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Epigenetic DNA methylation changes associated with headache chronification: A retrospective case-control study

Bendik S. Winsvold^{1,2*}, Priit Palta^{3,4*}, Else Eising^{5*}, Christian M. Page^{2,6*}, *The International Headache Genetics Consortium*, Arn M. J. M. van den Maagdenberg^{5,7}, Aarno Palotie^{3, 8-12}, John-Anker Zwart^{1,2}

1 FORMI and Department of Neurology, Oslo University Hospital, PO Box 4956 Nydalen, 0424 Oslo, Norway.

2 Institute of Clinical Medicine, University of Oslo, 0316, Oslo, Norway

3 Institute for Molecular Medicine Finland (FIMM), University of Helsinki, Tukholmankatu 8, Helsinki, Finland.

4 Estonian Genome Center, University of Tartu, Tartu, Estonia

5 Department of Human Genetics, Leiden University Medical Center, Leiden, The Netherlands

6 Norwegian Institute of Public Health, PO Box 4404 Nydalen, 0403 Oslo, Norway.

7 Department of Neurology, Leiden University Medical Center, Leiden, The Netherlands

8 Psychiatric and Neurodevelopmental Genetics Unit, Massachusetts General Hospital and Harvard Medical School, Boston, USA.

9 Medical and Population Genetics Program, Broad Institute of MIT and Harvard, Cambridge, USA.

10 Stanley Center for Psychiatric Research, Broad Institute of MIT and Harvard, Cambridge, USA.

11 Analytic and Translational Genetics Unit, Massachusetts General Hospital and Harvard Medical School,

12 Department of Neurology, Massachusetts General Hospital, Boston, USA

* The authors contributed equally to this work.

Supplemental Information: Supplemental Methods; Supplemental Figure 1;
Supplemental Table 1.

Correspondence should be addressed to:

Bendik S. Winsvold, Department of Neurology, Oslo University Hospital, Pb 4956

Nydalen, 0424 Oslo, Norway

Tel: +47 97604500

email: bendik.s.winsvold@gmail.com

Abstract

Background: The biological mechanisms of headache chronification are poorly understood. We aimed to identify changes in DNA methylation associated with the transformation from episodic to chronic headache.

Methods: Participants were recruited from the population-based Norwegian HUNT-Study. Thirty-six female headache patients who transformed from episodic to chronic headache between baseline and follow-up 11 years later were matched against 35 controls with episodic headache. DNA methylation was quantified at 485,000 CpG sites, and changes in methylation level at these sites were compared between cases and controls by linear regression analysis. Data were analyzed in two stages (Stages 1 and 2) and in a combined meta-analysis.

Results: None of the top 20 CpG sites identified in Stage 1 replicated in Stage 2 after multiple testing correction. In the combined meta-analysis the strongest associated CpG sites were related to *SH2D5* and *NPTX2*, two brain-expressed genes involved in

the regulation of synaptic plasticity. Functional enrichment analysis pointed to processes including calcium ion binding and estrogen receptor pathways.

Conclusion: In this first genome-wide study of DNA methylation in headache chronification several potentially implicated loci and processes were identified. The study exemplifies the use of prospectively collected population cohorts to search for epigenetic mechanisms of disease.

Key words: chronic headache; epigenetics; cohort studies

Introduction

In a number of patients who suffer from episodic headache attacks, their headache transforms into a chronic form (1, 2). Chronic headache, defined as headache occurring on at least 15 days per month, affects 3-5% of the general population and 40% of patients in headache clinics, and is associated with severe disability and impaired quality of life (1, 3). Approximately half of these patients have chronic migraine (1). Established risk factors for headache chronification include the overuse of acute headache medications and a high baseline attack frequency (4). The biological mechanisms involved in the transition from episodic to chronic headache are however unknown.

In recent years it has become evident that epigenetic processes play an important role in a range of multifactorial diseases (5). Epigenetics entail changes in gene expression that are not due to variation in the DNA sequence. A main epigenetic mechanism is DNA methylation, the covalent addition of a methyl group to the 5th carbon of cytosine residues, which is typically associated with gene silencing (5, 6). Epigenetic modifications can be stably inherited through cell divisions but can also be dynamic. They enable cell and developmental stage-specific regulation of gene expression, and play an important role in programming lasting responses to environmental cues (6, 7). Due to the ability of DNA methylation changes to shift a biological system from one stable state to another, their implication in headache chronification is of particular interest. It has been hypothesized that frequent headache attacks may lower the threshold for subsequent headache attacks through epigenetic mechanisms resulting in a feed-forward loop (6). Similarly, psychological stress and female sex hormones,

which have been implicated in migraine genesis, are known to exert their physiological effects partly through epigenetic mechanisms (6, 8).

