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## **Methylome and Transcriptome Profiling in Myasthenia Gravis Monozygotic Twins**

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## **Abstract**

**Objective:** To identify novel genetic and epigenetic factors associated with Myasthenia gravis (MG) using identical twins experimental study design.

**Methods:** The transcriptome and methylome of peripheral monocytes were compared between monozygotic (MZ) twins discordant and concordant for MG, as well as with MG singletons and healthy controls, all females. Sets of differentially expressed genes and differentially methylated CpGs were validated using RT-PCR for expression and target bisulfite sequencing for methylation on additional samples.

**Results:** >100 differentially expressed genes and ~1800 differentially methylated CpGs were detected in peripheral monocytes between MG patients and controls. Several transcripts associated with immune homeostasis and inflammation resolution were reduced in MG patients. Only a relatively few genes differed between the discordant healthy and MG co-twins, and both their expression and methylation profiles demonstrated very high similarity.

**Interpretation:** This is the first study to characterize the DNA methylation profile in MG, and the expression profile of immune cells in MZ twins with MG. Results suggest that numerous small changes in gene expression or methylation might together contribute to disease. Impaired monocyte function in MG and decreased expression of genes associated with inflammation resolution could contribute to the chronicity of the disease. Findings may serve as potential new predictive biomarkers for disease and disease activity, as well as potential future targets for therapy development. The high similarity between the healthy and the MG discordant twins, suggests that a molecular signature might precede a clinical phenotype, and that genetic predisposition may have a stronger contribution to disease than previously assumed.

**Keywords:** Autoimmunity; Biomarkers; Disease chronicity; Monocyte function; Predisposition; Resolution of inflammation

## 1. Introduction

Myasthenia gravis (MG) is a relatively rare autoimmune neuromuscular disorder, clinically characterized by weakness and fatigability of skeletal and extraocular muscles [1]. MG is a B-cell driven, T-cell dependent, complement- and antibody-mediated disease, resulting from autoantibodies directed against molecules at the post-synaptic membrane of the neuromuscular junction, including antibodies against autoantigens at the acetylcholine receptor (AChR) (85% of patients), the muscle-specific kinase (MuSK) or the lipoprotein-related protein 4 (LRP4) [2]. The binding of anti-AChR antibodies to their target impairs neuromuscular transmission by blocking the receptor, inducing its internalization and by complement-mediated destruction of the postsynaptic membrane [2]. MG predisposition is assumed to be influenced by both genetic and environmental factors [2,3]. Accumulating evidence supports the contribution of epigenetic modifications, DNA methylation in particular, in the development of autoimmune diseases such as Systemic Lupus Erythematosus (SLE), Rheumatoid Arthritis (RA), Sjögren's Syndrome and Multiple Sclerosis (MS) [4–6].

Monozygotic twin studies indicate a concordance rate in MG of 30-40%, similar to MS, Inflammatory Bowel Disease, and SLE, compared to 4-5% in dizygotic (DZ) twins [7]. The high discordant rate of MZ MG twins suggest that despite identical DNA and shared in-uterus and childhood environment, unknown factors contribute to disease development [7]. Thus, the study of discordant and concordant MZ twins provides an attractive model to investigate gene-environment interactions, including epigenetic contributions to disease pathogenesis. However, due to the rarity of autoimmune diseases such as MG, related publications have contributed only limited epidemiological data and brief clinical case reports.

The underlying pathogenic mechanisms of MG have mainly been attributed to anti-AChR autoreactive T cells, pathogenic autoantibody-producing B cells as well as impaired regulatory T-cell (Treg) function [8–10]. The function of immune cells is further modified by the inflammatory milieu, which is part of the autoimmune cascade, and associated cytokines from both the adaptive as well as innate immune system. Monocytes/macrophages (Mo/M $\phi$ ) of the innate system play an important role in regulating inflammation and induce immunity mainly through their hallmark function of phagocytosis and subsequent antigen presentation [11], and are important sources for cytokines and chemokines, which attract T and B cells and regulate the inflammatory response [12,13]. Dysregulation of Mo/M $\phi$  has been associated with several autoimmune diseases, but has scarcely been studied in MG. Reduced phagocytosis in monocytes in RA [14], defective phagocytosis and aberrant activation of the Mo/M $\phi$  system in SLE [12], and unbalanced pro-inflammatory macrophage (M1) and anti-inflammatory (M2) phenotype in diabetes [15] has been described. Thus, monocytes might have an important role in MG pathophysiology, yet to be fully elucidated.

The aim of this study was to analyze the transcriptome and methylome of five immune cell subsets in MZ twins discordant for MG compared with concordant MZ twins, MG singletons and healthy controls. We

hypothesized that by using the unique study design of MZ twins and purified immune cells, novel disease-associated genes would be detectable, including genes associated with predisposition and other genes associated with disease course. Due to data complexity, this article is focused on the analysis of data from monocytes.

## **2. Materials and methods**

### **2.1 Subjects**

7 female twin pairs diagnosed with MG, 4 discordant and 3 concordant, age 20-66 years (y) were recruited at the Institute of Myology, France. All twins were tested for auto-antibodies against AChR, and if negative, for antibodies against MuSK. Furthermore, 5 singleton MG patients, age 24-59y, 9 healthy females, age 23-52y and 6 healthy males, age 32-50y were recruited, and our previous obtained datasets from 5 healthy females, age 30-50y included [16]. The study was conducted following the approval of the institutional Ethical Committee (Ref afssaps B100384-30), and all participants signed a written informed consent.

### **2.2 Cell separation, DNA/RNA extraction and BeadChip hybridization**

Blood was collected from each female participant, 5 cell-subsets (CD4 and CD8 T cells, CD25<sup>+</sup> Tregs, B cells, CD14 monocytes) were purified, RNA and DNA extracted, and RNA quality control performed as previously described [16]. This multi-step protocol was done in parallel for each twin pair to reduce technical noise. Subsequently, RNA and DNA samples were hybridized to Illumina HumanHT-12 v4 (BD-103-0204 Illumina) or to HumanMethylation450 BeadChip (WG-314-1003 Illumina) respectively, according to manufacturer's protocol. Data generation was performed by the Genomics Core Facility at the Rappaport Faculty of Medicine, Technion, Israel. The data was submitted to Gene Expression Omnibus (GEO): GSE85649.

### **2.3 Statistics and pathway analysis**

The raw gene expression data from monocytes was exported from GenomeStudio and imported into JMP Genomics V7.1 software (SAS Institute Inc, Cary, NC). Quality control and analysis in JMP Genomics was done on log<sub>2</sub> transformed data, after filtering for non-expressed genes (detection p-value<0.01), and for low variance transcripts across samples (variance <5%). Initially, 3 discordant twin datasets were compared, however high similarity between the healthy discordant twins and the MG twins was found. Thus, in order to increase sample size, additional expression and methylation profiles were generated from 3 MG singleton patients and 3 healthy controls, and combined with the discordant and 3 concordant MZ MG datasets, as well as with previously collected monocyte datasets from 5 healthy controls [16], all females. In order to reduce batch effect between sets, two samples from the twin dataset and from the GSE71245 dataset were replicated, and data normalized accordingly. For gene expression the data was analyzed using one-way ANOVA, and MG associated differentially expressed genes (DEGs) were

defined as transcripts with more than 1.2-fold change of expression at a less than 0.05 False Discovery Rate (FDR) adjusted P-value [17]. For the methylation datasets, the methylation  $\beta$ -values were extracted directly from iDAT files using ChAMP R pipeline, that also performs extensive data normalization and reduction of systemic technical bias [18]. Statistical analysis was performed using both limma program within ChAMP pipeline and JMP-genomic software using ANOVA. Given the relatively small sample size and number of probes under consideration, we adopted a pragmatic approach and defined differentially methylated CpGs (DMGs) at  $\geq 5\%$  difference in methylation  $\beta$ -value at uncorrected p-values of  $< 0.01$ . The DMG list was evaluated against lists of defined regulatory elements, such as an enhancer annotation list from Broad Institute, ENSEMBL Regulatory Track, FANTOM and VISTA databases to define possible functional relevance [19]. Differentially methylated regions (DMRs) were defined using Probe lasso embedded with ChAMP R pipeline [20] and the relation between the DEGs list and DMRs list was evaluated using Genomic Regions Enrichment of Annotations Tool (GREAT) [21] and illumina annotation file. In addition, pathway analysis of DEGs was performed using Ingenuity Pathway Analysis tool (IPA 8.0, Ingenuity\_ Systems, and Redwood City, CA) and Advaita Bio's iPathwayGuide (<http://www.advaitabio.com/ipathwayguide>). We considered upstream regulatory or biological functions and diseases with Z score above or below 2 to be significant [22].

