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Quantitative trait loci in *ABCA1* modify cerebrospinal fluid amyloid- β_{1-42} and plasma apolipoprotein levels

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Abstract The ATP-binding cassette transporter A1 encoded by *ABCA1* plays an integral role in the efflux of cellular cholesterol and phospholipids, but may also be a central mediator of β -amyloid ($A\beta$) processing. Here, genetic association of the common R219K variant of *ABCA1* is shown with cerebrospinal fluid (CSF) $A\beta_{1-42}$ levels, reinforcing emerging evidence of a connection between lipid and $A\beta$ metabolism. In support of this

finding we demonstrate for the first time that CSF cholesterol and $A\beta_{1-42}$ are correlated. To affirm the plausible impact of *ABCA1* variation on cholesterol and related traits as well as to empower a survey of possible interactions (e.g. age, gender, and smoking), a large Swedish population consisting of over 2,700 individuals was enlisted and extensive measures of plasma lipid parameters carried out. These analyses revealed that R219K has a strong effect on apolipoprotein B (APOB) and LDL-cholesterol (LDL-C) among smokers ($P=0.000055$ and $P=0.00059$, respectively), but not among non-smokers. In contrast, no effect was evident with apolipoprotein A (APOA1) or HDL-cholesterol (HDL-C) levels. Plasma APOB and LDL-C, but not APOA1 and HDL-C, were shown to be markedly elevated in smokers versus non-smokers, affirming that smoking may selectively impact the former pathway. No other genetic markers in *ABCA1* exhibit effects as large as R219K, although a modest independent effect of R1587K was observed. Our data illuminate a possible genetic link between $A\beta$ and cholesterol metabolism, but also provide an intriguing example of an environmental exposure that may modify a genotype–phenotype relationship.

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Introduction

A perturbation of lipid metabolism is likely to underlie a number of human diseases, and the discovery of genes and/or environmental exposures that differentially modulate this process stands to greatly improve knowledge of their aetiologies. This is exemplified by the finding that variants of the gene encoding apolipoprotein E (*APOE*) modify lipid levels (Yue et al. 2005; Viiri et al. 2005), which in turn contributes to Alzheimer's disease

(AD OMIM 104300) and cardiovascular disease (CVD) (Corder et al. 1993; Poirier et al. 1993; Strittmatter et al. 1993; Eichner et al. 2002). As well as directly impacting plasma lipid levels, *APOE* also strongly affects β -amyloid ($A\beta$) deposition in the brain (Beffert et al. 1999), suggesting a connection between $A\beta$, the main component of plaques, and apolipoprotein metabolism. In further support of this, another prominent phenotypic effect of *APOE* variation is upon cerebrospinal fluid (CSF) levels of the 42 amino acid fragment of β -amyloid ($A\beta_{1-42}$) (Prince et al. 2004). In addition, other genes proven to influence AD (*APP*, *PSEN1*, and *PSEN2*) (Goate et al. 1991; Rogaeve et al. 1995; Sherrington et al. 1995) have been shown to be involved in $A\beta$ metabolism (Scheuner et al. 1996). On this basis, the search for genes that modify both $A\beta$ and lipid processing may aid in understanding potentially overlapping biological mechanisms underlying CVD and AD.

The gene encoding the ATP binding cassette A1 transporter (*ABCA1*) has become widely studied in connection with CVD and related quantitative traits (Clee et al. 2001; Tregouet et al. 2004). Since the discovery that mutations in *ABCA1* are the primary cause of Tangier disease (Brooks-Wilson et al. 1999; Bodzioch et al. 1999; Rust et al. 1999), in which HDL-cholesterol (HDL-C) levels are extremely low, several hundred articles have appeared describing the function of the protein, as well as attempts to associate common polymorphism in *ABCA1* to cholesterol-related traits. The *ABCA1* protein mediates the unidirectional efflux of free cholesterol and phospholipids from cells, facilitating the lipidation of most of the exchangeable apolipoproteins (including APOA1 and APOE) and the formation of nascent HDL molecules (Yancey et al. 2003). The *ABCA1* protein has a wide tissue distribution (Wellington et al. 2002), but has been most studied in peripheral tissues. Recent studies have shown that deficiency of *ABCA1* in the brain leads to a specific decrease in APOE levels (Hirsch-Reinshagen et al. 2004; Wahrle et al. 2004). Interestingly, *ABCA1* shares a commonality with APOE in that it appears to be involved in $A\beta$ metabolism (Fukumoto et al. 2002); this is supported by evidence suggesting a relationship between cholesterol metabolism and $A\beta$ metabolism (Simons et al. 1998, 2001; Kojro et al. 2001). Emerging data thus suggest that *ABCA1* plays an important role in lipid metabolism in both peripheral tissues and the central nervous system (CNS) and, like APOE, may also influence $A\beta$.

