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Antiphospholipid antibodies induce proinflammatory and procoagulant pathways in endothelial cells

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ABSTRACT

Antiphospholipid syndrome (APS) is an autoimmune thrombophilia characterized by recurrent thrombotic events and/or pregnancy morbidity in the presence of antiphospholipid antibodies detected either as anti-cardiolipin, anti-β2 Glycoprotein I (anti-β2GPI) or Lupus anticoagulant (LA). Endothelial deregulation characterizes the syndrome. To address gene expression changes accompanying the development of autoimmune phenotype in endothelial cells in the context of APS, we performed *transcriptomics* analysis in Human Umbilical Vein Endothelial Cells (HUVECs) stimulated with IgG from APS patients and β2GPI, followed by intersection of RNA-seq data with published microarray and ChIP-seq results (Chromatin Immunoprecipitation). Our strategy revealed that during HUVEC activation diverse signaling pathways such as TNF-α, TGF-β, MAPK38, and Hippo are triggered as indicated by Gene Ontology (GO) classification and pathway analysis. Finally, cell biology approaches performed side-by-side in naïve and stimulated cultured HUVECs, as well as, in placenta specimens derived from Healthy donors (HDs) and APS-patients verified the evolution of an APS-characteristic gene expression program in endothelial cells during the initial stages of the disease's development.

1. Introduction

Antiphospholipid syndrome (APS) is an autoimmune acquired thrombophilia characterized by recurrent arterial or venous thrombosis and/or pregnancy morbidity in the presence of serum auto-antibodies against phospholipid-binding plasma proteins such as β-2 glycoprotein-I (β2GPI) [1] and prothrombin [2,3], commonly described with the general term "anti-phospholipid antibodies" (aPL). Multiple mechanisms of action of aPL have been proposed to explain the pathogenesis of the syndrome [4], but several aspects, such as recurrent thrombosis [5], atherosclerosis, livedo reticularis, cognitive dysfunction, and multiple sclerosis-like symptoms [6] remain unresolved.

Endothelial deregulation is a characteristic of APS [7,8]. *In vitro* studies have demonstrated that incubation of Human Umbilical Vein Endothelial Cells (HUVECs) with aPL induces the increased expression of the adhesion molecules Intracellular Adhesion Molecule-1 (ICAM-1), Vascular Cell Adhesion Molecule 1 (VCAM-1) and E-selectin, as well as production of pro-inflammatory cytokines [Interleukin 1 (IL- 1), Interleukin 6 (IL-6), and Interleukin 8 (IL-8)] [9,10]. APS patients display increased circulating levels of these molecules as well [11,12]. Among the mechanisms proposed to explain the pro-thrombotic and pro-inflammatory properties of aPL, a process involving phosphorylation of p38 MAPK, nuclear translocation of Nuclear Factor Kappa B (NF-κB) and increased Tissue Factor expression has been described [13,14]. Moreover, anti-β2GPI/β2GPI complexes are known to bind to Annexin A2 [15] and ApoER2 [16], activate C5 [17–19] and activate innate immunity through TLR-2, TLR-4, and TLR-6 recruitment. The above phenomena drive HUVECs to procoagulant and proinflammatory pathways [9,20–24]. Previous work from our laboratory has shown that HUVECs treated with a mixture of anti-β2GPI plus β2GPI are activated, this activation being more prominent when the chemokine CXCL4 is added to the mixture [10]. In the plasma of APS patients, CXCL4 is elevated in the plasma of APS patients as well [25], a fact possibly attributed to platelet activation by complexes of anti-β2GPI antibodies

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and β2GPI, especially when the latter is dimerized by binding to CXCL4 chemokine [26,27].

Here, we aimed to address the molecular mechanisms operating inside HUVECs during their response to anti-β2GPI antibodies in the presence of β2GPI. To this end, we profiled the early transcriptional events that occur in HUVECs upon their activation by APS total IgG plus β2GPI. *Bioinformatics* analysis revealed critical signal transduction pathways such as TNF-α, TGF-β, MAPK38, and Hippo implicated in the alteration of the endothelial gene expression program and subsequently the generation of the APS phenotype, upon treatment with APS total IgG plus β2GPI.

Integrated analyses of our RNA-seq results along with published microarray-based and ChIP-seq studies in HUVECs [28–30] uncovered the orchestration of a specific gene expression program and the establishment of the "APS-state" transcriptome. Up-regulation of key APS target genes was also confirmed at the protein level in biopsy samples and cultured cells. Taken together, the aforementioned analyses provide a comprehensive view of the molecular mechanisms governing APS development.

2. Methods

2.1. Patients and specimens

HUVECs were isolated from 2 Healthy Donors (HDs) after uncomplicated pregnancy as previously described [31] and cultured in Endothelial Basal Medium-2 (EBM-2, Lonza CC-3162). Serum was isolated from 8 patients with APS. Placenta tissues were collected upon delivery from 2 APS patients and 5 HDs. All patient-derived specimens were obtained upon informed consent. This work was approved by the Ethics Committees of Aretaeion Hospital (B-7/24-09-2013) and National and Kapodistrian University of Athens (NKUA) (2539/4-11-2014).

2.2. Total IgG isolation and HUVEC stimulation

Total IgG was isolated from the sera of APS patients using Protein G Sepharose beads as previously described [10]. Specifically, total IgG was isolated from 8 patients with APS (numbered 1–8). To alleviate the effect of potential inter-individual variation, two pooled samples were prepared, i.e., one comprising total IgG derived from patients 1–4 and a second comprising total IgG derived from patients 5–8; the two pooled samples were used to stimulate HUVECs. HDs derived HUVECs were grown to confluency, serum-starved for 16 h and stimulated for 6 h using purified total IgG (1 mg/ml) in combination with human native β2GPI (20 μg/ml; Fitzgerald 30-AB23). HD-derived HUVECs were cultured in EBM, without addition of growth factors and serum, and were used as negative controls.

2.3. RNA-seq-library preparation and sequencing

HUVECs were cultured for 6 h in the presence (stimulated HUVECs) or absence (naïve HUVECs) of pooled IgG obtained from APS patients as previously described, and then subjected to RNA extraction. RNA was isolated with Trizol reagent (Thermo Fischer 15596018) and purified with the RNeasy MinElute Cleanup Kit (Qiagen 74204). Two naïve and two treated samples were processed for library preparation for RNA-sequencing according to the Illumina TruSeq RNA Library Prep Kit protocol (Illumina 15026495 F). In brief, poly-A selected RNA was fragmented, followed by first and second strand cDNA synthesis. Subsequently, DNA ends were repaired and subjected to A-tailing, sequencing adapters were ligated and samples were amplified by PCR for 11–12 cycles. Libraries were quantified using the Qubit Fluorometer and qualitatively assessed using the Agilent 2100 Bioanalyzer. Samples were sequenced using a 75 bp single end with the Illumina NextSeq500 sequencer at the Greek Genome Center (GGC) of the Biomedical Research Foundation of the Academy of Athens (Fig. 1A).

2.4. Quantitative Real-Time PCR

RNA derived from the same samples that were subjected to RNA-seq was reversed transcribed to produce cDNA using Superscript III (Invitrogen). For quantitative PCR KAPA SYBR FAST qPCR Master Mix was used and the reactions were run in duplicates in a Biorad CFX96 Real-Time PCR Detection System. Normalization was performed according to the Delta-Delta Ct method.

