Molecular Characterization of the *NLRC4* Expression in Relation to Interleukin-18 Levels

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Background—Interleukin-18 (IL-18) is a pleiotropic cytokine centrally involved in the cytokine cascade with complex immunomodulatory functions in innate and acquired immunity. Circulating IL-18 concentrations are associated with type 2 diabetes mellitus, cardiovascular events, and diverse inflammatory and autoimmune disorders.

Methods and Results—To identify causal variants affecting circulating IL-18 concentrations, we applied various omics and molecular biology approaches. By genome-wide association study, we confirmed association of IL-18 levels with a single nucleotide polymorphism in the untranslated exon 2 of the inflammasome component *NLRC4* (NLR family, caspase recruitment domain–containing 4) gene on chromosome 2 (rs385076; $P=2.4\times10^{-45}$). Subsequent molecular analyses by gene expression analysis and reporter gene assays indicated an effect of rs385076 on *NLRC4* expression and differential isoform usage by modulating binding of the transcription factor PU.1.

Conclusions—Our study provides evidence for the functional causality of single nucleotide polymorphism rs385076 within the *NLRC4* gene in relation to IL-18 activation. **(*Circ Cardiovasc Genet*. 2015;8:717-726. DOI: 10.1161/CIRCGENETICS.115.001079.)**

Key Words: gene expression ■ genetic variation ■ inflammasomes ■ interleukin-18 ■ transcription factors

Interleukin-18 (IL-18), a member of the IL-1 family, is a pleiotropic cytokine centrally involved in the cytokine cascade with complex immune-modulatory functions in innate and acquired immunity.[1,2] Circulating IL-18 levels are associated with diverse inflammatory and autoimmune disorders,[3] type 2 diabetes mellitus,[4] and cardiovascular events.[5,6] The mechanisms through which IL-18 predisposes to these disorders are unknown. Several studies have linked genetic variations in the IL-18 system to circulating biomarker concentrations, disease susceptibility, and progression. Common sequence variants in the 4 genes that constitute the IL-18 system including *IL18* (MIM 600953), *IL18R1* (IL-18 receptor 1, MIM 604494), *IL18RAP* (IL-18 receptor accessory protein, MIM 604509), and the gene coding for the soluble decoy receptor *IL18BP* (MIM 604113), have been comprehensively genotyped and related to circulating IL-18 levels and cardiovascular events.[7,8] Genome-wide findings confirm associations at the *IL18-BCO2* gene locus on chromosome 11 with circulating IL-18 levels.[9]

Clinical Perspective on p 726

In a recent study Matteini et al[10] identified additional genetic variants within an 8.8-Mb region of chromosome 2 spanning the genes *SRD5A2* (MIM 607306), *MEMO1* (MIM 611786), *DPY30* (MIM 612032), *SPAST* (MIM 604277), *SLC30A6* (MIM 611148), and *NLRC4* (MIM 606831) as being associated with circulating IL-18 levels. The *NLRC4* gene (NLR family caspase recruitment domain–containing protein 4) was discussed as the most likely inflammation and thus IL-18–related gene within this region. NLRC4 is a central component of the inflammasome, a multimeric protein complex initiating immune responses.[11] The NLRC4 inflammasome facilitates caspase-1–dependent inflammatory cytokine processing and pyroptosis.[11,12] In addition to sensing microbial pathogens, the function of the NLRC4 inflammasome is the activation of caspase-1 and subsequent proteolytic maturation of pro–IL-18 and pro–IL-1β into their active forms.[13,14] On the basis of involvement of NLRC4 in IL-18

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maturation, it can be hypothesized that *NLRC4* variants are most likely to influence circulating IL-18 levels via inflammasome action. Furthermore, associations of variants within the *NLRC4* locus with serum IL-18 levels were recently confirmed in an IL-18 genome-wide association study (GWAS) in patients with acute coronary syndrome.[15] In particular, the authors identified 1 single nucleotide polymorphism (SNP), rs385076, which was located in a region of the *NLRC4* locus with predicted regulatory relevance. However, both studies[10,15] do not provide molecular data supporting a possible causal association of genetic variants within *NLRC4* and circulating IL-18 levels.

The aims of the present study were (1) to validate genetic determinants of IL-18 by a GWAS and (2) to characterize identified loci on a molecular level by cloning the most promising candidate SNP and investigating transcriptomic and epigenetic (ie, methylation) mechanisms in relation to IL-18.

Materials and Methods

Genome-Wide Association Study

We assembled 3 cohorts for the discovery GWAS, comprising 9562 individuals of European ancestery: the Gutenberg Health Study (GHS), the Framigham Heart Study (FHS), and the Cooperative Health Research in the Region of Augsburg (KORA F4) study. For replication, 3 additional cohorts were included totaling 3348 individuals (Athero*Gene*, Monitoring of Trends and Determinants in Cardiovascular Diseases [MONICA/KORA S1/S2/S3], Prospective Epidemiological Study of Myocardial Infarction [PRIME] study). Study protocols were approved by the local ethics committees, and all participants provided written informed consent. Details on the GWAS discovery and replication cohorts are outlined in the Data Supplement and Table I in the Data Supplement.

Genotyping and Imputation

SNPs were genotyped on the Affymetrix Whole-Genome Human SNP Array 6.0 in GHS and KORA F4 and the Affymetrix Human Mapping 500K Array Set and 50K Human Gene Focused Panel in FHS. SNPs were imputed based on the 1000 Genome Phase 1 data set (version 3, build 37). Details of genotyping platform, quality controls, and imputation for each cohort are specified in Table II in the Data Supplement.

In AtheroGene, MONICA/KORA S1/S2/S3, and PRIME genotyping of rs385076 and rs11606049 (or the respective proxy SNP rs5744222 in MONICA/KORA S1/S2/S3), polymorphisms were performed using 5′ nuclease assay (TaqMan assay; Applied Biosystems, Darmstadt, Germany) for replication.

Laboratory Analyses

Measurement of Circulating IL-18 Levels

In all studies except MONICA/KORA S1/S2/S3, circulating IL-18 levels were measured using a commercially available ELISA (MBL Co, Ltd. Nagoya, Japan). The detection limit was 128 pg/mL. Intra-assay coefficients of variation was 6.9%; interassay coefficients of variation was 13%. In MONICA/KORA S1/S2/S3 serum levels of IL-18 were measured by bead-based multiple assay as described before[4] using an antibody pair and recombinant IL-18 protein from MBL (MBL Co, Ltd, Nagoya, Japan). The intra- and interassay coefficients of variation were <10.0% and <25.0%, respectively. Measurements were performed from deep-frozen samples (−80°C).

Cloning of NLRC4 rs385076 Luciferase Reporter Gene Constructs

To investigate the influence of the SNP rs385076 (T/C) on binding of the transcription factor PU.1, the 908-bp genomic region 32489378 to 32490286 on the antisense strand of the human chromosome 2 comprising the SNP rs385076 (T/C) and the putative PU.1 binding region within untranslated exon 2 of *NLRC4* was amplified by polymerase chain reaction (PCR) using genomic DNA from carriers of either the C allele or the T allele of rs385076 (T/C). The PCR products were cloned into the *Kpn*I-digested firefly luciferase reporter vector pGL4.10[luc2] (Promega GmbH, Mannheim, Germany). SNP alleles were confirmed by sequencing.

Cloning of PU.1 Overexpression Plasmid

For PU.1 overexpression, the PU.1 coding sequence was amplified by PCR using macrophage genomic DNA (Table III in the Data Supplement) and cloned into *Eco*RV- and *Sal*I-digested pVITRO2-MCS (InvivoGen, Toulouse, France). The insert was confirmed by sequencing.

Transfection of Cells and Measurement of Luciferase Activity

HEK293A cells were transfected with 0.5-µg/mL pGL4.10[luc2] containing either the rs385076 C or T allele and 0, 0.125, 0.25, or 0.5 µg/mL pVITRO2-PU.1 using 2-µL/mL Lipofectamine2000 (Life Technologies, Darmstadt, Germany). After 24 hours, Bright-Glo Reagent (Promega) was added and cells were immediately frozen at −80°C for 30 minutes. Cells were then thawed to room temperature, shaken at 14 000 rpm and luciferase activity was detected via luminescence reader.

Assessment of NLRC4 Isoforms Expression

Of 4 *NLRC4* isoforms, only isoforms 2 and 4 (ENST00000402280 and ENST00000404025) contain the 5′ untranslated region (UTR) exon 2. Therefore, the association between expression of *NLRC4* isoforms 2 and 4 and isoforms 1 and 3 (ENST00000360906 and ENST00000342905) with rs385076 genotype was determined. Expression of total *NLRC4* transcript expression, *NLRC4* isoforms 2 and 4, *NLRC4* isoforms 1 and 3, and *GAPDH* as reference gene was measured in monocytes from 1444 GHS subjects via real-time quantitative PCR using a 7900 TaqMan system (Applied Biosystems). Briefly, RNA was purified using TRIzol (Life Technologies) and reverse transcribed using High-Capacity cDNA Reverse Transcription Kit (Life Technologies) according to manufacturers' protocols. Real-time PCR was performed in a 10-µL reaction volume using 5-µL ABsolute quantitative PCR SYBR Green Mix (Life Technologies), 0.2-µL ROX Reference Dye (Jena Bioscience, Oxford, England), 0.25-ng cDNA and 150- to 500-nmol/L primers (Table III in the Data Supplement). After 15-minute activation at 95°C, 40 PCR cycles were run with 15-s denaturation at 95°C, 30-s annealing at 60/62°C, and 30-s extension at 72°C. Primer specificity was verified by melting curve analysis. *NLRC4* mRNA expression was normalized to *GAPDH* as an endogenous control.

Measurement of DNA Methylation

For assessement of DNA methylation sites (CpG sites) in relation to IL-18 levels, genome-wide methylation was performed using the Illumina HumanMethylation450 BeadChip in a subgroup of the KORA F4 cohort (n=1814; Data Supplement).

Statistical Analyses

Statistical Analysis of Genotyping Data

IL-18 levels showed a skewed distribution and were natural log-transformed for analyses. For genetic association analysis, age- and sex-adjusted linear regression models were applied in all discovery and replication cohorts separately, using an additive genetic model. In GHS, genetic relatedness was considered by identifying and removing outliers based on multidimensional scaling of genetic data before GWAS. In FHS, linear mixed-effect models were performed to account for relatedness among family members using the function *lmekin* from the R *kinship* package. KORA is a population representative subsample of the population in the study region. Cryptic relatedness in the study sample has been investigated based on genome-wide genotypic data. The degree of relatedness between samples is negligible. Therefore, no further adjustments have been made in this analysis. For meta-analysis,

individual estimates of allelic effects from GHS, FHS, and KORA F4 were combined after excluding genotyped and imputed SNPs not meeting the quality control filters. An inverse-variance weighted fixed-effects approach was applied as implemented in *METAL*.[16] The genome-wide significance level for the GWAS was set to $P<5\times10^{-8}$.

Additive effects of lead SNPs from different loci were tested in all discovery cohorts. SNPs were combined by adding the T allele dosages of rs385076 and rs11606049 for each individual resulting in many protective alleles between 0 and 4. The estimated percentage of explained variance for log-transformed IL_e-18 by SNPs was calculated by subtracting the explained variance of sex and age on log_e-transformed IL-18 from the multivariable model. For replication in Athero*Gene*, MONICA/KORA S1/S2/S3 and PRIME associations were calculated by applying the inverse-variance–based fixed-effects meta-analysis for the selected SNPs.

Conditional Analysis Within Genome-Wide Significant Loci

To determine whether the signals at each locus were independently associated with IL-18 levels, a conditional analysis was performed in the GHS data set using the variant with the lowest *P* value (with a region of ±500 kb) as a lead SNP using SNPTEST v2.5 (www.mathgen.stats.ox.ac.uk/genetics_software/snptest/snptest.html), built-in functions.

