DNA Methylation of Lipid-Related Genes Affects Blood Lipid Levels

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Background—Epigenetic mechanisms might be involved in the regulation of interindividual lipid level variability and thus may contribute to the cardiovascular risk profile. The aim of this study was to investigate the association between genome-wide DNA methylation and blood lipid levels high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, triglycerides, and total cholesterol. Observed DNA methylation changes were also further analyzed to examine their relationship with previous hospitalized myocardial infarction.

Methods and Results—Genome-wide DNA methylation patterns were determined in whole blood samples of 1776 subjects of the Cooperative Health Research in the Region of Augsburg F4 cohort using the Infinium HumanMethylation450 BeadChip (Illumina). Ten novel lipid-related CpG sites annotated to various genes including *ABCG1*, *MIR33B/SREBF1*, and *TNIP1* were identified. CpG cg06500161, located in *ABCG1*, was associated in opposite directions with both high-density lipoprotein cholesterol (β coefficient=–0.049; *P*=8.26E-17) and triglyceride levels (β=0.070; *P*=1.21E-27). Eight associations were confirmed by replication in the Cooperative Health Research in the Region of Augsburg F3 study (n=499) and in the Invecchiare in Chianti, Aging in the Chianti Area study (n=472). Associations between triglyceride levels and *SREBF1* and *ABCG1* were also found in adipose tissue of the Multiple Tissue Human Expression Resource cohort (n=634). Expression analysis revealed an association between *ABCG1* methylation and lipid levels that might be partly mediated by *ABCG1* expression. DNA methylation of *ABCG1* might also play a role in previous hospitalized myocardial infarction (odds ratio, 1.15; 95% confidence interval=1.06–1.25).

Conclusions—Epigenetic modifications of the newly identified loci might regulate disturbed blood lipid levels and thus contribute to the development of complex lipid-related diseases. **(*Circ Cardiovasc Genet*. 2015;8:334-342. DOI: 10.1161/CIRCRESAHA.116.000804.)**

Key Words: ABCG1 ■ DNA methylatio ■ epidemiology ■ gene expression ■ myocardial infarction

Coronary artery disease (CAD) is a major cause of death in industrialized countries.[1] Blood lipid levels, including high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), triglycerides, and total cholesterol (TC) levels, are considered heritable, modifiable risk factors for this disease.[2]

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Lipid levels can be influenced by drug therapy or lifestyle factors such as diet, physical activity, alcohol consumption, and smoking.[3] Several studies have also revealed a genetic impact on disturbed blood lipid levels. Genome-wide association studies identified a total of 157 genetic loci associated with lipid levels, explaining ≤12% of trait variance.[4] Beyond this, there is evidence that epigenetic mechanisms are also involved in interindividual lipid level variability and thus may contribute to the cardiovascular risk profile. One epigenome-wide analysis in patients with familial hypercholesterolemia identified *TNNT1* DNA methylation levels to be associated with HDL-C levels.[5] Another epigenome-wide analysis in a nonpopulation-based cohort observed an association between *CPT1A* DNA methylation levels and very-low-density lipoprotein cholesterol as well as triglyceride levels.[6]

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The aim of this study was to systematically investigate the association between main blood lipid levels (HDL-C, LDL-C, triglycerides, and TC) and genome-wide DNA methylation in whole blood of a large population-based cohort as well as in adipose tissue and skin samples. The identified associations were further explored through expression and functional studies and by investigation of genetic confounding. Finally, the relationship between observed DNA methylation changes and previous hospitalized myocardial infarction (MI) was explored.

Methods

The KORA study (Cooperative health research in the Region of Augsburg) consists of independent population-based samples from the general population living in the region of Augsburg, Southern Germany. The study has been conducted according to the principles expressed in the Declaration of Helsinki. Written informed consent has been given by each participant. The study was reviewed and approved by the local ethical committee (Bayerische Landesärztekammer). For the analysis, whole blood samples of the KORA F4 study were used (n=1776). The replication was done in whole blood samples of KORA F3 (n=499) and InCHIANTI (n=472) as well as in human adipose (n=634) and skin (n=395) samples of the Multiple Tissue Human Expression Resource (MuTHER) study. In the discovery and in the replication cohorts, genome-wide DNA methylation patterns were analyzed using the Infinium HumanMethylation450 BeadChip Array (Illumina). In KORA F4 and in the Invecchiare in Chianti, Aging in the Chianti Area (InCHIANTI) study, the analysis was performed using whole blood DNA of fasting participants; in KORA F3, nonfasting participants were also included. In KORA, blood was drawn in the morning (8:00–10:30 AM) and stored at −80°C until analysis. β-mixture quantile normalization[7] was applied to the DNA methylation data using the R package wateRmelon, version 1.0.3.[8] Table I in the Data Supplement provides a summary of normalized β values of the identified lipid-related CpGs in KORA F4. KORA F4/F3 samples were processed on 20/7 96-well plates in 9/4 batches; plate and batch effects were investigated using principle component analysis and eigenR2 analysis.[9] The plate variable explained 4.8% (F4), 6.3% (F3), and 8.1% (InCHIANTI) of variance in the DNA methylation data. Consequently, plate was included as a random effect in the analyses.

Lipid levels were determined in fasting fresh blood samples at most 6 hours after collection, except for KORA F3 which also includes nonfasting samples. In KORA F3 and F4, TC was measured using the cholesterol-esterase method (CHOL Flex, Dade-Behring, Germany). HDL-C and triglyceride levels were determined using the TGL Flex and AHDL Flex methods (Dade-Behring), respectively, and LDL-C was measured by a direct method (ALDL, Dade-Behring). In KORA F4/F3, the intra-assay coefficient of variation for repeated measurements was 1.85%/1.61% (TC), 2.75%/2.65% (triglycerides), 3.25%/2.89% (HDL-C), and 2.7%/3.02% (LDL-C). In InCHIANTI, TC was determined by the cholesterol-esterase method, HDL-C was measured with the Liquid Homogeneous HDL-C assay (Alifax S.p.A., Padova, Italy), and triglycerides through an enzymatic colorimetric test using lipoprotein lipase, glycerokinase, glycerol phosphate oxidase, and peroxidase. All 3 lipids were determined using the analyzer Modular P800 Hitachi (Roche Diagnostics, Mannheim, Germany). The intra-assay coefficient of variation was 0.8% (TC), 1.5% (triglycerides), and 0.8% (HDL-C). The level of LDL-C was calculated using the Friedewald formula (LDL-C=TC−[HDL-C+(triglycerides/5)]).

Detailed information about the cohorts, the process of DNA methylation analysis as well as data preprocessing, quality assessment, and further methods such as genotyping and gene expression are provided in the Data Supplement.

Statistical Analysis

Discovery Step

In the KORA F4 cohort, 26 of 1802 individuals were excluded from the analysis due to missing information in covariates or due to nonfasting status at the time point of blood collection, resulting in a final sample size of 1776 F4 subjects. Associations between DNA methylation β values and lipid levels were analyzed using linear mixed effects models implemented with the nlme package in R with lipid levels as response. To normalize lipid levels, square root (TC and LDL-C) and logarithmic (HDL-C and triglycerides) transformations were applied, followed by standardization to a mean of zero and an SD of 1. The following potential confounders were included as covariates: age, sex, body mass index, smoking, alcohol consumption, intake of lipid-lowering drugs, physical activity, history of MI, current hypertension, hemoglobin A1c levels, C-reactive protein levels, and white blood cell count. Experimental plate was included as a random effect. To correct for multiple comparisons, a genome-wide significance level of 1.1E-07 was used, determined according to the Bonferroni procedure. Because whole blood DNA samples were used, cell heterogeneity had to be considered as a confounder. As no measured cell count information was available for any cohort, sample-specific estimates of the proportion of the major white blood cell types were obtained using a statistical method described by Houseman et al.[10] The significant associations of the first model were recalculated, additionally adjusting for the estimated white blood cell proportions (CD8 T cells, CD4 T cells, natural killer cells, B-lymphocytes, monocytes, and granulocytes). To get a measure of the variance in the lipid levels explained by methylation levels, R^2 statistics were calculated according to Edwards et al,[11] using the R package pbkrtest, version 0.3–7.

Replication Step

Identified loci were replicated using the same statistical model in KORA F3 (n=499) as well as in InCHIANTI (n=472). In KORA F3, an adjustment for C-reactive protein was not possible because this variable was not available for this cohort. A fixed-effects meta-analysis of KORA F3 and InCHIANTI results was conducted with the R package metafor, version 1.9–2. Results were corrected according to the Bonferroni procedure (level of significance=4.5E-03).

For the MuTHER cohort, the Infinium HumanMethylation450 BeadChip Array signal intensities were quantile normalized and methylation β values were calculated using R 2.12 as previously described.[12] For cg06500161, no DNA methylation data were available as it did not pass the quality control filters. Data for n=634 adipose and n=395 skin samples were available for the final analysis. A linear mixed effects model was fitted for blood lipid values using the lme4 package in R. The model was adjusted for age, body mass index, smoking, statins, technical covariates (fixed effects), and family relationship and zygosity (random effects). A likelihood ratio test was used to assess significance, and the P value was calculated from the χ^2 distribution with 1 df using −2 log (likelihood ratio) as the test statistic. Results were corrected according to the Bonferroni procedure (level of significance=7.14E-03).

Single-Nucleotide Polymorphism Analysis

Investigation of genetic confounding was carried out to identify whether the observed associations between lipid and methylation levels in KORA F4 were due to single-nucleotide polymorphisms (SNPs) being associated with both lipid levels and DNA methylation. One hundred fifty-seven lipid-associated SNPs identified by the Global Lipids Genetics Consortium were included in the analysis.[4] SNP rs9411489 was excluded because genotype data were not available for the KORA F4 data set. Genotype data of 156 lipid-associated SNPs as well as DNA methylation data were available for 1710 KORA F4 participants. A preselection was done to reveal the lipid-associated SNPs which were at the same time nominally associated (P<0.05) with differentially methylated lipid-related CpG sites (CpGs; Table II in the Data Supplement). Next, models for each significant CpG–lipid pair were recalculated with additional adjustment for the respective preselected SNPs to see if the association was based on genetic confounding. Discovery, replication step, and SNP analysis were analyzed using the statistical package R, version 2.15.3.