Genetic association studies on *ABCA1* have been abundant, but have also left many questions unanswered. The accumulated literature supports the existence of functionally different alleles of *ABCA1* that affect both discrete disease states (Singaraja et al. 2003) and quantitative traits (Wang et al. 2000; Clee et al. 2001; Frikke-Schmidt et al. 2004). One of the principal problems has been that the sample sizes of most studies have been small, typically entailing less than 1,000 individuals. In the present study, we have employed two different samples to explore the possible influence of *ABCA1* sequence

variants on both $A\beta$ levels and on measures of lipid metabolism. For the latter in particular, we surveyed a large epidemiological sample to empower both the detection of genetic association and the illumination of possible interactions.

Materials and methods

Human subjects

Swedish subjects for CSF analyses were recruited as part of a longitudinal geriatric population study in Piteå, Sweden (mean age-at-exam \pm SD = 75.4 \pm 7.8, n = 226; 86 males and 140 females). More detailed descriptions of the subjects can be found elsewhere (Andreasen et al. 1999). This sample consists of both AD (n = 156) and frontotemporal dementia (FTD) cases (n = 70). This sample was not examined in our previous work on *ABCA1* (Katzov et al. 2004). For analyses involving ANOVA, a sample identifier was included as a covariate to adjust for differences in average $A\beta_{1-42}$ levels between AD and FTD groups. Clinical DNA samples employed in this study to explore for quantitative metabolic trait effects derive from individuals participating in the Stockholm Heart Epidemiology Program (SHEEP), a population based case-control study aimed at investigating the effects of various risk factors for myocardial infarction (MI) in men and women (Reuterwall et al. 1999). The subjects were aged 45–70 and were selected from the Stockholm County population registry (mean age-at-exam \pm SD = 59.6 \pm 7.1). The sample consists of healthy controls (n = 1,526; 1,033 males and 493 females) and individuals who have suffered a non-fatal, first MI event (n = 1,177; 829 males and 348 females) (Bennet et al. 2003). Exposure information was obtained from a detailed questionnaire, and a health examination that included blood sampling and anthropometric tests. The latter was conducted at least 3 months after the MI event to allow metabolic stability to be regained (Hamsten et al. 1986). Current smokers were defined as those who smoked tobacco regularly, or had stopped within the last 2-year period. Former smokers were subjects who used to smoke regularly but who had stopped 2 or more years previously. Non-smokers were defined as those who reported never having smoked, or having smoked regularly for less than 1 year.

Genotyping

Information on the R219K, I883M, and R1587K variants [representing cDNA (GenBank accession number NM_005502.2) rs2230806, c.658 G > A; rs4149313, c.2650 A > G; rs2230808, c.4762 G > A, respectively] may be found at dbSNP (<http://www.ncbi.nlm.nih.gov/SNP/>). Genotyping was performed using Dynamic Allele Specific Hybridization (DASH) (Prince et al. 2001). DASH and PCR were performed using previously

published oligonucleotide sequences and assay conditions (Katzov et al. 2004).

Cerebrospinal fluid collection and A β_{1-42} measurement

CSF samples were obtained by lumbar puncture in the L3/L4 or L4/L5 inter-space. Further details of CSF collection can be found elsewhere (Andreasen et al. 1999). CSF A β_{1-42} was determined using a sandwich ELISA (Innotest amyloid- $\beta_{(1-42)}$, Innogenetics, Ghent, Belgium) constructed to specifically measure A β_{1-42} (Vanderstichele et al. 1998).

Preparation for gas chromatography analysis of sterols

One microgram [26.26.26.27.27.27-²H₆] cholesterol (10 μ l from a stock solution of d₆-cholesterol in cyclohexane; 0.1 mg/ml) and 2 ng racemic [23.23.24.25-²H₄] 24-OH-cholesterol (Medical Isotopes, Pelham) and 4 ng [²H₅] 27-OH-Chol [50 μ l from a stock solution 40 ng 24S-hydroxycholesterol (24S-OH-Chol) and 80 ng 27-hydroxycholesterol per millilitre cyclohexane] were added as internal standards to 500 μ l CSF. This solution was evaporated under nitrogen gas. After alkaline hydrolysis with 1 ml 1 M sodium hydroxide in 90% ethanol for 1 h at 50°C and addition of 1 ml distilled water, the sterols and oxysterols were extracted twice with 3 ml cyclohexane. The combined organic phases were dried under nitrogen at 60°C. The residue was dissolved in 160 μ l *n*-decan. Eighty microlitres of the solution was transferred into a micro-vial for gas-liquid chromatography-mass spectrometric (GC-MS) analysis of cholesterol precursors, metabolites and plant sterols. Another 80 μ l was diluted with 400 μ l *n*-decan for analysis of cholesterol by gas-liquid chromatography-flame ionisation detection (GC-FID). The sterols and oxysterols were derivatised to trimethylsilyl-(TMSi) ethers by adding 40 μ l TMSi-reagent (pyridine:hexamethyldisilazane:trimethylchlorosilane; 9:3:1, by volume; all reagents were supplied by Merck, Darmstadt, Germany) into each micro-vial and incubated for 1 h at 60°C.

