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Differential CpG methylation of the promoter of interleukin 8 and the first intron of tissue factor in Antiphospholipid syndrome

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A B S T R A C T

Background: Antiphospholipid syndrome (APS) is an autoimmune thrombophilia characterized by recurrent thromboembolism and/or pregnancy morbidity in the presence of Antiphospholipid antibodies, mainly *anti*-β2 glycoprotein I (*anti*-β2GPI). The autoantibodies lead to monocyte and endothelial cell activation and subsequent secretion of tissue factor (F3) and proinflammatory cytokines, like interleukins 6 (IL6) and 8 (IL8). The etiology of the syndrome remains largely unknown, with the contribution of environmental, genetic and epigenetic factors considered significant.

Purpose: We aimed to identify epigenetic changes and factors potentially implicated in the pathophysiology of APS. To this end, we compared DNA methylation levels of the IL8 and F3 genes between healthy donors (HDs) and APS patients, using whole blood as a source.

Results: Methylation was significantly reduced in the IL8 promoter and significantly increased in the F3 gene body in APS patients compared to HDs and correlated with specific clinical parameters. In an *ex vivo* model partially mimicking APS, stimulation of monocytes with a mixture of β2GPI, *anti*-β2GPI and CXCL4 also induces DNA methylation changes in the above genes, along with increase of their expression. Stimulation of human umbilical vein endothelial cells (HUVECs) with the same mixture also results in transcriptional upregulation of epigenetic factors, including MECP2, DNMT3, TET1, HDAC9 and ARID5B.

Conclusions: The above data support that epigenetic alterations could be implicated in the pathophysiology of APS and prompt further investigation of their potential diagnostic or therapeutic utility.

1. Introduction

Antiphospholipid syndrome (APS) is an autoimmune thrombophilia characterized by recurrent thromboembolism and/or pregnancy morbidity in the presence of *anti*-β2 glycoprotein I (*anti*-β2GPI) and antiprothrombin antibodies or lupus anticoagulant (LA) [1,2]. Endothelial dysfunction along with innate and adaptive immunity activation has been shown to characterize the syndrome [3–6]. Experimental models indicate that endothelial cells treated with *anti*-β2GPI antibodies turn towards a procoagulant phenotype with overexpression of adhesive molecules such as e-selectin (SELE), Tissue Factor (F3) and VCAM [7–9]. Patients with APS have higher circulating levels of proinflammatory cytokines [10,11]. Toll Like Receptor (TLR) 2, 4 and 6 activation leads to monocyte and endothelial cell activation and subsequent expression of F3 and proinflammatory cytokines, such as IL-6 and IL-8 [12–18]. We have previously shown that platelet derived chemokines are elevated in the plasma of APS patients [19]. Platelets are activated by complexes of *anti*-β2GPI autoantibodies and β2GPI, especially when dimerized by binding to the CXCL4 chemokine [20–23].

Epigenetic modifications consist of structural adaptations of chromatin to the cellular environment and often correlate with altered activity state [24,25]. One of the major and perhaps the most extensively studied epigenetic modification is methylation of DNA on cytosine, which involves the covalent addition of a methyl group to the fifth position in the six-atom ring of the nucleotide cytosine (5-methylcytosine, 5 meC) and occurs mainly in the context of CpG dinucleotides [26].

Several studies have implicated epigenetic mechanisms in the development of autoimmunity. The methylation status of many genes has been linked to SLE pathogenesis and progression [27–29]. Genome-wide methylation analyses using Rheumatoid arthritis (RA) fibroblast-like synoviocytes and peripheral blood mononuclear cells have identified several differentially methylated loci on genes implicated in immune cell trafficking, cell adhesion, and extracellular matrix interactions [30,31]. A recent study has also shown epigenetic alterations in neutrophils isolated from patients with APS [32].

Based on the above we sought to explore the possible differential methylation of the IL8 and F3 genes, which encode proteins critical for the pathophysiology of APS. Moreover, in an *ex vivo* model partially mimicking APS, we assessed the transcript levels of a number of epigenetic factors. We identify differences in both specific CpG methylation levels as well as in the expression of several enzymes catalyzing epigenetic modifications and readers of epigenetic marks.

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2. Materials and methods

2.1. Patients and specimens

Patients with APS (n = 27) and healthy donors (HDs) (n = 25) were included. Patient and healthy donor demographic characteristics are depicted in Supplementary Table 1. The study protocol was approved by the Ethics committee of the National and Kapodistrian University of Athens. Whole blood was collected in EDTA tubes, and serum was isolated from all participants and kept frozen at −80 °C. Clinical events compatible with APS were extracted from patient medical records.

Human umbilical vein endothelial cells (HUVECs) were isolated from 3 HDs after uncomplicated pregnancy and cultured in Endothelial Basal Medium-2 (EBM-2, Lonza CC-3162).

2.2. Anti-β2GPI IgG isolation

Human native β2GPI was isolated from pooled citrated human plasma as previously described and immobilized on CNBr-Activated Sepharose 4B (GE Healthcare Life Sciences 17-0430-01) according to the manufacturer's instructions [33]. β2GPI Sepharose crosslinked beads were packed in centrifuge columns (Pierce 89897) for antibody affinity purification.

Total IgG was isolated from APS patient serum using Protein G Sepharose beads (GE Healthcare Life Sciences 17061801). Total IgG was eluted and concentrated using 30 K molecular weight cutoff (MWCO) concentrators (Pierce 88522) and subsequently incubated with the β2GPI conjugated beads overnight at 4 °C. Next day, columns were centrifuged at 1000 RCF and washed with 15 column volumes of PBS buffer. *Anti*-β2GPI IgG was eluted, titrated to neutral pH and concentrated with 2 resin bed volumes of Glycine-HCL buffer pH = 2.5 and 1 resin volume of 1 M Tris-HCl pH = 8 was added immediately. Samples were concentrated with 30 K MWCO concentrators.

