.

Hypertriglyceridemia is an independent risk factor for the development of CVD.[24] Despite its effect on plasma triglycerides, the role of *APOA5* in the development of CVD remains controversial.[20,25-29] In 372 Finnish men with CVD, who participated in the LOCAT study, it was demonstrated that only the rare allele of the S19W SNP (19W) was associated with increased progression of atherogenesis.[29] In contrast, in 2578 subjects from the general population in the Framingham Heart Study, an increased risk of CVD was found only for the rare allele of the – 1131T > C SNP (– 1131C).[20,29] The results for CVD in the Framingham Heart Study, however, conflicted with the results obtained for intima-media thickness (IMT), a surrogate marker of CVD. Differences in IMT were associated with the 19W allele, but an association of the – 1131C allele was only present in obese subjects.[25] In FCH patients, who have an increased risk of CVD, the relationship between variants in the *APOA5* gene and CVD was not previously examined.

The aim of the present study was to investigate the association of *APOA5* gene variants (– 1131T > C and S19W) with FCH and its associated phenotypes, including RLP levels, using a family-based SNP and haplotype approach in well-characterized FCH families. Furthermore, the suggested increased risk of CVD associated with variants in the *APOA5* gene was investigated in our FCH families by taking both intima-media thickness and CVD prevalence into account.

MATERIALS AND METHODS

Study Population

Back in 1994, we have recruited FCH families from the outpatient lipid clinic of the Radboud University Nijmegen Medical Centre, ascertained through probands, exhibiting a combined hyperlipidemia with both plasma TC and TG levels above the age- and gender-related 90th percentile,[30] during several periods in which they were not treated with lipid-lowering drugs, and despite dietary advice. Additionally, a first-degree relative possessed elevated levels of TC and/or TG above the 90th percentile and the proband, or a first-degree relative, suffered from premature (before the age of 60 years) cardiovascular disease. Families were excluded when probands were diagnosed with underlying diseases causing hyperlipidemia (i.e., diabetes mellitus type 1 and 2, hypothyroidism, and hepatic or renal impairment), a first-degree relative had tendon xanthomata or probands were homozygous for the *APOE2* allele. All included subjects were Caucasian and above the age of 10 years. The ascertained families had a mean size of 24 members from multiple (between 2 and 4) generations. The present FCH study population include the 5-year follow-up data of our original FCH families, consisting of 36 Dutch families, comprised of 611 subjects with known genealogic, phenotypic, and genotypic data, of whom 157 individuals were diagnosed as FCH patients.[31] In 1999, the diagnosis of FCH was based on the nomogram.[31] Plasma TG and TC levels, adjusted for age and gender, and absolute apoB levels, were applied to the nomogram to calculate a probability of being affected with FCH. When this probability of being affected with FCH is greater than 60%, the diagnostic phenotype is present in at least one first degree relative, and premature CVD (before the age of 60 years) is present in at least one individual in the family, the individual is defined as affected by FCH. Also included in the family-based analyses were the normolipidemic relatives ($n = 390$), the unaffected spouses of both FCH patients and normolipidemic relatives ($n = 64$), and subjects without known phenotypic and/or genotypic data ($n = 230$) to complete the pedigree structure. After the withdrawal of lipid-lowering medication for 4 weeks and an overnight fast, blood was drawn by venipuncture. Body mass index (BMI) was calculated as body weight (kilograms) divided by the square of height (meters).

Information concerning CVD was gathered through personal interviews and physical examinations performed by the clinical investigator. When the clinical investigator suspected the presence of CVD, further details and confirmation of the diagnosis were sought from the participant's general practitioner and hospital records. CVD was defined by angina pectoris (AP), myocardial infarction (MI), stroke, peripheral vascular disease or vascular surgery. In our study population ($n = 611$), 56 subjects were identified with CVD, including 26 subjects with AP, 25 with previous MI, 10 with peripheral vascular disease, seven with stroke and 23 who underwent vascular surgery. In total, 45% ($n = 25$) of these individuals were diagnosed with CVD based on the presence of two or more manifestations of CVD. The ethical committee of the Radboud University Nijmegen Medical Centre approved the study protocol and all procedures were in accordance with institutional guidelines. All subjects provided written informed consent.

Biochemical Analyses

Biochemical analyses were performed as previously described for this population.[31] In short, plasma TC and TG were determined by enzymatic, commercially available reagents (Boehringer-Mannheim, Germany, catalog No. 237574 and Sera Pak, Miles, Belgium, catalog No. 6639, respectively). Total plasma apoB concentrations were measured by immunonephelometry. HDL was quantified by the polyethylene glycol 6000 method. LDL subfractions were separated by single spin density gradient ultracentrifugation. A continuous variable, *K*, represented the LDL subfraction profile of each individual. A negative *K*-value ($K \leq -0.1$) reflected the presence of small dense LDL.[31] RLP levels were measured using an immuno-separation technique as described elsewhere.[32] Glucose concentrations were analyzed using the oxidation technique (Beckman®, Glucose Analyser2, Beckman Instruments Inc., Fullerton, CA 92634, USA). Plasma insulin concentrations were ascertained by a double antibody method. Insulin resistance was assessed by the homeostasis model assessment (HOMA) method.

DNA was extracted from peripheral blood lymphocytes using a standard technique.[33] Genotyping for the S19W and the − 1131T > C SNPs was carried out by PCR and restriction enzyme digestion, as previously described.[34] Genotyping of the S19W and − 1131T > C SNPs failed in 5% and 3% of the subjects, respectively.

Carotid IMT Measurements

All our FCH families were invited to participate in an on-going follow-up program. As part of the follow-up screening, non-invasive measurements of atherosclerosis were performed, including carotid intima-media thickness (IMT), in all family members, including both patients and normolipidemic relatives.[35] For the study population described in the present study, IMT data were available in 23 families, including 61 FCH patient and 155 normolipidemic relatives.

An AU5 Ultrasound machine (Esaote Biomedica) with a 7.5 MHz linear-array transducer was used to measure the IMT of both common carotid arteries. Longitudinal images of the most distal 10 mm of both the far and the near wall of the common carotid artery were obtained with the optimal projection (anterolateral, lateral or posterolateral). The actual measurement of IMT was performed offline by the sonographer using semi-automatic edge-detection software (M'Ath®Std version 2.0, Metris, Argenteuil, France).[36] All measurements were carried out in end-diastole using the R-wave of a simultaneously recorded ECG as a reference frame. From each frame the mean IMT was calculated over at least 7.5 mm of the aforementioned 10 mm segment (yielding a minimum quality index of 75%). The outcome variable was defined as the mean IMT of the near and far wall of both common carotid arteries.

Statistical Analyses

Descriptive statistics were compared using ANOVA for continuous traits and Pearson's chi-square test for discrete traits. Prior to further statistical analyses, extended Mendelian error-checking was performed with Pedcheck.[37] For families with Mendelian inconsistencies, problematic genotypes were set to missing for the complete nuclear family or the isolated problematic individual (1%). The parental data for both SNPs were tested for Hardy–Weinberg equilibrium by use of Fisher's exact-test. Variables with a skewed distribution, including TG levels, RLP levels and the HOMA-index, were logarithmically transformed. The HAPLOVIEW program[38] was used to estimate allele and haplotype frequencies in founders, representing the unrelated individuals of the study population, including probands and spouses. Linkage disequilibrium (LD) and allelic association (R^2) between the two SNPs were calculated with HAPLOVIEW using the confidence interval method of Gabriel et al.[39]

To study the relation of *APOA5* in FCH, we used both linkage and association analysis. The SOLAR 2.1.4 software was used to implement two-point linkage analysis. The presence of linkage was tested for FCH and its related phenotypes. As linkage analysis may have limited power to identify common variants with moderate effects for more complex diseases, such as FCH, we also performed association analysis.[40]

Associations between the individual polymorphisms (1131T > C and S19W), or haplotypes and FCH, or the related phenotypes, were analyzed using a family based approach, with the family based association test (FBAT) software.[41] FBAT is attractive because it can test for linkage and/or association while avoiding biases due to population admixture or stratification, misspecification of the trait distribution, and/or selection based on the trait. The FBAT test statistics utilizes a general approach to family-based association tests, as proposed by Rabinowitz and Laird (2000), and is based on the distribution of the offspring genotypes conditional on any trait information and on the parental genotypes.[41] An additive model of inheritance was used, as the mode of inheritance of FCH is unknown and this model is particularly robust. FBAT broke down the extended pedigrees into nuclear families (n = 219) and evaluated their

contribution to the test statistics. The –e option of FBAT, which computes the test statistic using an empirical variance estimator, was implemented to correct for the non-independence of the nuclear families and for the presence of linkage. The –p option, which performs a Monte-Carlo permutation procedure, was used to estimate empirical *P*-values. Adjustments for age, gender and BMI were done by calculation of the residuals. For the haplotype analysis, the haplotype-based association test (HBAT) command of FBAT was used, utilizing the –e and –p options. Differences were considered statistically significant at *P*-value < 0.05.

The explained variance in FCH by the *APOA5* variants was explored using the measured genotype method in the SOLAR Program.[42] The measured genotype analysis uses variance component methodology to estimate the proportion of variance due to environmental and additive genetic effects. By comparing models with and without the *APOA5* genotypes, estimations are calculated of what proportion of the FCH trait variance is attributable to the genotype by examining the changes to the estimates of environmental and additive genetic variance. *P*-values and the explicable proportion of variance are presented.

To test differences between the different genotypes, generalized estimating equation (GEE) analyses were performed in the STATA 8.0 software. The GEE analyze is especially designed to take into account possible correlated values within families. In the models used for the GEE analyses the link function 'canonical' was used and an equal-correlation population-averaged model was used as the working correlation matrix.

The analyses were conducted using SPSS 12.0.1, PEDCHECK 1.00, HAPLOVIEW 3.2, SOLAR 2.1.4, FBAT 3.2 and STATA 8.0 software.

RESULTS

Characteristics of study population

Anthropometric and metabolic characteristics of FCH patients, normolipidemic relatives and spouses are presented in Table 1. FCH patients were older than normolipidemic relatives, but younger than spouses. Compared to normolipidemic relatives and spouses, FCH patients had a higher prevalence of CVD and higher IMT values. When correcting the IMT values for the age difference between FCH patients and normolipidemic relatives, the difference in IMT remained significant (0.78 mm (SD 0.08) in FCH versus 0.73 mm (SD 0.08) in normolipidemic relatives, $P = 0.000$). FCH patients were characterized by increased lipid levels, including TC, TG, apoB, LDL and RLP concentrations, decreased HDL and an increased prevalence of sdLDL, as reflected by a *K*-value below – 0.1. Finally, FCH patients were more also obese, as reflected by a higher BMI, and insulin resistant, as reflected by a higher HOMA-index (Table 1). The anthropometric and metabolic characteristics of the subgroup of subjects with IMT data are presented in Supplementary Table 1. Characteristics of the subjects with IMT data did not

Table 1: Characteristics of patients with familial combined hyperlipidemia, normolipidemic relatives and spouses

	FCH patients (n = 157)	NL relatives (n = 390)	Spouses (n = 64)
Male Gender (n (%))	75 (47.8%)	177 (45.4%)	31 (48.4%)
Age (years)	47.0 (15.6)*,†	37.9 (15.7)‡	56.0 (10.5)
CVD (n (%))	32 (20.4%)*,†	20 (5.1%)	4 (6.3%)
IMT (mm)	0.80 (0.12)*	0.72 (0.12)	–
BMI (kg/m^2)	27.3 (4.1)*	24.1 (3.8)‡	26.8 (3.6)
TC (mmol/L)	6.5 (1.1)*,†	4.9 (0.9)‡	5.2 (0.8)
TG (mmol/L)	3.1 (1.5)*,†	1.2 (0.5)	1.3 (0.6)
Apo B (mg/L)	1370 (264)*,†	960 (220)	996 (175)
LDL (mmol/L)	4.1 (1.2)*,†	3.2 (0.9)‡	3.4 (0.7)
HDL (mmol/L)	0.98 (0.26)*,†	1.22 (0.30)	1.28 (0.40)
K-value	– 0.26 (0.26)*,†	0.05 (0.19)	0.05 (0.25)
RLP (mmol/L)	0.57 (0.40)*,†	0.26 (0.09)‡	0.29 (0.13)
HOMA	3.4 (1.8)*,†	2.3 (1.3)‡	2.7 (1.3)

Continuous variables are presented as mean (SD).

FCH, familial combined hyperlipidemia; NL relatives, normolipidemic relatives; CVD, cardiovascular disease; IMT, mean common carotid artery intima-media thickness was measured in 61 FCH patients and 155 normilipidemic relatives; BMI, body mass index; TC, total cholesterol; TG, triglycerides; Apo B, apolipoprotein B; LDL, LDL cholesterol; HDL, HDL cholesterol; *K*-value, a value < -0.1 represents the presence of small, dense LDL; RLP, remnant-like particle cholesterol; HOMA, homeostasis model assessment-index.

*$P < 0.05$, compared to NL relatives; †$P < 0.05$, compared to spouses; ‡$P < 0.05$, compared to spouses.

Supplementary table 1: Characteristics of the subjects with data on intima media thickness

	FCH Patients (n = 61)	NL Relatives (n = 155)
Male Gender (n (%))	28 (45.9%)	70 (45.2%)
Age (years)	47.4 (14.2)*	37.9 (14.7)
CVD (n (%))	9 (14.8%)*	9 (5.8%)
IMT (mm)	0.80 (0.12)*	0.72 (0.12)
BMI (kg/m^2)	28.3 (4.1)*	24.1 (3.6)
TC (mmol/L)	6.6 (1.3)*	5.4 (1.0)
TG (mmol/L)	2.9 (1.8)*	1.5 (1.1)
ApoB (mg/L)	1327 (297)*	973 (233)
LDL (mmol/L)	4.2 (1.2)*	3.2 (0.9)
HDL (mmol/L)	1.14 (0.26)*	1.33 (0.33)
K-value	-0.28 (0.25)*	0.05 (0.21)
RLP (mmol/L)	0.62 (0.45)*	0.25 (0.09)
HOMA	2.4 (1.3)	1.8 (1.0)

FCH, familial combined hyperlipidemia; NL relatives, normolipidemic relatives; CVD, cardiovascular disease; IMT, intima media thickness; BMI, body mass index; TC, total cholesterol; TG, triglycerides; Apo B, apolipoprotein B; LDL, LDL cholesterol; HDL, HDL cholesterol; K-value, a value < -0.1 represents the presence of small, dense LDL; RLP, remnant-like particle cholesterol; HOMA, homeostasis model assessment index.

*$P < 0.05$ compared to normolipidemic relatives.

Supplementary table 2: Characteristics of subjects with cardiovascular disease

	Subjects with CVD (n = 56)
Male Gender (n (%))	35 (62.5)
Age (years)	62.5 (9.3)
FCH (n (%))	24 (42.9)
IMT (mm)	0.88 (0.13)
BMI (kg/m^2)	27.3 (3.4)
TC (mmol/L)	6.8 (1.5)
TG (mmol/L)	2.9 (2.1)
ApoB (mg/L)	1339 (302)
LDL (mmol/L)	4.0 (1.2)
HDL (mmol/L)	1.19 (0.31)
K-value	-0.18 (0.31)
RLP (mmol/L)	0.46 (0.27)
HOMA	2.3 (1.4)

Continuous variables are presented as mean (SD).

FCH, familial combined hyperlipidemia; IMT, intima media thickness; BMI, body mass index; TC, total cholesterol; TG, triglycerides; Apo B, apolipoprotein B; LDL, LDL cholesterol; HDL, HDL cholesterol; K-value, a value < -0.1 represents the presence of small, dense LDL; RLP, remnant-like particle cholesterol; HOMA, homeostasis model assessment index.

differ significantly from the total group as presented in Table 1 (all $P < 0.05$). Anthropometric and metabolic characteristics of the subgroup of subjects with CVD are presented in Supplementary Table 2. As expected, patients with CVD were relatively old and predominantly men. Furthermore, patients with CVD had a relatively high IMT value and a more atherogenic lipid and lipoprotein profile reflected by high TC and TG levels, low HDL cholesterol levels and the presence of sdLDL. Forty-three percent of the subjects with CVD were diagnosed FCH.