Sterol analysis by GC-MS

Cholesterol, and the oxysterol 24S-OH-Chol, extracted from CSF samples was separated on a cross-linked methyl silicone DB-XLB 122-1232 fused silica capillary column (J&W Scientific, Folsom, CA) (30 m \times 0.25 mm i.d. \times 0.25 μ m film thickness) in a HP 5890 Series II (Hewlett Packard, Los Angeles, CA) gas-chromatograph combined with a HP 5972 Series mass selective detector. An aliquot of 2 μ l (or 3 μ l for the CSF aliquot) was injected by automated injection (HP 7673 auto sampler and automatic injection) in splitless mode at an injection temperature of 280°C. Helium was used as the carrier gas with an inlet pressure of 1.03 bar. The initial oven tem-

perature was kept at 150°C for 3 min; thereafter it was increased at a rate of 30°C/min to a final temperature of 290°C for 28 min. The temperature of the transfer line was kept at 280°C. Multiplier voltage was set to 2,650 eV (2,950 eV for CSF). Electron impact ionisation was employed at 70 eV. Selected-ion monitoring (SIM) was performed by cycling the quadrupole mass filter between the chosen *m/z* values at a rate of 2.5-5 (1.5-3 for CSF) cycles per second. TMSi-ether of 24S-OH-Chol was measured at *m/z* 413 (M⁺-OTMSi-CH(CH₃)₂) and deuterated 24S-OH-Chol at *m/z* 416 [M⁺-OTMSi-CD(CH₃)₂]. The concentrations of 24S-OH-Chol and cholesterol in CSF samples were calculated from standard curves using the corresponding deuterated compounds (isotope dilution method). Identity of all sterols was proven by comparison with the full scan mass spectra of authentic compounds. Additional qualifiers (characteristic fragment ions) were used for structural identification.

Statistical analysis

Deviation from Hardy-Weinberg equilibrium for genotypes at individual loci, as well as differences in genotype distributions between groups was assessed using the χ^2 statistic. Tests for association of single marker genotypes with CSF A β_{1-42} levels and other metabolic quantitative traits were performed using analysis of variance (ANOVA), and significance of pair-wise comparisons were assessed using Fisher's PLSD when *F* ratios were significant. Skewed traits (skewness > 1.0) were log-transformed prior to analysis. The above statistical analyses were performed using StatView version 5.0 (Abacus Concepts). Haplotypes were estimated using PHASE v2.1 which implements a Bayesian method of haplotype estimation for unrelated individuals (Stephens et al. 2001; Stephens and Donnelly 2003). For estimation of empirical *P* values in case-control tests, 1,000 permutations were used with all other settings at default.

Results

We began by testing *ABCA1* variants for a potential influence on CSF A β_{1-42} levels. We focussed on the R219K, I883M, and R1587K variants since these give rise to amino acid changes and because they are common in European populations (Clee et al. 2001). These are also among the most widely studied variants at this locus in relation to CVD phenotypes (Clee et al. 2001; Zwarts et al. 2002; Evans and Beil 2003; Srinivasan et al. 2003; Wollmer et al. 2003; Frikke-Schmidt et al. 2004; Tregouet et al. 2004). A significant effect of the R219K variant on CSF A β_{1-42} levels was observed whereby RK heterozygotes had the highest trait levels (547 \pm 19.2, *n* = 141; 658 \pm 35.1, *n* = 71; 443 \pm 60.6, *n* = 4; $F_{2,212}$ = 6.2, *P* = 0.0024, for RR, RK, and KK genotype classes, respectively; pg/ml \pm SEM). An age- and gender-adjusted model was not markedly different ($F_{2,210}$ = 5.9, *P* = 0.0031). The most

significant pair-wise comparison was between the RR and RK classes ($P=0.0007$, Fisher's PLSD post hoc test). Neither of the other markers exhibited significant effects on the CSF $A\beta_{1-42}$ trait (data not shown). We also note that an independent AD study suggested that the R variant was associated with earlier age-at-onset (AAO) in AD (Wollmer et al. 2003). However, we observed no AAO effect in this sample, consistent with our previous observations (Katzov et al. 2004) (data not shown).

