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RESEARCH ARTICLE

Genetically defined elevated homocysteine levels do not result in widespread changes of DNA methylation in leukocytes

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Data Availability Statement: We are not allowed to publish the full data on internet because of privacy policy. However, data can be accessed by individual researchers through these contacts for each individual study: Requests for the data from the Rotterdam study may be sent to: Frank van Rooij (f.vanrooij@erasmusmc.nl). Requests for the data from the Lothian Birth Cohort may be sent to Riccardo Marioni (rmarioni@exseed.ed.ac.uk). LBC methylation data have been submitted to the European Genome-phenome Archive -accession
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number EGAS00001000910. The informed consents given by KORA study participants do not cover data posting in public databases. However, data are available upon request from KORA-gen (https://epi.helmholtz-muenchen.de/). Data requests can be submitted online and are subject to approval by the KORA Board. Requests for the data from the Framingham Heart Study may be sent to Roby Jøeanes (robyjoeanes@phls.harvard.edu) or from the link: https://www.ncbi.nlm.nih.gov/projects/gap/cgi-bin/study.cgi?study_id=phs000724.v6.p10. Requests for the data from the Biobank-Based Integrative Omics Studies (BIOS) Consortium (RS, LLS, LL, CODAM and NTR cohorts) may be sent to Rick Jansen (R.Jansen@ggzingeest.nl) and from the accession number EGAC00001000277. Requests for the data from the MARTHA study may be sent to David-Alexandre Tréguoët (david.tregouet@upmc.fr). MARTHA Methylation data are already available in the Array Express database (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-3127. Request for the data from the F5L study may be sent to Roby Joehanes (robyjoehanes@hsl.harvard.edu) or from the link: https://www.ncbi.nlm.nih.gov/projects/gap/cgi-bin/study.cgi?study_id=phs000724.v6.p10. Requests for the data from the F5L study may be sent to France Gagnon (france.gagnon@utoronto.ca).

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Abstract

Background

DNA methylation is affected by the activities of the key enzymes and intermediate metabolites of the one-carbon pathway, one of which involves homocysteine. We investigated the effect of the well-known genetic variant associated with mildly elevated homocysteine: MTHFR 677C>T independently and in combination with other homocysteine-associated variants, on genome-wide leukocyte DNA-methylation.

Methods

Methylation levels were assessed using Illumina 450k arrays on 9,894 individuals of European ancestry from 12 cohort studies. Linear-mixed models were used to study the association of additive MTHFR 677C>T and genetic-risk score (GRS) based on 18 homocysteine-associated SNPs, with genome-wide methylation.

Results

Meta-analysis revealed that the MTHFR 677C>T variant was associated with 35 CpG sites in cis, and the GRS showed association with 113 CpG sites near the homocysteine-associated variants. Genome-wide analysis revealed that the MTHFR 677C>T variant was associated with 1 trans-CpG (nearest gene ZNF184), while the GRS model showed association with 5 significant trans-CpGs annotated to nearest genes PTF1A, MRPL55, CTDSP2, CRYM and FKBP5.

Conclusions

Our results do not show widespread changes in DNA-methylation across the genome, and therefore do not support the hypothesis that mildly elevated homocysteine is associated with widespread methylation changes in leukocytes.

Introduction

DNA methylation, an important epigenetic mechanism has gained interest in the field of cancer and aging over the last decade [1, 2]. DNA methylation is affected by the activities of the key enzymes and intermediate metabolites of the one-carbon pathway, one of which involves homocysteine (Hcy).

Our aim was to investigate the role of genetically defined Hcy levels on genome-wide DNA methylation. A number of earlier studies have reported a link between Hcy and DNA methylation [3]. In these studies, DNA methylation was quantified as a global measure, that represents the total methyl cytosine content of the DNA. In animal models, both diet- and genetically-induced elevated Hcy have been related to altered global methylation patterns in tissues of aorta, brain, liver and colon. In human subjects, global DNA methylation in blood was not consistently altered with elevated Hcy. The relationship between Hcy and methylation can be subject to substantial bias, given the strong relationship between several lifestyle factors, diseases and Hcy. A way to circumvent this bias is to use genetic factors determining Hcy concentrations as an instrument to study the relationship between Hcy and methylation. The use of
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Materials and methods