2.3. Monocyte activation

Peripheral blood mononuclear cells were isolated from 3 HDs through density gradient on Ficol-Paque 1.077 gr/ml and assayed in 4 separate experiments. Five hundred μL of cell suspension was seeded in 48-well plates in RPMI with 10% FBS at a final concentration of 4×10^6 cells/ml and left to adhere overnight. The following day, non-adherent cells were removed and new serum free medium was added in the wells for 1 h. The cells were then treated with 100 ng/ml LPS as a positive control, RPMI alone or every combination of IgG (50 μg/ml), β2GPI (20 μg/ml) and CXCL4 (40 ng/ml) for 4 h for RNA and DNA analysis. Monocytes were treated with IgG (50 μg/ml) and β2GPI (20 μg/ml) for 30 min, 1, 2, 4, 6, 8, 12 and 24 h for gene expression and methylation analysis.

2.4. HUVEC stimulation

HUVECs were stimulated with IgG isolated from APS patients and HDs. HUVECs isolated from 2 HDs were cultured to 70% confluence in 75 cm^2 flasks. Subsequently cells were trypsinized, seeded in 24-well plates and cultured in EBM-2 complete medium containing FGF-b, VEGF, IGF-1, EGF and 2.5% FBS. Upon confluence, the medium was removed and replaced with starvation medium containing EBM-2 and 1% FBS for 24 h in order to achieve cell synchronization before treatment and G0/G1 phase arrest [34]. Then every combination of *anti*-β2GPI IgG (50 μg/ml), β2GPI (20 μg/ml) and CXCL4 (40 ng/ml) was added to the wells for 4 h.

2.5. RNA isolation and Real Time PCR

Total mRNA was isolated from treated HUVECs using a commercially available kit (QIAGEN 74034). Five hundred ng of total RNA were reverse transcribed to cDNA (RR037B, PrimeScript™ RT Reagent Kit). Real Time PCR using SYBR green MM (Kapa Biosystems KK4618) was performed in order to determine the relative expression of SELE (selectin-5 leukocyte adhesion molecule), IL8, F3, VEGFC (vascular endothelial growth factor C receptor), TMBD (Thrombomodulin), DNMT3A (DNA methyltransferase 3 alpha), DNMTA3B (DNA methyltransferase 3 beta), SMYD4 (SET and MYND domain-containing protein 4), ARID3A (AT-rich interactive domain-containing protein 3A), ARID5B (AT-rich interactive domain-containing protein 3 B), KDM5C (Lysine-specific demethylase 5C), MeCP2 (methyl-CpG binding protein 2), HDAC4 (histone deacetylase 4), HDAC7 (histone deacetylase 7), HDAC9 (histone deacetylase 9), TET1 (Tet Methylcytosine Dioxygenase 1) and EZH2 (enhancer of zeste homolog 2) using β2 microglobulin as reference gene.

2.6. DNA isolation, bisulphite treatment and methylation specific PCR (MSPCR)

DNA from EDTA treated whole blood was isolated using a commercial DNA isolation kit according to the manufacturer's protocol (Macherey-Nagel 740951). Bisulfite conversion was performed using the EZ DNA Methylation-Gold Kit (Zymo Research D5005). Converted DNA was subsequently used in PCR using primers specific for either methylated or unmethylated template. IL8 primers have been previously reported and recognize 3 CpGs in the promoter region [35]. Primer sequences for F3 are: forward 5′GGAAAAAGATAAATTTATAC3′, reverse 5′ACATACCCCATATTTCTACG3′ for methylated template and forward 5′ GGAAAAAGATAAATTTTATAT3′, reverse 5′ACATACCCCAT ATTTCTACA3′ for unmethylated template and recognize 2 CpGs on positions Chr1:95006322 and Chr1:95006539 (hg19 assembly) in the first intron of the gene. For simplicity, we refer to the targets as IL8 promoter and F3 gene body or first intron CpGs respectively. Fully methylated and fully unmethylated bisulphite treated DNA were used as controls in order to assess the primer specificity. qPCR was performed with SYBR Green MM (Kapa Biosystems KK4618).

2.7. Immunocytochemical analysis for IL8 and F3 protein

Monocytes were isolated as previously described and seeded on coverslips for overnight adherence. Next day, cells were stimulated as described above for 4 h. Following treatment, the medium was aspirated and coverslips were washed three times with Phosphate-buffered saline (PBS). Cells were then fixed with 4% paraformaldehyde in PBS for 10 min. Coverslips were washed three times with PBS and cells were permeabilized with 0.1% Triton-X in PBS for 10 min. Coverslips were washed twice with PBS and nonspecific binding was blocked with buffer containing 10% goat serum in PBS for 30 min. Blocking buffer was aspirated and coverslips were incubated overnight at 4 °C with primary antibodies against IL8 (Cusabio, CSB-PA08327A0Rb, rabbit anti-human) and F3 (American Diagnostica, 4509, mouse anti-human) in blocking buffer at a final concentration 20 μg/ml and 10 μg/ml respectively. Next day primary antibody solution was removed and coverslips were washed three times with PBS. Coverslips were incubated for 1 h at RT with secondary antibody solution containing goat anti-rabbit CF-568 conjugated (Biotum, 20102, red) and goat anti-mouse CF-488 conjugated (Biotum, 20010, green) antibodies at a final concentration of 2.6 μg/ml. Secondary antibody solution was removed, coverslips were washed three times with PBS and cells were incubated with DAPI (4′,6-diamidino-2-phenylindole, ThermoFischer Scientific D1306) solution in water at a final concentration 100 ng/ml for 8 min at RT for nuclear visualization. Coverslips were washed three times with water and mounted on microscopy slides.

