The lipoprotein subfraction profile: heritability and identification of quantitative trait loci

Bernhard Kaess,[1,*] Marcus Fischer,[1,*] Andrea Baessler,[*] Klaus Stark,[*] Fritz Huber,[†] Werner Kremer,[†] Hans Robert Kalbitzer,[†] Heribert Schunkert,[§] Guenter Riegger,[*] and Christian Hengstenberg[2,*]

Klinik und Poliklinik für Innere Medizin II,* University of Regensburg, Regensburg, Germany; Institut für Biophysik und Physikalische Biochemie,† University of Regensburg, and Lipofit GmbH, Regensburg, Germany; and Medizinische Klinik II,§ University Hospital of Schleswig-Holstein, Lübeck, Germany

Abstract The HDL and LDL subclass profile is an emerging cardiovascular risk factor. Yet, the biological and genetic mechanisms controlling the lipoprotein subclass distribution are unclear. Therefore, we aimed *1*) to determine the heritability of the entire spectrum of LDL and HDL subclass features and *2*) to identify gene loci influencing the lipoprotein subfraction pattern. Using NMR spectroscopy, we analyzed the lipoprotein subclass distribution in 1,275 coronary artery disease patients derived from the Regensburg Myocardial Infarction Family Study. We calculated heritabilities, performed a microsatellite genome scan, and calculated linkage. HDL and LDL subclass profiles showed heritabilities ranging from 23% to 67% (all $P < 10^{-3}$) of traits using univariate calculation. After multivariate adjustment, we found heritabilities of 27–48% (all $P < 0.05$) for HDL and 21–44% for LDL traits. The linkage analysis revealed a significant logarithm of the odds (LOD) score (3.3) for HDL particle concentration on chromosome 18 and a highly suggestive signal for HDL particle size on chromosome 12 (2.9). After multivariate adjustment, we found a significant maximum LOD score of 3.7 for HDL size. Our study is the first to analyze heritability and linkage for the entire spectrum of LDL and HDL subclass features. Our findings may lead to the identification of genes controlling the lipoprotein subclass distribution.— Kaess, B., M. Fischer, A. Baessler, K. Stark, F. Huber, W. Kremer, H. R. Kalbitzer, H. Schunkert, G. Riegger, and C. Hengstenberg. **The lipoprotein subfraction profile: heritability and identification of quantitative trait loci. *J. Lipid Res.* 2008.** 49: **715–723.**

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The plasma lipoprotein profile plays a pivotal role in the pathogenesis of atherosclerosis and is a major predictor for coronary artery disease (CAD). In particular, LDL and HDL cholesterol levels have been established as important risk factors for CAD, and the decrease of LDL cholesterol is a principal target in cardiovascular preventive strategies (1). However, it has been known for several years that the major lipoprotein classes can be further characterized into subfractions. Indeed, there is growing evidence that the profile of these subclasses is a crucial prognostic factor for the manifestation and progression of CAD (2–4). With respect to the assessment of LDL particles, small dense particles have been shown to be much more atherogenic than large buoyant particles (5). Furthermore, several epidemiological studies demonstrated that mean LDL particle size and particle concentration provide more reliable prognostic information than the routine measurement of LDL cholesterol levels (6–9). Similar is true for HDL particles. Accumulating evidence shows that different subclasses have distinct functional properties and prognostic impact (10–12).

The cholesterol content per particle exhibits large interindividual variation. However, it is unclear which biological mechanisms control the lipoprotein subclass distribution. Specifically, it has not been clarified to what extent genetic factors contribute. Thus, the identification of genes influencing the lipoprotein subfraction profile is an important step toward understanding the underlying biological principles, which in turn may facilitate the development of novel preventive and therapeutic strategies. Therefore, we aimed *1*) to determine the heritability of the entire spectrum of LDL and HDL particle subclass features in families with high cardiovascular risk and the presence of CAD and *2*) to identify quantitative trait loci (QTLs) influencing the lipoprotein subfraction patterns, which we assessed by genome-wide linkage analysis.

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[1] B. Kaess and M. Fischer contributed equally to this work.
[2] To whom correspondence should be addressed.
e-mail: christian.hengstenberg@klinik.uni-regensburg.de

METHODS

Study population

The 1,275 patients for this study were derived from 590 families in the Regensburg Myocardial Infarction Family Study that has been described previously (13). Recruitment continued after the mentioned publication, so that in the present study we were able to include 77 additional families not included in our previous work. Briefly, CAD families were ascertained through index patients at 15 cardiac rehabilitation centers distributed throughout Germany. All index patients had suffered from myocardial infarction before 60 years of age. If at least one sibling presented with myocardial infarction or severe CAD before 70 years of age, defined by percutaneous coronary intervention or coronary artery bypass grafting, the entire nuclear family (index patient, available parents, and all affected and unaffected siblings) was contacted and invited to participate in the study. The Ethics Committee of the University of Regensburg approved the study protocol, and all participants gave written, informed consent. The investigation conforms with the principles outlined in the Declaration of Helsinki.