Expression Quantitative Trait Loci Analysis

Associations between selected SNPs and gene expression of transcripts in *cis* (±500 kb) were assessed for all samples with available gene expression data in GHS (n=1133),[17] and KORA F4 (n=699).[18] In both studies, gene expression analysis was performed using the Illumina Human HT-12 v3 BeadChip. Technical variables were used for adjustment as previously described.[18] In both studies, a linear model was used with log_2-transformed gene expression as dependent variable. For SNP-gene expression analyses, data were adjusted for age and sex. PheGenI (www.ncbi.nlm.nih.gov/gap/phegeni) and the Geuvadis Data Browser (www.ebi.ac.uk/Tools/geuvadis-das/) were used to compare significant expression quantitative trait loci from our study to publicly available data from large-scale studies.

Expression values of *NLRC4* and its isoforms measured by quantitative PCR were normalized for *GAPDH* Ct values before association analysis and are represented as deltaCt values (deltaCt_transcript=Ct_transcript – Ct_GAPDH). A linear mixed-effect model with the plate as random variable, transcript levels as dependent variable, and age and sex as covariates was used to calculate effects of rs385076 allele dosage on *NLRC4* isoform expression. Reported effect sizes refer to the β from the linear regression, which represents the change of deltaCt per rs385076 T allele.

Estimating the proportion of variance in IL-18 levels

The proportion of explained variance of log-transformed IL-18 levels attributable to genetic variants was calculated by subtracting the coefficients of variation estimated from a linear model with adjustments for sex and age from the multivariate model, which additionally included the allele dosage as independent variable.

Association With Cardiovascular Mortality

Hazard ratios for rs385076 and rs11606049 related to cardiovascular mortality were estimated using Cox proportional hazards regression in the AtheroGene study. Analyses were adjusted for age and sex and an additive genetic model was used.

Results

GWAS of Circulating IL-18 Levels

Characteristics of all discovery and replication cohorts participating in the GWAS are presented in Table I in the Data Supplement. Figure 1 depicts the Manhattan Plot of our initial GWAS approach demonstrating 2 regions of genome-wide significance for circulating IL-18 levels. Regional plots of these 2 regions including functional annotation are presented in Figure 2. Summary GWAS results are available in the Table IV in the Data Supplement. The strongest evidence of association was located within the *NLRC4* gene on chromosome 2 (lead SNP: rs385076; $P_{meta}=2.4\times10^{-45}$). On chromosome 11, the strongest signal was detected within the *IL18* gene (lead SNP: rs11606049; $P_{meta}=4.6\times10^{-35}$). Carriers of the respective T allele of both SNPs had lower IL-18 levels. Successful replication of these 2 SNPs (or the respective proxy SNP rs5744222 in MONICA/KORA S1/S2/S3) was performed in 3 independent cohorts (Athero*Gene* [n=1165], PRIME [n=440], and MONICA/KORA S1/S2/S3 [n=1743]). T allele frequencies ranged between 35.4% and 39.7% for rs385076 and between 19.9% and 26% for rs11606049 (Table 1). The associations of rs385076 and rs11606049 explained between 2.05% and 2.55% (rs385076) and 1.51% and 3.32% (rs11606049) of the interindividual variability of IL-18 levels among studies (Table 2). Combination of the lead SNP allele dosages from both loci led to an explained variance between 3.15% (FHS) and 5.49% (GHS; Table 2). After conditional analysis on the most significant SNP on chromosome 2 (rs385076) and chromsome 11 (rs11606049), no additional SNPs remained significant at the discovery *P* value threshold of $<5\times10^{-8}$ (Figure 1A and 1B).

Our meta-analysis data confirmed previous results of strong associations of circulating IL-18 levels with loci on chromosome 2 and 11.[9,10] For the *IL18* locus on chromosome 11, previous reports have already investigated the functional consequences of variants within this locus.[7,9,20] However, to our knowledge, for the locus on chromosome 2, no functional data in relation to circulating IL-18 levels have been presented. Therefore, we performed in silico and molecular characterization of the *NLRC4* locus.

In Silico Functional Analysis of the NLRC4 Locus

Regulatory and epigenetic features of the *NLRC4* locus from the Encyclopedia of DNA Elements (ENCODE)[21] and a data set by Pham et al[22] were explored using UCSC Genome Browser[23] and are displayed in Figure 3. The *NLRC4* gene encompasses 4 distinct isoforms and the SNP rs385076 lies within the 5′UTR of the *NLRC4* cDNA, in the untranslated exon 2 included in isoforms 2 and 4. This region directly falls with in a region enriched for the H3K27ac histone acetylation mark (often found near active regulatory elements) in human $CD14^+$ monocytes and several additional cell lines.[22] Furthermore, the region surrounding the SNP rs385076 lies within a DNase hypersensitivity cluster in multiple cell lines as indicated by ChIP-seq data. DNase hypersensitivity is characteristic for open chromatin regions, which are accessible for DNA-binding proteins. ChIP-seq data in monocytes[22] showed that strong binding of the transcription factor PU.1 occurs around rs385076. Together, these in silico data point toward regulatory region(s) within the *NLRC4* 5′UTR and suggest that different alleles of rs385076 might lead to different *NLRC4* expression levels.

NLRC4 expression may be affected in several ways: (1) a direct effect on *NLRC4* mRNA expression, (2) effects on differential mRNA isoform usage, (3) an effect on NLRC4 protein level, and (4) by modulated binding of transcription factor PU.1. These possibilities were investigated at the molecular level.

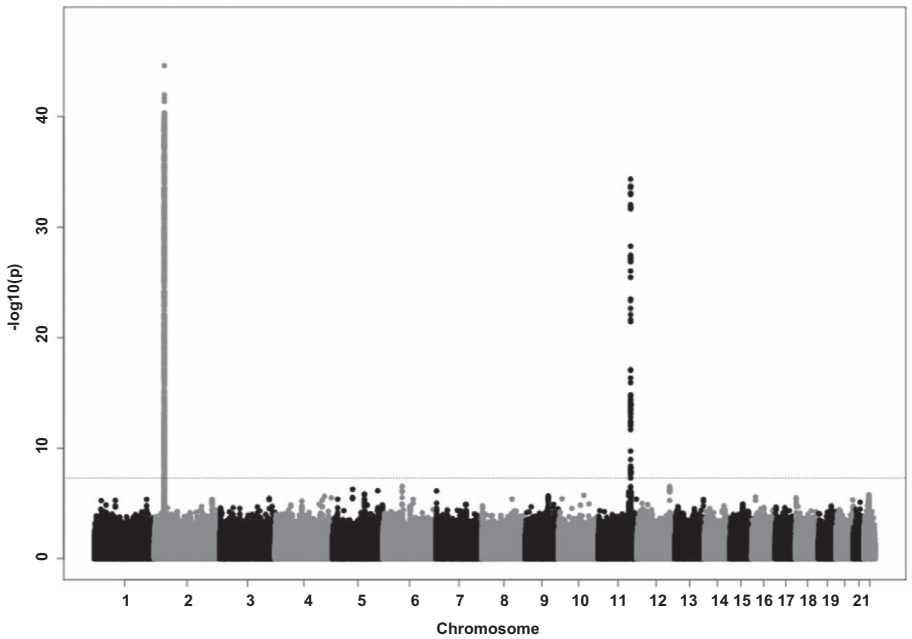

Figure 1. Results from the genome-wide association study meta-analysis of interleukin (IL)-18 concentrations in the Gutenberg Health Study (GHS) I, GHS II, the Framigham Heart Study (FHS), and the Cooperative Health Research in the Region of Augsburg (KORA F4). The Manhattan plot shows the associations between allele dosages of 1000 genomes imputed variants and $\log_e$-transformed IL-18 concentrations for all autosomes. The horizontal line indicates the genome-wide significance level ($P<5\times10^{-8}$).

Association of rs385076 and NLRC4 Gene Expression

To determine the association of NLRC4 mRNA expression and the SNP rs385076, the expression data sets of the GHS (monocytes) and KORA F4 (whole blood) studies were used. In monocytes, our data demonstrated an impact of rs385076 on *NLRC4* expression levels with a decrease of *NLRC4* mRNA in carriers of the T allele (β=–0.037; $P=3.95\times10^{-3}$). In contrast, in our whole blood data set, no association between rs385076 and *NLRC4* expression was observed (β=–0.085; $P=0.14$). We further compared our results with publicly available expression quantitative trait loci data of lymphoblastoid cell lines from 270 individuals[26] using PheGenI. The lymphoblastoid data were in line with our monocytic data, as decreased *NLRC4* mRNA expression for rs479333 C allele carriers (a proxy SNP of rs385076; $R^2=0.83$) was described (β=–0.133; $P=3.4\times10^{-8}$). In RNA-seq data from lymphoblastoid cell lines of 373 European individuals from the 1000 Genomes project,[27] which is publicly available at the Geuvadis Data Browser, rs385076 T allele was strongly associated with *NLRC4* gene expression (β=–0.373; $P=9.1\times10^{-14}$). A summary of rs385076 and rs479333 expression quantitative trait loci is provided in Table V in the Data Supplement.

Association of rs385076 and NLRC4 Isoform Expression

Expression of the *NLRC4* gene including all isoforms and 2 distinct groups of *NLRC4* mRNA isoforms was determined in monocytes of 1487 GHS participants by quantitative PCR. The group $NLRC4_{2,4}$ (encompassing isoforms 2 and 4) includes the rs385076 containing exon 2, whereas the group $NLRC4_{1,3}$ (encompassing isoforms 1 and 3) does not include the rs385076-exon 2 as depicted in Figure 3.

Consistent with our monocytic gene expression data, overall *NLRC4* expression was decreased in carriers of the rs385076 T allele (β=0.134; SE=0.05; $P=6.9\times10^{-3}$). Interestingly, expression of $NLRC4_{2,4}$ (β=0.305; SE=0.047; $P=2.0\times10^{-10}$) and $NLRC4_{1,3}$ (β=–0.103; SE=0.03; $P=7.4\times10^{-4}$) isoforms were associated with the T allele in opposite directions. Comparing the proportion of $NLRC4_{2,4}/NLRC4_{1,3}$ isoforms on overall *NLRC4* gene expression, we observed a shift toward usage of isoforms 1 and 3 in carriers of the rs385076 T allele as depicted in Figure 4. In relation to increased IL-18 concentrations, $NLRC4_{1,3}$ was significantly lower (β=0.027; SE=0.014; $P=0.049$), whereas overall *NLRC4* (β=0.014; SE=0.008; $P=0.092$) and $NLRC4_{2,4}$ (β=0.009; SE=0.008; $P=0.254$) expressions showed no significant change (Table 3).

PU.1 Binding and Influence on NLRC4 Expression in Relation to rs385076 Alleles

To evaluate whether the rs385076 variant influences binding of the transcription factor PU.1 and subsequently *NLRC4* expression, a luciferase reporter gene assay in the presence of overexpressed PU.1 levels was performed. Overexpression of PU.1 was confirmed on mRNA and protein levels (Figure II in the Data Supplement). Without PU.1 overexpression, no significant difference in luciferase activity between the rs385076 C and T alleles was observed. However, increasing PU.1 concentrations significantly raised luciferase activity in the presence of the rs385076 C allele but not the T allele (Figure 5).