2.8. Statistical analysis

Data are presented as mean ± Standard Deviation (SD). Statistical

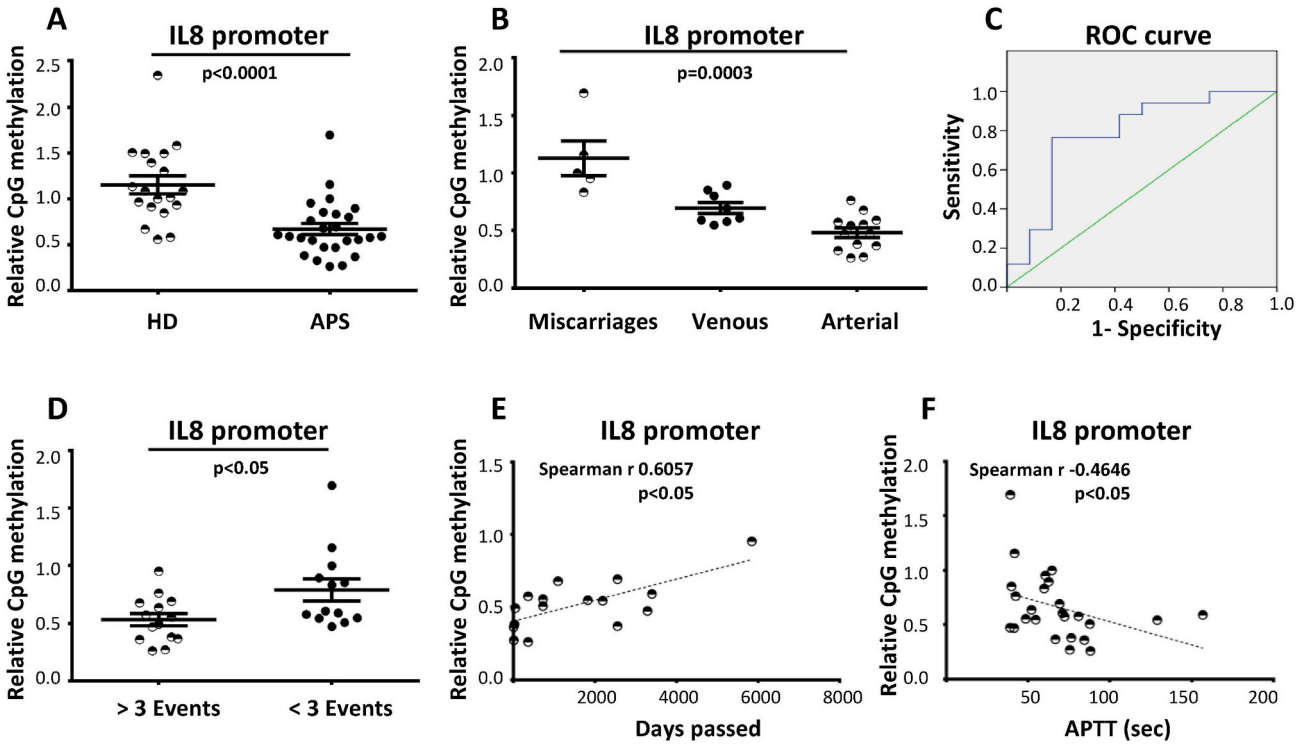

Fig. 1. Comparison of IL8 promoter methylation levels in DNA derived from whole blood of HD, APS patients and subcategories. A) APS patients have decreased relative IL8 promoter methylation compared to healthy donors (mean ± SD: APS 0.6715 ± 0.3090 vs HD 1.153 ± 0.4244, $p < 0.0001$). B) Differential IL8 promoter methylation levels among APS patients with a history of arterial thrombotic event, venous thromboembolism or pregnancy morbidity (mean: 0.4815 vs 0.6954 vs 1.128 respectively, Kruskal-Wallis analysis $p = 0.0003$). C) Discriminative power of IL8 methylation for APS patient history of arterial thromboembolism, venous thrombosis or pregnancy morbidity (area under the curve 0.786, $p = 0.0010$). D) Methylation levels of the IL8 gene promoter negatively correlate with the time since the last clinical event (Spearman r 0.6057, $p = 0.0129$). E) Differential IL8 methylation levels between APS patients having suffered more or less than 3 clinical events (mean: 0.533 vs 0.7907, $p = 0.0348$). F) Significant inverse correlation between IL8 methylation and APTT values in APS patients (Spearman r −0.4646, $p = 0.0193$).

analyses were performed with GraphPad Prism 6.0. Values and differences between groups were evaluated by Mann-Whitney, paired t tests or Kruskal-Wallis (One-Way ANOVA) tests for continuous variables. Spearman correlation as well as multivariate regression analysis was performed, as indicated in the figure legends. P-values < 0.05 were considered statistically significant.

3. Results

3.1. Decreased relative methylation levels of the IL8 promoter in APS patients

DNA isolated from APS patients had statistically significantly lower methylation levels in the IL8 promoter compared to healthy donors (mean ± SD: APS 0.6715 ± 0.3090 vs HD 1.153 ± 0.4244, $p < 0.0001$, Fig. 1A).