Genotyping

We extracted DNA from peripheral blood using a commercially available extraction kit (Gentra Puregene DNA Extraction Kit). A microsatellite genome-wide screen was carried out by the Mammalian Genotyping Service (Marshfield, Wisconsin), as reported previously, using the screening set 10 (405 markers, 378 autosomal markers) with an average marker spacing of 10 centimorgan (13).

Phenotyping

The phenotyping protocol has been described previously (13). In short, answers to a standardized questionnaire were obtained by specially trained telephone interviewers. This information was validated by retrospective analyses of medical records from hospital stays and primary physicians. Additionally, all patients underwent a medical examination during a visit scheduled at their primary care physician's office. Myocardial infarction was diagnosed and documented as described previously (13). Diabetes mellitus was defined by the use of antidiabetic medication or by increased glycosylated hemoglobin (>6.5%). Asservation of serum was carried out in a sitting position from nonfasting individuals. Routine lipid parameters were assessed during the recruitment phase of the Myocardial Infarction Family Study. Serum total cholesterol and HDL cholesterol were measured using a standard enzymatic method (CHOD-PAP), HDL cholesterol was determined after precipitation with phosphotungstate/Mg, and LDL cholesterol was determined after precipitation with dextrane sulfate in the supernatant.

NMR spectroscopy

NMR spectroscopy was carried out at Lipofit GmbH (Regensburg, Germany) from samples stored at −80°C. The technology was patented recently (WO 2005/119285 A1, DE 10 2004 026903 A1). Briefly, gradient-weighted NMR spectra of blood plasma were recorded, which led to characteristic overall profiles of the lipoprotein signals. Using the gradient-weighted spectra, spectral regions ranging from 1.5 to 0.7 ppm were modeled into a set of 15 lipoprotein subclasses. These lipoprotein subfractions were classified according to **Table 1**.

LDL and HDL levels computed by NMR spectroscopy showed good agreement with the values determined by routine enzymatic methods (**Fig. 1A**). For z-score-transformed LDL and HDL levels, the estimated mean differences (d) were $d = 7 \times 10^{-7}$ and $d = -0.0017$, respectively, and the standard deviations (s) of the differences were $s = 0.96$ and $s = 1.25$. Bland and Altman plots suggest that provided the differences within $d \pm 2s$ are not clinically important, the two measurements could be used interchangeably (Fig. 1B).

TABLE 1. Classification and properties of lipoprotein subclasses

Lipoprotein Subfraction	Particle Size	Density	Mass
	nm	*g/ml*	*g/nmol*
Chylomicrous B	>150	0.9600	59.86
Chylomicrous A	80–150	0.9800	17.74
VLDL	40–80	1.0060	4.87
Intermediate density lipoprotein	30–40	1.0150	0.58
LDL E	25–30	1.0190	0.39
LDL D	22–25	1.0270	0.26
LDL C	21–22	1.0350	0.20
LDL B	19–21	1.0450	0.15
LDL A	16–19	1.0600	0.10
HDL D	13–16	1.0630	0.05
HDL C	10–13	1.0900	0.03
HDL B	8.5–10	1.1200	0.02
HDL A	7–8.5	1.2000	0.01

Statistical analysis

Quantitative genetic analyses were conducted using the maximum likelihood-based, variance decomposition approach (14). The phenotypic variance of a trait is separated into additive genetic and environmental (nongenetic) effects. Heritability is defined as the proportion that the additive genetic effects contribute to the total phenotypic variance.

Multipoint variance components linkage analysis was applied as described previously (13) to test the linkage between marker loci and a given phenotype, which was based on specifying the expected genetic covariances between pairs of relatives as a function of their identity by descent at a marker linked to a QTL (14). These calculations were carried out using the SOLAR program package.

Adjustment for cholesterol synthethase enzyme (CSE) inhibitor use was performed in a product- and dose-dependent manner as described previously (15).

Bivariate multipoint linkage analysis was applied to exploit the additional information contained in the correlation pattern between two quantitative traits. The extension of the variance components linkage approach to the bivariate situation facilitates the testing of linkage of two correlated phenotypes (i.e., particle size and concentration) to a single genetic region simultaneously (16, 17).

