RESEARCH ARTICLE

Genome-Wide DNA Methylation Profiles Indicate CD8+ T Cell Hypermethylation in Multiple Sclerosis

Steffan D. Bos1,2‡, Christian M. Page1,2‡, Bettina K. Andreassen3,4, Emon Elboudwarej5, Marte W. Gustavsen1,2, Farren Briggs5, Hong Quach5, Ingvild S. Leikfoss1,2, Anja Bjølgerud1,2, Tone Berge1, Hanne F. Harbo1,2, Lisa F. Barcellos5

1 Department of Neurology, Oslo University Hospital, Oslo, Norway, 2 Institute of Clinical Medicine, University of Oslo, Oslo, Norway, 3 Oslo Centre for Biostatistics and Epidemiology, Department of Biostatistics, University of Oslo, Oslo, Norway, 4 Epi-Gen, Institute of Clinical Medicine, Akershus University Hospital, University of Oslo, Oslo, Norway, 5 Genetic Epidemiology and Genomics Laboratory, Division of Epidemiology, School of Public Health, University of California, Berkeley, United States of America

‡ These authors contributed equally to the manuscript.
* s.d.bos@medisin.uio.no

Abstract

Objective
Determine whether MS-specific DNA methylation profiles can be identified in whole blood or purified immune cells from untreated MS patients.

Methods
Whole blood, CD4+ and CD8+ T cell DNA from 16 female, treatment naïve MS patients and 14 matched controls was profiled using the HumanMethylation450K BeadChip. Genotype data were used to assess genetic homogeneity of our sample and to exclude potential SNP-induced DNA methylation measurement errors.

Results
As expected, significant differences between CD4+ T cells, CD8+ T cells and whole blood DNA methylation profiles were observed, regardless of disease status. Strong evidence for hypermethylation of CD8+ T cell, but not CD4+ T cell or whole blood DNA in MS patients compared to controls was observed. Genome-wide significant individual CpG-site DNA methylation differences were not identified. Furthermore, significant differences in gene DNA methylation of 148 established MS-associated risk genes were not observed.

Conclusion
While genome-wide significant DNA methylation differences were not detected for individual CpG-sites, strong evidence for DNA hypermethylation of CD8+ T cells for MS patients was observed, indicating a role for DNA methylation in MS. Further, our results suggest that large DNA methylation differences for CpG-sites tested here do not contribute to MS...
suscceptibility. In particular, large DNA methylation differences for CpG-sites within 148 estab-
established MS candidate genes tested in our study cannot explain missing heritability. Larger
studies of homogenous MS patients and matched controls are warranted to further eluci-
date the impact of CD8+ T cell and more subtle DNA methylation changes in MS develop-
ment and pathogenesis.

Introduction

Multiple sclerosis (MS) is a chronic, inflammatory disease of the central nervous system (CNS)
and the leading cause of disability in the young Western population[1]. The knowledge of the
underlying mechanisms is sparse, but points to a complex interplay between common genetic
and environmental factors. Genome-wide association studies (GWAS) and earlier genetic stud-
ies have identified 110 MS-associated loci and alleles of the HLA-DRB1 (most frequently
*15:01) and HLA-A (*02) loci[2, 3]. Immunologically relevant genes, particularly those in-
volved in T-helper cell differentiation, are significantly overrepresented among MS-associated
variants[4]. Clinical and para-clinical evidence indicate MS results at least in part from inflam-
matory reactions in the CNS[5]. CD4+ T cells predominate in acute CNS lesions[6], whereas
CD8+ T cells predominate in chronic lesions[7, 8], indicating an active role for these lympho-
cyte subclasses in MS.

Recently, epigenetic modifications have been shown to influence predisposition to complex
diseases[9]. DNA methylation, the addition a methyl group to the cytosine in C-G dinucleo-
tides (CpG-sites) modulates expression of nearby genes. DNA methylation associations have
been reported for several autoimmune diseases, including Sjogren’s syndrome, systemic lupus
erythematosus and rheumatoid arthritis[10–12]. Investigation of genome-wide DNA methyla-
tion can be performed by the Infinium HumanMethylation450 BeadChip (450K)[13]. DNA
methylation of different tissues is highly diverse and influenced by environmental factors, ther-
apy or on-going disease processes[14]. Therefore, sample homogeneity is a requirement for
successful investigations of the relationship between DNA methylation and phenotypes. How-
ever, in a clinical setting heterogeneous whole blood (WB) is easily accessible for MS patients,
and whether disease relevant changes can be reliably detected in WB has not been determined.

DNA methylation studies of WB, or purified blood cells from MS patients have been per-
formed for a small number of discordant twin pairs and siblings at genome-wide scale[15], or
for candidate genes and a limited numbers of CpG-sites[16, 17]. Huynh et al. have shown that
pathogen-free brain regions of MS patients have a different global and specific DNA methyla-
tion profile as compared to healthy donor brain samples[18]. More detailed DNA methylation
profile studies in carefully characterized, homogenous MS samples are highly warranted. Here
we present genome-wide DNA methylation results from purified CD4+ and CD8+ T cells and
WB of female MS patients and healthy controls.

Materials and Methods

Samples and genotyping

A homogenous collection of 16 untreated, female Norwegian MS patients with relapsing remit-
ting MS (RRMS) and 14 age-matched female controls were included (Table 1). All patients and
controls were of self-declared Nordic ancestry. Patients were between ages 18 and 63 and re-
cruited from the MS clinic at the Oslo University Hospital, Oslo, Norway. Controls were
recruited either through the patients or among hospital employees. None of the patients had ever received immune-modulatory drugs. Patients had not experienced a relapse or received steroids in the three months prior to enrollment and fulfilled the updated McDonald MS criteria[19]. MRI of the CNS was performed within four weeks of blood sampling and the number of lesions and contrast-enhancing lesions was counted. The Extended Disability Status Scale (EDSS) was performed on the day of blood sampling.

Genome-wide single nucleotide polymorphism (SNP) genotypes for patients and controls were assessed using the Human Omni Express BeadChip (Illumina, San Diego, CA, USA). A large Norwegian GWAS dataset published earlier[20] was used to confirm Nordic ancestry of our MS patients and controls by principal component analysis (PCA) as implemented in the R (version3.0.3) software package[21] (S1A Fig.). Genotypes were imputed against the European 1000-genomes data using IMPUTE2[22]. Details on procedures are provided in S1 Materials and Methods.

### Ethics statement

The Regional Committee for Medical and Health Research Ethics South East, Norway, approved this study. Written informed consent was obtained from all study participants.