In this study, subjects were recruited from the Norwegian population-based Nord-Trøndelag Health (HUNT) Study, where they gave blood samples and were assessed for headache at two time points 11 years apart. In order to identify changes in DNA methylation associated with headache chronification we compared cases, who transformed from episodic to chronic headache during the follow-up period, with controls who did not develop chronic headache. For all selected subjects, genome-wide DNA methylation levels were characterized at baseline and follow-up.

Materials and Methods

Participants

In the HUNT2 (1995–1997) and HUNT3 (2006–2008) studies, the entire population of the Norwegian county of Nord-Trøndelag, 20 years of age or older, was invited to complete extensive questionnaires concerning health-related issues, participate in a clinical examination and donate blood samples. Among 92,566 individuals in HUNT2 and 94,194 in HUNT3 invited to participate, 51,383 (56%) and 39,701 (42%), respectively, answered the headache questionnaire (9, 10).

The headache classification used in this study has been described in detail elsewhere (9-11). Briefly, in each survey those who answered 'yes' to the screening question 'Have you suffered from headache during the last year?' were defined as headache sufferers and asked to fill in the other headache questions. Participants indicated the number of days with headache per month, with the options < 7, 7-14 or > 14

days/month in HUNT2 and the options < 1, 1-6, 7-14 or > 14 days/month in HUNT3.

Chronic daily headache (CDH) was defined as headache occurring on ≥ 15 days/month. In HUNT2, acute medication overuse was defined as ‘analgesic use daily or almost daily for ≥ 1 month during the last 12 months’, and in HUNT3 as ‘over-the-counter-medication use for headache ≥ 4 days/week during the last month’.

Medication-overuse headache was defined as the combination of CDH and acute medication overuse (9, 11, 12). This headache classification has been validated by interview diagnoses (9, 10). In HUNT2, the sensitivity and specificity were, respectively 85% and 83% for headache suffering (Cohen's kappa $\kappa = 0.57$), and 91% and 56% for headache frequency < 7 days per month ($\kappa = 0.50$). In HUNT3, the sensitivity and specificity were, respectively 88% and 86% for headache suffering ($\kappa = 0.70$), 69% and 99% for CDH ($\kappa = 0.75$) and 75% and 100% for medication-overuse headache ($\kappa = 0.75$).

Cases for the current study were selected among participating women who at the time of HUNT2 (baseline) were ≤ 35 years of age and reported headache on < 7 days/month, and at the time of HUNT3 (follow-up) reported ≥ 15 headache days/month (CDH). To avoid sample heterogeneity resulting from different DNA methylation patterns in men and women, only women were included. Likewise, to avoid DNA methylation changes resulting from the onset of menopause during follow-up only women ≤ 35 years of age at baseline were included. In total 43 subjects fulfilled these criteria. After excluding samples with missing DNA from either baseline or follow-up, 40 cases were included in the study. We selected an equal number of controls, who were matched with cases on their headache frequency and age at baseline, but at follow-up either did not have headache anymore or had

headache on < 7 days/month. Average time between baseline and follow-up examinations was 11.4 years (range 10-12.6 years).

Ethics

Signed informed consent was obtained from all participants in the HUNT2 and HUNT3 surveys, and the present study was approved by the Regional Committee for Medical Research Ethics (2012/2316/REK midt).

Genome-wide quantification of DNA methylation

A detailed description of sample handling, quantification of DNA methylation, data normalization and quality control is given in **Supplemental Methods**. Briefly, we performed array quantification of DNA methylation on genomic DNA isolated from leukocytes, after bisulfite conversion, using the Illumina Infinium HumanMethylation450 BeadChip (Illumina, San Diego, CA, USA). For Stage 1, 96 samples from 24 cases and 24 controls were analyzed. For Stage 2, 64 samples from 16 cases and 16 controls were analyzed. The methylation level at each CpG site for each sample was calculated as a β -value, scaling from 0 (not methylated) to 1 (fully methylated). After sample-wise quality control steps, 36 cases and 35 controls contributed to the final analysis. An overview of the study design and participants in the final analysis are given in **Figure 1**. After marker-wise quality control steps, out of 485,775 CpG markers initially interrogated 431,282 and 436,517 markers were available for analysis in Stage 1 and Stage 2, respectively.