Gene Expression Analysis

For the gene expression analysis, 724 KORA F4 subjects were included, as for these participants both DNA methylation data and

expression data were available. We tried to disentangle the relationships between methylation at the CpGs, expression of the corresponding annotated gene, and lipid levels in an ad hoc approach based on a sequence of regression models with and without adjusting for the third of the 3 components. For each significant lipid-methylation pair, the association between lipid level and DNA methylation was recalculated for KORA F4 (n=724). Afterward we repeated the analysis, adjusting for the expression levels of the annotated gene (except for cg07504977 which has no annotation to a gene according to the University of California Santa Cruz [UCSC] Genome Browser; Table III in the Data Supplement). A *P* value for the association was determined through a likelihood ratio test. Similarly, the association between DNA methylation and transcript levels, and between lipid levels and transcript levels, were determined. All models were also adjusted for age, sex, body mass index, smoking, alcohol consumption, intake of lipid lowering drugs, physical activity, history of MI, current hypertension, hemoglobin A1c levels and C-reactive protein levels, as well as for white blood cell count and estimated white blood cell proportions. Models including expression data were additionally adjusted for the technical variables RNA integrity number, sample storage time, and RNA amplification batch.[13] The level of significance was set to 8.3E-04.

Association of DNA Methylation With Prevalent MI

To assess the association of the observed lipid-related CpGs with previous hospitalized MI in KORA F4, generalized linear mixed effects models were fitted with adaptive Gauss-Hermite quadrature using the R package lme4, version 1.0–4. Three models were analyzed. The first model was adjusted for age, sex, and estimated white blood cell proportions. In the second model, we additionally included body mass index, smoking, alcohol consumption, physical activity, current hypertension, hemoglobin A1c levels, C-reactive protein levels, and white blood cell count as covariates, and in the third model, the lipid variables (HDL-C, LDL-C, triglycerides, and TC) were also included. The Bonferroni correction was used with a significance level of 6.3E-03. The same analyses were done for KORA F3 and InCHIANTI. This statistical analysis and the gene expression analysis were performed using the statistical package R, version 3.0.2.

Results

Associations Between Genome-Wide DNA Methylation and Blood Lipid Levels

Characteristics of the discovery cohort (KORA F4) as well as the replication cohorts (KORA F3, InCHIANTI, and MuTHER cohorts) are shown in Table 1.

In KORA F4, DNA methylation levels at 1, 68, 17, and 80 CpGs were associated with HDL-C, triglycerides, LDL-C, and TC levels, respectively. When white blood cell proportions were included as covariates, the number of significant associations ($P<1.1E-07$) decreased, indicating the presence of blood cell confounding. The association of methylation level at 1 CpG with HDL-C and LDL-C remained significant, as well as the association of 10 CpGs with triglyceride levels. There were no longer any associations with TC. *P* values ranged from 1.21E-27 to 9.66E-08, with percentage of explained lipid level variance ranging from 1.6% to 6.5% (Table 2). CpG cg06500161, located in *ABCG1*, was associated in opposite

Table 1. Characteristics of Subjects of the Discovery Cohort and the Replication Cohorts

Characteristic	KORA F4 (n=1776)	KORA F3 (n=499)	InCHIANTI (n=472)	MuTHER cohort (n=856)
Age, y	60.8 (8.9)	52.9 (9.6)	71.2 (16.0)	59.4 (9.0)
Sex=male	867 (48.8%)	259 (51.9%)	215 (45.6%)	0 (0.0%)
BMI, kg/m^2	28.2 (4.8)	27.2 (4.5)	27.0 (4.3)	26.6 (4.9)
Current smoker	258 (14.5%)	249 (49.9%)	206 (43.7%)	84 (9.8%)
Physically active	1021 (57.5%)	249 (49.9%)	238 (50.5%)	NA
Alcohol consumption, g/d	15.5 (20.4)	16.1 (19.6)	12.6 (15.1)	NA
HDL-C, mg/dL	56.5 (14.6)	58.2 (17.8)	56.8 (14.8)	71.5 (18.2)
LDL-C, mg/dL	140.0 (35.1)	131.0 (33.2)	124.5 (32.4)	124.5 (37.9)
Triglyceride, mg/dL	133.1 (94.7)	164.6 (121.9)	119.7 (57.9)	99.2 (49.6)
Cholesterol, mg/dL	221.9 (39.3)	220.5 (38.2)	205.2 (37.9)	218.9 (38.7)
C-reactive protein, mg/L	2.5 (5.1)	NA	4.0 (8.0)	NA
Leukocytes count, per nL	5.9 (1.6)	7.3 (2.1)	6.3 (1.6)	6.5 (1.8)
HbA1c, %	5.6 (0.6)	5.3 (0.5)	4.9 (0.8)*	NA
Self-reported history				
Hypertension†	811 (45.7%)	211 (42.3%)	124 (26.3%)	172 (20.1%)
Hospitalized myocardial infarction	60 (3.4%)	8 (1.6%)	36 (7.6%)	NA
Intake of lipid-lowering drugs (excluding herbal substances)	290 (16.3%)	31 (6.2%)	61 (13.0%)	69 (8.1%)
Fasting at the time of blood collection‡	1776 (100.0%)	47 (9.4%)	472 (100.0%)	844 (98.5%)

Continuous and categorical characteristics are given as mean (SD) or absolute numbers and relative proportions, respectively. BMI indicates body mass index; HbA1c, hemoglobin A1c; HDL-C, high-density lipoprotein cholesterol; InCHIANTI, Invecchiare in Chianti, Aging in the Chianti Area study; KORA, Cooperative Health Research in the Region of Augsburg study; LDL-C, low-density lipoprotein cholesterol; MuTHER, Multiple Tissue Human Expression Resource; and NA, variable not available.

*In InCHIANTI, HbA1c levels were calculated using the formula (46.7+glucose level)/28.7; in KORA F3/F4, they were analyzed using the high-performance liquid chromatography method.

†>140/90 mm Hg or medically controlled.

‡Overnight fast of ≥8 hours.

Table 2. Associations Between Genome-Wide DNA Methylation and Lipid Levels

				KORA F4				KORA F3			InCHIANTI			Meta-Analysis*
Lipid	CpG	Chromosome No.	Gene	β Coefficient	SE	*P* Value	Exp Var, %	β Coefficient	SE	*P* Value	β Coefficient	SE	*P* Value	*P* Value
HDL-C	cg06500161	21	*ABCG1*	−0.049	0.006	8.26E-17	3.9	−0.065	0.014	2.97E-06	−0.071	0.016	1.13E-05	9.00E-11†
TG	cg06500161	21	*ABCG1*	0.070	0.006	1.21E-27	6.5	0.072	0.015	1.89E-06	0.063	0.016	1.03E-04	5.56E-10†
	cg19693031	1	*TXNIP*	−0.030	0.003	1.89E-17	4.1	−0.014	0.007	5.65E-02	−0.023	0.011	3.54E-02	5.67E-03
	cg11024682	17	*SREBF1*	0.059	0.008	5.54E-14	3.2	0.031	0.014	2.89E-02	0.030	0.013	2.36E-02	1.60E-03†
	cg00574958	11	*CPT1A*	−0.118	0.016	3.15E-13	3.2	−0.103	0.028	2.42E-04	−0.058	0.020	4.86E-03	7.88E-06†
	cg27243685	21	*ABCG1*	0.064	0.009	3.24E-13	3.0	0.050	0.014	5.87E-04	0.054	0.022	1.63E-02	2.49E-05†
	cg07504977	10	NA‡	0.026	0.004	3.93E-12	2.7	0.026	0.008	1.87E-03	0.027	0.009	3.74E-03	1.91E-05†
	cg20544516	17	*MIR33B/SREBF1*	0.043	0.007	2.84E-09	2.7	0.032	0.013	1.39E-02	0.032	0.018	7.16E-02	2.22E-03†
	cg12556569	11	*APOA5*	0.005	0.001	6.43E-09	1.9	0.002	0.002	2.56E-01	0.004	0.002	1.25E-02	1.20E-02
	cg07397296	21	*ABCG1*	0.027	0.005	9.48E-08	2.1	0.034	0.010	1.03E-03	0.008	0.011	4.63E-01	3.78E-03†
	cg07815238	15	NA‡	0.048	0.009	9.66E-08	1.6	0.015	0.017	3.69E-01	0.003	0.014	8.41E-01	4.61E-01
LDL-C	cg22178392	5	*TNIP1*	0.040	0.007	4.27E-09	2.1	0.049	0.015	1.11E-03	0.020	0.014	1.45E-01	1.04E-03†

Exp Var indicates explained variance; HDL-C, high-density lipoprotein cholesterol; InCHIANTI, Invecchiare in Chianti, Aging in the Chianti Area study; KORA, Cooperative Health Research in the Region of Augsburg study; LDL-C, low-density lipoprotein cholesterol; and TG, triglyceride.

*Meta-analysis of results of replication in KORA F3 and InCHIANTI.

†CpG with association confirmed by replication meta-analysis; level of significance: 1.1E-07 (discovery cohort) and 4.5E-03 (replication meta-analysis).

‡No gene annotation for this CpG according to the University of California Santa Cruz Genome Browser.

directions with HDL-C (β=–0.049; *P*=8.26E-17) and triglyceride levels (β=0.070; *P*=1.21E-27). Triglyceride levels were associated with 9 additional CpGs located in genes including *ABCG1*, *MIR33B*, *SREBF1*, and *CPT1A*. LDL-C showed a positive association with methylation status of 1 CpG located in *TNIP1* (β=0.040; *P*=4.27E-09).

The lipid-related CpGs were carried forward to replication in a meta-analysis of the KORA F3 and InCHIANTI cohorts. Nine of the 12 associations were confirmed (*P* values from 9.00E-11 to 3.78E-03; Table 2).

Tissue Expression of Candidate Genes and Replication in an Adipose Tissue Cohort

To address cell specificity and tissue specificity of *ABCG1*, *CPT1A*, and *SREBF1* expression, we quantified their expression in human blood cell types (peripheral blood mononuclear cells; CD14-, CD19-, CD3-, CD4-, and CD8-positive cells; and regulatory T cells) and human tissues (brain, heart, lung, kidney, small intestine, adipose tissue, and skeletal muscle; Figure I in the Data Supplement). All genes were expressed not only in blood cells but also in adipose tissue. Five of the replicated associations were also significant in adipose tissue of the MuTHER cohort (Table 3). Here, the CpG cg20544516 (*MIR33B/SREBF1*) showed the strongest association with triglyceride levels (β=0.012; *P*=1.20E-10), followed by CpGs located in *ABCG1* (cg27243685, cg07397296; β=0.013, *P*=5.86E-08 and β=0.008, 6.59E-07, respectively) and *SREBF1* (cg11024682, β=0.007, *P*=6.72E-04). The association between LDL-C and cg22178392 (*TNIP1*) was also found to be significant in adipose tissue (β=0.002; *P*=6.02E-03). In skin tissue, no associations could be determined except for triglyceride levels and cg11024682 (*SREBF1*) and cg00574958 (*CPT1A*; β=0.006, *P*=4.07E-04 and β=-0.005, *P*=2.81E-03, respectively). These results are in line with the strong expression of *MIR33B/SREBF1* in adipose tissue observed in our tissue panel (Figure I in the Data Supplement).