RESULTS

Study population

The clinical and anthropometric characteristics of the study population are described in **Table 2** and reflect the previously described risk factor profile of our myocardial infarction families (13).

Heritability estimates of lipoprotein subclasses

The heritability of each trait was calculated using variance component analysis. We computed heritability estimates in two different statistical models: first by univariate

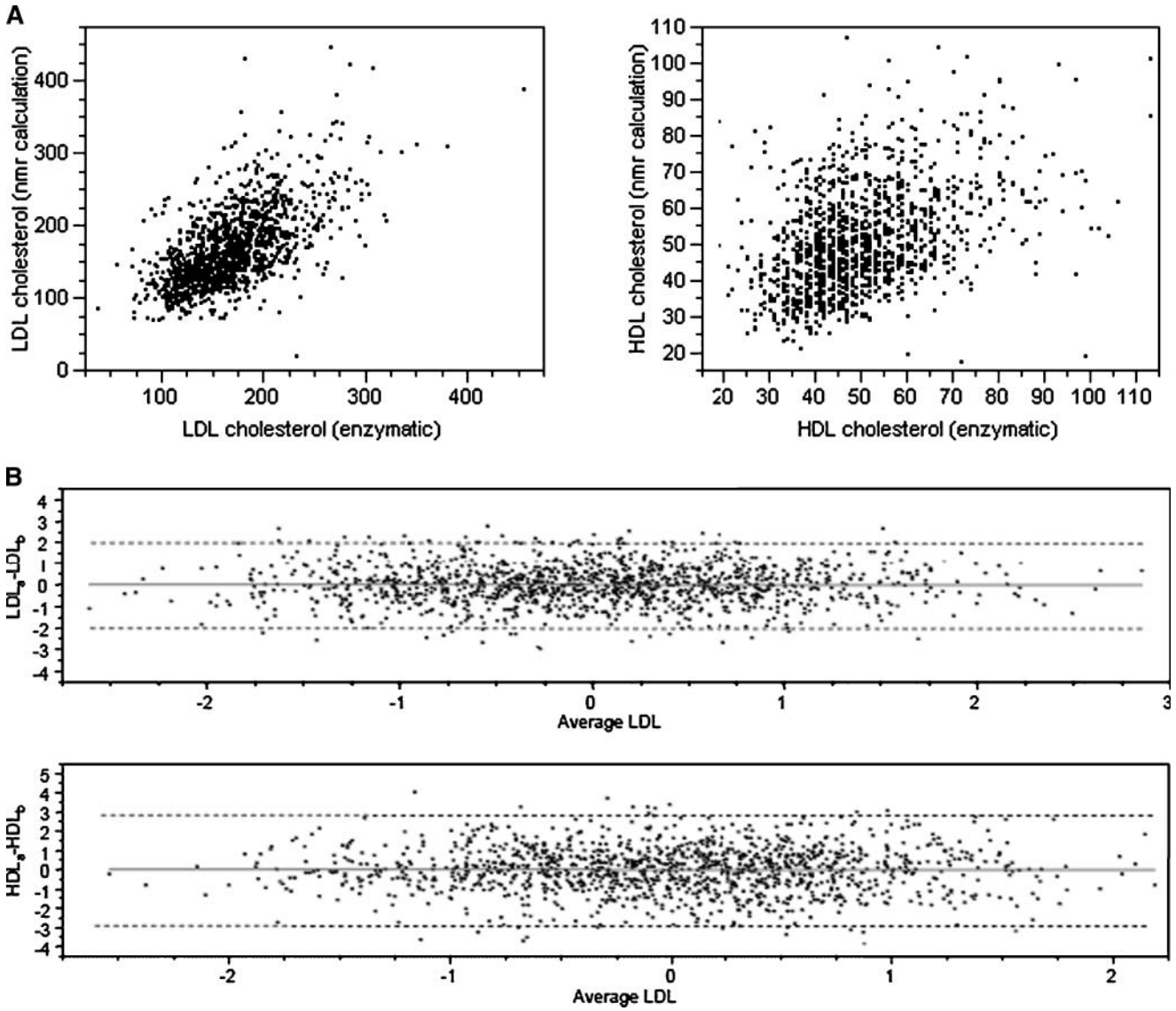

Fig. 1. Correlation of HDL and LDL cholesterol values assessed by enzymatic assays compared with calculation from NMR spectroscopy. A: Uncorrected values. B: Bland-Altman plot of z-score-transformed values of HDL and LDL cholesterol computed from NMR data (HDL_a/LDL_a) versus values determined by enzymatic assays (HDL_b/LDL_b).

calculation, and second in an adjusted model including as covariates age, gender, diabetes, body mass index, lipid-lowering therapy, smoking, and serum triglycerides. The resulting heritabilities are depicted in **Fig. 2**. In the unadjusted model, heritabilities ranged from 31% to 67% for HDL (all $P < 10^{-5}$) and from 23% to 48% for LDL traits (all $P < 10^{-3}$). After multivariate adjustment, we found heritability estimates of 27–48% for HDL (all $P < 0.05$) and 21–44% for LDL traits ($P < 0.05$ in LDL B, D, and E, particle concentration). The proportion of variance attributable to the inclusion of the covariates was found to be 16–25% in HDL and 7–30% in LDL traits (data not shown).