Group analysis showed that patients with a history of arterial thromboembolism had the lowest levels of IL8 promoter CpG methylation compared to patients with a history of venous thrombosis or pregnancy morbidity (Kruskal-Wallis analysis of means revealed difference of statistical significance between groups, $p = 0.0003$). Statistically significant difference was also observed when comparing levels in patients with arterial thrombotic events vs those with pregnancy morbidity, or those with venous thrombosis only. IL8 promoter methylation levels were also significantly different in patients with venous thrombosis vs those with pregnancy morbidity (Fig. 1B). IL8 methylation can discriminate with good specificity and sensitivity patients with a history of arterial thromboembolism from the others (1C).

APS patients who had suffered 3 or more clinical incidents had significantly lower methylation levels compared to the ones that had experienced less than 3 episodes (Fig. 1D). An inverse correlation was observed between the methylation levels of the examined CpGs in the IL8 gene promoter and the time since the last clinical event (Fig. 1E). Means ± SD of all comparisons are depicted in Table 1.

Within the APS group, a significant inverse correlation was found between IL8 promoter CpG methylation and APTT values (Fig. 1F).

Table 1
Association of clinical characteristics with IL8 promoter and F3 first intron relative methylation.

Clinical Characteristics	*Relative CpG methylated/unmethylated levels in patients positive for the clinical presentation expressed as fold over one healthy control sample (mean ± SD)*	
	IL8 promoter	**F3 first intron**
Clinical events		
Arterial	0.4815 ± 0.1545	1.832 ± 0.3916
Venous	0.6954 ± 0.1363	1.982 ± 0.5203
Pregnancy morbidity	1.128 ± 0.3377	1.876 ± 0.5177
Kruskal-Wallis test	p = 0.0003	p = 0.9331
Days since last incident		
Spearman r	0.6057	0.2363
	p = 0.0129	p = 0.1892
Number of events		
> 3	0.533 ± 0.199	1.832 ± 0.4234
< 3	0.7907 ± 0.3455	1.965 ± 0.5573
Mann-Whitney test	p = 0.0348	p = 0.8066

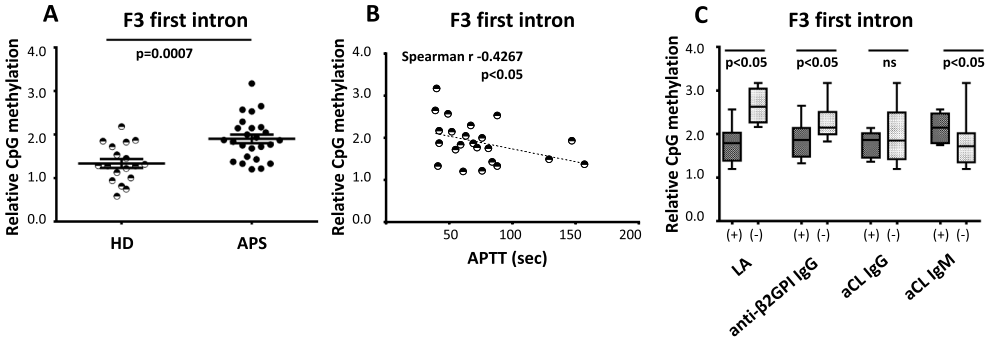

Fig. 2. A) APS patients show increased relative methylation levels in the F3 first intron compared to healthy donors (mean ± SD: APS 1.902 ± 0.4920 vs HD 1.335 ± 0.4356, $p < 0.0001$). B) Relative methylation of F3 first intron negatively correlates with APTT values (Spearman r −0.4267, $p = 0.0212$). C) Comparison of F3 methylation between APS patients positive or negative for *anti*-β2GPI IgG, LA, aCL IgM and IgG autoantibodies (*anti*-β2GPI IgG: 1.89 vs 2.278, LA: 1.786 vs 2.648, aCL IgM: 2.138 vs 1.79, $p < 0.05$).

3.2. Increased relative methylation levels of CpGs in the F3 gene body

Methylation of the examined CpGs in the first intron of F3 was statistically higher in whole blood derived from APS patients compared to healthy donors (mean ± SD: APS 1.902 ± 0.4920 vs HD 1.335 ± 0.4356, $p < 0.0001$, Fig. 2A).

Analysis between patient clinical subgroups revealed no difference in methylation among those that had suffered from arterial thromboembolism, venous thrombosis or pregnancy morbidity (Table 1). A negative correlation was observed between methylation levels and APTT values (Spearman r −0.4267, $p = 0.0212$, Fig. 2B). Association of CpG methylation levels was observed with LA, *anti*-β2GPI and aCL auto-antibody presence (Fig. 2C).

3.3. Stimulation of HD monocytes with anti-β2GPI IgG - β2GPI - CXCL4 leads to transcriptional activation and dynamic changes in methylation of the IL8 and F3 genes

Next, we assessed the involvement of epigenetic regulatory mechanisms in an *in vitro* model of monocyte stimulation with *anti*-β2GPI IgG/β2GPI/CXCL4. Initially, monocytes were stimulated with *anti*-β2GPI -β2GPI complex for 30 min, 1, 2, 4, 6, 8, 12 and 24 h in order to identify the optimal time point where maximum response is noted. Maximum effect for both IL8 and F3 genes following stimulation was noted at 4 h post stimulus (Supplementary Figs. 1A and 1C). IL8 mRNA levels remained persistently high whereas F3 returned to basal levels 24 h after the stimulus. Stimulation of HD monocytes for 4 h with *anti*-β2GPI, *anti*-β2GPI-β2GPI complex or a mixture of *anti*-β2GPI IgG-β2GPI-CXCL4 induces an activated phenotype. The mixture of *anti*-β2GPI IgG-β2GPI-CXCL4 had the maximum effect and upregulated the expression of the IL8 gene-a neutrophil chemotactic factor and F3 gene-which initiates the blood coagulation cascade. The observed increase was 200 fold for the IL8 gene (Fig. 3A) and 15 fold for the F3 gene compared to untreated cells (Fig. 3C). Stimulation also induced expression of IL8 and F3 at protein levels, as assessed by immunocytochemical analysis of monocytes treated with the activating factors for 4 h (Fig. 3E–H).