Analyses of the APOA5 gene with FCH and related lipid phenotypes

In founders, rare allele frequencies for the – 1131T > C and S19W SNPs were 0.08 and 0.11, respectively. In the probands, rare allele frequencies for the – 1131T > C and S19W SNPs were 0.10 and 0.16, respectively. The genotypic distributions of – 1131T > C and S19W were in Hardy–Weinberg proportions. The alleles of the – 1131T > C and the S19W SNPs were in complete linkage disequilibrium (D' = 1.00), but with very little correlation ($R^2 = 0.01$). A consequence of this linkage disequilibrium is that only three of the possible four different haplotypes were observed. Haplotype analyses can, therefore, provide additional information compared to the single SNP analyses, since it accounts for both sources of variation. The wildtype haplotype (APOA5*1) had a frequency of 0.83. The frequencies of the haplotype defined by a rare allele for the – 1131T > C (APOA5*2) or by a rare allele for the S19W SNP (APOA5*3) were 0.11 and 0.06, respectively. In total 394 individuals (92 FCH patients, 261 normolipidemic relatives and

41 spouses) carried only common alleles for both SNPs, who are referred to as "wildtypes". 64 subjects (8 FCH patients, 50 normolipidemic relatives and 6 spouses) carried one or two rare alleles for the – 1131T > C SNP and wildtype alleles for the S19W SNP and 115 subjects (38 FCH patients, 63 normolipidemic relatives and 14 spouses) carried one or two rare alleles for the S19W SNP and wildtype alleles for the – 1131T > C SNP. A rare allele for both the – 1131T > C SNP and the S19W SNP was present in 18 subjects (9 FCH patients, 9 normolipidemic relatives and 0 spouses), referred to as "compound heterozygotes".

Linkage analyses of the APOA5 region

Two-point linkage analyses performed utilizing the – 1131T > C and S19W SNPs in the *APOA5* gene did not provide evidence of linkage for FCH (– 1131T > C; lod score = 0.04 and S19W; lod score = 0.38). For TG levels a lod score of 1.6 was obtained for the S19W SNP, whereas a lod score of 0.05 was found for the – 1131T > C SNP. For all other phenotypes, including apoB, TC, RLP and HDL levels, and the presence of sdLDL, no linkage was present (all two-point lod scores < 0.5).

Association analyses of APOA5 variants with FCH and related phenotypes

The associations of the – 1131T > C and the S19W SNPs, individually and as haplotypes, with FCH and its related phenotypes are presented in Table 2A. These associations are corrected for age, gender and multiple testing. The APOA5*2 haplotype was not associated with FCH, while the APOA5*3 haplotype was overrepresented in patients with FCH ($P = 0.014$). Haplotype analyses showed that the *APOA5* haplotypes were associated with FCH ($P = 0.029$). This resulted in an increased risk of FCH for subjects ($n = 119$) with the rare allele for the S19W SNP (1.62 [95% CI: 1.03, 2.55]; $P = 0.026$) and for the compound heterozygotes ($n = 19$) carrying a rare allele for the – 1131T > C and the S19W SNPs (3.28 [95% CI: 1.38, 7.82]; $P = 0.008$). The APOA5*3 haplotype was also associated with individual lipid and lipoprotein levels, including TC ($P = 0.042$), TG ($P < 0.001$), apoB ($P = 0.024$), HDL ($P = 0.019$), *K*-value ($P = 0.024$) and RLP ($P = 0.001$). No associations were found for the obesity (BMI) and insulin resistance parameters (HOMA-index) (Table 2A). The APOA5*2 haplotype was not significantly associated with any of the characteristics of FCH. Comparable results were found when the non-independence of the families and the possible presence of linkage (–e option) were taken into account (data not shown).

Explained variance in FCH by the APOA5 gene

By measured genotype analyses, it was estimated that 2% of the genetic variance in FCH is attributable to the S19W SNP ($P < 0.001$). The S19W SNP also explained 3% of the variation in

Table 2: Association of rare alleles of the –1131T>C and the S19W SNPs and haplotypes in the *APOA5* gene with familial combined hyperlipidemia and its related phenotypes

		SNPs		Haplotypes		
	n	–1131C	19W	APOA5*2	APOA5*3	Overall Haplotype
A						
FCH	611	0.921	0.048	0.931	0.014	0.029
BMI (kg/m^2)	611	0.519	0.697	0.373	0.799	0.808
TC (mmol/L)	611	0.055	0.063	0.272	0.042	0.031
TG (mmol/L)§	611	0.041	0.000	0.141	0.000	0.000
Apo B (mg/L)	611	0.097	0.087	0.253	0.024	0.011
LDL (mmol/L)	608	0.403	0.320	0.596	0.630	0.787
HDL (mmol/L)	609	0.445	0.041	0.397	0.019	0.013
K-value	611	0.233	0.045	0.119	0.024	0.010
RLP (mmol/L)§	559	0.031	0.000	0.120	0.001	0.001
HOMA§	550	0.534	0.385	0.560	0.273	0.623
B						
CVD	611	0.482	0.379	0.066	0.607	0.202
IMT (mm)	216	0.437	0.494	0.797	0.656	0.542

P-values for the individual SNPs (FBAT) and haplotypes (HBAT) corrected for age, gender and multiple testing are presented.
§, Ln-transformed variable; *N*, number of subjects with available data for the relevant phenotype; APOA5*2, haplotype represented by the rare allele of – 1131T > C; APOA5*3, haplotype represented by the rare allele of S19W; Overall haplotype, the *P*-value obtained for the model including all haplotypes; FCH, familial combined hyperlipidemia was present in 157 subjects; BMI, body mass index; TC, total cholesterol; TG, triglycerides; Apo B, apolipoprotein B; LDL, LDL-cholesterol; HDL, HDL-cholesterol; *K*-value, a value < -0.1 represents the presence of small, dense LDL; RLP, remnant-like particle cholesterol; HOMA, homeostasis model assessment-index; CVD, cardiovascular disease was present in 56 subjects; IMT, mean common carotid artery intima-media thickness data were present in 61 FCH patients and 155 normolipidemic relatives.

TG levels ($P < 0.001$), 1% of the variance in HDLc levels ($P = 0.005$) and 1% of the variation in *K*-value ($P = 0.005$). The – 1131T > C SNP did not contribute to the variation in FCH or any of the related phenotypes (TC, TG, Apo B, LDL, HDL, *K*-value, RLP and HOMA-index); in fact, the point estimates for the combined effects of both genotypes, S19W and – 1131T > C, were identical to the point estimate obtained for S19W alone.

Association analyses of the APOA5 gene with IMT and CVD

The associations of the – 1131T > C and the S19W SNPs in *APOA5*, and their haplotypes, with IMT values and CVD prevalence, are presented in Table 2B. These associations are corrected for age, gender and multiple testing. The APOA5*3 haplotype was not associated with IMT or CVD. The APOA5*2 haplotype did show a trend towards significant association with CVD ($P = 0.07$), but no association with IMT was present. Overall, no association of the haplotypes with IMT values or CVD prevalence was evident. Further adjustment of IMT values for obesity did not change these results.

Effect of APOA5 variants on lipid and lipoprotein levels, IMT and prevalence of CVD

To determine the effect of the genetic variation in the *APOA5* gene on lipid levels in the general population, we combined the normolipidemic relatives and spouses in one control group, which was allowed because no difference in anthropometric and biochemical parameters, based on genotypes, were observed between relatives and spouses (data not shown). The results were standardized for age and gender and presented in Table 3. Compared to the wildtypes, individuals carrying a rare allele of the S19W SNP had increased TG levels (+ 22%) apoB levels (+ 5%) and decreased HDL levels (– 7%). Furthermore, in subjects with a 19W allele, a *K*-value below – 0.1 was present in 26% of the subjects, which was significantly higher than in wildtypes (16%; $P = 0.046$). Additionally, in carriers of the – 1131T > C rare allele, sdLDL was present in more individuals (29%; $P = 0.035$) compared to wildtypes. The compounds had approximately 15% lower HDLc levels and sdLDL was present in more individuals. Compared to the wildtypes, individuals carrying rare alleles for either or both SNPs had no increased IMT or increased prevalance of CVD (Table 3). The effect of *APOA5* on lipid levels among FCH patients were comparable (data not shown).

Table 3: Differences in phenotypic parameters in the control group including normolipidemic relatives and spouses with a rare allele for the – 1131T > C or the S19W SNP in the *APOA5* gene compared to wildtype individuals

	Wildtypes	– 1131C	19W	Compounds
	n = 302 (68.0%)	n = 56 (12.6%)	n = 77 (17.4%)	n = 9 (2.0%)
CVD (n (%))	10 (3.3%)	5 (8.9%)	6 (7.8%)	1 (11.1%)
IMT (mm)	0.74 (0.72–0.76)	– 1.4%	– 4.0%	+ 0.0%
TC (mmol/L)	4.99 (4.86–5.11)	+ 3.0%	+ 2.0%	– 3.4%
TG (mmol/L)	1.07 (1.01–1.14)	+ 8.4%	+ 22.4%*	+ 23.4%
ApoB (mg/L)	975 (949–1001)	+ 5.3%	+ 5.0%*	+ 4.6%
HDL (mmol/L)	1.24 (1.20–1.28)	– 2.4%	– 7.3%*	– 17.7%*
K-value < – 0.1 (n (%))	48 (15.9%)	16 (28.6%)*	20 (26.0%)*	4 (44.4%)*
RLP (mmol/L)	0.25 (0.24–0.26)	+ 0.0%	+ 4.0%	+ 8.0%

Presented are the data of the combined group of normolipidemic relatives and spouses. For the subjects wildtype for both SNPs, dichotomous variables are presented as number (%) and the continuous variables, standardized for age and gender, are presented as mean (95% CI); For the other groups, dichotomous variables are presented as number (%) and the continuous variables as percent difference compared to the wildtype individuals.

Rare allele – 1131T > C, subjects carrying either 1 or 2 rare alleles for the – 1131T > C SNP; Rare allele S19W, subjects carrying either 1 or 2 rare alleles for the S19W SNP; Compounds, subjects carrying 1 rare allele for both SNPs; CVD, cardiovascular disease; IMT, common carotid artery intima-media thickness; TC, total cholesterol; TG, triglycerides; ApoB, apolipoprotein B; HDL, HDL cholesterol; *K*-value, a value < – 0.1 represents the presence of small, dense LDL; RLP, remnant-like particle cholesterol.

*$P < 0.05$ compared to wildtypes.

DISCUSSION

The results from this study demonstrate that the S19W variant in the *APOA5* gene is associated with FCH. We show that the APOA5*3 haplotype is over-represented in patients with FCH. Furthermore, we provide evidence for the association of the APOA5*3 haplotype with a more atherogenic lipid profile. However, no association of the – 1131T > C and S19W variants in the *APOA5* gene with higher IMT values or an increased prevalence of CVD in patients with FCH was evident.

The 19W allele was previously associated with an increased risk of FCH and elevated levels of TG and apoB.[11,22] In the present study, we performed family-based haplotype analyses with the FBAT program, corrected either for multiple testing or for the presence of linkage in this region. We confirmed the independent association of the 19W allele with FCH and elevated TG levels. Additionally, we showed that the 19W allele is associated with an atherogenic lipid profile including elevated apoB levels, decreased HDL levels and the presence of sdLDL. This is in agreement with previously documented associations in general populations.[11,13,20,21] From the literature, it is also suggested that the haplotype containing the 19W is associated with the highest TG levels.[19,34]

In the present study, no independent association for the – 1131C allele was found with FCH or any of the related lipid phenotypes whereas the – 1131C allele was previously associated with an increased risk of FCH, and elevated levels of TG and sdLDL.[11,22,23] In the present study we defined FCH based on our nomogram.[31] To exclude that the lack of association of the – 1131T > C SNP with FCH in our population is due to different diagnostic criteria of FCH, we repeated the analyses, now applying the traditional diagnostic criteria of FCH, based on TC and/or TG levels above the 90th percentile. No association of the – 1131T > C SNP with FCH, based on the traditional diagnostic criteria, was found (Supplementary Table 3). Most likely, the small number of subjects carrying one or two C alleles of the – 1131T > C SNP contribute to the lack of involvement of this SNP to FCH. In contrast, compound heterozygotes did show a significant increased risk on FCH, despite the low number of subjects. However, as the lipid and lipoprotein levels among subjects who were compound heterozygotes did not differ from wildtype carriers, it may well be possible that the observed increased risk of FCH in compound heterozygotes is a false positive finding and therefore additional studies are required for confirmation of our observed increased risk on FCH in compound heterozygotes.

Previously, two studies investigated the association of variants in the *APOA5* gene and RLP levels. In the Framingham Heart Study population, both the 19W and – 1131C alleles were associated with RLP levels, while no association was found in the MONICA study population.[20,43] We report no association of the rare alleles of the – 1131T > C and the S19W SNPs in the *APOA5* gene with RLP levels in our FCH study population, which is in agreement with the findings of the MONICA study.[43]

Supplementary table 3: Association of rare alleles of the –1131 T>C and the S19W SNPs and haplotypes in the *APOA5* gene with familial combined hyperlipidemia based on the traditional diagnostic criteria

		SNPs		Haplotypes		
	n	-1131C	19W	APOA5*2	APOA5*3	Overall Haplotype
FCH	611	0.615	0.124	0.914	0.105	0.429

P-values for the individual SNPs (FBAT) and haplotypes (HBAT) corrected for age,
gender and multiple testing are presented.
N, number of subjects with available data for the relevant phenotype; APOA5*2, haplotype represented by the rare allele of –1131T>C; APOA5*3, haplotype represented by the rare allele of S19W; Overall haplotype, the *P*-value obtained for the model including all haplotypes. FCH, familial combined hyperlipidemia, based on the traditional diagnostic criteria, was present in 151 subjects.

Since the variants in the *APOA5* are suggested to be associated with plasma lipid levels, it is likely that it plays a role in the development of CVD. Several studies investigated possible associations between variants in the *APOA5* gene and the prevalence of CVD in Caucasians, however, these led to contradictory results.[20,25-29,44] The LOCAT study showed a trend toward increased progression of atherogenesis in male carriers of the rare allele of the S19W SNP; in contrast, no effect was found for the – 1131C allele.[29] In the Framingham Heart Study, an almost 2-fold increased risk of CVD was observed in females carrying the rare allele of the – 1131T > C SNP, but there was no effect in males. For the 19W allele, no effect was found in either gender.[20] In contrast, in the same Framingham Heart Study, the 19W allele was associated with IMT values in both genders. In obese subjects, this relationship with IMT was also observed for the rare allele of the – 1131T > C SNP.[25]

The association of variants in the *APOA5* gene with CVD and IMT values was not previously studied in FCH patients, who are known to have thicker IMT and an increased risk of CVD.[35] In the present study, we show that, although the rare allele of the S19W variant in the *APOA5* gene was associated with FCH and a disturbed lipid profile, no association with CVD or IMT was evident. This could be a result of the limited number of individuals with CVD in our study population, and the availability of IMT data in only a sub-set of the study population, resulting in limited power for studying these associations. Possible explanations for the contradictory results previously found for CVD could be that the association of variants in the *APOA5* gene with CVD is independent of TG levels, suggesting different mechanisms, or that the influence of *APOA5* gene variants on TG is relatively small and therefore not translated into differences in CVD.[25,27,29]

In the present study, the measured genotype method and FBAT were used to analyze the association of variants in the *APOA5* gene with FCH and its related phenotypes. The measured genotype method is more powerful, uses the whole pedigree and provides an effect estimate for the tested variables, but is not robust in the presence of population stratification. FBAT, however, is robust to stratification, but is less powerful and uses incomplete pedigree information. The results of both statistical analyses were highly concordant, thereby providing strong evidence for the role of the S19W SNP of the *APOA5* gene in FCH patients.