Although there is considerable literature suggesting a relationship between $A\beta$ and lipid metabolism, to our knowledge no study has ever specifically tested whether cholesterol levels and $A\beta$ are correlated, either in peripheral tissues or in the CNS. To address this question, we measured cholesterol and 24S-hydroxycholesterol (24S-OH-Chol) in CSF from 42 individuals from the above sample. Data depicting the correlation between $A\beta_{1-42}$ and both cholesterol and 24S-OH-Chol are shown in Fig. 1. The modest significance reflects the small sample size, but we note that the effect size is large, with approximately 10% of the variance in each trait being explained by the other trait. Age and gender adjustment improved model fits slightly ($P=0.016$ for $A\beta_{1-42}$ vs cholesterol, and $P=0.0091$ for $A\beta_{1-42}$ vs 24S-OH-Chol). Not shown in the figure is the regression between cholesterol and 24S-OH-Chol, which is highly

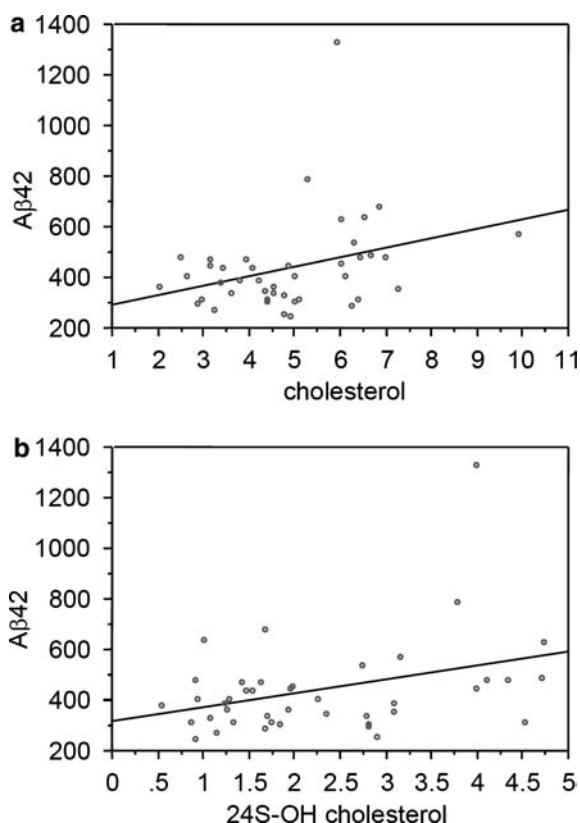


Fig. 1 Linear regression demonstrating a correlation between a cerebrospinal fluid (CSF) cholesterol and amyloid β ($A\beta_{1-42}$), and b 24S-OH-Chol and $A\beta_{1-42}$. In both cases there is a positive correlation between the two traits (a $r^2=0.11$, $P=0.035$; b $r^2=0.13$, $P=0.021$)

significant ($r^2=0.26$, $P=0.0006$, $N=42$). Given the size of this sample, we did not attempt to explore for effects of *ABCA1* variants on these measures of cholesterol.

We had difficulty obtaining additional CSF samples to replicate the finding of an effect of *ABCA1* on $A\beta_{1-42}$. However, presuming a possible fundamental relationship between cholesterol and $A\beta$ metabolism, we considered the alternative approach of examining lipid traits in a large CVD sample instead. From available phenotypes, we selected six traits that are commonly examined in CVD studies (Table 1). In an initial screen, we tested the R219K variant using ANOVA for effects upon these selected traits in the entire set of samples. A few modest signals emerged from this analysis, including significant findings for LDL-cholesterol (LDL-C), total cholesterol (TC), and APOB levels (Table 1). Interaction terms were then included for age, gender, disease status (MI or control), and smoking (current smokers or non-smokers) in second-order factorial ANOVA models, testing all three of these traits. Among these, the interaction term for smoking was significant for the APOB trait and thus stratified analyses were performed in smokers and non-smokers. The results of this analysis were highly significant in the smoking group but not in non-smokers (Table 2). On this basis, we also tested the LDL-C and TC traits, which were both significantly but modestly associated with R219K in smokers ($P=0.031$ and $P=0.019$, respectively), but not in non-smokers (Table 2). Notably, the data for the effect of R219K on APOB were similar to those for $A\beta_{1-42}$, whereby heterozygote levels differed from both homozygote groups, but in a non-additive fashion (i.e. the heterozygous class did not fall midway between opposite homozygotes).