2.5. RNA-seq and bioinformatics analyses

Deep-sequencing was performed to an average depth of 43 million reads per sample (ranging from 40,767,385 to 48,281,423). The quality of the raw sequencing reads was evaluated by the application of the FastQC algorithm (Galaxy version 0.69) [32] hosted in the Galaxy platform [33] with default parameters. Sequencing reads were mapped to the human genome (version hg19) using Tophat (Galaxy version 2.1.0) with default parameters [34]. Htseq-count (Galaxy Version 0.6.1galaxy3) was applied for reads counting [35]. Deseq2 was used for differential gene expression analysis between conditions [36]. Differentially expressed genes were called using a log fold change of 0.5 and a p value of 0.05 as a cutoff. BigWig coverage files were produced by applying the bam Coverage command from the DeepTools suite (Galaxy version 3.1.2.0.0) [37]. Heatmaps were constructed by submitting the Deseq2 normalized gene counts to the Morpheus software [38]. Gene Ontology (GO) analysis was performed using the Enrichr software [39]. Up-regulated genes were obtained directly from the lists described in the published microarray study [28] and aligned to our RNA-seq results. The microarray study followed a 4 h IgG plus β2GPI HUVECs stimulation protocol. To capture a sharp picture of common genes (described in Fig. 2B) between the two studies we compared the pair of our Naïve and 6 h IgG plus β2GPI treated HUVECs that exhibited the maximum response upon stimulation, to the microarray results. More specifically, we divided the sequencing counts derived from the most activated sample with those of the uninduced sample and applied a two-fold cutoff, in order to ensure for maximized specificity of the obtained results. This list of the differentially expressed genes was intersected with the published microarray results [28].

Published p65 ChIP-seq data derived from HUVECs treated with TNF-α for 1 h (GSM1154044) [29] were accessed through the Cistrome Data Browser [40]. Only strong binding sites displaying above 20-fold enrichment over background were kept. The GREAT algorithm was used to assign genes to p65 binding sites with the single closest gene within ±10 kb relative to the Transcription Start Site (TSS) option [41]. Published SMAD ChIP-seq data were downloaded (GSE27631) [30] and analyzed by mapping of the sequencing reads through application of Bowtie2 algorithm. Peaks were called using MACS2 algorithm.

Immunocytochemical analysis for IL-6, IL-8, NF-κB1, Tissue Factor, ICAM- 1, VCAM-1, E-selectin, P-selectin, TGF-β2 and TGFR1 protein.

HUVECs were isolated and cultured as previously described and then seeded on coverslips for overnight adherence. The next day, cells were treated as described above for 6 h. The detection of protein levels of IL-6, IL-8, NF-kB1, Tissue Factor, ICAM-1, VCAM- 1, E-selectin, P-selectin, TGF-β2 and TGFR1 was performed as previously. Coverslips were incubated overnight at 4 °C with primary antibodies against IL-6 (5 μg/ml, CSB-PA06757A0Rb, Cusabio), IL-8 (5 μg/ml, CSB-MA083271A0m, Cusabio), NF-κB1 (5 μg/ml, CSB-PA190132, Cusabio), TGF-β2 (5 μg/ml, CSB- PA07319A0Rb, Cusabio), Tissue Factor (5 μg/ml, 4509, American Diagnostica), ICAM-1 (5 μg/ml, AF796, R&D Systems), VCAM-1 (4 μg/ml, sc-18854, Santa Cruz Biotechnology), E-selectin (4 μg/ml, sc-271267, Santa Cruz Biotechnology), P-selectin (4 μg/ml,sc-137054, Santa Cruz Biotechnology) and TGFR1 (5 μg/ml,CSB-PA023451LA01HU, Cusabio). Coverslips were incubated for 1 h at RT with a secondary antibody solution containing goat anti-rabbit CF-568 conjugated (20102, Biotium, red signal) and goat anti-mouse CF-488 conjugated (20010, Biotium, green signal) antibodies at a final

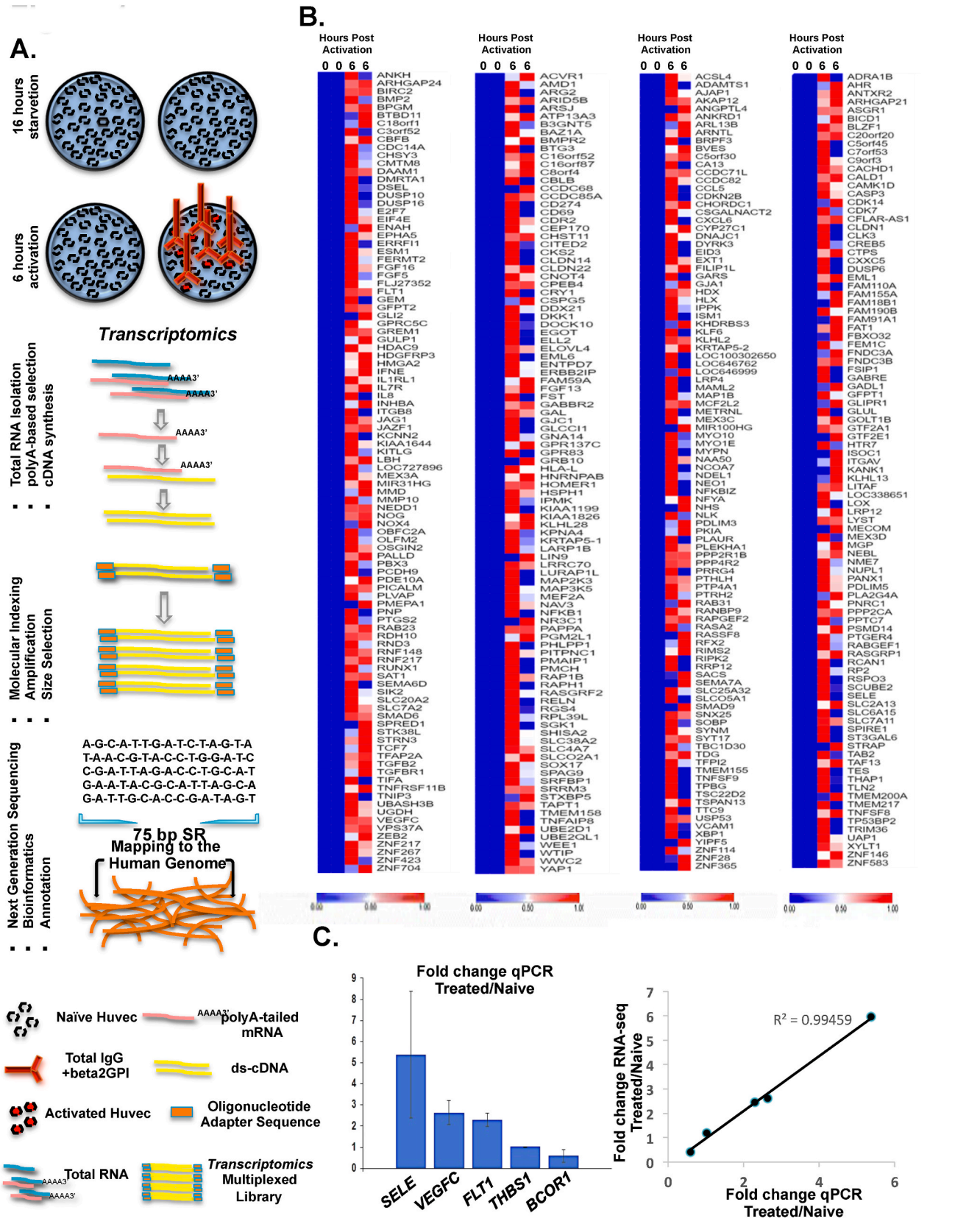
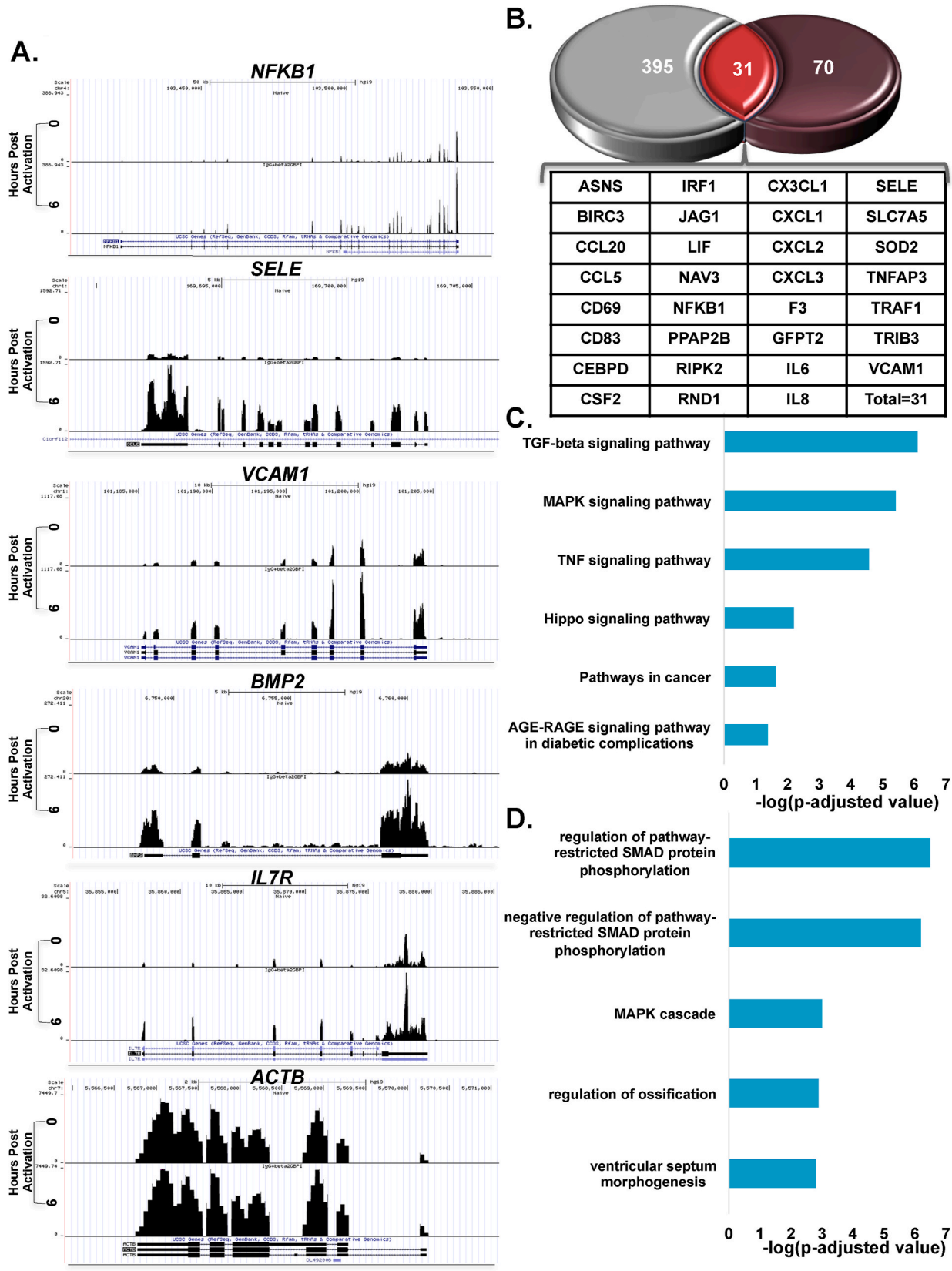