Analytic approach

Baseline characteristics

Baseline characteristics were compared between cases and controls by Fisher's exact test (for binary variables) or independent samples *t*-test (for continuous variables).

The examined variables included known or suggested risk factors for the development of chronic daily headache (4, 13); age (continuous); low educational level (upper secondary school or less as highest education); self-reported daily use of tranquilizers last year (yes, no); current daily smoking (yes, no); low physical activity (< 1 hour light exercise per week); body-mass index (continuous); Hospital Anxiety and Depression Scale (HADS) for each of anxiety and depression (continuous) (14), chronic musculoskeletal complaints (yes, no) (15); gastrointestinal complaints (yes, no) (16), self-reported diabetes mellitus (yes, no) and self-reported rheumatoid or osteoarthritis (yes, no). Of the participants included in the study, 3 had missing information on physical activity and 1 on chronic musculoskeletal complaints.

Association analysis and replication

The effect of headache chronification (case-control status) was modeled on changes in DNA methylation levels. For each individual, normalized methylation level estimate (β -value) at baseline was subtracted from methylation level estimate at follow-up for each CpG site. Resulting methylation level changes at each CpG site were then compared between cases and controls using linear regression model adjusted for age, using the *lm* package in R software (17). Data from each stage was analyzed separately and the 20 CpG sites showing strongest association with chronification in Stage 1 were taken forward for replication in Stage 2. A Bonferroni-corrected p-value threshold ($0.05/20 = 0.0025$) was used to consider statistically significant replication.

Meta-analysis

Subsequently, a fixed-effects meta-analysis was performed on DNA methylation data from Stage 1 and Stage 2, weighted by the inverse variance, using the GWAMA software (18). In total 436,549 CpG markers were present in either stage after quality control and could be used in the analysis, resulting in a Bonferroni-corrected p-value threshold of 1.15×10^{-7} ($0.05/436,549$).

Characterization of top-ranking CpG sites

Functional profiling of the meta-analysis top-ranking CpG sites was carried out using g:Profiler web-based software (19), see **Supplemental Methods** for a full description. Briefly, g:Profiler performs a statistical gene set enrichment analysis to find functional gene groups or biological pathways that are significantly over-represented in user-provided input. The results presented are based on the 200 top-ranking CpG sites from the meta-analysis, and results were considered significant if the multiple testing corrected p-value for the enriched group or pathway was ≤ 0.05 .

Characterization of gene expression in various human tissues was retrieved from the GTEx database (20). Although DNA methylation is largely tissue-specific, inter-individual variation in DNA methylation has been shown to correlate between blood and brain for a subset of CpG sites (21). Correlation of DNA methylation levels between blood and brain at the top CpG sites was analyzed using the Blood Brain DNA Methylation Comparison tool (<http://epigenetics.essex.ac.uk/bloodbrain>) that utilizes DNA methylation data of blood and four different brain regions from matched samples (21).

Analysis of medication overuse

Overuse of acute headache medications is a known risk factor for headache chronification. To evaluate to what extent medication overuse influenced the identified associations, the association analysis and meta-analysis was repeated stratified by medication overuse. Cases with or without medication overuse at follow-up were analyzed separately against all controls. Results were visualized as forest plots using the *metafor* R package (version 1.9-8) (22). Additionally, unsupervised clustering of the samples was performed using the *hclust* R package, based on their methylation levels at follow-up at each of the 200 top-ranking CpG sites from the initial meta-analysis.

Results

Demographic variables and potential risk factors for CDH did not differ between cases and controls at baseline (**Table 1**). However, four participants reported medication overuse at baseline, and all four later developed CDH. Likewise no difference between cases and controls was found for the listed variables when Stage 1 and Stage 2 were analyzed separately (data not shown). Average time between baseline and follow-up examinations was 11.4 years (range 10-12.6 years). At the time of follow-up 17 of 36 cases (47%) and 0 of 35 controls reported overuse of acute headache medication.