Study population

All participants provided a written informed consent, and each study was approved at the relevant organizations by their respective ethics review committees [RS, Institutional review board (Medical Ethics Committee) of the Erasmus Medical Center; LLS, Ethical committee of the Leiden University Medical Center; LL, Ethics committee of the University Medical Centre Groningen; NTR, Central Ethics Committee on Research Involving Human Subjects of the VU University Medical Centre; CODAM, Medical Ethical Committee of the Maastricht University; MARTHA, "Departement santé de la direction générale de la recherche et de l’innovation du ministère" (Projects DC: 2008–880 & 09.576); EGCUT; Research Ethics Committee of the University of Tartu; F5L, Research ethics boards of the University of Toronto and the Ottawa Hospital Research Institute; FHS, IRB (institutional review board); KORA, Local Ethics Committee; LBC1921, Lothian Research Ethics Committee (Wave 1: LREC/1998/4/183); LBC1936, Multi-Centre Research Ethics Committee for Scotland (Wave 1: MREC/01/0/56), and the Lothian Research Ethics Committee (Wave 1: LREC/2003/2/29)]. The analyses comprised of large population with 9,894 participants from 12 cohorts of European ancestry. Most of the cohorts were part of either the Cohorts for Heart and Aging Research in Genomic Epidemiology (CHARGE) consortium [11] and/or Biobank-based Integrative Omics Studies (BIOS) consortium [12]. All participants provided a written informed consent for the DNA collection and its use for genetic analyses. Each study was approved at the relevant organizations by their respective ethics review committees. Cohort-specific characteristics are provided in the S1 Text and S1 Table.

**MTHFR 677C>T and homocysteine-associated SNPs**

18 independent Hcy-associated SNPs from our GWAS meta-analysis [5], were selected to assess the relationship between mildly elevated Hcy concentrations and genome-wide DNA methylation. The genotypes of these SNPs were extracted from the genotyping data. Cohort-specific details of the quality control and the SNP imputation methods are provided in the S2 Table.
DNA methylation assessment

Whole blood samples were collected from the participants for DNA extraction. The genomic DNA was bisulfite converted using the Zymo EZ-96 DNA-methylation kit (Zymo Research, Irvine, CA, USA). Methylation profiling was performed using the Infinium Illumina Human-Methylation 450k BeadChip arrays (Illumina Inc., San Diego, USA) according to the manufacturers’ protocol. Beta values from 0 to 1, which represent the percentage of methylation, were calculated from the extracted raw methylated (M) and unmethylated (U) probe intensities and a default alpha (α) of 100. This is defined by the formula of β = M/(M+U+α). Normalization was performed on these raw beta values using DASEN [13] or SWAN [14] methods. Poor quality probes were excluded based on the detection p-values mostly >0.01 in >5% of samples. Cohort-specific data preprocessing methods are provided in the Table 1. Global methylation levels per sample was calculated by the mean of all CpGs as well as mean according to CpG islands, shores, shelves or non-coding regions [15].

Statistical analysis

Two models were run independently by each participating study. Firstly, an additive model for MTHFR 677C>T alone was used to investigate its independent association with genome-wide DNA methylation in a linear manner. For the MTHFR 677C>T variant, genotypes were coded as CC = 0, CT = 1 and TT = 2 to study the effect in methylation per MTHFR 677T allele.

In the second analysis, a weighted Genetic Risk Scores (GRS) was constructed from all the 18 Hcy-associated variants to investigate their combined and additive effect on genome-wide DNA methylation. Weighted GRS were calculated on the basis of their effect sizes [5] and number of corresponding risk alleles. The product of the two was calculated for each SNP and then summed up for all SNPs. The GRS was calculated using the equation below, where N is the number of elevated Hcy causing risk alleles for each SNP (0, 1 or 2 per genotype).