In the general population, the influence of variations in the *APOA5* gene on lipid levels is still controversial. Some studies show that the association of variations in the *APOA5* gene with increased TG levels is limited or not present in the general population.[11,22,23] Based on these studies, there is conjecture that *APOA5* gene variants modulate TG levels only when there is an altered genetic and metabolic background. In the present study, we show that the influence of the rare allele of the S19W SNP on lipid levels was not restricted to FCH patients, but also present in normolipidemic relatives and spouses.

In conclusion, in the present study of Dutch FCH families, the rare allele of the S19W SNP in the *APOA5* gene is associated with FCH and a more atherogenic lipid profile.

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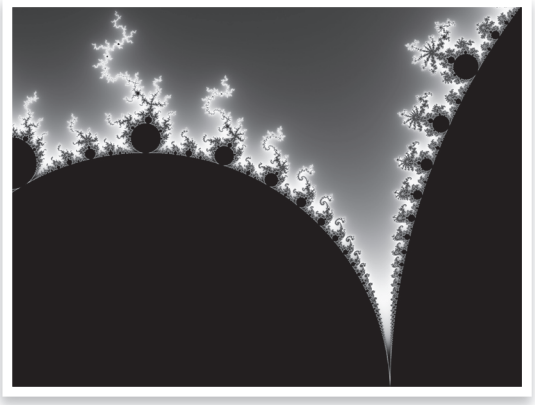
Chapter 2.5

Variation in the Apolipoprotein L-V Gene Is Associated with Plasma HDL and Triglyceride Levels



ABSTRACT

The Apolipoprotein L (ApoL) cluster is comprised of six genes encoding highly homologous proteins that are likely involved in lipid transport and metabolism, particularly of high-density lipoprotein cholesterol (HDL) and triglycerides (TG). The ApoL genes have not been studied in detail. In the current study, sixty-six single nucleotide polymorphisms (SNPs) spanning the ApoL cluster were typed in a randomly selected Dutch population (n = 188) and tested for association with HDL and TG. Findings were followed-up in a randomly sampled population cohort from Finland (n = 182). In the Dutch, five SNPs were nominally associated with HDL ($0.01 \leq P \leq 0.05$). When testing these SNPs in the sample from Finland, three ApoL-V SNPs were marginally associated with HDL and significantly associated with TG. Two of these ApoL-V variants were associated with TG/HDL ratio in both populations ($0.015 \leq P \leq 0.040$), while the third was marginally so ($0.015 \leq P \leq 0.068$). Combining the samples revealed that the ApoL-V SNPs were associated with all three outcomes. Haplotype analysis of ApoL-V showed that a shared haplotype, common in both populations, explained these associations. These analyses suggest that ApoL-V is involved in determining HDL and TG levels and TG/HDL ratio. Variation throughout the cluster, and particularly ApoL-V, may help explain individual variation in plasma lipids.

The role of high-density lipoprotein cholesterol (HDL) and triglycerides (TG) in determining cardiovascular disease risk are well established.[1] Increased HDL levels exert a protective effect, while increased TG is typically deleterious. These observations led to the suggestion that TG/HDL ratio might be a valuable clinical tool in assessing cardiovascular disease susceptibility,[2] and fostered considerable interest in environmental and genetic factors influencing the distributions of these lipid parameters.

Apolipoprotein L-I (ApoL-I) is a recently identified protein constituent of a subset of Apolipoprotein A-I (ApoA-I) containing particles (HDL);[3] subsequently, ApoL-I isoforms were also characterized as very low-density lipoprotein (VLDL) associated proteins.[4] This protein was correlated with plasma TG and cholesterol levels in normo- and hyperlipidemic individuals and diabetics,[5] and with plasma TG and hyperglycemia in coronary artery disease patients with perturbed lipid profiles.[6]

The search for the gene encoding ApoL-I led to the initial discovery of a cluster of four highly homologous genes located on chromosome 22q12.1-13.1.[7] These genes (ApoL-1 to ApoL-IV) are expressed in numerous tissues, including lung, pancreas, liver, spleen, prostate, and human vascular tissue.[8] Further study determined two additional members of the cluster, ApoL-V and ApoL-VI, and found all six genes to be ubiquitously expressed.[8] Phylogenetic analysis suggests that these genes are actually organized into two sub-clusters, one comprised of ApoL-I to ApoL-IV, and the other containing ApoL-V and ApoL-VI. The ApoL-I to ApoL-IV genes are thought to have arisen due to tandem duplication followed by rapid divergence, whereas the ApoL-V and ApoL-VI genes may have arisen independently.[8,9] Structural analysis showed that the ApoL proteins were highly ordered with conserved α helices and TATA-less promoters typical of other members of the apolipoprotein class.[7,8] Although these genes produce highly homologous proteins, there are substantial differences between the two sub-clusters in intronic sequences.[9] One report found ApoL homologues only in higher order primates, suggesting that the cluster arose recently, in evolutionary terms.[8]

Although of obvious interest in terms of lipid transport and metabolism, most explorations of ApoL to date focused on the discovery that ApoL-I is a trypanosome lytic factor.[10-13] Studies of genetic variation were similarly limited to a few single nucleotide polymorphisms (SNPs) in ApoL-I.[6,7] For a study on LD structure in isolated populations,[14] 2,486 SNPs were genotyped in a population sample from the Netherlands and a second population sample from Finland. These polymorphisms were selected to cover the entire chromosome with regular spacing. In the current study, sixty-six of these SNPs, spanning the ApoL cluster, were examined. These SNPs, and haplotypes, were tested for association with fasting plasma HDL, TG, and TG/HDL ratio, and represent the first study to attempt a cluster-wide analysis of genetic variation.

MATERIALS AND METHODS

Study Populations

Both populations used in this study were previously described.[14,15] Briefly, the Dutch population was sampled from a recent genetic isolate in the southwest Netherlands, founded by a limited number of individuals in the middle of the 18th century. Characterized by rapid growth and minimal immigration, the isolate now includes approximately 20,000 inhabitants. A large pedigree-based cohort (the ERF cohort) was selected from this population; the 188 individuals genotyped in this study were minimally related members of this population and had plasma lipid values available.

The Finnish population was sampled from the 1986 Northern Finland Birth Cohort (NFBC 1986), which ascertained all (99%) births with expected dates of birth between July 1, 1985 and June 30, 1986 that occurred in the two northernmost provinces in Finland. 182 individuals with fasting plasma lipid levels (measured at 16 years of age) from this population were selected. Overall attendance during blood sampling was 71% in this adolescent cohort.

The Medical Ethics Committees of Erasmus Medical Center Rotterdam and Oulu University Hospital approved these studies and protocols for the ascertainment of human subjects and informed consent was obtained from all participants.

Laboratory Analysis

In the ERF population, fasting blood samples were collected during the participant's visit to the research center. A Synchron LX20 (Beckman Coulter Inc., Fullerton, CA. U.S.A.) spectrophotometric chemical analyzer was utilized for the determination of plasma lipid values. In the NFBC 1986 population, blood samples were drawn after overnight fasting. Serum lipid levels were analyzed using a Cobas Integra 700 automatic analyzer (Roche Diagnostics, Basel, Switzerland).

DNA was extracted from whole blood using the salting-out method.[16] Genotyping was accomplished using Illumina BeadLab reagents and protocols.[17] The precise methods used were detailed earlier.[14]

Statistical Analysis

Hardy-Weinberg expectations were tested with an exact test, as some of the cell sizes were small.[18] Regression models were fitted in SPSSv11.0 to assess distributional assumptions of normality, which were tested using one-sample Kolmogorov-Smirnov tests. For further analyses, HDL, TG, and TG/HDL were natural log transformed to yield normally distributed residuals. Associations between ApoL SNPs and fasting plasma HDL, TG, and TG/HDL levels were exam-

ined using general linear models in the R base package (http://cran.r-project.org/). Linkage disequilibrium patterns were estimated using Haplo.view. Haplotype analyses, adjusted for posterior uncertainty in haplotype assignment, were conducted using the score test[19] implemented in the haplo.stats package for R. Empirical estimates of haplotype *P*-values were computed using permutation analysis.

No multiple testing corrections were implemented; any of the standard adjustments would be too punitive for several reasons. First of all, there was a valid *a priori* hypothesis. Additionally, a second, completely independent, population was available to test the limited number of nominally significant SNPs from the first sample. In addition, many of the SNPs, and the three outcomes tested, are strongly correlated, so these tests are not truly independent.

RESULTS

Table 1 shows general characteristics of the Dutch and Finnish populations. The gender distribution of the two samples was comparable, while the ages differed substantially (the NFBC 1986 sample was 16 years old when blood was drawn). Due largely to this age difference, lipid measures differed significantly between the populations. The Dutch possessed higher total cholesterol, low-density lipoprotein cholesterol, TG, and TG/HDL ratios, while the Finns exhibited higher HDL levels.

Sixty-six SNPs spanning the ApoL cluster were genotyped as part of a large study on linkage disequilibrium, which included these two samples.[14] Characteristics of these polymorphisms, including locations, call rates, minor allele frequencies, and conformation to Hardy-Weinberg expectations were assessed in the ERF population; three SNPs were removed from the sample on the basis of these analyses (Table 2). Two SNPs deviated from Hardy-Weinberg expectations (rs132700, $P_{HWE} = 0.01$; and rs132735, $P_{HWE} = 0.01$) and were not subsequently analyzed. A third was removed due to ineffective genotyping (rs9619573, call rate = 91%). The remaining 63 SNPs were analysed for association with plasma HDL and TG levels. No SNPs were determined

Table 1: General Characteristics of the Study Populations

	Netherlands	Finland	*P*-value
Male (%)	78 (41.5)	86 (47.3)	0.265
Age	55.51 (10.84)	16 (NA)	< 0.001
Total Cholesterol (mmol/L)	5.50 (1.16)	4.23 (1.07)	< 0.001
LDL (mmol/L)	3.68 (1.00)	2.21 (0.59)	< 0.001
HDL (mmol/L)	1.23 (0.35)	1.38 (0.27)	< 0.001
Triglycerides (mmol/L)	1.40 (0.81)	0.80 (0.38)	< 0.001
TG/HDL Ratio	1.29 (1.07)	0.62 (0.42)	< 0.001

LDL: Low-Density Lipoprotein Cholesterol; HDL: High-Density Lipoprotein Cholesterol; TG/HDL Ratio: Triglyceride – HDL Ratio.
Values for continuous variables are presented as mean (SD).

Table 2: Summary of SNPs and Their Relationship with Plasma HDL in the ERF Population

SNP	Gene	Call Rate	Minor Allele Frequency	Hardy-Weinberg *P*-value	β (s.e.)	Nominal *P*-value
rs5999894	Int.	1.00	0.07	1.00	-0.01 (0.06)	0.85
rs1894468	Int.	1.00	0.39	1.00	-0.03 (0.03)	0.36
rs5995133	ApoL-VI	1.00	0.11	0.14	0.03 (0.05)	0.46
rs738510	Int.	1.00	0.36	0.53	-0.05 (0.03)	0.10
rs972153	Int.	1.00	0.41	0.55	-0.04 (0.03)	0.17
rs2413367	Int.	1.00	0.35	0.75	-0.04 (0.03)	0.20
rs1540297	ApoL-V	1.00	0.35	0.34	-0.03 (0.03)	0.32
rs2076671	ApoL-V	1.00	0.29	0.38	0.07 (0.03)	0.03
rs2076672	ApoL-V	1.00	0.15	0.58	0.01 (0.04)	0.72
rs2073198	ApoL-V	1.00	0.29	0.38	0.07 (0.03)	0.03
rs2076673	ApoL-V	1.00	0.30	0.48	0.06 (0.03)	0.04
rs5750165	ApoL-V	0.98	0.38	0.76	-0.04 (0.03)	0.12
rs5755936	Int.	1.00	0.40	0.23	-0.05 (0.03)	0.05
rs926756	RBM9	1.00	0.37	0.53	-0.01 (0.03)	0.74
rs5750173	RBM9	1.00	0.16	0.41	-0.01 (0.04)	0.70
rs136055	RBM9	1.00	0.24	1.00	-0.03 (0.03)	0.34
rs5755951	RBM9	1.00	0.15	0.39	-0.02 (0.04)	0.61
rs6000006	RBM9	1.00	0.15	0.40	-0.02 (0.04)	0.56
rs5750178	RBM9	1.00	0.15	0.39	-0.02 (0.04)	0.61
rs9619573	*RBM9*	*0.91*	*0.16*	*0.25*		
rs2092786	RBM9	1.00	0.15	0.39	-0.02 (0.04)	0.61
rs2051579	RBM9	1.00	0.28	0.47	-0.01 (0.03)	0.65
rs970253	RBM9	1.00	0.13	0.50	0.00 (0.04)	0.91
rs10483192	RBM9	1.00	0.14	0.55	-0.02 (0.04)	0.53
rs5755974	RBM9	1.00	0.14	0.55	-0.02 (0.04)	0.53
rs5750197	RBM9	1.00	0.11	0.43	-0.03 (0.04)	0.49
rs6000036	RBM9	1.00	0.14	0.38	-0.02 (0.04)	0.55
rs5756002	RBM9	1.00	0.11	0.43	-0.03 (0.04)	0.49
rs2157258	RBM9	1.00	0.50	0.66	-0.01 (0.03)	0.60
rs5750204	RBM9	1.00	0.14	0.55	-0.02 (0.04)	0.53
rs5756032	RBM9	1.00	0.14	0.55	-0.02 (0.04)	0.53
rs737736	RBM9	1.00	0.11	0.44	-0.03 (0.04)	0.49
rs1573708	RBM9	1.00	0.43	0.77	0.00 (0.03)	0.87
rs5995197	Int.	1.00	0.29	0.59	-0.04 (0.03)	0.21
rs5995203	Int.	1.00	0.13	0.53	0.03 (0.04)	0.43
rs715550	Int.	1.00	0.23	1.00	-0.01 (0.03)	0.76
rs715546	Int.	1.00	0.24	0.84	-0.01 (0.03)	0.72
rs878847	Int.	1.00	0.32	0.87	0.04 (0.03)	0.14
rs876417	Int.	1.00	0.31	0.61	0.05 (0.03)	0.08

SNP	Gene	Call Rate	Minor Allele Frequency	Hardy-Weinberg *P*-value	β (s.e.)	Nominal *P*-value
rs5995224	Int.	1.00	0.39	0.76	0.05 (0.03)	0.08
rs132615	ApoL-III	1.00	0.35	0.75	0.00 (0.03)	0.91
rs132618	ApoL-III	1.00	0.49	0.66	0.04 (0.03)	0.17
rs3827346	ApoL-III	1.00	0.18	0.21	-0.02 (0.04)	0.65
rs132642	ApoL-III	1.00	0.18	0.46	0.00 (0.04)	0.91
rs132653	ApoL-III	1.00	0.18	0.46	0.00 (0.04)	0.91
rs132655	ApoL-III	1.00	0.46	0.11	0.02 (0.03)	0.53
rs132660	ApoL-III	1.00	0.45	0.14	0.02 (0.03)	0.52
rs132661	ApoL-III	1.00	0.31	1.00	-0.01 (0.03)	0.63
rs132663	ApoL-III	1.00	0.30	1.00	-0.01 (0.03)	0.65
rs916336	ApoL-IV	1.00	0.49	0.11	-0.02 (0.03)	0.33
rs12781	ApoL-IV	1.00	0.49	0.24	-0.02 (0.03)	0.35
rs2227167	ApoL-IV	1.00	0.49	0.15	-0.02 (0.03)	0.37
rs2227168	ApoL-IV	1.00	0.50	0.15	-0.02 (0.03)	0.35
rs132700	*ApoL-IV*	*1.00*	*0.28*	*0.01*		
rs2227169	ApoL-IV	1.00	0.49	0.11	-0.02 (0.03)	0.35
rs132734	ApoL-IV	1.00	0.42	0.46	-0.09 (0.03)	0.001
rs132735	*ApoL-IV*	*1.00*	*0.40*	*0.01*		
rs6518994	ApoL-IV	1.00	0.19	1.00	0.03 (0.04)	0.43
rs2010499	ApoL-II	1.00	0.30	0.60	-0.02 (0.03)	0.43
rs713929	ApoL-I	1.00	0.21	1.00	0.00 (0.03)	0.96
rs2239785	ApoL-I	1.00	0.21	1.00	0.00 (0.03)	0.93
rs136174	ApoL-I	1.00	0.21	1.00	0.00 (0.03)	0.96
rs136175	ApoL-I	1.00	0.21	1.00	0.00 (0.03)	0.96
rs136177	ApoL-I	1.00	0.21	1.00	0.00 (0.03)	0.96
rs4821475	Int.	1.00	0.36	0.34	0.04 (0.03)	0.17
rs735853	MYH9	1.00	0.45	0.30	-0.01 (0.03)	0.81

ApoL: Apolipoprotein L; RBM9: RNA Binding Protein 9; MYH9: Myosin 9; Int.: Intergenic

Hardy-Weinberg *P*-value calculated using an exact test. Values adjusted for age and sex.