The 20 CpG sites that demonstrated the strongest association with headache chronification in Stage 1 are listed in **Table 2**. Using the Bonferroni-corrected statistical significance level of 0.0025, none of these CpG sites reached statistical significance for replication in Stage 2. When data from the two stages were combined

in a fixed-effects meta-analysis, no CpG sites reached the multiple-testing corrected study-wide significance threshold of 1.15×10^{-7} . The most strongly associated CpG marker was cg01010870 (p-value = 6.17×10^{-6}), located near the transcription start site of the gene *SH2D5* (**Table 3**) that is highly expressed in various regions of the human brain (**Supplemental Figure 1**). The second most strongly associated CpG marker cg11086695 (p-value = 6.95×10^{-6}) is intergenic, located 76 kb downstream from *NPTX2* and 141 kb downstream from *TMEM130*. For 6 of the top 10 CpG markers (**Table 3**), methylation levels in blood showed high correlation with methylation levels in one or several regions of the human brain at p-value < 0.05 (21), exemplified for the most significantly associated CpG site (cg01010870) in **Figure 2**.

Genomic regions corresponding to the 200 top-ranking CpG sites from the meta-analysis were significantly enriched for genes belonging to specific biological functions (**Table 4, Supplemental Table 1**). Statistically strongest enrichment was observed for "calcium ion binding" (p-value = 1.21×10^{-5}), followed by "biological adhesion" (p-value = 4.23×10^{-5}), "intracellular estrogen receptor signaling" (p-value = 0.02), and "negative regulation of homotypic cell-cell adhesion" (p-value = 0.04).

When stratifying the meta-analysis for overuse of acute headache medications among cases, the associations for the 10 top-ranking CpG sites from the initial meta-analysis had the same direction and were of comparable strength in each of the two groups (**Figure 3**). Likewise, unbiased hierarchical cluster analysis based on the 200 top-ranking CpG sites did not discriminate between cases with and without medication overuse (**Figure 4**).

Discussion

This is, to our knowledge, the first study of epigenetic changes in headache chronification. Using a retrospective case-control design, DNA methylation changes were examined across the genome comparing patients who converted from episodic to chronic headache during a 11 year follow-up period with matched controls. In the discovery stage several CpG sites showed suggestive association to headache chronification, but these failed to replicate in the second stage. Combining association results from both stages in a fixed-effects meta-analysis we identified CpG sites that may point to genes involved in headache chronification. An enrichment was found for genes related to calcium ion binding, biological adhesion, and intracellular estrogen receptor signaling.

Since cases in this study reported episodic headache at baseline and CDH at the time of follow-up, it is likely that they represent *transformed* rather than *de-novo* chronic headache. A limitation is however that the questionnaire-based assessment did not allow distinction of chronic migraine from other types of chronic headache. Previous epidemiological studies suggest that approximately half of those with CDH fulfill criteria for chronic migraine (1), making it likely that migraineurs contribute substantially to our case group.

Medication overuse is a major risk factor for headache chronification, and it has previously been shown that medication overuse headache accounts for about half of the participants who report CDH in the HUNT population (13). A similar proportion was seen in this study with 17 of 36 cases (47%), but none of the controls, reporting medication overuse at the time of follow-up. Notably, all 4 participants who reported

medication overuse at baseline had developed chronic headache at follow-up. Given our design, it is feasible that at least some of the DNA methylation changes result from medication use. Still, when stratifying the meta-analysis results by cases with and without medication overuse, the strength and direction of association for the top 10 loci from the meta-analysis were similar between the groups (**Figure 3**). Also, unsupervised hierarchical clustering of the samples based on methylation levels for the top 200 CpG sites did not discriminate cases with from cases without medication overuse (**Figure 4**). The results therefore suggest that medication overuse is unlikely to explain the identified associations.

The most significant CpG site is at the *SH2D5* gene, which encodes the SH2 Domain Containing 5 protein. The second strongest associated CpG site is 76 kb downstream from the nearest gene *NPTX2*, which encodes the Neuronal Pentraxin II protein. Both proteins are highly expressed in adult human brain (23, 24) (**Supplemental Figure 1**), and DNA methylation levels at the associated CpG sites are highly correlated between brain and blood (**Table 3 and Figure 2**). Both proteins have presumed roles in regulating synaptic plasticity. The SH2 Domain Containing 5 protein regulates synaptic plasticity through the control of Rac-GTP levels (25). Neuronal Pentraxin II regulates synaptic plasticity by acting as an inhibitor of excitatory synapses, through binding and clustering of glutamatergic AMPA receptors. Neuronal Pentraxin II is induced by neuronal activity, and has an assumed function in rebalancing excitation-inhibition after periods of increased synaptic activity (23). Of interest, another of the top associated CpG markers is related to glutamate signaling. *GRID2* encodes the glutamate receptor $\delta 2$, an ionotropic glutamate receptor which is expressed in several brain regions, with highest expression in the cerebellum (20, 26). Epigenetic

