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Altered DNA methylation pattern characterizes the peripheral immune cells of patients with autoimmune hepatitis

Kalliopi Zachou[1] | Pinelopi Arvaniti[1] | Aggeliki Lyberopoulou[1] | Eirini Sevdali[2] | Matthaios Speletas[2] | Maria Ioannou[3] | George K. Koukoulis[3] | Yves Renaudineau[4,5] | George N. Dalekos[1]

[1]Department of Medicine and Research Laboratory of Internal Medicine, National Expertise Center of Greece in Autoimmune Liver Diseases, European Reference Network on Hepatological Diseases (ERN RARE-LIVER), General University Hospital of Larissa, Larissa, Greece

[2]Faculty of Medicine, Department of Immunology and Histocompatibility, School of Health Sciences, University of Thessaly, Larissa, Greece

[3]Faculty of Medicine, Department of Pathology, School of Health Sciences, University of Thessaly, Larissa, Greece

[4]INSERN U1291, CNR U5051, University Toulouse III, Toulouse Institute for infectious and inflammatory diseases, Toulouse, France

[5]Department of Immunology, Purpan University Hospital Toulouse, Toulouse, France

Correspondence
George N. Dalekos, Department of Medicine and Research Laboratory of Internal Medicine, National Expertise Center of Greece in Autoimmune Liver Diseases, General University Hospital of Larissa, 41110 Larissa, Greece.
Email: georgedalekos@gmail.com

Abstract

Background and Aims: Little is known about the impact of DNA methylation modifications on autoimmune hepatitis (AIH) pathogenesis and therapeutic response. We investigated the potential alterations of DNA methylation in AIH peripheral lymphocytes at diagnosis and remission.

Methods: Ten AIH patients at diagnosis (time-point 1; AIH-tp1), 8/10 following biochemical response (time-point 2; AIH-tp2), 9 primary biliary cholangitis (PBC) and 10 healthy controls (HC) were investigated. Peripheral CD19(+) and CD4(+) cells were isolated. Global DNA methylation ($5^{m}C$)/hydroxymethylation ($5^{hm}C$) was studied by ELISAs. mRNA of DNA methylation (DNMT1/3A/3B) and their counteracting hydroxymethylation enzymes (TET1/2/3) was determined by quantitative RT-PCR. Epigenome wide association study (EWAS) was performed in CD4(+) cells (Illumina HumanMethylation 850 K array) in AIH and HC. Total $5^{m}C/5^{hm}C$ was also assessed by immunohistochemistry (IHC) on paraffin-embedded liver sections.

Results: Reduced TET1 and increased DNMT3A mRNA levels characterized CD19(+) and CD4(+)-lymphocytes from AIH-tp1 compared to HC and PBC, respectively, without affecting global DNA $5^{m}C/5^{hm}C$. In AIH-tp1, CD4(+) DNMT3A expression was negatively correlated with serum IgG ($P = .03$). In remission, DNMT3A decreased in both CD19(+) and CD4(+) cells compared to AIH-tp1 ($P = .02$, $P = .03$ respectively). EWAS in CD4(+) cells from AIH patients confirmed important modifications in genes implicated in immune responses (HLA-DP, TNF, lnRNAs and CD86). IHC showed

List of abbreviations: $5^{hm}C$, 5-hydroxymethyl-cytosine; $5^{m}C$, 5-methyl-cytosine; AIZ, autoimmune hepatitis; ALT, alanine aminotransferase; AMA, antimitochondrial autoantibodies; ANA, antinuclear autoantibodies; AST, aspartate aminotransferase; DAVID, database for Annotation, Visualization and Integrated Discovery; DMPs, differentially methylated probes; DMRs, differentially methylated regions; DNMTs, DNA methyl-transferases; EWAS, epigenome-wide association studies; GAPDH, glyceraldehyde-3-Phosphate Dehydrogenase; GWAS, genome wide association studies; HC, health controls; HLA, human leucocyte antigen; MHC, major histocompatibility complex; miRNAs, micro-RNAs; MMF, mycophenolate mofetil; NASH, νon alcoholic steatohepatitis; PBC, primary biliary cholangitis; PBMCs, peripheral blood mononuclear cells; SjS, sjögren's syndrome; SLA, soluble liver antigen; SLE, systemic lupus erythematosus; SMA, smooth muscle cell antibodies; TETs, ten-eleven translocation deoxygenases; TSS, Transcription start sites; tp1, time point 1; tp2, time point 2; Tregs, T regulatory cells; TSS, transcription start site; UTR, untranslated region.

Note: Preliminary results of this project have been presented as an abstract in the 18th European Congress of Internal Medicine, Lisbon 29-31 August 2019, the 18th and 19th Hellenic Congresses of Hepatology, Athens 11-13 September 2020 and 6-9 May, 2021 as well as in the Digital Liver International Congress of EASL, June 23-26, 2021.

Kalliopi Zachou and Pinelopi Arvaniti, These authors contributed equally to this work.

Yves Renaudineau and George N. Dalekos, These authors contributed equally to this work as senior authors

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increased $5^{hm}C$ staining of periportal infiltrating lymphocytes in AIH-tp1 compared to HC and PBC.

Conclusion: Altered TET1 and DNMT3A expressions, characterize peripheral lymphocytes in AIH. DNMT3A was associated with disease activity and decreased following remission. Gene DNA methylation modifications affect immunological pathways that may play an important role in AIH pathogenesis.

KEYWORDS
autoimmune hepatitis, DNA methylation, DNA-methyltransferases, epigenetics, epigenome-wide association studies

1 | INTRODUCTION

Autoimmune hepatitis (AIH) is a chronic liver disease characterized by hypergammaglobulinaemia, autoantibodies, interface hepatitis and favourable response to immunosuppression.[1-6] Although its aetiology remains unknown, the interaction between genetic and environmental factors seems fundamental in AIH pathogenesis.[2]

Genetic predisposition to AIH in adults has been linked to genes within the human leucocyte antigen (HLA) region, particularly with the allelic variants *HLA DRB1*0301* and *DRB1*0401* of DRB1, while weaker associations have been found with non-HLA genes.[7] In children, apart from *HLA DRB1*0301* other HLA loci have also been implicated, but HLA *DRB1*0401* was underrepresentative.[8] Nevertheless, *HLA DRB1*0301* and *DRB1*0401* genotypes associations occur in only 51-55% of adult patients and *DRB1*0301* in 70% of children with AIH-type 1, indicating that additional factors, such as epigenetic changes, could contribute to its pathogenesis.[2,7-9]

Epigenetic modifications, consisting of DNA methylation, histone adjustment and micro-RNAs (miRNAs), influence gene expression without altering the DNA sequence. In eukaryotic cells, DNA methylation represents the central epigenetic process and refers to the methylation of carbon 5 of cytosine ($5^{m}C$) at the cytosine-phosphate-guanine dinucleotides (CpGs) by DNA methyl-transferases (DNMT1,3A,3B).[10-12] CpG-rich sequences (CpG islands) are mainly located within the promoter regions of many genes and when methylated, they usually block the accessibility of transcription factors, resulting in repression of gene transcription.[13] The latter is counterbalanced by an active DNA demethylation process comprising of the oxidization of $5^{m}C$ into 5-hydroxymethylcytosine ($5^{hm}C$) by Ten Eleven Translocation (TET1,2,3) deoxygenases.[14]

Regarding liver diseases, epigenetic studies have been performed mainly in liver fibrosis, non-alcoholic fatty liver disease, hepatocellular carcinoma and cholangiocarcinoma.[15,16] To date, epigenetic data in AIH is limited, with only one study reporting significant association of elevated miR21 with the biochemical and histological activity of AIH and decreased miR21 and miR122 in cirrhotic patients.[17]

As from the best of our knowledge, DNA $5^{m}C/5^{hm}C$ have not been explored in AIH, we investigated the potential presence of DNA methylation modifications in peripheral B and T cells from AIH patients by assessing alterations in DNA methylation through the analysis of global $5^{m}C$ and DNMT1/3A/3B expression as well as alteration in $5^{hm}C$ and TETs transcriptional expression levels. Such analysis was performed at diagnosis and remission. Differences in total $5^{m}C/5^{hm}C$ state between AIH patients at diagnosis and healthy controls (HC) were also assessed by immunohistochemistry (IHC) on paraffin-embedded liver sections. Finally, to evaluate the methylation alterations in specific CpG sites across the whole genome, we performed epigenome wide association study (EWAS) in CD4(+) cells from patients and controls using the Illumina HumanMethylation 850 K array.