Italicized SNPs removed from further analysis due to deviations from Hardy-Weinberg expectations or low call rate.

to affect TG levels at a nominal significance level of $P = 0.05$ (data not shown), however, five SNPs were nominally associated with HDL levels (Table 2).

These five SNPs were then analysed in the Finnish sample (Table 3). The SNP most significantly associated with HDL in ERF, rs132734 in ApoL-IV, did not affect HDL levels in the Finnish, nor did an intergenic SNP, rs5755936, located 3' of the ApoL-V gene. Three other SNPs, rs2076671, rs2073198, rs2076673, located in the ApoL-V gene, were marginally significant in the Finns. The direction of these associations was concordant with that observed in the Dutch

Table 3: Associations Between Selected SNPs and Plasma Lipid Outcomes

A. High-Density Lipoprotein Cholesterol (HDL)

SNP	Gene	Netherlands		Finland		Combined	
		β (s.e.)	*P*	β (s.e.)	*P*	β (s.e.)	*P*
rs132734	ApoL-IV	-0.092 (0.028)	0.001	0.005 (0.019)	0.791	-0.041 (0.017)	0.018
rs2076671	ApoL-V	0.067 (0.031)	0.034	0.038 (0.021)	0.071	0.052 (0.019)	0.006
rs2073198	ApoL-V	0.067 (0.031)	0.034	0.040 (0.021)	0.057	0.053 (0.019)	0.005
rs2076673	ApoL-V	0.063 (0.031)	0.044	0.040 (0.021)	0.057	0.051 (0.019)	0.007
rs5755936	Int.	-0.053 (0.028)	0.050	-0.002 (0.019)	0.908	-0.026 (0.017)	0.123

B. Triglycerides (TG)

SNP	Gene	Netherlands		Finland		Combined	
		β (s.e.)	*P*	β (s.e.)	*P*	β (s.e.)	*P*
rs132734	ApoL-IV	0.028 (0.052)	0.584	0.026 (0.041)	0.521	0.027 (0.033)	0.422
rs2076671	ApoL-V	-0.082 (0.056)	0.143	-0.089 (0.045)	0.049	-0.086 (0.036)	0.019
rs2073198	ApoL-V	-0.082 (0.056)	0.143	-0.091 (0.045)	0.044	-0.087 (0.036)	0.017
rs2076673	ApoL-V	-0.069 (0.056)	0.219	-0.091 (0.045)	0.044	-0.079 (0.036)	0.030
rs5755936	Int.	-0.003 (0.048)	0.952	0.032 (0.041)	0.438	0.009 (0.032)	0.783

C. TG/HDL Ratio

SNP	Gene	Netherlands		Finland		Combined	
		β (s.e.)	*P*	β (s.e.)	*P*	β (s.e.)	*P*
rs132734	ApoL-IV	0.120 (0.066)	0.071	0.020 (0.049)	0.687	0.067 (0.042)	0.108
rs2076671	ApoL-V	-0.150 (0.072)	0.040	-0.128 (0.054)	0.019	-0.138 (0.046)	0.003
rs2073198	ApoL-V	-0.150 (0.072)	0.040	-0.132 (0.054)	0.015	-0.140 (0.046)	0.002
rs2076673	ApoL-V	-0.132 (0.072)	0.068	-0.132 (0.054)	0.015	-0.130 (0.046)	0.004
rs5755936	Int.	0.050 (0.062)	0.421	0.036 (0.050	0.478	0.035 (0.041)	0.390

ApoL: Apolipoprotein L; β: Regression coefficient; s.e.: standard error; *P*: *P*-value.

Netherlands adjusted for age and sex; Finland adjusted for sex; Combined adjusted for age, sex and population.

population, and of the same order of magnitude. In a combined analysis of the Dutch and Finnish samples, these ApoL-V SNPs were more significant (Table 3A).

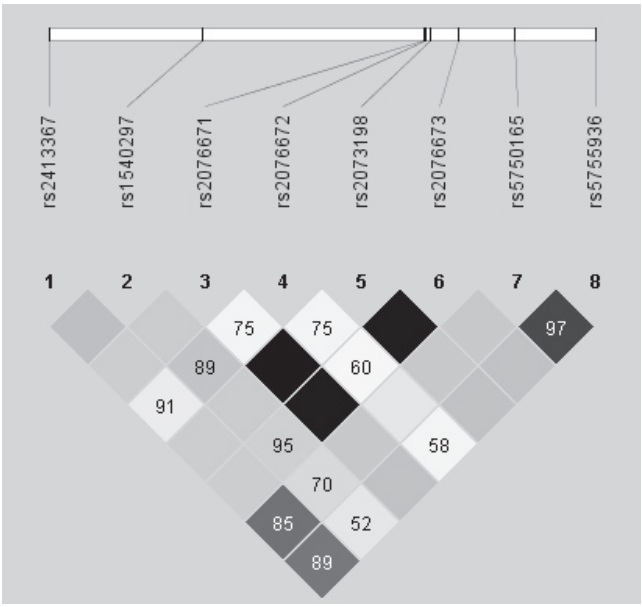
In terms of TG levels, these variants reached nominal significance in the Finnish and in the combined analysis. Although not significant in the Dutch, the effects were of similar magnitudes, and in the expected direction, in both populations and overall (Table 3B).

TG/HDL ratio was consistently associated with two SNPs in both populations and in combined analysis. Again, these effects were comparable in magnitude and direction (Table 3C) and reinforce the associations observed for HDL and TG.

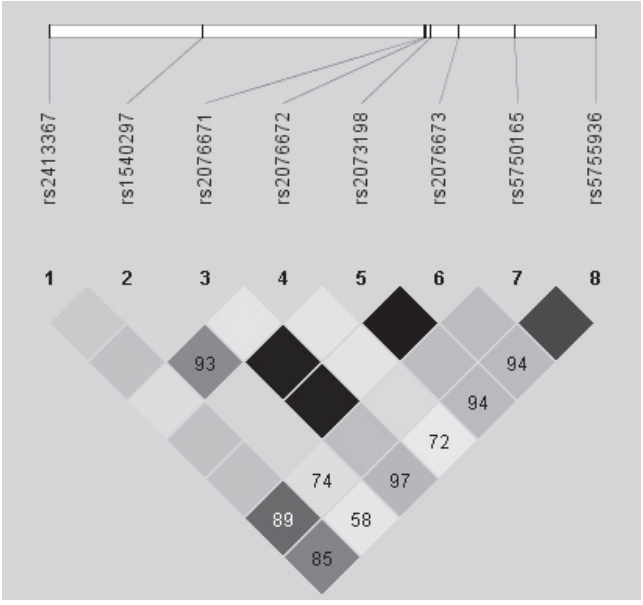
Linkage disequilibrium patterns were generally similar in the two populations (Figure 1). Haplotype analysis, using all SNPs in the ApoL-V gene, as well as one intergenic SNP on both the 5′ and 3′ ends, revealed similar patterns to those observed in the single SNP analyses (Table 4). The B haplotype was strongly associated with HDL levels in the Dutch sample ($P_{\text{simulated}}$ [P_s] = 0.008) and in the combined sample (P_s = 0.003) and marginally in the Finnish (P_s = 0.080).

Figure 1: Linkage disequilibrium patterns in the studied populations

A. ERF Population



B. Finland Population



Number indicate pair wise D'. Color saturation indicates strength of correlation (solid black: $R^2 = 1$; solid white: $R^2 = 0$).

Table 4: ApoL-V Haplotypes and Plasma Lipid Levels

	Netherlands				Finland				Combined			
Haplotype	Frequency	HDL	TG	TG/HDL	Frequency	HDL	TG	TG/HDL	Frequency	HDL	TG	TG/HDL
A (2-1-1-1-1-1-2-2)	0.317	0.494	0.800	0.605	0.345	0.585	0.078	0.088	0.331	0.382	0.160	0.156
B (1-1-2-1-2-2-1-1)	0.274	0.008	0.347	0.062	0.309	0.080	0.062	0.028	0.291	0.003	0.042	0.004
C (1-2-1-1-1-1-1-1)	0.166	0.929	0.811	0.804	0.066	0.087	0.103	0.425	0.112	0.353	0.603	0.959
D (1-2-1-2-1-1-1-1)	0.114	0.376	0.333	0.728	0.153	0.501	0.124	0.126	0.134	0.729	0.137	0.295
E (1-2-1-1-1-1-2-2)	0.033	0.011	0.786	0.174	NA	NA	NA	NA	0.028	0.132	0.271	0.787
F (1-2-1-2-1-1-1-2)	NA	NA	NA	NA	0.035	0.559	0.984	0.830	0.029	0.988	0.418	0.493
Overall		0.005	0.832	0.458		0.342	0.105	0.165		0.022	0.168	0.114

NA: Haplotype not estimated in that population.

P-values are simulated by permutation.

Netherlands adjusted for age and sex; Finland adjusted for sex; Combined adjusted for age, sex and population.

The same haplotype was associated with TG levels in the combined sample (P_s = 0.042) and nearly so in the Finns (P_s = 0.062). Although non-significant in the Dutch, the effect was of similar magnitude and in the proper direction. TG/HDL ratio and haplotype B were also associated, marginally in the Dutch (P_s = 0.062) and more strongly in the Finns (P_s = 0.028) and in combined analysis (P_s = 0.004).

DISCUSSION

In this study of ApoL gene cluster variation in Dutch and Finnish samples, SNPs in the ApoL-V gene were associated with fasting plasma HDL and TG levels, and TG/HDL ratio. The most consistent of these relationships were observed for TG/HDL ratio. The minor alleles were associated with modest (~ 0.05 mmol/L) increases in HDL, TG decreases of approximately 0.10 mmol/L, and decreases in TG/HDL ratio of 0.12. Haplotype analysis largely confirmed the single SNP findings. The B haplotype was associated with increased HDL, decreased TG, and decreased TG/HDL ratio.

Although this evidence, taken as a whole, is quite suggestive, there are some differences. In the Netherlands population, none of the SNPs, or haplotypes, were significantly associated with TG levels, while in the Finnish population, the associations with HDL levels were marginal. Despite this, the associations were very similar in terms of estimates of effect size. Combined analysis, and the analysis of TG/HDL ratio, strongly suggests an influence of ApoL-V variation on plasma lipids, detectable in the general population. Indeed, in combined analyses, all three outcomes (HDL, TG, and TG/HDL ratio) reached more significant levels.

A possible reason for these differences arises from the very different nature of the two study populations. Both come from genetically isolated populations, and have somewhat different patterns of linkage disequilibrium.[14] In the ApoL-V gene, the Finnish sample generally, although not always, exhibited stronger SNP-SNP correlations. This implies the possibility that a causative mutation, or mutations, either in the ApoL-V gene or elsewhere in the region, may be more tightly linked to the tested variants in one population than the other. This seems unlikely, however, as similar haplotypes were estimated in both populations, and the LD patterns were much more similar than different.

More importantly, the large disparity in age between the two populations may influence the effect of these allelic variants on lipid levels. A previous study of several variants known to affect lipid levels (including mutations in the hepatic lipase, lipoprotein lipase, and cholesteryl ester transfer protein genes), in parents and their offspring, demonstrated substantial differences between the adults and children.[20] The results from the current study demonstrate similar discrepancies.

Although not much is known regarding the function of ApoL proteins, other than ApoL-I, there is reason to suspect their involvement in lipid metabolism and transport. In addition to

the previously observed correlations between secreted ApoL-I levels and plasma lipids, there is evidence suggesting that ApoL-like proteins are expressed in both normal and atherosclerotic endothelial cells,[21] as well as other tissues known to be involved in lipid metabolism and transport, including pancreas and liver.[8] Further, structural features of the genes and proteins suggest similarities to other apolipoproteins. ApoL-V, specifically, is thought to be a cytoplasmic protein.[8] This implies that it may be involved in intracellular lipid metabolism.

The association of ApoL-I with both HDL and VLDL particles further suggests the potential involvement of ApoLs in the TG-HDL axis,[22] perhaps by facilitating the CETP-mediated pathway that exchanges TG from TG-rich particles, such as VLDL, for HDL cholesteryl esters.[23] This process leads to more rapid catabolism of TG-enhanced HDL;[24] if ApoL-V enhances this process, it would explain the associations observed here.

The SNPs studied here were did not provide optimal coverage of portions of the ApoL cluster, making it impossible to rule out other potential associations between members of the cluster and plasma lipids. However, these results suggest that ApoL-V is involved in determining HDL and TG levels.

In summation, variants in the ApoL-V gene were nominally associated with increased HDL, decreased TG, and, most consistently, with decreased TG/HDL ratio. Haplotype analysis of the gene showed concordant results, with the B haplotype demonstrating consistent association. In combined analyses, all three SNPs were significantly associated with all three outcome measures.

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Chapter 3

Genome-wide Studies

Chapter 3.1

Genome-Wide Association Analysis of Plasma Lipids Determines Novel Loci Affecting Total Cholesterol, Triglycerides and Triglyceride/HDL Ratio: the ERF Study

ABSTRACT

Objectives: It is presumed that many unknown genetic variations contribute to circulating lipid levels. A genome-wide association analysis was conducted to determine genes involved in influencing plasma total cholesterol (TC), high-density lipoprotein cholesterol (HDL), low-density lipoprotein cholesterol (LDL), triglycerides (TG), TC/HDL ratio, and TG/HDL ratio.

Design: 197 members of the Erasmus Rucphen Family (ERF) Study were genotyped with the Affymetrix 250K Nsp array from the GeneChip® Human Mapping 500K Array Set. The association between SNPs and fasting plasma lipids was assessed using the GenABEL R library. Genome-wide significance was determined using whole genome permutation. General linear models and haplo.stats were used to evaluate SNPs and haplotypes in regions flanking the genome-wide significant SNPs.

Results: Three SNPs reached genome-wide significance for TC levels, although two were in perfect LD ($R^2 = 1$) ($P_{\text{Genome Wide}}$ [P_{GW}] = 0.012 and P_{GW} = 0.020). These SNPs were located 5′ of the ERR3 gene. A number of other SNPs in the region were nominally significant, and sliding-window haplotype analysis revealed an extended region surrounding these polymorphisms. A fourth SNP reached genome-wide significance for both TG and TG/HDL ratio (P_{GW} = 0.042 and P_{GW} = 0.041). Again, surrounding SNPs and haplotypes were significant, although this region is largely uncharacterized.

Conclusions: There is promising evidence to support the involvement of ERR3 in determining plasma TC levels. This gene codes for a nuclear orphan receptor; other members of this protein family are known to be involved in lipid levels. The TG region occurs in a putative processed transcript site and will require further study.