---

Table 1. Characteristics of individual MS patients and summaries of patients and controls.

| Patient | Age category1 | Years MS2 | EDSS2 | MSSS2 | OCB3 | MRI lesions | Contrast lesions MRI4 |
|---------|---------------|-----------|-------|-------|------|-------------|------------------------|
| 1       | 3             | 11        | 3.50  | 4.13  | Yes  | >20         | No                     |
| 2       | 3             | 11        | 2.00  | 2.11  | Yes  | >20         | No                     |
| 3       | 6             | 33        | 2.50  | 1.14  | No   | >20         | No                     |
| 4       | 1             | 2         | 0.00  | 0.53  | Yes  | 10–20       | No                     |
| 5       | 5             | 1         | 0.00  | 0.64  | Yes  | >20         | No                     |
| 6       | 2             | 8         | 1.50  | 1.90  | Yes  | >20         | No                     |
| 7       | 2             | 11        | 1.50  | 1.38  | No   | >20         | No                     |
| 8       | 4             | 6         | 5.00  | 7.61  | Yes  | 10–20       | Yes                    |
| 9       | 5             | 11        | 0.00  | 0.17  | Yes  | >20         | Yes                    |
| 10      | 3             | 9         | 1.00  | 0.86  | Yes  | >20         | No                     |
| 11      | 2             | 6         | 2.00  | 3.51  | Yes  | >20         | Yes                    |
| 12      | 4             | 16        | 1.00  | 0.38  | Yes  | 10–20       | No                     |
| 13      | 2             | 6         | 1.50  | 2.30  | Yes  | >20         | Yes                    |
| 14      | 5             | 3         | 2.50  | 5.98  | Yes  | 10–20       | No                     |
| 15      | 2             | 6         | 2.00  | 3.51  | Yes  | 10–20       | No                     |
| 16      | 4             | 1         | 1.00  | 2.34  | Yes  | 10–20       | No                     |

Summarized

| Patients Mean (S.D.; range) | 38.9(25–63) | 8.8(7.7; 1–33) | 1.7(1.3; 0–5) | 2.4(2.1; 0.2–7.6) | 14/16(87.5%) | N/A | 4/16(25%) |
|----------------------------|-------------|----------------|----------------|-------------------|---------------|-----|-----------|
| Controls Mean (S.D.; range) | 39.2(28–58) | N/A            | N/A            | N/A               | N/A           | N/A | N/A       |

1Age category: 1 = 25–29, 2 = 30–34, 3 = 35–39, 4 = 40–44, 5 = 45–49, 6 = 60–64.
2At inclusion in this study.
3Oligoclonal bands present in cerebrospinal fluid taken at time of diagnosis.
4Contrast enhancing lesions on MRI.

Abbreviations: EDSS = Expanded Disability Status Scale, MSSS = Multiple Sclerosis Severity Score, OCB = oligoclonal bands, MRI = Magnetic Resonance Imaging, S.D. standard deviation

doi:10.1371/journal.pone.0117403.t001
DNA methylation profiling and data normalization

CD4+ and CD8+ T cells from WB were isolated for MS patients and controls in a semi-automated manner using the autoMACS Pro Separator (Miltenyi Biotec, Germany). DNA from WB and purified CD4+ and CD8+ T cell samples was extracted and treated with bisulphite. DNA methylation levels were assessed using the 450K (Illumina, USA). Raw data were exported from Illumina’s BeadStudio and normalized using the ‘BMIQ’ algorithm described previously[23]. Analyses were performed using beta values of methylation[24]. The CD4+ sample from donor 8 and both the CD8+ and WB sample from donor 3 had technical issues and were excluded before further analysis.

In order to prevent false positive signals due to genetic variation other than DNA methylation at probes, all probes that had an observed SNP in their target sequence (N = 60,106; see S1 Materials and Methods) in our data were removed before analysis[25] (S1B Fig.). To assess consistency of cell type specific methylation profiles, PCA of overall DNA methylation was applied (Fig. 1).

**Fig 1. Principal component analyses.** For samples in analyses a PCA was performed on overall methylation levels of CpG-sites that passed both quality controls and SNP filtering in (A) whole blood (Red), CD4+ T cells (Blue) and CD8+ T cells (Magenta) for all cases (squares) and controls (triangles). (B) PCA of DNA methylation data from whole blood only. (C) PCA of DNA methylation data from CD4+ T cells only. (D) PCA of DNA methylation data from CD8+ T cells only.

doi:10.1371/journal.pone.0117403.g001
To account for cellular heterogeneity of WB, we adjusted for cell type distribution in our regression models. Sample-specific estimates of the cell type proportions were obtained by adapting the algorithm from Houseman et al.\[26\] using reference information on cell-specific methylation signatures\[27\]. Details on the procedures above are provided in S1 Materials and Methods.

CpG-site differential methylation analysis

Two regression models were used in the analysis CpG-sites. In the first model we analyzed CD4+ T cell, CD8+ T cell or WB data separately, with 'case-control' status as a factor. Secondly, a two-way interaction model that utilized data from both CD4+ and CD8+ T cells was applied. In this model three factors were included; the 'cell type', the 'group' effect (case-control status), and an 'interaction' factor, which tested for statistical interaction between the cell type and case-control status. In case of statistical interaction between these two main factors, the DNA methylation directions are different between cell types across groups. To account for multiple testing we employed the Benjamini and Hochberg false discovery rate (FDR)\[28\]. CpG-sites with the lowest nominal p-values and at least 5% absolute difference in methylation \[29\] between MS patients and controls were examined. We examined the differences prioritized by lowest p-values to ensure the most consistently changing CpG-sites between MS cases and controls were considered. Fisher’s exact test was used to test for differences in distribution of all CpG-sites that reached nominal significance.

For the 5% of probes with the lowest p-values in the CD4+ and CD8+ T cell specific analyses, we determined whether support for any observed signal was present at neighboring CpG-sites. Our approach was based on the method described recently by Jaffe et al.\[30\]. Briefly, we defined a neighbor probe to be of interest if its p-value was also in the 5% of probes with lowest p-values for the respective cell type analyses, and the maximum distance between CpG-sites was not greater than 500 base pairs. If a neighbor hit was identified the algorithm then extended over the next 500 base pairs until no additional hits were present. We then grouped these individual CpG-sites into differentially methylated regions (DMRs). By permutation testing based on the area under the curve with respect to the test statistic we calculated p-values for these DMRs.

Per-gene differential methylation analysis

The recently published list of MS-associated SNPs was used to define candidate genes (N = 148) for methylation differences given their putative role in the genetic predisposition to MS \[4\]. To account for multiple testing we also applied the FDR procedure\[28\]. CpG-sites were assigned to specific genes (N = 21,115) based on the provided Illumina manifest for the 450K. CpG-sites that mapped to multiple genes were included in analyses of all these genes. We used a permutation test based on the sum of the test statistics for each CpG-site within a gene.