Investigation of Genetic Confounding

Known lipid-related SNPs, which were nominally associated with DNA methylation at the identified lipid-related CpGs and thus acting as potential confounders, are shown in Table II in the Data Supplement. *P* values ranged between 4.99E-02 and 3.42E-05, except for 1 SNP (rs964184), which was highly significantly associated with DNA methylation of cg12556569 (*APOA5*; *P*=3.75E-289). After recalculation of the models for each CpG–lipid pair with additional adjustment for the respective preselected SNP, only the association between methylation of *APOA5* and triglycerides was considerably genetically confounded (Table IV in the Data Supplement). Further analyses showed that rs964184, from the preselected SNPs for *APOA5*, caused the genetic confounding (data not shown).

Gene Expression Analysis

Gene expression analysis revealed a negative association between methylation at *ABCG1* and mRNA levels of the 6 *ABCG1* transcripts (cg06500161: *P*=5.42E-14, cg27243685: *P*=1.86E-07). Here, transcript ILMN_2329927 showed the highest association (cg06500161: β=–0.151, *P*=5.22E-15; cg27243685: β=–0.185, *P*=4.34E-06) of the 6 *ABCG1* transcripts. Adjusted for lipids, the association became less significant. Detailed results of the analyses are shown in Table V in the Data Supplement.

The association between cg06500161 and HDL-C (*P*=4.10E-07) weakened (*P*=1.30E-02) after adjusting for *ABCG1* transcripts. Similar results were observed for the association between cg06500161 and triglyceride levels and between cg27243685 and triglyceride levels.

Table 3. Association Between Blood Lipid Levels and Lipid-Related CpGs in Adipose and Skin Tissue of the Multiple Tissue Human Expression Resource Cohort

			Adipose (n=634)			Skin (n=395)		
Lipid	CpG	Gene	β Coefficient	SE	*P* Value	β Coefficient	SE	*P* Value
HDL-C	cg06500161	*ABCG1*	NA*	NA*	NA*	NA*	NA*	NA*
TG	cg06500161	*ABCG1*	NA*	NA*	NA*	NA*	NA*	NA*
	cg11024682	*SREBF1*	0.007	0.002	6.72E-04	0.006	0.002	4.07E-04
	cg00574958	*CPT1A*	0.0001	0.001	8.16E-01	−0.005	0.002	2.81E-03
	cg27243685	*ABCG1*	0.013	0.002	5.86E-08	0.003	0.003	3.91E-01
	cg07504977	NA†	0.006	0.003	4.60E-02	0.004	0.002	1.56E-01
	cg20544516	*MIR33B/ SREBF1*	0.012	0.002	1.20E-10	−0.001	0.002	5.04E-01
	cg07397296	*ABCG1*	0.008	0.002	6.59E-07	0.001	0.002	6.64E-01
LDL-C	cg22178392	*TNIP1*	0.002	0.001	6.02E-03	0.0003	0.001	7.49E-01

HDL-C indicates high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; and TG, triglyceride.
*No DNA methylation data were available for this CpG site.
†No gene annotation for this CpG according to the UCSC Genome Browser. Level of significance: 7.14E-03.

ABCG1 transcript levels showed a strong positive association with HDL-C (*P*=7.76E-13) and a negative association with triglyceride levels (*P*=1.25E-33). Significance was reduced when adjusting for *ABCG1* DNA methylation (see also Figure 1; Figure II in the Data Supplement).

Functional Analysis of cg06500161 (*ABCG1*) and cg20544516 (*MIR33B/SREBF1*)

To assess the biological relevance of the DNA methylation status of CpGs found to be associated with lipid levels, electrophoretic mobility shift assays were carried out for cg06500161 (*ABCG1*). This CpG was chosen because it showed the strongest association with both HDL-C and triglycerides. cg20544516 was also included in the analysis because of its functionally interesting location in *SREBF1* in a region coding for a microRNA (MIR33b).

The electrophoretic mobility shift assay for cg06500161 identified a higher binding affinity of a protein complex for the unmethylated status of cg06500161 compared with the methylated status. For cg20544516, a strong protein binding affinity was detected in the methylated status, which was not detectable in the unmethylated status (Figure III in the Data Supplement).

DNA Methylation and Prevalent MI

The CpGs associated with lipid levels were tested for an association with previous hospitalized MI in the discovery cohort KORA F4 (n=1776 with n=60 cases). Three models were analyzed and CpG cg06500161, located in the *ABCG1* gene, showed an association with MI independent of lipid levels in all 3 models (eg, model 3: β=0.141, *P*=1.30E-03; Table VI in the Data Supplement). The results could not be replicated in KORA F3 and InCHIANTI, possibly due to the low number of MI cases (n=8 in KORA F3; n=36 in InCHIANTI).

Discussion

DNA Methylation of Genes Involved in Lipid Metabolism Is Associated With HDL-C, Triglycerides, and LDL-C Levels

Our results indicated that DNA methylation of cg06500161 in *ABCG1* was associated in opposite directions with HDL-C and triglyceride levels. Integrating gene expression data revealed an association between cg06500161 methylation and lipid levels which might be partly mediated by *ABCG*1 expression. DNA methylation at this CpG was also elevated in cases of MIs compared to healthy individuals.

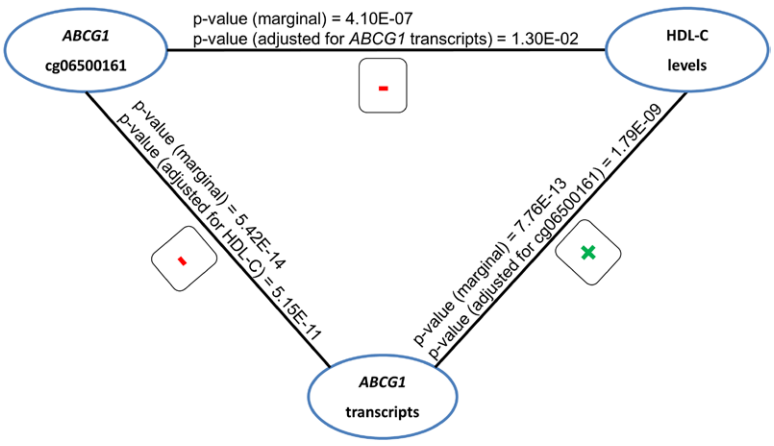

Figure 1. Results of expression analysis between cg06500161 (*ABCG1*) and high-density lipoprotein cholesterol (HDL-C) levels. *ABCG1* methylation (cg06500161) was negatively associated with *ABCG1* mRNA levels. *ABCG1* mRNA levels were positively associated with HDL-C levels. Adjustment analysis indicated that the association between *ABCG1* methylation and HDL-C might be partly mediated by the expression of *ABCG1*. Detailed association results can be found in Table V in the Data Supplement. n=724, level of significance=8.3E-04.

One challenge of genome-wide DNA methylation analyses in blood samples is the difference in methylation patterns between different blood cell types.[10,14] In our blood cell expression panel of *ABCG1*, *CPT1A*, and *SREBF1*, varying expression patterns were also detectable (Figure I in the Data Supplement), which underline the issue of cell heterogeneity. After adjustment for estimated blood cell proportions using the method proposed by Houseman et al,[10] the number of significant CpGs decreased from 166 to 12. Therefore, in all further analyses, cell proportions were included as covariates to correct for cell heterogeneity.

We identified 7 new lipid-related CpGs located in *ABCG1* (HDL-C, triglycerides), *MIR33B/SREBF1*, in an intergenic region (triglycerides), and in *TNIP1* (LDL-C). In addition, we replicated 1 CpG (cg00574958 in *CPT1A*), which was found to be associated with triglyceride levels in $CD4^+$ T cells in the GOLDN study (n=991).[6] Five of the associations were also found in adipose tissue, of which the strongest associations were observed between triglyceride levels and *MIR33B/SREBF1* as well as *ABCG1* DNA methylation. Both genes are highly expressed in adipose tissue (>1.0E07 copies/µg RNA; Figure I in the Data Supplement). In skin, triglyceride levels were associated with *SREBF1* and *CPT1A* DNA methylation, but there was no significant association with *ABCG1* methylation. These results indicate a tissue-specific association between triglyceride levels and *MIR33B/SREBF1* and *ABCG1* DNA methylation.

Additionally, we examined whether the observed associations between lipid and methylation levels in KORA F4 were based on confounding by lipid-associated SNPs. Most associations remained significant after additional adjustment for SNPs which were nominally associated with DNA methylation at the respective CpG site. Only one CpG-lipid association was found to be confounded. The association between DNA methylation of cg12556569 (located in the promoter region of *APOA5*) and triglyceride levels was confounded by rs964184, which is known to primarily affect triglyceride levels.[4] One study had previously identified this SNP as an mQTL (cytosine modification quantitative trait loci).[15] Our results indicate that the nominal associations between trait-associated SNPs and DNA methylation of lipid-related CpGs were dependent on lipids and that the identified lipid–DNA methylation associations were not due to genetic confounding.