Plasma lipid levels, including total cholesterol (TC), high- and low-density lipoprotein cholesterol (HDL and LDL), and triglycerides (TG), are important determinants of cardiovascular disease. Long accepted treatment guidelines recommend aggressive lowering of LDL levels,[1] while increasing focus is being paid to HDL as a potentially valuable target of therapy.[2] Elevated TG levels are another important risk factor for cardiovascular disease, comprising one aspect of atherogenic dyslipidemia on the "TG-HDL axis", which also includes small, dense LDL,[3] and is increasingly being viewed as an independent risk factor.[4]

Lipid levels are known to be highly heritable, which indicates a substantial genetic contribution to the variation of these traits.[5] Considerable effort has been expended trying to locate the genes contributing to plasma lipid variation, with varying degrees of success. Some rare variants leading to genetic lipid disorders, such as the LDL receptor mutations leading to familial hypercholesterolemia[6] and the genetic CETP deficiencies leading to hyperalphalipoproteinemia,[7] were previously detected. Additionally, a number of variants affecting lipids in the general population were identified (such as in the genes coding for hepatic lipase, lipoprotein lipase, Apolipoprotein A-I, cholesteryl ester transfer protein, etc.), but many of the genes influencing these traits are currently unknown.

Most previous attempts to locate important genes have relied on either candidate gene approaches, which require a strong *a priori* hypothesis, or linkage analysis, which is typically most powerful in the case of rare variants with strong effects. Recent advances in high-throughput, dense genotyping,[8] however, are allowing for a new type of analysis: genome-wide association. This type of analysis may offer substantial benefits in terms of identifying common variants[9,10] that are likely to explain a substantial portion of the variation of lipid levels in the general population.

Using genetic isolates in association studies offers several advantages, compared to outbred populations. These include a reduction in genetic complexity for rare variants, a reduction in environmental variability, and an increased extent of linkage disequilibrium (LD).[11,12] The longer stretches of LD make it possible to conduct genome-wide association studies with fewer markers than would be necessary in a general population,[13] while reduced genetic heterogeneity simplifies the task of identifying associated variants.

In the current study, a genome-wide analysis of the relationship between plasma lipids, including TC, HDL, LDL, TG, TC/HDL ratio, and TG/HDL ratio, and genetic variation was conducted. Approximately 250,000 single nucleotide polymorphisms (SNPs) were genotyped in 197 members of a recent genetic isolate from the Netherlands for whom lipid data was available.

MATERIALS AND METHODS

Study Population

197 individuals from the Erasmus Ruchpen Family (ERF) population were selected for this study. This population, which was previously described, is a recent genetic isolate in the southwestern Netherlands.[5] Briefly, this population was founded in the middle of the 18th century by approximately 150 individuals and was isolated until the last few decades. Characterized by rapid growth and minimal immigration, the isolate now includes approximately 20,000 inhabitants. Twenty couples living in the region in the 19th century were chosen. These couples parented a minimum of 6 children, each of whom was baptized in the latter half of the nineteenth century in the community church. All living descendants of these pairs (as well as their spouses), ascertained on the basis of municipal and baptismal records, were traced and invited to participate ($n \approx 3{,}000$). This population exhibits reduced genetic heterogeneity and increased linkage disequilibrium, compared to general populations.[13,14]

Subjects completed an interview with a physician and a thorough medical examination during a visit to the research center. All participants gave informed consent, and the Medical Ethics Committee of Erasmus Medical Center approved the study protocol.

Laboratory Analysis

Fasting blood samples were collected during the participant's visit to the research center. A Synchron LX20 (Beckman Coulter Inc., Fullerton, CA. U.S.A.) spectrophotometric chemical analyzer was utilized for the determination of plasma lipid values.

Genomic DNA was extracted from whole blood samples drawn at the baseline examination, utilizing the salting out method.[15] The 250K Nsp array from the GeneChip® Human Mapping 500K Array Set (Affymetrix) was utilized to determine genome-wide genotypes. The chips were run and analysed according to the manufacturer's protocols.

Statistical Analysis

Prior to running analyses, genotypes for all autosomal SNPs were screened according to minor allele frequency (< 2.5%) and call rate (> 90%). The resulting SNPs were screened for association with lipid levels, adjusted for age and sex, using the emp.qtscore function of the GenABEL package for R.[16] 1,000 genome-wide permutations allowed for estimation of genome-wide empirical significance levels.

SNPs that reached genome-wide significance were selected for more in-depth analysis. Regions surrounding these SNPs were selected by including SNPs 150,000 base pairs up- and downstream from the identified variants. These regions were more strictly quality controlled

(minor allele frequency > 5%, call rate > 95%, and Hardy-Weinberg exact test $P > 0.05$), and then analyzed using age and sex adjusted generalized linear models. These analyses were also conducted in GenABEL.

Linkage disequilibrium patterns for the selected regions were determined with Haplo.View. Sliding window haplotype analyses were also used to characterize these regions, using two- and five-SNP sliding windows. The haplotypes were estimated with an E-M algorithm and association was tested using Schaid's score test,[17] as implemented in the haplo.stats package for R. The haplotype analyses implemented permutation to estimate empirical significance levels.

RESULTS

Of the 256,554 autosomal SNPs that were genotyped, 45,471 (~ 18%) were excluded due to low minor allele frequency. The vast majority of these were non-polymorphic in this sample. Since a high percentage of low MAF SNPs were observed, the data from the 197 ERF samples were compared with the 120 Hapmap CEU samples to check for potential population effects. Pearson correlations were high (> 0.93) for all chromosomes ($P < 0.0001$). Of the genotypes observed to be non-polymorphic in ERF, 94.9% were also genotyped in Hapmap; 99.7% of these were non-polymorphic in the Hapmap Europeans. An additional 2% of markers were excluded due to low call rates (< 90%).

Baseline characteristics are described in Table 1. Tests for association revealed several SNPs that were significant at the 5% level, genome-wide (Figure 1). Three SNPs on chromosome 1 were significantly associated with plasma TC levels (SNP_A-2125755, P_{Nominal} [P_N] = $1.1\text{x}10^{-7}$, $P_{\text{Genome Wide}}$ [P_{GW}] = 0.012; SNP_A-2296608, $P_N = 2.1\text{x}10^{-7}$, $P_{GW} = 0.020$; and SNP_A-2221449, $P_N = 2.1\text{x}10^{-7}$, $P_{GW} = 0.020$), although the last two of these were perfectly concordant ($R^2 = 1$) adjacent SNPs. An additional SNP, SNP_A-2155094 on chromosome 6, was associated with plasma TG ($P_N = 4.5\text{x}10^{-7}$, $P_{GW} = 0.042$) and TG/HDL ratio ($P_N = 7.0\text{x}10^{-7}$, $P_{GW} = 0.041$). No SNPs reached the level of genome-wide significance for HDL, LDL, or TC/HDL ratio.

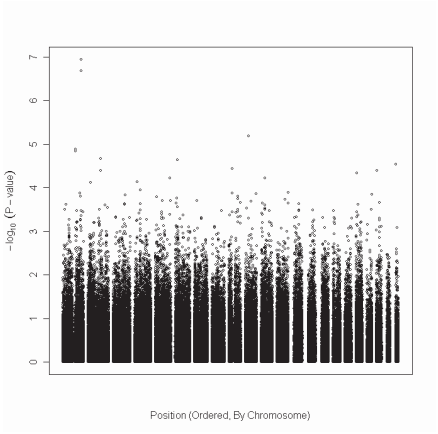
Table 1: Descriptive Statistics

	Mean (S.D.)
Age	31.22 (6.49)
Sex [Male, n (%)]	97 (49.2)
BMI	25.48 (4.60)
TC	5.27 (1.00)
LDL	3.53 (0.93)
HDL	1.26 (0.35)
TG	1.14 (0.69)
TC/HDL	4.44 (1.35)

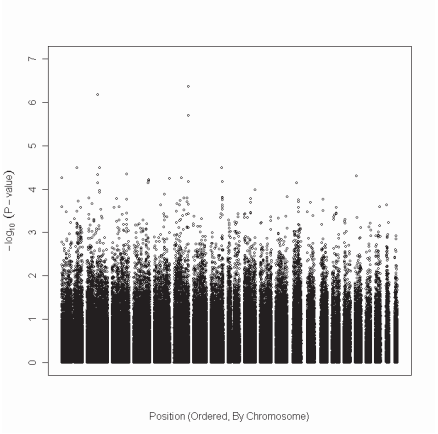
Values for continuous variables are presented as mean (SD).

Figure 1: Genome-wide analysis of lipid levels

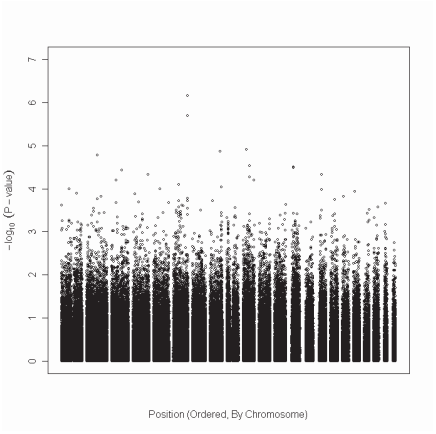
A. Total Cholesterol



B. Triglycerides



C. TG/HDL Ratio



Points are plotted as $-\log_{10}$ (*P*-value) *vs.* ordered positions.

SNPs flanking those significant at the genome-wide level were selected for further analysis. In the chromosome 1 region originally associated with total cholesterol, 34 SNPs, including the reference SNPs, were selected. A total of three were eliminated due to call rates below 95% or for deviations from Hardy-Weinberg ($P_{exact} < 0.05$). The remaining 31 SNPs were tested for association with generalized linear regression models. Several SNPs flanking the index SNPs were also nominally associated with TC. Additionally, these SNPs were associated with LDL, and, in some instances, with HDL (Table 2).

The region surrounding the chromosome 6 SNP initially associated with TG and TG/HDL ratio contained 46 SNPs. Of these, seven were removed from further consideration due to

Table 2: Chromosome 1 region containing SNPs that reached genome-wide significance for TC levels

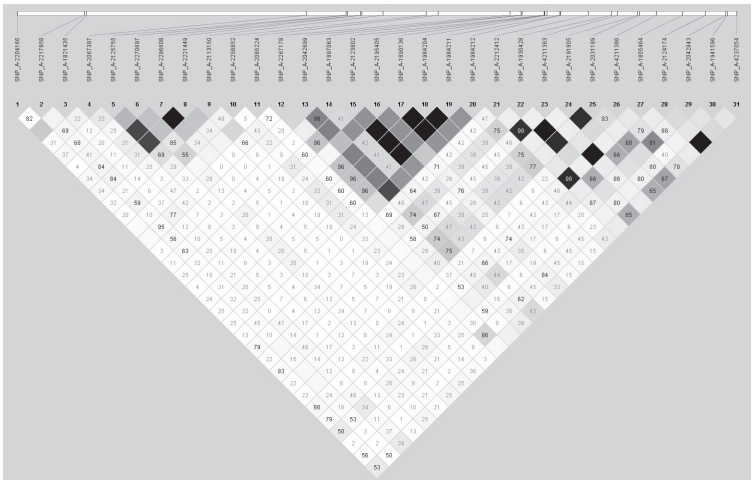
Affy. #	Rs #	Gene	Position	TC β	TC *P*	LDL β	LDL *P*	HDL β	HDL *P*	TG β	TG *P*	TC/HDL β	TC/HDL *P*	TG/HDL β	TG/HDL *P*
SNP_A-2204166	rs12030122	USH2A	212893863	-0.3460	0.0286	-0.3862	0.0070	0.0350	0.3815	-0.0443	0.6165	-0.4578	0.0190	-0.0794	0.4460
SNP_A-2217958	rs17026598	USH2A	212920677	-0.2284	0.2507	-0.1552	0.3949	-0.0633	0.2043	0.0671	0.5463	0.0952	0.7021	0.1304	0.3181
SNP_A-1821435	rs6604658	USH2A	212921519	0.1800	0.0741	0.1777	0.0528	-0.0008	0.9754	-0.0261	0.6423	0.1772	0.1559	-0.0253	0.7017
SNP_A-2067397	rs12043707	Int.	213009555	0.3267	0.0003	0.2812	0.0007	-0.0077	0.7466	0.1313	0.0109	0.2944	0.0099	0.1390	0.0224
SNP_A-2125755	rs6697616	Int.	213025345	-0.5103	0.0000	-0.4060	0.0000	-0.0578	0.0151	-0.1126	0.0327	-0.1612	0.1745	-0.0548	0.3817
SNP_A-2270697	rs4540735	Int.	213026045	0.3223	0.0020	0.2936	0.0020	0.0197	0.4620	0.0444	0.4523	0.1825	0.1653	0.0247	0.7223
SNP_A-2296608	rs4520477	Int.	213031092	0.4752	0.0000	0.3587	0.0000	0.0656	0.0037	0.1059	0.0357	0.1050	0.3556	0.0403	0.5013
SNP_A-2221449	rs4579828	Int.	213031765	0.4752	0.0000	0.3587	0.0000	0.0656	0.0037	0.1059	0.0357	0.1050	0.3556	0.0403	0.5013
SNP_A-2113150	rs7411184	Int.	213040199	0.3527	0.0007	0.2064	0.0330	0.0609	0.0219	0.1007	0.0879	0.0086	0.9482	0.0399	0.5686
SNP_A-2258852	rs12041726	Int.	213057433	-0.1839	0.0708	-0.1618	0.0804	-0.0314	0.2194	0.0063	0.9111	-0.0264	0.8354	0.0377	0.5677
SNP_A-2085224	rs11806070	ERR3	213062217	0.3754	0.0516	0.2275	0.1946	0.0887	0.0659	0.0547	0.6071	-0.0634	0.7919	-0.0340	0.7858
SNP_A-2267178	rs4846514	ERR3	213091080	0.0785	0.5975	-0.0728	0.5913	0.0101	0.7869	0.2153	0.0077	0.0602	0.7436	0.2052	0.0321
SNP_A-2042699	rs1833036	ERR3	213095407	0.0778	0.4039	0.0332	0.6961	0.0129	0.5811	0.0535	0.2990	-0.0057	0.9604	0.0406	0.5036
SNP_A-1897063	rs6604631	ERR3	213095914	0.0702	0.4879	0.0633	0.4929	0.0162	0.5283	-0.0177	0.7480	-0.0477	0.7058	-0.0339	0.6049
SNP_A-2123802	rs12145418	ERR3	213104715	0.0166	0.8727	0.0104	0.9122	-0.0148	0.5686	0.0325	0.5710	0.1028	0.4221	0.0473	0.4834
SNP_A-2195405	rs12757147	ERR3	213104890	-0.0058	0.9628	0.0765	0.5002	-0.0230	0.4611	-0.0964	0.1600	0.1024	0.5060	-0.0734	0.3646
SNP_A-1890136	rs12757165	ERR3	213104932	0.0193	0.8530	0.0159	0.8668	-0.0143	0.5844	0.0287	0.6137	0.1039	0.4198	0.0430	0.5208
SNP_A-1984204	rs4147271	ERR3	213105733	0.0166	0.8727	0.0104	0.9122	-0.0148	0.5686	0.0325	0.5710	0.1028	0.4221	0.0473	0.4834
SNP_A-1984211	rs10495027	ERR3	213111017	0.0166	0.8727	0.0104	0.9122	-0.0148	0.5686	0.0325	0.5710	0.1028	0.4221	0.0473	0.4834
SNP_A-1984212	rs6604635	ERR3	213111245	-0.1096	0.2466	-0.0683	0.4292	-0.0163	0.4931	-0.0507	0.3337	0.0032	0.9785	-0.0344	0.5778
SNP_A-2212412	rs17042848	ERR3	213122396	0.1709	0.1905	0.1741	0.1423	0.0059	0.8569	-0.0188	0.7967	0.1216	0.4502	-0.0247	0.7730
SNP_A-1930428	rs11117625	ERR3	213131659	-0.0557	0.6216	-0.0363	0.7243	0.0160	0.5724	-0.0885	0.1544	-0.0966	0.4888	-0.1045	0.1532
SNP_A-4211383	rs10495030	ERR3	213133994	-0.2153	0.0889	-0.2267	0.0498	-0.0046	0.8864	0.0376	0.5972	-0.1591	0.3162	0.0422	0.6144
SNP_A-2191805	rs10863257	ERR3	213141996	-0.0557	0.6216	-0.0363	0.7243	0.0160	0.5724	-0.0885	0.1544	-0.0966	0.4888	-0.1045	0.1532
SNP_A-2031189	rs17669502	ERR3	213142948	0.0432	0.7020	0.0298	0.7725	-0.0167	0.5554	0.0756	0.2251	0.0838	0.5482	0.0922	0.2082
SNP_A-4211386	rs10218694	ERR3	213149502	-0.1144	0.2822	-0.0695	0.4745	0.0123	0.6441	-0.1308	0.0249	-0.1809	0.1684	-0.1431	0.0374
SNP_A-1805464	rs1833039	ERR3	213160044	0.1682	0.1979	0.1865	0.1162	0.0034	0.9186	-0.0414	0.5697	0.1205	0.4570	-0.0448	0.6015
SNP_A-2124174	rs11590373	ERR3	213169104	-0.2169	0.0421	-0.1852	0.0572	-0.0263	0.3290	-0.0060	0.9200	-0.0609	0.6479	0.0203	0.7725
SNP_A-2042843	rs1498276	ERR3	213177322	-0.0085	0.9379	-0.0344	0.7305	-0.0284	0.2993	0.1257	0.0359	0.1277	0.3444	0.1541	0.0286
SNP_A-1941596	rs6673703	ERR3	213177958	-0.0529	0.6521	-0.0406	0.7045	-0.0371	0.2053	0.0597	0.3572	0.1453	0.3161	0.0968	0.2038
SNP_A-4237054	rs11117633	ERR3	213181714	-0.2619	0.0140	-0.2293	0.0164	-0.0311	0.2652	0.0119	0.8388	-0.0772	0.5671	0.0429	0.5411