Results

MS patient and control characteristics

Study characteristics are provided in Table 1. There were no significant differences between mean age or smoking status of MS patients compared to controls. All patients were diagnosed having RRMS, and the mean duration of disease was 8.8 years. The majority of patients had oligoclonal bands in their cerebrospinal fluid. All patients had modest EDSS and MSSS scores, and more than 10 typical MS lesions on cerebral MRI.
Cell type specific DNA methylation profiles

PCA analysis of the DNA methylation profiles of CD4+ and CD8+ T cells as well as WB samples identified differences in the overall DNA methylation patterns between these cell types (Fig. 1A). Within each cell type, we did not observe clustering of the MS patients and controls, indicating that on a global level there are no large, consistent DNA methylation differences that distinguish individuals according to disease status. (Fig. 1B-D)

Single CpG-site methylation analyses

In total 424,990 CpG-sites were considered after removal of CpG-sites with a low detection signal or SNPs in the probe sequence. Complete results from the per-CpG-site analysis using linear regression models are provided in S1 Table. We examined whether methylation differences observed in the T cell subsets were correlated with WB. Correlation of absolute mean differences from the WB data and either CD4+ and CD8+ T cell data was only moderate (respectively \(R^2 = 0.51\) and \(R^2 = 0.56\)), whereas a higher correlation coefficient (\(R^2 = 0.70\)) was observed for CD4+ and CD8+ T cells (S1C Fig.).

The 40 CpG-sites with the lowest nominal p-values and \(>5\%\) absolute difference in methylation between MS patients and controls are listed in Table 2–4. For CD4+ and CD8+ T cells we also listed whether associated CpG-sites were in a DMR as defined above. All DMRs are provided in S2 Table. Two CpG-sites occurred in the top-40 for all three analyses, both were hypermethylated in MS patients compared to controls. The first of these two probes, cg05821046, is annotated at \(TMEM48\), 622 base pairs upstream from the gene transcription start site. This CpG-site is located in a DMR of three CpG-sites, which was identified in both CD4+ and CD8+ T cell analyses (S2 Table, Chr1:54304846–54305115). \(TMEM48\) encodes a protein involved in the nuclear pore complex formation. The second probe, cg22560193, is located in the first exon of \(APC2\), a gene predicted to be involved in microtubule and beta-catenin binding. Furthermore, several CpG-sites within \(DNHD1\) were also among the top 40 most differentially methylated in all three datasets. This gene encodes the dynein heavy chain domain like 1, which is a protein complex that is involved in microtubule movement. We note that after adjustment for multiple testing, none of these findings reached a genome-wide significance level (lowest adjusted p-value = 0.88, S1 Table).

Interestingly, for CD8+ T cells, 38 of the 40 most differentially methylated CpG-sites (95%) showed evidence for hypermethylation in MS patients when compared to controls. The \(DNHD1\) gene contained one of the only two hypomethylated CpG-sites in CD8+ T cells (Table 3). In contrast, a more balanced pattern was observed for both CD4+ T cells and WB; a much lower number of CpG-sites, 55% and 52.5%, respectively showed evidence for hypermethylation in MS patients, compared to controls (Table 2 and Table 4 respectively). When considering all CpG-sites with nominal p-values below 0.05 from the patient-control comparison, the proportion of hypermethylated CD8+ T cell CpG-sites in MS patients is significantly greater than hypomethylated CpG-sites (Fisher’s exact test p-value < 0.01, Fig. 2A). DNA methylation of CpG-sites at different genomic features with respect to genes may provide additional insights in specific roles of the observed DNA hypermethylation in CD8+ T cells. When we considered genomic features for CpG-sites with p-values below 0.05, an overrepresentation of hypermethylated CpG-sites was slightly more frequent in 1,500 base pair regions upstream of the transcription start site (TSS-1500) and 1st exon of genes (\(\geq 76\%\) hypermethylated sites) whereas the gene body and 3’-UTR show less evidence for hypermethylation; the lowest proportion (63%) of hypermethylated CpG-sites was observed in the 3’-UTR (data not shown). Furthermore, when we compared the more recently diagnosed patients (<7 years from diagnosis) with patients diagnosed earlier (>8 years from diagnosis) the more recently diagnosed patients showed a slightly higher proportion of
DNA hypermethylation of their CD8+ T cells (proportion of hypermethylated sites 73% in recently diagnosed patients vs. 68% in the earlier diagnosed patients). We also examined CpG-sites for which patient-control comparisons did not yield p-values below 0.05, and the observation that CD8+ T cells are more likely to be hypermethylated remained, although less significant

Table 2. Top 40 results sorted by p-values from linear regression analysis models of DNA methylation in CD4+ T cells.