\[
GRS = 0.1583 x N(rs1801133 : A) + 0.0542 x N(rs2275565 : G) + 0.0718 x N(rs234709 : C) + 0.0435 x N(rs466306 : T) + 0.0453 x N(rs1801222 : A) + 0.101 x N(rs12134663 : C) + 0.0529 x N(rs12750845 : A) + 0.056 x N(rs2851391 : T) + 0.0449 x N(rs9369898 : A) + 0.0422 x N(rs831333 : A) + 0.0864 x N(rs7422339 : A) + 0.1242 x N(rs7130284 : C) + 0.0963 x N(rs1514657 : A) + 0.0597 x N(rs548987 : C) + 0.0395 x N(rs42648 : G) + 0.0512 x N(rs2251468 : C) + 0.045 x N(rs957140 : G) + 0.090 x N(rs12921383 : C)
\]

Both the analyses were based on linear mixed models of lme4 package in R. We also analyzed the effect of MTHFR 677C>T and GRS on global methylation levels, where we calculated the overall mean levels per individual as well as categorized the means as per CGI annotations [15]. The models were adjusted for technical covariates and biological covariates like age, sex and differential white blood cell (WBC) counts (see S1 Table for details about covariates for each cohort). The technical covariates were cohort-specific and treated as random effects. WBC counts were either used as measured counts, or they were imputed based on the Houseman method as implemented in the minfi package [17], or the modified version of the Houseman method (Documentation and R script: https://github.com/mvaniterson/wbccPredictor) that uses partial least-squares [18] to handle multivariate responses and high-dimensional covariates and has been previously used [19]. This method from van Iterson used the R package pls [18] to fit the linear model based on the DNA methylation data, to predict the white blood cell composition as percentages that sum up to almost 100%. Age and gender were used as covariates.
Table 1. Details of methylation 450k pre-processing. Quality control, normalization and association model.

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Cohorts</th>
<th>Detection p-value criteria</th>
<th>Cross-reactive &amp; polymorphic</th>
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<th>Criteria (Method)</th>
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<th>Dye bias correction</th>
<th>Normalization method</th>
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<td></td>
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<td>No</td>
<td>None</td>
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<td>Array, array position, plate, hybridization date</td>
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<td>No</td>
<td>None</td>
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<td>SWAN</td>
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</table>

Probes with SNPs at SBE & probes with improper binding [16], probes that were absent in 8 or more studies, cis-probes with <5 Mb distance from Hcy-SNPs.
Meta-analysis

Summary statistics for the two models were obtained from each study. Because of the different probe exclusions in each cohort [Table 1], we removed probes that were present in ≤4 studies. We also excluded probes with SNPs at single base extension site, and probes with improper binding [12], leaving a total of 465,694 probes for the meta-analysis. Meta-analysis was performed using the fixed effect model in METAL [20], with the classical approach that uses effect size estimates and standard errors as input obtained from the individual study summary statistics for each CpG probe. The output of meta-analysis gave the combined effect size estimates, standard errors and p-values per probe. These p-values were corrected using the Benjamini Hochberg method of false discovery rate (FDR), where FDR < 0.05 was considered statistically significant. For the MTHFR 677C>T model, positive effect sizes correspond to percentage increase in methylation per MTHFR 677T allele. For the GRS model, positive effect sizes correspond to percentage increase in methylation per unit increase in GRS. We also took into account heterogeneity of the meta-analysis by $I^2$ which was calculated using METAL per probe and excluded significant probes if $I^2 < 40$. We also calculated the genomic inflation factor ($\lambda$) [21] to estimate the inflation in test statistics that may be caused by population structure or other unknown confounding factors. This $\lambda$ was estimated for the distribution of p-values using the median method, which is defined as the ratio of the observed median of the test statistic distribution and the expected median 0.455 [21, 22].

Genomic Regions Enrichment of Annotations Tool (GREAT) was used for annotating CpGs for nearby genes, that assigns a basal regulatory region to extend up to 5 kb upstream and 1 kb downstream from its transcription start site and a maximum extension distance up to 1 Mb [23], as defined by UCSC [24]. Furthermore, strength of the instrument or allele score was calculated using the F-statistics, using the tool, mRnd [25]. We took into account cohort heterogeneity $I^2$ and excluded significant probes if $I^2 < 40$.