Values adjusted for age and sex.

low minor allele frequencies (< 5%), low call rates (< 95%) or deviations from Hardy-Weinberg expectations ($P_{exact} < 0.05$). GLM-based results for the remaining 39 SNPs are presented in Table 3. A number of SNPs were nominally associated with TG levels. These associations were generally stronger when looking at the TG/HDL ratio. Additionally, some SNPs were marginally associated with HDL.

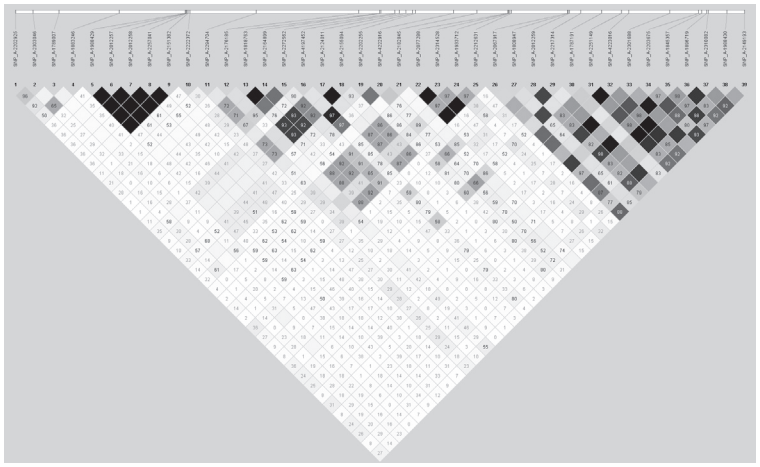
Linkage disequilibrium was assessed for both the chromosome 1 (Figure 2) and chromosome 6 (Figure 3) regions. The chromosome 1 SNPs that were significant fell into a strong LD block located just 5′ of the ERR3 gene. A second strong block, encompassing the gene itself, was also observed. In the chromosome 6 region, three blocks were seen. The significant single

Figure 2: Linkage disequilibrium structure for chromosome 1 region



Color density is R^2 (Black: $R^2 = 1$; White: $R^2 = 0$) and numbers are D′.

Figure 3: Linkage disequilibrium structure for chromosome 6 region



Color density is R^2 (Black: $R^2 = 1$; White: $R^2 = 0$) and numbers are D′.

Table 3: Chromosome 6 region containing SNPs that reached genome-wide significance for TG and TG/HDL ratio

Affy. #	Rs #	Gene	Position	TG β	TG *P*	TG/HDL β	TG/HDL *P*	HDL β	HDL *P*	TC β	TC *P*	LDL β	LDL *P*	TC/HDL β	TC/HDL *P*
SNP_A-2202925	rs4574608	Int.	163991814	0.0465	0.3719	0.0664	0.2781	-0.0199	0.3981	0.0431	0.6473	0.0201	0.8153	0.1598	0.1683
SNP_A-2303046	rs9364695	Int.	163997316	-0.0996	0.1076	-0.1334	0.0664	0.0339	0.2338	-0.0195	0.8647	0.0101	0.9224	-0.2228	0.1123
SNP_A-1789007	rs6455908	Int.	164005554	-0.0706	0.3691	-0.0852	0.3570	0.0146	0.6819	-0.0056	0.9685	-0.0090	0.9446	-0.0694	0.6934
SNP_A-1883246	rs6942363	Int.	164024496	-0.0601	0.3143	-0.1096	0.1174	0.0495	0.0633	-0.0410	0.7016	-0.0580	0.5526	-0.2755	0.0371
SNP_A-1988429	rs1407658	Int.	164045253	0.1048	0.3663	0.1719	0.2072	-0.0670	0.2006	-0.0107	0.9594	0.0480	0.8023	0.3883	0.1328
SNP_A-2012257	rs1407659	Int.	164045394	0.0816	0.4903	0.1611	0.2471	-0.0795	0.1358	-0.0255	0.9054	0.0588	0.7638	0.4260	0.1061
SNP_A-2012258	rs1407660	Int.	164045505	-0.1431	0.2220	-0.2030	0.1423	0.0599	0.2627	-0.0345	0.8729	-0.0671	0.7329	-0.4064	0.1238
SNP_A-2257041	rs4709002	Int.	164045651	-0.1067	0.3572	-0.1772	0.1954	0.0705	0.1786	0.0195	0.9257	-0.0398	0.8336	-0.3952	0.1261
SNP_A-2151382	rs2146464	Int.	164046022	0.1048	0.3663	0.1719	0.2072	-0.0670	0.2006	-0.0107	0.9594	0.0480	0.8023	0.3883	0.1328
SNP_A-2222372	rs9458879	Int.	164046693	0.0339	0.5273	0.0485	0.4429	-0.0145	0.5547	-0.1167	0.2345	-0.1307	0.1417	-0.0143	0.9065
SNP_A-2294704	rs1475161	Int.	164067512	-0.1291	0.0215	-0.1501	0.0230	0.0210	0.4134	-0.0611	0.5524	-0.0140	0.8811	-0.1419	0.2630
SNP_A-2176185	rs9365600	Int.	164099769	-0.2128	0.0054	-0.2682	0.0028	0.0554	0.1147	-0.0839	0.5520	-0.0473	0.7131	-0.3077	0.0756
SNP_A-1818763	rs4709747	Int.	164106354	-0.1828	0.0003	-0.2259	0.0001	0.0430	0.0639	-0.0576	0.5383	-0.0258	0.7620	-0.2363	0.0390
SNP_A-2164899	rs9456881	Int.	164106700	0.1774	0.0006	0.2238	0.0002	-0.0464	0.0471	0.0304	0.7490	0.0076	0.9309	0.2210	0.0554
SNP_A-2272562	rs9347780	Int.	164111102	-0.1516	0.0054	-0.1892	0.0031	0.0376	0.1328	-0.0064	0.9491	0.0099	0.9142	-0.1495	0.2269
SNP_A-4197452	rs6931768	Int.	164112524	-0.1749	0.0259	-0.2095	0.0234	0.0346	0.3357	-0.1507	0.2930	-0.0889	0.4974	-0.3223	0.0675
SNP_A-2124011	rs7753445	Int.	164114575	-0.2489	0.0000	-0.2928	0.0000	0.0439	0.0617	-0.1405	0.1367	-0.0742	0.3904	-0.3138	0.0064
SNP_A-2155094	rs7740431	Int.	164116590	0.2666	0.0000	0.3091	0.0000	-0.0424	0.0748	0.1653	0.0806	0.0887	0.3083	0.3313	0.0043
SNP_A-2202255	rs9458892	Int.	164117628	-0.1531	0.0533	-0.1899	0.0400	0.0369	0.3044	-0.1437	0.3155	-0.1064	0.4130	-0.3389	0.0486
SNP_A-4222916	rs1570409	Int.	164129544	-0.0209	0.7809	-0.0151	0.8642	-0.0057	0.8660	-0.0620	0.6483	-0.0483	0.6966	-0.0299	0.8577
SNP_A-2182845	rs6938954	Int.	164136944	-0.2525	0.0305	-0.2844	0.0387	0.0319	0.5504	-0.3403	0.1086	-0.2215	0.2538	-0.4899	0.0612
SNP_A-2077288	rs6928576	Int.	164146677	-0.1309	0.0316	-0.1611	0.0245	0.0302	0.2796	0.0976	0.3786	0.1213	0.2292	-0.0611	0.6565
SNP_A-2314528	rs6902153	Int.	164146867	-0.1370	0.0212	-0.1678	0.0163	0.0308	0.2567	0.0928	0.3931	0.1176	0.2343	-0.0684	0.6115
SNP_A-1933712	rs9356140	Int.	164147353	-0.0747	0.1612	-0.0614	0.3269	-0.0133	0.5835	-0.0129	0.8942	0.0447	0.6124	0.0330	0.7846
SNP_A-2212631	rs10945918	Int.	164147732	-0.1370	0.0212	-0.1678	0.0163	0.0308	0.2567	0.0928	0.3931	0.1176	0.2343	-0.0684	0.6115
SNP_A-2067917	rs9364701	Int.	164165395	0.1033	0.0490	0.1286	0.0371	-0.0253	0.2902	0.0588	0.5388	0.0566	0.5168	0.1557	0.1869
SNP_A-1808947	rs6908861	Int.	164165695	-0.0735	0.2470	-0.0828	0.2683	0.0093	0.7481	-0.0986	0.3894	-0.0856	0.4120	-0.1191	0.4022
SNP_A-2012259	rs6455921	Int.	164165980	-0.0398	0.4617	-0.0617	0.3316	0.0219	0.3699	-0.0192	0.8446	-0.0360	0.6862	-0.1132	0.3485
SNP_A-2217314	rs10945921	Int.	164169635	-0.0230	0.6790	-0.0529	0.4177	0.0299	0.2323	0.0030	0.9763	-0.0351	0.7011	-0.1213	0.3275
SNP_A-1787191	rs6922730	Int.	164176158	0.1190	0.0709	0.1254	0.1067	-0.0064	0.8326	0.1622	0.1759	0.1143	0.2971	0.1504	0.3111
SNP_A-2251149	rs1111705	Int.	164182384	-0.0892	0.0865	-0.1168	0.0560	0.0276	0.2427	-0.0983	0.2982	-0.1005	0.2427	-0.1870	0.1080
SNP_A-4223016	rs7765258	Int.	164184707	0.0883	0.0942	0.1176	0.0571	-0.0293	0.2163	0.1054	0.2615	0.1091	0.1997	0.1974	0.0843
SNP_A-2301688	rs6455922	Int.	164197635	-0.0230	0.6790	-0.0529	0.4177	0.0299	0.2323	0.0030	0.9763	-0.0351	0.7011	-0.1213	0.3275
SNP_A-2203675	rs9347792	Int.	164206673	-0.0862	0.1018	-0.1177	0.0569	0.0316	0.1866	-0.0378	0.6935	-0.0541	0.5358	-0.1578	0.1812
SNP_A-1845357	rs10945924	Int.	164207648	0.0278	0.6132	0.0550	0.3948	-0.0272	0.2730	0.0064	0.9485	0.0388	0.6683	0.1188	0.3328
SNP_A-1896719	rs9347793	Int.	164209177	-0.0886	0.0869	-0.1162	0.0564	0.0276	0.2443	-0.0980	0.3006	-0.1004	0.2445	-0.1866	0.1094
SNP_A-2310082	rs9355439	Int.	164209686	0.0900	0.0796	0.1166	0.0531	-0.0267	0.2530	0.0825	0.3768	0.0790	0.3528	0.1705	0.1381
SNP_A-1988430	rs6935481	Int.	164215559	0.0241	0.6483	0.0510	0.4118	-0.0269	0.2597	-0.0166	0.8624	0.0121	0.8898	0.0978	0.4074
SNP_A-2149193	rs9364703	Int.	164221085	-0.0876	0.0956	-0.1268	0.0396	0.0392	0.0995	-0.0103	0.9144	-0.0258	0.7670	-0.1576	0.1810

Values adjusted for age and sex.

SNPs were located in a block of moderately high LD, which was flanked on either side by blocks of somewhat stronger LD.

Sliding window haplotype analysis reinforced the previous observations of both single SNP association and LD patterns (Figure 4). In the chromosome 1 region, a sustained peak covering 7 (2 SNP windows) or 9 (5 SNP windows) SNPs reached statistical significance. These peaks covered the area observed to have high LD and were, in several cases, highly significant ($P < 1 \times 10^{-7}$).

In the chromosome 6 region, haplotypes again showed broad peaks overlying the central LD block in Figure 3. Associations with TG were significant for 7 (2 SNP window) or 10 (5 SNP window) consecutive haplotypes. A similar pattern existed for TG/HDL ratio; these peaks actually covered a slightly broader region.

DISCUSSION

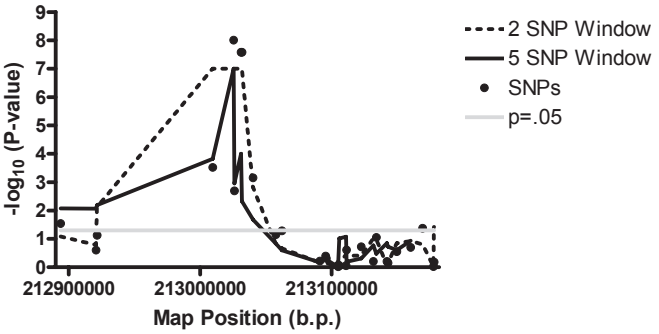
In this genome-wide association study of plasma lipid levels, several variants were found which reached genome-wide significance levels. Two SNPs (plus a third perfectly correlated to one of the two) closely spaced on chromosome 1 were significantly associated with plasma TC. Another SNP, located on chromosome 6, was significantly associated with TG and TG/HDL ratio. These findings were strongly reinforced by haplotype analysis of the flanking regions. These may represent novel loci involved in the determination of circulating lipid levels.

Previous studies reported evidence for linkage of LDL and LDL-ApoB to the chromosome 1 region identified here.[18,19] This region is in between the Usher Syndrome type 2A (USH2A) gene and the estrogen receptor-related protein 3 (ERR3) gene. Nine SNPs were nominally associated with TC in this area, while significant haplotypes spanned approximately 120 kb. The most significant SNPs were located close to the 5′ end of ERR3. This is an eminently logical candidate gene, as ERR3 is a member of the nuclear orphan receptor family of proteins. Numerous of these proteins were previously associated with lipid metabolism and transport, including the liver X receptors[20] and the peroxisome proliferator activated receptors (PPAR).[21] These receptors are typically involved in cholesterol homeostasis; variations in such a gene may explain the results observed here.