| probelD1 | Gene2 | p-value3 | Effectsize4 | stdev5 | p-value DMR (# probes in DMR)6 |
|----------|-------|----------|-------------|--------|--------------------------------|
| cg20585410 | DCX | 3.86E-05 | -0.074 | 0.015 | - |
| cg13988338 | No gene | 7.30E-05 | -0.093 | 0.020 | - |
| cg15552461 | RDH13 | 9.58E-05 | -0.069 | 0.015 | - |
| cg01833234 | DNHD1 | 1.49E-04 | 0.145 | 0.033 | - |
| cg07937631 | No gene | 1.51E-04 | 0.144 | 0.033 | - |
| cg24637308 | DNHD1 | 1.63E-04 | 0.108 | 0.025 | - |
| cg27419327 | No gene | 2.29E-04 | -0.073 | 0.017 | - |
| cg26477117 | TEKT5 | 2.57E-04 | -0.242 | 0.058 | - |
| cg02336026 | No gene | 2.78E-04 | -0.065 | 0.016 | - |
| cg24431033 | TXNL1 | 2.78E-04 | 0.072 | 0.017 | 5.5E-02 (3) |
| cg12543766 | MAGI2 | 2.84E-04 | -0.194 | 0.046 | - |
| cg03700679 | TTC30B | 2.94E-04 | 0.053 | 0.013 | - |
| cg06346838 | APC2 | 3.88E-04 | -0.062 | 0.015 | - |
| cg05820146 | TMEM48 | 4.03E-04 | -0.065 | 0.016 | 7.0E-04 (3) |
| cg11213150 | ANGPTL2/RALGPS1 | 4.06E-04 | -0.054 | 0.013 | - |
| cg08633479 | USP29 | 4.11E-04 | 0.066 | 0.016 | - |
| cg12243267 | USP29 | 5.40E-04 | 0.064 | 0.016 | - |
| cg06154311 | C20orf151 | 5.68E-04 | -0.075 | 0.019 | - |
| cg27246129 | DLL1 | 6.50E-04 | -0.095 | 0.025 | - |
| cg15627136 | No gene | 6.65E-04 | -0.060 | 0.016 | - |
| cg16288318 | No gene | 6.81E-04 | -0.096 | 0.025 | - |
| cg16259355 | DACH2 | 7.49E-04 | 0.064 | 0.017 | - |
| cg17332091 | No gene | 8.03E-04 | -0.051 | 0.013 | 1.0E-03 (3) |
| cg23023970 | INPP5A | 8.82E-04 | -0.061 | 0.016 | - |
| cg08682625 | LOC727677 | 9.72E-04 | 0.116 | 0.031 | - |
| cg04587084 | No gene | 1.03E-03 | -0.070 | 0.019 | - |
| cg10208301 | DNHD1 | 1.08E-03 | 0.129 | 0.035 | - |
| cg07733481 | SEMA5B | 1.15E-03 | 0.148 | 0.041 | - |
| cg14667685 | No gene | 1.34E-03 | -0.078 | 0.022 | 2.0E-03 (5) |
| cg22560193 | APC2 | 1.39E-03 | -0.089 | 0.025 | - |
| cg14759977 | SUGT1L1 | 1.44E-03 | 0.051 | 0.014 | - |
| cg01413790 | No gene | 1.45E-03 | -0.057 | 0.016 | 1.0E-03 (3) |
| cg09742183 | HOXB2 | 1.51E-03 | 0.068 | 0.019 | - |
| cg20954971 | No gene | 1.53E-03 | -0.067 | 0.019 | - |
| cg15051426 | OR10J5 | 1.64E-03 | -0.074 | 0.021 | - |
| cg19285525 | RBMS1 | 1.65E-03 | -0.395 | 0.113 | - |
| cg07019386 | No gene | 1.66E-03 | -0.080 | 0.023 | 5.0E-02 (3) |
| cg17976205 | C20orf151 | 1.74E-03 | -0.052 | 0.015 | - |
| cg22687569 | No gene | 1.79E-03 | -0.120 | 0.035 | - |

(Continued)
For blood and CD4+ T cells, the distributions of hyper vs. hypomethylated CpG-sites were nearly identical (~50%) and not significantly different (Fig. 2A).

Methylation differences between cell types

As expected, we observed large differences in DNA methylation profiles between CD4+ and CD8+ T cells. This was illustrated by the high total number of CpG-sites showing significant differences and the large differences of beta levels for these sites. Table 5 shows the 20 most significantly different CpG-sites among cell types, adjusted for disease status and possible interaction between disease status and cell type. Among these 20 CpG-sites none showed a case-control or interaction effect in the combined model. The CpG-sites showing the greatest differences among cell types had beta differences of up to 0.85, translating to an almost full switch of methylation status. Furthermore, the genes near or containing these CpG-sites have known roles in CD4+ T cell and CD8+ T cell regulation.

MS candidate genes and exploratory per-gene analyses

Analysis of MS patients versus controls was performed at gene-level using a per-gene DNA methylation summary statistic for either CD4+ or CD8+ T cells. When considering CpG-sites annotated to genes of all established MS-associated SNPs[2], we observed no significant differences between MS patients and controls following correction for multiple testing (S3 Table). Similarly, no significant genes were observed when all genes covered by the 450K were taken into consideration (S3 Table).

Discussion

Using a robust genome-wide DNA methylation profiling approach, we show no consistent large-effect DNA methylation differences for CD4+ T cells, CD8+ T cells or WB in a homogeneous collection of MS patients and controls. However, while nominally significant methylation differences were small, CD8+ T cell DNA from MS patients showed strong evidence for hypermethylation at a large number of these CpG-sites. Furthermore, we confirmed large-effect,
genome-wide significant DNA methylation differences between CD4+ T cells and CD8+ T cells, underscoring the importance of separating different immune cell subpopulations in DNA methylation studies. Although none of the MS patient-control DNA methylation analyses reached genome-wide significance, we observed two CpG-sites with low p-values for all the three different sample types. We cannot exclude the possibility that genetic variation other

Table 3. Top 40 results sorted by p-values from linear regression analysis models of DNA methylation in CD8+ T cells.

| probeID   | Gene  | p-value | Effectsize | stdev | p-value | DMR (# probes in DMR) |
|-----------|-------|---------|------------|-------|---------|------------------------|
| cg06346838 | APC2  | 2.91E-06 | -0.087     | 0.015 | -       |                        |
| cg22560193 | APC2  | 2.16E-05 | -0.101     | 0.020 | -       |                        |
| cg17332091 | No gene | 2.22E-05 | -0.066     | 0.013 | 2.0E-05 (3) |
| cg13988338 | No gene | 4.61E-05 | -0.093     | 0.019 | -       |                        |
| cg10673318 | No gene | 5.39E-05 | -0.062     | 0.013 | -       |                        |
| cg19432993 | HOXA2 | 6.94E-05 | -0.066     | 0.014 | 1.3E-02 (5) |
| cg21995652 | HANBP3| 1.43E-04 | -0.055     | 0.012 | -       |                        |
| cg24998110 | HEDC  | 1.47E-04 | 0.060      | 0.014 | -       |                        |
| cg18772882 | NTRK3 | 1.74E-04 | -0.051     | 0.012 | -       |                        |
| cg20971998 | No gene | 1.79E-04 | -0.078     | 0.018 | -       |                        |
| cg12580893 | No gene | 2.00E-04 | -0.066     | 0.015 | -       |                        |
| cg20585410 | DCX   | 2.18E-04 | -0.088     | 0.021 | -       |                        |
| cg13560901 | TRIL  | 2.86E-04 | -0.072     | 0.017 | -       |                        |
| cg20864214 | ARHGEF17 | 2.95E-04 | -0.090     | 0.022 | -       |                        |
| cg07311615 | ESRRP | 2.95E-04 | -0.068     | 0.016 | 2.0E-03 (2) |
| cg02225599 | HOXA2 | 2.99E-04 | -0.064     | 0.016 | 1.3E-02 (5) |
| cg09309261 | LH5   | 3.68E-04 | -0.063     | 0.016 | -       |                        |
| cg11902995 | No gene | 3.80E-04 | -0.063     | 0.016 | -       |                        |
| cg26477117 | TEK5  | 4.59E-04 | -0.241     | 0.061 | -       |                        |
| cg19225422 | No gene | 4.80E-04 | -0.052     | 0.013 | -       |                        |
| cg09213964 | LRR43 | 4.82E-04 | -0.051     | 0.013 | -       |                        |
| cg10173124 | CYP27C1| 5.21E-04 | -0.052     | 0.013 | -       |                        |
| cg05821046 | TMEM48| 5.36E-04 | -0.097     | 0.025 | 2.2E-01 (3) |
| cg18782774 | No gene | 5.59E-04 | -0.052     | 0.013 | -       |                        |
| cg24938727 | HHATL | 6.39E-04 | -0.061     | 0.016 | -       |                        |
| cg00402910 | AMMECR1| 6.54E-04 | -0.062     | 0.016 | -       |                        |
| cg08065835 | No gene | 6.67E-04 | -0.051     | 0.013 | -       |                        |
| cg04764898 | C19orf45 | 6.77E-04 | -0.056     | 0.015 | -       |                        |
| cg21686577 | SRRM3 | 6.81E-04 | -0.058     | 0.015 | -       |                        |
| cg08387780 | No gene | 6.90E-04 | -0.058     | 0.015 | 2.0E-05 (3) |
| cg01573321 | PSD3  | 7.23E-04 | -0.064     | 0.017 | -       |                        |
| cg14531668 | No gene | 7.23E-04 | -0.050     | 0.013 | -       |                        |
| cg22970003 | PTPRN2 | 7.63E-04 | -0.073     | 0.019 | -       |                        |
| cg14828182 | LOC654342 | 7.63E-04 | -0.062     | 0.016 | -       |                        |
| cg20692922 | No gene | 7.65E-04 | -0.078     | 0.021 | -       |                        |
| cg16017089 | ARHGEF17 | 7.79E-04 | -0.059     | 0.016 | -       |                        |
| cg24637308 | DNHD1 | 7.84E-04 | 0.086      | 0.023 | -       |                        |
| cg09307264 | KIF1C/INCA1 | 8.08E-04 | -0.052     | 0.014 | -       |                        |
| cg05280762 | VSIQ1 | 8.08E-04 | -0.054     | 0.014 | -       |                        |