Lay summary

- Autoimmune hepatitis (AIH) is a non-resolving chronic liver disease of unknown aetiology with favourable response to immunosuppression. Since genetic studies are insufficient to explain the observed risk for AIH, epigenetic modifications may affect its occurrence and outcome.
- We found alterations of DNA methylation in peripheral immune cells and liver histology of AIH patients, which were associated with disease activity and modified by immunosuppression.
- These results provide the first evidence that epigenetics play a role in AIH pathogenesis, which may lead to therapeutic implications for the management of the disease.

2 | PATIENTS AND METHODS

2.1 | Study samples and peripheral mononuclear cells (PBMCs) preparation

PBMCs from 10 AIH patients were isolated from peripheral blood collected at the time of diagnosis (AIH time-point 1; AIH-tp1), by gradient centrifugation (Histopaque-1077, Sigma-Aldrich, St. Louis, MI, USA). PBMCs were then mixed with freezing medium (FBS with 10% DMSO Sigma-Aldrich) and cryopreserved in liquid nitrogen until use.

In eight of these patients, PBMCs were also isolated at a second time-point when they had complete biochemical response under immunosuppression (AIH time-point 2; AIH-tp2). According to the guidelines of the Hellenic Association for the Study of the Liver[5] and our published protocols,[18,19] AIH-tp2 patients were receiving at the time of investigation either combination therapy with prednisolone 0.5-1 mg/kg/day and mycophenolate mofetil 1.5-2 g/day (MMF; n = 6) or MMF maintenance monotherapy (n = 2). Ten healthy served as HC and 9 primary biliary cholangitis (PBC) patients at diagnosis before treatment initiation served as the disease control group. Patients and controls were age- and sex- matched (Table S1).

As in our previous reports,[18,19] all biopsies were assessed using the Knodell histological/activity index score[20] and patients were divided into two groups according to inflammation: minimal-mild and moderate–severe and according to fibrosis: minimal/mild–moderate and severe fibrosis-cirrhosis. In PBC, the Ludwig staging system was applied.[21]

All patients consented to participate in this study. The ethical committee of the General University Hospital of Larissa approved the protocol which conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the institution's human research committee (March 21, 2016/2258).

2.2 | CD19(+) B- and CD4(+) T-cell isolation

CD19(+) and CD4(+)-lymphocytes were isolated by ROBOSEP™-S platform (Stemcell Technologies, Vancouver, USA). PBMCs were incubated with specific antibodies for magnetic selection using the EasySep Human CD19 Positive Selection Kit II and Human CD4 Negative Selection Kit respectively (Stemcell Technologies). Purity of the isolated B and T cells was assessed by flow cytometry using PE-Cy5-anti-CD19 and FITC-anti-CD3/PE-anti-CD8 antibody (Biolegend Inc., San Diego, CA, USA) on a Coulter FC-500 flow cytometer (Beckman-Coulter, Brea, CA, USA) (Figure S1A-D). A total average of 1×10^6 CD19(+) and 2×10^6 CD4(+) cells were isolated per sample and stored at −80°C.

2.3 | DNA/RNA extraction and quantification

Details for the genomic DNA and total RNA extraction and quantification are shown in Supplementary Patients and Methods section.

2.4 | Determination of $5^{m}C$ and $5^{hm}C$ DNA levels

$5^{m}C$ and $5^{hm}C$ DNA levels were determined using MethylFlash™ Global DNA Methylation (5-mC) and Hydroxymethylation (5-hmC) ELISA Easy Kit (EpiGentek, NY, USA), according to the manufacturer's instructions. Briefly, 100 ng of DNA was bound in duplicates to strip-wells with high DNA affinity, the methylated and hydroxymethylated fraction was detected by $5^{m}C$ and $5^{hm}C$ antibody-based detection complex in one-step manner and quantified colorimetrically at 450 nm in a microplate spectrophotometer (Dynex OpsysMR™ Microplate Reader, Aspect Scientific Ltd, UK). The percentage of $5^{m}C$ and $5^{hm}C$ in the cytosine content was proportional to the optical density (OD) measured and was calculated by generating a logarithmic standard curve based on positive, negative controls and seven standard samples supplied by the manufacturer.

2.5 | DNMT1, DNMT3A, DNMT3B, TET1, TET2 and TET3 mRNA quantification

One μg of total RNA was reversed transcribed using random hexamer primers and 10 Units of Transcriptor Reverse Transcriptase according to the Transcriptor First Strand cDNA synthesis kit (Roche Diagnostics, Basel, Switzerland) in a 20 μL reaction for 10 minutes at 25°C, 60 minutes at 50°C and 5 minutes at 85°C. Quantitative polymerase chain reaction was carried out using FastStart DNA Master SYBR Green I (Roche Diagnostics) in a total volume of 20 μL containing 250 nM specific forward and reverse primers. Amplification and detection were performed in a Lightcycler^{R} 96 Instrument (Roche Life Sciences, Bavaria, Germany) under the following conditions: 1 cycle at 50°C for 2 minutes and 95°C for 10 minutes and 40 cycles at 95°C for 15 seconds and 60°C for 1 minutes. The primers used for DNMT3A, DNMT3B, TET1 and TET2 were as previously described.[22] For the quantification of human DNMT1, TET3 and Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) genes, commercially specific primers were used: DNMT1: Hs00945875_m1, TET3: Hs00896441_m1, GAPDH: Hs02758991_g1 (Thermo Fisher Scientific, Waltham, MA, USA). Human GAPDH mRNA was chosen as internal control and for quantification of gene expression the comparative CT method [2 − (ΔCT target −ΔCT calibrator) or 2 − ΔΔCT] was used. Validation experiments were carried out in duplicates and each run was completed with a melting curve analysis to confirm the specificity of the amplification and the lack of primer dimmers.

2.6 | EWAS

Five hundred nanoggram of DNA from CD4(+)-lymphocytes from AIH (10 tp1 and 5 tp2) and 9 HC were bisulfite-converted (Zymo Research, Irvine, CA, USA) and DNA methylation was evaluated by hybridizing bisulfite-converted DNA to the Human Methylation EPIC array Bead Chip (Diagenode SA, Belgium), which allows the interrogation of over 850000 methylation sites throughout the

genome at single-nucleotide resolution (Supplementary Patients and Methods; EWAS). These steps were performed by NXT-DX Company (Gent, Belgium) according to manufacturer's instructions. Methylation data were provided as β-values: $\beta = M/(M + U)$, where M was the fluorescent signal of methylation and U the respective signal of the unmethylated probe. The β-values ranged from 0 (no methylation) to 1 (100% methylation). A quality control on the output of the Illumina Infinium EPIC array was performed with the Bioconductor R package, Chip Analysis Methylation Pipeline (ChAMP), according to which no sample had a proportion of failed probes >0.1. After normalization, potential batch effects were evaluated with the singular value decomposition method, which did not identify any significant source of variations that needed corrections.

2.7 | Liver immunohistochemistry (IHC)

Paraffin-embedded sections from six AIH-tp1 patients, three PBC and seven HC obtained during cholecystectomy, were investigated. Staining for $5^{m}C$ and $5^{hm}C$ was performed with anti-5-methylcytosine (5-mC) and anti-5-hydroxymethylcytosine (5-hmC) antibodies [Abcam, 33D3 (ab10805) and RM236 (ab214728)]. Five micrometre liver sections were deparaffinized by immersing the slides in xylene and in concentration decreasing alcohol grades solution. Antigen retrieval was performed in a Tris- EDTA (pH = 9) solution at 98°C for 20 minutes, while endogenous peroxidase activity was blocked by quenching the tissue sections with 3.0% hydrogen peroxide in methanol for 10 minutes. The sections were then washed with 1% donkey serum in PBS-0,4% Triton X-100 (TBST) solution for 5 minutes for permeabilization, followed by incubation with primary antibody (dilution 1/150) in room temperature for 30 minutes. After washing the sections with TBST solution, they were incubated with Linker and Polymer HRP (Ready to use reagent, Envision Flex, Dako) for 15 and 30 minutes respectively. Finally, sections were incubated with 3,3′-diaminobenzidin (DAB^{+}) and washed with distilled water. Haematoxylin Harris was used for nuclear counterstaining, and dehydration was performed in increasing concentration alcohol solutions and xylene. Negative controls consisted of substitution of primary antibody with pre-immune serum.