Linkage analysis

To identify gene loci potentially affecting the lipoprotein subclass pattern, we conducted a genome-wide linkage scan for QTLs underlying lipoprotein subclasses as well as the mean size and concentration of LDL and HDL particles. Using univariate models, we found a highly suggestive signal for HDL size [logarithm of the odds (LOD) score of 2.9] (**Fig. 3A**) on chromosome 12 as well as significant linkage for HDL particle number on chromosome 18 (LOD score of 3.3; Fig. 3B). The maximum LOD scores of all assessed lipoprotein traits (in univariate calculation) are listed in **Table 3**.

After adjusting for age and sex, the signal for HDL size became stronger, showing a significant LOD score of 3.3, whereas the peak for HDL particle concentration on chromosome 18 was slightly weaker, but still significant with a LOD score of 3.2. Adjusting also for body mass index, diabetes mellitus, lipid-lowering therapy, and smoking, we found LOD scores of 3.7 for HDL size on chromosome 12 and 3.1 for HDL particle concentration on chromosome 18 (**Fig. 4**). After additional adjustment for triglycerides,

TABLE 2. Clinical and anthropometric characteristics of the study population

Characteristic	Index Patients	Affected Sibs
No.	458	817
Age (years)	59.5 ± 7.7	61.5 ± 9.0
Age of onset (years)	53.9 ± 8.2	54.0 ± 9.1
Sex (% male)	79.7	66.2
Total cholesterol (mg/dl)	225 ± 42	233 ± 47
LDL cholesterol (mg/dl)	158 ± 41	167 ± 47
HDL cholesterol (mg/dl)	50 ± 14	49 ± 13
Triglycerides (mg/dl)	209 ± 143	207 ± 155
One-vessel CAD (%)	21.3	21.2
Two-vessel CAD (%)	35.7	29.1
Three-vessel CAD (%)	40.2	43.3
Hypertension (%)	61.0	60.4
Body mass index	26.9 ± 3.4	26.8 ± 3.2
Diabetes mellitus (%)	20.4	20.5
Lipid-lowering therapy (%)	62.8	39.9

CAD, coronary artery disease. Values are means ± SD or percentage.

the peak on chromosome 12 decreased to 3.1, hence remaining significant, whereas the signal for HDL particle concentration on chromosome 18 was not affected.

We next investigated whether the two traits HDL size and particle concentration are dependent on each other. Covariate adjustments showed that HDL size explains 17% of HDL concentration variance ($P = 5.1 \times 10^{-53}$), whereas HDL particle concentration accounts for 17% of HDL size variance ($P = 4.3 \times 10^{-43}$). To address the question of whether this phenotypic interdependence has a genetic basis, we performed a bivariate genetic analysis. We found a significant negative genetic correlation ($\rho_G = -0.29 \pm 0.09$; $P = 0.005$) and a significant negative environmental correlation ($\rho_E = -0.58 \pm 0.088$; $P = 4.5 \times 10^{-6}$) between HDL size and concentration. Therefore, a group of genes might contribute to the variations of both HDL size and HDL particle concentration. Multipoint linkage analysis was performed to detect and localize QTLs jointly influencing variation in HDL size and particle concentration. However, the bivariate linkage scan did not reveal significant LOD score improvements.

DISCUSSION

Heritability of lipoprotein subfractions

In the present study, we investigated the heritability of the LDL and HDL subclass profile and performed genome-wide linkage analyses. The heritability of the major lipoprotein classes is undoubted, but quantitative results vary broadly (18). Less is known regarding the genetic influence on lipoprotein subclass distribution and heterogeneity. Regarding LDL, most studies, for analytical reasons, have been carried out on either mean or peak LDL particle size, reporting heritabilities of 30–60% (19), in concordance with our findings.