#### **2.4 RNA Expression and DNA methylation validation**

Selected MG-associated DEGs were validated by RT-PCR using the microfluidic dynamic arrays (BMK-M10-96.96-EG/1 Fluidigm) and ABI-7300 on samples from 15 MG female patients (6 concordant, 4 discordant, and 5 singleton), 4 healthy discordant twin samples and 15 healthy singletons (9 females and 6 males). Relative quantification was calculated by the comparative CT method ( $2^{-\Delta\Delta CT}$ ) with Ubiquitin-conjugating enzyme E2D2 and Ribosomal Protein L4 used for normalization.

For validation of selected DMGs, target bisulfite sequencing (TBS), an assay that combines target bisulfite amplification with next generation sequencing assay, was used as previously described [16]. We validated the methylation levels of 15 amplicons in female samples only, from 15 MG patients, 9 healthy singletons and 4 healthy discordant twin samples. For all samples, the 15 amplicons were pooled together and ligated to Ion Xpress™ Barcode Adapters and the 28 barcoded libraries quantified and pooled into one tube. 10pM DNA was subjected to emulsion PCR (E-PCR) onto the IonSpheres (ISPs; Life Technologies) using PGM™ Sequencing 400 Kit on OneTouch system (Life Technologies) [23]. The reaction efficiency was estimated using the Qubit Ion Sphere quality control kit (Life Technologies). Enriched ISPs were sequenced on Ion 316 Chip using Ion PGM™ Hi-Q™ Sequencing Kit, and FASTQ files generated [23]. The FASTQ files were aligned to the target amplicons sequences and methylations sites, i.e. cytosine to thymidine SNPs at a CpG site were flagged, using Bismark [24] and visualized using Integrative Genomics Viewer (IGV). The average coverage per amplicon-sample was over 2000 reads with large variation between amplicons, thus amplicons with  $< 200$  reads were excluded. The read count was normalized using Upper Quartile Scaling normalization to overcome the variation in sample

coverage. Both methylation percentage, i.e. the number of cytosine in a specific position out of total reads, and methylation count, i.e. the number of reads with cytosine in a specific position per sample-amplicon, were calculated. Normalized methylation counts were analyzed using a binomial mixed model and Fisher exact test [25].

### 3. Results

#### 3.1 Identification and recruitment of MZ MG twins cohort

Through a European cooperation within the FIGHT-MG consortium, we identified 15 MZ MG twin pairs from Europe and US, of which 10 pairs were females, 5 pairs were concordant and 10 pairs discordant to MG, and the age range was 18 - 88y. Since age and gender affects DNA methylation [5,26,27], only female twins, age 20-66y, were recruited for the current study, i.e. 3 concordant and 4 discordant pairs. Furthermore, samples from 5 female MG singletons, and 15 healthy controls, 9 females and 6 males were collected. Demographic and clinical details of MG twins and singletons are summarized in **Table 1**. The average age of the concordant twins was ~60 y, while only ~30 y in the discordant twins. All twin participants were tested for anti-AChR antibodies and if negative, for anti-MUSK antibodies: 10 out of 15 MG patients were positive for anti-AChR antibodies, 1 patient was positive for LRP4 antibodies, while in 4 patients no MG-antibodies were detected. All concordant twins showed the same antibody profile as their twin. Surprisingly, in two of the discordant twin pairs, the healthy twin was also positive for anti-AChR antibodies, despite having no clinical phenotype. Due to the rarity of MZ MG patients, recruitment was not restricted by treatment, and most, but not all, of the MG patients were treated with Pyridostigmine Bromide.

#### 3.2 High similarity in expression and methylation profiles between discordant twins

Initially, monocyte expression and methylation profiles were characterized using HumanHT-12 chip for expression and HumanMethylation450 chip for methylation in 6 MZ twin pairs, 3 concordant and 3 discordant for MG. Both expression and methylation signatures were found to be highly similar between the healthy discordant twins (DTH) and the MG discordant twins (DTMG), revealing only a few, relatively small differences (**Figures 1A & 1D**). Comparing DTH with the concordant twins (CTMG) resulted in more variation (**Figures 1B & 1E**), but to a similar extent as observed between DTMG and CTMG (**Figures 1C & 1F**), suggesting that these differences are mainly due to variations between twin pairs, and less likely to be related to disease phenotype. We suspected that the small number of detectable disease-associated differences is due to the small sample size and therefore modified the experimental design: a third dataset was created using samples from 3 singleton MG patients and 3 healthy controls, all females. Additionally, a dataset previously obtained using HumanHT-12 and HumanMethylation450 chips on monocytes from 5 healthy females was included in the analysis. Batch effect was minimized by normalization according to two repeated samples from the primary chips. The combined datasets were analyzed using one-way ANOVA for three defined groups: 1) singleton healthy controls (HH), 2)

discordant twins without clinical phenotype (DTH) and 3) MG patients (MG) including MG concordant and discordant twins (CTMG and DTMG) and MG singleton patients (MGS). Results and validation steps are described below.

### 3.3 Detection of MG-associated differentially expressed genes

Using a criteria of  $\geq 1.2$  fold change (FC) in expression at a FDR corrected p-value of  $< 0.05$ , 134 DEGs were detected between MG patients and HH controls, 104 genes were downregulated while 30 were upregulated in MG patients (**Supplemental Table S1**). Ingenuity Pathway analysis (IPA) of these 134 genes showed high connectivity between transcripts with activation of immune function, inflammation, and enrichment of canonical pathways, such as TREM1 and Toll-like receptor signaling (**Table 2**). 30 DEGs with FC  $> 1.3$  and detailed annotation were selected and further validated by RT-PCR using RNA samples from 15 MG patients (6 CTMG, 4 DTMG and 5 singleton samples), 15 healthy controls (9 females and 6 males), and 4 DTH samples. Out of the 30 DEGs evaluated, 16 were confirmed at a p-value  $< 0.05$ , 2 at a p-value  $< 0.07$ , between cases and controls, of which 4 significantly differed similarly between the discordant twins, i.e. between the MG twin and her healthy co-twin (**Table 3**). Four representative transcripts, namely IL1B, SERPINB2, EGR2, FPR2, are visualized in **Figure 2A**, of which two, namely EGR2 and FPR2, also statistically differ between the discordant twins. Cluster analysis based on the expression of the validated disease-associated transcripts shows two clusters of MG and HH, with 3 out of 4 DTH clustering with the MG samples and the remaining DTH with the HH group (**Figure 2B**). This indicates<sup>7</sup> that despite the lack of clinical phenotype, DTH individuals share some disease-associated monocyte expression profile with MG patients. The 6 samples from healthy males were included in the transcription validation analysis in order to increase the sample size, and as can be seen in **Figure 2B**, samples from the healthy males clustered together with samples from the healthy females.

### 3.4 Pathway analysis suggests that TREM-1 signaling pathway might be impaired in MG

In order to determine any functional relationship between the 18 validated MG-associated DEGs, the gene list was uploaded into an IPA pathway analysis. Despite the relatively low number of DEGs, several pathways with z-score below -2 were identified, of which three are presented in **Figure 3**. Triggering receptor expressed on myeloid cells (TREM1) is the upstream regulator of six of the transcripts, EGR2 and EGR3 transcription factors, IL1B cytokine, CXCL2 chemokine, CKS2 a cyclin dependent kinase and SERPINB2 protease inhibitor (**Figure 3A**); inhibition of immune cell proliferation is linked to downregulation of 6 transcripts; EGR2, EGR3, IL1B, CXCL2, ANPEP and CDKN2D (**Figure 3B**); and inhibition of chemotaxis of myeloid cells is linked to downregulation of 5 transcripts, IL1B, CXCL2, PLAUR, C3AR1 and FPR2 (**Figure 3C**). These results suggest a functional connection between monocyte MG-associated DEGs, immune cell function and the TREM-1 signaling pathway, which is known as an important mediator of innate inflammatory responses [28].