In order to evaluate the epigenetic regulation of IL8 and F3 gene expression, genomic DNA from monocytes treated for 30 min, 1, 2, 4, 6, 8, 12 or 24 h was assessed with MSPCR for the determination of relative methylation levels in the first intron of F3 and the IL8 promoter. Upon treatment with *anti*-β2GPI IgG-β2GPI, DNA methylation levels of the IL8 promoter exhibited an initial increase reaching a peak at 4 h followed by a decrease at 6 h and 8 h and a return to basal levels at 24 h post stimulus. F3 first intron methylation also exhibited an initial increase at 4 h, a trend for decrease at 6 h and 8 h and returned to basal levels at 24 h. The difference between the untreated cells at the 6 h and 8 h hour time point was not significant for the F3 first intron (Supplementary Figs. 1B and 1D). At 4 h post treatment with *anti*-β2GPI IgG-β2GPI, both regions examined showed statistically significant increased relative methylation compared to untreated cells (Fig. 3B and D). The same stimulus for 6 h resulted in a statistically significant decrease of relative methylation in the IL8 promoter, whereas in the F3 first intron the difference was not significant compared to untreated cells. The strongest effect on methylation was observed with the triple *anti*-β2GPI IgG-β2GPI-CXCL4 mixture (Supplementary Fig. 2). The results are derived from 5 independent experiments and support that transcriptional activation of both genes is accompanied by dynamic changes in their methylation status.

3.4. Stimulation of HD HUVECs with anti-β2GPI IgG - β2GPI - CXCL4 induces an activated phenotype and transcriptionally regulates multiple epigenetic factors

Next, we assessed the involvement of epigenetic regulatory mechanisms in an *in vitro* model implicating interactions of HUVECs with *anti*-β2GPI IgG/β2GPI/CXCL4. For this reason, HUVECs derived from healthy donors (HD HUVECs) were stimulated *in vitro*. When HD HUVECs were stimulated with *anti*-β2GPI, *anti*-β2GPI -β2GPI complex or a mixture of *anti*-β2GPI IgG-β2GPI-CXCL4, they acquired an activated and procoagulant phenotype. The mixture of *anti*-β2GPI IgG-β2GPI-CXCL4 induced the expression of SELE - a leukocyte cell adhesion molecule, IL8, F3, and VEGFC - an endothelial growth factor which regulates the inflammatory microenvironment, and reduced the expression of TMBD-a cofactor in the anticoagulant pathway of the thrombin-induced activation of protein C. The triple mixture of *anti*-β2GPI IgG-β2GPI-CXCL4 had the strongest effect (Fig. 4A–E).

In vitro stimulation of HD HUVECs with combinations of *anti*-β2GPI IgG - β2GPI - CXCL4 resulted in the differential expression of genes encoding epigenetic factors. Compared to untreated cells, significant differences were observed in the relative expression of MECP2 (2 fold), DNMT3B (2 fold), TET1 (2.5 fold increase) HDAC9 genes (3fold) and ARID5B (3fold) (Fig. 4F–J). No difference was noted for the DNMT3A, ARID3A, SMYD4, EZH2, HDAC4, HDAC7 and KDM5C genes (Fig. 4K).

3.5. Stimulation of HUVECs with anti-β2GPI IgG - β2GPI - CXCL4 induces changes in methylation of the F3 gene

DNA isolated from HD HUVECs treated in an *in vitro* model of Antiphospholipid syndrome was assessed by MSPCR in the promoter of IL8 gene and the first intron of the F3 gene. We did not observe any change under any treatment in the relative methylation levels in the IL8 promoter in HD treated HUVECs, or any association with gene expression (Fig. 5A and C). Treatment of HUVECS with *anti*-β2GPI IgG, β2GPI and CXCL4 resulted in decreased relative methylation levels of the F3 first intron CpGs examined (Fig. 5B). A strong inverse correlation between methylation and expression of F3 was also noted (Spearman r −0.8000, $p = 0.0002$, Fig. 5D).

4. Discussion

The present report demonstrates for the first time that relative methylation levels in the IL8 promoter are decreased in whole blood of patients with APS compared to HDs. This decrease is more pronounced