Fig. 1. Methodology, results and validation of RNA-seq in HUVECs treated with IgG and β2GPI isolated from APS patients A. Schematic representation of HUVECs' treatment and RNA sequencing. B. Transcriptomics and Bioinformatics analysis uncovered 395 genes that were activated during the first 6 h of treatment. Inflammatory and immune response genes as well as known APS-markers including genes that encode for transcription factors (e.g NF-κB, SMAD, YAP1), chemokines (e.g IL-8), growth factors (TGFβ-2), adhesion molecules (e.g E-Selectin, VCAM-1, ICAM-1), signal transduction molecules (e.g MAP2K3, MAP3K5) are upregulated. C. Verification of the results from transcriptomics analyses using gene-specific qPCR assays for selected genes (left panel). High correlation of the RNA-seq/qPCR results is detected (right panel).



ASNS	IRF1	CX3CL1	SELE
BIRC3	JAG1	CXCL1	SLC7A5
CCL20	LIF	CXCL2	SOD2
CCL5	NAV3	CXCL3	TNFAP3
CD69	NFKB1	F3	TRAF1
CD83	PPAP2B	GFPT2	TRIB3
CEBPD	RIPK2	IL6	VCAM1
CSF2	RND1	IL8	Total=31

Fig. 2. RNA-seq intersection with published data and gene ontology analysis. A. Snapshots from Genome Browser show that sequencing reads (signal) covering exon sequences of NFKB1, SELE, VCAM1, BMP2, IL7R are increased in treated samples compared to control samples whereas signals derived from ACTB exons, remain unaffected B. Direct intersection of previously published microarray results with our RNA-seq results revealed 31 common upregulated genes (2 B)·C-D KEGG pathway and Gene Ontology analysis (Biological Process) for the upregulated genes reveals that signal transduction pathways such as TNF-signaling, TGF-β signaling, MAPK38- signaling and Hippo pathway operate simultaneously upon HUVECs activation.

concentration of 2.6 μg/ml. Nuclear visualization was achieved with DAPI (4′,6-diamidino-2-phenylindole, D1306, Thermo Fischer Scientific) solution in water at a final concentration 100 ng/ml for 8 min at RT and coverslips were mounted on microscopy slides and visually analyzed.

2.6. Immunohistochemical analysis of placenta specimens

Snap-frozen tissue specimens were cut at 10 nm thickness and fixed with methanol for 10 min followed by acetone for 2 min at −20 °C. Nonspecific antibody binding was blocked by incubation with 2% non-immune fetal bovine serum in Phosphate Buffer Saline. Staining was performed by incubation with the above primary and isotype-matched controls overnight at 4 °C, followed by 30 min incubation with fluorochrome-conjugated secondary antibodies at room temperature in a humidified chamber. Primary and secondary antibodies and DAPI were used at the concentrations described above. Slides were visually analyzed.

2.7. Image processing and statistical analysis

Data are presented as mean ± Standard Deviation (SD). Statistical analyses were performed with GraphPad Prism 5.0. Values and differences between groups were evaluated by Mann-Whitney (One-Way ANOVA) tests for continuous variables. P- values < 0.05 were considered statistically significant.

2.8. Data sharing statement

RNA-seq data are deposited at the GEO under the accession number GSE143506. Reviewers can access our data using the following token: wlgtgouunjmtxol.

3. Results

3.1. Transcriptomics analysis in an APS model-system using mock or total IgG plus β2GPI-induced HUVEC cells

Treatment with APS patient-derived total plus β2GPI for 6 h significantly altered the gene expression profile of HUVECs as assessed by the results of RNA-seq experiments in biological replicates (Fig. 1A). Direct comparison of gene expression levels between HUVECs treated for 6 h and untreated controls revealed a multitude of Differentially Expressed Genes (DEGs) (Fig. 1B, Supplementary Tables 1 and 2) (Fig. 1B). DEGs include both up-regulated and down-regulated genes, indicating substantial alteration of the gene expression profile of HUVECs upon stimulation (Fig. 1B, Supplementary Table 2). In total, 395 genes were captured up-regulated at the 6 h time-point following stimulation, including known APS-markers and critical regulators of immune response pathways, as well as characteristic genes that have been previously linked to human inducible gene expression in various model-systems. A variety of genes that encode for transcription regulators (e.g. NF-κB, SMAD, YAP1), chemokines (e.g. IL-8), growth factors (TGFβ-2), adhesion molecules (e.g. E-Selectin, VCAM-1), signal transduction molecules (e.g. MAPK2K3, MAP3K5) and a variety of alternative molecules are induced. Next, we verified the results of the RNA-seq analysis by applying gene-specific qPCR assays for selected genes such as *SELE*, *VEGF*, *FLT1*, *BCOR1* (Fig. 1C left panel). Direct alignment of the RNA-seq/qPCR independent methodologies revealed high correlation of the derived results ($R^2 = 0.994$) (Fig. 1C right panel).