Interaction of Genes of Lipid-Associated CpGs

Interestingly, 3 of the genes where the lipid-related CpGs are localized—*ABCG1*, *MIR33B/SREBF1*, and *CPT1A*—and their gene products interact with one another (Figure 2). *SREBF1* and *SREBF2* (sterol regulatory element-binding transcription factor 1 and 2) code for the membrane-bound transcription factors SREBP1 and SREBP2, which activate the synthesis of fatty acid and the synthesis and uptake of cholesterol.[16,17] The intronic microRNAs 33a and 33b (MIR33a/b) are located within *SREBF2* and *SREBF1*, respectively. Coincident with transcription of *SREBF2/1*, the embedded MIR33a/b is cotranscribed.[18] MIR33a/b act as negative regulators, repressing many genes involved in fatty acid oxidation and cholesterol transport,[18–23] such as carnitine palmitoyltransferase 1A (CPT1A), which is important for the transport of fatty acids into the mitochondria for their oxidation.[24] Studies also identified a role for MIR33a/b in the repression of the ABC transporters ABCA1 and ABCG1.[20,25] *ABCG1* encodes the ABC-transporter G1, a cholesterol transporter which plays a role in cellular lipid homeostasis. It has been shown that ABCG1 functions cooperatively with ABCA1.[26] ABCA1 transports phospholipids and cholesterol to lipid-poor HDL subclasses such as apolipoprotein A–I, whereas ABCG1 has more mature HDL particles as its acceptor.[27,28]

In the present study, the methylation levels of these genes, *MIR33B/SREBF1*, *ABCG1*, and *CPT1A*, are associated with blood triglyceride levels, suggesting an epigenetic modulation of lipid and fatty acid metabolism. Here, *ABCG1* might play a key role because 1 CpG (cg06500161) located in this gene is associated with both HDL-C and triglyceride levels. The function of *ABCG1* in HDL-C metabolism has been recorded in several studies and reviews[29–31]; however, no report yet exists about a direct role of *ABCG1* in connection with triglyceride levels. One study showed that genetic variants in the *ABCG1* promoter were associated with *ABCG1* expression, which showed an influence on the bioavailability of lipoprotein lipase. Accordingly, ABCG1 regulates the bioavailability of macrophage-secreted lipoprotein lipase, thereby promoting lipid accumulation, primarily in the form of triglycerides, in primary human macrophages.[32]

TNIP1, the methylation of which was associated with LDL-C levels in this study, encodes the tumor necrosis factor-α-induced protein 3–interacting protein 1. This protein seems to be important in regulating multiple receptor–mediated transcriptional activity of peroxisome proliferator–activated receptors[33] and retinoic acid receptors.[34] Interestingly, ligand-activated retinoic acid receptor increases *ABCA1* and *ABCG1* expression in human macrophages, modulating *ABCG1* promoter activity via LXR responsive elements-dependent mechanisms.[35] Additionally, studies revealed that peroxisome proliferator–activated receptor α/γ-activators induce *ABCA1* expression in macrophages[36] and peroxisome proliferator–activated receptor γ induce *ABCG1* expression.[37] Therefore, *TNIP1* might have an indirect impact on the expression of *ABCA1/G1*.

Methylation of *ABCG1* Is Associated With *ABCG1* Transcripts

The identified negative association between *ABCG1* methylation (cg06500161, cg27243685) and *ABCG1* mRNA levels is

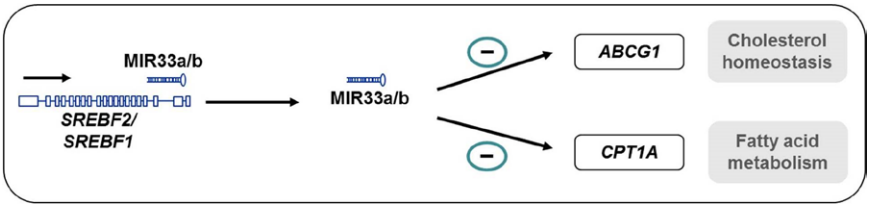

Figure 2. Interrelation of genes whose DNA methylation level is associated with lipid levels. When *SREBF1/2* is transcriptionally activated, MIR33a/b are cotranscribed, which play a role in repression of *ABCG1* and *CPT1A*.

possibly mediated by methylation-dependent transcription factor binding, as observed in the electrophoretic mobility shift assay experiments. *ABCG1* mRNA levels were additionally associated with HDL-C and triglyceride levels in opposite directions. The negative association between *ABCG1* methylation (cg06500161) and HDL-C might be partly mediated by the expression of *ABCG1*. These results demonstrate the complexity of the relationship between DNA methylation and gene expression.

Our findings could provide the missing link between disturbed blood lipid levels and changed expression patterns of *ABCG1*. Studies have shown that in patients with type 2 diabetes mellitus, the *ABCG1* expression in macrophages is reduced, leading to decreased cholesterol efflux to HDL.[38] Interestingly, a recent study shows an association between the methylation status of cg06500161 (*ABCG1*) and fasting insulin as well as with HOMA-IR (homeostatic model assessment), a surrogate marker of insulin resistance.[39] All results indicate a key role of DNA methylation of *ABCG1* in the development of complex lipid-related diseases.

ABCG1—An Epigenetic Link Between Blood Lipid Levels and MI?

DNA methylation has been linked to biological processes of cardiovascular disease such as atherosclerosis.[40] An association between *ABCA1* methylation and HDL-C levels as well as CAD in patients with familial hypercholesterolemia has been reported.[41]

We identified a positive association between cg06500161 (*ABCG1)* and MI in the KORA F4 cohort: DNA methylation levels of cg06500161 are higher in subjects with previous hospitalized MI compared with healthy people. Because the number of subjects with self-reported hospitalized MI was low in KORA F3 and InCHIANTI, no replication was achieved. These results need further confirmation by prospective genome-wide DNA methylation studies.

Genetic variants in *ABCG*1 were shown to be associated with CAD.[42,43] However, nothing is yet known about an epigenetic impact of *ABCG1* on the development of MI. A human cell culture study showed a reduction of macrophage *ABCG1* expression when higher triglyceride levels were present in the culture media. The author suggests that hypertriglyceridemia may increase the risk of CAD via direct actions on macrophages favoring foam cell formation, thus leading to the development of atherosclerotic plaque.[44] Changes in *ABCG1* DNA methylation might mediate the development of atherosclerotic plaques in response to high triglyceride levels. Thus, with this study, we found hints for a new perspective on the molecular background of CAD.

Conclusions

We found associations between DNA methylation and lipid levels for genes contributing to the modulation of cholesterol and fatty acid metabolism. Epigenetic modification of *ABCG1* and its regulatory network could play a key role on the path from disturbed blood lipid levels to the development of complex lipid-related diseases. These results indicate an epigenetic impact on metabolic regulation in humans and give new insights into the complex picture of lipid-related complex diseases.

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Disclosures

None.

Appendix

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CLINICAL PERSPECTIVE

Blood lipid levels, including high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, triglycerides, and total cholesterol levels, are considered heritable modifiable risk factors for coronary artery disease. In addition to drug therapy and lifestyle factors, the genetic background also has an influence on lipid levels. Genome-wide association studies have identified a total of 157 genetic loci associated with lipid levels, explaining ≤12% of trait variance. Beyond this, epigenetic mechanisms may also contribute to the interindividual variation in circulating lipid levels and thereby may contribute to cardiovascular risk. In this study, first the association between genome-wide DNA methylation in whole blood and blood lipid levels (high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, triglycerides, and total cholesterol) was systematically investigated; second, the relationship between observed DNA methylation changes and prior hospitalization for myocardial infarction was explored. We observed associations between blood lipid levels and DNA methylation of genes involved in cholesterol and fatty acid metabolism. Further analyses reveal that changes in *ABCG1* DNA methylation might mediate the association of high triglyceride levels with the risk of developing a myocardial infarction. These results lead to the possibility that epigenetic modification of *ABCG1* and its regulatory network may play a key role in the path from altered blood lipid levels to the development of lipid-related disorders, including myocardial infarction. Also, showing that epigenetic changes are associated with both dyslipidemia and myocardial infarction may support the development of new classes of pharmacological agents for the treatment of lipid-related disorders.

SUPPLEMENTAL MATERIAL

Supplemental Methods

Discovery cohort

The KORA study (Cooperative health research in the Region of Augsburg) consists of independent population-based samples from the general population living in the region of Augsburg, Southern Germany. The KORA F4 study, a seven-year follow-up study of the KORA S4 survey (examined between 1999 and 2001), was conducted between 2006 and 2008. The standardized examinations applied in the survey have been described in detail elsewhere[1, 2]. A total of 3080 subjects with ages ranging from 32 to 81 years participated in the examination. At the time of the KORA F4 visit, blood samples for metabolic analyses, DNA and RNA extraction were collected. All participants signed a written informed consent form and the studies were approved by the local ethics committee (Bayerische Landesärztekammer). In a random subgroup of 1802 subjects the genome-wide DNA methylation patterns were analyzed with the Infinium HumanMethylation450 BeadChip (Illumina, San Diego, USA).

Replication cohorts

The KORA F3 study is a ten-year follow-up of the population-based KORA S3 survey (examined in 1994/95), conducted in 2004/05. KORA F4 and KORA F3 are two independent cohorts; they were conducted at different time points with no overlap of individuals[2]. InCHIANTI is a population-based study initiated in 1998 in the Chianti region of Tuscany, Italy. Data for this analysis were taken from the participants´ third follow-up visit, in 2007/2008. In KORA F3 DNA methylation data for 499 subjects

(comprised of 250 smokers and 250 non-smokers[1]; one sample is missing due to data preprocessing) and for InCHIANTI data for 472 subjects were available, and both were analyzed using the Infinium HumanMethylation450 BeadChip.

The Multiple Tissue Human Expression Resource (MuTHER) study includes 856 female twins recruited from the TwinsUK Adult twin registry (154 monozygotic twin pairs, 232 dizygotic twin pairs and 84 singletons)[3]. Infinium HumanMethylation450 BeadChip array data on adipose tissue biopsies were available for 648 individuals[4] and for 469 skin biopsies. Due to missing phenotype information the final sample size for the analysis was 634 for adipose samples and 395 for skin samples. Blood lipids and other phenotypes were measured at the same time point as the biopsies as previously described[5].

Genome-wide DNA methylation analysis with Infinium HumanMethylation450 BeadChip

Genome-wide DNA methylation patterns were analyzed in the discovery cohort as well as in the replication cohorts using the Infinium HumanMethylation450 BeadChip Array (Illumina, San Diego, USA) as described elsewhere[1]. The percentage of methylation at a given cytosine is reported as a beta-value, which is a continuous variable between 0 and 1 corresponding to the ratio of the methylated signal over the sum of the methylated and unmethylated signals of the particular cytosine site.

Data preprocessing and quality assessment

Raw methylation data were extracted with Illumina Genome Studio (version 2011.1) with methylation module (version 1.9.0) and preprocessed using R (version 3.0.1). Probes with signals being summarized from less than three functional beads and

probes with a detection p-value larger than 0.01 were defined as low-confidence probes. Since probe binding might be affected by SNPs in the binding area, sites representing or being located in a 50 bp proximity to SNPs with a minor allele frequency of at least 5% were also excluded from the data set[6]. Using the R package lumi (version 2.12.0)[7], a color bias adjustment using smooth quantile normalization was conducted as well as a background correction based on negative control probes present on the Infinium HumanMethylation BeadChip, separately for the two color channels and chips. In a further step beta values corresponding to low-confidence probes were set to missing, and samples as well as CpG sites (CpGs) were subjected to a 95% call rate threshold, where samples and CpGs with more than 5% low-confidence probes were removed from the analysis. Finally, beta-mixture quantile normalization (BMIQ) was applied to correct the shift in the distribution of the beta values of the InfI and InfII probes[8]. This was done using the R package wateRmelon, version 1.0.3[9]. Supplemental Table 1 shows a summary of normalized beta values of the identified lipid-related CpGs in KORA F4. The entire dataset is available upon request from KORA-gen (http://epi.helmholtz-muenchen.de/kora-gen). Requests should be sent to kora-gen@helmholtzmuenchen.de and are subject to approval by the KORA board to ensure that appropriate conditions are met to preserve patient privacy.