The model for the effect of R219K on APOB and CSF $A\beta_{1-42}$ suggested by the above data implies either heterosis (Comings and MacMurray 2000) or a possible interaction with other variants in *ABCA1* (or a nearby gene). To begin exploring this latter possibility in the CVD sample (since it affords the most power to detect such effects), genotyping of the I883M and R1587K variants was performed, and ANOVA used to model the potential independent main effects in a joint analysis of all three markers (including R219K). We noted that LD is very weak between these sites (Katzov et al. 2004) and therefore assumed that collinearity would likely not be a problem. In smokers, the main effect for R219K improved when adjusting for the other sites from $F_{2,943}=8.4$, $P=0.00024$ in isolation to $F_{2,898}=9.9$, $P=0.000055$ when all three markers were included. An independent effect for the R1587K variant was also noted in the full model ($F_{2,898}=3.8$, $P=0.023$). The I883M variant was not associated with the APOB trait either in isolation or in the full model. A final model was also constructed including all three markers and age, gender, and case-control status as covariates. The main effect of the R219K variant was not markedly changed in this analysis ($F_{2,895}=9.6$, $P=0.000077$). None of the markers were significantly associated with APOB levels in non-smokers.

Table 1 Quantitative trait analyses for the R219K variant. Values represent the mean \pm SEM for each trait according to genotype. The number of individuals in each category is shown in parenthesis. Comparisons were performed by ANOVA models. Non-normally

distributed traits were log transformed. *HDL-C* High density lipoprotein cholesterol, *LDL-C* low density lipoprotein cholesterol, *TC* total cholesterol, *TG* triglycerides

Traits	KK	RK	RR	F value	P value
APOA1 ^a (g/l)	1.46 \pm 0.02 (157)	1.48 \pm 0.01 (966)	1.47 \pm 0.01 (1,439)	0.23	0.79
APOB (g/l)	1.64 \pm 0.03 (157)	1.53 \pm 0.01 (966)	1.55 \pm 0.01 (1,439)	5.4	0.0046
HDL-C (mmol/l)	1.16 \pm 0.26 (156)	1.20 \pm 0.01 (961)	1.20 \pm 0.01 (1,427)	1.1	0.34
LDL-C (mmol/l)	4.26 \pm 0.86 (154)	4.08 \pm 0.03 (951)	4.05 \pm 0.03 (1,415)	0.26	0.038
TC (mmol/l)	6.24 \pm 0.09 (157)	6.04 \pm 0.04 (969)	6.01 \pm 0.03 (1,439)	3.02	0.049
TG ^a (mmol/l)	1.87 \pm 0.11 (157)	1.71 \pm 0.04 (969)	1.72 \pm 0.03 (1,441)	1.38	0.25

^aLog-transformed traits

Table 2 Phenotypic effects of the R219K variant in smokers and non-smokers

ApoB	KK	RK	RR	F value	P value
Non-smokers	1.56 \pm 0.04 (102)	1.51 \pm 0.02 (573)	1.50 \pm 0.01 (878)	1.1	0.32
Smokers	1.80 \pm 0.06 (53)	1.56 \pm 0.02 (366)	1.62 \pm 0.02 (527)	8.4	0.00024
TC					
Non-smokers	6.06 \pm 0.11 (102)	5.97 \pm 0.04 (574)	5.93 \pm 0.04 (879)	0.84	0.43
Smokers	6.62 \pm 0.16 (53)	6.14 \pm 0.06 (367)	6.15 \pm 0.05 (526)	4.0	0.019
LDL-C					
Non-smokers	4.14 \pm 0.10 (102)	4.03 \pm 0.04 (568)	3.99 \pm 0.03 (868)	1.34	0.26
Smokers	4.54 \pm 0.16 (50)	4.13 \pm 0.05 (355)	4.17 \pm 0.05 (516)	3.5	0.031

Values are the mean \pm SEM according to R219K genotype. The number of individuals in each category is shown in parenthesis. Comparisons were performed by ANOVA. *TC* total cholesterol, *LDL-C* low density lipoprotein cholesterol

Having provided evidence of their potential independent effects, we used a diplotype strategy (Gu et al. 2004) to test for interactions between the R219K and R1587K variants, focussing on the smoking population. This strategy allows testing of haplotype effects, and can expose hybrids (individuals with two unlike haplotypes) that may contribute to trait variance. For this, we used PHASE v2.1 (Stephens et al. 2001; Stephens and Donnelly 2003), which produced estimates of approximately 0.7 probability for all heterozygous individuals. We note for reference that this strategy is essentially identical to the strategy that has long been used with *APOE*, whereby two sites distinguish the ϵ 2, ϵ 3, and ϵ 4 alleles and their diplotype configurations. The results from this analysis were highly significant ($F_{7,905} = 4.7$, $P = 0.000037$) and are shown in Fig. 2. Given the uncertainty of the double heterozygote class, it was excluded in an additional model, which did not markedly alter significance ($F_{6,776} = 4.9$, $P = 0.000057$).

We also tested the R219K, I883M, and R1587K markers for association with MI risk in the whole sample and in smokers and non-smokers. Among these tests, the only significant finding to emerge was modest significance for R1587K in the smoking population ($P = 0.025$) (Table 3). Diplotype distributions (constructed with R219K and R1587K) between cases and controls in the smoking population were not significantly different (data not shown). We further tested whether *ABCA1* haplotype distributions were different in MI versus control samples using PHASE v2.1 but

found no significant effects in the whole sample or in either smokers and non-smokers (data not shown).