(Continued)
than DNA methylation could underlie such consistent results; however, given the dense genotype information we obtained, and lack of a known SNP in the probe sequences[31], our evidence strongly suggests a consistent DNA methylation difference between MS patients and controls is present. The first CpG-site, measured by probe cg05821046 resides in a DMR including two additional probes for both CD4+ and CD8+ T cells (Tables 2 and 3). The lead CpG-site is localized upstream of\textit{TMEM48}, a gene encoding the nuclear pore complex protein NDC1. Little is known about this protein and its potential role in MS. The second consistent CpG-site difference was measured by probe cg22560193 and is annotated to the last exon of gene\textit{APC2}. This CpG-site is not located in a DMR when considering the CpG-sites covered by the 450K. \textit{APC2} encodes the protein adenomatosis polyposis coli 2, which is mainly expressed in neuronal tissue. The relevance of increased DNA methylation of CpG-sites within this gene in immune cells from MS patients is unclear.

Remarkably, the CD8+ T cells of MS patients showed a predominantly higher level of DNA methylation compared to controls for those CpG-sites with the lowest p-values. Since the canonical role of DNA methylation at gene promoters is gene silencing and we observed a slightly higher percentage of hypermethylated sites in these promoter regions, it is possible that gene silencing in circulating CD8+ T cells of MS patients may be present. Whether this observation persists in a larger study warrants further investigation.

After correcting for multiple testing, we did not find significant evidence for association between per-gene DNA methylation within specifically candidate genes[2], or when all genes on the 450K were considered. It is important to note that the 450K covers only a portion of the CpG-sites present in the human genome. Although the array is gene centric and largely encompasses potential regulatory regions, it is possible that MS-associated DNA methylation differences exist outside the CpG-sites covered by this array. Given the complex disease aetiology in MS, at individual patient level, changes in DNA methylation may still contribute to disease-risk.

While the sample size in this study is modest, we had at least 80% power to detect beta-value differences of 0.05 and larger, assuming per-CpG-site median standard deviations (S1D Fig.). Thus, for half of the CpG-sites, the power to detect a beta difference over 0.05 was over

### Table 3. (Continued)

**CD8+ T cells**

| probeID | Gene  | p-value | Effectsize | stdev | p-value DMR (# probes in DMR) |
|---------|-------|---------|------------|-------|-------------------------------|
| cg25512439 | CNTN4 | 9.38E-04 | -0.060 | 0.016 | - |

\(^1\)Probe ID on 450K chip.
\(^2\)Gene annotated to probe.
\(^3\)p-value for specified probe in CD8+ T cells.
\(^4\)Effect size of beta difference for specified probe. Positive values indicate hypomethylation of MS samples (i.e. controls DNA methylation higher than MS patients).
\(^5\)Standard deviation for specified probe.

Permutation-derived p-values for DMR in case the indicated probes is located in a DMR, in brackets we provided the number supportive CpG-sites in the respective DMRs.

Formatting legend

**Bold probeID** Specific probe occurs in all three data top-40 (see Tables 2, 4)

**Bold Italic Gene** Gene occurs in all three data top-40 (see Tables 2, 4)

**Bold Effectsize** Hypermethylation of probe in MS patients

Results shown are restricted to methylation differences of at least 5% (absolute beta difference). Full lists are provided in S1 Table.

doi:10.1371/journal.pone.0117403.t003
80%. Therefore, our study had power to detect large-effect, consistent methylation differences between MS patients and controls. The observed hypermethylation in CD8+ T cells has small effect sizes and none of the CpG-sites reached genome-wide significance individually. A PCA of genome-wide SNP data[20] allowed us to verify Nordic ancestry and excluded systematic genetic differences between patients and controls in the study. Methylation levels for specific loci

Table 4. Top 40 results sorted by p-values from linear regression analysis models of DNA methylation in whole blood samples.