### 3.5 Distinct DNA methylation profiles were found in MG patients versus healthy controls

DNA methylation profile using HumanMethylation450 BeadChip showed similar results to the gene expression described above: a relatively small number of 23 DMGs were identified between the MG discordant twins and the healthy discordant twins at  $\geq 5\%$  difference in methylation  $\beta$  at uncorrected p-values of  $< 0.01$  (**Supplemental Table S2**). However, 1846 MG-associated DMGs were identified between healthy controls and MG patients at the above cutoff, of which 1242 were hypermethylated and 604 were hypomethylated (**Supplemental Table S3**). Two chromosomes, 6 and 19, seem to be particularly enriched with DMGs, with 64 out of 99 DMGs on chromosome 6 located within the 4Mbp HLA locus (data not shown). The genomic location of most of the MG-associated DMGs in general, and the hypermethylated DMGs in particular, were highly enriched in intergenic regions, 42% compared to the expected 25% coverage of the chip (**Figure 4A**) (Chi-test p-value 0.001), thus not annotated to specific genes. This might explain why no clear links between the MG-associated DEGs and the MG-associated DMGs datasets could be established, despite extensive search using different tools and databases, even though most of the DEGs showed reduced expression and most DMGs were hypermethylated, consistent with the central dogma of a negative correlation between expression and DNA methylation. It is possible that at least part of these DMGs are located in distal regulatory elements within intergenic regions or linked to transcripts not included in HumanHT-12 beadarray, such as long non-coding RNA. Since cell-specific mapping of enhancer elements of the transcriptome is not fully defined, the link and biological relation between the two datasets remains to be discovered.

The results of a two-way unsupervised hierarchical cluster analysis of the top 58 MG-associated DMGs (**Supplemental table S4**) are visualized in **Figure 4B**. The analysis separates the hypermethylated and hypomethylated sites on the horizontal scale, and patients and control samples on the vertical scale, with DTH samples clustering with MG samples. Thus, similarly to the gene expression profile, the methylation profile of the MG-associated DMGs in the discordant healthy twins is more similar to MG patients than to healthy controls. To validate DMGs, the target bisulfite sequencing (TBS) method was used as described previously [16]. Primers for 14 amplicons selected from the top 58 DMGs and 1 internal control were synthesized and used to amplify bi-sulfide converted DNA from 15 MG cases, 4 DTH, and 9 healthy controls, all females. No male samples were included in the validation of the DMGs due to the well-known sex effect on DNA methylation [16,26,29]. Each of the amplicons, ranging in size between 200-400bp, were designed to be centered around the MG-associated illumina beadarray CpG site, but CpG sites in the vicinity of the target CpG were sequenced as well. Out of the 14 target amplicons, 2 were excluded because of poor read coverage. Of the remaining, 6 amplicons were found to differ statistically between MG cases and controls, (**Table 4**). Visualization of normalized methylation counts in 4 representative amplicons, namely *BTN3A3*, *CAMKK2*, *FAM196B* and *KCNMB2*, is depicted in **Figure 4C**. In most, the difference in methylation count extends over several CpG sites within the amplicon (**Table 4**).

The 1846 MG-associated DMGs (Supplemental Table S3) were compared to a list of CpG site-related SNPs (cgSNPs), i.e. a SNP that leads to gain or loss of CpG sites [30], kindly provided by Zhou [31] and to MG-associated SNPs previously published [32–37]. 12 DMGs were found to be cgSNPs (Supplemental Table S5), and 8 DMGs overlap with MG-associated SNPs on chromosome 6, from the whole genome study of Renton et al. [37] (Supplemental Table S6). Interestingly, one of the overlapping DMG/MG-associated SNPs, namely rs3117109, located closely to C6orf10, is also a cgSNP, based on the list of Zhou et al. [31].

## 4. Discussion

In this study, we aimed to unravel genetic and epigenetic involvement in MG by obtaining the expression and methylation profiles of immune cells, using unique and extremely rare samples of MZ, female twins discordant and concordant for MG, recruited through a comprehensive, collaborative international effort of patients and physicians. The great potential value of MZ twin studies is widely recognized. The ability to control for many potential confounders encountered in general population studies, such as genetic background, maternal effects, early-life environmental exposure, age, gender and cohort effects, makes MZ twin studies considerably more powerful in detecting relatively small genetics and epigenetics effects even with relatively small cohorts. The value of MZ twins studies has been shown in numerous studies such as aging, obesity, autoimmune and heart diseases, psychiatric and neurological disorders, cancer and many more [38–42]. We believe that by using this MZ MG twin approach, we were able to identify disease-associated genes and epigenetic modifications, which may not be possible to detect in a case-control MG study. Therefore results presented here, supported by the first validation stage performed by us, may provide unique information for better comprehension and future investigation of MG pathogenesis.

### 4.1 Monocyte function in MG seems to be impaired

The analysis presented in this manuscript focused upon the data from monocytes, and identified several MG-associated DEGs and DMGs, suggesting that dysregulated monocytes may contribute to MG pathology. The detected MG-expression profile was enriched with monocyte functions and inflammatory responses, such as recognition of bacteria and viruses, Toll-like receptor and NFkB signaling, dendritic cell maturation, IL-6 signaling and Inflammasome pathway. Mo/M $\phi$  are major players of the innate immune response, inducing immunity by presentation of antigens and production of cytokines and chemokines, and altered Mo/M $\phi$  function may be involved in autoimmunity [12,15]. Only a few studies on monocytes in MG have been published, reporting elevated numbers of monocytes in the MG thymus, increased percentages of CD86- and CD80-expressing circulating monocytes, and increased Mo/M $\phi$  - dependent cytokine production from T cells [43–45]. This, taken together with results of our study, points to an important, previously relatively overlooked role of monocytes in MG, in line with their role in a spectrum of autoimmune diseases [12,14,15]. No overlap was found between the detected monocyte

DEGs and recently published MG-specific DEGs in PBMCs [46], emphasizing the cell specificity and the importance of analysis of isolated cell types.

A puzzling result was the observation that most DEGs were down-regulated in MG patients compared to controls. We expected a more equal ratio of down- and up-regulated genes, including upregulation of genes of the inflammatory milieu, similar to findings in the MG thymus [47]. However, several of our detected DEGs are involved in resolution of inflammation and homeostasis, and their reduced expression has previously been associated with autoimmune diseases. For example, EGR2 is important for the maintenance of T cell homeostasis and induction of T cell anergy [48], and negatively regulates differentiation of Th17 cells [49]. In fact, EGR2 has been suggested as a biomarker for recovery from the autoimmune disease Guillain-Barré Syndrome [50]. In a murine model, deletion of Egr2 and Egr3 in lymphocytes resulted in a lethal autoimmune syndrome with increased levels of pro-inflammatory cytokines [51]. SerpinB2 is associated with several human inflammatory diseases, and SerpinB2-depleted mice showed increased Th1 responses [52]. Furthermore, Mo/M $\phi$  isolated from synovial fluid from RA patients showed reduced expression of SERPINB2 and other genes associated with homeostasis and inflammation resolution [53]. FPR2 is an anti-inflammatory receptor, with a role in dampening the inflammatory response. Depletion of Fpr2 in a murine model caused acute inflammatory responses, and Fpr2 agonists are being evaluated as potential anti-inflammatory therapeutics [54,55]. Thus, the obtained lower expression of FPR2, SERPINB2, EGR2 and EGR3 in monocytes in MG patients may indicate decreased suppressor activity, which could underlie chronic inflammation in MG patients, a hypothesis to be further assessed in functional studies.

