**Epigenome-Wide Study of Circulating TNFα Identifies Robust Methylation Signals Linked to Incident Coronary Heart Disease**

**Aslibekyan: Epigenome-wide association study of TNFα**

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Word count: 4523

**Key Points**

**Question:** Is DNA methylation in peripheral blood cells associated with circulating tumor necrosis factor alpha (TNFα) levels and the risk of coronary heart disease?

**Findings:** Top methylation loci associated with circulating TNFα in whole blood or CD4+ T-cells were located in or near *DTX3L-PARP9* complex, *NLRC5*, *TRIM69*, *ABO*, *OAS2*, and *MX1*. The findings in *NLRC5* and one in *DTX3L-PARP9* were successfully replicated and linked to gene expression. Furthermore, methylation at these loci was robustly inversely associated with the risk of incident coronary heart disease.

**Meaning:** Upon further validation, these epigenetic associations may be leveraged in the pursuit of new or improved therapeutic applications.

**Abstract**

**Importance:** Tumor necrosis factor alpha (TNFα) is a proinflammatory cytokine with manifold consequences for mammalian pathophysiology, including cardiovascular disease. Strategies for therapeutic inhibition of TNFα have produced mixed results, necessitating a deeper understanding of TNFα biology to enhance treatment precision.

**Objective:** To conduct an epigenome-wide analysis of blood-derived DNA methylation and TNFα levels; to interrogate the functional relevance of findings.

**Design:** Discovery meta-analysis of epigenome-wide associations with cross-sectional circulating TNFα from six studies with external replication. Follow-up analyses investigated associations of identified methylation loci with gene expression and incident coronary heart disease (CHD).

**Setting:** Discovery and replication in eight population-, community-, region-, or occupation-based cohorts and one interventional trial.

**Participants:** Discovery N=4,794; replication N=816. Individuals with autoimmune diseases or taking immune-modulating agents were excluded. CHD meta-analysis included N=11,461 with 1,895 events.

**Exposure:** Circulating TNFα concentration.

**Main Measures:** DNA methylation at ~450,000 loci, neighboring DNA sequence variation, gene expression, and incident CHD.

**Results:** In the discovery stage, circulating TNFα levels were associated with methylation of ten cytosine-phosphate-guanine (CpG) sites (P≤2.52**×**10-6), located in or near *DTX3L-PARP9* (cg00959259, cg08122652, cg22930808), *NLRC5* (cg16411857, cg07839457), *TRIM69* (cg22107533, cg05439368), *ABO* (cg13683939), *OAS2* (cg21549285), and *MX1* (cg11601443) after accounting for multiple testing. Of those, negative associations between TNFα and the methylation of two loci in *NLRC5* (cg16411857 and cg07839457) and one in *DTX3L-PARP9* (cg08122652) externally replicated (P≤0.003). Methylation at the replicated TNFα loci was negatively associated with neighboring gene expression in two of the three participating cohorts; in turn, expression of *NLRC5*, *DTX3L*, and *PARP9* was strongly (P≤0.003) positively associated with TNFα. Methylation of cg07839457 in *NLRC5* was weakly associated with neighboring sequence variant on chromosome 16 (rs17369768), located at a transcriptionally active region in multiple tissues, and nominally associated with metabolic traits (visceral adipose tissue volume, waist circumference, weight) and inflammatory conditions (psoriasis, rheumatoid arthritis). All replicated TNFα-related CpGs were associated with a lower risk of incident CHD (10-21% decreased risk per 10% higher methylation, P<0.005).

**Conclusions and Relevance:** We identified and replicated novel epigenetic correlates of circulating TNFα in blood samples and linked these loci to CHD risk, opening opportunities for validation and therapeutic applications.

**Introduction**

Tumor necrosis factor alpha (TNFα) is a proinflammatory cytokine with pleiotropic effects in human health and disease. In addition to its well-characterized pathogenic contributions to inflammatory and autoimmune diseases, atherosclerosis, type 2 diabetes, and cancer, TNFα also plays a key homeostatic role in pathogen defense, tissue repair and regeneration, and organ development (reviewed in Kalliolias, et al.1). Therapeutic inhibition of TNFα is used in clinical settings with both successes (e.g. in various forms of autoimmune diseases) and failures (e.g. in multiple sclerosis2). Furthermore, treatment with TNF inhibitors has long been known to lower the risk of cardiovascular disease among autoimmune disease patients,3 and currently several trials (e.g. NCT01893996) are assessing cardioprotective effects of inhibiting inflammatory cytokines, yet the underlying mechanisms have remained elusive.

 Circulating levels of TNFα have a moderate genetic determinant, with heritability estimates ranging from 17%4 to 39%5 in large-scale European twin studies to 68% in a Ugandan community with a high prevalence of tuberculosis.6 Notably, known common polymorphisms account for a relatively minor fraction of that heritable component, explaining <4% of the trait variance in a recent meta-analysis of genome-wide association studies of circulating TNFα levels (personal correspondence; see letter attached). There is emerging evidence suggesting that epigenetic processes like DNA methylation, which reflect changes in gene expression that occur without sequence mutations, may offer promising clues in the search for missing TNFα heritability. For example, methylation of two cytosine-phosphate-guanine (CpG) loci in the *TNF* promoter was associated with lower plasma TNFα levels in a small cohort of young women;7 a subsequent larger study found similar relationships, and confirmed the inverse association between *TNFα* methylation and expression.8 *In vitro*, experimental manipulation of DNA methylation has been shown to alter the cells’ ability to produce TNFα,9 offering causal support for the association observed in population studies. To date, however, no study has comprehensively examined the DNA methylation across the entire genome in relation to circulating levels of TNFα in large human populations, or has interrogated TNFα epigenetics with regards to cardiovascular risk.