2.8 | Immunohistochemical evaluation

Immunostaining was semi-quantitatively evaluated in a blinded fashion regarding any of the histological and clinical characteristics of the patients by two independent observers. The degree of staining was determined according to its amount and intensity, using a four-point scoring system, as follows: 0 = no staining; 1 = positive nuclear staining in less than 20% of cells; 2 = 21–50% of positive cells and 3 = positive nuclear staining in more than 50% of cells.

2.9 | Statistical analysis

Analysis was made using the SPSS 20 and GraphPad-Prism 7.0 software. Results were expressed as median (range) and mean ± standard deviation. Data were compared with Kruskall-Wallis and Mann–Whitney U-test for the detection of differences between independent samples and Wilcoxon test for paired samples. Pearson coefficient (R) and Spearman's coefficient (r) were used for correlations, where applicable. Two-sided p-values <0.05 were considered as statistically significant in 95% confidence interval.

Statistical analysis of the EWAS data was performed with the Bioconductor R package ChAMP.[23] After normalization, identification of differentially methylated positions (DMPs) between groups was performed using Benjamini-Hochberg adjusted P-value <.05. Differentially methylated regions (DMRs) between groups, were identified with BumpHunter method and adjusted P-value <.05.[24] Gene set enrichment analysis using GOmeth method was performed on the genes associated with the DMRs to determine potential enriched pathways.[25] Finally, to identify if differentially methylated genes shared common functional properties between AIH-tp2 and AIH-tp1, we used the Database for Annotation, Visualization and Integrated Discovery (DAVID), which builds clusters of genes with significantly similar ontologies as tested against a complete list of genes in the database.[26] Medium stringency was used to yield a broader set of ontological groups.

3 | RESULTS

Global $5^{m}C$ and $5^{hm}C$ in CD19(+) and CD4(+) cells and their association with clinical, biochemical and serological parameters.

Global $5^{m}C$ and $5^{hm}C$ levels in CD4(+) and CD19(+) cells did not differ between the study groups (Figure 1A-D). Since $5^{m}C$ and $5^{hm}C$ are opposite procedures, we also investigated whether the ratio $5^{m}C/5^{hm}C$ could give any additional information regarding the global methylation status of each group or differences between groups. However, all the ratios in all groups in both CD19(+) and CD4(+) cells were > 1 and there were no differences between groups (Figure 1E, F).

In addition, $5^{m}C$ and $5^{hm}C$ levels in AIH-tp1 and AIH-tp2 patients were not correlated with age, AST, ALT, IgG, ANA and SMA (data not shown). However, $5^{m}C$ levels in CD4(+)-lymphocytes were negatively correlated with disease duration ($r = -.76$; $P = .01$) in AIH-tp1 patients.

3.1 | DNMTs and TETs in CD19(+) and CD4(+)-lymphocytes from AIH-tp1 patients

AIH-tp1 patients had significantly lower TET1 mRNA levels in CD19(+) and CD4(+) lymphocytes compared to HC ($P = .03$; $P = .01$ respectively; Figure 2A,B). AIH-tp1 had significantly higher

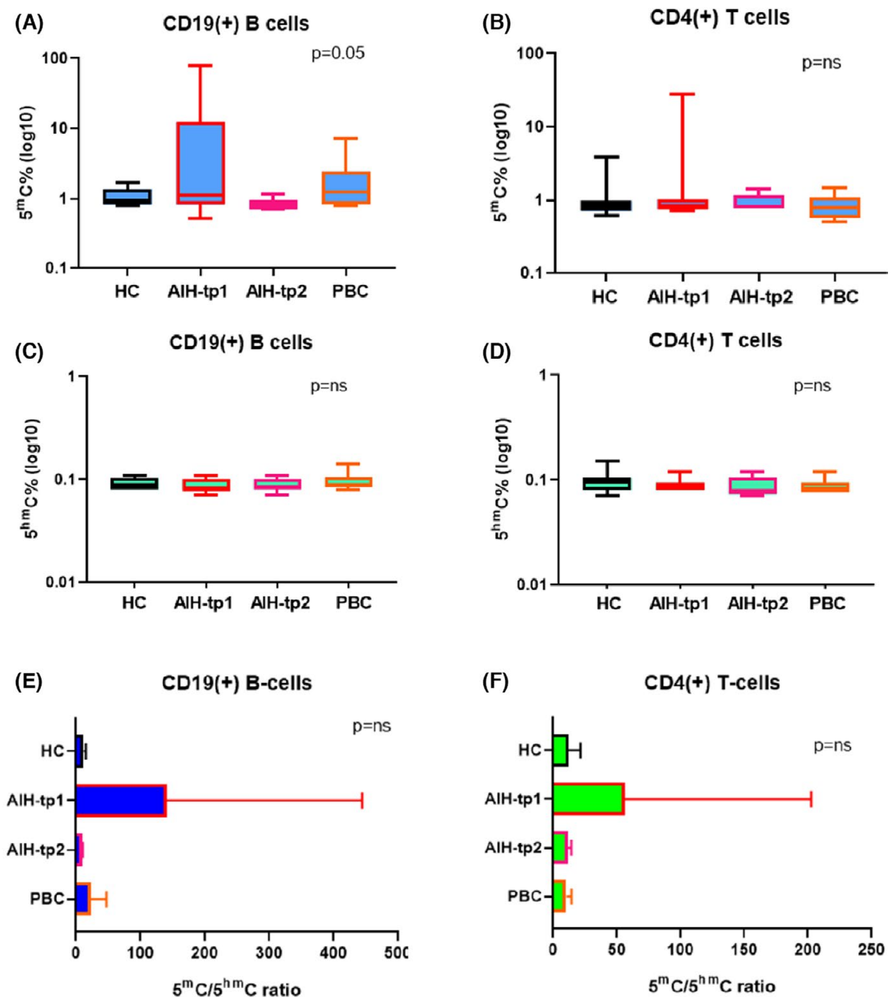


FIGURE 1 No differences were found in $5^{m}C$ (A and B) and $5^{hm}C$ levels (C and D) in CD19(+) and CD4(+) cells from AIH-tp1, AIH-tp2, PBC and HC. Furthermore, $5^{m}C/5^{hm}C$ ratios did not differ between groups neither in CD19(+) nor in CD4(+) cells (E and F). $5^{m}C$, 5-methylcytosine; $5^{hm}C$, 5-hydroxymethylcytosine; AIH-tp1, autoimmune hepatitis time-point 1; AIH-tp2, autoimmune hepatitis time-point 2; HC, healthy controls; ns, not-significant; PBC, primary biliary cholangitis

DNMT3A in CD19(+) and CD4(+) lymphocytes compared to PBC ($P = .04$; $P = .002$ respectively; Figure 2C,D). When we calculated DNMT3A/TET1 ratio for each group, 4/10 HC had ratio < 1 in both CD19(+) and CD4(+) cells but the median was >1 [1.6 (0.29–6.2) and 1.025 (0.3–4.5) respectively], while all AIH-tp1 and PBC patients had ratios >1 in both cell types (data not shown). In addition, AIH-tp1 patients had significantly higher DNMT3A/TET1 ratios compared to HC and PBC patients in both cell types (Figure 1E). No differences in DNMT1, TET2 and TET3 in CD19(+) or CD4(+) cells were observed between AIH-tp1 and HC or PBC. Of note, PBC had significantly higher DNMT1 and TET3 in CD19(+) cells compared to HC ($P = .02$; $P = .005$ respectively; Figures S2A,B). DNMT3B levels were beyond the detection threshold so no further analysis was made.

3.2 | Effect of immunosuppression on DNMTs and TETs expression in AIH

AIH-tp2 patients had significantly lower DNMT3A mRNA in CD19(+) ($P = .02$) and CD4(+)-lymphocytes ($P = .03$) compared to AIH-tp1 (Figure 3A, B). This supports that immunosuppression controls DNMT3A overexpression as DNMT3A levels in AIH-tp2 did not differ from HC, either in CD19(+) or in CD4(+) lymphocytes (Figure S3). In addition, DNMT3A/TET1 ratio did not differ between AIH-tp2 and AIH-tp1 groups (Figure 3C, D). The mRNA levels of DNMT1 and TETs did not differ between the two groups (data not shown).