DNA Methylation in Relation to Circulating IL-18 Levels and NLRC4 Expression

We screened for genome-wide associations between DNA methylation in whole blood and circulating IL-18. As shown in Figure III in the Data Supplement, 6 CpG–IL-18 associations

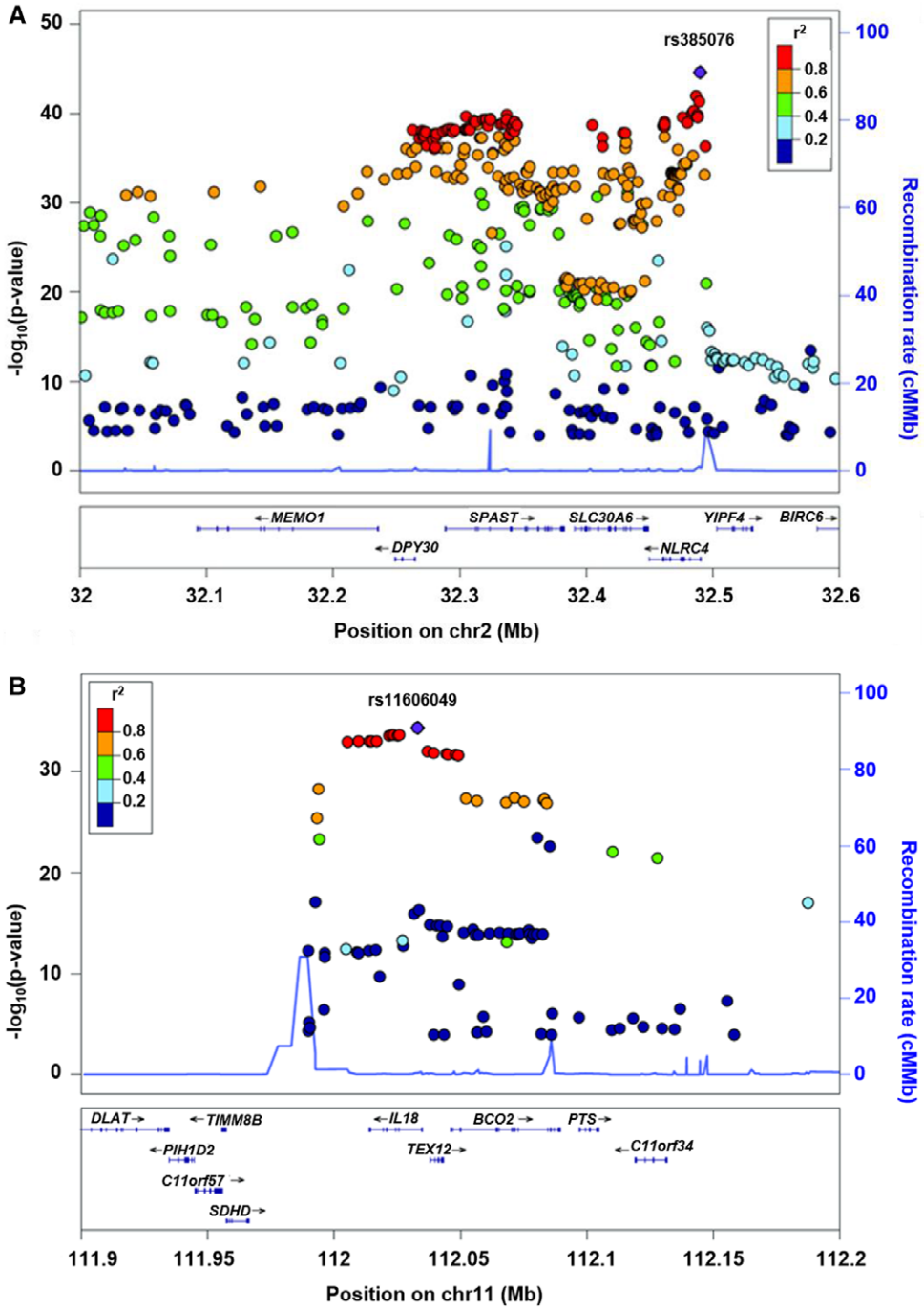

Figure 2. Regional plot for the *NLRC4* locus (**A**) and the *IL18* locus (**B**). *P* values of single nucleotide polymorphisms (SNPs) from $\log_e$ interleukin (IL)-18 meta-analysis were plotted as –log10 values against their physical position on chromsome 2 (*NLRC4*) and chromosome 11 (*IL18*), respectively (NCBI build 37). **A**, The lead SNP rs385076 on chromsome 2 is represented by a blue diamond and lies within the 5′ region of *NLRC4*. **B**, The lead SNP rs11606049 on chromsome 11 is represented by a blue diamond and lies within an intronic region of the *IL18* gene. The color code for the pairwise linkage disequilibrium structure is based on 1000 genomes 2012 European.

at the Bonferroni threshold 1×10^{-7} were identified. No genome-wide significant CpG sites were found for the chromosome 2 (*NLRC4*) locus. However, 2 CpG sites cg07055315 and cg22805603 within the *NLRC4* locus showed moderate associations ($P=2.25\times10^{-5}$ and $P=8.5\times10^{-3}$, respectively). Genome-wide significance was found for the 2 CpG sites on chromosome 16 (cg07839457; $P=2.85\times10^{-12}$ and cg16411857; $P=8.67\times10^{-08}$), both located within the *NLRC5* gene, another member of the NLRC inflammasome protein family.

Clinical Relevance of the IL-18 GWAS Loci

As circulating IL-18 levels have been associated to cardiovascular disease (MIM 611139) in previous studies,[5,6,28] a possible link between the SNPs in *NLRC4* and *IL-18* and cardiovascular outcome was investigated. For this, we tested the association of rs385076 and rs11606049 with cardiovascular death in 2585 patients with coronary heart disease of the Athero*Gene* cohort (median follow-up, 4.9 years; maximum, 7.6 years; 159 cardiovascular deaths). The rs385076 T allele, related to lower

Table 1. Effects of Lead SNPs on Circulating IL-18 Levels in Discovery and Replication

SNP (Coding/ Noncoding Allele)	Chr	Gene	Meta-Analysis β	Meta-Analysis SE	Meta-Analysis *P* Value	Meta-Analysis AF†	Athero*Gene* β	Athero*Gene* SE	Athero*Gene* *P* Value	Athero*Gene* AF†	PRIME β	PRIME SE	PRIME *P* Value	PRIME AF†	MONICA/KORA S1/S2/S3 β	MONICA/KORA S1/S2/S3 SE	MONICA/KORA S1/S2/S3 *P* Value	MONICA/KORA S1/S2/S3 AF†
rs385076 (T/C)	2	*NLRC4*	−0.093	0.007	2.4×10^{-45}	35.6	−0.107	0.017	9.0×10^{-10}	39.7	−0.098	0.032	0.002	35.4	−0.107	0.026	4.0×10^{-5}	36.5
rs11606049 (T/C)	11	*IL18*	−0.089	0.007	4.6×10^{-35}	24.5	−0.060	0.020	0.0027	26.0	−0.109	0.029	0.004	19.9	…	…	…	…
rs5744222 (A/C)*			−0.085	0.007	9.2×10^{-32}	75.1	…	…	…	…	…	…	…	…	−0.110	0.030	3.0×10^{-4}	…

β refers to the effect estimate and SE to the standard error from the linear regression model after adjustment for sex and age. For each SNP, the chromosome (Chr) and nearest gene (Gene) are shown. Discovery meta-analysis in the Gutenberg Health Study (GHS) I and II, the Framigham Heart Study (FHS), and the Cooperative Health Research in the Region of Augsburg (KORA F4). Athero*Gene*, PRIME, and MONICA/KORA S1/S2/S3 were used as independent replication cohorts. AF indicates allele frequency; IL, interleukin; MONICA, Monitoring of Trends and Determinants in Cardiovascular Diseases; PRIME, Prospective Epidemiological Study of Myocardial Infarction; and SNP, single nucleotide polymorphism.

*Proxy SNP for rs11606049 with R^2>0.9 according to SNAP.[19]

†AF of the coding allele in %.

IL-18 concentration, was associated with a protective effect on cardiovascular mortality with an age- and sex-adjusted hazard ratio of 0.78 (95% confidence interval, 0.62–0.98; *P*=0.03). However, the IL-18–lowering allele of rs11606049 was not significantly associated with cardiovascular mortality.

Discussion

Our study aimed to confirm and functionally characterize genetic determinants of circulating IL-18 levels by molecular approaches. Our molecular data show that the SNP rs385076 affects *NLRC4* expression and differential *NLRC4* isoform usage by influencing the binding of the transcription factor PU.1. Our data suggest that the IL-18–lowering T allele of rs385076 associates with cardiovascular events in a protective manner, thereby providing a link for clinical relevance.

Molecular Analyses of Circulating IL-18 Levels

Genetic Determinants of Circulating IL-18 Levels

Our GWA meta-analysis and replication study revealed 2 loci, on chromsomes 2 and 11, which associated with circulating IL-18 levels at genome-wide significance. Previous GWAS reported significant associations of several SNPs on both of these chromosomes with circulating IL-18 levels.[9,10] The *IL18-BOC2* locus associated with IL-18 levels in 2 independent GWAS samples with the strongest association found for SNP rs2115763, located in intron 2 of *BCO2*.[9] Our data confirmed the strong association of this *BCO2* SNP and showed additional SNPs with even stronger association in the *IL18* gene region, including our top SNP rs11606049 ($P=4.6\times10^{-35}$). Moreover, our GWAS and conditional studies confirm and extend findings on further genetic determinants of circulating IL-18 identified by Matteini et al[10] and Johansson et al[15] on chromosome 2 and point toward *NLRC4* as the determinant locus within this region. SNP rs385076 showed the strongest association of all SNPs in our data (β=−0.093; SE, 0.007; $P=2.4\times10^{-45}$) and is located in the untranslated exon 2 (also designated as 5′UTR) of *NLRC4*, suggesting a functional effect of this SNP. This assumption was further strengthened by our in silico investigation of the *NLRC4* 5′UTR and our experimental data.

Effect of SNP rs385076 on NLRC4 Gene Expression and Function

NLRC4 encodes a cytosolic protein with a caspase recruitment domain found primarily in monocytes and macrophages. On activation, NLRC4 assembles into a multiprotein complex, the inflammasome, which in turn leads to activation of caspase-1 and subsequent maturation of IL-18.[13,14] Our in silico functional analyses of the *NLRC4* 5′UTR/exon 2 region point toward the presence of a binding region of the transcription factor PU.1 within the exact region, where the top GWAS SNP (rs385076) is located.

Our molecular data suggested that rs385076 influences the binding of the transcription factor PU.1 to the *NLRC4* 5′UTR region, with reduced PU.1 binding to the *NLRC4* 5′UTR region, in the presence of the IL-18–lowering T allele. The most obvious effect of this influence would be an impact on *NLRC4* mRNA expression. Indeed, our data demonstrated that carriers of the IL-18–lowering T allele of rs385076 had a

Table 2. Variance of Circulating IL-18 Levels Explained by SNPs

Variant (Allele)	Gene	Explained Variance (R^2) GHS I	GHS II	KORA F4	FHS
Sample size	…	2743	1073	1802	2940
rs385076 (T)	*NLRC4*	2.05%	2.22%	2.55%	2.19%
rs11606049 (T)	*IL18*	1.51%	3.32%	1.89%	1.03%
rs385076 (T)+rs11606049 (T)	*NLRC4+IL18*	3.68%	5.49%	4.50%	3.15%

SNPs were combined by adding the allele dosages of rs385076 T and rs11606049 T for each individual resulting in many protective alleles between 0 and 4. The R^2 is the estimated percentage of explained variance for $\log_e$-transformed IL-18 by SNPs. It is calculated by subtracting the explained variance of sex and age on log-transformed IL-18 from the multivariate model. FHS indicates the Framigham Heart Study; GHS, The Gutenberg Health Study; IL, interleukin; KORA F4, the Cooperative Health Research in the Region of Augsburg; and SNP, single nucleotide polymorphism.