Fig. 2A illustrates representative examples of expression events occurring prior or upon HUVEC activation across the genomic loci of *NF-κB1*, *SELE*, *VCAM-1*, *BMP2*, *IL7R* and *ACTB* as visualized in the Genome Browser. Moreover, direct intersection of the data derived from the previously published microarray experiments [28] with our RNA-seq results revealed that both studies share an extended spectrum of common up-regulated genes (Fig. 2B), which includes among others several APS-related genes such as *SELE*, *VCAM-1*, *IL-6, IL-8*, captured to become activated upon HUVEC stimulation (Fig. 2B). To gain further insight into the molecular pathways implicated in the response of HUVECs to treatment, we performed Gene Ontology (GO) analysis of the up-regulated genes. Induction of HUVECs seems to affect diverse cellular pathways that presumably coordinate distinct functions, since the repertoire of the inducible genes identified is heterogeneous (Fig. 4A). Prominent pathways include the TNF-α signaling, TGF-β signaling, MAPK38-signaling and Hippo pathways (Fig. 2 C; KEGG Pathway Analysis) (Fig. 2D; GO Biological Process) indicating that the observed transcriptional output is the result of the concerted action of multiple chemokines, signal transduction molecules and transcription factors (TFs) (Fig. 4A). A series of transcription factors among the DEGs become up-regulated, a fact consistent with the broad gene expression changes observed in our inducible cell model system of APS study. Subsequent analyses revealed that several up-regulated TFs belong to the aforementioned signal transduction pathways (Fig. 2C and D), presumably influencing the transition from the naïve to the aberrant cell state, through the activation of a unique gene expression program that leads to the generation of a "new transcriptome".

3.2. Stimulation of HD HUVECs with total IgG derived from APS patients plus β2GPI results in increased protein levels of pro-inflammatory and pro-coagulant mediators

To address whether the upregulation of the specific genes could also be observed at the protein level, we performed immunostaining in naïve and stimulated HUVECs. HDs-derived HUVECs were *in vitro* stimulated with the same mix of pooled total IgG from APS patients with anti-β2GPI reactivity plus β2GPI for 6 h, as described above. The mixture of APS-patient total IgG plus β2GPI induced a substantial increase of the protein levels of the proinflammatory cytokines IL-6, IL-8 as well the transcription factor NF-κB1 (Fig. 3A-3C). The same changes although to a somewhat lesser degree, were observed for the cell adhesion molecules ICAM-1, E-selectin, P-selectin, Tissue Factor and TGF-β2 (Fig. 3D–I). There was no significant difference in the levels of TGFR1 in the 6 h-time frame between the treated and the untreated cells (Fig. 3J.). Fluorescence intensity analysis revealed that all the inflammatory mediators and adhesion molecules presented statistically significant difference between untreated and stimulated endothelial cells (Fig. 3K).

3.3. Intersection of the RNA-seq with published Chip-seq data reveals NF-κB and SMAD targets among the DEGs

To identify significant regulators of the HUVEC response upon APS patients-derived total IgG and β2GPI stimulation, we examined the transcription factors that become activated upon treatment. As shown in Fig. 4A, a variety of genes encoding for transcriptional regulators are activated after 6 h of treatment. Interestingly, these factors belong to distinct families and include known downstream effectors of different signal transduction pathways, some of which have been implicated in APS. In particular, among the up-regulated transcription factors, NF-κB1, SMADs, BMP2, and YAP1 are captured, a result consistent with the implication of TNF, TGF-β, MAPK38- and Hippo pathways in HUVEC activation upon with total IgG derived from APS patients plus β2GPI for 6 h (Fig. 2C and D). In order to provide additional elements of the molecular mechanism of APS pathogenesis and to identify putative direct targets of the key transcriptional regulators implicated, we performed combined *bioinformatics* meta-analyses of available published ChIP-seq results on TNF-α activated HUVECs [29] with our RNA-seq data. This intersection of published NF-κB-ChIP-seq [29] with our RNA-seq data highlights NF-κB binding-events neighboring (±10 kb) the transcription start site (TSS) of 28 up-regulated genes, including targets of the above transcription factor such as *SELE*, *VCAM1*, *NF-κB1* which have been linked to APS development (Fig. 4B). Moreover, intersection of the

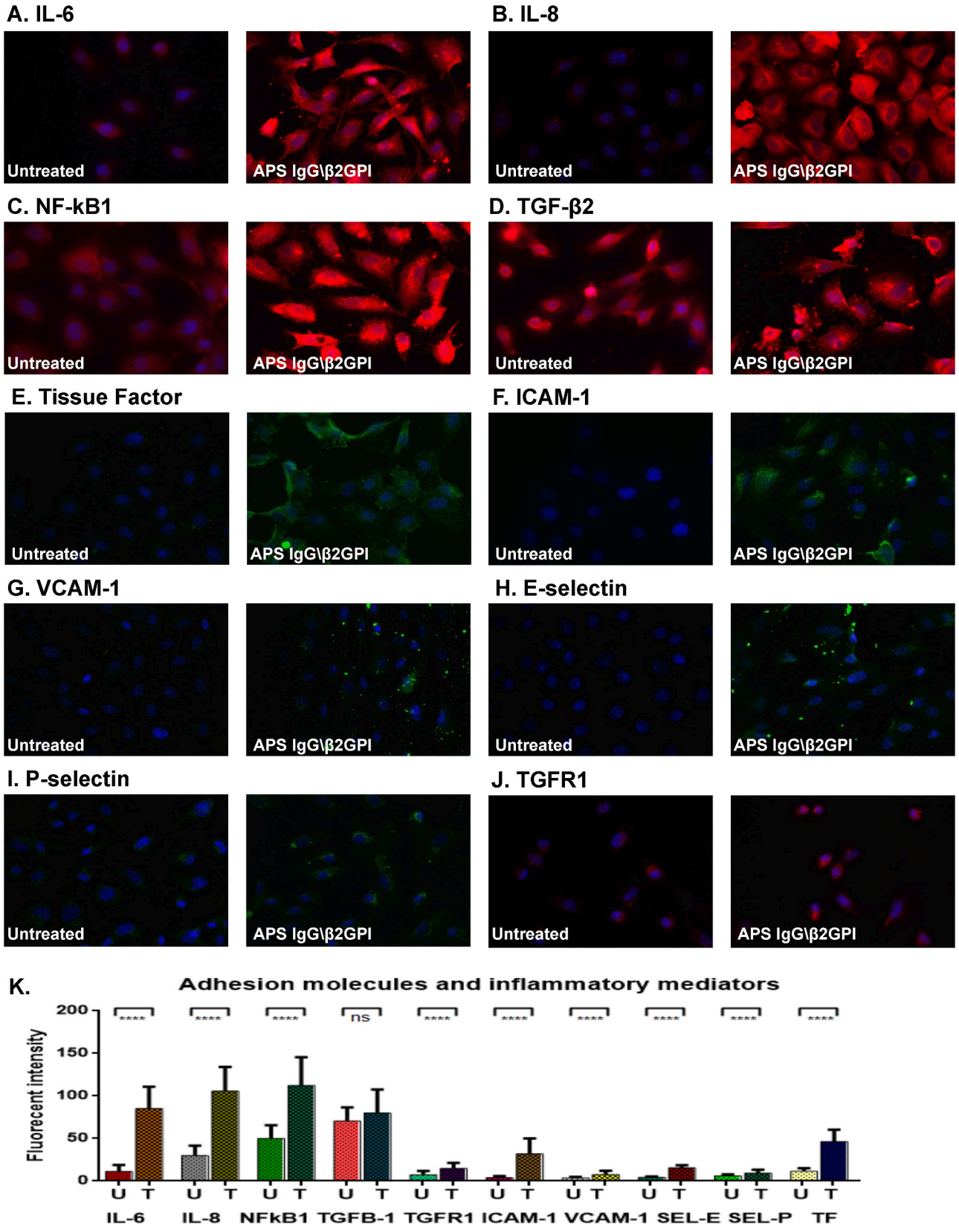
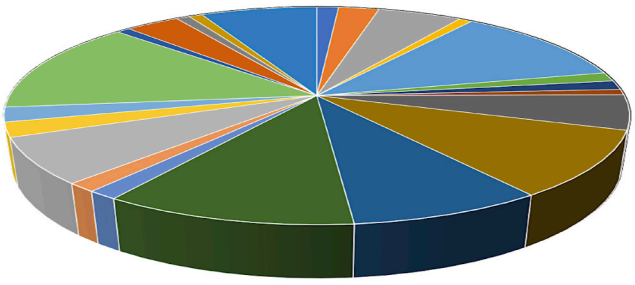