3.3 | Association of DNMTs and TETs mRNA with clinical, biochemical and serological parameters in AIH-tp1 and AIH-tp2 patients

DNMT3A mRNA in CD4(+) and CD19(+) cells of AIH-tp1 was negatively correlated with IgG ($R = -0.68$; $P = .03$ and $R = -0.57$; $P = .08$ respectively) (Figure 3E, F). In contrast, DNMT3A levels in CD4(+) cells from AIH-tp2 were positively correlated with IgG ($r = 0.8$; $P = .02$).

3.4 | EWAS in CD4(+) lymphocytes from AIH-tp1 patients compared to HC

Since AIH activity as attested by IgG serum levels, was correlated with DNMT3A expression only in CD4(+) cells and AIH is genetically linked

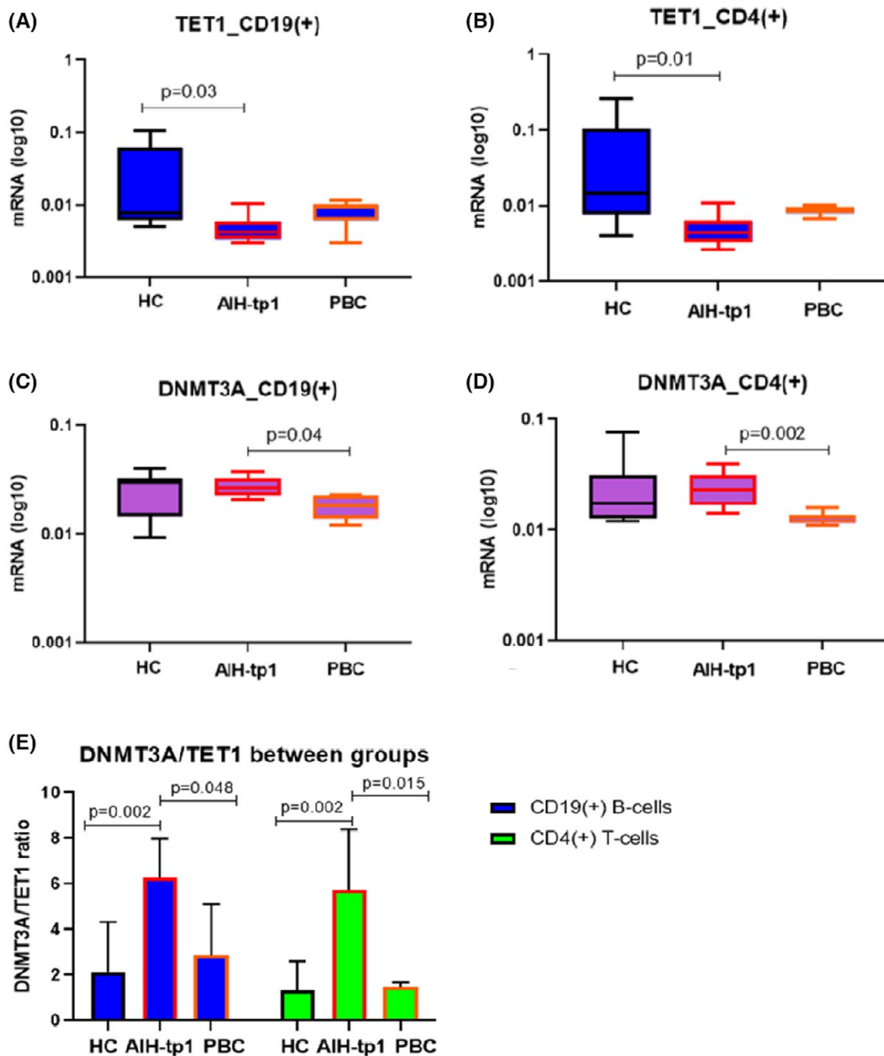


FIGURE 2 A, CD19(+) cells from AIH-tp1 characterized by reduced TET1 compared to HC ($P = .03$) B, CD4(+) cells from AIH-tp1 characterized by reduced TET1 compared to HC ($P = .01$). C, Increased DNMT3A was found in CD19(+) cells from AIH-tp1 compared to PBC ($P = .04$). D, Increased DNMT3A was found in CD4(+) cells from AIH-tp1 compared to PBC ($P = .002$). E, Increased DNMT3A/TET1 ratio was observed in AIH-tp1 compared to HC and PBC both in CD19(+) ($P = .002$ and $P = .048$ respectively) and CD4(+) cells ($P = .002$ and $P = .015$ respectively). AIH-tp1, autoimmune hepatitis time-point 1; AIH-tp2, autoimmune hepatitis time-point 2; DNMT3A, DNA methyltransferase 3A; HC, healthy controls; PBC, primary biliary cholangitis; TET1, ten-eleven translocation methylcytosine dioxygenase 1

to the major histocompatibility complex (MHC) class-II antigens orchestrating adaptive humoral and cell-mediated *immune responses*, the CD(4+) cells were further selected to investigate DMRs, which are more highly associated with diseases as compared to a single CpG analysis. To this end, we have used the BumpHunter method and have identified 287 CpG motifs corresponding to 29 DMRs between 10 AIH-tp1 and 9 HC (Figure 4A). These DMRs corresponded to unique and annotated genes present on 26 autosomes and 3 on sex chromosomes. Regarding functional genomic distribution, the majority of DMRs (14/29, 48.3%), corresponded to gene promoters and transcription start sites (TSS), while 6/29 (20.7%) corresponded to introns (Figure 4B). Regarding methylation status, 17/29 (58.6%) genes were hypomethylated (Table 1). Interestingly, 7/8 differentially methylated genes located on chromosome 6 are located on MHC. Among them, two are encoded by MHC class-II molecules: HLA-DPA1 (hypermethylated, $P = .003$.) and HLA-DPB2 (hypomethylated, $P = .01$). In addition, the long intergenic non-protein coding RNA 2571 gene (LINC02571), which belongs to the group of long noncoding RNAs (lncRNAs), was retrieved hypermethylated ($P = .02$), while the promoter of the tumour necrosis factor (TNF) gene was hypomethylated ($P = .01$; Table 1) and ZFP57 was hypermethylated ($P = .02$).

One of the most differentially methylated genes ($P = .0002$) was the promoter of platelet-derived growth factor receptor like gene. Finally, the RhoH gene was found hypermethylated ($P = .01$; Table 1).

Using the GOmeth method, no pathways were identified as enriched based on the genes associated with the identified DMR.

3.5 | EWAS in CD4(+) lymphocytes from AIH-tp1 patients compared to AIH-tp2

Next, we investigated whether methylation of the CpG motifs is affected by immunosupression in CD4(+) lymphocytes from AIH (10 AIH-tp1 and 5 AIH-tp2). In AIH-tp2 and at CpG level, 831 differentially methylated probes (DMPs; 11.2% hypomethylated and 88.2% hypermethylated) were identified compared to AIH-tp1, corresponding to 576 unique and annotated genes (Figure 5A). DMPs functional genomic distribution retrieved an enrichment in intergenic regions at isolated CpG or open sea regions as well as in gene bodies within shores regions present up to 2 kb from CpG islands (Figure 5B,C).

Although GOmeth did not reveal any enriched pathway, DAVID could categorize 376/576 genes in 12 clusters and 38 subgroups (Table S2).