Our pathway analysis suggests that TREM-1 signaling pathway is enriched in MG. TREM-1 is known to amplify inflammation, to induce production of inflammatory cytokines and chemokines, to activate antigen-presenting cells, [56] and to inhibit macrophage apoptosis [57]. While the expression levels of TREM-1 did not differ between cases and controls, the identification of several DEGs regulated by the TREM-1 pathway suggests, that monocyte function may be impaired in MG. The three major circulating monocyte subsets: classical, intermediate and non-classical monocytes, were recently shown by single cell analysis to have distinct gene expression signature, and different expression levels of TREM-1, TREM-2 and SERPINB2 [58]. It remains to be determined, if an altered TREM-1 pathway in MG is specific to one of these monocyte subsets. The expression of TREM-1 was shown to be regulated by NF- $\kappa$ B, PU.1. and Nrf2 transcription factors, but also by enzymatic processes by MMPs [59]. While the endogenous and exogenous TREM-1 ligand is still unknown, TREM-1 initiates a signaling event through its constitutively association with DAP12, which, when phosphorylated by Src family kinases, activates multiple effector pathways and molecules, including the production of chemokines and cytokines [59,60]. TREM-1 also indirectly modulates signaling pathways induced by Toll Like Receptors (TLRs) and NOD-Like Receptors (NLRs), by a yet unknown mechanism [59]. Thus, the differences found in expression of several downstream genes of the TREM-1 pathway in MG could be due to altered membrane expression of TREM-1 or modifications in the phosphorylation of DAP12, or due to alterations in TLR/NLR pathways.

While TREM-1 was shown to overlap with LPS in up-regulation of a number of immunomodulatory factors, TREM-1 activation antagonized other LPS-induced genes, including IL1-2p40 and IL-23 cytokine production, indicating that the TREM-1 signaling innate response is complex [60]. We plan to study the functional impairment of this pathway, however this is beyond the scope of the current manuscript

#### **4.2 Genetic predisposition in MG: lesson from discordant twins**

Being aware of the small sample size of MZ MG twins, we expected to detect some disease-associated genes differing between discordant twins, both at the transcription and the DNA methylation levels. However, extremely high similarity was found between the healthy and the MG discordant twins, and an unexpected high similarity between the healthy discordant twins and the whole MG patients group, which includes also genetically unrelated patients. Remarkably, detectable levels of anti-AChR antibodies, a MG hallmark [61], were detected in two out of four DTH twins, without any clinical phenotype. While it is not possible to predict whether these individuals will eventually develop MG, it shall be noticed that the average age of the discordant twins was much lower than the average age of the concordant twins recruited. Ongoing follow up of these healthy twins will allow us in the future to determine whether their healthy clinical state remains. In general, the unique use of discordant twins such as in this study, may allow to distinguish between candidate disease-associated genes, found only in MG patients, and candidate genes of pre-disposition, present also in healthy discordant twins. Markers preceding clinical diagnostic by years have been described in Alzheimer disease, type I and type 2 diabetes, and cardiovascular disease [62,63], but have not been translated to a preclinical treatment approach, mainly due to insufficient predictive values and considerable economic burden. Our results from MZ twins indicate that clinical and para-clinical follow-up of siblings to patients with MG should be considered, as has also previously been suggested [64].

Integrative analysis of whole genome association (GWAS) and DNA methylation association (EWAS) results in specific traits, suggests that many of the associated sites might be CpG site-related SNPs (cgSNPs), i.e. a SNP that leads to gain or loss of CpG sites [30]. Such cgSNPs and consequently differentially methylated sites are expected to be present in both MZ twins, and thus could explain the high similarity between the healthy and the MG discordant twin. Thus, the estimated 30-40% MG concordance rate [65] might be set too low, mainly due to the small number of MZ MG twins available, lack of adequate follow-up of the healthy co-twins and lack of zygosity determination [3]. A comparison of the 1846 MG-associated DMGs with a list of cgSNPs provided by Zhou [31] revealed that at least 12 of these methylation changes occurs in sites of cgSNPs, and thus are expected to occur in both discordant twins. Furthermore, 8 of the MG-associated DMGs overlapped with MG-associated SNPs [37], of which at least one has been shown to be a cgSNP [31], as presented in Supplemental Tables 5+6. These results point to that at least some of the changes in methylation are due to cgSNPs, including SNPs previously associated with MG. A functional study of the biological relevance of alterations in these sites and genes could shed new light on the mechanism of MG pathogenesis.

### 4.3 DNA methylation changes between cases and controls are modest

Complex diseases such as MG are proposed to arise from an interplay between genetics and environmental, epigenetic factors [40]. In the current study, we found numerous but small delta differences in DNA methylation between cases and controls, with enrichment for CpG changes in intergenic regions without known gene annotation, making biological interpretation of these findings difficult with the currently available databases. Interestingly, while pathway analysis of the 136 DEGs created a highly cohesive and interconnected network, enriched for the expected monocytes/macrophages functions (**Table 2**), the 1846 DMGs did not show such connectivity. Nevertheless, the enrichment of DMGs within the HLA locus, a locus strongly implicated in MG susceptibility [3], suggests that these differences may have biological relevance. The small magnitude of methylation differences is similar to publications on MZ twins discordant for T1D [62] and RA patients [5], however none of these published DMGs overlap with our data in MG. Understanding transcriptional regulation is key to comprehend normal and abnormal phenotypes. About two third of the DMGs in the current project were hyper-methylated in MG, while most of the DEGs were down-regulated. However since no correlation was found between DEGs and DMGs, despite extensive database search such as The Ensembl Regulatory Build and others [19], the biological impact remains to be elucidated. Although the ENCODE project [66] has mapped hundreds of thousands of enhancers elements, the limited resolution of high-throughput chromatin interaction assays hampers efforts to connect regulatory elements to specific genes [67,68]. Moreover, the relation between the enhancer and the gene it regulates is highly dynamic, tissue specific, and cell-development dependent [68]. Thus, improved analysis tools and more comprehensive mapping of immune cell regulatory elements may allow in the future to better define the relation between MG-associated expression and methylation profiles found in the current study.

## 5. Conclusion

The current study is the first to characterize the expression and methylation profiles of peripheral monocytes from MZ twins with MG. Results suggest that numerous small changes in gene expression or methylation might together contribute to disease onset. Several transcripts highlighted in the current study have been associated with dysregulated immune homeostasis and inflammation resolution, suggesting that impaired monocyte function may have an important role in MG disease susceptibility and disease course, and thus could contribute to the chronicity of the disease. Hence, the current study, despite the limited number of MZ twin pairs available, points to: A distinct involvement of the innate immune response in MG pathogenesis; Suggests an additional direction of research for deciphering the molecular basis of MG pathology; Provides a list of novel potential genes for further functional analysis, as well as potential candidates for biomarkers and targeted therapy, following further replication. In addition, the high similarity between the healthy and the MG discordant twins, both at the expression and the methylation level,

indicates that genetic predisposition may have a stronger contribution to disease phenotype than previously assumed.