alteration of synaptic plasticity has been previously hypothesized as a possible mechanism of migraine chronification (6). Support for this comes from the experimental finding that synchronous neuronal activity, which also occurs in cortical spreading depression of the migraine aura (27), results in DNA methylation changes in brain-specific genes related to neuronal plasticity (28). One may envisage that as neuronal activity can cause epigenetic changes altering synaptic plasticity, migraine attacks in a feed-forward loop may promote subsequent migraine attacks leading to chronification. Our findings seem to provide at least some support for a role of synaptic plasticity in headache chronification. Regardless, it is far from clear whether such changes are a cause or consequence of frequent headache attacks. Furthermore, our findings may implicate that glutamatergic activity is implicated not only in migraine pathogenesis (29), but also in the transition from episodic to chronic headache.

When considering the more extensive list of 200 top-ranking CpG sites, there is a statistically significant enrichment in genes related to several specific functional groups and pathways. The strongest enrichment was seen for calcium ion binding. Calcium ion binding and signaling is important in a wide range of cellular systems, including the synaptic release of neurotransmitters (30). While this presents a plausible mechanism for calcium ion imbalance in migraine pathogenesis, no genetic association to this or other calcium channels have been found for the common forms of migraine (31). The second strongest enrichment was found for genes involved in biological cell adhesion. Cell adhesion molecules have multiple roles in tissue development and function. In the human brain they play a major role at synapses, where they control synaptic morphology and plasticity, and at the neurovascular unit

they form the molecular interface between the vascular and the central nervous system and are involved in the complex signaling between these systems (32, 33). Lastly, in this study of female subjects, we found enrichment for genes involved in estrogen receptor signaling. This corresponds well with lines of evidence suggesting a role of estrogen in migraine pathogenesis (34). Estrogen receptor signaling has also been discussed specifically in relation to possible epigenetic mechanisms in migraine, since the estrogen receptors predominantly assert their action through the epigenetic programming of target genes (6).

Strengths of this study include the genome-wide assessment of DNA methylation sites, which allowed us to search for genetic loci associated with headache chronification without relying on previous hypotheses. Also, the headache classification used in this study has been validated by clinical interview. Given the longitudinal design we could determine DNA methylation levels in both cases and controls at two time points, before and after the development of chronic headache, which gave the possibility to detect changes in DNA methylation levels occurring during the natural course of the disease.

Our study has clear limitations. Despite a large baseline population of 51,000 individuals with available DNA who were characterized with respect to headache, and a follow-up time of 11 years, only 36 cases fulfilled inclusion criteria and could be used in the final analysis. This resulted in limited statistical power, as indicated by the failure to replicate signals identified in the discovery stage in the smaller replication sample. We therefore focused our subsequent analyses on the combined dataset of both stages in order to maximize the power to detect true signals. The loci identified

from the combined analysis aggregate in functionally meaningful pathways, indicating the presence of true signals. Nevertheless, the reported associations will require replication in independent samples, but given our complex design this will be challenging. This clearly demonstrates the difficulty of designing studies of epigenetic changes during the natural course of incident disease, and suggests that international collaboration is required to achieve robust sample sizes when using such a study design. Another limitation was that we determined DNA methylation in leucocytes from whole blood, whereas DNA methylation patterns are to a considerable extent tissue-specific, so our findings may not be representative of changes occurring in the more relevant tissue in headache, in particular the brain (8). However, for the majority of the top associated markers, DNA methylation levels in blood correlated well with DNA methylation levels in one or more brain regions, suggesting that our design has good potential to detect DNA methylation relevant to migraine. This is also in line with previous studies that have found that DNA methylation patterns in non-affected tissues such as blood can be of value, even leading to treatment decisions (35).