Identifying cis- and trans-CpG effects

We defined the CpGs as “cis” when the CpG was annotated within 1Mb upstream or downstream of the SNP. Trans-CpGs were defined as CpGs that were associated with the SNP, and were annotated >1Mb apart. We defined the CpGs in the GRS model the same way by accounting for the bp distance of the CpGs from each of the 18 SNPs. For the significant trans-CpGs that were 1-5Mb apart from any of the 18 SNPs, we performed a conditional analysis adjusting for that SNP to investigate whether they were trans-CpGs associated with Hcy GRS or long range cis-CpGs driven by the nearby SNPs that were part of the GRS. In the conditional analysis, if the bonferroni corrected p-values were no longer significant, we considered those trans-CpGs as long-range cis-CpGs. For the significant trans-CpGs that were >5Mb apart from any of the 18 SNPs, we looked up for their tested individual association with each of the 18 SNPs in our previous trans-CpG mapping analysis [12]. This is to see whether the association of these trans-CpGs was Hcy GRS driven or driven by a single SNP that was a part of GRS. In order to confirm these trans-CpG effects, we performed a similar conditional analysis by including the respective SNP as a covariate in the model. Both conditional analyses were performed on a subset of 3,786 samples from 6 cohorts, and the results were compared with the unconditional analysis in the same subset.

H19/IGF locus

Three Differentially Methylated Regions (DMRs) of IGF2/H19 locus at chromosome 11 have been reported to be related with homocysteine [26, 27]. We identified seven CpGs on the 450k array that were underlying the three DMRs of this locus, for their association with MTHFR
677C>T variant or GRS. Bonferroni method was applied on these 7 CpGs to check for multiple testing.

**Enrichment of folate-associated CpGs**
We further focused our analysis on the 443 previously identified CpGs, of which methylation in cord blood of newborns were associated with maternal plasma folate levels [28]. We compared the p-values of these 443 CpGs from the MTHFR 677C>T and the GRS results, and compared them to the p-values of 100 random CpGs with 1000 permutations, to check for their significant enrichment, using the Fisher’s exact test.

**Results**

**Population characteristics**
The meta-analysis included 9,894 adults from 12 cohorts. Studies were population-based, except for the Cohort on Diabetes and Atherosclerosis Maastricht, MARseille THrombosis Association study and the French-Canadian family study, where individuals were selected based on mildly increased diabetes mellitus type 2 and cardiovascular risk factors, cases of venous thrombosis and probands with venous thromboembolism, respectively.

**Meta-analysis of MTHFR 677C>T and GRS model**
The explained variance in Hcy by MTHFR 677C>T is 5.3% [4] and by GRS is 5.9% [5]. For a sample size of 9,894, the F-statistics of the additive MTHFR 677C>T and GRS was 554 and 621, respectively. Meta-analysis of 456,694 probes identified 35 cis- [S3 Table] and 1 trans-[S5 Table, Table 2] CpGs for the MTHFR model [Fig 1] and 113 cis- [S4 Table] and 30 trans-

<table>
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<tr>
<th>CpG N Beta</th>
<th>SE</th>
<th>P</th>
<th>FDR</th>
<th>I²</th>
<th>Beta</th>
<th>SE</th>
<th>P</th>
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Beta: Regression coefficients
SE: Standard errors of the regression coefficients
FDR: False discovery rate adjusted P-value, threshold = 0.05
I²: Heterogeneity I² parameter
*Promoter-associated
*Enhancer-associated, Enhancer annotation from Illumina 450k annotation

https://doi.org/10.1371/journal.pone.0182472.t002
The \( \lambda \) was 1.01 for the MTHFR 677C>T SNP and 0.92 for GRS [S1 Fig]. Cis-CpGs Meta-analysis on 465,694 CpGs of the MTHFR 677C>T variant showed association with 35 cis-CpGs on chromosome 1 with FDR < 0.05 [Fig 1, S3 Table]. These cis-CpGs showed a range from 2.4% increase to 1.7% decrease in methylation per MTHFR 677T allele. The nearest genes associated with this cis-region included MTHFR itself, AGTRAP, CLCN6, NPPA, NPPB, PLOD1, MFN2 and TNFRSF8. For the GRS model, we observed 113 cis-CpGs with FDR < 0.05 [Fig 2, S4 Table]. Out of the 113, 16 cis-CpGs showed overlap with the MTHFR 677C>T analysis, which involved a smaller region of 238 Kb [Fig 3].