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**Table 1: Demographic and clinical data on the experimental cohort**

<b><i>MZ Twin</i></b>	<b><i>Age</i></b>	<b><i>Country</i></b>	<b><i>Clinical phenotype</i></b>	<b><i>Auto-antibodies</i></b>	<b><i>Other autoimmune diseases</i></b>	<b><i>Treatment</i></b>
<b><i>Concordant</i></b>	59	Czech R	+	AChR+	Autoimmune Thyroiditis	Pyridostigmine
			+	AChR+	Autoimmune Thyroiditis	<u>A</u> mbenonium
	54	USA	+	AChR+	SLE, chronic immune thrombocytopenia	Pyridostigmine
			+	AChR+	no	No treatment
	66	France	+	AChR- MUSK-	no	Pyridostigmine
			+	AChR- MUSK-	no	Pyridostigmine
<b><i>Discordant</i></b>	35	UK	+	AChR+	no	Pyridostigmine
			-	AChR+	no	-
	34	Netherlands	+	AChR- MUSK-	no	Pyridostigmine
			-	AChR- MUSK-	no	-
	21	France	+	AChR- MUSK- LRP4+	no	No treatment
			-	AChR- MUSK-	no	-
	33	France	+	AChR+	no	Pyridostigmine Prednisone Azathioprine
			-	AChR+	no	-
<b><i>Singleton</i></b>	59	France	+	AChR+	no	Pyridostigmine
	34	France	+	AChR+	no	Pyridostigmine Azathioprine
	44	France	+	AChR-	no	No treatment
	48	France	+	AChR+	no	<u>A</u> mbenonium Azathioprine
	53	France	+	AChR+	no	Pyridostigmine Prednisone

**Table 2: Enriched canonical pathways between MG and healthy control**

<b>Ingenuity Canonical Pathways</b>	<b>z-score</b>
TREM1 Signaling	-2.45
Toll-like Receptor Signaling	-2.24
IL-6 Signaling	-2.24
NF-kB Signaling	-2.24
Inflammasome pathway	-2
Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses	-2
Dendritic Cell Maturation	-2
Acute Phase Response Signaling	-2
cAMP-mediated signaling	-2
PPAR Signaling	2
LXR/RXR Activation	2.24

IPA identified enriched canonical pathways with a Z-score of above and below 2 in 134 DEGs between MG and healthy controls



**Table 3: Summary of validated MG-associated transcripts**

Gene Name	FC HH-MG	p-value (T test)	FC DTH-DTMG	p-value (paired Wilcoxon test)
ANPEP	1.4	0.0208	-	-
C3AR1	2.1	0.0379	1.5	0.06
CDKN2D	0.5	0.0003	1.4	0.0304
CKS2	2.3	0.0001	-	-
CSTF2	1.54	0.0115	-	-
CXCL2	2.1	0.0369	-	-
EGR2	3.2	0.0003	1.97	0.0304
EGR3	4.2	0.0001	-	-
EREG	2.5	0.0412	-	-
FPR2	2.6	0.0025	1.4	0.0304
IL1B	1.3	0.008	-	-
LEPROTL1	1.54	0.00088	-	-
MCL1	1.3	0.0162	-	-
MRPS28	1.32	0.0132	-	-
PIGF	1.32	0.063	1.15	0.0304
PLAUR	1.5	0.056	1.4	0.0304
SERPINB2	4.4	0.0001	-	-
ZNF296	0.6	0.0013	-	-

**Validation of MG-associated transcripts by qRT-PCR.** Gene symbols, fold change (FC) and p-value < 0.05 based on t-test between MG patients and healthy (HH), as well as FC and p-value < 0.05 based on Wilcoxon paired test between discordant twins are presented.

**Table 4: Summary of validated MG-associated DMGs**

Gene Symbol	BP <sup>a</sup>	HH (N=9)		MG (N=15)		DTH (N=4)		P-value <sup>d</sup>	
		% Methyl <sup>b</sup>	Methyl_ Count <sup>c</sup>	% Methyl <sup>b</sup>	Methyl_ Count <sup>c</sup>	% Methyl <sup>b</sup>	Methyl_ Count <sup>c</sup>	ANOVA	Chi-Square
<b>BTN3A3</b>	68	34.9	489	44.0	676	38.8	509	0.0207	0.03
<b>BTN3A3</b>	85	40.8	590	51.9	824	45.3	619	0.0205	0.0262
<b>BTN3A3</b>	106	54.0	943	69.8	1404	62.3	1052	0.0054	0.0114
<b>BTN3A3</b>	190	72.3	1265	81.2	1670	77.3	1283	0.0105	0.0105
<b>CAMKK2</b>	61	33.6	71	15.3	36	16.6	26	0.0096	0.0286
<b>CAMKK2</b>	106	32.85	92	17.3	47	9.9	20	0.0131	0.037
<b>CAMKK2</b>	207	15.1	146	3.5	24	4.5	29	0.006	0.0902
<b>CYP2U1</b>	95	3.9	51	2.3	31	3.0	34	0.01	0.0172
<b>EID2</b>	46	13.6	571	9.4	221	14.5	462	0.004	0.0055
<b>EID2</b>	131	61.4	2900	55.9	857	48.9	1696	0.0004	0.0009
<b>FAM196B</b>	106	34.2	1593	29.6	858	33.7	1082	0.0046	0.0101
<b>FAM196B</b>	126	30.6	1449	25.9	764	30.6	996	0.0051	0.0086
<b>FAM196B</b>	139	14.0	675	12.7	375	16.5	541	0.0399	0.0289
<b>KCNMB2</b>	233	91.4	167	93.4	260	92.7	116	0.0074	0.0065

**Validation of MG-associated DMGs by TBS between MG patients, healthy (HH) and discordant healthy twins (DTH).**

<sup>a</sup>BP – Base pair position within specific amplicon

<sup>b</sup>Methylation percentage calculated as number of cytosine read at a position out of total read count.

<sup>c</sup>Normalized Methylation Count - Normalized CpG count at a position within an amplicon. Mean count is presented for each of the three groups HH, MG, and DTH

<sup>d</sup>p- Value calculated on normalized methylation count between the three groups.

## Figures legend

### Figure 1: Both CD14 expression and methylation signatures are highly similar between the healthy and the MG discordant twins

Expression and methylation scatter plots. Average expression signal of 47K probes in a log<sub>2</sub> scale in A-C, and average  $\beta$ -methylation value in D-F. **A.** DTH versus DTMG on the x-axis and y-axis respectively. **B.** DTH versus CTMG twins on the x-axis and y-axis respectively. **C.** DTMG versus CTMG twins on the x-axis and y-axis respectively. **D.** DTH versus DTMG on the x-axis and y-axis respectively. **E.** DTH versus CTMG twins on the x-axis and y-axis respectively. **F.** DTMG versus CTMG twins on the x-axis and y-axis respectively. DTH- healthy discordant twins; DTMG – MG discordant twin; CTMG – MG concordant twin

### Figure 2: DTH expression signature is more similar to MG patients than to healthy control

**A.** Four representative genes of the DEGs validated by qRT-PCR in samples from healthy controls (HH), MG patients (MG) and healthy discordant twins (DTH) - Box plots presenting  $10^{-2^{\Delta\text{dCT}}}$  normalized to reference genes, i.e. lower value represents lower expression. The middle line within a box represents the mean value of the group, and the middle line of the graph represents the mean value of the three groups. Each discordant twin pair shares a symbol and is connected with a line. **B.** Expression heat plot - Samples clustering based on expression similarity of the validated transcripts shown as heat plot, with red to blue color scale indicating high to low similarity between samples. Sample name appears next to the plot, with MG patients in blue, healthy discordant twins (DTH) in red, and healthy controls (HH) in green, number indicating twin pair number with A or B for co-twins, F-female, M-male.

### Figure 3: Ingenuity pathway analysis (IPA) identifies functional connections between monocyte DEGs.

18 validated monocyte disease-associated DEGs were uploaded into IPA, and functional enrichment is presented. The different shapes surrounding the gene name indicate the molecular family (enzyme, cytokine, peptidase, transcription regulator, transporter, kinase and other). Downregulated transcripts are in green and upregulated transcripts in red in MG patients compared to healthy controls. **A.** TREM1 is the upstream regulator of the six DEGs indicated. **B.** Down-regulation of immune cells proliferation links six DEGs. **C.** Down-regulation of myeloid cell chemotaxis links five transcripts.

**Figure 4: DTH methylation signature is more similar to MG patients than to healthy controls**

**A.** Hypermethylated MG-associated CpGs are enriched in Intergenic region- CpGs sites are divided by genomic location: Intergenic region (blue), Promoter (orange), Gene body (grey) and 3 UTR (yellow). **(I)** Percent CpGs sites in the different locations covered in the whole chip. **(II)** Percent hypermethylated MG-associated CpGs per location. **B.** Two-ways unsupervised hierarchical clustering of  $\beta$ -methylation values of top 58 CpG sites in beadchip samples, with red to blue color scale indicating  $\beta$ -methylation values scaling. Sample name appears under the plot, with MG patients in blue, discordant healthy twins (DTH) in red, and healthy controls (HH) in green, number indicating twin pair number with A or B for co-twins. **C.** Representative validation of CD14 DMGs by Target bisulfide sequencing - Normalized methylation count on the y-axis of 4 representative DMGs of three experimental groups on the x-axis, healthy controls (HH), healthy discordant twins (DTH), and MG patients, with p-value between HH and MG presented. Each discordant twin pair shares a symbol and is connected with a line. The middle line within a box represents the mean value of the group, and the middle line of the graph represents the mean value of the three groups.