In all statistical models in which DNA methylation was used as response (including the gene expression and SNP analysis) beta values were transformed to M values to achieve approximate homoscedasticity and normality of the data. M values are defined as the binary logit of the beta values (M value=log2[beta value/(1-beta value)])[10].

Genotyping and gene expression

For a total number of N=1710 KORA F4 samples both DNA methylation and genotype data were available. KORA F4 samples were genotyped using the Affymetrix Axiom chip array. Genotypes were called with the Affymetrix software and were annotated to NCBI build 37. We excluded individuals with call rates <97%, mismatches of phenotypic and genetic gender as well as heterozygosity rates +/-5 SD from the mean. Before imputation we excluded SNPs with call rates <98%, HWE p-values <5E-6, and minor allele frequency <1%. Pre-phasing was performed with SHAPEIT v2. Imputation was performed with IMPUTE v2.3.0 using the 1000G phase1 (v3) reference panel.

For N=724 KORA F4 subjects with DNA methylation data gene expression data were also available. Gene expression profiling was performed using the Illumina Human HT-12 v3 Expression BeadChip as described elsewhere[11]. Briefly, total RNA was extracted from whole blood under fasting conditions using the PAXgene Blood miRNA Kit (Quiagen). Purity and integrity of the RNA was assessed on the Agilent Bioanalyzer with the 6000 Nano LabChip reagent set (Agilent Technologies, Germany). 500 ng of RNA was reverse transcribed and bioting-UTP-labeled into cRNA using the Illumina TotalPrep-96 RNA Amp Kit (Ambion, Darmstadt, Germany). A total of 3000 ng of cRNA was hybridized to the Illumina Human HT-12 v3 Expression BeadChip, followed by washing steps as described in the Illumina protocol. The raw intensity data were exported from Illumina software Genome Studio to R and processed (log transformation and quantile normalization[12]) using the lumi:1.12.4 package[7] from the Bioconductor open source software (http://www.bioconductor.org). Gene expression data are available for download at

ArrayExpress (E-MTAB-1708) (http://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-1708/).

Expression of ABCG1, CPT1A and SREBF1 in different blood cell types and human tissues

Total RNA (1 µg) from human liver (pool of 10 donors; HTCR Stiftung[13]) and human brain, heart, lung, kidney, small intestine, adipose tissue, skeletal muscle, peripheral blood mononuclear cells (PBMC), CD14-, CD19-, CD3-, CD4-, CD8-positive cells, and regulatory T-cells (pool of 2-3 donors; Clontech) was reverse transcribed according to published protocols[14]. qRT-PCR was performed in quadruplicate as described[14] using the following primers and probes for *ABCG1* (5'-primer: 5'-gcagttctgcatcctcttcaa-3'; 3'-primer: 5'-gatgcgcaggtgtgtcag-3'; probe: 5'FAM-ccttcctcagcatcatgagggactcgg-3'TAMRA) and *SREBF1* (5'-primer: 5'-CACAACGCCATTGAGAAGC-3'; 3'-primer: 5'- GCGCAAGACAGCAGATTTATT-3'; probe: 5'FAM-CTCAAGGATCTGGTGGTGGGCACTGAG-3'TAMRA). Quantification of *CPT1*A expression was performed with the KAPA SYBR FAST qPCR Kit (Kapabiosystems) according to the manufacturer's instructions using the following primers: 5'-primer: 5'-TCGTCACCTCTTCTGCCTTT-3'; 3'-primer: 5'-ACACACCATAGCCGTCATCA-3'. Absolute copies were determined using plasmid standard curves and normalized to µg input RNA.

Electrophoretic mobility shift assays (EMSAs)

THP1 nuclear extracts (Active Motif, Rixensart, Belgium, catalogue number 36076) were used for EMSA. Cy5-labeled and unlabeled oligonucleotides (Metabion) containing the methylated or unmethylated CpG (cg06500161, cg20544516) were

annealed and purified in a 12% polyacrylamide gel. Sequences of used oligonucleotides are shown in Suppl. Table 7. For the binding reaction, 5 µg of nuclear extract, 1 ng of labeled probe and either no or rising concentrations (10 and 100 ng) of unlabeled competitor oligonucleotides were incubated in binding buffer (4% v/v glycerol, 1 mM MgCl2, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 10 mM TrisHCl pH 7.5) with 0.05 mg poly(dI-dC) (Roche Diagnostics) in a total volume of 10 µl for 20 min at 4°C. DNA-protein complexes were separated by electrophoresis using a 5.3% polyacrylamide gel and a running buffer (0.5 x tris-borate-EDTA (TBE) buffer) at 4°C. The gels were scanned on the Typhoon Trio+ Imager (GE Healthcare).

Ethics Statements

The KORA study has been conducted according to the principles expressed in the Declaration of Helsinki. Written informed consent has been given by each participant. The study, including the protocols for subject recruitment and assessment and the informed consent for participants, was reviewed and approved by the local ethical committee (Bayerische Landesärztekammer).

Ethical approval was granted by the Instituto Nazionale Riposo e Cura Anziani institutional review board in Italy. Participants received a detailed description of the study protocols and signed an informed participation consent.

Tissue samples were obtained and experimental procedures were performed within the framework of the nonprofit foundation HTCR, including the informed patient's consent[13]. All the procedures followed in the MuTHER cohort were in accordance with the ethical standards of the St. Thomas' Research Ethics Committee (REC

reference 07/H0802/84) at St. Thomas' Hospital in London, and all study subjects provided written informed consent.

Supplemental Tables

Suppl. Table 1

Summary of normalized beta values of lipid-related CpGs in KORA F4

CpG	Gene	Min	Max	Mean
cg06500161	*ABCG1*	0.37	0.78	0.63
cg07397296	*ABCG1*	0.09	0.43	0.29
cg27243685	*ABCG1*	0.77	0.98	0.91
cg11024682	*SREBF1*	0.36	0.67	0.48
cg20544516	*MIR33B/SREBF1*	0.60	0.91	0.73
cg00574958	*CPT1A*	0.01	0.15	0.05
cg19693031	*TXNIP*	0.43	0.97	0.78
cg22178392	*TNIP1*	0.07	0.38	0.19
cg12556569	*APOA5*	0.01	0.97	0.24
cg07504977	NA*	0.13	0.61	0.37
cg07815238	NA*	0.26	0.65	0.45

For each CpG site the minimum and maximum of the beta values is shown, as well as the mean value. *no gene annotation for this CpG according to the UCSC Genome Browser

Suppl. Table 2

Pre-selected SNPs showing nominal associations (p<0.05) with DNA methylation of lipid-related CpGs

CpG	annotated gene	SNP	β coefficient	p-value
cg00574958	*CPT1A*	rs964184	0.06	9.41E-03
cg00574958	*CPT1A*	rs605066	-0.04	1.14E-02
cg00574958	*CPT1A*	rs11603023	-0.03	3.00E-02
cg00574958	*CPT1A*	rs4846914	0.03	4.59E-02
cg06500161	*ABCG1*	rs964184	-0.05	3.42E-05
cg06500161	*ABCG1*	rs7255436	0.03	1.18E-03
cg06500161	*ABCG1*	rs2814982	-0.04	1.42E-02
cg06500161	*ABCG1*	rs9488822	-0.02	1.99E-02
cg06500161	*ABCG1*	rs10128711	-0.02	2.88E-02
cg06500161	*ABCG1*	rs4299376	0.02	3.06E-02
cg06500161	*ABCG1*	rs2000999	0.02	4.60E-02
cg06500161	*ABCG1*	rs5756931	0.02	4.93E-02
cg07397296	*ABCG1*	rs2954029	-0.03	1.23E-02
cg07397296	*ABCG1*	rs1260326	-0.03	1.50E-02
cg07397296	*ABCG1*	rs12967135	-0.03	1.64E-02
cg07397296	*ABCG1*	rs11776767	-0.03	2.14E-02
cg07397296	*ABCG1*	rs4983559	0.04	2.48E-02
cg07397296	*ABCG1*	rs12027135	-0.02	3.13E-02
cg07397296	*ABCG1*	rs7255436	0.03	3.80E-02
cg07397296	*ABCG1*	rs12670798	-0.03	4.24E-02

cg07397296	*ABCG1*	rs11563251	0.04	4.33E-02
cg07504977	NA*	rs1260326	-0.05	7.69E-04
cg07504977	NA*	rs1367117	-0.04	2.48E-03
cg07504977	NA*	rs11649653	0.04	7.97E-03
cg07504977	NA*	rs11776767	-0.04	1.04E-02
cg07504977	NA*	rs6805251	-0.04	1.20E-02
cg07504977	NA*	rs6450176	-0.04	1.23E-02
cg07504977	NA*	rs964184	-0.04	2.34E-02
cg07504977	NA*	rs970548	-0.03	2.89E-02
cg07504977	NA*	rs4420638	0.04	2.97E-02
cg07504977	NA*	rs4253772	-0.05	2.97E-02
cg07504977	NA*	rs2287623	-0.03	3.28E-02
cg07504977	NA*	rs1077514	-0.04	3.55E-02
cg07504977	NA*	rs3822072	0.03	3.66E-02
cg07504977	NA*	rs12916	-0.03	3.94E-02
cg07504977	NA*	rs2602836	0.03	4.84E-02
cg07815238	NA*	rs4765127	-0.03	7.78E-04
cg07815238	NA*	rs1532085	0.02	6.34E-03
cg07815238	NA*	rs2954029	-0.02	1.24E-02
cg07815238	NA*	rs492602	-0.02	2.04E-02
cg07815238	NA*	rs7134375	0.02	3.14E-02
cg07815238	NA*	rs2929282	0.04	3.35E-02
cg07815238	NA*	rs4660293	-0.02	3.66E-02
cg07815238	NA*	rs13315871	-0.03	4.53E-02
cg07815238	NA*	rs1564348	0.02	4.99E-02