Therefore, we conducted the first epigenome-wide meta-analysis of associations between circulating TNFα levels and DNA methylation in whole blood samples or isolated lymphocytes from 4,794 individuals representing six cohorts in the Cohorts for Heart and Aging Research in Genetic Epidemiology (CHARGE) consortium. We subsequently sought and achieved replication of the top CpG loci in an independent population, evaluated the associations between DNA methylation and *cis*-gene expression, and assessed the contributions of DNA sequence variation to the observed CpG methylation variation in the regions of interest. Finally, we investigated the association of the top epigenetic correlates of circulating TNFα with incident coronary heart disease (CHD) in a meta-analysis comprising 11,461 participants with 1,895 CHD events.

**Methods**

*Discovery and Replication Populations*

In the discovery phase, the epigenome-wide study included 4,794 individuals of European descent from six studies participating in the CHARGE consortium:10 Framingham Heart Study (FHS), Genetics of Lipid Lowering Drugs and Diet Network (GOLDN) study, The Invecchiare in Chianti Study (InCHIANTI), Kooperative Gesundheitsforschung in der Region Augsburg (KORA), Lothian Birth Cohort 1921 (LBC1921), and Normative Aging Study (NAS). Replication analyses were conducted in the Northern Finland Birth Cohort 1966 (NFBC66) and the Helsinki Birth Cohort Study (HBCS). Individuals who reported an autoimmune diagnosis or taking immune-modulating agents (e.g. TNFα blockers) were not included in the analyses. Further details about each study are included in Table 1 and eMethods 1. All study protocols were approved by Institutional Review Boards of the participating study sites, and all participants provided written informed consent.

*Laboratory Measurements*

Circulating TNFα levels were measured in pg/ml using the approaches listed in Table 1. In all but one cohort (FHS), TNFα was measured at the same time and center visit as blood was drawn for the quantification of DNA methylation. In the FHS, TNFα was measured in the same individuals during the preceding examination cycle, approximately seven years prior to the DNA methylation assay. Circulating TNFα was natural log-transformed (lnTNFα) to reduce skewness of the distribution. Individuals whose lnTNFα measurements were more than four standard deviations away from the cohort mean were excluded from subsequent analyses.

*DNA Methylation Measurements, Normalization, and Quality Control*

All studies used the Illumina Infinium Human Methylation450 Beadchip (Illumina Inc, San Diego, CA) to quantify epigenome-wide DNA methylation. In all studies but one, these measurements were performed on DNA extracted from whole blood samples; the GOLDN study isolated and quantified DNA methylation on CD4+ T-cells. Detection P values were estimated by proprietary Illumina software (Genome Studio) and defined as the probability that the total observed signal fell within the background intensity. The ratio of the methylated probe intensity to the overall signal intensity (the β score), estimated by Illumina Genome Studio software, was used as the quantitative measure of DNA methylation.11 Studies varied in their approaches to correction for background values, accounting for white blood cell (WBC) subtypes, and normalization of the β scores, as summarized in eTable 1.

*Statistical Analyses*

In the discovery phase, each cohort fit three linear mixed effect regression models to assess associations between lnTNFα (predictor) and normalized methylation β scores (outcomes). The base model adjusted for age and sex as fixed effects and technical covariates (array, row, and/or column number) as a random effect. The second model additionally adjusted for measured or estimated white blood cells (WBC) subtypes for studies reporting methylation in whole blood samples. The third model adjusted for the same covariates as the second model, additionally including smoking (current, former, or never) and body mass index (BMI) in kg/m2. All covariates were selected based on their known associations with DNA methylation. Cohorts additionally adjusted for relatedness or other study-specific covariates as necessary (eTable 2). Results from the six cohorts participating in the discovery phase were meta-analyzed using a fixed effects, inverse-variance weighted approach in METASOFT.12 To account for potential undue influence of the NAS measurements (which had substantially higher TNFα levels), we performed a sensitivity meta-analysis excluding that cohort from the discovery stage. Because GOLDN was the only cohort that used CD4+ T-cells and not whole blood samples, we also ran a sensitivity meta-analysis excluding GOLDN.

 Findings were carried forward to the replication phase if the false discovery rate (FDR) for the specific CpG was below 0.05. Models used in the replication analysis were identical to those implemented in the discovery phase and the statistical significance threshold was defined using the stringent Bonferroni correction: 0.05/number of statistically significant hits from the discovery meta-analysis.

*Gene Expression Measurements and Analysis*

The CpG sites that significantly replicated in the independent replication sample were further tested for association with *cis*-gene expression in whole blood in 3,738 participants with available gene expression measurements: FHS, KORA, and RS. Methods for the quantification of gene expression in each of the three cohorts are described in eMethods 2. All three cohorts conducted the expression-methylation association (eQTM) analyses in two stages. First, both DNA methylation β scores and gene expression values were residualized with adjustment for age, sex, imputed cell count proportions, as well as study-specific and technical covariates detailed in eMethods 2. Second, the eQTM linear regression models were fit with the gene expression residual as the dependent variable and the methylation residual as the independent variable. FHS models were additionally adjusted for 25 methylation surrogate variables (SVs) and 25 expression SVs to account for unmeasured technical and batch effects. Statistical significance was established using the Bonferroni threshold of 0.05/number of hypotheses tested, i.e. the number of CpG-gene transcript pairs. mRNA transcripts that achieved statistical significance in at least two cohorts were further evaluated for association with circulating TNFα in FHS, using regression models adjusted for age, sex, imputed WBC counts, smoking, BMI, and technical covariates.