Figure 1

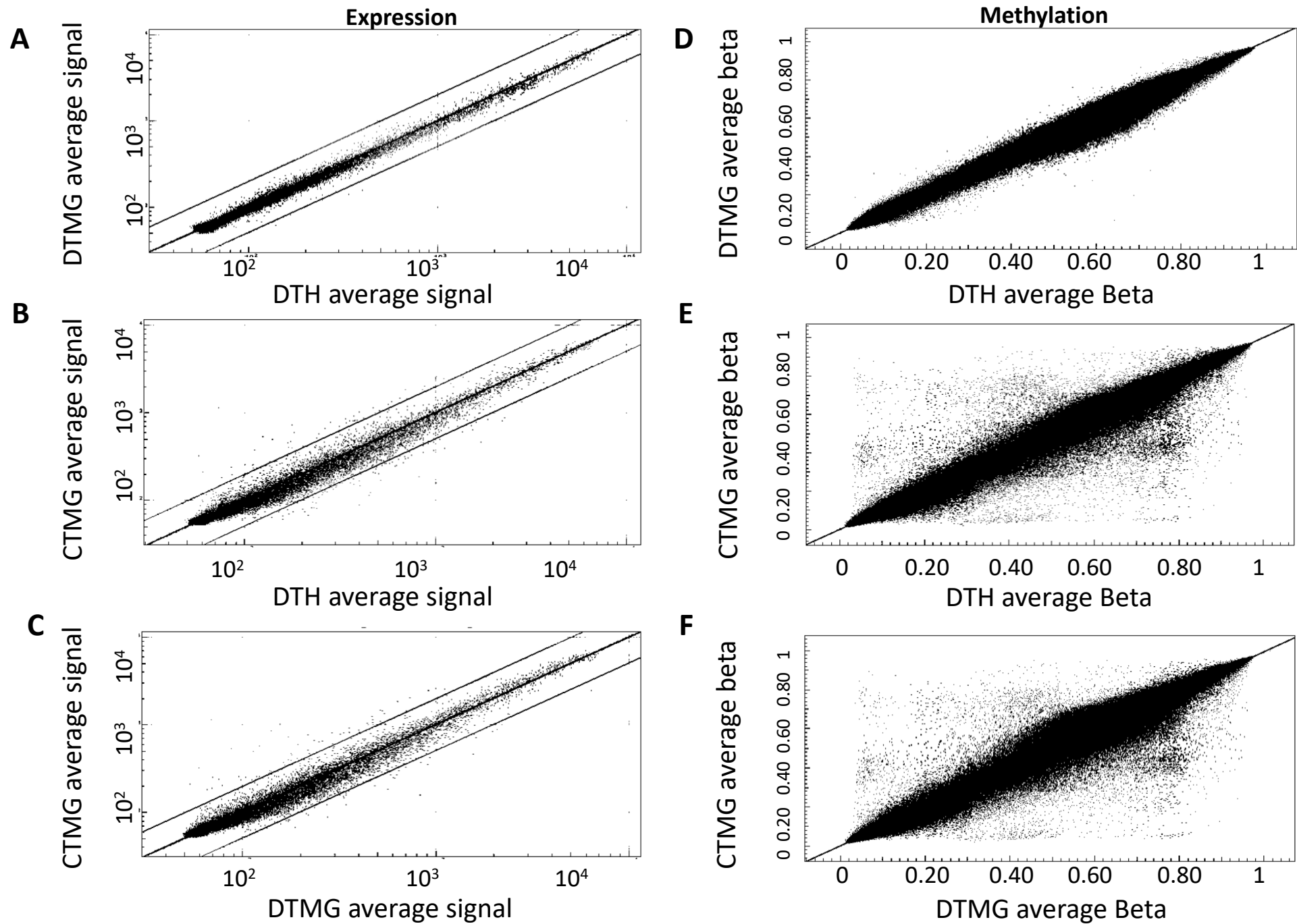


Figure 2A

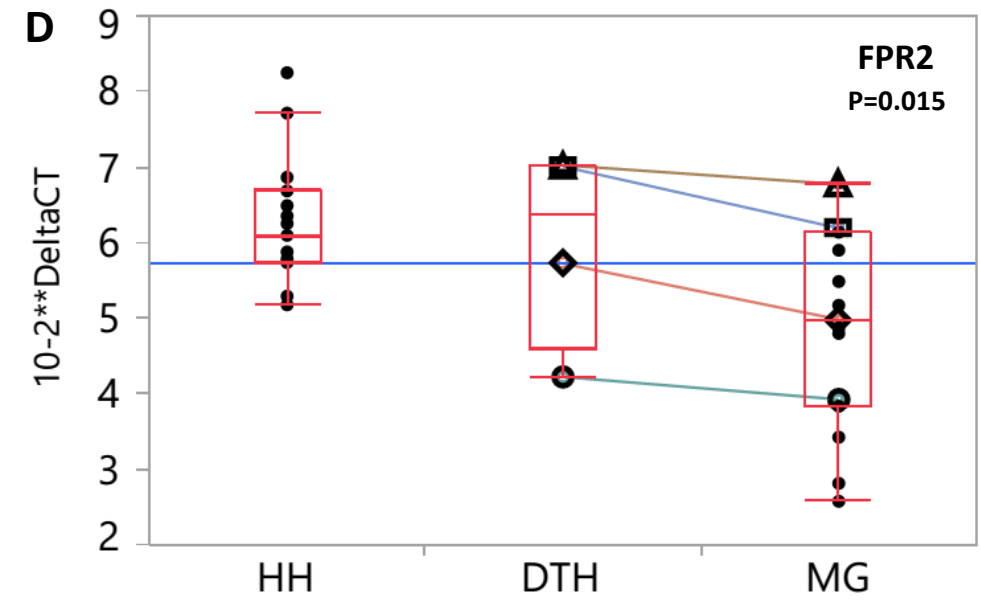
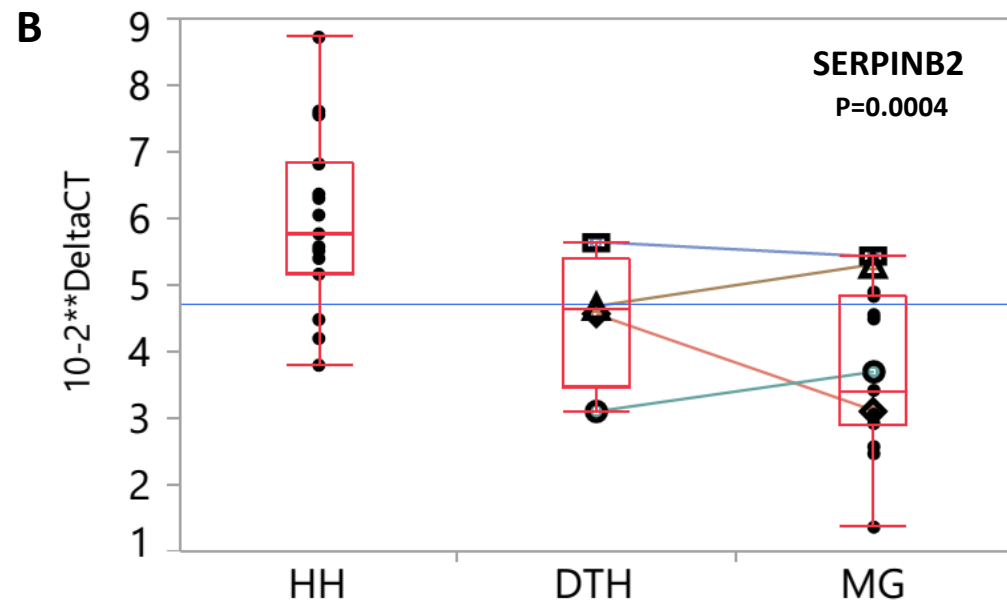
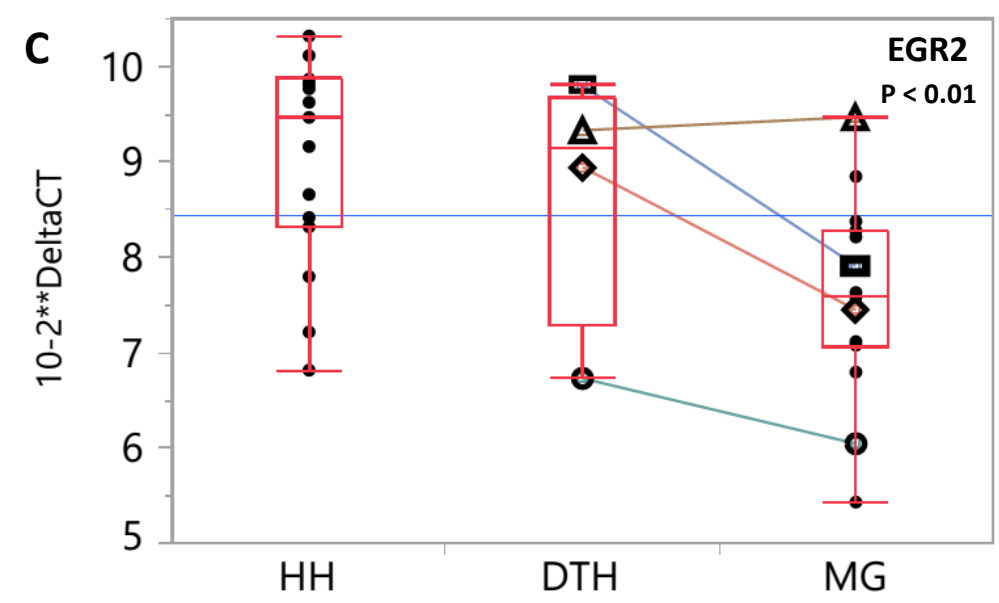
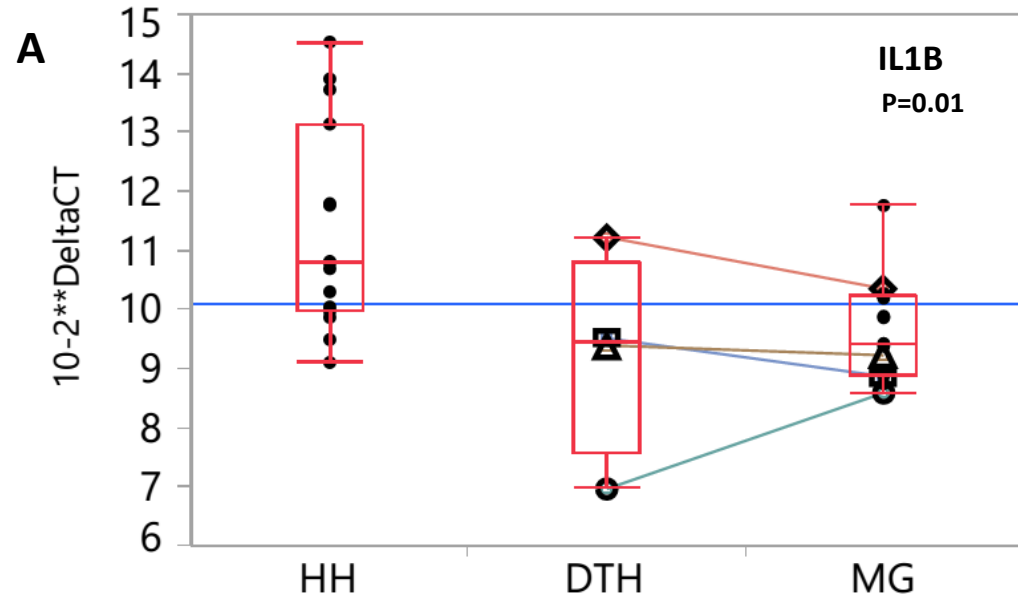