**Fig 1. Manhattan plot.** Association between MTHFR 677C>T (rs1801133) and genome-wide DNA methylation in 9,894 samples, with 35 cis-meQTLs at chromosome 1 (black/grey) and 1 trans-meQTL at chromosome 6 (green) with FDR<0.05.

https://doi.org/10.1371/journal.pone.0182472.g001
Trans-CpGs

For the MTHFR 677C>T model, meta-analysis of 465,694 CpGs identified 1 significant trans-CpG which was located on chromosome 6 [Fig 1, S5 Table]. This trans-CpG (cg05411165) showed 1% decrease in methylation per MTHFR T allele. It was annotated near ZNF184 (25414 bp upstream) and HIST1H2BL (309398 bp downstream). For the GRS model, we observed 30 significant trans-CpGs [Fig 2]. These trans-CpGs showed a range from 5.6% increase to 5.1% decrease in methylation per 0.1 unit increase in GRS. Of these 30 trans-CpGs, 23 were negatively associated with the GRS model. To assess overlap between two models, we evaluated association of the trans-CpG of the MTHFR 677 C>T model within the GRS model. This trans-CpG (cg05411165) showed a 10% decrease in methylation in the GRS model but was not FDR significant (raw p-value = 0.01).

Critical evaluation of the 30 trans-CpGs of GRS model demonstrated that 14 trans-CpGs were located in a large region of 3.08 Mb length within chromosome 6. The GRS model consists of 18 SNPs including a SNP on chromosome 6. The 14 trans CpG identified with the GRS

Fig 2. Manhattan plot. Association between GRS of 18 Hcy-associated SNPs and genome-wide DNA methylation in 9,894 samples, with 113 cis-meQTLs (black/grey) and 30 trans-meQTLs (green), at FDR<0.05.

https://doi.org/10.1371/journal.pone.0182472.g002
model were at a distance between 1 and 5 Mb away from the Hcy-associated variant rs548987 of the \textit{SLC17A3} gene at chromosome 6 [Table a in S6 Table].

**Conditional analysis: Chromosome 6 region near rs548987.** To investigate whether the 14 \textit{trans}-CpGs on chromosome 6 near rs548987 were influenced by this variant, we performed conditional analysis on a subset of 3,786 samples from 6 cohorts. After correction of the model
for rs548987 as a covariate, none of the 14 trans-CpGs were significant at a Bonferroni threshold of 3.57E-03 [Table b in S6 Table].

**Conditional analysis: Influence of SNPs within the GRS model.**

To further investigate the remaining 16 trans-CpGs from the 30, whether any of them were driven by a single variant, rather than the combined effect of the 18 homocysteine-associated variants, we checked the trans-CpG mapping analysis of the single SNPs using the BIOS dataset [12]. We observed that 7 of the 16 remaining trans-CpGs located >5 Mb from the Hcy-associated variants, were directly associated with either rs548987 SNP of SLC17A3 gene at chromosome 6, or rs154657 SNP of DPEP1 gene at chromosome 16 [Table a in S7 Table]. After correction for these 7 trans-CpGs by including the respective SNP as a covariate in the model, none of the 7 trans-CpGs remained significant at a Bonferroni threshold of 7.14E-03 [Table b in S7 Table]. After correction for cis-effects of Hcy-associated SNPs in the GRS model, we identified a remaining list of 9 Hcy-associated trans-CpGs, 4 of which had substantial heterogeneity I² [S8 Table].

**Overlapping trans-CpG between MTHFR and GRS models**

When doing a lookup in the MTHFR 677C>T model for the finally identified 5 trans-CpGs, all of them showed similar direction of effect, but did not achieve genome-wide significance (Lowest raw p-value = 3.36E-03) [Table 2].

**Trans-CpGs affecting Gene expression**

We evaluated whether methylation levels of the observed trans-CpG from the MTHFR 677C>T model and 5 trans-CpGs from the GRS model were associated with expression levels of the nearby genes, in the BIOS dataset [12]. None of the trans-CpGs was associated with mRNA expression differences of nearby genes.