*Integrating DNA Methylation and Sequence Data*

To establish genetic contributions to the observed methylation of the top loci, we studied genetic associations with DNA methylation in *cis* (±20kb) using the GOLDN study as the discovery cohort and RS as the replication population. Specifically, we tested for associations between the CpGs that were significantly associated with circulating TNFα in both discovery and replication stages and *cis* DNA sequence variation in RS (meQTL discovery) and GOLDN (meQTL replication). Genotyping and imputation procedures for both cohorts are described in eMethods 3. In the discovery phase, we created residuals by regressing inverse-normal transformed methylation β scores on the first ten methylation principal components and up to the first ten ancestry principal components. The residuals then served as the dependent variables in a linear regression model with SNPs as predictors. SNPs with low imputation quality (r2 < 0.4) and low frequency variants (MAF < 0.01) were removed from the analyses. SNPs with the FDR < 0.05 were carried forward to replication. In the replication phase, we fit linear mixed models with the methylation β scores as outcomes, adjusted for age, sex, study site, four CD4+ T-cell purity principal components (fixed effects) and family relatedness (random effect). Due to limited evidence of population stratification in GOLDN,13 we did not adjust for ancestry. The two variants that achieved nominal significance in the replication phase were subsequently tested for association with circulating TNFα in GOLDN using linear mixed models adjusting for age, sex, study site (fixed effects) and family (random effect).

 In addition to meQTL analyses, we searched for overlap with the genomic regions containing the replicated sites in two genome-wide association study (GWAS) catalogs (<http://www.ebi.ac.uk/gwas/search>, accessed 4/25/2017, and <http://www.phenoscanner.medschl.cam.ac.uk/phenoscanner>, accessed 8/7/2017) to assess previously reported associations of sequence variants in the regions of interest and disease traits.

*Associations with Incident CHD*

We tested associations between the replicated epigenetic correlates of circulating TNFα and incident CHD in a CHARGE consortium fixed effects meta-analysis that included 470,346 CpGs, 1,895 disease events, and 11,641 participants from the following cohorts: Atherosclerosis Risk in Communities (ARIC), Cardiovascular Health Study (CHS), Long-term Follow-up of Antithrombotic Management Patterns In Acute Coronary Syndrome Patients (EPICOR), FHS, InCHIANTI, KORA, NAS, and Women’s Health Initiative (WHI). The definition of CHD events included coronary insufficiency, coronary revascularization, recognized MI (hospitalization with diagnostic ECG changes and/or biomarkers of MI), and coronary death. Participants with prevalent CHD at enrollment were not included. Each cohort study obtained written informed consent from participants and ethics approval from its respective institutional review boards and ethics committees. In each cohort, associations were adjusted for age, sex, smoking status, education, BMI, differential WBC counts (either directly measured or imputed), and technical covariates. Data were meta-analyzed using an inverse-variance weighted fixed effects method. This lookup was restricted to the top four CpG sites from the circulating TNFα meta-analysis; findings were considered statistically significant if P met the Bonferroni threshold of α=0.05/4=0.0125. Further details about the incident CHD meta-analysis14 are available in eMethods 4.

*Functional Annotation*

We used Hudson Alpha Institute for Biotechnology - ENCODE project custom methylation tracks implemented in the UCSC genome browser as well as the Illumina annotation file to visualize and annotate the functional potential of the top CpGs, including such indicators of regulatory activity as H3K27Ac marks, DNAseI hypersensitivity elements in relevant cell types, and genomic location of the CpGs (promoter vs gene body, exon vs intron, etc).

**Results**

*General Characteristics*

Demographic and TNFα characteristics of all participating cohorts are summarized in Table 1. The cohorts varied in age, ranging from the mean age of 31(±0.3) years in NFBC66 to 87(±0.4) years in the LBC1921 cohort. The cohorts were approximately half female with the exception of NAS and HBCS, which only recruited men. Circulating TNFα values were consistent for all cohorts except NAS, which reported exceptionally high concentrations with considerable variance. The observed discrepancy may be due to technical differences between approaches to TNFα quantification, as documented in Table 1.

*Meta-analysis and Replication*

Of the three models fit to test associations between epigenome-wide methylation and TNFα levels, the third model exhibited the least inflation (λ=1.14 vs. 1.19 in the first model and 1.15 in the second model). Based on the λ statistics as well as prior evidence in support of adjusting epigenetic models for smoking and BMI, this discussion and the sensitivity analyses focused on the third model; however, the other two models yielded similar top hits (eTables 3 and 4).

The results of the epigenome-wide analyses are summarized in Table 2 and visualized in Figure 1. Ten CpG sites located in six genomic regions emerged as the top hits in the discovery stage (FDR < 0.05). Of those, three CpG sites—two located in the NLR family CARD domain containing 5 gene (*NLRC5*) and one in the deltex E3 ubiquitin ligase 3L/ poly(ADP-ribose) polymerase family member 9 gene complex (*DTX3L/PARP9*)—replicated in 677 individuals from the NFBC66 cohort. All replicated associations were in the similar direction and had comparable effect sizes. Another locus in *DTX3L/PARP9* was not able to undergo replication due to failing quality control procedures in NFBC66; however, due to its proximity and similarity of associations to the replicated cg08122652, we included it in subsequent analyses. The *DTX3L/PARP9* and the tripartite motif containing 69 gene(*TRIM69*) loci approached statistical significance for replication in NFBC66 but failed to meet the Bonferroni correction. Nevertheless, the direction of effect and magnitude of association were similar across cohorts.None of the associations, however, replicated in the much smaller (N=149) HBCS cohort or in the meta-analysis of HBCS and NFBC66 (data not shown). Sensitivity analyses removing either NAS or GOLDN did not appreciably change the top findings (eTables 5 and 6). Namely, cg16411857 in *NLRC5* remained the top hit in all analyses.