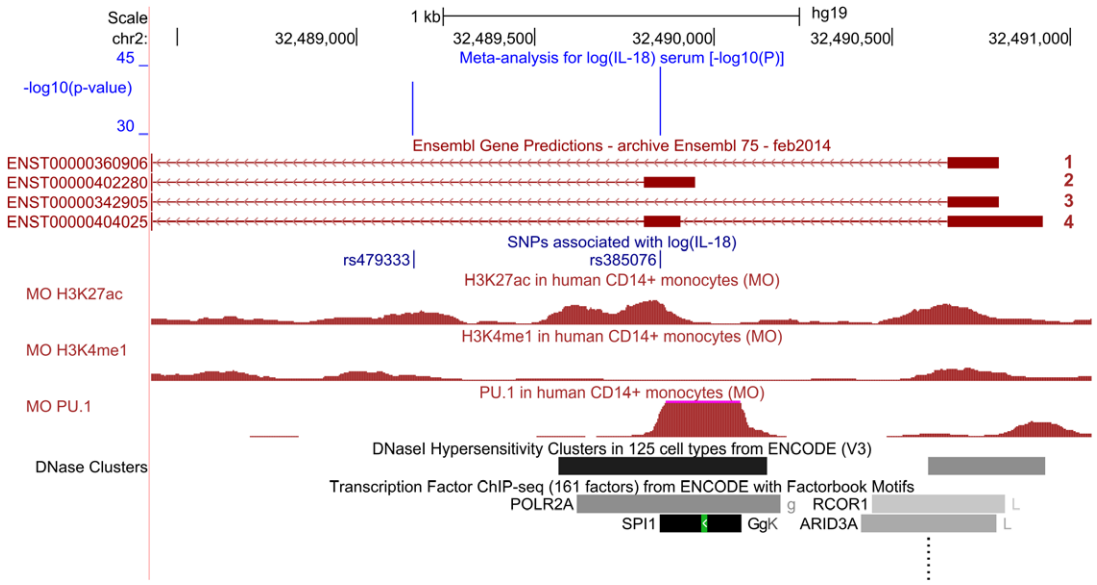

Figure 3. In silico functional analyses for rs385076 within the *NLRC4* gene region. Colocalization of interleukin (IL)-18–associated variants and regulatory and epigenetic features were investigated in the UCSC Genome Browser (http://www.genome-euro.ucsc.edu) using genome release hg19. Only single nucleotide polymorphisms (SNPs) with $P < 5\times10^{-8}$ for the association with circulating IL-18 levels are shown (green track). Tracks showing histone modifications and PU.1 binding sites were uploaded into the UCSC Genome Browser from an external study[22] (red tracks). The PU.1 track represents binding sites of the transcription factor PU.1 in monocytes (MO) from a CHIP-seq experiment. The H3K27ac track shows histone modification by acetylation from CHIP-seq experiments in monocytes, which can be considered as a mark for active enhancers.[24] The H3K4me1 mark shows regions undergoing histone methylation, which are likely to reflect cell-type–specific regulation of a gene.[25] Enhancer regions are highlighted by DNaseI hypersensitivity clusters from the ENCODE project.[21] In the bottom track, CHIP-seq results for transcription factors (TF) from the ENCODE project are shown, indicating TF binding regions. rs385076 falls into the 5′ untranslated region exon of *NLRC4* and a binding region of PU.1. DNase hypersensitivity clusters around rs385076 suggest a good DNA accessibility in monocytes and histone modification data highlights the region as an enhancer. ChIP-seq indicates chromatin immunoprecipitation DNA-sequencing.

decreased *NLRC4* mRNA expression in monocytes. Evidence for expression quantitative trait loci was corroborated in silico in 2 publicly available studies using lymphoblastoid cell lines.[26] In monocytes, we observed decreased expression of *NLRC4* isoforms containing the 5′UTR exon 2 in carriers of rs385076 T alleles (isoforms 2 and 4) and a switch in isoform usage toward those without exon 2 (isoforms 1 and 3). We detected an association between isoforms without the 5′UTR exon 2 and decreased circulating IL-18, but it still needs to be investigated in more detail, whether an enhanced expression of these isoforms leads to reduced activation of IL-18. When compared with monocytes, our analyses in whole blood showed no significant association between rs385076 and *NLRC4* gene expression. Because the inflammasome is mainly active in the innate immune system and is thus more prominent in monocytes, monocyte-specific gene expression might be narrowed by other cell types present in whole blood.

In addition to an influence on PU.1 binding, ENCODE data suggest characteristics for an open chromatin region (hypersensitivity cluster) and a binding site for the polymerase 2 (Pol2) around the region of rs385076. Also, phosphorylation of the NLRC4 protein on a specific serine residue also has been described to play a critical role, possibly by driving conformational changes necessary for inflammasome activation.[29] In our data, SNPs located near this serine residue in the exon 5 also showed a strong association with circulating IL-18 levels (rs408813, $P=2.5\times10^{-40}$ and rs455060, $P=5.04\times10^{-34}$). Epigenetic modifications mirrored by methylation status showed no statistically significant relation of NLRC4 with IL-18 levels in our data. However, methylation analysis identified an additional inflammasome-linked gene, *NLRC5*, known to play a role in the regulation of IL-18 levels.[30,31] Thus, further experimental work is required to assess the relation between regulatory elements, inflammasome orchestration, and IL-18 levels. Our results of the molecular analyses indicate a decreased *NLRC4* gene expression and a switched isoform usage for the rs385076 T allele, putatively modulated by differential PU.1 binding. Because the NLRC4 inflammasome plays a role in IL-18 activation, our observations are consistent with the finding that rs385076 T alleles lead to lower circulating IL-18 concentrations.

Genetic Variation and Cardiovascular Mortality

Because it had previously been shown that circulating IL-18 levels are a marker of cardiovascular mortality in coronary heart disease,[5,32,33] we evaluated the association of SNPs rs385076 (*NLRC4*) and rs11606049 (*IL18*) with incident fatal cardiovascular events in 2585 patients with coronary heart disease in the Athero*Gene* study. The IL-18–lowering allele of rs385076 was associated with a lower risk of cardiovascular mortality (odds ratio, 0.78; 95% confidence interval, 0.62–0.98; P=0.03). This observation of an association of the genotype with IL-18 concentrations and potentially clinically relevant outcomes could support a causal relationship in disease evolution. Although variations of the *IL18* gene had previously been shown to influence circulating IL-18 levels and clinical outcome in patients with coronary artery disease,[7] our study did not show an association of our lead *IL-18* SNP rs11606049 with cardiovascular

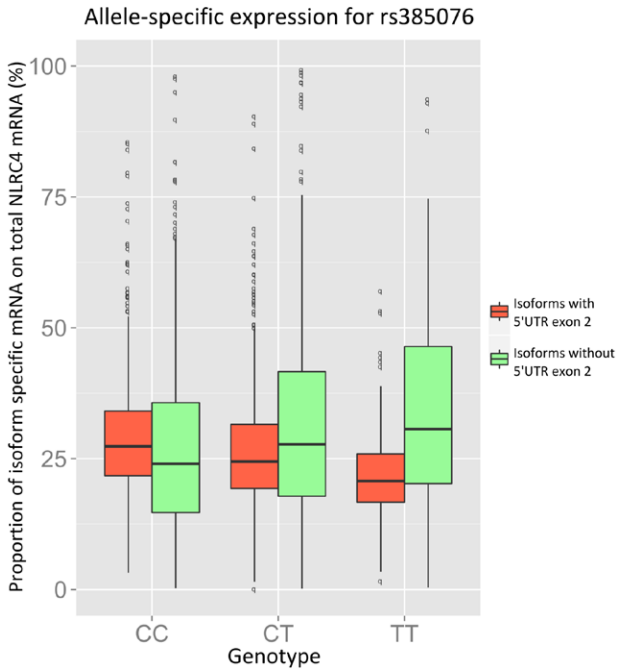

Figure 4. Allele-specific *NLRC4* isoform expression in relation to the rs385076 genotype. Box plots show the distribution of different isoform usage, which is defined as proportion of isoform expression on overall *NLRC4* gene expression, for each allele of rs385076. All transcripts were quantified by quantitative polymerase chain reaction and normalized for Ct values of the housekeeping gene GAPDH. Isoforms including 5′ untranslated region (UTR) exon (isoforms 2 and 4) are marked red, others (isoforms 1 and 3) are marked green. We can observe a switch in isoform usage with a higher number of T alleles leading to a higher proportion of transcripts, which do not include 5′UTR exon.

mortality. Because the inflammasome is a central component to inflammatory activity and immune response, our findings may be applicable beyond the cardiovascular system to a broad range of inflammatory and autoimmune diseases[3] and diabetes mellitus.[4] The detailed work-up of the pathophysiological pathways involved in the genetic and post-transcriptional *NLRC4* regulation will show whether causal relations with disease can be identified. Such work may reveal risk indicators and, more importantly, potential therapeutic targets.

Strengths and Limitations

The present study applies a combination of genetic, molecular, and methylation approaches to gain insights into the regulation of circulating IL-18 levels in large, well-characterized studies. Nevertheless, it is important to address some limitations. The present data are restricted to individuals of European descent and associations may be different in other ethnicities. The question, whether NLRC4 protein levels associate with *NLRC4* gene expression in relation to the SNP rs385076, still remains to be answered. We were not able to show a strong relation of SNP rs385076 with NLRC4 protein levels in peripheral blood mononuclear cells in 200 individuals from the community-based Gutenberg Health Study (data not shown). This null finding could be because of the low number of samples investigated in rather healthy individuals. Furthermore, the NLRC4 protein was measured in peripheral blood mononuclear cells, whereas an effect of rs385076 on *NLRC4* gene expression was observed in monocytes. Further in-depth experimental studies and sequencing approaches are needed to better understand the implications of these newly identified genetic variants in relation to the exact regulation of inflammatory and autoimmune pathways. Finally, DNA methylation data were only available from whole blood. Here, we did not see a significant association of CpG sites with *NLRC4*. To assess whether *NLRC4* expression in relation to IL-18 might be influenced by methylation in a cell-type–specific manner, additional methylation analyses, for example, in monocytes, need to be performed.

In summary, using multiple molecular and genetic approaches, we confirmed and extended previous knowledge in relation to circulating IL-18 levels within *NLRC4*. Genetic variants in *NLRC4* affect binding of the transcription factor PU.1, suggesting thereby a mediation of the influence on circulating IL-18 levels. The clinical importance of our results is underlined by an association between rs385076 T allele and a lower risk of cardiovascular mortality. The exact mechanisms in relation to cardiovascular disease and other inflammatory and autoimmune diseases need to be established and should stimulate further epidemiological and experimental studies.

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Table 3. Allele-Specific *NLRC4* Isoform Expression in Relation to the rs385076 Genotype

			Association Between mRNA (deltaCt) and rs385076 T Alleles				Association Between mRNA (deltaCt) and log(IL-18)			
Isoforms	Description	deltaCt	β	SE	*P* Value	n	β	SE	*P* Value	n
2 and 4	Including exon 2	5.1±1.4	0.305	0.047	2.0×10^{-10}	1175	0.009	0.008	0.254	1459
1 and 3	Excluding exon 2	5.2±0.8	−0.103	0.030	7.4×10^{-4}	1171	0.027	0.014	0.049	1438
1, 2, 3, and 4	Total *NLRC4* mRNA	3.2±1.3	0.134	0.050	6.9×10^{-3}	1180	0.014	0.008	0.092	1467

Expression levels of mRNA are represented as deltaCt values (deltaCt(transcript)=Ct(transcript)−Ct(*GAPDH*)), which are normalized for the housekeeping gene *GAPDH*. β estimates were calculated in a linear mixed-effect model and refer to the change in deltaCt for each T allele of rs385076 and the change in log(IL-18), respectively. Therefore, a negative β implies an increase in gene expression. n refers to the number of individuals with nonmissing values used for a test.

