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.

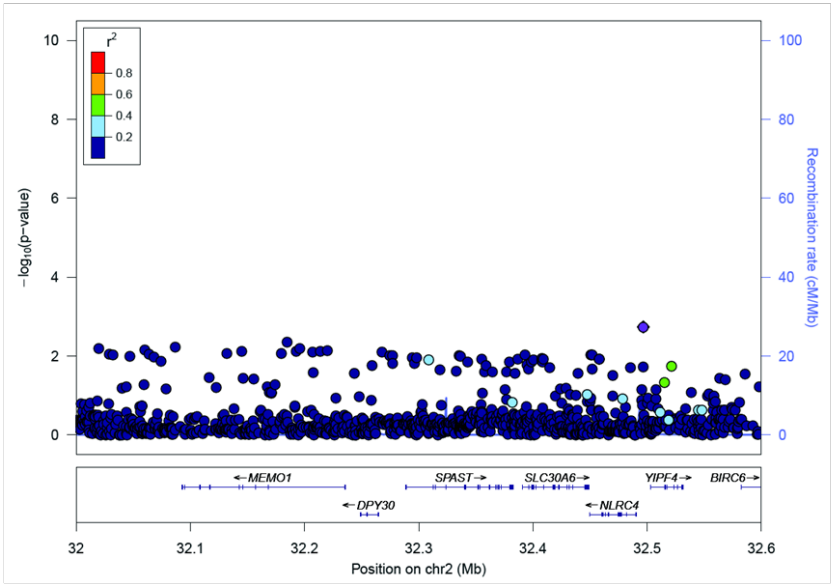
Please see separate Excel file for Supplemental Table 4.

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

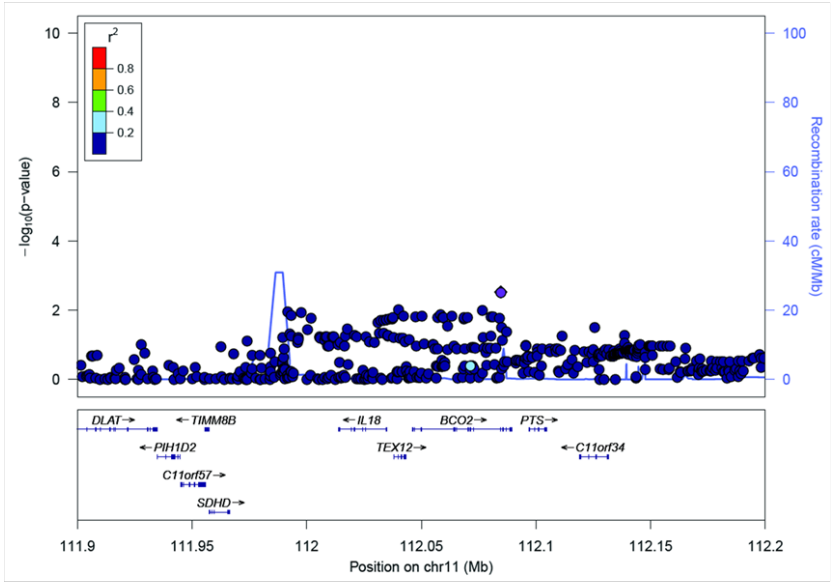
					Association between IL-18 lowering allele and NLRC4 mRNA	
Platform (Reference)	**SNP (allele)**	**Cell type**	**N**	**Method**	**Beta**	**P**
Geuvadis Data Browser (ebi.ac.uk/Tools/geuvadis-das/)	rs385076 (T)	lymphoblastoid cell lines	373	RNA-seq	-0,373	$9{,}1\times10^{-14}$
PheGenI (ncbi.nlm.nih.gov/gap/phegeni)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