| probelD1 | Gene2   | p-value3 | Effectsize4 | stdev5 |
|----------|---------|----------|-------------|--------|
| cg16259355 | DACH2  | 6.95E-05 | 0.109       | 0.023  |
| cg24493834 | LAMA2   | 8.65E-05 | 0.059       | 0.012  |
| cg23023844 | TTLL8   | 1.16E-04 | 0.138       | 0.030  |
| cg04903509 | GALNT9  | 1.27E-04 | 0.058       | 0.013  |
| cg20373036 | POU3F4  | 2.25E-04 | -0.059      | 0.014  |
| cg00827196 | No gene | 3.63E-04 | -0.051      | 0.012  |
| cg16288318 | No gene | 3.98E-04 | -0.147      | 0.035  |
| cg00420742 | NLRP12  | 5.07E-04 | 0.051       | 0.013  |
| cg02336026 | No gene | 5.78E-04 | -0.076      | 0.019  |
| cg05052271 | PLS3    | 5.87E-04 | -0.070      | 0.018  |
| cg01262952 | ANKRD1  | 5.88E-04 | 0.078       | 0.020  |
| cg02313554 | No gene | 7.35E-04 | -0.138      | 0.036  |
| cg13834112 | No gene | 7.86E-04 | -0.051      | 0.013  |
| cg25031670 | No gene | 8.17E-04 | -0.084      | 0.022  |
| cg25671428 | CLSTN2  | 8.26E-04 | -0.051      | 0.013  |
| cg05141400 | MAGEB4  | 8.60E-04 | -0.086      | 0.023  |
| cg01281231 | No gene | 8.85E-04 | -0.054      | 0.014  |
| cg25488749 | No gene | 8.92E-04 | -0.052      | 0.014  |
| **cg22560193** | **APC2** | **9.08E-04** | **-0.091** | **0.024** |
| cg27571374 | No gene | 9.31E-04 | 0.137       | 0.036  |
| cg06076512 | No gene | 9.76E-04 | 0.054       | 0.014  |
| cg11837293 | No gene | 1.02E-03 | 0.058       | 0.015  |
| cg02851397 | PCDHA7  | 1.06E-03 | -0.081      | 0.022  |
| cg17140469 | No gene | 1.08E-03 | -0.066      | 0.018  |
| cg20410114 | No gene | 1.08E-03 | 0.053       | 0.014  |
| cg11336696 | TMEM27  | 1.15E-03 | -0.064      | 0.017  |
| cg11185456 | DNHD1   | 1.19E-03 | 0.152       | 0.041  |
| cg06833709 | LGI1    | 1.19E-03 | -0.061      | 0.017  |
| cg08243619 | PTCHD2  | 1.19E-03 | 0.081       | 0.022  |
| cg18618432 | No gene | 1.22E-03 | -0.382      | 0.104  |
| cg25523580 | MMD2    | 1.24E-03 | -0.089      | 0.024  |
| cg24938727 | HHATL   | 1.33E-03 | -0.063      | 0.017  |
| **cg05821046** | **TMEM48** | **1.37E-03** | **0.087** | **0.024** |
| cg00399951 | NXPH1   | 1.39E-03 | -0.085      | 0.023  |
| cg14336566 | TDRD9   | 1.44E-03 | 0.072       | 0.020  |
| cg23266594 | CDX1    | 1.48E-03 | -0.078      | 0.022  |
| cg07465864 | YTHDC2  | 1.51E-03 | 0.066       | 0.018  |
| cg22351833 | No gene | 1.52E-03 | -0.069      | 0.019  |
| cg02778467 | RGD1/PLGB2 | 1.58E-03 | -0.091      | 0.025  |

(Continued)
might change with age and differ between gender[32]; therefore, only female MS patients and female, age matched controls were included in this study. The clinical data show these MS patients are representative of an average MS population with a relative benign disease course. Importantly, since medication may influence DNA methylation[33], the MS patients selected for this study had never used immune-modulatory drugs at time of sampling or received steroids for at least three months prior to inclusion. Furthermore, since tobacco smoke is a known driver of methylation differences in peripheral blood cells[34], we also performed an analysis including smoking status as a covariate; however, this did not substantially change the results (data not shown).

A recent study by Graves et al. reported significant DNA methylation changes within CD4+ T cells of the MHC region in MS patients using the 450K[35]. In our study, we noted 18 of 19 (95%) of these CpG-sites within the MHC were compromised by the presence of at least one
SNP in the probe sequence[25]. For the remaining CpG-site in the MHC, we did not observe a nominally significant difference. Furthermore, a SNP was present in the probes for 8 of 55 associated CpG-sites outside the MHC region. None of the remaining 47 non-MHC CpG-sites reached significance in our study. Therefore, we could not confirm the findings reported by Graves et al.[35]. Notably, our sample was smaller, though more clinically homogeneous with respect gender and disease course. The high number of excluded CpG-sites due to the presence of a SNP in the probe sequence underscores the need for genotype-based filtering of chip-based DNA methylation data. Alternatively, probes that might contain SNPs[25] can be identified by utilizing publicly available data[36].

Our results are in agreement with Baranzini et al., who applied reduced bisulphite sequencing covering over 2 million CpG-sites, and showed no consistent large-scale methylation differences in MS discordant twins and siblings[15]. The reported switch of methylation from 20% to 80% for CpG-sites close to the TMEM1 or PEX14 genes between discordant twins could not be examined, since these CpG-sites are not included on the 450K.

Table 5. Distinct differences between CD4+ and CD8+ T-cells observed in the 'cell type' term when applying a linear regression two-way interaction model including both the CD4+ and CD8+ T cell methylation data, including the terms 'cell type', 'group' (case-control status), and 'interaction' (case-control status x cell type).

| probeID | Gene   | Effect size | SD  | Cell Type | Cell Type BH corrected | Group | Interaction |
|---------|--------|-------------|-----|-----------|------------------------|-------|-------------|
| cg22505006 | ZBTB7B | 0.849 | 0.008 | 1.61E-40 | 6.85E-35 | 0.992 | 0.502 |
| cg24955196 | ZBTB7B | 0.724 | 0.007 | 3.27E-40 | 6.94E-35 | 0.408 | 0.799 |
| cg16871561 | SLC25A3 | 0.709 | 0.010 | 4.03E-35 | 5.71E-30 | 0.918 | 0.290 |
| cg25939861 | CD8A | -0.754 | 0.012 | 1.29E-34 | 1.37E-29 | 0.314 | 0.824 |
| cg06935361 | BRCA2 | -0.669 | 0.011 | 1.97E-34 | 1.67E-29 | 0.779 | 0.602 |
| cg00219921 | CD8A | -0.764 | 0.013 | 3.41E-33 | 2.41E-28 | 0.870 | 0.904 |
| cg01782486 | ZBTB7B | 0.656 | 0.012 | 6.61E-33 | 4.01E-28 | 0.718 | 0.632 |
| cg06449334 | No gene | -0.533 | 0.010 | 2.74E-32 | 1.46E-27 | 0.299 | 0.557 |
| cg25350872 | LOC154822 | -0.530 | 0.010 | 4.35E-32 | 1.87E-27 | 0.314 | 0.062 |
| cg17343167 | N4BP3 | -0.448 | 0.009 | 4.82E-32 | 1.87E-27 | 0.503 | 0.467 |
| cg24345747 | CD8A | -0.638 | 0.012 | 4.85E-32 | 1.87E-27 | 0.370 | 0.396 |
| cg19453665 | SERPINH1 | -0.309 | 0.006 | 9.20E-32 | 3.16E-27 | 0.392 | 0.891 |
| cg03318654 | CD8A | -0.559 | 0.011 | 9.66E-32 | 3.16E-27 | 0.408 | 0.947 |
| cg03505866 | KIAA0247 | 0.437 | 0.009 | 1.14E-31 | 3.46E-27 | 0.092 | 0.769 |
| cg08934126 | CTNNBIP1 | -0.309 | 0.006 | 1.42E-31 | 4.04E-27 | 0.829 | 0.264 |
| cg10837404 | DCP2 | 0.574 | 0.012 | 2.33E-31 | 6.19E-27 | 0.460 | 0.357 |
| cg26986871 | No gene | -0.565 | 0.011 | 3.33E-31 | 7.96E-27 | 0.400 | 0.664 |
| cg14477767 | No gene | 0.716 | 0.015 | 3.37E-31 | 7.96E-27 | 0.144 | 0.386 |
| cg24462702 | CD40LG | 0.378 | 0.008 | 4.38E-31 | 9.80E-27 | 0.749 | 0.191 |
| cg13798679 | No gene | -0.446 | 0.010 | 1.22E-30 | 2.59E-26 | 0.326 | 0.835 |