The primary outstanding question from this study is how smoking and *ABCA1* might be interacting to affect lipid profiles. We note that smoking has long been known to influence lipid metabolism (Doll and Hill 1996; Talmud and Humphries 2002) but to specifically address this question here we tested for differences in six lipid-related traits between smokers and non-smokers. We adjusted for gender, age, and disease status (MI or control), and found highly significant differences for APOB, LDL-C, TC, and TG (triglycerides), but not for APOA1 and HDL-C. The strongest difference was observed for APOB levels ($F_{1,2624} = 25.1$, $P = 0.00000058$; 1.514 ± 0.009 mean g/l \pm SEM for non-smokers and 1.608 ± 0.013 for smokers). Importantly, in examining the data for the effect of *ABCA1* on APOB levels shown in Table 2, we note that there is no significant difference in the heterozygote class between smokers and non-smokers ($F_{1,923} = 2.1$, $P = 0.14$). This contrasts with the differences in the rare allele homozygous class ($F_{1,150} = 9.4$, $P = 0.0025$) and the common-allele homozygous class ($F_{1,1400} = 20.4$, $P = 0.0000068$). Thus, the lack of effect in heterozygotes does not appear to derive from the smaller number of individuals in this category compared to common-allele homozygotes, since rare-allele homozygotes do exhibit a highly significant difference between smokers and non-smokers.

To explore the possible functional effects of *ABCA1* variants, we used the PolyPhen tool (<http://www.bor>

Table 3 Case-control single marker tests in smokers and non-smokers. Genotype frequencies for the three studied SNPs in *ABCA1* are presented. For differences between cases and controls, allele frequencies were tested in 3x2 contingency tables. *MI* Myocardial infarction

R219K (rs2230806)		KK	RK	RR	<i>P</i> value		
Smokers	MI	29 (0.05)	205 (0.39)	293 (0.56)	0.98		
	Controls	24 (0.06)	162 (0.38)	236 (0.56)			
Non-smokers	MI	35 (0.06)	198 (0.36)	314 (0.57)		0.87	
	Controls	67 (0.67)	377 (0.37)	566 (0.56)			
I883M (rs4149313)		II	IM	MM			
Smokers	MI	418 (0.76)	127 (0.23)	4 (0.01)			0.14
	Controls	349 (0.81)	77 (0.18)	3 (0.01)			
Non-smokers	MI	445 (0.78)	115 (0.2)	12 (0.02)	0.31		
	Controls	843 (0.81)	184 (0.18)	16 (0.02)			
R1587K (rs2230808) (rs2230808)		KK	RK	RR			
Smokers	MI	29 (0.05)	160 (0.30)	342 (0.64)		0.025	
	Controls	14 (0.03)	159 (0.38)	250 (0.59)			
Non-smokers	MI	25 (0.04)	182 (0.33)	346 (0.62)			0.66
	Controls	57 (0.05)	329 (0.32)	650 (0.63)			

k.embl-heidelberg.de/PolyPhen/) (Ramensky et al. 2002) for five coding SNPs (cSNPs) in *ABCA1* (R219K rs2230806; V771M rs2066718; V825I rs4149312; I883M rs2066714; R1587K rs2230808) (Frikke-Schmidt et al. 2004). As a control we also tested the *APOE-ε4* (R130C rs428358) variant. We found the predicted effects of all the tested cSNPs in *ABCA1* to be benign (i.e. most likely lacking any phenotypic effect), whilst the functional effect of the *APOE-ε4* (rs428358) variant was predicted to be probably damaging.

Finally, we tallied the number of statistical hypothesis tested in this study in its entirety. There were 57 independent tests; note that applying a strict Bonferroni correction for multiple testing does not eliminate the significance of the primary findings for either CSF $A\beta_{1-42}$ ($P=0.0007_{\text{uncorrected}}$ and $P=0.04_{\text{corrected}}$) or APOB levels ($P=0.000037_{\text{uncorrected}}$ and $P=0.0021_{\text{corrected}}$).