Fig. 3. Immunofluorescent antibody staining in naïve and treated HUVECs. AK. The mixture of APS total IgG and β2GPI induces a pronounced increase of the protein levels of the proinflammatory cytokines IL-6, IL-8 as well the transcription factor NF-κB1 and cell adhesion molecules Tissue Factor, ICAM-1, VCAM-1, Eselectin, P-selectin and TGFR1. There was no significant difference for the TGFR1 molecule (3 J). Visual analysis revealed that all the inflammatory mediators and adhesion molecules presented statistically significant difference between the untreated and treated endothelial cells (3 K).

A.



Extracellular matrix protein	0.80 %	Cytoskeletal protein	1.50 %
Transporter	3.10 %	Transmembrane receptor regulatory/adaptor protein	0.50 %
Transferase	6.90 %	Oxidoreductase	0.80 %
Lyase	0.80 %	Cell adhesion molecule	0.50 %
Ligase	3.10 %	Nucleic acid binding	5.90 %
Signaling molecule	5.10 %	Enzyme modulator	6.60 %
Calcium – binding protein	0.80 %	Defense/immunity protein	0.80 %
Hydrolase	4.10 %	Transfer/carrier	1.30 %
Membrane traffic protein	1.30 %	Transcription factor	8.20 %
Chaperone	0.50 %	Cell junction protein	2 %
Structural protein	0.50 %	Isomerase	0.50 %
Receptor	4.30 %		

Upregulated Transcription Factors

AHR	ARID5B	ARNTL	BMP2	CBFB	CNOT4	CREB5	DMRTA1	DNAJC1	E2F7	GLI2
HDX	HLX	HMGA2	JAZF1	KLF6	MECOM	MEF2A	NCOA7	NFKB1	NFKBIZ	NFYA
NR3C1	PBX3	RFX2	RUNX1	SMAD6	SMAD9	SOX17	TCF7	TFAP2A	THAP1	YAP1

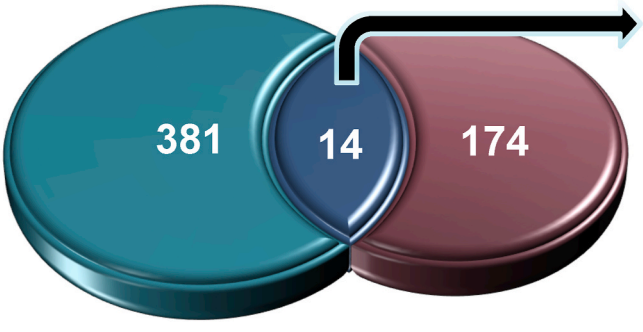
B.

p65-ChIP-seq Huvec/ RNA-seq Huvec

BMP2	BAZ1A	NFKBIZ	PANX1
FLT1	PMAIP1	CXCL6	PTGER4
IL1RL1	MAP2K3	IPPK	SELE
BIRC2	RAPH1	SEMA7A	FAM110A
TIFA	C8orf4	MAML2	RSPO3
ELL2	RIPK2	CACHD1	CREB5
NFKB1	VCAM1	PPTC7	PNRC1

C.

Smad-ChIP-seq Huvec/ RNA-seq Huvec



ANKRD1	CLDN14	MECOM
BICD1	CMTM8	OSGIN2
BIRC2	EXT1	SMAD9
CBFB	IPPK	TES
CCDC68	JAG1	Total=14

Fig. 4. Upregulated transcription factors and their putative targets. A. (Upper panel) Classification of upregulated genes into families. (Bottom panel) Genes that encode for transcriptional regulators are activated in HUVECs upon treatment. B. Combined bioinformatics meta-analyses of published NF-κB-ChIP-seq results on HUVECs with our RNA-seq data reveals several NF-kB binding-events in 28 upregulated genes that includes known target genes of the above transcription factor C. Intersection of the RNA-seq results with the already published Smad1/5-ChIP-seq experiment in HUVECs identifies 14 direct target genes of Smad1/5 such as SMAD9, IPPK, CCDC68.

RNA-seq results with published Smad1/5-ChIP-seq data in HUVECs activated by BMP-6 or BMP-9 [30] identified 14 direct target genes of *Smad1/5* that have been captured up-regulated in our *transcriptomics* experiments, including among others SMAD9, IPPK and CCDC68 (Fig. 4C). The above integrated *bioinformatics* approach verifies the initial discovery of simultaneous activation of multiple signal transduction pathways that presumably orchestrates the evolution of an APS-characteristic gene expression program, instructed by the operation of sequence-specific transcription factors.