cg11024682	*SREBF1*	rs12670798	-0.03	1.87E-03
cg11024682	*SREBF1*	rs11649653	0.02	3.50E-03
cg11024682	*SREBF1*	rs1260326	-0.02	7.28E-03
cg11024682	*SREBF1*	rs8017377	-0.02	1.61E-02
cg11024682	*SREBF1*	rs6805251	-0.02	1.68E-02
cg11024682	*SREBF1*	rs7255436	0.02	1.77E-02
cg11024682	*SREBF1*	rs2954029	-0.02	1.84E-02
cg11024682	*SREBF1*	rs3780181	0.04	2.70E-02
cg11024682	*SREBF1*	rs13326165	0.02	2.87E-02
cg11024682	*SREBF1*	rs1800562	-0.04	3.15E-02
cg12556569	*APOA5*	rs964184	-3.94	3.75E-289
cg12556569	*APOA5*	rs2030746	0.35	2.89E-04
cg12556569	*APOA5*	rs9488822	0.25	1.17E-02
cg12556569	*APOA5*	rs12145743	0.26	1.91E-02
cg12556569	*APOA5*	rs2929282	0.47	2.34E-02
cg12556569	*APOA5*	rs2131925	0.22	2.41E-02
cg12556569	*APOA5*	rs11613352	-0.24	3.54E-02
cg12556569	*APOA5*	rs6065906	-0.25	3.68E-02
cg12556569	*APOA5*	rs6029526	0.20	3.87E-02
cg12556569	*APOA5*	rs10128711	-0.22	4.05E-02
cg12556569	*APOA5*	rs7241918	0.25	4.96E-02
cg19693031	*TXNIP*	rs1936800	-0.06	1.53E-03
cg19693031	*TXNIP*	rs6450176	0.06	9.85E-03
cg19693031	*TXNIP*	rs2602836	0.04	2.64E-02
cg19693031	*TXNIP*	rs1260326	0.04	4.26E-02

cg20544516	*MIR33B/SREBF1*	rs7515577	0.03	2.51E-03
cg20544516	*MIR33B/SREBF1*	rs964184	-0.04	3.58E-03
cg20544516	*MIR33B/SREBF1*	rs1689800	-0.02	6.47E-03
cg20544516	*MIR33B/SREBF1*	rs5763662	0.08	1.42E-02
cg20544516	*MIR33B/SREBF1*	rs12967135	0.02	1.70E-02
cg20544516	*MIR33B/SREBF1*	rs11649653	0.02	2.93E-02
cg20544516	*MIR33B/SREBF1*	rs2954029	-0.02	3.07E-02
cg20544516	*MIR33B/SREBF1*	rs7134375	0.02	3.40E-02
cg20544516	*MIR33B/SREBF1*	rs12678919	-0.03	4.33E-02
cg20544516	*MIR33B/SREBF1*	rs2929282	0.04	4.81E-02
cg22178392	*TNIP1*	rs964184	-0.06	8.23E-03
cg22178392	*TNIP1*	rs5763662	0.14	1.78E-02
cg22178392	*TNIP1*	rs1800562	-0.09	1.95E-02
cg22178392	*TNIP1*	rs2287623	-0.04	3.59E-02
cg22178392	*TNIP1*	rs386000	0.04	4.16E-02
cg27243685	*ABCG1*	rs838880	0.06	4.89E-04
cg27243685	*ABCG1*	rs2131925	0.04	1.23E-03
cg27243685	*ABCG1*	rs4148008	0.04	3.05E-03
cg27243685	*ABCG1*	rs3822072	0.03	5.26E-03
cg27243685	*ABCG1*	rs4983559	0.05	6.30E-03
cg27243685	*ABCG1*	rs4420638	0.04	7.41E-03
cg27243685	*ABCG1*	rs2081687	0.03	7.78E-03
cg27243685	*ABCG1*	rs12670798	-0.04	1.25E-02
cg27243685	*ABCG1*	rs138777	-0.03	1.34E-02
cg27243685	*ABCG1*	rs4942486	-0.03	1.53E-02

cg27243685	*ABCG1*	rs13107325	-0.05	2.00E-02
cg27243685	*ABCG1*	rs2954029	-0.03	2.20E-02
cg27243685	*ABCG1*	rs11776767	-0.03	2.73E-02
cg27243685	*ABCG1*	rs11869286	0.03	2.88E-02

*no gene annotation for this CpG according to the UCSC Genome Browser

Suppl. Table 3

Probes of the Illumina Human HT-12 v3 Expression BeadChip used for expression analysis

Probe ID	Array Address ID	Annotated gene	Chr*	Probe Chr Orientation
ILMN_1658176	460593	*ABCG1*	21	+
ILMN_2262362	3440292	*ABCG1*	21	+
ILMN_1743638	4290020	*ABCG1*	21	+
ILMN_2329927	5860377	*ABCG1*	21	+
ILMN_1794782	6060377	*ABCG1*	21	+
ILMN_1695968	6450059	*ABCG1*	21	+
ILMN_1687589	2680458	*CPT1A*	11	-
ILMN_1710052	4150091	*CPT1A*	11	-
ILMN_1696316	5290358	*CPT1A*	11	-
ILMN_1663035	3390343	*SREBF1*	17	-
ILMN_1695378	4230521	*SREBF1*	17	-
ILMN_2328986	6840044	*SREBF1*	17	-
ILMN_1697448	1240440	*TXNIP*	1	+
ILMN_1703650	2680100	*TNIP1*	5	-

*Chr = chromosome

Suppl. Table 4

Investigation of genetic confounding in KORA F4

Lipid	CpG	Gene	β coef*	p-value SNP analysis	Discovery p-value†
HDL-C	cg06500161	*ABCG1*	-0.048	5.61E-15	8.26E-17
TG	cg06500161	*ABCG1*	0.065	7.41E-24	1.21E-27
	cg19693031	*TXNIP*	-0.029	1.86E-16	1.89E-17
	cg11024682	*SREBF1*	0.054	1.83E-11	5.54E-14
	cg00574958	*CPT1A*	-0.112	6.35E-12	3.15E-13
	cg27243685	*ABCG1*	0.062	3.10E-12	3.24E-13
	cg07504977	NA‡	0.023	1.08E-09	3.93E-12
	cg20544516	*MIR33B/SREBF1*	0.039	1.23E-07	2.84E-09
	cg12556569	*APOA5*	-0.001	2.05E-01	6.43E-09
	cg07397296	*ABCG1*	0.024	3.74E-06	9.48E-08
	cg07815238	NA‡	0.049	1.95E-07	9.66E-08
LDL-C	cg22178392	*TNIP1*	0.042	1.42E-09	4.27E-09

Genetic confounding was examined for each significant CpG–lipid pair. *β coef: β coefficient †p-value of CpG-lipid associations in KORA F4 ‡no gene annotation for this CpG according to the UCSC Genome Browser.

Suppl. Table 5

Results of the gene expression analyses

a) Associations between lipid levels and *ABCG1* DNA methylation with and without adjusting for *ABCG1* expression (N=724)

Without adjustment					***adjusting for ABCG1 transcripts***			
Lipid	**CpG**	**β coef***	**SE†**	**p-value**	***ABCG1* transcript**	**β coef***	**SE†**	**p-value**
					all 6 transcripts	-0.025	0.01	1.30E-02
					ILMN_2329927	-0.029	0.01	4.10E-03
					ILMN_1794782	-0.043	0.01	2.31E-05
HDL	cg06500161	-0.050	0.01	4.10E-07	ILMN_2262362	-0.047	0.01	2.94E-06
					ILMN_1658176	-0.047	0.01	2.83E-06
					ILMN_1695968	-0.046	0.01	3.45E-06
					ILMN_1743638	-0.047	0.01	2.62E-06

					all 6 transcripts	0.044	0.01	1.36E-05
					ILMN_2329927	0.049	0.01	1.34E-06
					ILMN_1794782	0.073	0.01	4.69E-12
TG	cg06500161	0.075	0.01	5.56E-13	ILMN_2262362	0.078	0.01	1.81E-13
					ILMN_1658176	0.078	0.01	1.56E-13
					ILMN_1695968	0.077	0.01	2.63E-13
					ILMN_1743638	0.078	0.01	1.82E-13
					all 6 transcripts	0.040	0.01	3.24E-03
					ILMN_2329927	0.049	0.01	3.30E-04
					ILMN_1794782	0.067	0.01	7.23E-06

TG	cg27243685	0.075	0.01	2.36E-07	ILMN_2262362	0.074	0.01	6.21E-07
					ILMN_1658176	0.074	0.01	5.73E-07
					ILMN_1695968	0.073	0.01	8.04E-07
					ILMN_1743638	0.074	0.01	5.46E-07

b) Associations between *ABCG1* DNA methylation and *ABCG1* transcripts with and without adjusting for blood lipids

Without adjustment ***adjusting for blood lipids***

CpG	***ABCG1* transcript**	**β coef***	**SE†**	**p-value**	**CpG**	**Lipid**	***ABCG1* transcript**	**β coef***	**SE†**	**p-value**
cg06500161	all transcripts			5.42E-14	cg06500161	HDL	all transcripts			5.15E-11
	ILMN_2329927	-0.151	0.02	5.22E-15			ILMN_2329927	-0.14	0.02	3.42E-12
	ILMN_1794782	-0.135	0.04	9.98E-05			ILMN_1794782	-0.12	0.04	6.72E-04
	ILMN_2262362	0.016	0.05	7.41E-01			ILMN_2262362	0.02	0.05	7.37E-01

	ILMN_1658176	0.011	0.04	7.57E-01		ILMN_1658176	0.02	0.04	6.26E-01
	ILMN_1695968	0.037	0.04	3.25E-01		ILMN_1695968	0.03	0.04	3.82E-01
	ILMN_1743638	-0.013	0.05	8.06E-01		ILMN_1743638	-0.02	0.05	6.48E-01
					TG	all transcripts			1.23E-06
						ILMN_2329927	-0.11	0.02	7.53E-08
						ILMN_1794782	-0.10	0.04	3.48E-03
						ILMN_2262362	0.00	0.05	9.94E-01
						ILMN_1658176	0.02	0.04	6.02E-01
						ILMN_1695968	0.02	0.04	5.81E-01
						ILMN_1743638	-0.02	0.05	7.42E-01

cg27243685	all transcripts			1.86E-07	cg27243685	TG	all transcripts			8.11E-04
	ILMN_2329927	-0.185	0.04	4.34E-06			ILMN_2329927	-0.11	0.05	8.98E-03
	ILMN_1794782	-0.286	0.07	5.72E-05			ILMN_1794782	-0.23	0.07	1.00E-03
	ILMN_2262362	0.124	0.10	1.99E-01			ILMN_2262362	0.10	0.10	2.93E-01
	ILMN_1658176	-0.064	0.08	3.88E-01			ILMN_1658176	-0.05	0.08	4.64E-01
	ILMN_1695968	0.139	0.08	7.21E-02			ILMN_1695968	0.11	0.08	1.35E-01
	ILMN_1743638	0.118	0.11	2.80E-01			ILMN_1743638	0.11	0.11	2.94E-01

c) Associations between *ABCG1* transcripts and lipids with and without adjusting for *ABCG1* DNA methylation