Little data exist about the heritable aspect of HDL subclasses. By ultracentrifugation techniques, HDL can been divided into two major fractions: large lipid-rich HDL_2 (corresponding to HDL B/C in our study) and small, dense lipid-poor HDL_3 (corresponding to HDL A). Using this classification, O'Connell et al. (20) reported heritabilities of 4–69% for HDL_2 and −34–20% for HDL_3 (depending on the statistical model) in a study of ~200 Danish twins. Bo et al. (21) found heritabilities of 90% for HDL_2 and 80% for HDL_3 in 50 Italian twins, while Kuusi et al. (22) reported heritability estimates of 56% for HDL_2 and <0% for HDL_3 in 35 Finnish twins. To conclude, the results of these studies display some variability, but they support the range of our findings.

Together, the heritabilities observed in our study are in conformity with the published data. However, to our knowledge, our study is the first to examine the heritability of the entire spectrum of LDL and HDL subclasses in the same large cohort.

Linkage analysis

After investigating heritability, we aimed to identify gene loci influencing the lipoprotein subfraction profile, which we assessed by conducting a genome-wide linkage scan for QTLs on each lipoprotein subclass as well as particle concentration and mean size. We found significant linkage for HDL particle concentration on chromosome 18 and for HDL size on chromosome 12.

Although a large number of studies provide linkage data for lipoprotein traits [summarized by Bosse et al. (23)], there is very limited knowledge regarding distinct HDL particle features. Using candidate gene approaches, the HDL subclass profile has been associated with variations in the genes for hepatic lipase (22, 24–26), endothelial lipase (27), apolipoprotein A-II (APOA-II) (28), APOA-IV (29), APOC3 (30), cholesteryl ester transfer protein (CETP) (31), APOε (32), and LCAT (in baboons) (33). Transgenic mouse models showed effects of CETP overexpression on HDL size (34). Performing genome-wide linkage analysis, Almasy et al. (35) described a locus on chromosome 8 influencing a component of HDL cholesterol, namely unesterified HDL_{2a}, and a locus on chromosome 15 affecting HDL size pattern. Recently, Yang et al. (36) reported linkage between HDL_3 and a locus on chromosome 6, in which fine-mapping of six candidate genes within the region revealed a significant single nucleotide polymorphism in the pleiomorphic adenoma gene-like 1 gene.

Hence, to our knowledge, the two gene loci identified in our study have not been reported before in the context of the HDL subfraction profile. Nevertheless, the locus on chromosome 12 was described previously by Bielinski et al. (37). Those authors found significant linkage to total HDL cholesterol in a cohort of ~1,200 Asians. A possible candidate gene residing within that region is the insulin-like growth factor-1 gene, the translation product of which is crucial in glucose and fat metabolism. Another prominent candidate is ACBCB, a gene that encodes acetyl-CoA carboxylase-β (Acc2). Acc2 plays a pivotal role in regulating mitochondrial fatty acid oxidation. Homozygous Acc2 knockout mice eat more food, gain less weight, and continuously metabolize fatty acids compared with wild-type mice. Furthermore, Acc2 knockout mice are resistant