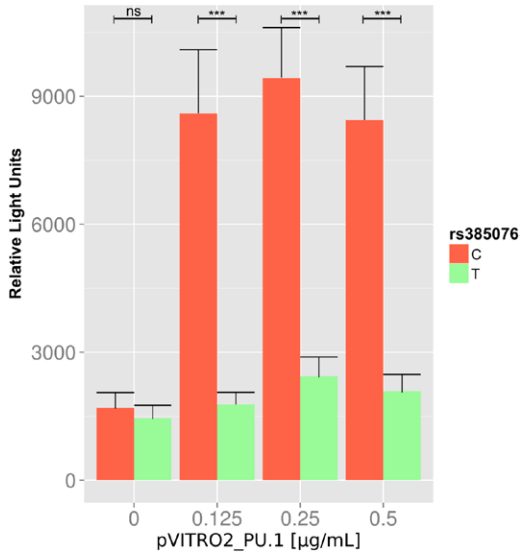

Figure 5. PU.1 overexpression increases *NLRC4* gene expression in relation to the rs385076 C allele. HEK293A cells were transfected with pGL4.10[luc2] plasmid, containing either rs385076 C (shown in red) or T allele (shown in green) and the putative PU.1 binding site, as well as the pVITRO2-PU.1 plasmid. Expression of yellow fluorescent protein after transfection of an empty pEYFP-N1 plasmid was used to control for transfection efficiency. PU.1 overexpression significantly increased luciferase activity of the rs385076 C allele but not the T allele in the plasmids. Without PU.1 overexpression, there was no significant difference between C and T alleles. Cells with 0.125 µg/mL (4.24-fold, $P=3\times10^{-4}$), 0.25 µg/mL (3.34-fold, $P=3\times10^{-4}$), and 0.5 µg/mL (3.5-fold, $P=5\times10^{-4}$) of the pVITRO2-PU.1 plasmid showed significant increases of luciferase activity for the C allele when compared with that for the T allele. ns indicates not significant, ***$P<0.001$; n=4.

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Disclosures

None.

Appendix

From the Clinic for General and Interventional Cardiology, University Heart Center Hamburg, Hamburg (T.Z., T.H., C.M., D.L., S.Z., J.K., S.B., R.B.S.); German Center for Cardiovascular Research (DZHK e.V.), partner site Hamburg, Lübeck, Kiel, Germany (T.Z., T.H., C.M., S.B., R.B.S.); Institute of Epidemiology II (H.R., J.B., M.W., S.Z., A.P., C.M., B.T.), Research Unit of Molecular Epidemiology (M.W., S.Z., C.M.), Institute of Genetic Epidemiology (K.S.), Institute of Human Genetics, (H.P.), Helmholtz Zentrum München, Neuherberg, Germany; Department of Internal Medicine II–Cardiology, University of Ulm Medical Center, Ulm, Germany (H.R., W.K.); Fraunhofer IME ScreeningPort, Hamburg, Germany (O.P.); National Heart, Lung, and Blood Institute's and Boston University's Framingham Heart Study, Framingham, MA (J.D., E.J.B.); Department of Biostatistics (J.D.) and Department of Epidemiology (E.J.B.), Boston University School of Public Health, MA; Cardiology Section, Preventive Medicine Section, Evans Memorial Medicine Department, Whitaker Cardiovascular Institute, School of Medicine, Boston University, MA (E.J.B.); Center for Thrombosis and Hemostasis, Preventive Cardiology and Preventive Medicine, Department of Medicine II (P.S.W.), Department of Medicine II (M.E., T.M.), Institute for Clinical Chemistry and Laboratory Medicine (K.J.L.), University Medical Center, Mainz, Germany; German Center for Cardiovascular Research (DZHK e.V.), partner site Rhine Main, Mainz, Germany (P.S.W., T.M.); Institute of Medical Biometry and Statistics, University Hospital Schleswig-Holstein, Lübeck, Germany (A.Z.); Center for Clinical Trials, University of Lübeck, Lübeck, Germany (A.Z.); Sorbonne Universités, Université Pierre et Marie CURIE Univ Paris 06, Paris, France (L.T., C.P.); INSERM, UMR_S 1166, Team Genomics and Pathophysiology of Cardiovascular Diseases, Paris, France (L.T., C.P.); ICAN Institute for Cardiometabolism and Nutrition, Paris, France (L.T., C.P.); Institute of Medical Informatics, Biometry and Epidemiology, Chair of Genetic Epidemiology, Ludwig-Maximilians-Universität, München, Germany (K.S.); Institute of Human Genetics (H.P.), Deutsches Herzzentrum (W.K.), Technische Universität München, München, Germany; Institute for Clinical Diabetology, German Diabetes Center, Leibniz Center for Diabetes Research at Heinrich Heine University Düsseldorf, Düsseldorf, Germany (C.H.); German Center for Diabetes Research (DZD e.V.), partner site Düsseldorf, Düsseldorf (C.H.); partner site Munich, Germany (C.M.); and German Center for Cardiovascular Research (DZHK e.V.), partner site Munich Heart Alliance, Munich, Germany (W.K.).

References

1. Okamura H, Tsutsi H, Komatsu T, Yutsudo M, Hakura A, Tanimoto T, et al. Cloning of a new cytokine that induces IFN-gamma production by T cells. *Nature*. 1995;378:88–91. doi: 10.1038/378088a0.
2. Dinarello CA, Fantuzzi G. Interleukin-18 and host defense against infection. *J Infect Dis*. 2003;187(suppl 2):S370–S384. doi: 10.1086/374751.
3. Mühl H, Pfeilschifter J. Interleukin-18 bioactivity: a novel target for immunopharmacological anti-inflammatory intervention. *Eur J Pharmacol*. 2004;500:63–71. doi: 10.1016/j.ejphar.2004.07.012.
4. Thorand B, Kolb H, Baumert J, Koenig W, Chambless L, Meisinger C, et al. Elevated levels of interleukin-18 predict the development of type 2

diabetes: results from the MONICA/KORA Augsburg Study, 1984-2002. *Diabetes*. 2005;54:2932–2938.
5. Blankenberg S, Tiret L, Bickel C, Peetz D, Cambien F, Meyer J, et al; AtheroGene Investigators. Interleukin-18 is a strong predictor of cardiovascular death in stable and unstable angina. *Circulation*. 2002;106:24–30.
6. Blankenberg S, Luc G, Ducimetière P, Arveiler D, Ferrières J, Amouyel P, et al; PRIME Study Group. Interleukin-18 and the risk of coronary heart disease in European men: the Prospective Epidemiological Study of Myocardial Infarction (PRIME). *Circulation*. 2003;108:2453–2459. doi: 10.1161/01.CIR.0000099509.76044.A2.
7. Tiret L, Godefroy T, Lubos E, Nicaud V, Tregouet DA, Barbaux S, et al; AtheroGene Investigators. Genetic analysis of the interleukin-18 system highlights the role of the interleukin-18 gene in cardiovascular disease. *Circulation*. 2005;112:643–650. doi: 10.1161/CIRCULATIONAHA.104.519702.
8. Grisoni ML, Proust C, Alanne M, Desuremain M, Salomaa V, Kuulasmaa K, et al. Lack of association between polymorphisms of the IL18R1 and IL18RAP genes and cardiovascular risk: the MORGAM Project. *BMC Med Genet*. 2009;10:44. doi: 10.1186/1471-2350-10-44.
9. He M, Cornelis MC, Kraft P, van Dam RM, Sun Q, Laurie CC, et al. Genome-wide association study identifies variants at the IL18-BCO2 locus associated with interleukin-18 levels. *Arterioscler Thromb Vasc Biol*. 2010;30:885–890. doi: 10.1161/ATVBAHA.109.199422.
10. Matteini AM, Li J, Lange EM, Tanaka T, Lange LA, Tracy RP, et al. Novel gene variants predict serum levels of the cytokines IL-18 and IL-1ra in older adults. *Cytokine*. 2014;65:10–16. doi: 10.1016/j.cyto.2013.10.002.
11. Lamkanfi M, Dixit VM. Inflammasomes and their roles in health and disease. *Annu Rev Cell Dev Biol*. 2012;28:137–161. doi: 10.1146/annurev-cellbio-101011-155745.
12. Leavy O. Inflammasome: NAIPs: pathogen-sensing proteins. *Nat Rev Immunol*. 2011;11:644. doi: 10.1038/nri3069.
13. Abdul-Sater AA, Saïd-Sadier N, Ojcius DM, Yilmaz O, Kelly KA. Inflammasomes bridge signaling between pathogen identification and the immune response. *Drugs Today (Barc)*. 2009;45(suppl B):105–112.
14. Strowig T, Henao-Mejia J, Elinav E, Flavell R. Inflammasomes in health and disease. *Nature*. 2012;481:278–286. doi: 10.1038/nature10759.
15. Johansson Å, Eriksson N, Becker RC, Storey RF, Himmelmann A, Hagström E, et al; PLATO Investigators. NLRC4 Inflammasome Is an Important Regulator of Interleukin-18 Levels in Patients With Acute Coronary Syndromes: Genome-Wide Association Study in the PLATelet inhibition and patient Outcomes Trial (PLATO). *Circ Cardiovasc Genet*. 2015;8:498–506. doi: 10.1161/CIRCGENETICS.114.000724.
16. Willer CJ, Li Y, Abecasis GR. METAL: fast and efficient meta-analysis of genomewide association scans. *Bioinformatics*. 2010;26:2190–2191. doi: 10.1093/bioinformatics/btq340.
17. Zeller T, Wild P, Szymczak S, Rotival M, Schillert A, Castagne R, et al. Genetics and beyond–the transcriptome of human monocytes and disease susceptibility. *PLoS One*. 2010;5:e10693. doi: 10.1371/journal.pone.0010693.
18. Schurmann C, Heim K, Schillert A, Blankenberg S, Carstensen M, Dörr M, et al. Analyzing illumina gene expression microarray data from different tissues: methodological aspects of data analysis in the metaxpress consortium. *PLoS One*. 2012;7:e50938. doi: 10.1371/journal.pone.0050938.
19. Johnson AD, Handsaker RE, Pulit SL, Nizzari MM, O'Donnell CJ, de Bakker PI. SNAP: a web-based tool for identification and annotation of proxy SNPs using HapMap. *Bioinformatics*. 2008;24:2938–2939. doi: 10.1093/bioinformatics/btn564.
20. Barbaux S, Poirier O, Godefroy T, Kleinert H, Blankenberg S, Cambien F, et al. Differential haplotypic expression of the interleukin-18 gene. *Eur J Hum Genet*. 2007;15:856–863. doi: 10.1038/sj.ejhg.5201842.
21. Consortium EP, Bernstein BE, Birney E, Dunham I, Green ED, Gunter C, et al. An integrated encyclopedia of DNA elements in the human genome. *Nature*. 2012;489:57–74.
22. Pham TH, Benner C, Lichtinger M, Schwarzfischer L, Hu Y, Andreesen R, et al. Dynamic epigenetic enhancer signatures reveal key transcription factors associated with monocytic differentiation states. *Blood*. 2012;119:e161–e171. doi: 10.1182/blood-2012-01-402453.
23. Kent WJ, Sugnet CW, Furey TS, Roskin KM, Pringle TH, Zahler AM, et al. The human genome browser at UCSC. *Genome Res*. 2002;12:996–1006. doi: 10.1101/gr.229102. Article published online before print in May 2002.
24. Creyghton MP, Cheng AW, Welstead GG, Kooistra T, Carey BW, Steine EJ, et al. Histone H3K27ac separates active from poised enhancers and predicts developmental state. *Proc Natl Acad Sci U S A*. 2010;107:21931–21936. doi: 10.1073/pnas.1016071107.
25. Tian Y, Jia Z, Wang J, Huang Z, Tang J, Zheng Y, et al. Global mapping of H3K4me1 and H3K4me3 reveals the chromatin state-based cell type-specific gene regulation in human Treg cells. *PLoS One*. 2011;6:e27770. doi: 10.1371/journal.pone.0027770.
26. Stranger BE, Nica AC, Forrest MS, Dimas A, Bird CP, Beazley C, et al. Population genomics of human gene expression. *Nat Genet*. 2007;39:1217–1224. doi: 10.1038/ng2142.
27. Lappalainen T, Sammeth M, Friedländer MR, 't Hoen PA, Monlong J, Rivas MA, et al; Geuvadis Consortium. Transcriptome and genome sequencing uncovers functional variation in humans. *Nature*. 2013;501:506–511. doi: 10.1038/nature12531.
28. Kaptoge S, Seshasai SR, Gao P, Freitag DF, Butterworth AS, Borglykke A, et al. Inflammatory cytokines and risk of coronary heart disease: new prospective study and updated meta-analysis. *Eur Heart J*. 2014;35:578–589. doi: 10.1093/eurheartj/eht367.
29. Qu Y, Misaghi S, Izrael-Tomasevic A, Newton K, Gilmour LL, Lamkanfi M, et al. Phosphorylation of NLRC4 is critical for inflammasome activation. *Nature*. 2012;490:539–542. doi: 10.1038/nature11429.
30. Kobayashi KS, van den Elsen PJ. NLRC5: a key regulator of MHC class I-dependent immune responses. *Nat Rev Immunol*. 2012;12:813–820. doi: 10.1038/nri3339.
31. Kofoed EM, Vance RE. Innate immune recognition of bacterial ligands by NAIPs determines inflammasome specificity. *Nature*. 2011;477:592–595. doi: 10.1038/nature10394.
32. Espinola-Klein C, Rupprecht HJ, Bickel C, Lackner K, Schnabel R, Munzel T, et al; AtheroGene Investigators. Inflammation, atherosclerotic burden and cardiovascular prognosis. *Atherosclerosis*. 2007;195:e126–e134. doi: 10.1016/j.atherosclerosis.2007.01.021.
33. Mallat Z, Corbaz A, Scoazec A, Besnard S, Lesèche G, Chvatchko Y, et al. Expression of interleukin-18 in human atherosclerotic plaques and relation to plaque instability. *Circulation*. 2001;104:1598–1603.