Without adjustment ***adjusting for ABCG1 DNA methylation***

Lipid	***ABCG1* transcript**	**β coef***	**SE†**	**p-value**	**Lipid**	**CpG**	***ABCG1* transcript**	**β coef***	**SE†**	**p-value**
HDL-C	all transcripts			7.76E-13	HDL-C	cg06500161	all transcripts			1.79E-09

	ILMN_2329927	0.15	0.02	2.45E-13			ILMN_2329927	0.13	0.02	3.89E-10
	ILMN_1794782	0.13	0.04	2.22E-04			ILMN_1794782	0.11	0.04	2.10E-03
	ILMN_2262362	0.01	0.05	8.65E-01			ILMN_2262362	0.01	0.05	8.65E-01
	ILMN_1658176	0.05	0.04	2.07E-01			ILMN_1658176	0.05	0.04	1.99E-01
	ILMN_1695968	-0.04	0.04	2.89E-01			ILMN_1695968	-0.03	0.04	3.73E-01
	ILMN_1743638	-0.05	0.06	3.44E-01			ILMN_1743638	-0.06	0.06	2.85E-01
TG	all transcripts			1.25E-33	TG	cg06500161	all transcripts			1.20E-25
	ILMN_2329927	-0.49	0.04	1.55E-27			ILMN_2329927	-0.43	0.04	1.42E-20
	ILMN_1794782	-0.32	0.08	2.51E-05			ILMN_1794782	-0.25	0.08	9.82E-04
	ILMN_2262362	0.16	0.11	1.33E-01			ILMN_2262362	0.15	0.10	1.45E-01
	ILMN_1658176	-0.09	0.08	2.66E-01			ILMN_1658176	-0.10	0.08	2.01E-01

ILMN_1695968	0.15	0.09	6.38E-02			ILMN_1695968	0.13	0.08	9.86E-02
ILMN_1743638	0.04	0.12	7.09E-01			ILMN_1743638	0.05	0.12	6.50E-01
				TG	cg27243685	all transcripts			7.08E-30
						ILMN_2329927	-0.47	0.04	9.56E-25
						ILMN_1794782	-0.27	0.08	4.55E-04
						ILMN_2262362	0.14	0.11	1.72E-01
						ILMN_1658176	-0.08	0.08	3.14E-01
						ILMN_1695968	0.13	0.09	1.13E-01
						ILMN_1743638	0.02	0.12	8.92E-01

Results of the expression analyses a) The association between lipid level and *ABCG1* DNA methylation was recalculated in N=724 KORA F4 participants. b) Investigation of the association between *ABCG1* DNA methylation and *ABCG1* transcripts. c) Analysis of the association between *ABCG1* transcripts and lipids. Results of models with adjustment are shown on the right side of the table. Level of significance: 8.3E-04. *β coef: β coefficient †SE: standard error

Suppl. Table 6

Association of DNA methylation with prevalent myocardial infarction in KORA F4 (N=60)

Model 1 (covariates: age, sex and CD8 T-cells, CD4 T-cells, natural killer cells, B-lymphocytes, monocytes and granulocytes)

CpG	Chr*	gene	β coef†	SE‡	p-value	OR (95% CI)§
cg00574958	11	*CPT1A*	0.007	0.10	9.42E-01	1.01 (0.83/1.23)
cg06500161	21	*ABCG1*	0.198	0.04	3.72E-07‖	1.22 (1.13/1.32)
cg07397296	21	*ABCG1*	0.017	0.03	5.74E-01	1.02 (0.96/1.08)
cg07504977	10	NA#	0.024	0.02	2.97E-01	1.02 (0.98/1.07)
cg11024682	17	*SREBF1*	-0.018	0.05	8.01E-03	1.13 (1.03/1.23)
cg20544516	17	*MIR33B/ SREBF1*	0.120	0.04	6.36E-01	1.02 (0.94/1.11)
cg22178392	5	*TNIP1*	0.005	0.04	6.31E-01	0.98 (0.91/1.06)
cg27243685	21	*ABCG1*	-0.003	0.06	2.39E-03‖	1.21 (1.07/1.38)

Model 2 (covariates: as in model 1 plus body mass index, smoking, alcohol consumption, physical activity, current hypertension, HbA1c levels, C-reactive protein levels, white blood cell count)

CpG	Chr*	gene	β coef†	SE‡	p-value	OR (95% CI)§
cg00574958	11	*CPT1A*	0.09	0.104	3.95E-01	1.09 (0.89/1.34)

cg06500161	21	*ABCG1*	0.16	0.041	1.01E-04[‖]	1.17 (1.08/1.27)
cg07397296	21	*ABCG1*	0.01	0.032	7.98E-01	1.01 (0.95/1.07)
cg07504977	10	NA[#]	0.01	0.023	5.78E-01	1.01 (0.97/1.06)
cg11024682	17	*SREBF1*	0.09	0.048	6.05E-02	1.09 (1.00/1.20)
cg20544516	17	*MIR33B/ SREBF1*	0.02	0.044	6.70E-01	1.02 (0.93/1.11)
cg22178392	5	*TNIP1*	0.00	0.040	9.30E-01	1.00 (0.93/1.09)
cg27243685	21	*ABCG1*	0.16	0.067	1.52E-02	1.18 (1.03/1.34)

Model 3 (covariates: as in model 2 plus the lipid variables (HDL-C, LDL-C, TG, TC)

CpG	**Chr**[*]	**gene**	**β coef**[†]	**SE**[‡]	**p-value**	**OR (95% CI)**[§]
cg00574958	11	*CPT1A*	0.063	0.11	5.59E-01	1.06 (0.86/1.31)
cg06500161	21	*ABCG1*	0.141	0.04	1.30E-03[‖]	1.15 (1.06/1.25)
cg07397296	21	*ABCG1*	0.005	0.03	8.82E-01	1.00 (0.94/1.07)
cg07504977	10	NA[#]	0.015	0.02	5.30E-01	1.02 (0.97/1.06)
cg11024682	17	*SREBF1*	0.089	0.05	7.14E-02	1.09 (0.99/1.20)
cg20544516	17	*MIR33B/ SREBF1*	0.008	0.05	8.63E-01	1.01 (0.92/1.10)
cg22178392	5	*TNIP1*	0.022	0.04	5.91E-01	1.02 (0.94/1.11)
cg27243685	21	*ABCG1*	0.133	0.07	4.83E-02	1.14 (1.00/1.30)

Lipid-related CpGs were tested for an association with previous hospitalized MI in KORA F4. [*]Chr: chromosome [†]β coef: β coefficient [‡]SE: standard error [§]OR: Odds Ratio CI: confidence interval [‖]significant; level of significance: 6.3E-03 [#]no gene annotation for this CpG according to the UCSC Genome Browser

Suppl. Table 7

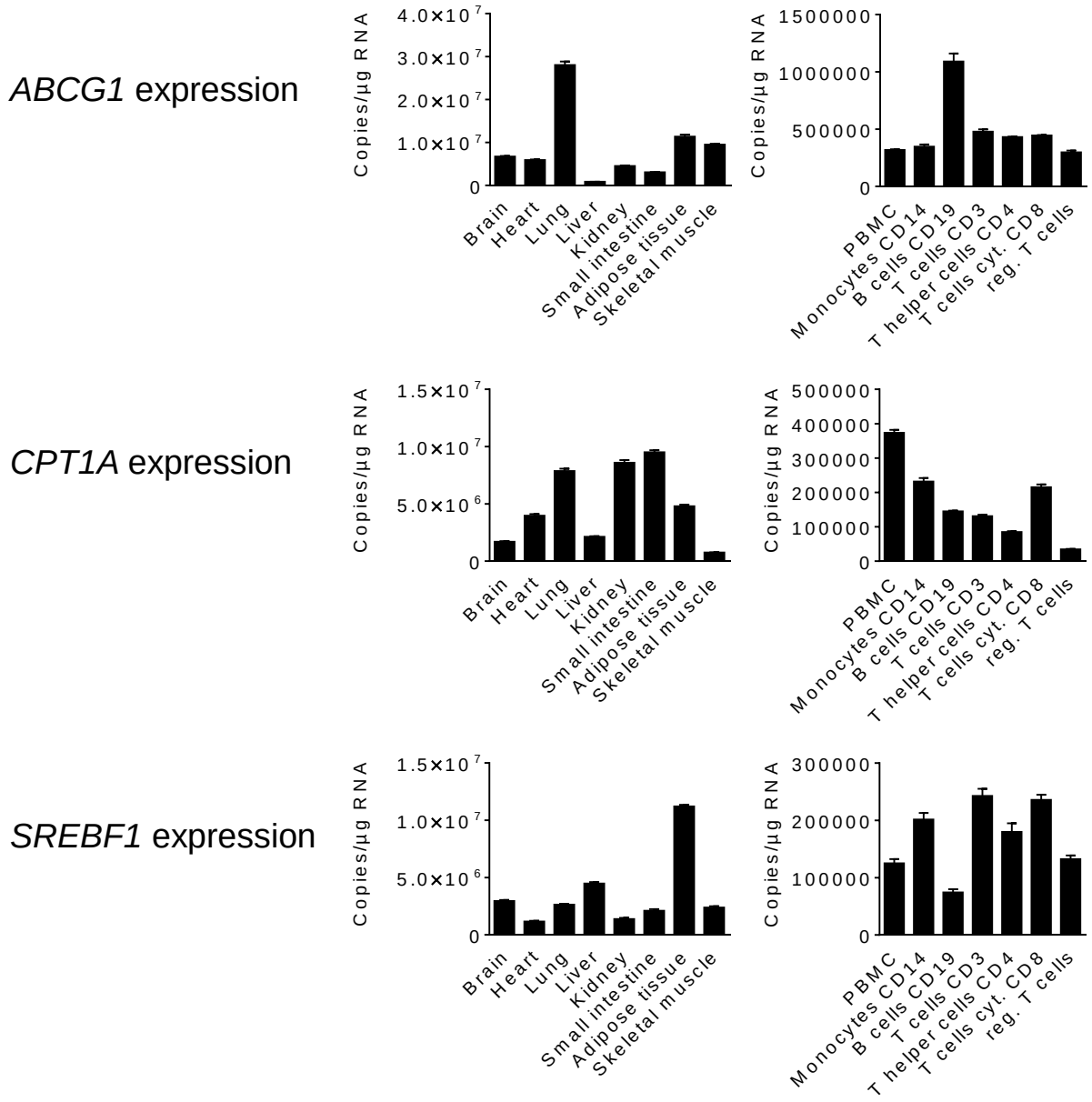
Sequences of oligonucleotides used for EMSAs