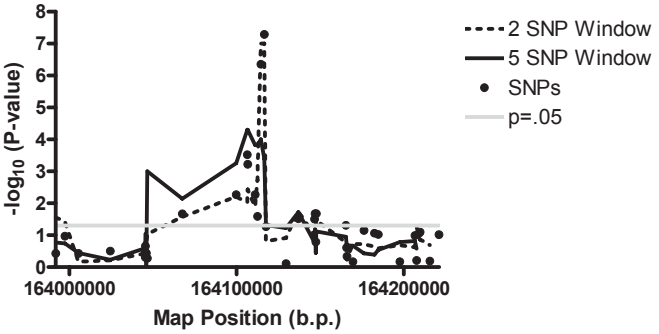
The chromosome 6 region does not offer such an obvious candidate. Although linkage of TG and HDL to chromosome 6 were observed in some studies,[22,23] the loci identified in those studies were several cytogenetic bands removed from the area found here. In the ERF sample, 13 SNPs in the region flanking the index SNP were nominally associated with TG and 15 with TG/HDL ratio. Haplotypes were significant over broad regions. Most of the SNPs tested lie in a putative processed transcript, RP1-230L10.1, about which little is known. The closest known gene, QK1, the homologue of the mouse quaking gene, which is a member of a family of proteins that are involved in signal transduction and activation of RNA (STAR).[24] These proteins

Figure 4: Sliding window haplotype analysis for chromosome 1 and 6 regions (permutation)

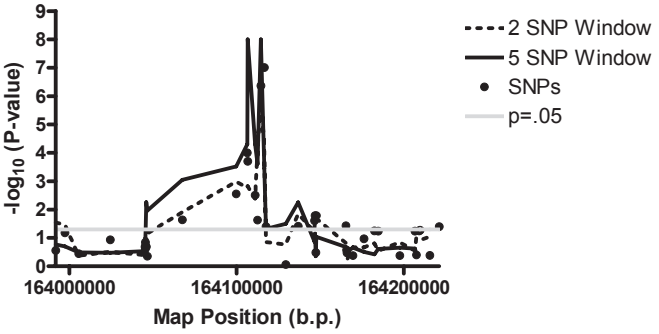
A. Chromosome 1 region – Total Cholesterol



B. Chromosome 6 region – Triglycerides



C. Chromosome 6 region – TG/HDL Ratio



Points are plotted as – $\log_{10}$ (*P*-value) *vs.* ordered positions.

are conserved from yeast to human, and appear to play an important role in development.[25] Their involvement in determining lipid levels may be plausible, given there role in mRNA activation.

A limitation in this type of study is the multiple testing dilemma. In this study, full genome-wide permutation was performed. This helps to limit false positives, and provides an empirical estimate of significance, but may still be quite punitive. In the context of the lipid metabolism system, it is likely that hundreds of genes are involved, which suggests that numerous true significant findings did not reach genome-wide significance, particularly in this relatively small sample of 197 individuals. Another limitation of this study is that no replication cohort was available. Even with multiple testing corrections, the possibility of false positives cannot be overruled.

In summation, in this genome-wide association study of plasma lipid levels, two areas reached genome-wide significance levels. On chromosome 1, SNPs near the ERR3 gene affected TC levels. These SNPs also affected LDL. On chromosome 6, a non-characterized region contained SNPs associated with TG levels. These regions require more in-depth study, especially the chromosome 1 region, which include several significant SNPs and lies under previously identified linkage peaks.

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Chapter 4

Discussion

Chapter 4.1

General Discussion

DISCUSSION

It is generally accepted that a large number of genes, as well as gene-gene and gene-environment interactions, play a role in determining complex phenotypes, such as circulating lipid levels.[1] The complexity of the various lipid pathways suggests that it will require intensive efforts to fully comprehend lipid formation, transport, metabolism and etiological role in clinical outcomes. Despite these overarching intricacies, substantial progress has been made thus far. Further strides will require interdisciplinary input from a wide variety of scientists, including clinical researchers, geneticists and genetic epidemiologists, experimentalists using cell and animal models, and bioinformaticians.

Underlying Genetic Component of Lipid Levels

In chapter 1.2 of this thesis, a study of heritabilities, inbreeding effects, and the well-described Apolipoprotein E (ApoE) ε2/3/4 isoforms[2] was conducted in a genetically isolated population. Statistically significant, and substantial, polygenic contributions to plasma lipid levels were found. Heritabilities ranged from 0.24 for TG to 0.56 for HDL. These findings suggest the presence of common variants that are likely to have individually small effects. These results were of added benefit due to the fact that the population was not selected on the basis of phenotype; in other words, these heritability estimates represent a comparatively unbiased estimate of the proportion of lipid trait variance attributable to additive genetic factors in the general population. The inbreeding analysis demonstrated that a number of rare, recessive alleles are also likely to play a role in determining lipid levels. The role of ApoE, which has a fairly large effect (larger than many other common variants), was estimated to account for a relatively minor portion (ranging from 0.008 for TG to 0.053 for LDL) of lipid variance.

Candidate Gene Analyses

Genetic variations in a large number of genes that were previously identified require further efforts to fully elucidate their functions. Hepatic lipase, an important protein involved in the lipolysis of HDL particles,[3] provides a good example. One variant, the –514 C>T single nucleotide polymorphism (SNP) in the promoter region of the hepatic lipase gene, received considerable attention in the literature, although consistent associations were not observed.[4-9] In chapter 1.3, a meta-analysis of this polymorphism sought to address these inconsistent findings. In an analysis of 25 published reports, the –514C>T SNP was demonstrated to significantly decrease hepatic lipase activity *in vivo* by 5.83 mmol/L·h [95% confidence interval (CI): -8.48, -3.17; $P < 0.001$] in heterozygotes and 11.05 mmol/L·h [95% CI: -14.74, -7.36; $P < 0.001$] in TT homozygotes. This reduction in lipolytic activity, in turn, led to significant increases in HDL levels. These raised HDL levels, 0.04 mmol/liter [95% CI: 0.02, 0.05; $P < 0.001$] and 0.09

mmol/liter [95% CI: 0.07, 0.12; $P < 0.001$] for the CT and TT genotypes, respectively, exhibited an allele-dosage effect and remained unaffected by stratifications for gender, ethnicity, and risk category.

This hepatic lipase promoter SNP was subsequently analyzed in the Rotterdam Study, a prospective follow-up study of an elderly (aged 55 years and older) cohort (chapter 2.2).[10] The effects of this genotype were analyzed with respect to lipid levels, atherosclerosis measures (intima media thickness[11] and plaque score), and incidence of myocardial infarction (MI). The findings with respect to raised HDL levels were precisely in the range estimated in the meta-analysis (0.11 mmol/L in both genders, $P < 0.001$). These increased HDL levels led to decreased TC/HDL ratio, as expected. Increased HDL, however, did not translate into a reduction in atherosclerosis. Although no change in incidence of MI could be detected in females, a significant increase in risk of incidence of MI was seen in males. This increase (1.30 [95% CI: 1.03, 1.65] for CT and 1.70 [95% CI: 1.35, 2.15]) was independent of serum HDL levels; although the influence of changes in net HDL turnover cannot be excluded, this finding implies an effect via a different pathway than HDL metabolism, possibly due to reduced clearance of apolipoprotein B containing particles,[12] increased oxidation of LDL,[13] or through the creation of functionally defective HDL particles.[14]

Another important protein involved in HDL metabolism is cholesteryl ester transfer protein (CETP), which enriches HDL particles with TG from TG-rich lipoproteins (particularly very-low-density lipoprotein cholesterol) in exchange for cholesteryl esters.[15] A missense polymorphism in the CETP gene, the I405V SNP, leads to an isoleucine to valine shift in the 405th residue of the mature CETP protein. This SNP, which garnered widespread interest following an association with longevity, was shown in a meta-analysis to decrease CETP mass and activity and increase HDL levels.[16] In chapter 2.1, the role of this SNP in determining lipid levels, atherosclerosis proxies and incidence of MI was assessed, again in the Rotterdam Study population. The V allele was significantly associated with increased serum HDL; individuals with the VV genotype possessed HDL levels 0.06 mmol/L higher than II individuals. As with the hepatic lipase variant, this increase in HDL was accompanied by a decrease in TC/HDL ratio. No changes in atherosclerosis proxies were observed. The V allele, however, was associated with decreases in MI incidence in male patients. Heterozygotes saw their risk of MI reduced by a factor of 0.76 [95% CI: 0.60, 0.96] while V homozygotes reduced their risk by a factor of 0.57 [95% CI: 0.45, 0.73]. This effect, which went in the anticipated direction,[17] was independent of HDL levels. Recently, a clinical trial of torcetrapib, a strong CETP inhibitor that markedly raised HDL levels, was stopped, because it was associated with excess mortality.[18] Our findings suggest that more modest CETP inhibition may be beneficial and that CETP is still a suitable target for intervention.

The hepatic lipase and CETP polymorphisms were jointly analyzed in chapter 2.2; gene-gene interactions are thought to be important for determining complex phenotypes.[19] In this analysis, the joint effect of the two SNPs was markedly larger than expected, given the in-

dividual effect sizes. Individuals homozygous for both mutations exhibited HDL levels 0.29 mmol/L higher than those who were wild-type homozygotes for both SNPs. This epistatic effect was statistically significant. No discernable effect on measures of atherosclerosis was noted, and the sample sizes were too small to adequately examine the relationship with risk of incidence of MI. Despite this, this finding in humans is of considerable interest because our current knowledge of HDL metabolism is based, in large part, on observations made using mouse models. Additionally, identifying the effects of both gene-gene and gene-environment interactions on complex phenotypes, such as lipid levels,[20] is becoming an increasingly important goal in genetic studies.

Studies in Familial Combined Hyperlipidemia

Two other candidate gene studies, presented in chapters 2.3 and 2.4, involved a cohort comprised of families ascertained on the basis of presence of familial combined hyperlipidemia (FCH). FCH is the most frequent lipid disorder of unknown genetic etiology, affecting 2–5% of the general population.[21,22] It is a complex dislypidemia characterized by increased ApoB, TG, and TC levels, and often accompanied by decreased HDL, increased small, dense LDL (sdLDL), and increased incidence of cardiovascular disease.[23] In these studies, the contributions of the upstream stimulatory factor 1 (USF1) and apolipoprotein A-V (ApoA-V) genes were examined. USF1 was recently suggested as the gene underlying a replicated linkage signal for FCH.[24-26] The tested USF1 variants were marginally associated with the traditional diagnostic criteria of FCH, as well as some important quantitative lipid parameters, including TC, ApoB, and the presence of small, dense LDL. These results suggest that, although it may not be a major gene for FCH, USF1 plays an important role in modulating the lipid characteristics of FCH. Interestingly, although USF1 is known to regulate hepatic lipase transcription,[27] no associations were observed with respect to HDL levels.

The variants in ApoA-V, by contrast, seem to play a greater role in both FCH and it's associated lipid characteristics.[28,29] These include significant single SNP and haplotype associations with FCH, TC, TG, ApoB, HDL, sdLDL, and remnant-like particle cholesterol levels. These variants were also tested for association in the subset of individuals who were either normolipidemic relatives or spouses of FCH patients. In this "control" sample, ApoA-V was significantly associated with increased TG, ApoB, and sdLDL, and decreased HDL. In addition to being a risk factor for FCH, then, ApoA-V plays a role in the determination of lipid levels in non-affected individuals. Although human ApoA-V levels are remarkably low, it may be an unexpectedly important determinant of the "TG-HDL axis," a set of linked characteristics (increased TG, decreased HDL, and increased sdLDL) that are frequently observed in clinical coronary heart disease, metabolic syndrome, and type II diabetes.[30]

Micro-Array Based SNP Studies

In contrast to the studies discussed previously, which utilized either one or two SNPs, the analysis of the Apolipoprotein L (ApoL) gene cluster (chapter 2.5) took advantage of recent developments in high-density SNP genotyping using micro-array technology.[31] The ApoL cluster is located on chromosome 22 and includes six closely related genes, ApoL-I to ApoL-VI.[32] In the analysis, sixty-six SNPs spanning the cluster (out of 2486 SNPs typed chromosome-wide), which were originally determined as part of study on linkage disequilibrium patterns in isolated populations,[33] were tested for association with TG and HDL levels in a sample (n = 188) randomly selected from the ERF population. Five SNPs were nominally associated with HDL; these five SNPs were subsequently analyzed in an independent population randomly selected from the 1986 Northern Finland Birth Cohort (n = 182). Three SNPs, located in the ApoL-V gene, were determined to influence TG, HDL, and TG/HDL levels. Haplotype analysis of ApoL-V suggested that a single frequent haplotype accounted for these associations. While a role for ApoL proteins in lipid transport and metabolism is generally accepted,[34] the function of ApoL-V is almost completely unexplored; however, it seems plausible that it is involved in the TG-HDL axis, possibly by facilitating CETP-mediated transfers of TG to HDL particles.

In chapter 3.1, the true potential of micro-array based genotyping was explored in a genome-wide association study to determine novel variants involved in lipid levels. Approximately 250,000 SNPs, spanning the genome, were determined, using the Affymetrix 250K Nsp array from the GeneChip® Human Mapping 500K Array Set,[35] in a sample of 197 individuals from the ERF population. These genotypes were analyzed using GenABEL, a genome-wide association analysis package developed by Yurii Aulchenko and his colleagues in the Genetic Epidemiology Unit.[36] This package implements a rapid test for analyzing associations between a genotype and a phenotype, and additionally allows for whole genome permutation, which is considered the most efficacious method for combating the multiple testing issue.[37]

Three SNPs on chromosome 1 were associated with TC at the genome-wide level, although two of these were perfectly correlated ($R^2 = 1$). All three were located just 5′ of the estrogen-related receptor gamma (ERR3) gene, which codes for a nuclear orphan receptor.[38] A number of flanking SNPs were also strongly associated; these SNPs were also associated with LDL, suggesting that the changes in levels observed are through an LDL pathway. Haplotypes of two- and five-SNP sliding windows supported the single SNP analyses. One other SNP, located on chromosome 6, was associated at the genome level with TG and TG/HDL ratio. Several flanking SNPs were also associated, and, again, sliding window haplotype analysis supported the individual SNP analyses. This SNP is located in a putative processed transcript site. Although very little is known about this region, it may be promising for follow-up.

The ERR3 region, by comparison, is a good candidate for lipid metabolism. Several other members of the nuclear orphan receptor family, such as the liver X receptor, play important roles in the system.[39] The strength of the associations observed in this region, coupled with

the supporting evidence from flanking SNPs, haplotypes, and a priori knowledge all lead to the conclusion that ERR3 is a promising candidate region.

These genome-wide studies demonstrate the exciting potential to identify novel genes, with no a priori hypotheses, other than that genes play a role in the phenotype of interest. Even with small samples, it may be possible to identify previously unknown variants, such as the complement factor H polymorphism identified for age-related macular degeneration.[40] With larger sample sizes, it should be possible to identify common genetic variants with smaller effect sizes.

In conclusion, both candidate gene and genome-wide approaches offer a wide variety of tools to assess genotype-phenotype relationships. Candidate gene analyses benefit from well-founded assumptions about the biological plausibility of the gene in question, comparatively low cost, and ease of analysis. Genome-wide approaches, although they are expensive and introduce the somewhat intractable problem of multiple testing, offer the opportunity to identify completely novel genes involved in determining the phenotype. Given the difficulty of establishing genes involved in complex phenotypes, it is likely that both candidate and genome-wide methods will continue to be implemented. Both types of analysis can be utilized in families, general populations, or specifically selected (case-control) samples. Families may increase statistical power, due to known genealogy, but are difficult to ascertain (especially in the case of late onset disease states). Only a few years ago, genome-wide approaches (i.e. linkage) were only available in family-based studies. Since the advent of high-throughput, high-density genotyping methods, genome-wide approaches can be done in non-related individuals and should yield interesting results in the years to come.