A better understanding of the mechanisms underlying headache chronification will be important for developing better strategies to treat and prevent headaches in these severely affected patients. Epigenetic changes represent modifiable factors, and thereby offer potential targets for novel drug therapies (5). Although candidate regions identified in this study require independent replication and further characterization, this is a first step towards understanding epigenetic changes occurring in headache chronification.

Article highlights

- The two CpG sites with strongest association to headache chronification were found in relation to genes that regulate synaptic plasticity.
- Associated CpG sites also showed enrichment for genes involved in calcium ion binding, cell adhesion and estrogen receptor pathways.
- The results suggest possible mechanisms by which epigenetic DNA methylation may be involved in the chronification of headache.

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Conflict of interest Statement

None declared.

Figure legends

Figure 1: Diagram of the study design and participants. A total of 36 female headache patients who transformed from episodic to chronic headache ('cases') between examinations at baseline and follow-up were matched against 35 female headache patients who did not transform to chronic headache ('controls').

Figure 2: Correlation of DNA methylation in blood and four brain regions for cg01010870 (SH2D5). Top: Boxplot of the distribution of DNA methylation values for cg01010870 split by tissue. Bottom: Four scatterplots demonstrating the relationship between DNA methylation in whole blood and four brain regions: PFC=pre-frontal cortex; EC=entorhinal cortex; STG=superior temporal gyrus; CER=cerebellum. Plots are retrieved using the Blood Brain DNA Methylation Comparison tool (21).

Figure 3. Forest plots showing meta-analysis results separately for cases with and without medication overuse. Meta-analysis beta-value (Beta) and its 95% confidence intervals (95% CI) for the top 10 CpG sites (cg01010870 to cg14118171) from the meta-analysis.

Figure 4: Unsupervised clustering of cases based on the top 200 CpG sites from the meta-analysis. Unsupervised hierarchical clustering using methylation status at

follow-up did not discriminate between cases with (marked in yellow) and without medication overuse in either Stage 1 (A) or Stage 2 (B).

Supplemental Figure 1. Relative expression of SH2D5 in various human tissues.

Retrieved from the GTEx database (20). RPKM=Reads Per Kilobase of transcript per Million mapped reads. Box plots are shown as median and 25th and 75th percentiles; points are displayed as outliers if they are above or below 1.5 times the interquartile range.

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Epigenetic DNA methylation changes associated with headache chronification: A retrospective case-control study

SUPPLEMENTAL METHODS

Blood sampling and DNA isolation

Blood samples were donated from subjects in the HUNT2 or HUNT3 studies at the time of inclusion. Serum was separated from whole blood within 2 hours by centrifugation, placed in a refrigerator together with a blood clot or EDTA whole blood, and transported in a cooler within 1 - 3 days to the HUNT Biobank, where it was stored at -70°C . DNA was isolated from leukocytes from thawed blood clots or EDTA whole blood by using Gentra Puregene Blood Kit (Qiagen Diagnostics GmbH, Hamburg, Germany) or MasterPure DNA purification Kit for blood, Buffy Coat DNA Purification Protocol (Epicentre) and stored at -20°C . DNA concentrations were quantified using PicoGreen reagents, and every eight normalized sample was spectrophotometrically tested a second time using NanoDrop (ND1000 or ND8000) (Thermo Fisher Scientific Inc., Waltham, MA, USA). Purity was assessed based on the A260/A280 ratios.

Genome-wide quantification of DNA methylation

Bisulfite conversion and array quantification of DNA methylation was performed in two separate stages. In Stage 1, 96 samples from 24 cases and 24 controls were analyzed at the Institute for Molecular Medicine Finland (FIMM), Helsinki, Finland; in Stage 2, 64 samples from 16 cases and 16 controls were analyzed at the Wellcome Trust Sanger Institute, Cambridge, UK. Bisulfite conversion of 1 μg of the DNA sample was performed using the EZ-96 DNA Methylation Kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's protocol. 5 μl of the bisulfite converted DNA were used for genome-wide methylation profiling using the Illumina Infinium HumanMethylation450 BeadChip (Illumina, San Diego, CA, USA) according to manufacturer's protocol. After hybridization and washing, the BeadChips were scanned using the Illumina HiScan SQ scanner. The efficiency of the bisulfite conversion was checked according to the manufacturer's protocol.