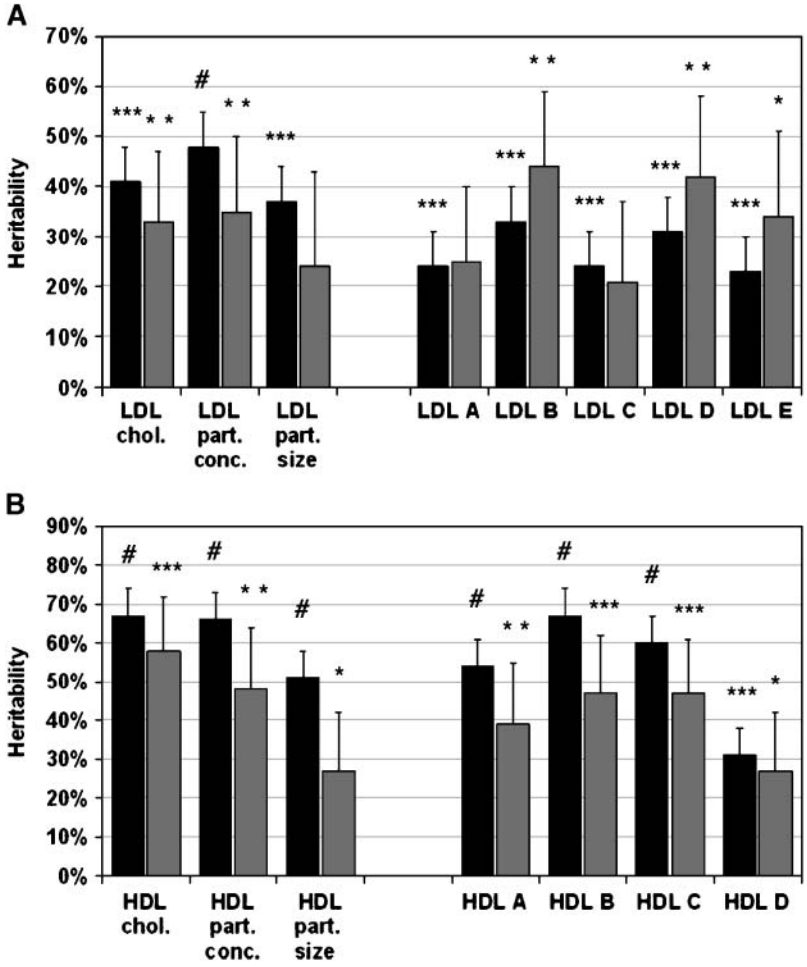

Fig. 2. Heritability estimates of LDL (A) and HDL (B) subclass features as obtained by variance component analysis (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, $^{\#}$ $P < 10^{-10}$). chol., cholesterol as computed from NMR spectroscopy; part. conc., particle concentration; part. size, mean particle size). Black bars show the univariate model, and gray bars show the adjusted model. Error bars indicate ± SD.

to obesity and diabetes, remain sensitive to insulin, and have normal glucose levels when fed a high-fat, high-carbohydrate diet compared with wild-type mice (38). These findings led to the development of Acc inhibitors as a promising new treatment of the metabolic syndrome (39).

Regarding the locus on chromosome 18, we did not find reports in the context of lipoproteins. However, there is evidence that the locus might be involved in metabolic traits, because Chen et al. (40) reported a positive association of marker GATA11A06 with fasting glucose in the Framingham offspring cohort. Possible candidate genes in the region are the melanocortin receptor genes 2 and 5. The melanocortin system plays a pivotal role in energy homeostasis, obesity, and cachexia (41).

Biological implications and prognostic impact of lipoprotein subclass distribution

Historically, cardiovascular risk attributed to the plasma lipoprotein profile, for reasons of analytical simplicity, has been assessed by measuring LDL and HDL cholesterol levels as surrogate parameters of particle concentrations. However, there is growing evidence that lipoprotein subclass determinations improve the prediction of coronary disease risk. For LDL, the predominance of small, dense LDL particles (termed subclass pattern B) has been associated with a 3-fold increased risk for CAD (42). Although the predominance of small, dense LDL is probably closely connected to other lipid markers such as triglycerides, HDL cholesterol, and apoB (9, 42–46), this trait has been accepted as an emerging cardiovascular risk factor (1).

For HDL, the situation is more complex. Initially, the data on the prognostic impact of HDL subclasses were controversial. Some authors described a major protective effect of small, dense HDL_3 particles (47–49), whereas others found the main protective properties in large HDL_2 particles (50) or even a positive correlation between small HDL particles and CAD (3). However, in the last years, there seems to be growing epidemiological evidence that large HDL particles harbor the protective properties of HDL cholesterol, whereas small, poorly lipidated HDL particles might indeed be positively correlated with cardiovascular events (4, 8, 11, 12, 51, 52).