**H19/IGF2 locus**

We specifically focused on the IGF2-H19 region for differential methylation, since methylation at this locus has repeatedly been linked to the homocysteine metabolism in a number of studies [29–31]. S4 Fig shows the results of the whole IGF2-H19 region, and the 7 CpGs annotated to 3 DMRs of the IGF2/H19 gene that had previously been reported to be differentially methylated (DMR0, DMR2, H19-DMR3). Data from our 450k arrays contained 2 CpGs at DMR0, 4 CpGs at DMR2 and 1 CpG at H19-DMR3. None of them showed an association with MTHFR 677C>T or GRS with a Bonferroni cut off of 7.14E-03 [S9 Table, S4 Fig].

**Enrichment of previously found folate-associated CpGs**

Next we focused on a set of 443 CpGs that were identified to be differentially methylated in children at birth according to the folate levels in the mothers [28]. We found a highly significant enrichment for significant p-values in the MTHFR model in the 443 CpGs as compared to a random set of other CpGs (>3 times enrichment of significant p-values, enrichment p = 0.0079). However, we did not find a significant enrichment for the GRS model.

**Global DNA methylation changes**

In addition to genome-wide DNA methylation changes we analyzed the effect of MTHFR 677C>T and GRS models on overall mean methylation levels. There was no significant association between the MTHFR 677C>T or GRS on global methylation overall or mean methylation of CpG islands, shores, shelves or non-coding regions [Table 3] [15].
This is the first large-scale study to investigate the effect of Hcy-associated SNPs on genome-wide DNA methylation using the Illumina 450k arrays in 9,894 individuals. The results showed no widespread trans-effects of the MTHFR 677C>T SNP on DNA methylation, apart from 1 trans-CpG at chromosome 6. The GRS model showed 5 trans-CpGs, after carefully examining the direct effects of individual SNPs with conditional analyses.

In this current study, we used MTHFR 677C>T independently and the combined weighted GRS of the 18 Hcy-associated variants [5], to test whether mildly elevated Hcy concentrations induce DNA methylation changes in blood cells. Our goal was to investigate genetically defined elevated Hcy on genome-wide DNA methylation. The use of genetic variants is less sensitive to confounding and bias as compared to classical epidemiological studies [32].

We calculated the strength of our exposure variables: MTHFR 677C>T and GRS using the F-statistics. For a strong exposure, the value of the F-statistics is expected to be greater than 10 [33]. With our large sample size (n = 9,894) and proportion of variance explained being 5.3 to 5.9%, the F-statistics was 554 and 621 respectively, indicating very high strength and enough power of our analysis. However, we did not observe widespread trans-effects despite of having strong additive MTHFR 677C>T and GRS.

We observed a single trans-CpG for the MTHFR 677C>T variant. This CpG is located near ZNF184 and HIST1H2BL. Both these genes are thought to play a role in transcriptional regulation. For the GRS, we found 30 trans-CpGs associated, 14 of which are spread over a region of 3,08 Mb at chromosome 6. These CpGs were annotated to genes that included ZNF322 and HIST1H2BJ, HLA-J, HLA-A, HLA-G, but also the proximal region of ZFP57 gene, which was previously identified as a folate-sensitive region in a genome-wide methylation study of 23 women [34]. However, when we performed a conditional analysis on these 14 CpGs in this region, by adjusting for the nearby variant rs548987 of the SLC17A3 gene, the effect sizes significantly attenuated and the nominal p-values were no more significant. The results indicate that this region was influenced by the rs548987 SNP of SLC17A3 gene and was not Hcy-associated.
We finally observed 5 trans-CpGs associated to genetically defined Hcy using the GRS, after carefully examining the direct effects of individual SNPs with conditional analyses and discarding CpGs that showed substantial cohort heterogeneity. A total of 3 CpGs showed hypo-methylation, one of which was annotated to the FKBP5 gene. FKBP5 encodes for the FK506-binding protein 51 (FKBP51) whose expression has recently been shown to decrease DNMT1 activity and thereby decreasing global methylation [35].

Furthermore, when looking at our methylation-expression results [12], none of the 5 trans-CpGs was associated with mRNA expression differences of nearby genes. The possible explanation for these negative findings could be that these CpG sites might have an effect further away on trans-genes. Conversely, it has been shown that the methylation-expression correlation in cis are not best predicted using the CpG position alone, but by using specific chromatin marks [36]. Furthermore, it could also be that these correlations are specific to other tissues, but not in blood.