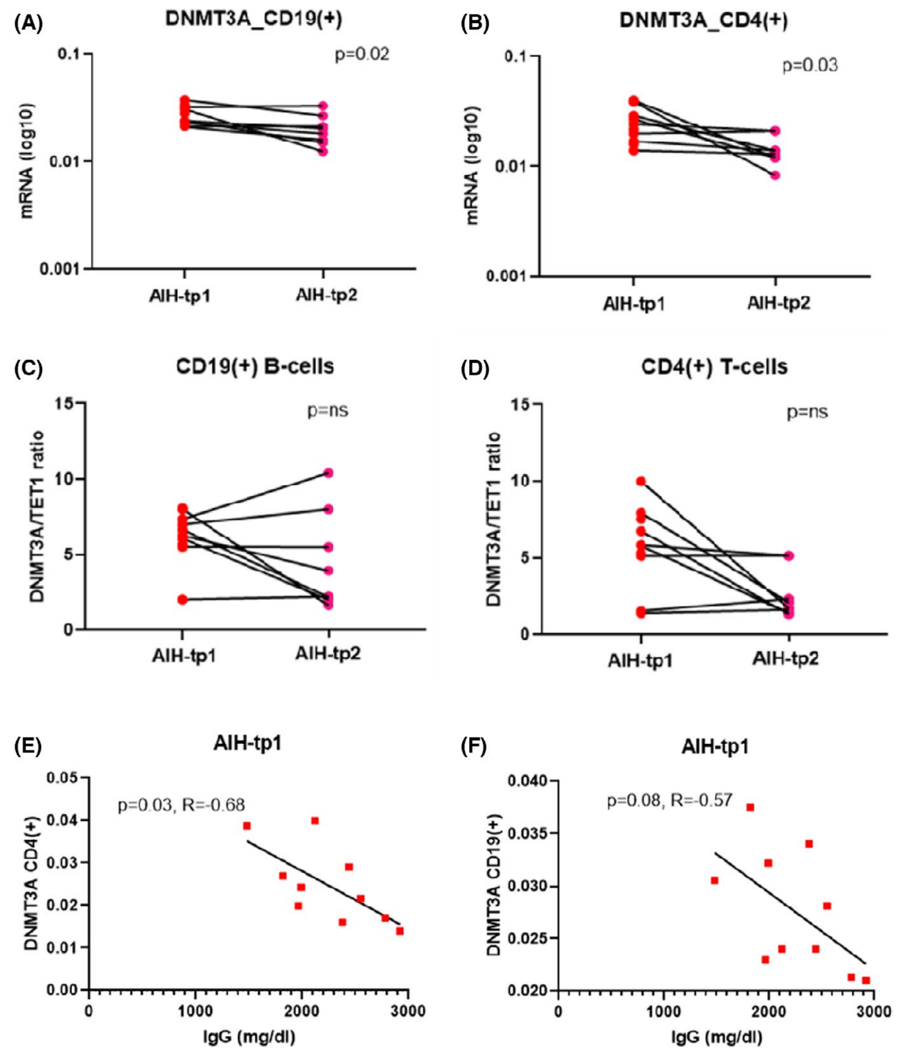


FIGURE 3 CD19(+) and CD4(+) cells after complete remission (AIH-tp2) characterized by decreased DNMT3A mRNA levels (logarithmic scale) compared to AIH-tp1 (A; $P = .02$ and B; $P = .03$). DNMT3A/TET1 ratio did not differ between AIH-tp2 and AIH-tp1 in both cell types (C and D). E, DNMT3A in CD4(+) cells was negatively correlated with IgG in AIH-tp1 ($R = -0.68$, $P = .03$). F, a negative trend was observed between DNMT3A and IgG in CD19(+) cells from AIH-tp1 ($R = -0.57$, $P = .08$). DNMT3A, DNA methyltransferase 3A; AIH-tp1, autoimmune hepatitis time-point 1; AIH-tp2, autoimmune hepatitis time-point 2

The main functional annotations over-represented were those classified as 'nucleotide-binding kinases', 'metal-binding proteins', 'phospholipid-metabolism', 'motor-proteins', 'membrane-proteins' ($P < .05$; Table S2). Of note, the annotation cluster 'immunity', comprised of genes most of which were hypermethylated in AIH-tp2 (Table S3).

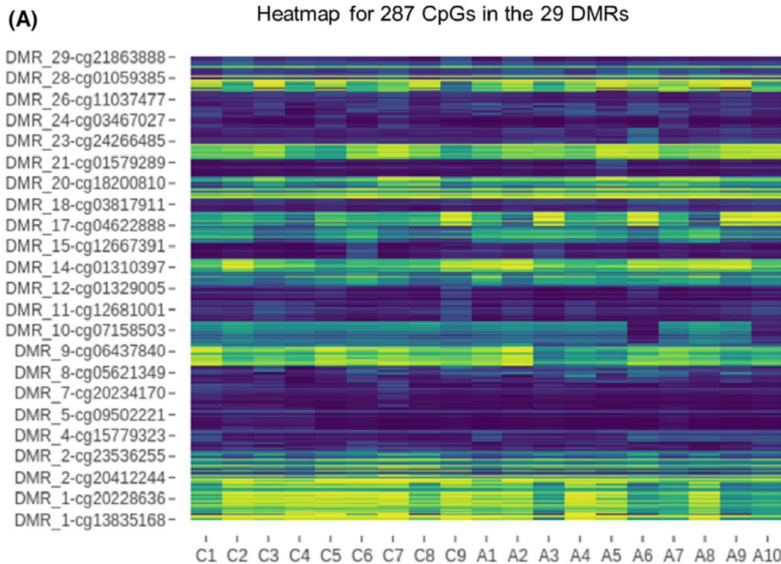
To go further in the analysis of immunosuppression on DNA methylation in CD4(+) lymphocytes, the 14 DMRs between AIH-tp1 and AIH-tp2 (Table S4) as well as the top 25 differentially methylated genes (among genes with 2 DMPs) were explored (Table 2). DMRs surrounding promoter regions were predominantly retrieved in 9/14 (64.3%) of AIH-tp2 compared to AIH-tp1. Among the key immune genes differentially methylated between AIH-tp2 and AIH-tp1, the activation marker CD86, the miRNA processing enzyme DROSHA and lncRNAs (LINC00211 and LINC01140) were hypermethylated in AIH-tp2 ($P < .05$; Table 2).

3.6 | $5^{m}C/5^{hm}C$ staining in liver sections

Finally, representative intense and diffuse nuclear $5^{hm}C$ immunohistochemical staining in most of the lymphocytes infiltrating the portal tract of AIH-tp1 cases is shown in Figure 6A. In addition, strong nuclear immunoreaction was observed in the limiting plate hepatocytes and bile duct epithelial cells of AIH-tp1 cases (Table S5, Figure 6A). On the contrary, there was absence of positive lymphocytes in the portal tract of HC cases, which also showed lack of immunoreactivity in periportal hepatocytes, and weak immunostaining of few bile duct epithelial cells (Table S5, Figure 6B). In addition, $5^{hm}C$ immunostaining showed absence of nuclear positivity in most of lymphocytes infiltrating the portal tracts with no immunostaining of the bile duct epithelial cells in three representative PBC cases (Table S2, Figure 6C).

Hepatocytes of both AIH-tp1 and control cases showed similar nuclear $5^{hm}C$ and $5^{m}C$ immunostaining (Tables S5, S6 respectively). $5^{m}C$ staining of liver infiltrating lymphocytes showed no differences in localization and intensity between patients and controls (Table S6).

$5^{m}C$ and $5^{hm}C$ immunostaining of Kupffer cells showed slightly reduced scores between AIH and HC (Tables S5, S6). However, the number of cases studied is small to allow further interpretation.