Fig2B

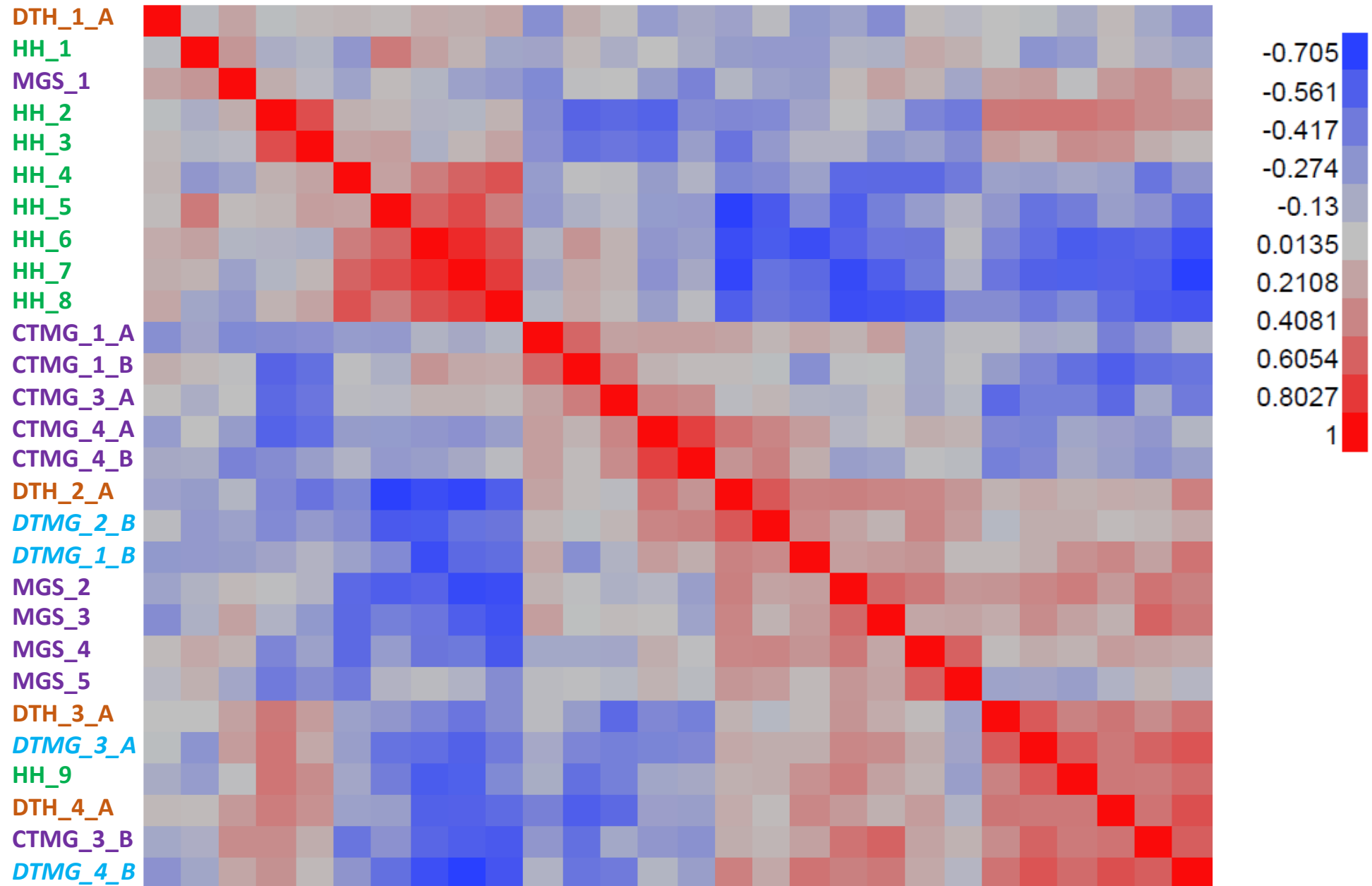


Figure 3

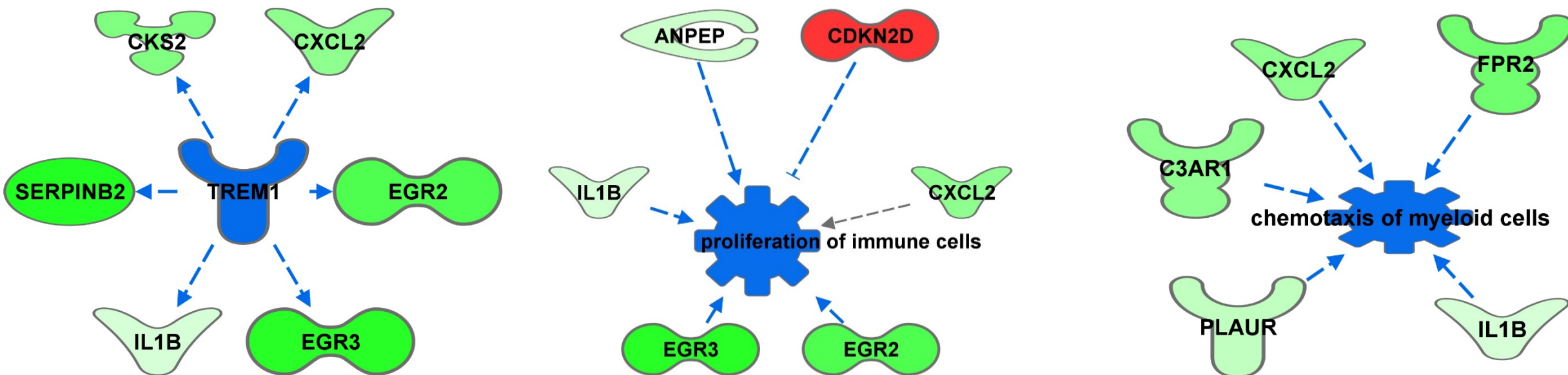


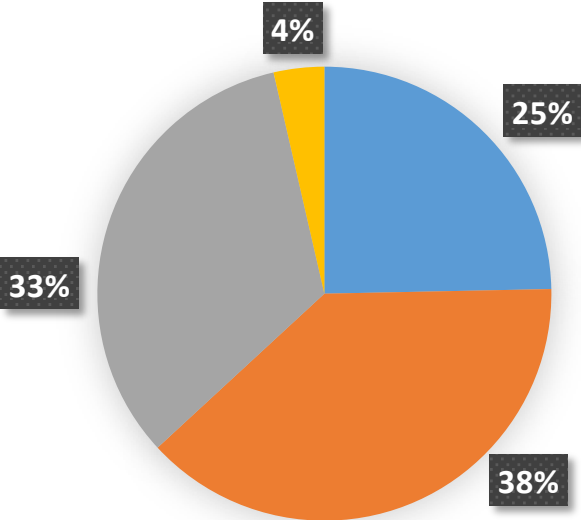


Figure 4 A

- Intergenic Region
- Promoter
- Gene Body