CLINICAL PERSPECTIVE

Interleukin-18 (IL-18) has been related to cardiovascular disease pathogenesis and outcome. Genetic determinants of circulating IL-18 are less well established. Our genome-wide association study in community cohorts identified loci in the *IL18* gene and the untranslated exon 2 of the *NLRC4* (NLR family, caspase recruitment domain–containing 4) gene on chromosome 2. NLRC4 is a central component of the inflammasome, a multimeric protein complex initiating immune responses. Functional data of the region around the top single nucleotide polymorphism (rs385076) within the *NLRC4* 5′ untranslated region /exon 2 locus indicate the presence of a binding region of the transcription factor PU.1. Our molecular analyses revealed a reduced PU.1 binding to the *NLRC4* 5′ untranslated region and a decreased *NLRC4* mRNA expression related to the IL-18–lowering allele. Altered binding of the transcription factor may help explain the association with circulating IL-18 concentrations. Clinically, the IL-18–lowering allele of rs385076 was associated with a lower risk of cardiovascular mortality in individuals with manifest coronary artery disease. Overall, our results provide evidence for a causal relationship between the genetic variant at the *NLRC4* 5′ untranslated region/exon 2 locus, IL-18 concentrations, and clinical outcome. A better understanding of the determinants of the inflammasome and the regulation of circulating IL-18 concentrations may shed light on the pathophysiology of atherosclerosis and other chronic inflammatory diseases. Our results offer pathways that need further evaluation for the identification of possible targets to modulate the IL-18 system in a broad range of inflammatory and autoimmune diseases.

SUPPLEMENTAL MATERIAL

Supplemental Methods

Cohort descriptions

Gutenberg Health Study (GHS)

Individuals of the Rhine-Main area in Germany were enrolled in the ongoing GHS, a community-based, prospective, observational single-center cohort study as described earlier (1, 2). The sample was stratified 1:1 by sex and residence (urban and rural) and in equal strata for decades of age. Individuals between 35 and 74 years of age were enrolled, and written, informed consent was obtained from all participants. Exclusion criteria were insufficient knowledge of German language and physical or psychological inability to participate in the examinations at the study centre. The study protocol and sampling design were approved by the local ethics committee and by the local and federal data safety commissioners. Baseline examination of 15,000 study participants was performed between 2007 and 2012. Genome-wide genotyping was performed in two successive rounds: cohort GHS I (n = 3,500; first GWA subsample performed in 2008-2009) and cohort GHS II (n =1,500; an independent subsample with GWA performed in 2010). Genome-wide association (GWA) data on IL-18 concentrations were available in 2,743 individuals of GHS I and 1,073 individual of GHS II.

Framingham Heart Study (FHS)

The FHS, an observational community-based cohort study, was implemented in 1948 to assess risk factors for cardiovascular disease (3). Offspring of the Original cohort and their spouses were invited for participation in 1971 and seen for clinic visits every four to eight years. GWAS data on IL-18 concentrations were available in 2,940 individuals from examination cycle 7 of the

Framingham Offspring cohort.

Cooperative Health Research in the Region of Augsburg (KORA F3 and KORA F4) Study

The KORA Study consisted of a series of independent population-based epidemiological surveys of participants living in the region of Augsburg, Southern Germany (4). All survey participants are residents of German nationality identified through the registration office. The study followed the recommendations of the Declaration of Helsinki and was approved by the local ethical committees. All participants underwent standardized examinations including blood withdrawals for plasma and DNA (5). GWAS data on IL-18 concentrations were available in 2,806 individuals from the KORA F4 study conducted between 2006 and 2008. For replication of the methylation discovery findings, a subsample of the KORA F3 study conducted in 2004/05 was used. Initially, a number of 250 current smokers were randomly chosen and matched by sex and age classes (with five-year range) with 250 non-smokers. After excluding 12 participants with missing information on phenotype or methylation data, a number of 488 participants were included in the replication analyses.

Athero*Gene*

The Athero*Gene* study is a cohort of patients with documented coronary heart disease (CHD) enrolled at the Johannes Gutenberg University, Mainz, Germany and Bundeswehrzentralkrankenhaus, Koblenz, Germany who have been followed up for cardiovascular death by questionnaire and telephone interview over a median of 4.9 (maximum 7.6) years (6). Participants with information on IL-18 concentrations and DNA were used to assess the association with IL-18 levels in 1,165 participants as replication, and with cardiovascular death in 2,585 participants.

Monitoring of Trends and Determinants in Cardiovascular Diseases (MONICA)/KORA S1/S2/S3

Within MONICA/KORA a case-cohort study was performed using surveys S1-S3 conducted between 1984 to 1995 to assess associations between inflammation-related biomarkers, genotypes and incident CHD disease outcomes, denoted as MONICA/KORA S1/S2/S3 in the present analysis (7). For this study, samples including participants with information on IL-18 concentrations and DNA were used to assess the association with IL-18 levels in 1,743 participants as replication and with incident CHD events in 1,945 participants from the complete case-cohort study (n=307 cases) with a mean follow-up of 10.5 years. In MONICA/KORA S1/S2/S3, CHD was defined as incident fatal or non-fatal myocardial infarction or sudden cardiac death occurring before the age of 75 years and was identified through the MONICA/KORA Augsburg coronary event registry and through follow-up questionnaires for subjects who had moved out of the study area. For deceased subjects information on causes of death was obtained from local Health Departments.

Prospective Epidemiological Study of Myocardial Infarction (PRIME)

The PRIME study is a European prospective cohort constituted of men aged 49-60 years at enrolment (1991-94) and prospectively followed up during 10 years to assess cardiovascular events (8). Analysis was performed using a nested case/cohort design (568 cases with an acute coronary event / 572 age-matched controls) of whom 518 had IL-18 measurements.

The study participant characteristics of all cohorts are given in Table S1.

Genotyping and quality control

GHS

In GHS, genotyping and quality control has been described (1). Genomic DNA was extracted

from buffy coats prepared from EDTA blood samples. Genotyping was performed using the Affymetrix Genome-Wide Human SNP Array 6.0, as described by the Affymetrix user manual. Genotypes were called using the Affymetrix Birdseed-V2 calling algorithm and quality control was performed using GenABEL (http://mga.bionet.nsc.ru/nlru/GenABEL/). Because genotyping was performed in two successive rounds (cohort GHS I (n = 3,500) and cohort GHS II (n =1,500)), the two cohorts were analyzed separately. Individuals with a call rate below 97% or an autosomal heterozygosity higher than 3 SD around the mean were excluded. After applying standard quality criteria (minor allele frequency 1%, genotype call rate 98% and P value for deviation from Hardy-Weinberg equilibrium), 662,405 SNPs in 2996 subjects (GHS I) and 673,914 SNPs in 1179 subjects (GHS II), respectively, remained for analysis. In GHS, multidimensional scaling was used prior to GWAS to detect population stratification. Outliers were identified based on comparison of the first two components and were excluded from further analysis. Imputations based on 1000 Genomes Integrated Phase 1 integrated release version 3 were performed separately in GHS I and GHS II using IMPUTE v2.1.0.

KORA F4

In KORA F4, all samples were genotyped with the Affymetrix Human SNP Array 6.0. Hybridization of genomic DNA was done in accordance with the manufacturer's standard recommendations. Genotypes were determined using Birdseed2 clustering algorithm (Affymetrix Array 6.0). For quality control purposes, we applied a positive control and a negative control DNA every 96 samples. On chip level only subjects with overall genotyping efficiencies of at least 93% were included. In addition the called sex had to agree with the sex in the KORA study database. Imputation of genotypes was performed using maximum likelihood method with the software MACH v1.0.15. Association analyses were performed using PROBABEL.

FHS

In FHS, all samples were genotyped with the Affymetrix GeneChip Human Mapping 500K Array Set and the 50K Human Gene Focused Panel. FHS tested for population stratification using the first 10 principal components of IL-18 concentrations in relation to the genotypes using EIGENSTRAT (http://www.broad.mit.edu/tools/software.html) (9). Imputations of genotypes were performed with the software MACH v1.0.16 based on 1000G Phase 1 integrated release version 3.

An overview about the genotyping and quality control characteristics is provided in Table S2.

Measurement of DNA methylation

Array-based DNA methylation measurements

Genome-wide methylation was assessed using the Illumina HumanMethylation450 BeadChip in a subgroup of the KORA F4 cohort (n=1,802), following the Illumina Infinium HD Methylation protocol. Briefly, this included a whole genome amplification step using 4 µl of each bisulfite converted sample, followed by enzymatic fragmentation and application of the samples to BeadChips (Illumina). The arrays were fluorescently stained and scanned with the Illumina HiScan SQ scanner. Details about data processing and quality control have been described in Zeilinger *et al*. . The percentage of methylation of a given locus is reported as a ß-value, which is a continuous variable between 0 and 1. The ß-value corresponds to the ratio of the methylated signal over the sum of the methylated and unmethylated signals. Association analyses were performed using R 3.0.2.

Statistical analysis of methylation data

IL-18 levels were log-transformed and observations deviating more than three standard

deviations from the mean were excluded. Individuals with acute inflammation, based on CRP levels >10 mg/L, were excluded from the analysis. To allow for a more straightforward interpretation, ß-values, defined as the ratio of the methylated signal intensity divided by the overall signal intensity (10), were used in all analyses. Associations between log-transformed IL-18 levels and methylation beta-values in KORA F4 were assessed using multivariable linear regression. IL-18 served as the outcome variable and was regressed on each methylation site, adjusting for age, sex, smoking status, body mass index (BMI) and diabetes. The Bonferroni method was used to correct for multiple comparisons, yielding a global significance level of $P=1x10^{-07}$ (0.05/450.000 CpG sites). For methylation sites with a significant p-value in the initial analysis, random effects models were used to check for possible plate effects. All analyses were performed using R 3.0.2 (http://www.R-project.org/.)