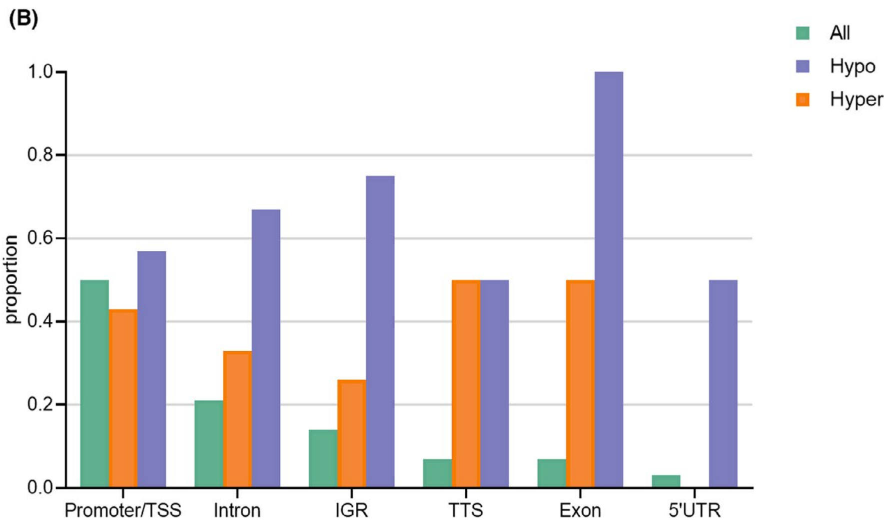


FIGURE 4 A, Heatmap of significant CpGs corresponding to 29 DMRs detected between AIH-tp1 and HC (C1-C9: HC, A1-A10: AIH-tp1). B, Genomic distribution of 29 DMRs. AIH-tp1, autoimmune hepatitis time-point 1; CpGs, cytosine-phosphate-guanine dinucleotides; DMRs, differentially methylated regions; 5′′UTR, 5′ ′untranslated region; hyper, hypermethylated; hypo, hypomethylated; IGR, Intergenic regions; TSS, Transcription start sites

4 | DISCUSSION

We evaluated the DNA methylation status in liver sections and peripheral B and T cells from AIH patients. The following points arise from the present investigation: first, altered TET1 and DNMT3A expression characterizes both CD19(+) and CD4(+)-lymphocytes from AIH-tp1 patients compared to HC and PBC; second, after induction of remission, DNMT3A expression is decreased; third, in AIH-tp1 patients, DNMT3A was negatively correlated with IgG, while the opposite was observed in AIH-tp2 patients; forth, although changes in DNMT3A and TET1 expression were not associated with global and major $5^{m}C/5^{hm}C$ changes, using an EWAS approach we found, however, differences in DNA methylation of diverse genes in CD4(+)-lymphocytes from AIH-tp1 patients compared to HC and AIH-tp2 patients and fifth, we observed strong nuclear $5^{hm}C$ staining at the histological level of the periportal infiltrating lymphocytes in AIH-tp1 compared to controls. Taken together, these findings suggest that epigenetic modifications may play an important role in AIH pathogenesis and therapeutic response.

TETs are the main enzymes involved in active DNA demethylation associated with the modification and removal of $5^{m}C$. In mice, it has been shown that TETs play important role in mature B-cell antibody production, but also in facilitating the in vitro differentiation of naïve CD4(+) cells to T-regulatory cells (T-regs) by demethylating Foxp3 enhancer CNS2, while in-vivo seem to stabilize the expression of Foxp3 in T-regs.[27] Therefore, TET-deficient phenotypes are characterized by reduced class switch recombination capacity and decreased Foxp3 stability.[27] Our findings of decreased transcriptional expression of TET1 in CD19(+)- and CD4(+)-lymphocytes from AIH patients with active disease may reflect the abovementioned immune dysregulation.

In AIH-tp1, DNMT3A expression in CD19(+)- and CD4(+)-lymphocytes was increased compared to PBC. This finding together with the increased DNMT1 and TET3 levels, which characterized CD19(+) cells of PBC compared to HC, points to a different epigenetic profile between the two diseases. In addition, increased DNMT3A and DNMT1 transcriptional levels have been reported in CD4(+)-lymphocytes from patients with either clinically or serologically active systemic lupus erythematosus (SLE).[28] In SLE, both

TABLE 1 Differentially methylated genes corresponding to 29 DMRs detected in AIH-tp1 patients and healthy controls (HC)

Gene	Chromosome	Description	Location	Methylation difference (AIH-tp1 vs HC)	P value
CAT	11p13	Catalase	Promoter-TSS	0.59	.001
EID3	12q23.3	EP300 interacting inhibitor of differentiation 3	promoter-TSS	−0.65	.007
GOLGA3	12q24.33	Golgin A3	intron	−0.65	.01
HTR2A	13q14.2	5-Hydroxytryptamine receptor 2A	Intergenic	−0.64	.01
RHOJ	14q23.2	Ras homologue family member J	5′ UTR	−0.56	.01
ADAM21P	14q24.2	ADA metallopeptidase domain 21 pseudogene 1	intron	12148	.0002
BISPR	19p13.11	BST2 interferon stimulated positive regulator	Promoter-TSS	0.72	.004
TYW3	1p31.1	tRNA-yW synthesizing protein 3 homologue	Promoter-TSS	0.88	.0004
PM20D1	1q32.1	Peptidase M20 domain containing 1	Promoter-TSS	−0.58	.005
WBP2NL	22q13.2	WBP2 N-terminal like	TTS	−0.56	.01
PAIP2B	2p13.3	Poly(A) binding protein interacting protein 2B	Intergenic	−0.47	.01
MUC-20	3q29	Mucin 20, Cell Surface Associated	Intron	−0.69	.007
RHOH	4p14	Ras homologue family member H	Promoter-TSS	0.57	.01
STPG2	4q22.3	Sperm tail PG-rich repeat containing 2	Promoter-TSS	0.58	.02
EIF4E	4q23	Eukaryotic translation initiation factor 4E	Promoter-TSS	−0.69	.005
VTRNA2-1	5q31.1	Vault RNA 2–1	TTS	0.64	.002
HLA-DPA1	6p21.32	Major histocompatibility complex class II	Intron	0.58	.003
HLA-DPB2	6p21.32	Major histocompatibility complex class II	Intron	−0.56	.01
LINC02571	6p21.33	long intergenic non-protein coding RNA 2571 (MHC locus)	Intergenic	0.54	.02
TCF19	6p21.33	transcription factor 19 (MHC locus)	Exon	−0.93	.001
TNF	6p21.33	tumour necrosis factor (MHC locus)	Promoter-TSS	−0.49	.01
HCG4B	6p22.1	HLA complex group 4B (MHC locus)	Promoter-TSS	10439	.008
ZFP57	6p22.1	ZFP57 zinc finger protein (MHC locus)	Intergenic	0.52	.02
DDX43	6q13	DEAD-box helicase 43	Exon	0.56	.001
PDGFRL	8p22	platelet-derived growth factor receptor like	Promoter-TSS	−11703	.0002
LHX6.1	9q33.2	LIM Homeobox 6	Intron	−0.65	.01
CDK16	Xp11.3	cyclin dependent kinase 16	Promoter-TSS	−0.68	.006
TXLNGY	Yq11.222	taxilin gamma pseudogene	promoter-TSS	−0.59	.01
EIF1AY	Yq11.223	eukaryotic translation initiation factor 1A	Promoter-TSS	−0.82	.001

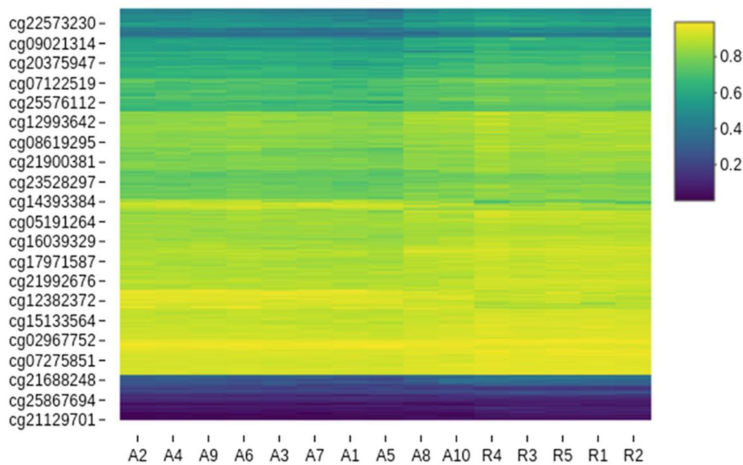
Abbreviations: AIH-tp1, autoimmune hepatitis time-point 1; AIH-tp2, autoimmune hepatitis time-point 2; TSS, transcription start site; Methylation difference between AIH-tp1 and HC: negative value means hypomethylation.