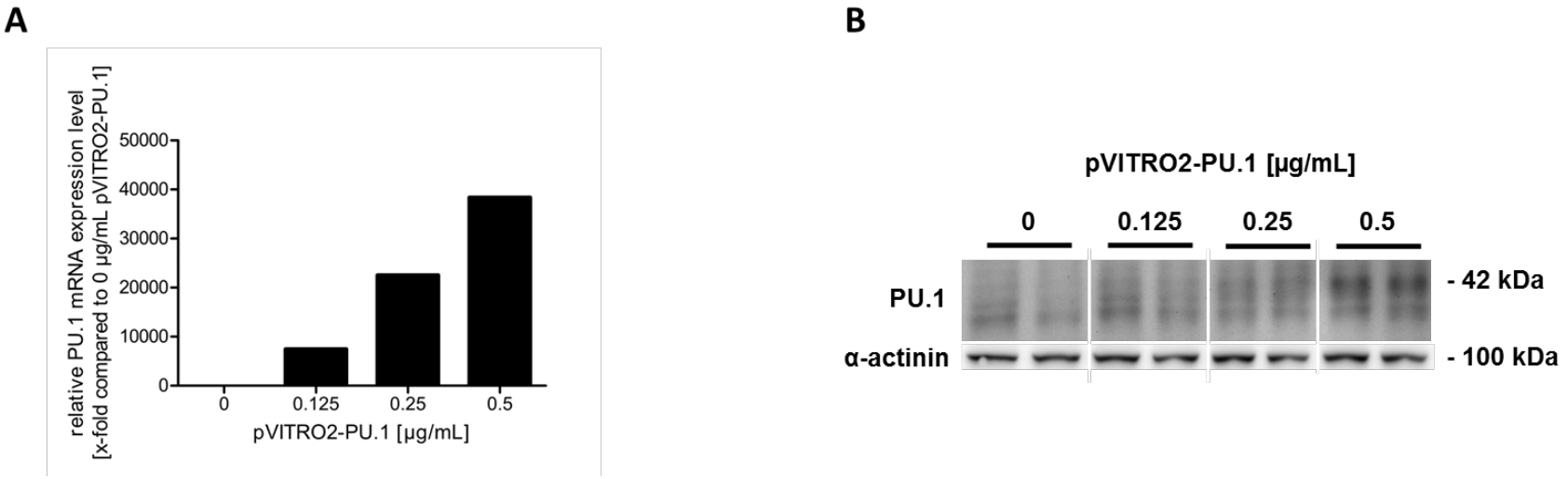
A



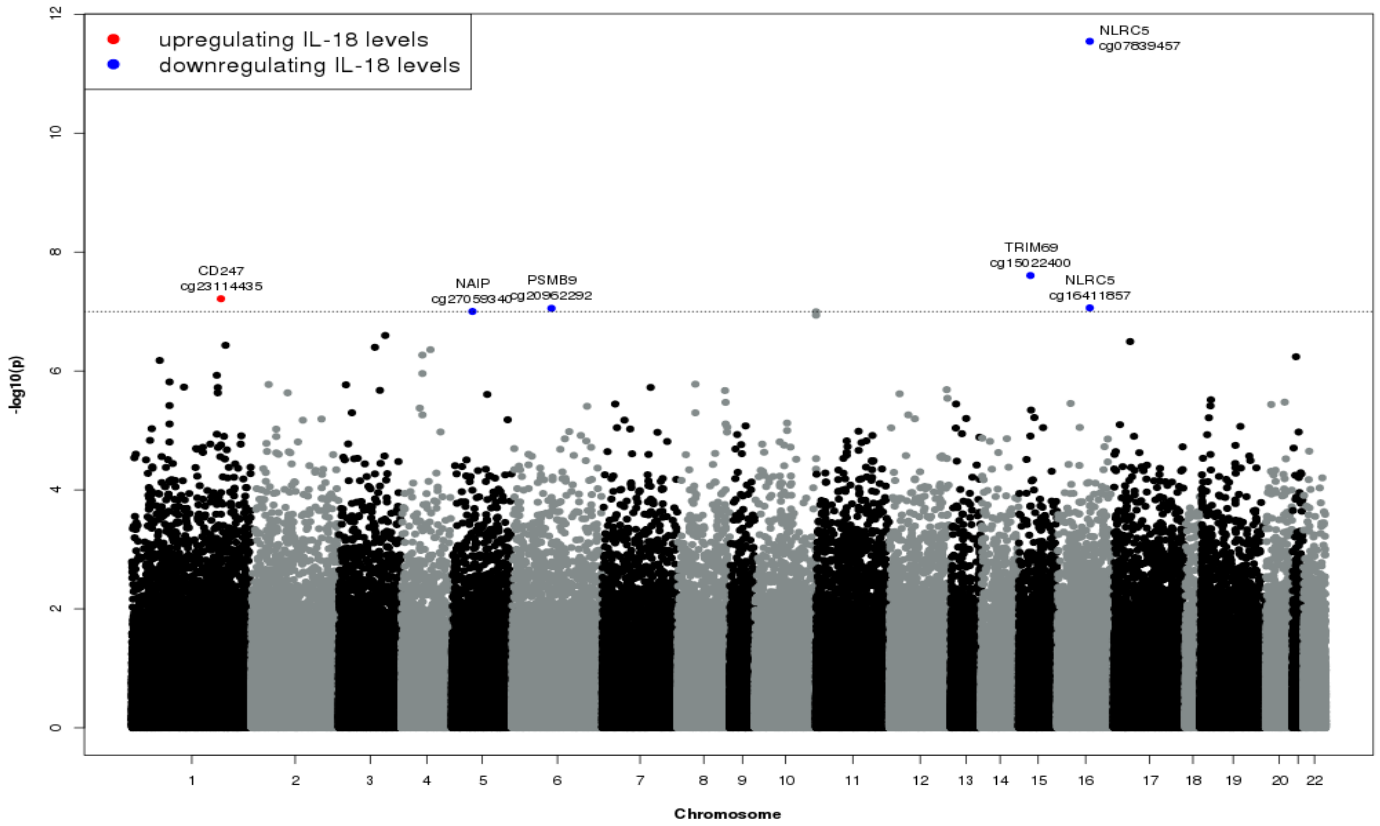
B



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.



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