We observed that the IGF2-H19 locus did not show association with methylation according to genetically defined elevated homocysteine. This is in contrast to the previous findings in mice, where tissue-specific changes in H19 DMR methylation were found in liver, brain and aorta, and increased expression of H19 was found in aorta [26, 27]. Similar to what we found, the H19-DMR3 between CBS deficient patients and controls also did not show a significant difference, in a previous study [29]. Our results show that this imprinted locus is not deregulated by long-term genetically defined mildly elevated homocysteine. However, previously it was reported that MTHFR 677C>T variant shows changes in DNA methylation in peripheral blood mononuclear cells, only through an interaction with folate [7]. Hence, further studies are needed to study the effect of MTHFR 677C>T variant in the presence of blood folate levels.

We did not see widespread methylation changes associated to mildly elevated plasma Hcy concentrations. This result is not in line with a number of earlier reports, which have shown global methylation changes in association with the MTHFR 677C>T variant and Hcy concentrations [3]. Previous two studies on this topic have shown contradictory results. There has been reports that showed a lower circulating global methylation level in individuals with the MTHFR 677TT genotype [6,7]. However, there are also a few negative studies that showed no relation between the MTHFR 677TT genotype and global methylation levels [8–10]. All these studies had modest sample sizes (upto 300 individuals were studied), and measured methylation on a global level using the LINE-1 assay, which measures a repetitive sequence, of which the function is unknown. In contrast, we here studied a genome wide site-specific analysis focused on functional regions of the genome [15]. We here show convincing evidence that there is no association between the MTHFR 677C>T or GRS on overall methylation levels, nor is there a relationship between methylation of CpG islands, shores, shelves or non-coding regions separately, which supports the previous null associations.

Furthermore, in order to test for causal effect in a mendelian randomization study, an instrument, which is in our case MTHFR 677C>T or GRS, should satisfy the 3 basic assumptions [37, 38]. One, the instrument should be associated with the exposure, which is in our case Hcy. Two, the instrument should not affect the outcome, which is in our case DNA methylation, except through the exposure Hcy. Three, the instrument should not be associated with any confounder of the exposure-outcome association. Although assumptions one and two are satisfied in our case [5], the GRS model might violate assumption three [37, 38].

The GRS model contains a few SNPs which are, in addition to the association with Hcy, also associated with other traits. For example, the variants near to HNF1A gene have been associated with a number of other traits [39–44]. This could also be the reason why the results of the MTHFR 677C>T and GRS models are quite different. Nevertheless, the MTHFR
677C>T variant explains most of the variation in Hcy, as compared to the other variants in
the GRS model and is therefore a strong instrument to examine the effect of deregulation
of the one-carbon metabolism on methylation.

The relationship between MTHFR 677C>T and DNA methylation is modified by folate lev-
els. Only in individuals with low folate status, the effect of the MTHFR 677TT genotype is seen
[7]. Unfortunately, we were unable to study this interaction, since folate levels were not avail-
able in our study. Another prerequisite to be able to perform MR is that the relationship
between Hcy and methylation is known. The relationship between Hcy and DNA methylation
is only known in studies until now where methylation is measured at a global level. Therefore,
the estimation of the causal effect could not be done. Unfortunately, we also did not have Hcy
data available in all cohorts of this study, and therefore were unable to perform a full mendel-
lian randomization study. We rather focused on the association of genetically defined elevated
Hcy levels with DNA methylation.

We did not find widespread differences in methylation related to genetically defined homo-
cysteine levels. The association was not observed in global methylation levels nor in wide-
spread CpGs including the previously known H19/IGF2 locus. There are a number of possible
explanations for this finding. First, it is known that the relationship between MTHFR 677C>T
and DNA methylation is modified by folate levels, as described above. The effect of MTHFR
677C>T is seen in individuals with low folate status [7], which could have masked possible
relationships between the MHTFR variant and methylation. A second possible explanation for
the relative low number of identified CpGs, is that we have studied the wrong tissue. Most
methylation measures are conducted in blood leukocytes as this tissue is readily available.
However, the causal effect of Hcy could be specific to other tissues like liver, heart and brain.
Therefore, the possible effect of mildly elevated Hcy on such specific tissues cannot be
excluded. Third, there is little variation in the one-carbon metabolism in the normal popula-
tion. This metabolism is pivotal to cell survival and function and therefore tidily regulated. It
could be that there is a correlation between homocysteine and more pronounced effects on
methylation when homocysteine levels are more extreme.