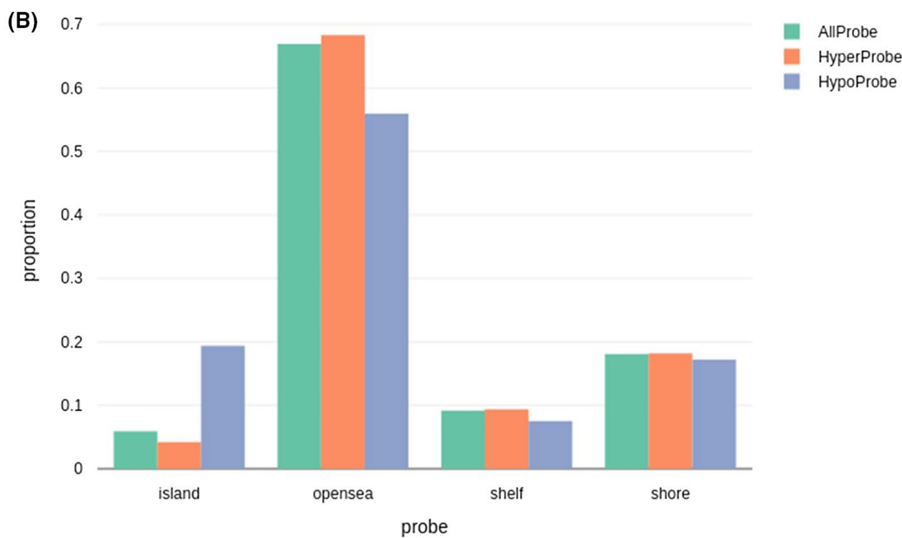
methyltransferases were inversely correlated with markers of disease activity such as, C3 complement component and anti-dsDNA antibody.[28] Accordingly, we found that DNMT3A levels in active AIH were negatively associated with IgG, a serological marker of AIH activity. This correlation between DNMT3A mRNA levels and IgG may reflect a DNMT3A dependent plasma cell dysregulation, which increases autoantibody production as indicated by the elevated IgG values.[29]

Corticosteroids administration has been reported to alter DNMT1 expression in PBMCs from SLE patients, while MMF has also been shown to induce epigenetic changes.[30] This is in accordance with our findings, as immunosuppression with MMF seems to decrease DNMT3A expression in both CD19(+)- and CD4(+)-lymphocytes. The decrease in DNMT3A in responders compared to active AIH together with its positive correlation with IgG in patients at remission suggests that immunosuppression probably restores the epigenetic deregulations at least in B cells.

EWAS in CD4(+) cells showed methylation alterations of diverse genes. Actually, DMRs analysis between AIH-tp1 and HC revealed that most DMRs located on gene promoters indicating their potential effect on the expression of implicated genes. However, recent studies have shown that the hyper/hypomethylation of the regions downstream of promoter-TSS are also highly involved in the regulation of gene expression.[31]

Methylation changes in CD4(+) cells affected, among others, HLA-DP genes. The association of AIH with the HLA class-II alleles was retrieved from early studies and confirmed from recent genome wide association studies (GWAS).[7] In this context, HLA-DP





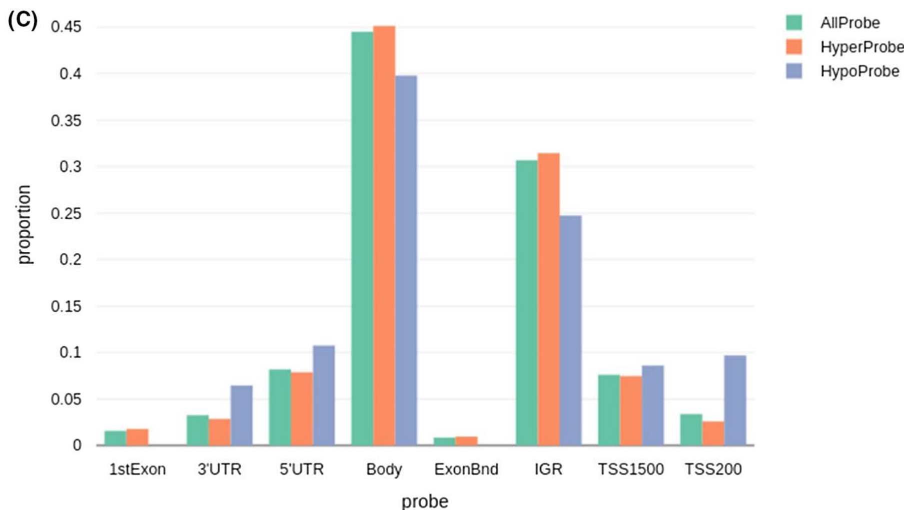


FIGURE 5 A, Heatmap of significant CpGs corresponding to 831 DMPs detected between AIH-tp2 (n = 5) and AIH-tp1 (n = 10) (R1-5: AIH-tp2, A1-10: AIH-tp1). B, Genomic distribution of significant DMPs. DMPs are enriched in open sea regions and shores. C, Most DMPs are located on gene bodies and IGR. 3′'UTR, 3' ′untranslated region; 5' ′UTR, 5' ′untranslated region; AIH-tp1, autoimmune hepatitis time-point 1; AIH-tp2, autoimmune hepatitis time-point 2; CpGs, cytosine-phosphate-guanine dinucleotides; DMPs, differentially methylated probes; hyper, hypermethylated; hypo, hypomethylated; IGR, intergenic regions; TSS, transcription start sites

polymorphisms can modulate interactions with the invariant chain chaperone, resulting in presentation of both exogenous and endogenous antigens in CD4(+) cells.[32] In addition, ZFP57 gene was found differentially methylated. ZFP57 belongs to the Methyl-CpG Binding Zinc Finger Proteins (a large family of methyl-binding proteins) and is associated with several cellular processes including regulation of gene expression, genomic imprinting, cell signalling and transcriptional repression.[33]

TABLE 2 Top 25 differentially methylated genes between AIH-tp2 and AIH-tp1 patients

Gene	Chromo-some	CpG	Methylation difference (AIH-tp2 vs. AIH-tp1)	Description	P value
ANKRD11	16q24.3	cg00169122 cg08423714	0.07 0.06	Ankyrin Repeat Domain 11	.03 .04
ATP11A	13q34	cg16628188 cg14354398	−0.025 0.03	ATPase Phospholipid Transporting 11A	.04 .05
CARS1	11p15.4	cg09827966 cg04137890	0.03 0.02	Cysteinyl-TRNA Synthetase 1	.04 .05
CD86	3q13.33	cg13617155 cg20753131	0.098 0.075	CD86 Molecule	.04 .04
COL15A1	9q22.33	cg14259208 cg07251446	−0.028 0.063	Collagen Type XV Alpha 1 Chain	.04 .04
DNAH2	17p13.1	cg22573230 cg26524256	0.087 0.02	Dynein Axonemal Heavy Chain 2	.04 .04
DPH6	15q14	cg12382372 cg26149924	−0.05 0.08	Diphthamine Biosynthesis 6	.05 .05
DROSHA	5p13.3	cg15628639 cg09622076	−0.063 0.015	Drosha Ribonuclease III	.03 .05
ENOX1	13q14.11	cg24171628 cg10448831	0.04 0.026	Ecto-NOX Disulfide-Thiol Exchanger 1	.03 .04
TAF5/FAM19A5	22q13.32	cg12555819 cg13520744	0.054 0.04	FAM Chemokine Like Family Member 5	.04 .05
H6PD	1p36.22	cg09021539 cg13943024	0.046 −0.03	Hexose-6-Phosphate Dehydrogenase/ Glucose 1-Dehydrogenase	.03 .04
INPP5B	1p34.3	cg01300277 cg23310078	0.063 0.07	Inositol Polyphosphate-5-Phosphatase B	.03 .05
LINC00211	2p22.2	cg12176155 cg00832367	0.039 0.019	Long Intergenic Non-Protein Coding RNA 211	.009 .04
LINC01140	1p22.3	cg10277282 cg23492309	0.06 0.08	Long Intergenic Non-Protein Coding RNA 1140	.04 .04
LMF1	16p13.3	cg10301401 cg03796797	0.019 0.048	Lipase Maturation Factor 1	.04 .04
MAGEC2	Xq27.2	cg07549474	0.065	MAGE Family Member E2	.04
MS4A15	11q12.2	cg27474534 cg01372278	0.017 0.031	Membrane Spanning 4-Domains A15	.03 .04
MSRA	8p23.1	cg07122519 cg12712618	0.073 0.023	Methionine Sulfoxide Reductase A	.03 .04
PLCB1	20p12.3	cg13788583 cg20272813	0.072 0.052	Phospholipase C Beta 1	.04 .03
SNORD115-15	15q11.2	cg20375947 cg10963511	0.076 0.05	Small Nucleolar RNA, C/D Box 115–15	.04 .04
ST3GAL4	11q24.2	cg06534890 cg08080418	0.07 0.027	ST3 Beta-GalactosideAlpha-2,3-Sialyltransferase 4	.02 .04
ST6GALNAC4	9q34.11	cg17139858 cg20936291	0.04 −0.05	ST6 N-Acetylgalactosaminide Alpha-2,6-Sialyltransferase 4	.009 .03
STRA8	7q33	cg25778497 cg15066191	0.044 0.07	Stimulated By Retinoic Acid 8	.04 .04
TMEM218	11q24.2	cg27596275 cg19174059	0.029 0.028	Transmembrane Protein 218	.04 .04
WDR27	6q27	cg01393964 cg04445182	0.087 0.036	WD Repeat Domain 27	.04 .04

Abbreviations: AIH-tp1, autoimmune hepatitis time-point 1; AIH-tp2, autoimmune hepatitis time-point 2; CpG, cytosine-phosphate-guanine dinucleotide.