3.4. Increased protein levels of inflammatory and adhesion molecules in APS placenta biopsies

To address whether the results obtained in cultured endothelial cells

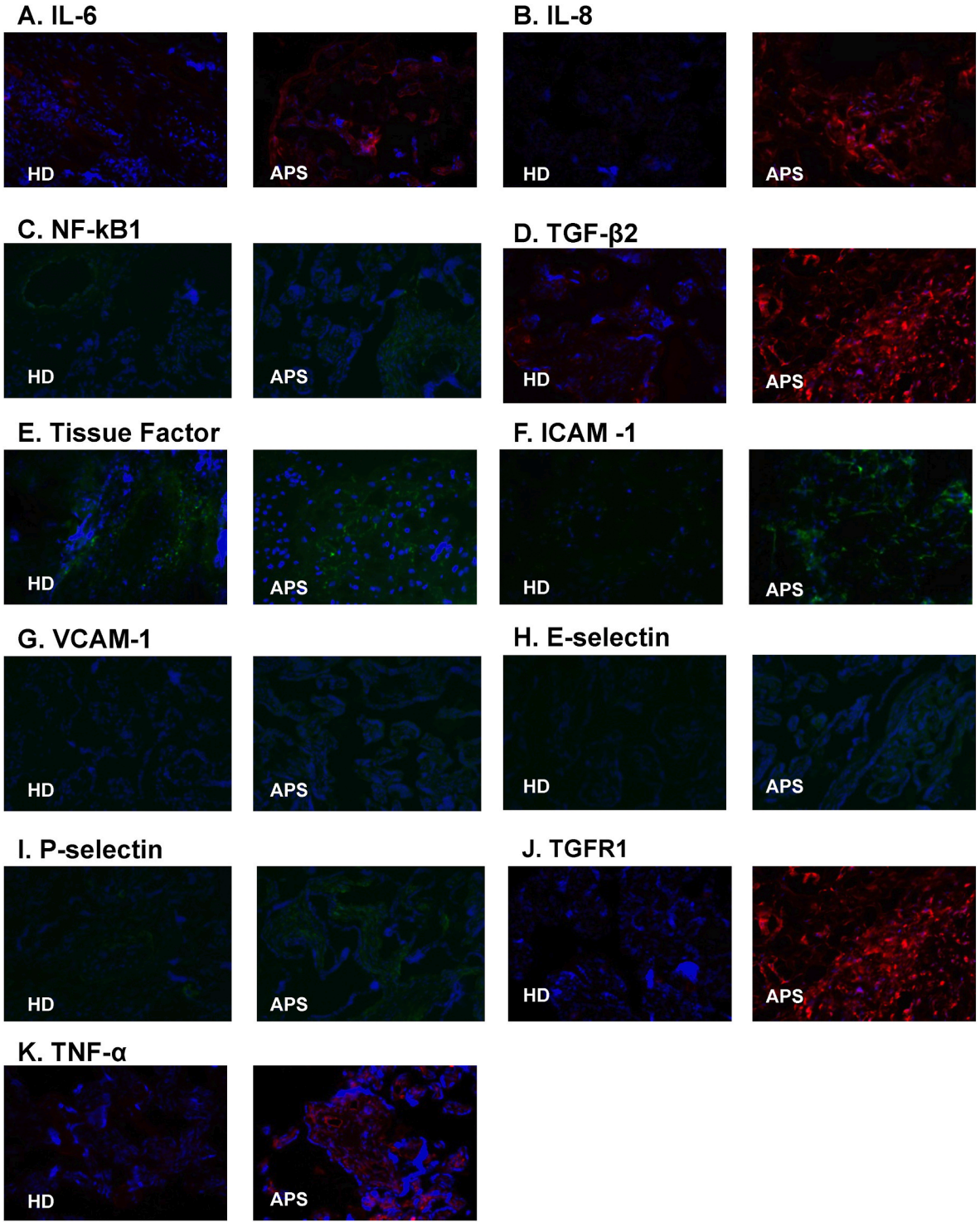


Fig. 5. Immunofluorescent antibody staining in placenta biopsies from APS patients and healthy women. A-K Placenta biopsies derived from APS patients as well as Healthy Donors show increased signal intensity for IL-6, IL-8, NF-κB1, ICAM1, VCAM-1, E-selectin, P-selectin, TGF-β2, and TGFR1 (5A-5D, 5F-5J). Slight difference in fluorescence intensity between HD and APS patient was observed for Tissue Factor (5E). Increased signal intensity was observed as well for the TNF-α molecule in the APS placenta biopsies (5 K).

describe gene expression changes that occur in human tissue in the context of APS, we performed immunostaining in placenta biopsies isolated both from APS patients and HDs. Placenta biopsies derived from APS patients show increased signal intensity for IL-6, IL-8 as well as NF-κB1, TGF-β2, and TGFR1 (Fig. 5A–D, 5J). Slight difference was observed in Tissue Factor staining intensity (Fig. 5E). The cell adhesion molecules ICAM-1, VCAM-1, E-selectin and P-selectin were found up-regulated in placentas derived from APS patients compared to those of healthy donors (Fig. 5F–I), while increased signal intensity was also observed for TNF-α in the APS placenta specimens (Fig. 5K).

4. Discussion

The present study describes the gene expression profile of endothelial cells treated with IgG isolated from APS patients plus β2GPI in a model mimicking APS. *Transcriptomics* analysis of treated HUVECs with APS sera plus β2GPI revealed 906 differentially expressed genes, among which 395 were up-regulated and 511 were down-regulated. A multitude of the up-regulated genes such as *IL-8, VCAM-1, E-selectin, IL-6, TGF-β2* and *TGFR1* are included, consistent with reported studies. Interestingly, a similar phenotype has been described in sepsis [42] yet in APS patients it seems to be rather localized and weak. Our data support that the endothelial phenotype following antibody stimulation is the result of the concerted action of several diverse signaling pathways and transcriptional networks presumably instructed by the synchronized action of a variety of up-regulated transcription factors. Our analysis highlights that the TNF, TGF-β, MAPK38 and Hippo pathways are critical components of the activation process. Our experimental strategy posits for a model according to which 'APS conditions' convert the naïve phenotype of endothelial cells from the ground state to an activated phenotype via the simultaneous activation of a spectrum of transcriptional regulators capable for the fine-tuning of a large number of genes contributing to APS pathology. Our results indicate that aberrant transcriptional reprogramming, which is presumably caused by increased levels of diverse transcription factors (Fig. 4A), coordinates the APS phenotype. These findings are further supported by the observed increased protein levels of relevant transcription factors, signaling molecules and APS marker-genes on both in vitro treated endothelial cells and placenta biopsies from APS patients.

Endothelial cell activation in APS is characterized by the surface expression of adhesion molecules, chemokines such as *IL-8, IL-6* and *Tissue Factor* [10,43–45]. Unveiling the pathophysiology of endothelial dysfunction in APS would contribute to providing treatments beyond anticoagulants [46–48] especially in severe clinical cases, or in cases complicated with hemorrhage and those with non-typical APS symptoms, such as multiple sclerosis-like lesions, migraine or the catastrophic form of the syndrome. Analyses of endothelial dysfunction in APS patients *in vivo* have not been reported. Significant data come from studies with *in vitro* models constituted with endothelial cells exposed to either patient serum and plasma, monoclonal aPL, or IgGs isolated from patients with APS [16,49,50]. The transcriptional profile of the activated endothelial cells in APS has been described in part [28] by an elegant Affymetrix microarray-based analysis, providing critical regulatory insights were emerged. Specifically, endothelial cells were induced for 4 h by patient-derived anti-β2GPI antibodies [28], several chemokines, receptors, and growth factors were captured to become up-regulated. In the present study, we complement the above data as well as findings derived from our previous studies [10] by applying a holistic approach based on *transcriptomics* technology coupled with *bioinformatics* analyses and computational biology tools. Our combined *bioinformatics* rationale utilizes analysis of gene expression, gene ontology (GO) classification, signal transduction pathway identification and intersection of the RNA-seq results with published microarray and ChIP-seq data [28–30].

Taking into account the above results, we propose that the HUVECs-APS-activated transcriptome become progressively constructed upon APS total IgG and β2GPI induction of endothelial cells, a phenomenon which indicates coordinated and fine-tuned action of inducible genes that are regulated by distinct signal transduction pathways. Combinatorial mechanisms have been described to regulate transcription of inducible genes [51]. This type of regulation is in alignment with the characterized generation of a pathogenic phenotype in endothelial cells, since as shown by our results, hundreds of genes alter their expression status rapidly, a fact that poises in part for a non-ordinal cellular response. Several signal transduction cascades are switched on upon treatment of HUVECs and subsequently lead to the activation of TFs and the induction of their target genes. Thus, activation of endothelial cells induced by APS total IgG and β2GPI promotes transcriptional reprogramming or/and deregulation of the naïve tissue-specific gene expression programs a fact that in turn can be translated into the evolvement of an aggressive pathogenic autoimmune phenotype.