Probe name	**5´sequence 3´**
cg20544516_um	TCCGGGCTGCACTGC**CG**AGGCACTGCACCCGC
cg20544516_m	TCCGGGCTGCACTGC-(me)**C-G**AGGCACTGCACCCGC
cg06500161_um	CCTTCTCTAGACACC**CG**GCGGGACTAGTTCCT
cg06500161_m	CCTTCTCTAGACACC-(me)**C-G**GCGGGACTAGTTCCT
Oct1	TGTCGAATGCAAATCACTAGAA

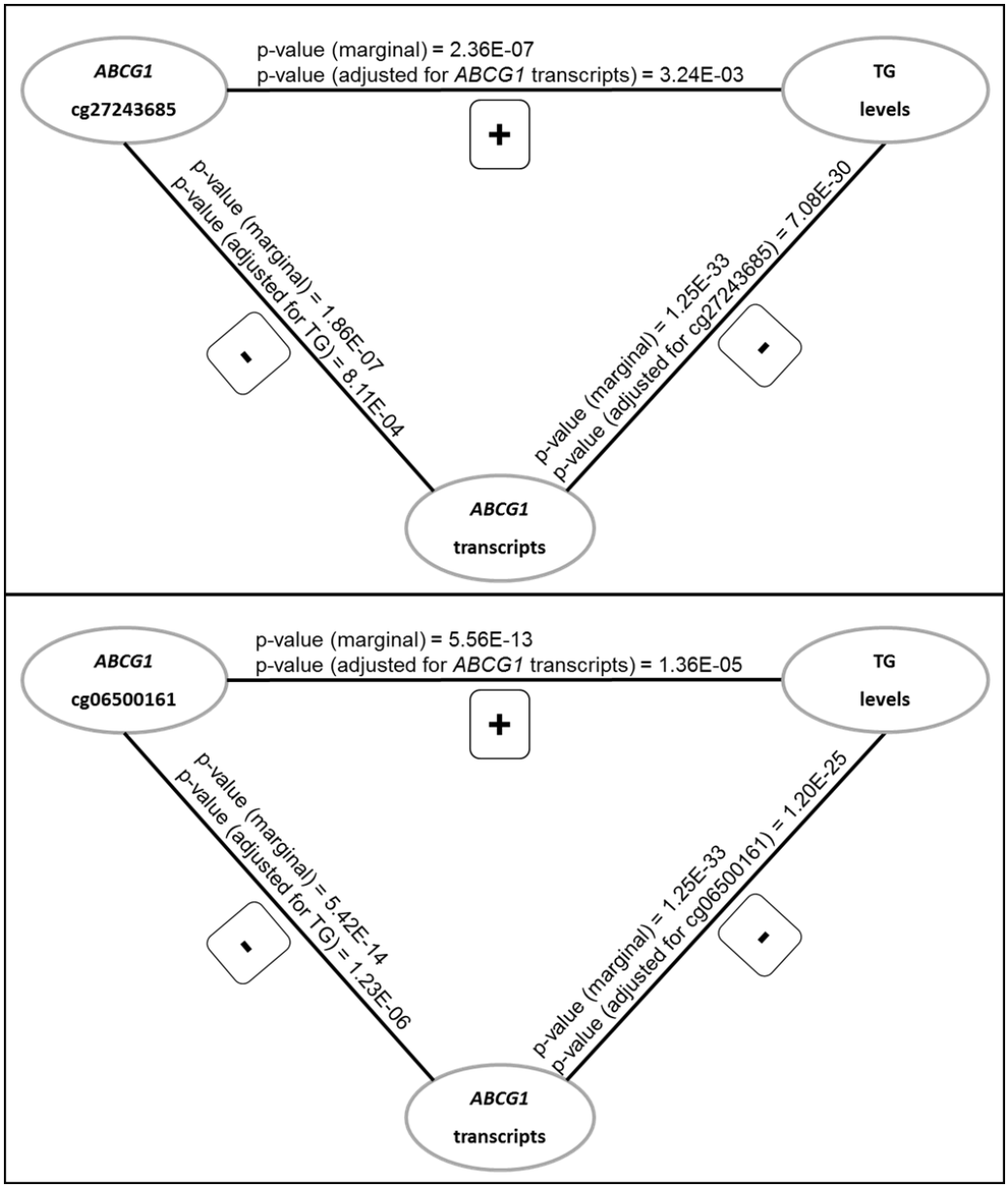
Only forward oligonucleotides are shown. Methylated (m) and unmethylated (um) Cy5-labelled oligonucleotides carrying the respective CpG site were used. The appropriate reverse oligonucleotides were complementary and unlabeled. For competition EMSAs, unlabeled oligonucleotides were used. For verification of specificity competitions with unlabeled Oct1 consensus oligonucleotides were done; underlined sequence = consensus sequence for transcription factor[15].

um = unmethylated status of CpG, m = methylated status of CpG, Oct1 = octamer binding transcription factor 1

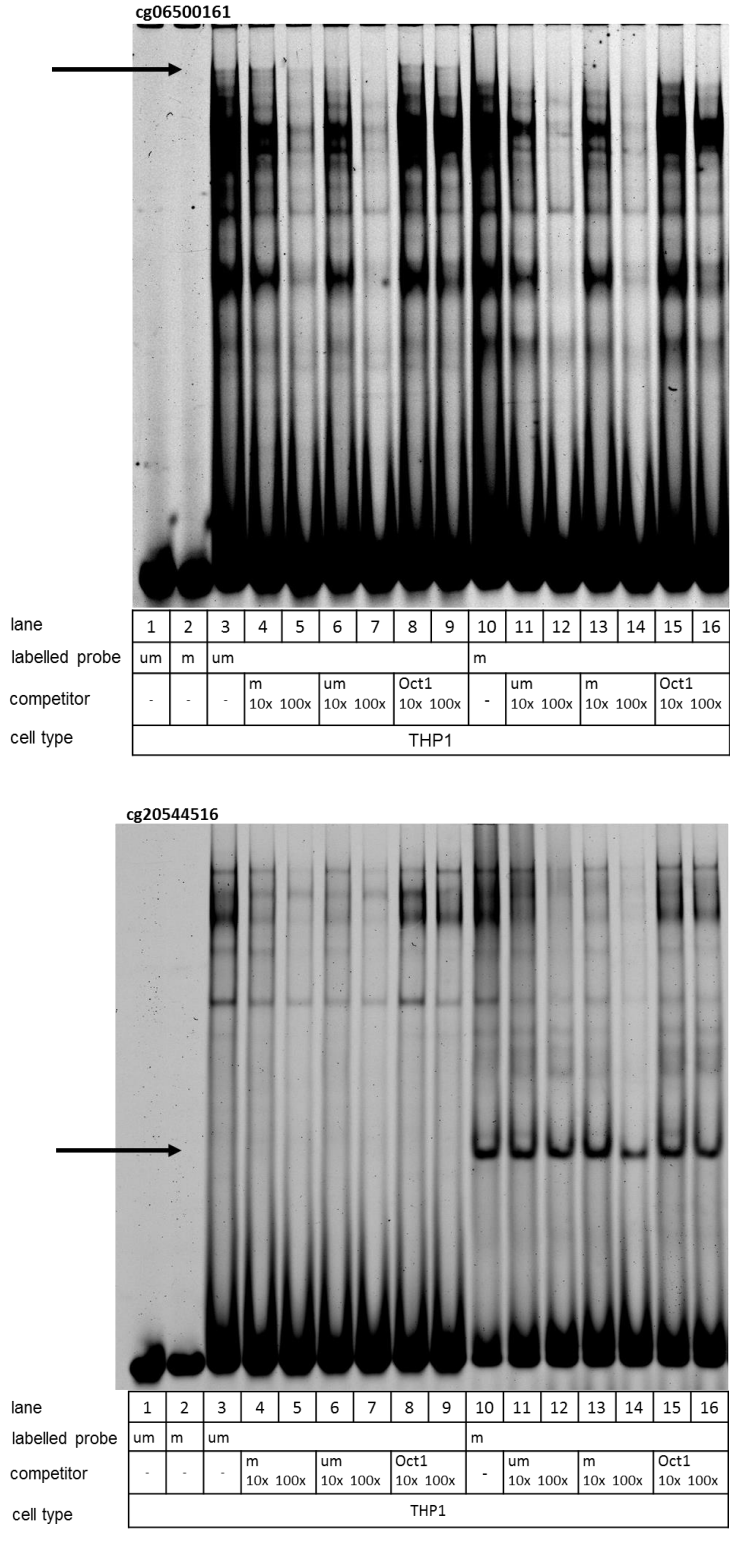
Supplemental Figures



Suppl. Figure 1: Expression of *ABCG1*, *CPT1A* and *SREBF1* in different tissues and in blood cell types. To address cell- and tissue-specificity of *ABCG1*, *CPT1A* and *SREBF1* expression, we quantified their expression in human blood cell types (peripheral blood mononuclear cells (PBMC), CD14-, CD19-, CD3-, CD4-, CD8-positive cells, and regulatory T-cells) and human tissues (human brain, heart, lung, kidney, small intestine, adipose tissue, skeletal muscle).



Suppl. Figure 2: Results of the expression analyses. The CpGs cg06500161 and cg27243685 (both located in *ABCG1*) showed an association with *ABCG1* mRNA levels which were in turn associated with blood TG levels. Recalculation of the associations adjusted for the third of the three components leads to the likely conclusion that the association between DNA methylation of *ABCG1* and TG levels is based on the expression of *ABCG1*. The results suggest a feedback regulation as the association between *ABCG1* methylation and *ABCG1* mRNA levels is less significant adjusted for TG levels. N=724, level of significance=8.3E-04



Suppl. Figure 3: Methylation-specific protein binding patterns of the CpGs cg06500161 (*ABCG1*; top) and cg20544516 (*MIR33B/SREBF1*; bottom). Methylated (m) and unmethylated (um) Cy5-labelled probes carrying the cg06500161 site and

the cg20544516 site, respectively, were used in competition EMSAs using THP1 nuclear extracts. Arrows indicate methylation specific binding patterns. Lanes 1 and 2 show free oligonucleotides without incubation with nuclear extract. Lanes 3 and 10 show patterns for unmethylated and methylated status of the CpGs. In lanes 4, 5, 13, 14 competition with unlabeled methylated oligonucleotides was performed, whereas lanes 6, 7, 11, 12 show the results of the competition with the unlabeled unmethylated oligonucleotides. For competition different concentrations were used (10 and 100 ng/µl). For verification of specificity competitions with unlabeled Oct1 consensus oligonucleotides (lanes 8, 9, 15, 16) were done. um = unmethylated status of CpG, m = methylated status of CpG, Oct1 = octamer binding transcription factor 1

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