*Methylation vs. Expression vs. Circulating TNFα*

We observed nine cis-eQTMs (methylation-expression pairs) between methylation at the four TNFα-associated loci and cis-gene expression in FHS. All were robust in RS, while none reached significance in KORA. CpG-transcript pairs that satisfied the Bonferroni threshold in at least two cohorts are presented in Table 3. Direction (negative for all transcripts except the methylation with karyopherin subunit alpha 1 gene (*KPNA1*) pair) and magnitude of associations were consistent between FHS and RS. Of the five transcripts that were significantly associated with TNFα-linked CpGs in both FHS and RS (*NLRC5*, *DTX3L*, *KPNA1*, *PARP9*, and the poly(ADP-ribose) polymerase family member 14 gene (*PARP14*)), three were positively associated with circulating TNFα in FHS, with respective Pof 5.47**×**10-5 (*NLRC5*), 0.003 (*DTX3L*), and 0.003 (*PARP14*) meeting the Bonferroni threshold.

*Methylation vs. Sequence Variation vs. Circulating TNFα*

Of all significant methylation correlates of TNFα, only cg07839457 showed nominally significant (P < 0.05) replication of associations with two neighboring *NLRC5* sequence variants: rs17369768 and a deletion at the 57042641 position on chromosome 16 (eTable7). Neither locus was significantly associated with circulating TNFα in GOLDN (P =0.60 for rs17369768 and P=0.61 for the deletion at the 57042641 position on chromosome 16). However, rs17369768 was nominally associated with visceral adipose tissue volume, waist circumference, weight, psoriasis, and rheumatoid arthritis in public databases (<http://www.phenoscanner.medschl.cam.ac.uk/phenoscanner>).

*Associations with Incident CHD*

Methylation at all four TNFα-associated loci was robustly negatively associated with the risk of incident CHD in the meta-analysis of CHARGE cohorts (Table 4). Adjusted for the appropriate covariates, each 10% increase in methylation of a given TNFα-associated locus was associated with a 10-21% decrease in the risk of an adverse CHD event.

*GWAS Catalog Look-Up and Functional Annotation*

Of the three common SNPs in or near *NLRC5* that were reported in the GWAS catalog, one (rs821470) was associated with the risk of schizophrenia in a Swedish sample,15 corroborated by the association of another variant (rs17290922) with mood symptoms in the context of schizophrenia.16 The third polymorphism (rs1532624) was reported to be associated with HDL cholesterol,17 but was mapped to the neighboring cholesteryl ester transfer protein gene (*CETP*)—a known lipid locus—rather than *NLRC5* by Ensembl algorithms. The closest reported variant to the *DTX3L/PARP9* locus (rs2173763) was associated with major depressive disorder.18 Conversely, a search for SNPs previously reported to be associated with circulating TNFα yielded no results located in the regions harboring the replicated epigenetic hits, although an *ABO* polymorphism (from a region that emerged as a top hit yet did not replicate) was identified as a TNFα protein quantitative trait locus in an earlier analysis of KORA data.19

 Bioinformatic regulatory annotations for the *DTX3L/PARP9* and *NLRC5* regions are presented in eFigures 1 and 2, respectively. Both sets of loci are adjacent to or overlap regulatory elements, specifically H3K27Ac peaks, DNAse I hypersensitivity sites, and transcription factors. The two *NLRC5* loci are positioned within 200bp of each other in the promoter region, with cg07839457 mapping to the north shore of the CpG island containing cg16411857.The two *DTX3L/PARP9* methylation variants lay within 40bp of each other, on the north shore of a CpG island in the first intron of *PARP9*; both are methylated in B-lymphocytes but not in embryonic stem or umbilical vein endothelial cells.

**Discussion**

Using epigenome-wide data from adult participants of European descent, we have identified and replicated novel associations between leukocyte DNA methylation loci in two genomic regions—mapping to *NLRC5* and *DTX3L/PARP9*—and circulating TNFα. Concordant with the bioinformatic annotation of the *NLRC5* and *DTX3L/PARP9* methylation loci, which offered evidence of regulatory potential, we observed associations between the top methylation correlates of TNFα and the expression of neighboring genes: *NLRC5*, *DTX3L*, *PARP9*, *PARP14*, and *KPNA1*. Of those, expression of *NLRC5*, *DTX3L*, and *PARP9* was robustly associated with circulating TNFα. Moreover, DNA methylation at the same loci that were correlated with lower plasma TNFα levels was also strongly associated with a decreased risk of incident CHD in a multi-ethnic meta-analysis. On balance, our findings support a relationship between DNA methylation and expression of immune-related genes, circulating cytokine concentrations, and CHD.

 Both genomic regions that were discovered and validated in our analysis encode proteins that play a pivotal role in the immune response. *NLRC5* is a specific transactivator of major histocompatibility complex (MHC) class I genes,20 which encode human leukocyte antigens (HLA) proteins that set off the adaptive immune reaction by presenting antigens to CD8+ T-lymphocytes.21 These processes are induced chiefly by interferon-gamma (IFNγ) stimulation although also by toll-like receptor ligands, other interferons, and viral infections.22 NLRC5 binds to promoters of MHC class I and related genes (major histocompatibility complex, class I, A, B, C, E, and F genes (*HLA-A*, *-B*, *-C*, *-E*, *-F*) as well as the transporter 1, ATP binding cassette subfamily B member gene (*TAP1*), *PSMB9*, the beta-2-microglobulin gene (*B2M*)23) and activates their expression through an SXY module.24 By activating CD8+ T-cells via MHC class I proteins, NLRC5 has also been shown to upregulate IFNγ, creating a positive feedback loop ensuring an effective response to intracellular pathogens.25