In our present analyses, even though consistent data were derived on the expression of some factors, such as IL-8, NF-κB, TGFBR1, VCAM-1, and E-selectin, the proinflammatory cytokine, transcription factor and cytokine receptor profile detected by immunihistochemistry in APS patients' placenta samples did not fully recapitulate the up-regulated genes identified through *transcriptomics*. Several differences between the two systems could account for this observation. For instance, the *transcriptomics* model is only dependent on the patients' IgG and exogenous β2GPI, while the APS placenta is a much more complex system and some molecules may be induced from other signals existing in the tissue. In addition, post-translational modifications after gene expression may alter protein expression.

Our findings are also of clinical importance especially in the following circumstances:

a) in patients with recurrent thrombotic events despite adequate anticoagulation [5]; b) in patients with APS-related clinical findings non-related directly to thrombosis, such as premature atherosclerosis [52] and multiple sclerosis-like findings and or symptoms [53] and
c) thrombosis combined with hemorrhage [54]. In all these circumstances anti-coagulation might be a dangerous [54], or inadequate therapy [5,52,53] and abrogation of the pathways discovered in this report might be important to halt the disease process.

In conclusion, IgG from APS patients in the presence of β2GPI stimulates HUVECs of HDs to a procoagulant and pro-inflammatory phenotype by activating TNF, TGF-β, MAPK38 and Hippo pathways, which influence the expression of hundreds of genes. A detailed analysis of the above pathways will probably identify novel therapeutic targets for this disease. Major questions on that issue are the following: what is the molecular mechanism by which IgG from APS patients leads to differential expression of several cytokines and transcription factors and secondly how could the abrogation of these pathways contribute to control of the disease process? Anti-β2GPI antibodies can activate receptors such as TLR4, TLR2 and ApoER2 expressed on endothelial cells [55], leading to the activation of several transcription factors, such as NFκB, phosphatidylinositol 3-kinase (PI3K) signaling pathway and p38 MAPK. Furthermore, monocytes and neutrophils from APS patients display increased reactive oxygen species production, increased expression of pro-inflammatory and prothrombotic molecules and mitochondrial dysfunction associated with activation of PI3K pathway and mammalian target of rapamycin (mTOR). Interestingly, inhibition of the mTOR pathway led to improved survival of renal allografts in patients with antiphospholipid syndrome [56,57]. Taken together, the above mechanistic insights could provide for potential pathways and molecules that could serve as drug targets to investigate as a means to control difficult cases of APS.

Author authorship and conflict-of-interest statements

M. P. wrote the first draft of the paper, designed a part of the study and performed the experiments concerning HUVEC isolation and

stimulation by IgG from APS patients, as well as immunocytochemistry in HUVECs and declares no competing financial interests, E.A. performed *transcriptomics* experiment and analysis of data and wrote the first draft of the paper and declares no competing financial interests, S.F. performed *transcriptomics* experiment and analysis of data and wrote the first draft of the paper and declares no competing financial interests, E.T performed immunohistochemistry experiments on HUVECS and placenta sections and image processing and statistical analysis writing also the respective parts of the manuscript and declares no competing financial interests, P.K. supervised some experiments and wrote the first draft of the paper and declares no competing financial interests, M.A. designed the *transcriptomics* study, supervised the experiments, analyzed the data and wrote the manuscript and declares no competing financial interests. P.G.V. designed the whole study, supervised the experiments wrote the paper, corrected the first draft and analyzed the data and declares no competing financial interests.

Credit author statement

Markos Patsouras: Methodology, Validation, Investigation, Data Curation, Writing - Review & Editing. **Eirini Alexopoulou:** Methodology, Validation, Formal analysis, Investigation, Data Curation, Writing - Review & Editing. **Spyros Foutadakis:** Software, Validation, Formal analysis, Investigation, Data Curation, Writing - Review & Editing. **Eirini Tsiki:** Validation, Formal analysis, Investigation, Writing - Review & Editing. **Panagiota Karagianni:** Validation, Formal analysis, Investigation, Writing - Review & Editing. **Marios Agelopoulos:** Methodology, Validation, Investigation, Resources, Data Curation, Writing - Review & Editing, Supervision, Project administration, Funding acquisition. **Panayiotis G. Vlachoyiannopoulos:** Conceptualization, Methodology, Validation, Investigation, Resources, Writing - Original Draft, Writing - Review & Editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Professor Panayiotis G Vlachoyiannopoulos reports financial support was provided by State Scholarships Foundation.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jtauto.2023.100202.