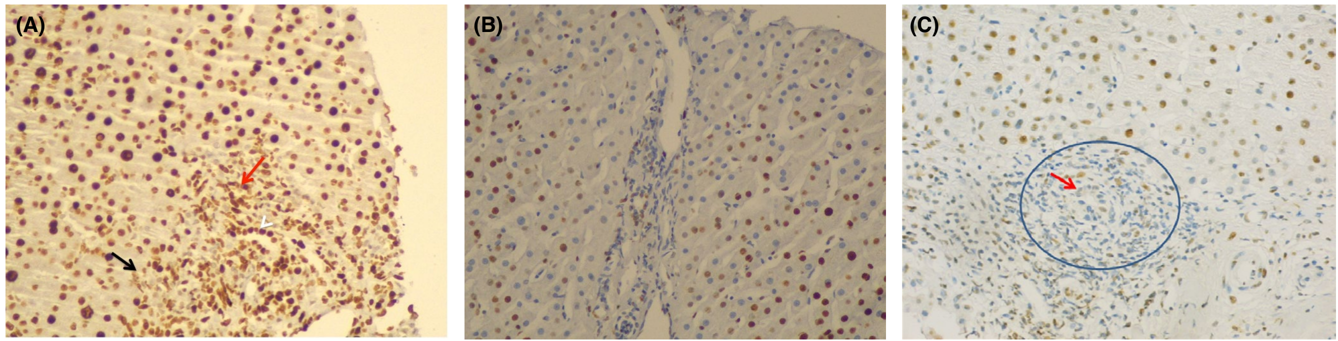


FIGURE 6 $5^{hm}C$ immunostaining, original magnification X200. A, AIH-tp1: $5^{hm}C$ immunostaining of liver tissue sections shows strong nuclear positivity in the majority of the lymphocytes infiltrating the portal tract (red arrow). Portal inflammatory infiltrate disrupts limiting plate and surrounds individual hepatocytes. The latter show nuclear immunoreaction as well (black arrow). The bile duct epithelial cells show strong immunoexpression (arrowlead). B, Controls: $5^{hm}C$ staining shows absence of positive lymphocytes in the portal tract. Note the absence of immunoreactivity in periportal hepatocytes. In addition, a few bile duct epithelial cells show a weaker positive immunoreaction than in AIH. $5^{hm}C$, 5-hydroxymethylcytosine; AIH-tp1, autoimmune hepatitis time-point 1. C, PBC: $5^{hm}C$ immunostaining of liver tissue sections shows absence of nuclear positivity in most lymphocytes infiltrating the portal tract (circle-red arrow). In addition, the bile duct epithelial cells show negative immunostaining. $5^{hm}C$, 5-hydroxymethylcytosine; AIH-tp1, autoimmune hepatitis time-point 1, PBC, primary biliary cholangitis

LINC02571 gene was also found hypermethylated. Recent evidence indicates that lncRNAs play important roles in controlling the development of diverse immune cells and the mechanisms of immune cell activation.[34] Of note, another gene located on MHC class-III, the TNF gene promoter, was found hypomethylated. This finding keeps up with a recent study of Bovensiepen et al,[35] who found that TNF gene expression in liver-infiltrating lymphocytes was strongly upregulated and that the proportion of TNF-producing CD4(+) cells was elevated both in blood and the liver of AIH patients compared to HC. Among the rest of the genes found hypo/hypermethylated in AIH-tp1, RhoH (promoter hypermethylation) belongs to the Rho family of small GTPases and plays an important role in positive and negative thymic selection, in the functional differentiation of T cells and in T-cell activation through TCR signalling.[36] Interestingly, most of the genes (88%) of CD4(+) cells were hypermethylated in AIH-tp2 patients, suggesting a shift in methylation profile after achievement of remission, probably towards a less activated state. Furthermore, some of these genes such as, ANXA1 and PYCARD are known to play diverse roles in immune responses, especially in the activation of inflammasome.[37]

Among the top differentially methylated genes, CD86 encodes CD86/B7.2 molecule, which is a central costimulatory molecule mainly expressed on antigen-presenting cells. However, recently it has been shown that CD86 is also expressed in humans on CD4(+) cells in response to activation, suggesting a functional role of B7 molecules in the regulation of a T-cell response.[38] The hypermethylation of CD86 gene in AIH at remission could signify a central role of CD86 molecule in -downregulating the immunological response in AIH. Interestingly, SorCS1 promoter was found hypomethylated in AIH at remission. This is in line with the role of the family of Vps10p receptors in the regulation of production and exocytosis of pro-inflammatory cytokines as well as the immune functions of T and NK cells during adaptive immune responses.[39]

To explore 'in situ' modifications of methylation, that might contribute to AIH molecular phenotype and might influence the methylation status of circulating immune cells, we assessed the $5^{m}C$ and $5^{hm}C$ protein expression in paraffin-embedded liver sections. Interestingly, the findings confirmed the results of EWAS since most of the lymphocytes infiltrating the portal tract, and the surrounding individual periportal hepatocytes, in AIH-tp1 liver sections, were hypomethylated (stained strongly for $5^{hm}C$) compared to HC and PBC patients. Of note, periportal hepatocytes and biliary duct epithelial cells seemed to follow the same pattern of hypomethylation, pointing towards an altered methylation milieu in the liver of AIH patients. To our knowledge, this is the first report of the $5^{hm}C$ and $5^{m}C$ liver tissue-mapping of AIH. These findings merit further investigation in large-scale studies including large number of liver biopsies, in order to correlate the cellular hypomethylation with the pathogenesis and/or the progression of the disease.

Our study has some limitations as the number of patients and controls was limited. However, the fact that we studied epigenetic modifications in pure peripheral B- and T-helper cells but also in liver sections, increases the reliability of our findings, as epigenetic changes are cell-type specific and studies on PBMCs may lead to ambiguous results. Another limitation, which however needs to be addressed in future studies, is that we did not investigate epigenetic changes in specific B- or T-cell subtypes such as naïve, memory cells, B and T-regs, since different epigenetic alterations may be implicated in each of these lymphocyte subtypes. However, this was beyond of the aims of the present study as it was the first exploratory study on DNA methylation in AIH compared mainly to the healthy state. Finally, we chose to perform EWAS only in CD4(+) cells because AIH is genetically linked strongly to MHC II antigens (e.g. HLADRB1*03 and − 04) that interact with CD4(+) cells orchestrating adaptive humoral and cell-mediated *immune responses. Moreover,* AIH activity

as attested by the IgG serum levels was correlated with DNMT3A expression only in CD4(+) cells.

In conclusion, we showed that altered expression of DNMT3A and TET1, as well as altered DNA methylation of diverse genes characterize immune cells in periphery and at the histological level of AIH patients, supporting the implication of epigenetic modifications in disease pathogenesis. Notably, epigenetic modifications were associated with disease activity and modified by immunosuppression. These findings open new insights in understanding of disease pathophysiology and may lead to novel therapeutic interventions.

CONFLICTS OF INTEREST

The authors have nothing to disclose.

DATA AVAILABILITY STATEMENT

Data Availability: All data used to support the findings of this study are included within the article and supplementary files.

ORCID

George N. Dalekos https://orcid.org/0000-0001-7075-8464

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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