Molecular and functional analyses

In silico analyses using ENCODE data

To determine putative functional effects of the SNPs and CpG sites we checked whether these fall into regulatory regions indicated by DNase hypersensitivity clusters, CHIP-seq validated transcription factor binding sites, histone acetylations and methylations from the Encyclopedia of DNA Elements (ENCODE) project (11) using the UCSC Genome Browser (12). We additionally added a data set containing putative PU.1 binding sites, H3K27 acetylation and H3K4me1 from publicly available CHIPseq data ((13), http://www.ag-rehli.de/NGSdata.htm) to highlight monocyte specific epigenetic features.

Confirmation of PU.1 overexpression

HEK293A cells were transfected with 0 µg/mL, 0.125 µg/mL, 0.25 µg/mL or 0.5 µg/mL pVITRO2-PU.1 using 2 µL/mL Lipofectamine2000 (Life Technologies). *PU.1* mRNA

expression was determined via real-time qPCR using a 7900 TaqMan system (Applied Biosystems). Twenty-four hours after transfection, RNA was purified using RNeasy Mini Kit (Qiagen) and reverse transcribed using High-Capacity cDNA Reverse Transcription Kit (Life Technologies) according to manufacturers' protocols. Real-time PCR was performed in a 10 µl reaction volume using 5 µL TaqMan® Gene Expression Master Mix (Life Technologies), 5 ng cDNA and 0.5 µL of the PU.1 and 18S gene expression assay (Hs02786711_m1 and Hs99999901_s1, Life Technologies), respectively. *PU.1* mRNA expression was normalized to 18S as an endogenous control and normalized to basal *PU.1* expression in cells transfected with 0 µg/mL pVITRO2-PU.1using the formula 2-ΔΔCt. PU.1 protein expression was determined via western blot. Twenty-four hours after transfection, cells were lysed with Cell Lysis Buffer (Cell Signaling Technology) and boiled for 5 min in SDS sample buffer. Samples were separated by 12 % SDS-PAGE, transferred to nitrocellulose membranes and blocked with 5 % BSA. Membranes were incubated with rabbit anti-PU.1 (2266, Cell Signaling Technology) and rabbit anti-α-actinin (3134, Cell Signaling Technology) bands were detected via chemiluminescence. Specific signals were detected via chemiluminescence.

Acknowledgment

GHS

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PRIME

The PRIME Study is organized under an agreement between INSERM and the Merck, Sharpe and Dohme-Chibret Laboratory, with the following participating laboratories: The Strasbourg MONICA Project, Department of Epidemiology and Public Health, Faculty of Medicine, Strasbourg, France (D. Arveiler, B. Haas); The Toulouse MONICA Project, INSERM U558, Departmentof Epidemiology, Paul Sabatier-Toulouse Purpan University, Toulouse, France (J.

Ferrières, J. B. Ruidavets); The Lille MONICA Project, INSERM U508, Pasteur Institute, Lille, France (P. Amouyel, M. Montaye); The Department of Epidemiology and Public Health, Queen's University Belfast, Northern Ireland (A. Evans, J. Yarnell, F. Kee); The Department of Atherosclerosis, INSERM UR545, Lille, France (G. Luc, J.M. Bard); The Laboratory of Haematology, La Timone Hospital, Marseilles, France (I. Juhan-Vague); The Laboratory of Endocrinology, INSERM U326, Toulouse, France (B. Perret); The Vitamin Research Unit, The University of Bern, Bern, Switzerland (F. Gey); The Trace Element Laboratory, Department of Medicine, Queen's University Belfast, Northern Ireland (J. Woodside, I. Young); The DNA Bank, INSERM U937, Paris, France (F. Cambien); The Coordinating Center, INSERM U258, Paris-Villejuif, France (P. Ducimetière, A. Bingham). The following organizations allowed the enrollment of the PRIME subjects: the Health screening centers organized by the Social Security of Lille (Institut Pasteur), Strasbourg, Toulouse and Tourcoing; Occupational Medicine Services of Haute-Garonne and of the Urban Community of Strasbourg; the association Inter-entreprises des Services Médicaux du Travail de Lille et environs; the Comité pour le Développement de la Médecine du Travail; the Mutuelle Générale des PTT du Bas-Rhin; the Laboratoire d'Analyses de l'Institut de Chimie Biologique de la Faculté de Médecine de Strasbourg; the Department of Health (NI) and the Northern Ireland Chest Heart and Stroke Association.

Supplemental Table 1: Characteristics of study participants in the GWAS discovery and replication

	Discovery				**Replication**		
	GHS I	**GHS II**	**FHS**	**KORA F4**	**AtheroGene**	**PRIME**	**MONICA/KORA S1/S2/S3**
N	2,743	1,073	2,940	1,802	2,585†	1,140†	1,743†
Age, years	56.1±10.8	55.5±10.8	61.1±9.5	60.9±8.9	61.6 ± 9.8	55.2 ± 2.9	52.7 ± 10.6
Women, %	46.4	47.9	53.6	51.3	22.4	0	51.0
Body mass index, kg/m^2	27.3±4.7	27.5±5.0	28.1±5.3	28.2±4.8	27.5 ± 3.8	26.9 ± 3.4	27.2 ± 4.1
Interleukin-18, pg/mL							
Median ($25^{th}/75^{th}$ percentile)	227 (181/296)	224 (180/284)	235 (174/313)	299 (231/391)	298 (225/394)	205 (154/285)	177 (118/251)
Mean $\log_e$(IL-18) ± SD	5.46±0.36	5.44±0.35	5.48±0.41	5.71±0.42	5.71 ± 0.42	5.35 ± 0.51	5.09 ± 0.74*

Numbers are presented as mean±SD for continuous, or percent for categorical variables. †Individuals with IL-18: N = 1,165 in Athero*Gene*, N = 518 in PRIME, N = 1,743 in MONICA/KORA S1/S2/S3 case-cohort

Supplemental Table 2: Genotyping and quality control characteristics of the discovery cohorts

	GHS I	GHS II	KORA F4	FHS
Array type	Affymetrix 6.0	Affymetrix 6.0	Affymetrix 6.0	Affymetrix 500K MIPS 50K
Genotype calling	Birdseed	Birdseed		BRLMM
QC Filter for genotyped SNPs used for imputation	call rate < 95%, MAF <1%, pHWE<10^{-4}	call rate < 95%, MAF <1%, pHWE<10^{-4}	callrate < 98% , pHWE ≥ 0.000005 , MAF ≥ 1%	call rate <97%, MAF <1%, pHWE<10^{-6}
No. of SNPs used for imputation	662.405	673.914	508,532	411,643
Prephasing	SHAPEIT	SHAPEIT	SHAPEIT v2	MaCH
Imputation	IMPUTE v2.1.0	IMPUTE v2.1.0	IMPUTE v2.3.0	minimac
Imputation backbone (NCBI build)	1000 Genomes Phase 1 version 3 (build 37)	1000 Genomes Phase 1 version 3 (build 37)	1000 Genomes Phase 1 version 3 (build 37)	1000 Genomes Phase 1 version 3 (build 37)
QC filter for imputed SNPs	IMPUTE info score >0.4, MAF >1%	IMPUTE info score >0.4, MAF >1%	MAF ≥ 1%	MAF ≥ 1% , imputation quality (MACH R-square) ≥ 0.3

Supplemental Table 3: Sequences of PCR-tagged oligonucleotides used for *NLRC4* isoform quantification

Target	**Primer**	**Sequence 5' - 3'**	**Concentration in qPCR**	**Annealing temperature**
NLRC4 rs385076	Forward	TGCCCTCCAGCTTGGGTACCAGAACAAGAC	-	-
	Reverse	TAGCTGGGGCGCGGTGCCGGGTACCTGTAA		
PU.1	Forward	GGACCAGATATCGCCACCATGTTACAGGCCTGCAAAATG		
	Reverse	CTTATTGTCGACTCAGTGGGGCGGG		
NLRC4 isoforms 2 and 4	Forward	GGCCTCACTGAAACGGAAAGC	150 nM	60 °C
	Reverse	CCTTTATGAAATTCATTGTTCTGGATGAAAGC	150 nM	
NLRC4 isoforms 1 and 3	Forward	GTTTATACTCCGGAGGGTGTCCC	300 nM	62 °C
	Reverse	CTACTCTTCATTCCCTGTACCTTGAATCC	300 nM	
NLRC4 all isoforms	Forward	GCTCTTCATGAACTGATCGACAGG	500 nM	60 °C
	Reverse	CTCCAAATGTTTCAACAGGCTGCTC	500 nM	
GAPDH	Forward	CGAGATCCCTCCAAAATCAA	300 nM	62 °C
	Reverse	TTCACACCCATGACGAACAT	300 nM	

Supplemental Table 4: Genome-wide significant SNPs from IL-18 GWAS meta-analysis

GWAS on log-transformed IL-18 levels were performed separately in GHS I, GHS II, FHS and KORA under adjustment for sex and age. A meta-analysis was performed by inverse variance method. All SNPs with $p<5\times10\text{-}8$ are considered genome-wide significant. SNP annotation is based on genome release GRCh37.

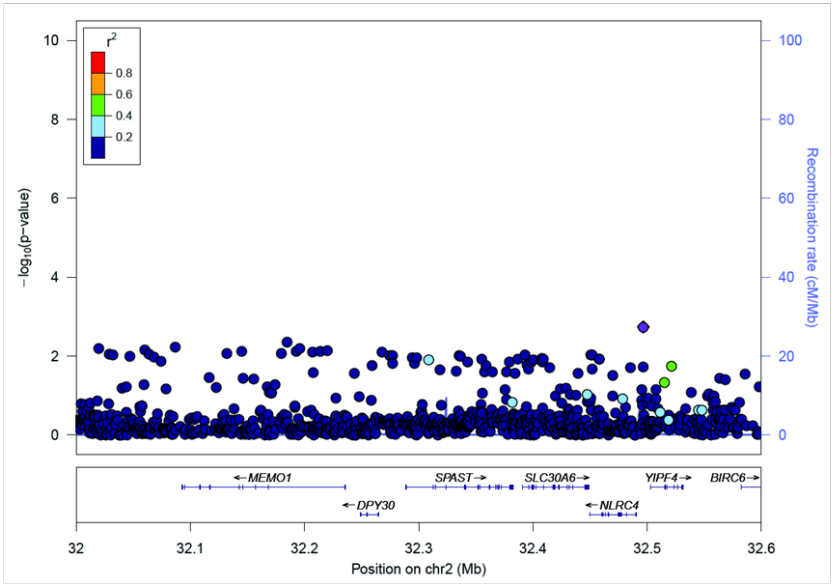
Please see separate Excel file for Supplemental Table 4.

Supplemental Table 5: Association between alleles, lowering IL-18 levels and *NLRC4* mRNA in public data.