The role of *NLRC5* as a master regulator of the immune response, combined with its remarkable specificity, has positioned it as a promising therapeutic target in multiple clinical settings. The specific methylation loci that emerged as our top findings, cg16411857 and cg07839457, have been shown to be significantly hypomethylated in blood from immune-suppressed HIV-infected individuals, also correlating negatively with viral load.26 In another whole blood DNA methylation study, both CpGs were linked to circulating IL-18, further highlighting the role of *NLRC5* in inflammation and offering a possible mechanism for the association we observed with CHD incidence.26 Of interest, the *NLRC5* promoter (and specifically the cg16411857 locus) was shown to be hypermethylated in 13 distinct cancer types, with a corresponding reduction in expression of not only *NLRC5* but also of other genes in the MHC class I family—providing a mechanism for evasion of CD8+ T-lymphocyte antitumor activity.27 By replicating the previously reported associations between *NLRC5* promoter methylation and gene expression, and identifying novel associations with circulating TNFα and CHD, our study adds to the robust body of evidence in support of *NLRC5* involvement in a wide range of pathophysiologic conditions.

Similarly to *NLRC5*, increased expression of *DTX3L-PARP9* has been shown to enhance IFNγ signaling and therefore host immune response.28 In the setting of infection, DTX3L-PARP9 ubiquitin ligase complex promotes immunity by both upregulating IFN-induced gene expression and initiating the degradation of viral proteases.28 Recent evidence suggests that DTX3L-PARP9 may play a key role in other pathophysiologic conditions, particularly vascular inflammation and atherosclerosis. In macrophage-like cell lines stimulated with IFNγ, experimental silencing of *PARP9* has suppressed the induction of TNFα (consistently with the directions of association observed in our analyses) while silencing of *PARP14* has had opposite effects (in contrast with our observations); additionally, PARP14 deficiency was shown to promote atherogenesis in mice.29 Possible explanations for the discrepancy in the direction of association may include cell type (macrophages vs. T-lymphocytes or whole blood), tightly controlled experimental conditions in cell culture/murine models vs. observational data from free-living humans, chance, or other factors. Therapeutic inhibition of other PARP enzymes—specifically PARP1—has also been shown to confer cardioprotective effects30 as well as to reduce circulating TNFα *in vivo*.31 Although the inconsistency of the PARP14 finding across studies merits close attention in future investigations, our analysis contributes to growing evidence linking PARP enzymes with systemic inflammation and CHD.29

In follow-up analyses, we found only limited evidence of genotype contributions to the methylation of the CpG sites of interest, suggesting the importance of environmental determinants. A prior analysis of DNA methylation heritability in the GOLDN study reported h2 < 0.4 for cg16411857, cg08122652, and cg00959259 and 0.4 < h2 < 0.5 for cg07839457, without strong (P<10-7) evidence of associations with either *cis* or *trans*-SNPs.32 In the same dataset, CpGs like cg07839457—moderately heritable yet genotype-independent—were shown to be enriched in the genomic regions that evade erasure during embryogenesis, as well as near genes related to T cell activation.32 It is therefore possible that the methylation of cg07839457 in *NLRC5* could be programmed by environmental exposures (notably pathogens) and transmitted across generations, although further targeted studies are needed to rigorously test this hypothesis.

To date, the presented analysis is the largest epigenetic study of circulating TNFα, both in sample size and scope, with previous analyses largely confined to candidate gene or repetitive element methylation studies. Specifically, a number of whole blood studies have interrogated relationships between methylation in the tumor necrosis factor gene (*TNF*)gene promoter, corresponding gene expression (where available), and circulating TNFα levels in various disease contexts, e.g. rheumatoid arthritis, chronic periodontitis,33,34 type 1 diabetes,35 or obesity.7 Interestingly, *TNF* was not among the top regions associated with circulating TNFα in our meta-analysis or in published GWAS of TNFα.19 Furthermore, there was little overlap between our epigenome-wide findings and previous studies of DNA sequence determinants of TNFα. The only exception is the absence of a replicated association with ABO, alpha 1-3-N-acetylgalactosaminyltransferase and alpha 1-3-galactosyltransferase gene (*ABO*) methylation in our study that was also observed in a previous protein quantitative trait loci GWAS,19 which presented evidence that the effect was assay-specific and may be driven by cross-reactivity with ABO antigens. Finally, to the best of our knowledge, the *NLRC5* and *DTX3L-PARP9* findings have not been reported in epigenetic studies of other proinflammatory cytokines, although a recent large-scale meta-analysis of C-reactive protein reported multiple associations with methylation loci in other interferon pathway genes,36 illustrating distinct yet related epigenetic determinants of the human immune response.