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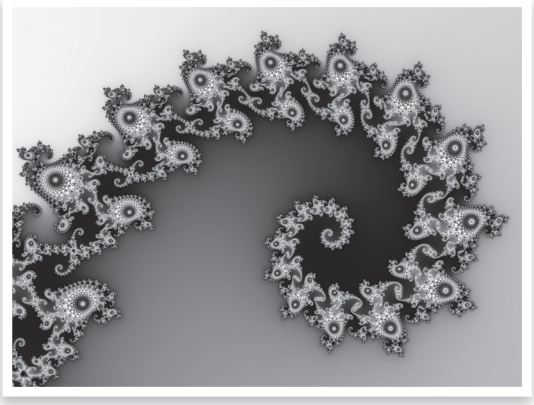
Chapter 5

Summary (Samenvatting)



SUMMARY

Cardiovascular disease, despite decades of research, remains the leading cause of mortality in much of the world. Circulating (serum and plasma) lipid levels are important determinants of cardiovascular disease risk. Total cholesterol, low-density lipoprotein cholesterol (LDL), and triglyceride levels lead to increased incidence of disease, while high-density lipoprotein cholesterol (HDL) leads to decreased incidence. The levels of these lipid particles are determined in large measure by genetic factors. Despite years of searching for these genes, and the vast strides made thus far, there are still many unknown genetic factors affecting lipid levels.

In this thesis, a large number of genetic epidemiology tools are applied to the task of identifying and characterizing genes involved in lipid transport, metabolism, and homeostasis. Two types of approaches are implemented: candidate-gene analysis and genome-wide analysis. These broad classes of analyses were utilized in both population-based and family-based studies, in both general and selected populations (including a Dutch genetic isolate), and yielded a number of important insights.

The first chapter begins with a general overview of both lipids and genetic epidemiological methods. It continues, in chapter 1.2, with a study designed to estimate the proportion of lipid parameter variation attributable to genetic factors. This study also examined the effects of inbreeding on lipid levels and the influence of the well-characterized Apolipoprotein E ε 2/3/4 isoforms. These analyses determined that between a quarter (triglycerides) and a half (HDL) of the variance in these measurements are due to genetic causes. Inbreeding contributes to this variation, particularly for total cholesterol and LDL. Apolipoprotein E isoforms, while important, do not explain a large portion of lipid variance.

The influence of a promoter polymorphism in the hepatic lipase gene promoter was studied in chapter 1.3. Using meta-analysis to synthesize the data from 25 publications, the –514C>T single nucleotide polymorphism (SNP) was determined to significantly decrease hepatic lipase activity and, subsequently, increase HDL levels.

In chapter 2, candidate gene approaches were implemented to study a variety of genes, or regions, known, or suspected, of influencing lipid levels. In chapter 2.1, the effects of an amino acid shift polymorphism in the cholesteryl ester transfer protein gene was explored. The mutation caused an increase in serum HDL levels, which, in turn, led to a decreased risk of myocardial infarction in men. The joint effect of this polymorphism and the hepatic lipase promoter polymorphism were tested in chapter 2.2. The hepatic lipase variant was associated with higher HDL levels overall, and an increased risk of heart attack in males; together, the two polymorphisms exerted a strong effect on HDL.

In chapters 2.3 and 2.4, polymorphisms in two candidate genes, upstream stimulatory factor one (USF1) and Apolipoprotein A-V (ApoA-V), were evaluated in a set of families ascertained for familial combined hyperlipidemia (FCH). USF1, previously reported to be a major player in FCH, did not play a role in the disease in these Dutch families, although the SNPs,

both individually and in haplotypic analysis, influenced total cholesterol levels. ApoA-V, by contrast, affected FCH risk, as well as numerous lipid phenotypes, including total cholesterol; triglycerides; apolipoprotein B; HDL; small, dense LDL; and remnant-like particle cholesterol. The effects of the tested genetic variants extended to the normo-lipidemic relatives and spouses of the FCH probands, suggesting that ApoA-V is an important determinant of lipid levels in a general population.

An interesting set of genes located on chromosome 22, the apolipoprotein L (ApoL) cluster, was studied in chapter 2.5. A large number of SNPs (n = 66) were analyzed in a sample of Dutch individuals, and followed-up in a sample of Finns. Three SNPs, located in the ApoL-V gene, affected triglyerides, HDL, and their ratio. Haplotype analysis revealed a shared haplotype in both populations that led to these associations. As these phenotypes form an important component of atherogenic dyslipidemia, this finding is of particular interest.

Chapter 3.1 presents results from a genome-wide association study of lipid levels, utilizing a recently published piece of software developed in-house (GenABEL). This study reported two novel loci that reached genome-wide significance. The first, located on chromosome 1, suggests that the estrogen receptor-related protein 3 gene (ERR3) influences total cholesterol and LDL. Other nuclear orphan receptors, similar to ERR3, were previously implicated in lipid metabolism. The second region, located on chromosome 6, affected triglycerides and triglyceride/HDL ratio. Although the region is not well characterized, the polymorphisms are located in a putative transcript site, which may harbor an as yet unknown gene.

In chapter 4, the results from these studies are discussed in a more general manner, and findings are placed in a broader context. In total, they suggest that genetics play an important role in determining lipid levels, and that these genetic variants are also involved in subsequent disease. Although the numbers of genes involved, and their complex interactions with each other and with environmental factors, make the goal of fully elucidating these systems a distant dream, substantial progress has been, and will continue to be, made.

SAMENVATTING

Hart- en vaatziekten blijven, ondanks decennia van onderzoek, de belangrijkste oorzaak van sterfte in grote delen van de wereld. Lipiden spiegels in serum en plasma zijn belangrijke determinanten van hart- en vaatziekten. Totaal cholesterol, *low-density* lipoproteïne cholesterol (LDL) en triglyceride spiegels verhogen de incidentie van hart- en vaatziekten, terwijl *high-density* lipoproteïne cholesterol (HDL) deze incidentie juist verlaagt. De spiegels van deze lipiden-partikels worden in grote mate bepaald door genetische factoren. Ondanks jaren van onderzoek naar deze genetische factoren, en de grote vooruitgang die er al geboekt is, zijn er nog altijd veel onbekende genetische factoren die lipiden spiegels beïnvloeden.

In dit proefschrift wordt een groot aantal verschillende genetisch-epidemiologische technieken gebruikt om genen te vinden en karakteriseren die betrokken zijn bij lipiden transport, metabolisme en homeostase. Twee verschillende benaderingswijzen worden gevolgd: kandidaat gen studies en genoomwijde analyse. Deze technieken werden toegepast in populatie-gebaseerde zowel als in familie-gebaseerde studies, in de algemene populatie maar ook in genetisch geïsoleerde populaties (waaronder een Nederlandse), en leverden een aantal belangrijke inzichten op.

Het eerste hoofdstuk begint met een algemeen overzicht van de lipiden en van genetisch-epidemiologische methoden. Vervolgens wordt in hoofdstuk 1.2 een studie beschreven die werd opgezet om te onderzoeken wat de proportie is van de variatie in de verschillende lipiden parameters die verklaard kan worden door genetische factoren. Ook werd in deze studie het effect van bloedverwantschap op lipiden spiegels onderzocht, evenals de invloed van de goed gekarakteriseerde isovormen (ε 2/3/4) van Apolipoproteïne E. Deze analyses toonden aan dat tussen een kwart (triglyceriden) en de helft (HDL) van de variantie in deze bepalingen het gevolg is van genetische factoren. Bloedverwantschap draagt bij tot deze variatie, met name voor totaal cholesterol en LDL. De Apolipoproteïne E isovormen, alhoewel van belang, blijken geen groot deel van de variantie van lipiden spiegels te verklaren.

De invloed van een promoter polymorfisme in de promoter regio van het hepatische lipase gen werd bestudeerd in hoofdstuk 1.3. Een meta-analyse waarin de data van 25 publicaties werden gecombineerd, toonde aan dat het –514C>T *single nucleotide* polymorfisme (SNP) de hepatische lipase activiteit significant verlaagde, en de HDL spiegels verhoogde.

In hoofdstuk 2 werd de kandidaat gen benadering toegepast om een aantal genen, of chromosomale regio's, te onderzoeken die een bekende of vermoedde invloed op lipiden spiegels zouden kunnen hebben. In hoofdstuk 2.1 werden de effecten van een aminozuur substitutie polymorfisme in het cholesteryl ester transferase proteïne gen onderzocht. De mutatie veroorzaakte een verhoging van serum HDL spiegels, wat vervolgens leidde tot een verlaagd risico voor myocard infarct in mannen. Het gecombineerde effect van dit polymorfisme en het hepatische lipase promoter polymorfisme werd onderzocht in hoofdstuk 2.2. De variant in hepatische lipase was geassocieerd met verhoogde HDL spiegels, en in mannen met een

verhoogd risico op het krijgen van een hartaanval; in combinatie hadden de twee polymorfismen een sterk effect op HDL.

In hoofdstuk 2.3 en 2.4 werden polymorfismen in twee kandidaatgenen, te weten *upstream stimulatory factor 1* (USF1) en Apolipoproteïne A-V (ApoA-V) geëvalueerd in een groep families met familiale gecombineerde hyperlipidemie (FCH). USF1, dat voorheen werd gerapporteerd als belangrijk gen voor FCH, bleek geen rol te spelen in deze ziekte in de Nederlandse families, hoewel de SNPs, zowel afzonderlijk als in haplotype analyse, van invloed bleken op de totale cholesterol spiegels. ApoA-V daarentegen beïnvloedde het risico voor FCH wel, evenals een aantal lipiden fenotypes, waaronder totaal cholesterol, triglyceriden, apolipoproteïne B, kleine, dichte LDL, en *remnant-like* partikel cholesterol. Het effect van de geteste genetische varianten bleek ook aantoonbaar in de normolipidemische familieleden en partners van FCH patiënten, wat erop duidt dat ApoA-V een belangrijke determinant is van lipiden spiegels in de algemene populatie.

Een interessante groep genen gelokaliseerd op chromosoom 22, te weten het apolipoproteïne L (ApoL) cluster, werd onderzocht in hoofdstuk 2.5. Een groot aantal SNPs (n = 66) werd geanalyseerd in een Nederlandse populatie, en vervolgens in een Finse populatie. Drie SNPs, gelegen in het ApoL-V gen, hadden een effect op triglyceriden, HDL, en hun onderlinge ratio. Haplotype analyse toonde aan dat een gedeeld haplotype in beide populaties deze associaties kon verklaren. Aangezien deze fenotypes een belangrijke component vormen van atherogene dyslipidemie, is deze bevinding van bijzonder belang.

In hoofdstuk 3.1 worden de resultaten van een genoomwijde associatie studie van lipiden spiegels gepresenteerd. In deze studie werd gebruik gemaakt van een recent gepubliceerd, in-huis ontwikkeld software programma (GenABEL). Twee nieuwe loci werden ontdekt die genoomwijd significant geassocieerd waren. De eerste locus, gelegen op chromosoom 1, duidt op een invloed van het oestrogeen receptor gerelateerde proteïne 3 gen (ERR3) op totaal cholesterol en LDL. Andere *nuclear orphan* receptoren vergelijkbaar met ERR3 zijn al eerder in verband gebracht met lipide metabolisme. De tweede regio, op chromosoom 6, beïnvloedde triglyceriden en de triglyceride/HDL ratio. De regio is nog niet goed gekarakteriseerd, maar de polymorfismen zijn gelokaliseerd in een mogelijke transcriptie eenheid, wat kan duiden op de aanwezigheid van een nog onbekend gen.

In hoofdstuk 4 worden de resultaten van deze studies in meer algemene termen bediscussieerd en in een bredere context geplaatst. Alle studies wijzen erop dat genen een belangrijke rol spelen in het bepalen van lipiden spiegels, en dat deze genen ook betrokken zijn bij de ziekten die het gevolg zijn van verstoringen in de lipiden spiegels. Ondanks dat door het aantal betrokken genen, en hun complexe interacties met andere genen en omgevingsfactoren, de volledige opheldering van de onderliggende pathomechanismen voorlopig een verre droom blijft, is er een aanzienlijke vooruitgang geboekt, en deze ontwikkeling zal zeker voortgezet worden.

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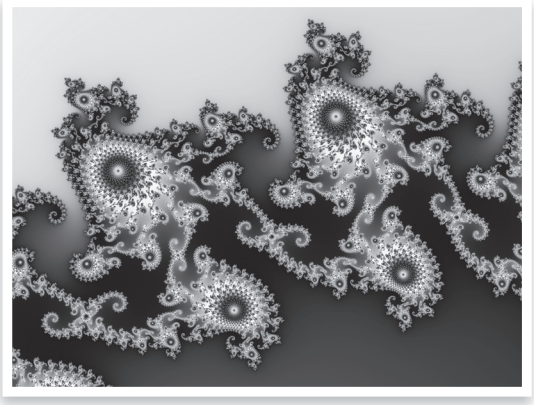

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Jacob and Sophie are my adorable little muses. Their photo, stuck just behind my monitor and always in view, is a guaranteed smile-maker and mood-lifter, no matter the adversity at hand!

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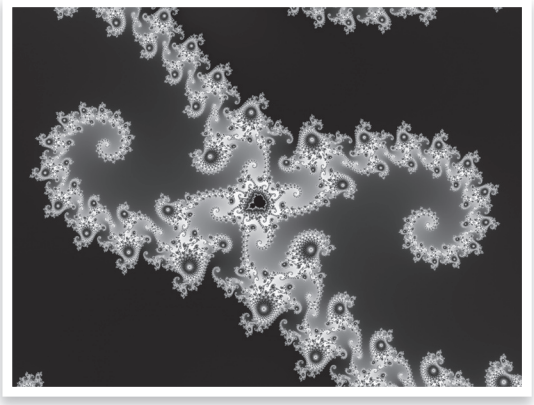
And then there is Kristel. I do not even know where to begin! You have been my rock, supporting me through both my failures and triumphs. You are my best friend, my companion, my colleague, and my partner. To paraphrase Winston Churchill, "I have got tunes in my head for every [place] I have been to, and indeed for every critical or exciting phase in my life. Some day when [our] ship comes home, [we are] going to have them all collected in gramophone records, and then [we] will sit in a chair and [drink a glass of wine], while pictures and faces, moods and sensations long vanished return; and pale but true there gleams the light of other days."

List of Publications



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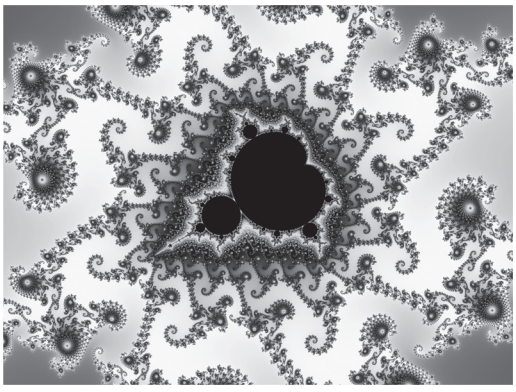
About the Author



When the author was born, the world was gripped by a seismic shift in it's thinking about the unbounded potential of science and technology. Of course, these two events were completely unrelated; the latter was due to the unprecedented, televised success of the Apollo 11 moon landing. After graduating from high school, he spent several years studying flight technology (learning to fly airplanes) before completing his baccalaureate degree in Physical and Human Geography, with university distinctions, at the University of California, Berkeley. He later relocated to New York City, where he accumulated a substantial amount of lipid and laboratory knowledge working under the auspices of Professor Ira J. Goldberg at Columbia University. He moved to the Netherlands in 2002 to pursue a Master's degree in genetic epidemiology in the Department of Epidemiology & Biostatistics at Erasmus University in Rotterdam. Finding Europe to his favor, he stayed in Holland to complete a Doctor of Science degree in 2004, and, subsequently, continued working towards a Doctor of Philosophy. His studies have been presented in a variety of forums, and, in 2004, he won a Young Investigator Award at the European Society of Human Genetics' annual congress. After completing his Ph.D., he plans to continue his endeavors in the field of lipid genetics.