1Probe ID on 450K chip.
2Gene annotated to probe.
3Effect size of beta difference for specified probe.
4standard deviation for specified probe.
5p-value for specified probe in respective models.
6Benjamini-Hochberg corrected p-values for factor "cell type".

The top 20 highest-ranking probes sorted by p-values for differences of the 'cell type' term are listed, full lists are provided in S1 Table.

doi:10.1371/journal.pone.0117403.t005
Temporality must be considered in DNA methylation studies. It remains possible that MS patient DNA methylation profiles deviated from healthy controls at disease onset and are no longer detectable. When we consider the more recently diagnosed patients these showed a high proportion of DNA hypermethylation of their CD8+ T cells. The patients that were diagnosed earlier also show a profound DNA hypermethylation, though the proportion is slightly lower as compared to the recently diagnosed patients. We cannot exclude the possibility that the disease process in itself affects DNA methylation. This possibility must be investigated in a longitudinal cohort of MS patients.

For use as possible biomarkers of MS in the clinic, characteristic DNA methylation profiles should preferably be identified in easily obtainable WB. After correction of the WB methylation profiles in our dataset according to Houseman et al., the correlation coefficients of WB compared to T cells remained moderate (S1C Fig.). Therefore, we cannot conclude that WB will reliably reflect disease relevant changes in T cells, however additional studies on the biomarker value of DNA methylation profiles derived from WB are warranted.

In conclusion, this is the first study of genome-wide DNA methylation profiles derived from WB, CD4+ and CD8+ T cells, in homogenous, untreated female MS patients and matched controls. We identified strong evidence for DNA hypermethylation in CD8+ T cells of MS patients. The significant methylation differences observed between CD4+ T cells, CD8+ T cells and WB underscore the importance of considering cell-based profiles. Further, more sophisticated algorithms for correction of individual variability in cell proportions are needed, if DNA methylation profiles from WB are to be used reliably. Based on available power, we excluded large-scale individual and per-gene DNA methylation differences between patients and controls, for CpG-sites tested here. In particular, large DNA methylation differences for CpG-sites within 148 established MS candidate genes tested in the current study do not explain missing heritability. Larger studies of homogenous MS patients and controls are warranted to further elucidate the impact of smaller DNA methylation changes that may be important in MS pathogenesis.

Supporting Information

S1 Fig. Supplementary figures S1A-D. A. Principal component analysis (PCA) of MS patients and controls used in the methylation analyses (respectively triangles and squares in color). The principal components for samples in current study were plotted against those derived from an earlier large GWAS study of Norwegian MS patients and controls. Results show the samples in the DNA methylation study cluster within the Nordic population. B. SNPs in methylation probes influence reported beta values; example of a SNP located in the sensing probe sequence of CpG-site cg21139150 resulting correlation between reported beta-values and sample genotype. C. Scatterplot of –log(p-values) of the per-probe patient-control analysis for CD8+ T cell test statistics against CD4+ T cell test statistics, resulting in a correlation coefficient \( R^2 = 0.70 \). D. Post-hoc power calculations for increasing quintiles of observed probe variance.

(TIF)

S1 Materials and Methods. Detailed materials and methods for procedures briefly described in manuscript.

(OCX)

S1 Table. Per-probe analyses details.

(ZIP)

S2 Table. All DMR analyses details.

(XLSX)
S3 Table. Per-gene analyses details.
(XLSX)

Acknowledgments
The authors acknowledge Elisabeth Gulowsen Celius, Xiaorong Shao, Gro Nygård, Aslaug Muggerud, Piotr Sowa, Dag Undlien, Anne Spurkland, Hanne Saether, Kristina Gervin and the Norwegian Metacenter for Computational Science for their contributions in patient recruitment, data collection, use of facilities and/or technical assistance.

Author Contributions
Conceived and designed the experiments: SDB BKA TB HFH LB. Performed the experiments: SDB MWG ISL AB HQ EE. Analyzed the data: SDB CMP BKA EE. Contributed reagents/materials/analysis tools: SDB CMP BKA TB HFH LB. Wrote the paper: SDB CMP BKA EE MWG FB HQ ISL AB TB HFH LB. Technical assistance and quality assurance: HQ AB.