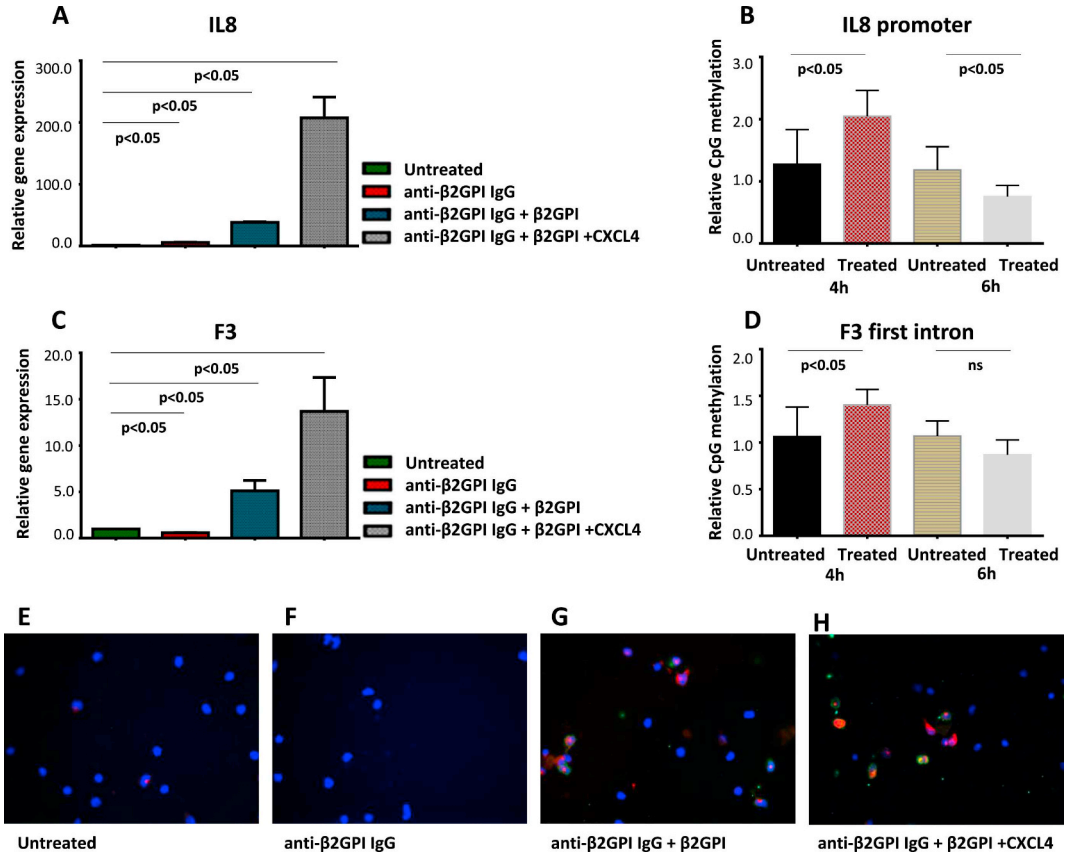

Fig. 3. Quantitation of IL8 and F3 expression and methylation levels in HD monocytes stimulated with *anti*-β2GPI, *anti*-β2GPI -β2GPI complex and a mixture of *anti*-β2GPI IgG-β2GPI-CXCL4. A) Stimulation causes induction of IL8 expression and (B) dynamic changes in IL8 promoter methylation. The triple mixture of *anti*-β2GPI IgG-β2GPI-CXCL4 has the maximum effect. C) Upregulation of F3 gene expression and (D) dynamic changes in F3 methylation upon treatment. Results derived from 5 independent experiments. E-H) Higher fluorescent intensity upon staining for Tissue factor and IL8 in monocytes upon stimulation. Green fluorescent signal indicates Tissue Factor and red IL8 protein. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

in patients with a history of arterial thrombosis, those who are chronically closer to a clinical event at the time of assessment or those who have experienced more than three clinical events. Moreover, IL8 relative methylation negatively correlates with APTT values, which is indicative of Lupus Anticoagulant, a strong risk factor for recurrent thrombosis and increased mortality [36,37]. Concerning the F3 first intron, the CpGs examined showed differential methylation between patients and healthy individuals. Methylation levels were higher in APS patients and seemed to associate with low antibody titers, a finding that might be attributed to antibody consumption [38]. Given that F3 expression has been reported to be higher in APS patients [39,40], our findings agree with the general view that CpG methylation in the first intron positively correlates with, or may even mediate increased gene expression [41–43].

Cell culture based experiments were in concert with the clinical data. We focused on two different cell types, crucial for the clinical presentation of the syndrome. In an *in vitro* model of APS, upon stimulation with a combination of *anti*-β2GPI, β2GPI, and CXCL4, both monocytes and HUVECs acquire an activated phenotype and express higher levels of IL8 and F3 mRNA and protein. Transcriptional activation of the two genes was accompanied by changes in their DNA methylation status. Monocyte stimulation resulted in an initial methylation increase in both the IL8 promoter and the F3 first intron, followed by a decrease to almost basal or somewhat lower levels. The initial increase in IL8 promoter methylation levels is puzzling, as we generally observe reduced methylation when the gene is transcriptionally induced. One possible explanation is that the increase we observe represents rather Cytosine hydroxymethylation levels, at the specific CpGs. The sites are also protected by bisulfite conversion and are, thus, indistinguishable from methylated sites by this approach. Dynamic changes in Cytosine hydroxymethylation have been proposed as an intermediate step in the removal of the methyl mark in mammals [44,45]. Moreover, previous studies have demonstrated that dynamically increased hydroxymethylation levels in promoters and enhancers correlate with transcription factor binding during lineage specification in human embryonic stem cells [46]. A similar increase could be taking place in our model system upon stimulation, correlating with the recruitment of transcription factors to the IL8 promoter region to mediate transcriptional activation. A potential role of this epigenetic mark in response to stimulation is also supported by the increased transcript levels of TET1, the enzyme that catalyzes hydroxymethylation, that we observe upon treatment.

Recent research has shown that HUVEC stimulation with atherogenic and prothrombotic stimuli leads to endothelial deregulation in a way which is dependent on DNA methylation and histone posttranslational modifications [47,48]. We wanted to examine this interaction in the context of APS. To this end, in our *in vitro* model, comprising of HUVEC activation by the *anti*-β2GPI-β2GPI-CXCL4 complex we assessed the relative expression of genes involved in the major epigenetic regulatory mechanisms. We observed that several genes such as DNMT3B, MECP2, TET1, HDAC9 and ARID5B showed differential expression levels between treated and untreated cells. Interestingly, the stronger the stimulus and the activation status of the cells, as noted by activation markers such as e-selectin, the more intense the change in the levels of epigenetic regulatory genes observed.