Discussion

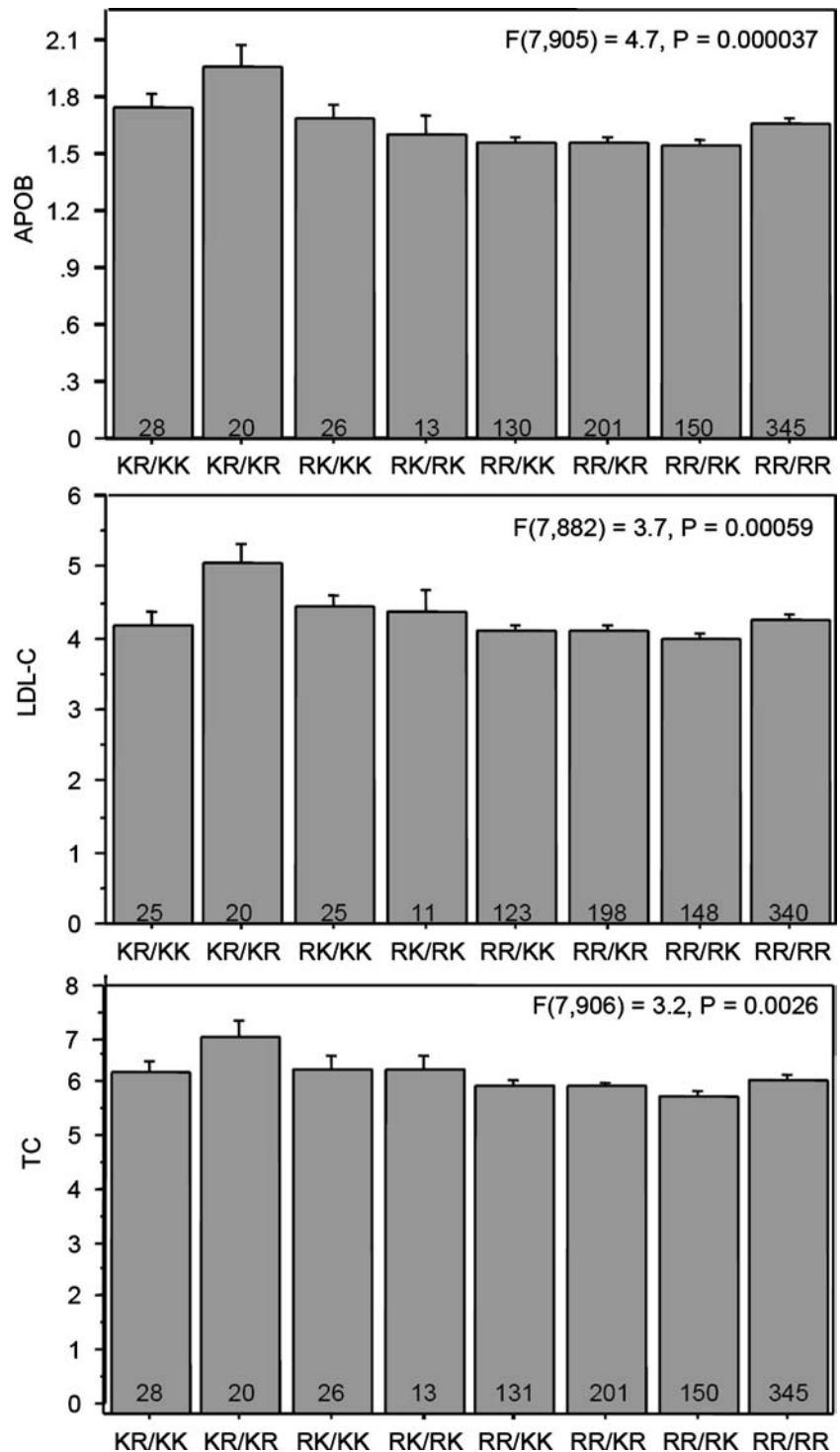
We have previously shown that CSF $A\beta_{1-42}$ is robustly affected by polymorphism in *APOE* (Prince et al. 2004), the most widely replicated genetic risk factor for AD, and have thus presumed that other true AD-modifying genes might also exhibit effects upon $A\beta$ metabolism. That *ABCA1* can directly influence $A\beta$ has been demonstrated (Fukumoto et al. 2002), and more recent evidence suggests that *ABCA1* also interacts with *APOE* in the CNS (Hirsch-Reinshagen et al. 2004). In the present study, we suggest that genetic variants of *ABCA1* may influence CSF $A\beta_{1-42}$ levels, possibly reflecting its involvement with $A\beta$ metabolism. This is supported by the finding here that $A\beta_{1-42}$ and cholesterol levels are correlated in CSF. Thus, *ABCA1* shares a similarity with *APOE* in that both appear to be involved in lipid and $A\beta$ metabolism.