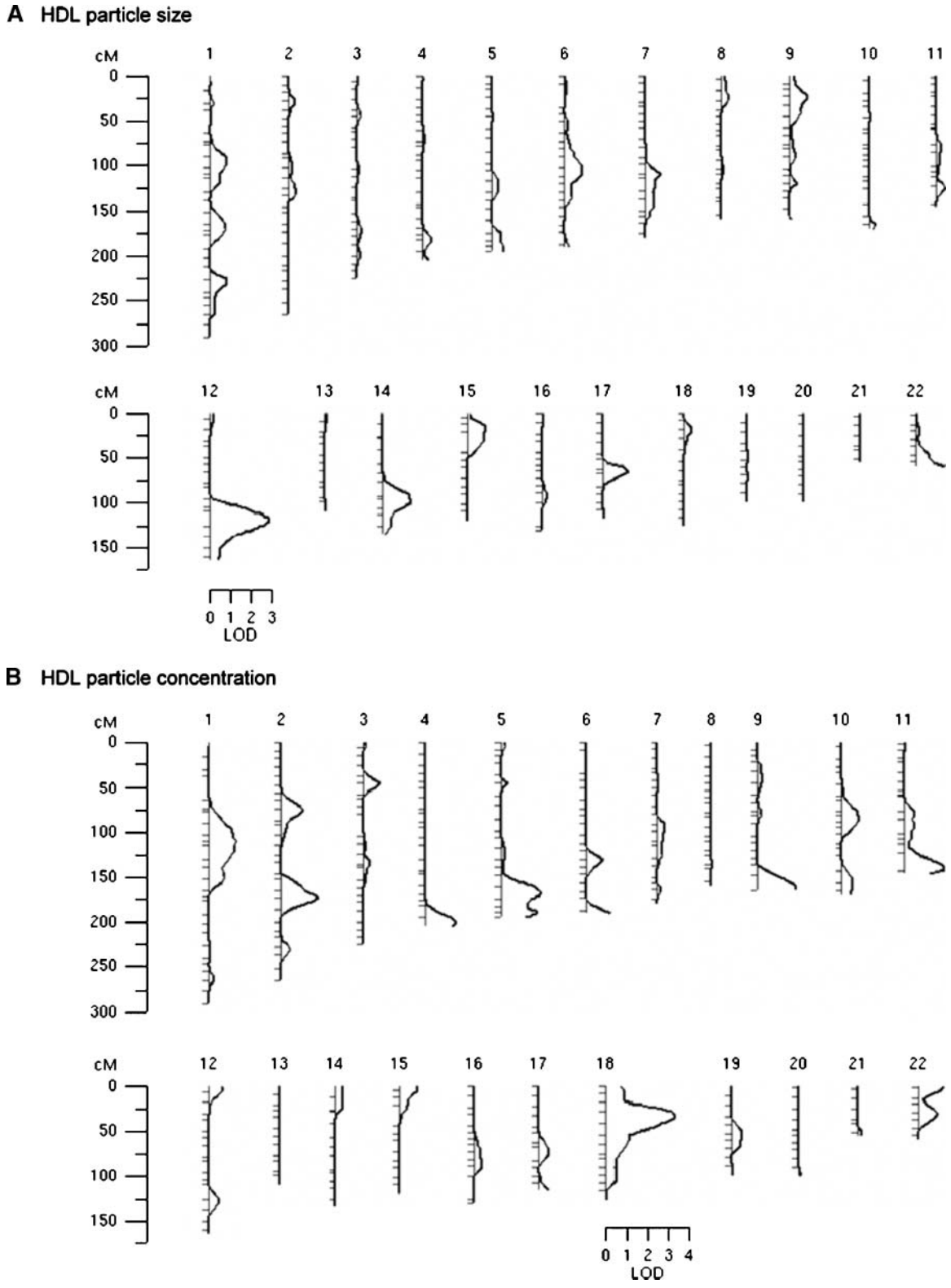

Fig. 3. Genome-wide linkage scan for mean HDL particle size (A) and HDL particle concentration (B) using univariate calculation. cM, centimorgan; LOD, logarithm of the odds.

Limitations of this study

Some limitations of the present study should be mentioned. Our heritability estimates may overestimate the true genetic contribution, because we did not account for shared environmental exposures among family members in our model. Although most siblings (>90%) reported living in separate households from one another and their parents, it is possible that a shared environ-

TABLE 3. Maximum LOD scores for all assessed lipoprotein traits in univariate calculation

Trait	LOD	Chromosome	Position (centimorgan)
LDL			
LDL cholesterol[a]	(1.5)	(11)	(0)
LDL particle size	1.9	11	99
LDL particle concentration	(2.5)	(11)	(2)
LDL A (16–19 nm)	2.4	1	259
LDL B (19–21 nm)	1.2	9	160
LDL C (21–22 nm)	1.3	4	145
LDL D (22–25 nm)	2.3	17	109
LDL E (25–30 nm)	1.3	9	20
HDL			
HDL cholesterol[a]	1.1	11	74
HDL particle size	2.9	12	119
HDL particle concentration	3.3	18	33
HDL A (7–8.5 nm)	2.7	12	124
HDL B (8.5–10 nm)	(2.4)	(3)	(0)
HDL C (10–13 nm)	1.9	12	113
HDL D (13–16 nm)	1.4	10	125

Logarithm of the odds (LOD) scores in parentheses describe telomeric peaks.

[a] Cholesterol values as computed from NMR spectroscopy.

ment early in life contributes to specific phenotypes seen in adulthood.

Second, the technology of NMR spectroscopy must be discussed. NMR spectroscopy analyzes particle sizes and concentrations, but it is not capable of determining the cholesterol content of lipoprotein particles. Therefore, the reconstruction of LDL and HDL cholesterol values is performed by assuming an average cholesterol content for each particle size, multiplying these values by the particle number for each subclass, and finally adding all of the subclass cholesterol values. This algorithm works fine in normolipidemic individuals but can be problematic in dyslipidemic, particularly hypertriglyceridemic, patients, as the proportional cholesterol content of lipoprotein subclasses varies under these conditions. These considerations at least partly explain why the computed LDL and HDL cholesterol values do not show perfect correlation with the values obtained by routine enzymatic methods.