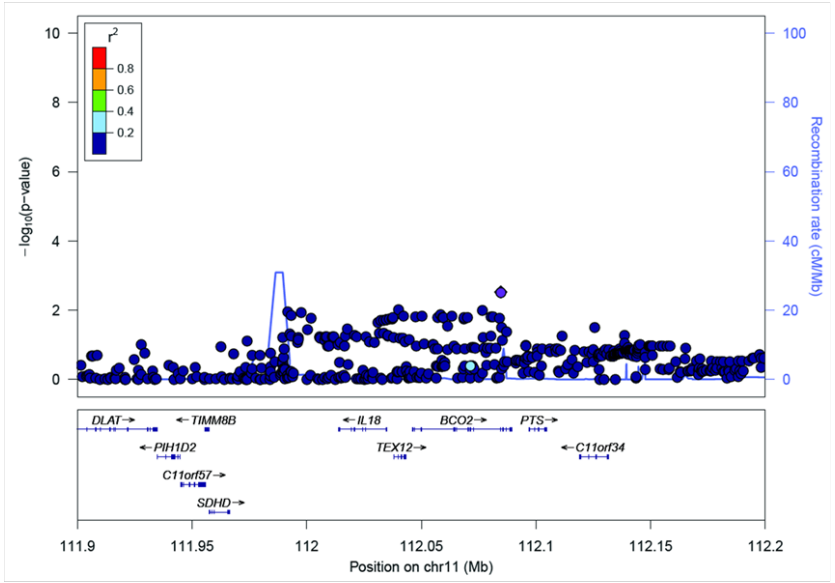
					Association between IL-18 lowering allele and NLRC4 mRNA	
Platform (Reference)	**SNP (allele)**	**Cell type**	**N**	**Method**	**Beta**	**P**
Geuvadis Data Browser (ebi.ac.uk/Tools/geuvadis-das/)	rs385076 (T)	lymphoblastoid cell lines	373	RNA-seq	-0,373	$9{,}1\times10^{-14}$
PheGenI (ncbi.nlm.nih.gov/gap/phegeni)	rs479333 (C)	lymphoblastoid cell lines	270	Microarray	-0,133	$3{,}4\times10^{-8}$

Supplemental Figure 1: Regional plot for the *NLRC4* locus (A) and *IL18* locus (B) from conditional analysis. Associations between variants from the *NLRC4* and *IL18* locus and $\log_e$(IL-18) levels were conditioned on the allele dosage of rs385076 (*NLRC4*) (A) and rs11606049 (IL18) (B), respectively. P-values are plotted as –log10 values against their physical position on chromosomes 2 (*NLRC4*) and 11 (IL18), respectively (NCBI build 37). The color code for the pairwise linkage disequilibrium (LD) structure is based on 1,000 Genomes 2012 EUR. No SNP reached the significance level of $P<5\times10^{-8}$ indicating independent signals within each locus.

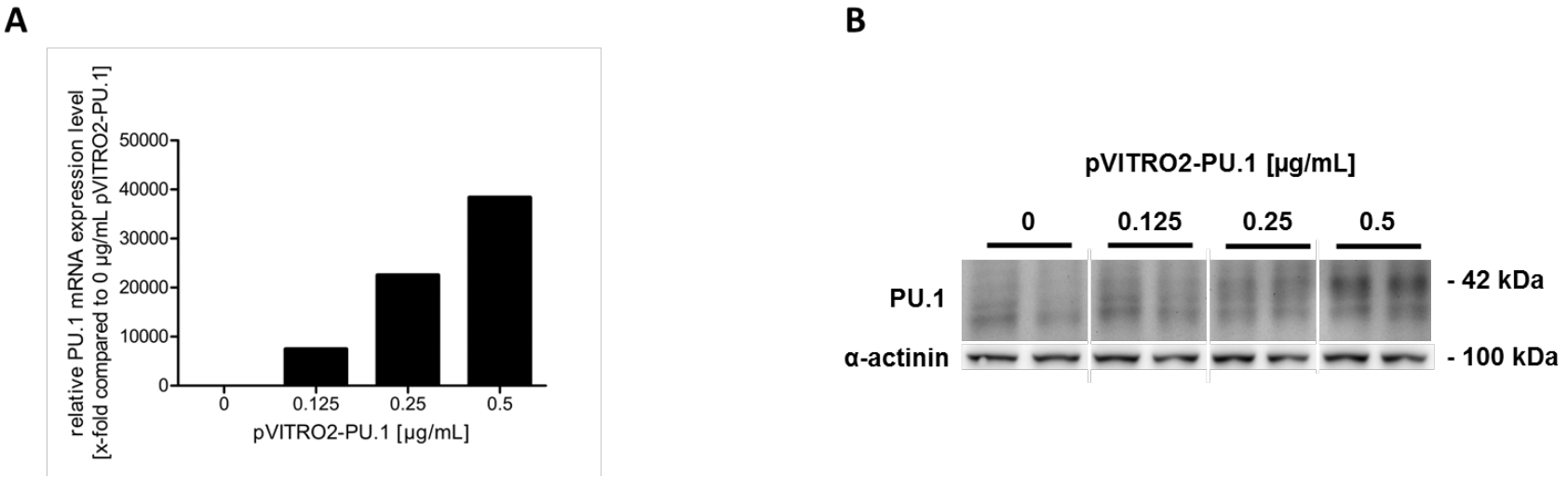
A



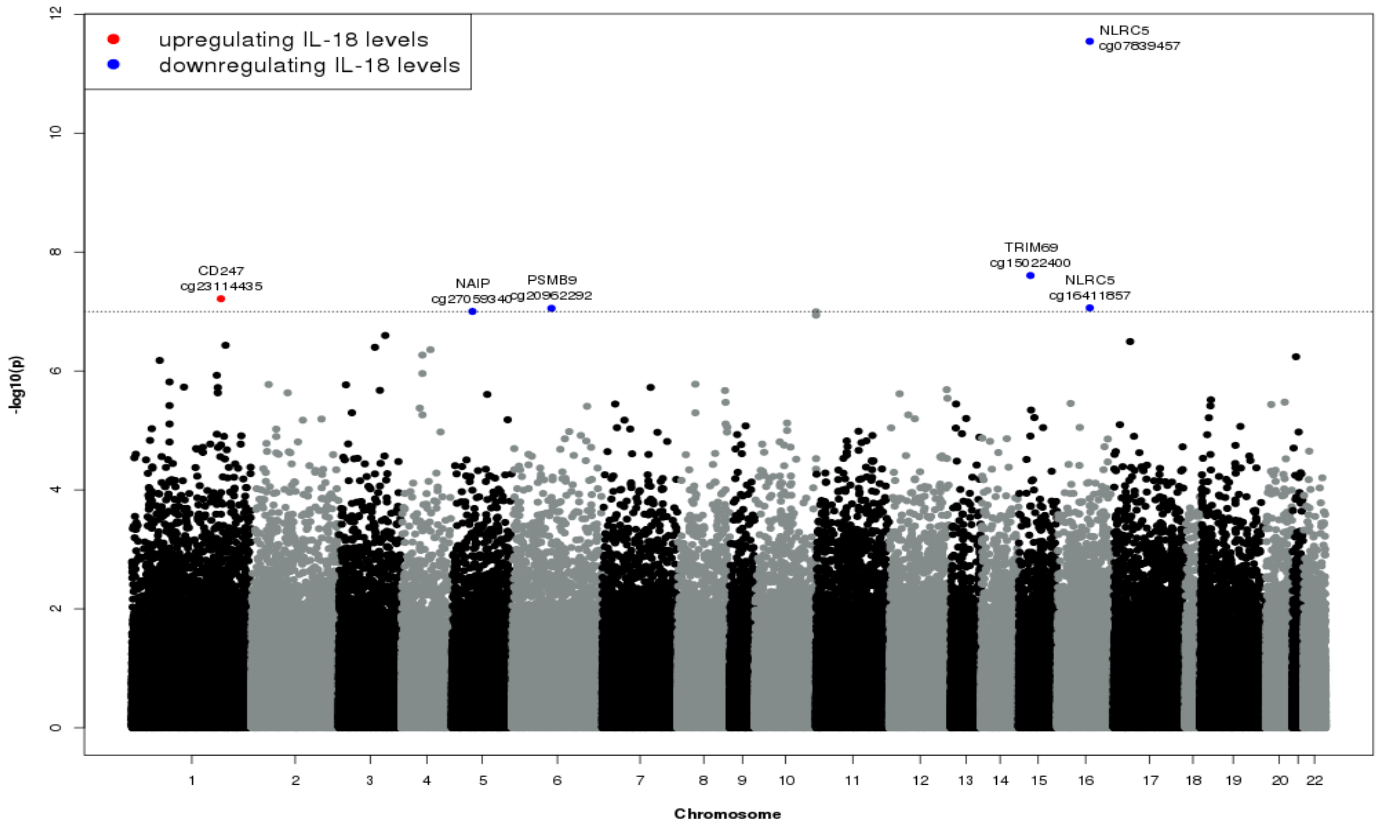
B



Supplemental Figure 2: PU.1 overexpression. **A)** PU.1 mRNA expression and **B)** PU.1 protein expression was increased in HEK293A cells 24 h after transfection with pVITRO2-PU.1.



Supplemental Figure 3: Results from the genome-wide DNA-methylation analysis in KORA F4. The Manhattan plot shows the association between DNA methylation sites and $\log_e$(IL-18) which were adjusted for age, sex, smoking status, BMI and diabetes.



Supplemental References

1. Zeller T, Wild P, Szymczak S, Rotival M, Schillert A, Castagne R, et al. Genetics and beyond--the transcriptome of human monocytes and disease susceptibility. PLoS One. 2010;5(5):e10693. PubMed PMID: 20502693. Pubmed Central PMCID: 2872668.

2. Wild PS, Zeller T, Beutel M, Blettner M, Dugi KA, Lackner KJ, et al. [The Gutenberg Health Study]. Bundesgesundheitsblatt Gesundheitsforschung Gesundheitsschutz. 2012 Jun;55(6-7):824-9. PubMed PMID: 22736163. Die Gutenberg Gesundheitsstudie.

3. Oppenheimer GM. Framingham Heart Study: the first 20 years. Prog Cardiovasc Dis. 2010 Jul-Aug;53(1):55-61. PubMed PMID: 20620427.

4. Holle R, Happich M, Lowel H, Wichmann HE, Group MKS. KORA--a research platform for population based health research. Gesundheitswesen. 2005 Aug;67 Suppl 1:S19-25. PubMed PMID: 16032513.

5. Wichmann HE, Gieger C, Illig T, Group MKS. KORA-gen--resource for population genetics, controls and a broad spectrum of disease phenotypes. Gesundheitswesen. 2005 Aug;67 Suppl 1:S26-30. PubMed PMID: 16032514.

6. Blankenberg S, Rupprecht HJ, Bickel C, Torzewski M, Hafner G, Tiret L, et al. Glutathione peroxidase 1 activity and cardiovascular events in patients with coronary artery disease. N Engl J Med. 2003 Oct 23;349(17):1605-13. PubMed PMID: 14573732.

7. Koenig W, Khuseyinova N, Baumert J, Thorand B, Loewel H, Chambless L, et al. Increased concentrations of C-reactive protein and IL-6 but not IL-18 are independently associated with incident coronary events in middle-aged men and women: results from the MONICA/KORA Augsburg case-cohort study, 1984-2002. Arterioscler Thromb Vasc Biol. 2006 Dec;26(12):2745-51. PubMed PMID: 17008587.

8. Yarnell JW. The PRIME study: classical risk factors do not explain the severalfold differences in risk of coronary heart disease between France and Northern Ireland. Prospective Epidemiological Study of Myocardial Infarction. QJM. 1998 Oct;91(10):667-76. PubMed PMID: 10024924.

9. Price AL, Patterson NJ, Plenge RM, Weinblatt ME, Shadick NA, Reich D. Principal components analysis corrects for stratification in genome-wide association studies. Nat Genet. 2006 Aug;38(8):904-9. PubMed PMID: 16862161.

10. Bibikova M, Barnes B, Tsan C, Ho V, Klotzle B, Le JM, et al. High density DNA methylation array with single CpG site resolution. Genomics. 2011 Oct;98(4):288-95. PubMed PMID: 21839163.

11. Consortium EP, Bernstein BE, Birney E, Dunham I, Green ED, Gunter C, et al. An integrated encyclopedia of DNA elements in the human genome. Nature. 2012 Sep 6;489(7414):57-74. PubMed PMID: 22955616. Pubmed Central PMCID: 3439153.

12. Kent WJ, Sugnet CW, Furey TS, Roskin KM, Pringle TH, Zahler AM, et al. The human genome browser at UCSC. Genome Res. 2002 Jun;12(6):996-1006. PubMed PMID: 12045153. Pubmed Central PMCID: 186604.

13. Pham TH, Benner C, Lichtinger M, Schwarzfischer L, Hu Y, Andreesen R, et al. Dynamic epigenetic enhancer signatures reveal key transcription factors associated with monocytic differentiation states. Blood. 2012 Jun 14;119(24):e161-71. PubMed PMID: 22550342.

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Molecular Characterization of the *NLRC4* Expression in Relation to Interleukin-18 Levels

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