Data normalization and quality control

Raw image data were imported into the GenomeStudio (Illumina version 2011.1) Methylation module (version 1.9), which was used to extract the fluorescent signal intensities and pre-normalize the data. Briefly, background signal intensity was subtracted from the raw signal intensity values and data were normalized with respect to the Illumina internal control probes. The methylation level was calculated as a β -value (*AVG_Beta*), scaling from 0 (not methylated) to 1 (fully methylated), representing the fraction of methylated DNA molecules at the corresponding CpG site in the assayed sample. These data were exported from the GenomeStudio as standard Illumina report files and further normalized and analyzed with custom scripts. Data for stages 1 and 2 were jointly quantile-normalized (1) with R software (version 3.2.3) (2) as described by Grundberg et al. (3), followed by the PCA analysis of β -values to detect any potential outliers and other quality control steps.

In sample-wise quality control steps, altogether 9 samples were excluded. Of the 40 cases and 40 controls selected for methylation typing (24 cases and 24 controls

in Stage 1; 16 cases and 16 controls in Stage 2), samples were excluded because of low DNA concentration ($n = 2$); because they were red staining outliers ($n = 2$); had gender mismatch between methylation typing and phenotype annotation ($n = 1$); or were sample outliers as identified by PCA analysis ($n = 4$). Samples were also excluded if after above-mentioned exclusions they did not have data for both time points available, resulting in 36 cases (23 in Stage 1 and 13 in Stage 2) and 35 controls (21 in Stage 1 and 14 in Stage 2) contributing to the final analysis.

Marker (CpG site) wise quality control was carried out similarly to Grundberg and colleagues (3). Briefly, out of 485,775 CpG markers initially interrogated by the HumanMethylation450 chip, all markers were excluded that had a call rate ≤ 0.9 ; overlapped with single nucleotide variants with minor allele frequency $\geq 5\%$ in the CEU panel of the 1000Genomes Project (4); or overlapped with copy number variants in 1000Genomes Project. After quality control 431,282 and 436,517 markers were available for analysis in Stage 1 and Stage 2, respectively.

Genomic annotation of interrogated CpG sites

To assign each assayed CpG marker to their corresponding gene, genomic and gene-level annotations provided by Illumina (HumanMethylation450_15017482_v.1.1.csv, based on the human reference sequence assembly version GRCh37/hg19) were used. In order to get the latest genomic annotation of the meta-analysis top-ranking CpG sites for functional profiling, chromosomal coordinates of the top 1000 CpG sites were converted to the current version of the human reference sequence assembly (GRCh38/hg38) with the liftOver web-based batch conversion tool (<http://genome.ucsc.edu/cgi-bin/hgLiftOver>, by using default parameters).

Blood cell composition estimates

White blood cell (WBC) composition is regarded a main source of variation in DNA methylation analyses of whole blood. To check for possible WBC composition biases in our samples, the WBC composition was estimated from the DNA methylation profiles using the R-package minfi (version 1.8.9) developed by Houseman et al. (5). No significant differences were found in white blood cell composition between cases and controls in either Stage 1 or Stage 2.

Functional profiling of top-ranking CpG sites using g:profiler

Functional profiling of the meta-analysis top-ranking CpG sites was carried out by utilizing g:Profiler web-based software (version r1536, <http://biit.cs.ut.ee/gprofiler>) using Ensembl 83 genomic annotations, Gene Ontology release 2016-01-21 and KEGG biological pathways release 2016-01-25 (6). Considering all genes in user-provided gene lists or chromosomal regions, g:Profiler performs statistical gene set enrichment analysis to find which functional gene groups or biological pathways are significantly over-represented in user-provided input. As our input CpG site coordinates were ordered by their meta-analysis association p-values, the ‘Ordered query’ option was used, and we also required at least 3 CpG site-related genes in any tested functional group or pathway (option ‘Size of query / term intersection’). Only CpG markers that were successfully called and showed the same effect direction in both stages were included, in order to enrich the input for true positive signals. The optimal size of the input set of CpG sites for the gene set enrichment analysis depends on a balance between including a sufficient number of sites to enable the detection of enrichment, while limiting the input set so that the highest possible proportion of true signals is obtained. To this end, the analysis was run using incrementally larger input

sets, including 10, 20, 50, 100 and 200 meta-analysis top-ranking CpG markers respectively. The sets including 10, 20, 50 and 100 markers did not return significant enriched functional groups or pathways and are therefore not presented. The results were considered significant if the multiple testing corrected p-value for enriched group or pathway was ≤ 0.05 .

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