References
1. Compston A, Coles A (2008) Multiple sclerosis. Lancet 372: 1502–1517. doi:10.1016/S0140-6736(08)61620-7 PMID: 18970977
2. International Multiple Sclerosis Genetics Consortium (2013) Analysis of immune-related loci identifies 48 new susceptibility variants for multiple sclerosis. Nat Genet 45: 1353–1360. doi:10.1038/ng.2770 PMID: 24076602
3. Gourraud PA, Harbo HF, Hauser SL, Baranzini SE (2012) The genetics of multiple sclerosis: an up-to-date review. Immunological reviews 248: 87–103. doi: 10.1111/j.1600-065X.2012.01134.x PMID: 22725956
4. International Multiple Sclerosis Genetics Consortium (2013) Network-based multiple sclerosis pathway analysis with GWAS data from 15,000 cases and 30,000 controls. Am J Hum Genet 92: 854–865. doi:10.1016/j.ajhg.2013.04.019 PMID: 23731539
5. Maurano MT, Humbert R, Rynes E, Thurman RE, Haugen E, et al. (2012) Systematic localization of common disease-associated variation in regulatory DNA. Science 337: 1190–1195. doi: 10.1126/science.1222794 PMID: 22955828
6. Chitnis T (2007) The role of CD4 T cells in the pathogenesis of multiple sclerosis. International review of neurobiology 79: 43–72. PMID: 17531837
7. Huseby ES, Huseby PG, Shah S, Smith R, Stadinski BD (2012) Pathogenic CD8 T cells in multiple sclerosis and its experimental models. Frontiers in immunology 3: 64. doi: 10.3389/fimmu.2012.00064 PMID: 22566945
8. Broux B, Stinissen P, Hellings N (2013) Which immune cells matter? The immunopathogenesis of multiple sclerosis. Critical reviews in immunology 33: 283–306. PMID: 23971528
9. Nielsen HM, Tost J (2012) Epigenetic changes in inflammatory and autoimmune diseases. Sub-cellular biochemistry 61: 455–478.
10. Altorki N, Coit P, Hughes T, Koelsch KA, Stone DU, et al. (2014) Genome-Wide DNA Methylation Patterns in Naive CD4+ T Cells From Patients With Primary Sjogren’s Syndrome. Arthritis & rheumatology 66: 731–739. doi: 10.1001/bone.2015.01.007 PMID: 25603545
11. Absher DM, Li X, Waite LL, Gibson A, Roberts K, et al. (2013) Genome-wide DNA methylation analysis of systemic lupus erythematosus reveals persistent hypomethylation of interferon genes and compositional changes to CD4+ T-cell populations. PLoS genetics 9: e1003678. doi: 10.1371/journal.pgen.1003678 PMID: 23950730
12. Whitaker JW, Shoemaker R, Boyle DL, Hillman J, Anderson D, et al. (2013) An imprinted rheumatoid arthritis methylome signature reflects pathogenic phenotype. Genome medicine 5: 40. doi: 10.1186/gm444 PMID: 23961487
13. Bibikova M, Barnes B, Tsan C, Ho V, Klotzle B, et al. (2011) High density DNA methylation array with single CpG site resolution. Genomics 98: 288–295. doi: 10.1016/j.ygeno.2011.07.007 PMID: 21839163
14. Feinberg AP (2007) Phenotypic plasticity and the epigenetics of human disease. Nature 447: 433–440. PMID: 17522877
15. Baranzini SE, Mudge J, van Velkinburgh JC, Khankhanian P, Khrebtukova I, et al. (2010) Genome, epigenome and RNA sequences of monozygotic twins discordant for multiple sclerosis. Nature 464: 1351–1356. doi: 10.1038/nature08990 PMID: 20428171

16. Kumagai C, Kalman B, Middleton FA, Vyshtina T, Massa PT (2012) Increased promoter methylation of the immune regulatory gene SHP-1 in leukocytes of multiple sclerosis subjects. Journal of neuroimmunology 246: 51–57. doi: 10.1016/j.jneuroim.2012.03.003 PMID: 22458980

17. Calabrese R, Zampieri M, Mechelli R, Annibali V, Guastafierro T, et al. (2012) Methylation-dependent PAD2 upregulation in multiple sclerosis peripheral blood. Multiple sclerosis 18: 299–304. doi: 10.1177/1352458511421055 PMID: 21878453

18. Huynh JL, Garg P, Thin TH, Yoo S, Dutta R, et al. (2014) Epigenome-wide differences in pathology-free regions of multiple sclerosis-affected brains. Nature neuroscience 17: 121–130. doi: 10.1038/nn.3588 PMID: 24270187

19. Polman CH, Reingold SC, Banwell B, Clanet M, Cohen JA, et al. (2011) Diagnostic criteria for multiple sclerosis: 2010 revisions to the McDonald criteria. Annals of neurology 69: 292–302. doi: 10.1002/ana.22366 PMID: 21387374

20. International Multiple Sclerosis Genetics Consortium (2007) Risk alleles for multiple sclerosis identified by a genomewide study. The New England journal of medicine 357: 851–862. PMID: 17660530

21. R Core Team (2013) A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, Available: http://www.R-project.org/. doi:10.1007/978-3-319-70252-9

22. Howie BN, Donnelly P, Marchini J (2009) A flexible and accurate genotype imputation method for the next generation of genome-wide association studies. PLoS genetics 5: e1000529. doi: 10.1371/journal.pgen.1000529 PMID: 19543373

23. Teschendorff AE, Marabita F, Lechner M, Bartlett T, Teager J et al. (2013) A beta-mixture quantile normalization method for correcting probe design bias in Illumina Infinium 450 k DNA methylation data. Bioinformatics 29: 189–196. doi: 10.1093/bioinformatics/bts680 PMID: 23175756

24. Dedeurwaerder S, Defrance M, Calonne E, Denis H, Sotiriou C, et al. (2011) Evaluation of the Infinium Methylation 450K technology. Epigenomics 3: 771–784. doi: 10.2217/epi.11.105 PMID: 22126295

25. Price ME, Cotton AM, Lam LL, Farre P, Emberly E, et al. (2013) Additional annotation enhances potential for biologically-relevant analysis of the Illumina Infinium HumanMethylation450 BeadChip array. Epigenetics & chromatin 6: 4. doi: 10.1038/tp.2014.145 PMID: 25603415

26. Houseman EA, Molitor J, Marsit CJ (2014) Reference-free cell mixture adjustments in analysis of DNA methylation data. Bioinformatics 30: 1431–1439. doi: 10.1093/bioinformatics/btu029 PMID: 24451622

27. Reinius LE, Acevedo N, Joerink M, Pershagen G, Dahlen SE, et al. (2012) Differential DNA methylation in purified human blood cells: implications for cell lineage and studies on disease susceptibility. PLoS one 7: e41361. doi: 10.1371/journal.pone.0041361 PMID: 22848472

28. Benjamini Y, Hochberg Y (1995) Controlling the False Discovery Rate—a Practical and Powerful Approach to Multiple Testing. J Roy Stat Soc B Met 57: 289–300.

29. Dayeh T, Volkov P, Salo S, Hall E, Nilsson E, et al. (2014) Genome-wide DNA methylation analysis of CD8+ T-Cells are associated with multiple sclerosis. Multiple sclerosis 20: 1033–1041. PMID: 24336351

30. Graves M, Benton M, Lea R, Boyle M, Tajouri L, et al. (2013) Methylation differences at the HLA-DRB1 locus in CD4+ T-Cells are associated with multiple sclerosis. Multiple sclerosis 20: 1033–1041. PMID: 24336351

31. International HapMap Consortium (2003) The International HapMap Project. Nature 426: 789–796. PMID: 14685227