DNMT3B is one of the enzymes catalyzing Cytosine methylation. It

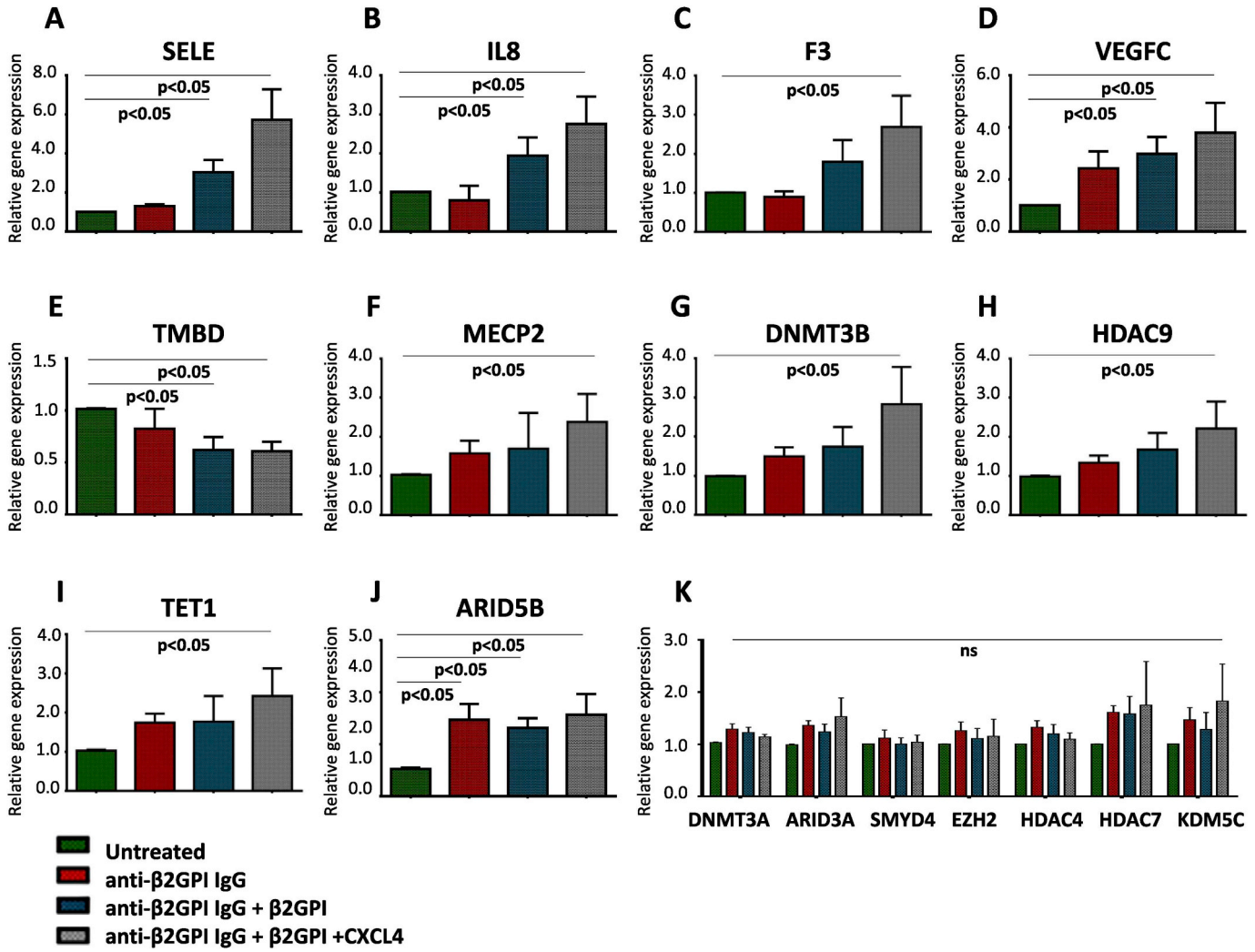

Fig. 4. Stimulation of HD HUVECs with *anti*-β2GPI, *anti*-β2GPI -β2GPI complex and a mixture of *anti*-β2GPI IgG-β2GPI-CXCL4 induces an activated and procoagulant phenotype (A–E) as well as increased expression of MECP2 (F), DNMT3B (G), TET1 (I), HDAC9 (H) and ARID5B (J) genes compared to untreated cells. All differences vs untreated were statistically significant, $p < 0.05$. K) No statistical difference was noted for the DNMT3A, ARID3A, SMYD4, EZH2, HDAC4, HDAC 7 and KDM5C genes.

is a *de novo* DNA methyltransferase, critical for the establishment of methylation patterns in the genome. Methyl cytosine binding protein 2 (MECP2), selectively binds to methylated CpG and is involved in regulating both the repression and the activation of methylation-sensitive genes. TET1 is a methylcytosine dioxygenase, converting methyl-Cytosine to hydroxymethyl-Cytosine. Its activity is independent of DNA replication, while studies implicate it in autoimmune conditions [49,50]. Expression of the above genes is upregulated in HUVECs upon stimulation. In other words, besides the methyl mark itself, enzymes that catalyze its addition or removal from Cytosine and proteins that recognize it are also significantly altered upon stimulation, highlighting a significant role of DNA methylation in the orchestration of this response.

Finally, given that DNA methylation is in cross-talk with histone modifications, we sought to identify histone modifying enzymes that could be implicated in the response to *anti*-β2GPI-β2GPI-CXCL4 antibody complex stimulation, as additional epigenetic modifiers. We find levels of HDAC9 and ARID5B to be increased upon stimulation. HDAC9 generally functions as a transcriptional repressor. It has been reported to have a positive effect in TLR signaling and innate immunity, rendering macrophages more sensitive to LPS, as well as in atherosclerosis and metabolic disease [51,52]. On the other hand, ARID5B plays a role in histone demethylation and can function as a transcriptional activator by removal of the repressive histone H3 lysine 9 dimethyl mark. Recently ARID5B was shown to be upregulated in monocytes from patients with cardiovascular disease and critical for the complete inflammatory response in LPS stimulated macrophages [53]. ARID5B silencing led to down regulation of proinflammatory cytokines such as tumor necrosis factor (TNF) and IL-1a and type I interferon signaling pathway activator and effector cytokines.