To further investigate the potential consequences of *ABCA1* variation on lipid metabolism, we examined a large CVD sample, focussing on six commonly studied lipid traits. Since its discovery as the cause of Tangier

disease (Brooks-Wilson et al. 1999; Bodzioch et al. 1999; Rust et al. 1999), many genetic association studies have been performed providing evidence that common polymorphism in *ABCA1* impacts cholesterol-related phenotypes, the most replicable of which is plasma HDL-C level (Clee et al. 2001). However, not all studies have demonstrated effects upon HDL-C levels (Kakko et al. 2003). One of the primary weaknesses of prior genetic association studies on *ABCA1* has been that sample sizes have been relatively small. The results of the present investigation suggest that variants of *ABCA1* primarily affect APOB levels given that there was no evidence for effects upon HDL-C or APOA1 levels, the two principal phenotypes for which several prior studies suggest association (Wang et al. 2000; Srinivasan et al. 2003; Tregouet et al. 2004). Studies in animal models point to several phenotypes that are affected by *ABCA1* modification, including plasma APOB levels, which has been shown both in transgenic mice (Vaisman et al. 2001) and more recently in studies exploiting RNA interference (Ragozin et al. 2005). In the present study, significant effects were also evident for LDL-C and TC levels, but this is perhaps unsurprising given the strong correlations of these traits with APOB. Our findings nonetheless illuminate the question of what specific phenotypes may be best suited to provide evidence that a particular pathway is affected by a gene. The consideration of multiple lipid-related traits in future studies will probably be essential to resolving this question.

An intriguing aspect of these findings was that effects appear to occur only in individuals who smoke. One prior study did suggest stronger effects of R219K upon coronary heart disease in smokers (Cenarro et al. 2003). In contrast, one of the most comprehensive studies to date on *ABCA1* variation found an association with MI exclusively in non-smokers (Tregouet et al. 2004). Thus, it appears possible that smoking modifies the phenotypic effects of *ABCA1*, but the nature of the interaction is unclear at present. This may have a bearing on the success or failure of future studies that attempt to relate *ABCA1* variation to other disease phenotypes, but may

Fig. 2 Diplotype analysis of quantitative traits considering the joint contributions of the R219K and R1587K variants. Categories represent the diplotype configurations of each individual from among four possible haplotypes (RR, KK, RK, and KR, where R219K is at the first position). Values shown are mean \pm SEM. *F* ratios from ANOVA omnibus tests and associated *P* values are shown in the upper-right corner of each panel. The number of individuals in each category is shown below each column. The highest trait levels occur in individuals that are homozygous for the K variant of R219K and homozygous for the R variant of R1587K. *LDL-C* Low density lipoprotein cholesterol



also serve as a point to revisit in large published studies, such as that of Frikke-Schmidt et al. (2004). In particular, our own results on AD (Katzov et al. 2004) and on CSF $A\beta_{1-42}$ levels in the present study may be influenced by this potential modification. Unfortunately, it is uncommon for AD sample collection strategies to include information about smoking or other lifestyle data, and this is admittedly true for the majority of our own samples. Larger studies on *ABCA1* in AD are ongoing in

samples from the Swedish Twin Registry (Lichtenstein et al. 2002), for which these kinds of data are available, and should help to resolve this issue. This and other studies that consider smoking should help illuminate an appropriate genetic model to facilitate further replication efforts. This will be necessary in order to corroborate findings across phenotypes (for example, earlier observations of modest effects on senile plaque load in the AD brain) (Katzov et al. 2004).

The extent of local sequence variation in *ABCA1* is fairly well resolved after a number of exhaustive DNA sequencing efforts (Wang et al. 2000; Tregouet et al. 2004). Among discovered and examined variable sites, the best evidence for potentially functional polymorphism is for the commonly studied R219K and R1587K variants (Tregouet et al. 2004). Interestingly, pair-wise linkage disequilibrium for variants in *ABCA1* is fairly weak (Katzov et al. 2004; Tregouet et al. 2004), and on this basis it is intriguing that studies have found sporadic effects of these particular markers on different phenotypes. Discrepancies of this nature may reflect low power in a study but might also indicate allelic heterogeneity in the region (i.e. two or more functional variants). In support of this latter possibility, we obtained evidence that R219K and R1587K have independent effects upon plasma APOB levels and our data indicate that there may be complex interactions between the two variants. In addition to these common variants, there is also evidence that rare mutations in *ABCA1* can often be a cause of extremely low HDL-C levels in the population (Cohen et al. 2004), but the extent to which these contribute to more normal variation is still unknown. We acknowledge that promoter or other regulatory regions may also be important, but more comprehensive studies would be needed to test these effects.

In summary, substantial evidence that A β and cholesterol metabolism are tightly linked exists (Hartmann 2001; Simons et al. 2001), and the data presented here on a potential role for genetic variants of *ABCA1* supports this. We believe that continued efforts to map AD and CVD susceptibility genes will benefit from the joint consideration of both AD- and CVD-related phenotypes, especially those connected to cholesterol metabolism. Given the extent of positive published data on *ABCA1*, there is a high likelihood that functionally relevant sequence variation occurs in this genomic region, but it may most appropriately be explored further in the context of potential modification by allelic and environmental heterogeneity.

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