Given the relevance of the TNFα phenotype to the inflammatory response, the use of leukocyte-derived DNA for methylation measurements constitutes a clear strength of the study. Furthermore, the accessibility of blood facilitates future translational applications of our findings. DNA methylation measurements were available in multiple cohorts that also offered genotype and expression data, enabling us to follow up on our top findings for a deeper understanding of the mechanisms underlying the association between methylation and circulating TNFα. However, several limitations of our integrative analyses must be noted. First, the expression findings replicated robustly between FHS and RS, but not in KORA. Possible reasons include discrepancies in population characteristics, gene expression measurements, or chance. Second, none of the methylation vs. TNFα findings replicated in HBCS, likely due to insufficient power, driven by the limited sample size of that cohort (N=149); other reasons could include the differences in TNFα measurement approach (Table 1). The consistency in both magnitude and direction of associations across the discovery cohorts and their successful replication in NFBC66 increase confidence in the validity of our findings. Third, FHS measurements of methylation and TNFα were taken several years apart, while all other cohorts performed them contemporaneously. However, the FHS findings were similar to those derived from cross-sectional studies, indirectly indicating temporal stability of the observed associations in adults. Also, the difference in time between the measurements would bias the effect estimates towards the null, further reassuring our findings. Finally, the reported associations may not be interpreted as causal because they were established in observational data that do not preclude bias, e.g. due to residual confounding. Causal inference methods such as Mendelian randomization, used widely to corroborate findings of epigenome-wide studies, are not optimal for our study because strong genetic instruments for either 1) the methylation at the top loci, which we showed to be only weakly related to the genotype and 2) TNFα itself are not currently available. Future studies may consider directly interrogating the relationship between DNA methylation in *NLRC5* and *PARP9-DTX3L* and systemic inflammation in experimental models.

 In summary, we report novel evidence linking DNA methylation in two immune response-related regions—*NLRC5* and *PARP9-DTX3L*—with corresponding gene expression, circulating TNFα, and incident CHD in a population-based meta-analysis, highlighting the potential of these regions as translational targets. Further, our findings illustrate the utility of agnostic methylome-wide studies in identifying physiologically meaningful phenomena. In concert with evidence from *in vitro* and *in vivo* functional studies, our findings yield valuable insights into immunopathology of CHD.

**Acknowledgments**

The participating studies acknowledge the following sources of funding:

FHS: The Framingham Heart Study is funded by National Institutes of Health (NIH) contract N01-HC-25195 and HHSN268201500001I and administered by Boston University. The DNA methylation and gene expression assays for this investigation was funded by the Division of Intramural Research, National Heart, Lung, and Blood Institute (NHLBI), NIH, and an NIH Director’s Challenge Award (Levy). The analytic component of this project was funded by the Division of Intramural Research, NHLBI, and the Center for Information Technology, NIH, Bethesda, MD. This study used the computational resources of the Biowulf system at the NIH, Bethesda, MD ([https://hpc.nih.gov/](https://mail.nih.gov/owa/redir.aspx?C=BnFcCCa8hWVJIpMb8L6Ubn5yJ8cqrbJn06vxHuYc_jRNzbTbBW_UCA..&URL=https%3a%2f%2furldefense.proofpoint.com%2fv2%2furl%3fu%3dhttps-3A__hpc.nih.gov_%26d%3dDwMGaQ%26c%3dqS4goWBT7poplM69zy_3xhKwEW14JZMSdioCoppxeFU%26r%3dwPfdwvVfqrAQ1YY0RjTHhabrBGGA0H59RZqBKa2kiyO7e3qrtiWhKlKhVItqBvuX%26m%3dAV3wUaG8NYJzvKWGGwUPhbTGNbtXaa1owkUGypSRM-0%26s%3dujfYvNfF-0dGH0dGlwCn8t_Rui2iCjT6ttR-uUMynyg%26e%3d)). Dr. Mendelson was partly supported by the Tommy Kaplan Fund, Boston Children’s Hospital and the NIH NHLBI K99 HL136875. The TNFα assay was supported by a Career Development Award from the American Diabetes Association (Meigs). Dr. Meigs is supported by NIDDK K24 DK080140.

GOLDN: The GOLDN project was funded by NIH NHLBI R01 HL104135 (Arnett) and this analysis was enabled by NIH NHLBI K01 136700 (Aslibekyan).

HBCS: The Helsinki Birth Cohort Study has been supported by grants from the Academy of Finland (1284859 and 12848591), the Finnish Diabetes Research Society, Folkhälsan Research Foundation, Novo Nordisk Foundation, Finska Läkaresällskapet, Signe and Ane Gyllenberg Foundation, University of Helsinki, Ministry of Education, Ahokas Foundation, Emil Aaltonen Foundation.

InCHIANTI: The InCHIANTI study baseline (1998-2000) was supported as a "targeted project" (ICS110.1/RF97.71) by the Italian Ministry of Health and in part by the U.S. National Institute on Aging (Contracts: 263 MD 9164 and 263 MD 821336).

KORA: The KORA study was initiated and financed by the Helmholtz Zentrum München – German Research Center for Environmental Health, which is funded by the German Federal Ministry of Education and Research (BMBF) and by the State of Bavaria. Furthermore, KORA research was supported within the Munich Center of Health Sciences (MC-Health), Ludwig-Maximilians-Universität, as part of LMUinnovativ. The research leading to these results has received funding from the European Union Seventh Framework Programme (FP7/2007-2013) under grant agreements # 261433 (BioSHaRe) and # 603288 (sysVASC). The German Diabetes Center is funded by the German Federal Ministry of Health (BMG) and the Ministry of Innovation, Science, Research and Technology (MIWF) of the State North Rhine-Westphalia. This study was supported in part by a grant from the German Federal Ministry of Education and Research (BMBF) to the German Center for Diabetes Research (DZD e.V.)

LBC1921: Phenotype collection in the Lothian Birth Cohort 1921 was supported by the UK’s Biotechnology and Biological Sciences Research Council (BBSRC), The Royal Society and The Chief Scientist Office of the Scottish Government. Methylation typing was supported by Centre for Cognitive Ageing and Cognitive Epidemiology (Pilot Fund award), Age UK, The Wellcome Trust Institutional Strategic Support Fund, The University of Edinburgh, and The University of Queensland. REM, JMS, and IJD are members of the University of Edinburgh Centre for Cognitive Ageing and Cognitive Epidemiology (CCACE). CCACE is supported by funding from the BBSRC, the Economic and Social Research Council (ESRC), the Medical Research Council (MRC), and the University of Edinburgh as part of the cross-council Lifelong Health and Wellbeing initiative (MR/K026992/1).