Another issue to be considered is the fact that the families studied here were ascertained for early myocardial infarction, such that we have no data on healthy subjects. However, we have no reason to believe that the overall heritability of lipoprotein particle features is affected by a myocardial infarction in the patients' history.

Furthermore, the use of lipid-lowering therapy has to be mentioned. Almost 50% of our study population was on statin therapy. HDL cholesterol increases slightly under CSE inhibitor therapy, which some authors have attributed to an increasing HDL particle concentration, whereas others observed an increased mean particle size (53–55). In our data, statin therapy had a weak effect on both HDL particle size and particle concentration, explaining <1% of the variance of the two traits (0.38% and 0.54%, respectively). To further assess the effect of statin use, we calculated heritabilities excluding all patients on CSE inhibitors. Despite poor statistical power, the results were similar to those obtained from the entire cohort (data not shown). In our multivariate models, we adjusted for the use of CSE inhibitors in a product- and dose-dependent manner; nevertheless, this issue may leave some uncertainty.

Generally, the adjustment for covariates should be discussed. Plasma lipoproteins are not only influenced by a number of external factors such as age, sex, medication, and nutrition but are also highly dependent on each other. We adjusted for triglyceride levels to account for the fact that our blood samples were taken from nonfasting individuals. We decided not to adjust for other lipid traits, because in the complex interdependence of the lipoprotein profile the nature of any genetic influence is likely to be pleiotropic. Therefore, adjusting for other lipid traits

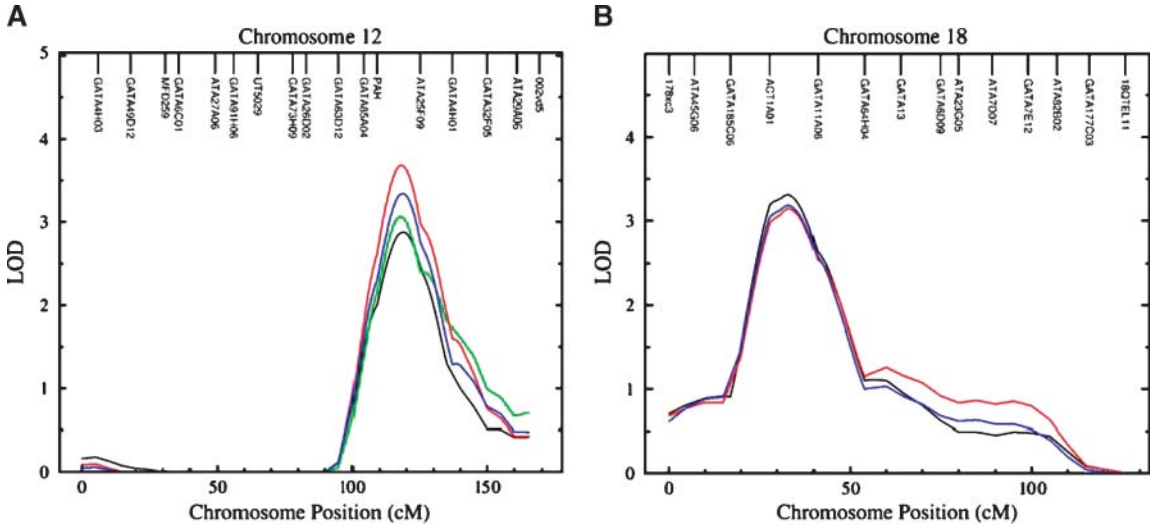

Fig. 4. LOD plots for HDL size (A) and HDL particle concentration (B) after adjustment. Black, no adjustment; blue, adjustment for age and sex; red, additional adjustment for diabetes, body mass index, statins, and smoking; green, further adjustment for triglycerides (significant only for HDL particle size).

would mask genetic effects rather than correct confounders. Hence, the adjustment for triglycerides may also represent an overcorrection, as (fasting) triglyceride levels have a heritable component as well (56).

Conclusion

We present the first study to investigate the heritability of the entire LDL and HDL subfraction profile using NMR spectroscopy. Moreover, we found significant evidence of linkage for HDL size on chromosome 12 and for HDL particle concentration on chromosome 18. While the latter locus has been reported previously in the context of fasting plasma glucose, the former region has been described as a QTL influencing HDL cholesterol. Therefore, both loci are likely to harbor genes influencing metabolic traits. Identifying these genes may provide new insight into the biological mechanisms underlying the lipoprotein subfraction profile and may possibly allow the development of novel therapeutic and preventive strategies.

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