In our model mimicking APS, transcriptional regulation of the above genes indicates a critical epigenetic component in response to stimulation which would orchestrate the gene expression program. For this reason, we wanted to assess whether this deregulation leads to epigenetic changes in the loci we evaluated in peripheral blood DNA isolated from APS patients. Endothelial cells treated in conditions simulating Antiphospholipid syndrome had decreased F3 first intron methylation levels compared to controls, and relative methylation strongly correlated with gene expression levels. This finding shows that upon treatment the methyl mark is removed from this CpG, a process that probably facilitates the binding of transcription factors to the region inducing the transcriptional activation of the F3 gene in HUVECs. The observed difference in the pattern of methylation between whole blood DNA and treated HUVECs could be possibly attributed to the fact that these are distinct cell types with differential regulation of gene expression.

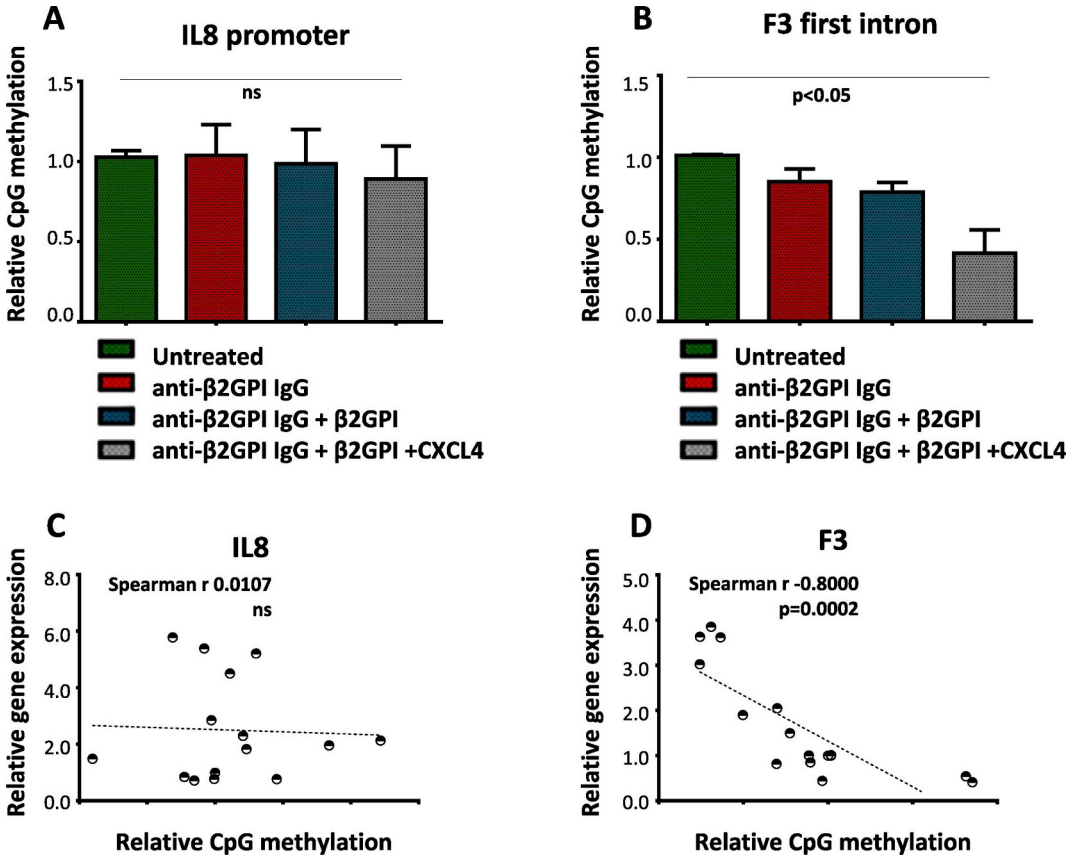

Fig. 5. Quantitation of IL8 (A) and F3 (B) methylation levels in HD HUVECS treated with *anti*-β2GPI IgG, β2GPI and CXCL4. C) Relative methylation of the IL8 promoter does not correlate with IL8 relative gene expression levels (Spearman r −0.01071, p = 0.9698). D) Relative methylation of the F3 promoter has a strong inverse correlation with the relative F3 gene expression in HUVECs (Spearman r −0.8000, p = 0.0002).

5. Conclusions

Taken together, our data combined with previous studies support a model for APS in which *anti*-β2GPI-β2GPI-CXCL4 antibody complexes activate monocytes and endothelial cells leading to a proinflammatory and procoagulant phenotype, as marked by IL8, F3, SELE and VEGFC overexpression as well as downregulation of TMBD. This activated status is characterized by increased expression of MECP2, DNMT3B, TET1, HDAC9 and ARID5B which might influence the epigenetic alterations observed in the IL8 promoter and F3 first intron in APS patients.

From a clinical view, IL8 promoter methylation could serve as a discriminating factor for clinical presentation, and differential IL8 and F3 methylation levels could possibly signify an underlying active prothrombotic and inflammatory process. Future studies could address the potential utility of IL8 promoter and F3 first intron methylation as biomarkers or therapeutic targets in the context of APS.

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Appendix A. Supplementary data

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