NAS: This study is supported by grants from the National Institute of Environmental Health Sciences (NIEHS) (R01ES021733, R01ES025225-01A1, ES015172, ES014663 and ES020010) and Environmental Protection Agency (EPA) grant RD832416. The US Department of Veterans Affairs (VA) Normative Aging Study (NAS) is supported by the Cooperative Studies Program/ERIC, US Department of Veterans Affairs, and is a research component of the Massachusetts Veterans Epidemiology Research and Information Center (MAVERIC). The views expressed in this paper are those of the authors and do not necessarily represent the views of the US Department of Veterans Affairs. Additional support was provided by the US Department of Agriculture, Agricultural Research Service (contract 53-K06-510).

NFBC66: The present analysis was supported by the European Union’s Horizon 2020 research and innovation programme under grant agreement #633212 (Wielscher). NFBC1966 received financial support from the Academy of Finland (project grants 104781, 120315, 129269, 1114194, 24300796, Center of Excellence in Complex Disease Genetics and SALVE), University Hospital Oulu, Biocenter, University of Oulu, Finland (75617), NHLBI grant R01 HL087679 through the STAMPEED program (R01 MH083268), NIH/NIMH (R01 MH63706), ENGAGE project and grant agreement HEALTH-F4-2007-201413, EU FP7 EurHEALTHAgeing-277849, the Medical Research Council, UK (G0500539, G0600705, G1002319, PrevMetSyn/SALVE) and the MRC, Centenary Early Career Award. The program is currently being funded by the H2020-633595 DynaHEALTH action, academy of Finland EGEA-project (285547) and EU H2020 ALEC project (Grant Agreement 633212).

RS: The generation and management of the methylation array data for the Rotterdam Study was executed by the Human Genotyping Facility of the Genetic Laboratory of the Department of Internal Medicine, Erasmus MC, the Netherlands. The methylation data were funded by the Genetic Laboratory of the Department of Internal Medicine, Erasmus MC, and by the Netherlands Organization for Scientific Research (NWO; project number 184021007). The Rotterdam Study is funded by Erasmus Medical Center and Erasmus University, Rotterdam, Netherlands Organization for the Health Research and Development (ZonMw), the Research Institute for Diseases in the Elderly (RIDE), the Ministry of Education, Culture and Science, the Ministry for Health, Welfare and Sports, the European Commission (DG XII), and the Municipality of Rotterdam.

Data access: Participant-level phenotype and genotype data from the Framingham Heart Study are accessible from the U.S. National Center for Biotechnology Information (NCBI) database of Genotypes and Phenotypes (dbGaP) at <https://dbgap.ncbi.nlm.nih.gov/> to approved scientific investigators pursuing research questions that are consistent with the informed consent agreements provided by individual research participants. The FHS methylation data are available at dbGaP under the accession number phs000724.v2.p9 and gene expression data at accession number phs000363.v3.p6.

**Disclosures**

OHF works in ErasmusAGE, a center for aging research across the life course funded by Nestlé Nutrition (Nestec Ltd.), Metagenics Inc., and AXA. Nestlé Nutrition (Nestec Ltd.), Metagenics Inc., and AXA had no role in the design and conduct of the study; collection, management, analysis, and interpretation of the data; and preparation, review or approval of the manuscript.

Other authors report no conflict of interest.

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Figure 1 [title]. Epigenome-wide associations between DNA methylation and circulating TNFa in the discovery stage (N=4,794).

Figure 1 [legend]. The X-axis displays the chromosome number, and the Y axis displays –log10(P) for each of the CpG sites (colored dots). The red horizontal line denotes the FDR = 0.05 threshold for statistical significance.



Table 1. Characteristics of the participating cohorts.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Study** | **N** | **Country** | **Mean Age, years ± SD** | **% Female** | **Mean TNFα pg/ml ± SD** | **TNFα Assay** |
| Discovery Phase |
|  FHS | 1730 | USA | 67.0 ± 9.0 | 52 | 1.4 ± 1.2 | ELISA (R&D Systems) |
|  GOLDN | 970 | USA | 47.8 ± 16.3 | 52 | 3.2 ± 1.7 | ELISA (R&D Systems) |
|  InCHIANTI | 498 | Italy | 62.8 ± 15.8 | 55 | 4.3 ± 2.2 | ELISA (R&D Systems) |
|  KORA | 800 | Germany | 69.0 ± 4.3 | 49 | 2.5 ± 2.2 | ELISA (R&D Systems) |
|  LBC1921 | 165 | UK | 86.7 ± 0.4 | 54 | 1.5 ± 1.6 | Immunonepheometry (Dade-Behring) |
|  NAS | 631 | USA | 74.6 ± 6.8 | 0 | 55.0 ± 155.8 | Milliplex Human Cytokine/Chemokine Panel (EMD Millipore) |
| Replication Phase |
|  NFBC66 | 667 | Finland | 31.0 ± 0.3 | 56 | 7.8 ± 9.0 | ELISA (Merck) |
|  HBCS | 149 | Finland | 63.3 ± 2.7 | 0 | 16.7 ± 47.6 | Milliplex Map Human Metabolic Hormone Panel Kit (HMH-34K) |

FHS, Framingham Heart Study; GOLDN, Genetics of Lipid Lowering Drugs and Diet Network; HBCS, Helsinki Birth Cohort Study; InCHIANTI, Invecchiare in Chianti Study; KORA, Kooperative Gesundheitsforschung in der Region Augsburg Study; LBC1921, Lothian Birth Cohort 1921; NAS, Normative Aging Study; NFBC66, Northern Finland Birth Cohort 1966; TNFα, tumor necrosis factor α