- 3 UTR

A

% CpG sites-whole chip



Hypermethylated Monocyte MG associated DMRs %

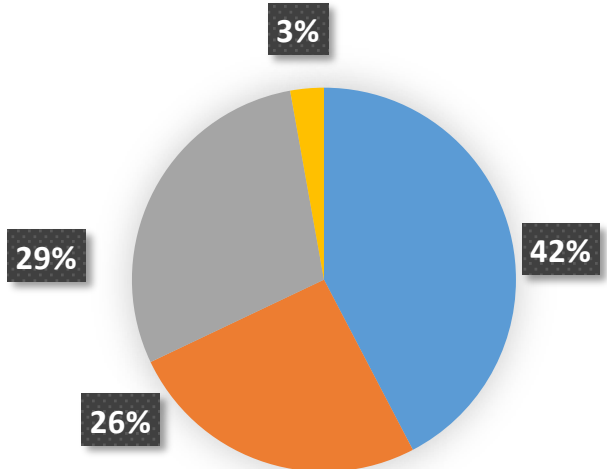


Figure 4B

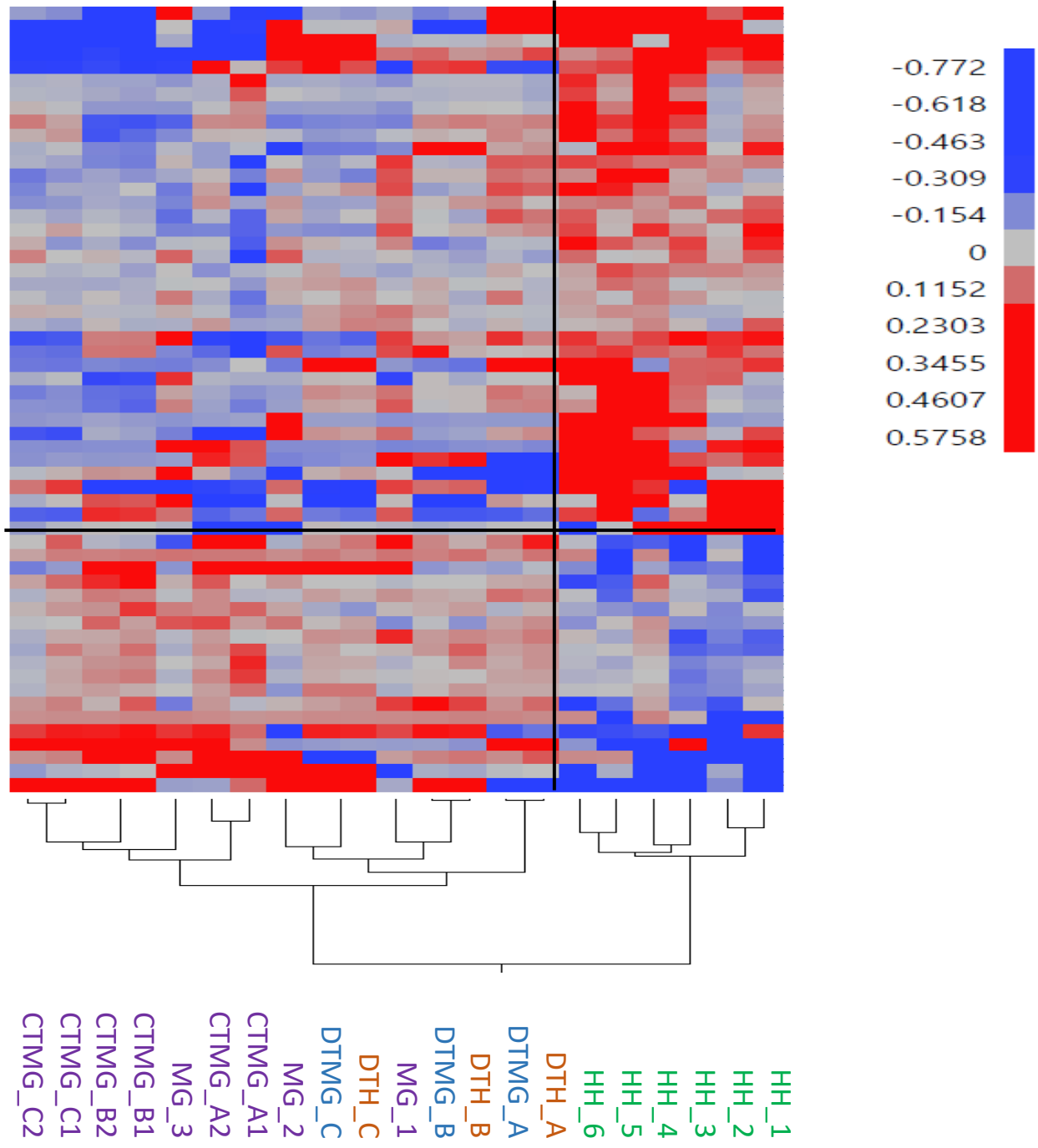


Figure 4C