Conclusions
We observed 1 \textit{trans}-CpG (nearest genes ZNF184 and \textit{HIST1H2BL}) on chromosome 6 associ-
ated with the MTHFR 677C>T variant. The GRS model showed 5 significant \textit{trans}-CpGs,
which do not overlap with the MTHFR \textit{trans}-CpG. In conclusion, our results do not show
widespread statistically significant \textit{trans}-effects of MTHFR and GRS models, and therefore do
not support the hypothesis that genetically defined mildly elevated Hcy concentrations are
associated with widespread methylation changes in leukocytes. More studies with measured
Hcy concentrations are needed to confirm this.

Supporting information
\textbf{S1 Fig. Quantile-quantile plots.} Association of (a) MTHFR 677C>T and (b) Genetic risk
score of all the 18 Hcy-associated variants with genome-wide DNA methylation.
(PDF)

\textbf{S2 Fig. Forest plot.} \textit{Trans}-meQTL of the MTHFR 677C>T model across 12 cohorts.
(PDF)

\textbf{S3 Fig. Forest plots.} \textit{Trans}-meQTLs of the Genetic risk score model across 12 cohorts.
(PDF)
S4 Fig. Regional manhattan plots. (a) MTHFR 677 C>T variant or (b) Genetic risk score associated 3 DMRs of IGF2/H19 genes containing 7 CpGs (green) from the 450k data; DMR0 “Chr.11:2,170,380–2,170,517” with 2 CpGs, DMR2 “Chr.11:2,154,113–2,154,414” with 4 CpGs and H19-DMR3 “Chr.11:2,021,072–2,021,273” with 1 CpG.

(PDF)

S1 Table. Cohort characteristic.
(PDF)

S2 Table. Details of genotyping methods. Quality control of SNPs and imputation.
(PDF)

S3 Table. 35 cis-meQTLs with FDR<0.05 that are associated with MTHFR 677C>T variant (rs1801133) in a samples size of 9,894. The CpGs are sorted in base pairs.
(PDF)

S4 Table. 113 cis-meQTLs with FDR<0.05 that are associated with Genetic Risk Score of 18 Hcy-associated variants in a sample size of 9,894. The CpGs are sorted in chromosomes and base pairs.
(PDF)

S5 Table. Trans-meQTLs with FDR<0.05 that are associated with MTHFR 677C>T variant (rs1801133) in a sample size of 9,894.
(PDF)

S6 Table. (a) 14 trans-meQTLs of chromosome 6 with FDR<0.05 that are associated with Genetic Risk Score of 18 Hcy-associated variants in a sample size of 9,894, and were 1Mb-5Mb away from the SNP rs548987 of SLC17A3 gene. (b) Conditional analysis for the 14 trans-meQTLs of chromosome 6 with adjustment for the SNP rs548987 of SLC17A3 gene in a subset of 3,786. The CpGs are sorted in base pairs.
(PDF)

S7 Table. (a) 7 genome-wide trans-meQTLs with FDR<0.05 that are associated with Genetic Risk Score of 18 Hcy-associated variants in a sample size of 9,894 and were a direct trans-meQTL of either SNP rs548987 of SLC17A3 gene or rs154657 SNP of DPEP1 gene. (b) Conditional analysis for the 7 genome-wide trans-meQTLs with adjustment for their respective SNP rs548987 of SLC17A3 gene at chromosome 6 or rs154657 SNP of DPEP1 gene at chromosomes 16 in a subset of 3,786.
(PDF)

S8 Table. 9 genome-wide trans-meQTLs with FDR<0.05 that are associated with Genetic Risk Score of 18 Hcy-associated variants in a sample size of 9,894. The CpGs are sorted in chromosomes and base pairs.
(PDF)

S9 Table. (a) MTHFR 677C>T variant associated DMPs at the 3 IGF2/H19 DMR regions at chromosome 11. (b) GRS associated DMPs at the 3 IGF2/H19 DMR regions at chromosome 11.
(PDF)

S1 Text. Description of cohorts.
(PDF)
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Rotterdam Study

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Biobank-Based Integrative Omics Studies (BIOS) Consortium

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References