Table 2. Associations of methylation sites and circulating TNFα.a

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  |  |  |  | **Discovery** | **Replication** |
| **CpG site** | **Chr** | **Position**b | **Gene** | **β ± SE** | P | **β ± SE (NFBC66)** | **P (NFBC66)** | **β ± SE** **(HBCS)** | **P (HBCS)** |
| **cg16411857** | **16** | **57023191** | **NLRC5** | **-0.009 ± 0.001** | **2.95×10-10** | **-0.009 ± 0.003** | **0.003** | 0.002 ± 0.005 | 0.62 |
| cg13683939 | 9 | 136152547 | intergenic; proximal to *ABO* | 0.04 ± 0.008 | 1.31**×**10-8 | -0.02 ± 0.01 | 0.18 | Did not pass QC |
| **cg07839457** | **16** | **57023022** | **NLRC5** | **-0.01 ± 0.002** | **2.49×10-8** | **-0.01 ± 0.004** | **0.0003** | -0.009 ± 0.007 | 0.23 |
| cg21549285 | 21 | 42799141 | *MX1* | -0.01 ± 0.002 | 6.09**×**10-8 | -0.006 ± 0.003 | 0.09 | -0.00004 ± 0.005 | 0.99 |
| cg11601443 | 12 | 113415930 | *OAS2* | -0.004 ± 0.0008 | 2.78**×**10-7 | -0.001 ± 0.001 | 0.39 | -0.003 ± 0.002 | 0.25 |
| cg00959259 | 3 | 122281975 | *DTX3L;PARP9* | -0.01 ± 0.002 | 4.39**×**10-7 | Did not pass QC | 0.0009 ± 0.007 | 0.90 |
| **cg08122652** | **3** | **122281939** | ***DTX3L;PARP9*** | **-0.006 ± 0.001** | **4.75×10-7** | **-0.007 ± 0.002** | **0.003** | -0.0003 ± 0.003 | 0.92 |
| cg22107533 | 15 | 45028083 | *TRIM69* | -0.006 ± 0.001 | 9.13**×**10-7 | -0.005 ± 0.002 | 0.01 | 0.001 ± 0.004 | 0.77 |
| cg05439368 | 15 | 45028098 | *TRIM69* | -0.006 ± 0.001 | 1.02**×**10-6 | -0.006 ± 0.002 | 0.01 | 0.002 ± 0.004 | 0.67 |
| cg22930808 | 3 | 122281881 | *DTX3L;PARP9* | -0.008 ± 0.002 | 2.52**×**10-6 | -0.008 ± 0.004 | 0.04 | -0.004 ± 0.005 | 0.50 |

aModel adjusted for age, sex, white blood cell proportions, technical covariates, smoking, and BMI with FDR < 0.05 in the discovery stage. Results that met the Bonferroni threshold in the replication stage (P=0.05/10=0.005) are in bold.

bhg19

Chr, chromosome; CpG, cytosine-phosphate-guanine; HBC, Helsinki Birth Cohort, NFBC66, Northern Finland Birth Cohort 1966; TNFα, tumor necrosis factor α

Table 3. Associations between methylation status of top TNFα CpG sites and neighboring gene expression.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **CpG site** | **Chr** | **Transcript** | **P RS** | P FHS | **P KORA** | **Direction of Association** |
| N |  |  | 750 | 2,262 | 726 |  |
| cg16411857 | 16 | NLRC5 | 0.0002 | 2.56**×**10-8 | 0.16 | --- |
| cg07839457 | 16 | NLRC5 | 2.10**×**10-7 | 1.85**×**10-8 | 0.44 | --- |
| cg00959259 | 3 | *DTX3L* | 1.07**×**10-6 | 1.80**×**10-9 | 0.80 | --+ |
| *PARP9* | 2.86**×**10-22 | 2.58**×**10-13 | 0.81/0.07a | ---- |
| *PARP14* | 9.21**×**10-23 | 6.51**×**10-17 | n/a | --? |
| cg08122652 | 3 | *DTX3L* | 2.91**×**10-7 | 2.13**×**10-6 | 0.61 | --- |
| *KPNA1* | 0.0003 | 0.00004 | n/a | ++? |
| *PARP9* | 1.04**×**10-25 | 1.09**×**10-9 | 0.12/0.26a | ---- |
| *PARP14* | 1.25**×**10-24 | 8.48**×**10-15 | n/a | --? |

aTwo probes (ILMN\_1731224 and ILMN\_2053527) corresponded to *PARP9* in KORA.

Chr, chromosome; CpG, cytosine-phosphate-guanine; FHS, Framingham Heart Study; KORA, Kooperative Gesundheitsforschung in der Region Augsburg Study; TNFα, tumor necrosis factor α

Table 4. Associations between incident CHD and methylation status of top TNFα CpG sites in a meta-analysis of 8 cohorts with 1,895 disease events and 11,641 participants.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **CpG site** | **Chr** | **Position**a | **Gene** | **β ± SE** | **P** |
| cg16411857 | 16 | 57023191 | NLRC5 | -0.15 ± 0.05 | 0.003 |
| cg07839457 | 16 | 57023022 | NLRC5 | -0.12 ± 0.03 | 3.1×10-5 |
| cg00959259 | 3 | 122281975 | *DTX3L;PARP9* | -0.10 ± 0.03 | 0.002 |
| cg08122652 | 3 | 122281939 | *DTX3L;PARP9* | -0.21 ± 0.05 | 2.0×10-5 |

ahg19

Chr, chromosome; CpG, cytosine-phosphate-guanine