References

[1] H.P. McNeil, C.N. Chesterman, S.A. Krilis, Immunology and clinical importance of antiphospholipid antibodies, Adv. Immunol. 49 (1991) 193–280.
[2] E.M. Bevers, M. Galli, T. Barbui, P. Comfurius, R.F. Zwaal, Lupus anticoagulant IgG's (LA) are not directed to phospholipids only, but to a complex of lipid-bound human prothrombin, Thromb. Haemostasis 66 (6) (1991) 629–632.
[3] R. Cervera, J.C. Piette, J. Font, et al., Antiphospholipid syndrome: clinical and immunologic manifestations and patterns of disease expression in a cohort of 1,000 patients, Arthritis Rheum. 46 (4) (2002) 1019–1027.
[4] A.A. Mehdi, I. Uthman, M. Khamashta, Antiphospholipid syndrome: pathogenesis and a window of treatment opportunities in the future, Eur. J. Clin. Invest. 40 (5) (2010) 451–464.
[5] S.S. Pierangeli, M.E. Vega-Ostertag, E.B. Gonzalez, New targeted therapies for treatment of thrombosis in antiphospholipid syndrome, Expet Rev. Mol. Med. 9 (30) (2007) 1–15.
[6] S. Miyakis, M.D. Lockshin, T. Atsumi, et al., International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (APS), J. Thromb. Haemostasis 4 (2) (2006) 295–306.
[7] K. Ma, R. Simantov, J.C. Zhang, R. Silverstein, K.A. Hajjar, K.R. McCrae, High affinity binding of beta 2-glycoprotein I to human endothelial cells is mediated by annexin II, J. Biol. Chem. 275 (20) (2000) 15541–15548.
[8] G. Kaplanski, P. Cacoub, C. Farnarier, et al., Increased soluble vascular cell adhesion molecule 1 concentrations in patients with primary or systemic lupus erythematosus-related antiphospholipid syndrome: correlations with the severity of thrombosis, Arthritis Rheum. 43 (1) (2000) 55–64.
[9] M. Vega-Ostertag, K. Casper, R. Swerlick, D. Ferrara, E.N. Harris, S.S. Pierangeli, Involvement of p38 MAPK in the up-regulation of tissue factor on endothelial cells by antiphospholipid antibodies, Arthritis Rheum. 52 (5) (2005) 1545–1554.
[10] M.D. Patsouras, P. Karagianni, P. Kogionou, P.G. Vlachoyiannopoulos, Differential CpG methylation of the promoter of interleukin 8 and the first intron of tissue factor in Antiphospholipid syndrome, J. Autoimmun. 102 (2019) 159–166.
[11] P. Soltesz, H. Der, K. Veres, et al., Immunological features of primary antiphospholipid syndrome in connection with endothelial dysfunction, Rheumatology 47 (11) (2008) 1628–1634.
[12] R.R. Forastiero, M.E. Martinuzzo, G.F. de Larranaga, Circulating levels of tissue factor and proinflammatory cytokines in patients with primary antiphospholipid syndrome or leprosy related antiphospholipid antibodies, Lupus 14 (2) (2005) 129–136.
[13] S. Dunoyer-Geindre, P. de Moerloose, B. Galve-de Rochemonteix, G. Reber, E. K. Kruithof, NFkappaB is an essential intermediate in the activation of endothelial cells by anti-beta(2)-glycoprotein 1 antibodies, Thromb. Haemostasis 88 (5) (2002) 851–857.
[14] J.G. Hanly, C. Hong, A. Issekutz, Beta 2-glycoprotein I and anticardiolipin antibody binding to resting and activated cultured human endothelial cells, J. Rheumatol. 23 (9) (1996) 1543–1549.
[15] J. Zhang, K.R. McCrae, Annexin A2 mediates endothelial cell activation by antiphospholipid/anti-beta2 glycoprotein I antibodies, Blood 105 (5) (2005) 1964–1969.
[16] A. Sacharidou, K.L. Chambliss, V. Ulrich, et al., Antiphospholipid antibodies induce thrombosis by PP2A activation via apoER2-Dab2-SHC1 complex formation in endothelium, Blood 131 (19) (2018) 2097–2110.
[17] V.M. Holers, G. Girardi, L. Mo, et al., Complement C3 activation is required for antiphospholipid antibody-induced fetal loss, J. Exp. Med. 195 (2) (2002) 211–220.
[18] M.Y. Kim, M.M. Guerra, E. Kaplowitz, et al., Complement activation predicts adverse pregnancy outcome in patients with systemic lupus erythematosus and/or antiphospholipid antibodies, Ann. Rheum. Dis. 77 (4) (2018) 549–555.
[19] D. Erkan, J.E. Salmon, The role of complement inhibition in thrombotic angiopathies and antiphospholipid syndrome, Turk. J. Haematol. 33 (1) (2016) 1–7.
[20] M. Sorice, A. Longo, A. Capozzi, et al., Anti-beta2-glycoprotein I antibodies induce monocyte release of tumor necrosis factor alpha and tissue factor by signal transduction pathways involving lipid rafts, Arthritis Rheum. 56 (8) (2007) 2687–2697.
[21] N. Satta, E.K. Kruithof, C. Fickentscher, et al., Toll-like receptor 2 mediates the activation of human monocytes and endothelial cells by antiphospholipid antibodies, Blood 117 (20) (2011) 5523–5531.
[22] K.L. Allen, F.V. Fonseca, V. Betapudi, B. Willard, J. Zhang, K.R. McCrae, A novel pathway for human endothelial cell activation by antiphospholipid/anti-beta2 glycoprotein I antibodies, Blood 119 (3) (2012) 884–893.
[23] A.M. Curtis, P.F. Wilkinson, M. Gui, T.L. Gales, E. Hu, J.M. Edelberg, p38 mitogen-activated protein kinase targets the production of proinflammatory endothelial microparticles, J. Thromb. Haemostasis 7 (4) (2009) 701–709.
[24] E. Raschi, C.B. Chighizola, C. Grossi, et al., beta2-glycoprotein I, lipopolysaccharide and endothelial TLR4: three players in the two hit theory for anti- phospholipid-mediated thrombosis, J. Autoimmun. 55 (2014) 42–50.
[25] M.D. Patsouras, M.P. Sikara, E.P. Grika, H.M. Moutsopoulos, A.G. Tzioufas, P. G. Vlachoyiannopoulos, Elevated expression of platelet-derived chemokines in patients with antiphospholipid syndrome, J. Autoimmun. 65 (2015) 30–37.
[26] M.P. Sikara, J.G. Routsias, M. Samiotaki, G. Panayotou, H.M. Moutsopoulos, P. G. Vlachoyiannopoulos, {beta}2 Glycoprotein I ({beta}2GPI) binds platelet factor 4 (PF4): implications for the pathogenesis of antiphospholipid syndrome, Blood 115 (3) (2010) 713–723.
[27] P.G. Vlachoyiannopoulos, J.G. Routsias, A novel mechanism of thrombosis in antiphospholipid antibody syndrome, J. Autoimmun. 35 (3) (2010) 248–255.
[28] C. Hamid, K. Norgate, D.P. D'Cruz, et al., Anti-beta2GPI-antibody-induced endothelial cell gene expression profiling reveals induction of novel pro-inflammatory genes potentially involved in primary antiphospholipid syndrome, Ann. Rheum. Dis. 66 (8) (2007) 1000–1007.
[29] F. Jin, Y. Li, J.R. Dixon, et al., A high-resolution map of the three-dimensional chromatin interactome in human cells, Nature 503 (7475) (2013) 290–294.
[30] M. Morikawa, D. Koinuma, S. Tsutsumi, et al., ChIP-seq reveals cell type-specific binding patterns of BMP-specific Smads and a novel binding motif, Nucleic Acids Res. 39 (20) (2011) 8712–8727.

[31] B. Baudin, A. Bruneel, N. Bosselut, M. Vaubourdolle, A protocol for isolation and culture of human umbilical vein endothelial cells, Nat. Protoc. 2 (3) (2007) 481–485.
[32] Andrews S. FastQC, A Quality Control Tool for High Throughput Sequence Data, 2010.
[33] E. Afgan, D. Baker, B. Batut, et al., The Galaxy platform for accessible, reproducible and collaborative biomedical analyses: 2018 update, Nucleic Acids Res. 46 (W1) (2018) W537–W544.
[34] C. Trapnell, L. Pachter, S.L. Salzberg, TopHat: discovering splice junctions with RNA-Seq, Bioinformatics 25 (9) (2009) 1105–1111.
[35] S. Anders, P.T. Pyl, W. Huber, HTSeq–a Python framework to work with high-throughput sequencing data, Bioinformatics 31 (2) (2015) 166–169.
[36] M.I. Love, W. Huber, S. Anders, Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2, Genome Biol. 15 (12) (2014) 550.
[37] F. Ramirez, F. Dundar, S. Diehl, B.A. Gruning, T. Manke, deepTools: a flexible platform for exploring deep-sequencing data, Nucleic Acids Res. 42 (2014) W187–W191. Web Server issue).
[38] Morpheus Software: https://software.broadinstitute.org/morpheus/.
[39] M.V. Kuleshov, M.R. Jones, A.D. Rouillard, et al., Enrichr: a comprehensive gene set enrichment analysis web server 2016 update, Nucleic Acids Res. 44 (W1) (2016) W90–W97.
[40] R. Zheng, C. Wan, S. Mei, et al., Cistrome Data Browser: expanded datasets and new tools for gene regulatory analysis, Nucleic Acids Res. 47 (D1) (2019) D729–D735.
[41] C.Y. McLean, D. Bristor, M. Hiller, et al., GREAT improves functional interpretation of cis-regulatory regions, Nat. Biotechnol. 28 (5) (2010) 495–501.
[42] C. Ince, P.R. Mayeux, T. Nguyen, et al., The endothelium in sepsis, Shock 45 (3) (2016) 259–270.
[43] S. Siddique, J. Risse, G. Canaud, S. Zuily, Vascular manifestations in antiphospholipid syndrome (APS): is APS a thrombophilia or a vasculopathy? Curr. Rheumatol. Rep. 19 (10) (2017) 64.
[44] B. Engel, G. Muller, B. Roch, et al., Serum of patients with antiphospholipid syndrome induces adhesion molecules in endothelial cells, Atherosclerosis Suppl. 30 (2017) 141–148.
[45] M. Velasquez, M. Rojas, V.M. Abrahams, C. Escudero, A.P. Cadavid, Mechanisms of endothelial dysfunction in antiphospholipid syndrome: association with clinical manifestations, Front. Physiol. 9 (2018) 1840.
[46] G. Espinosa, H. Berman, R. Cervera, Management of refractory cases of catastrophic antiphospholipid syndrome, Autoimmun. Rev. 10 (11) (2011) 664–668.
[47] T. Scoble, S. Wijetilleka, M.A. Khamashta, Management of refractory anti-phospholipid syndrome, Autoimmun. Rev. 10 (11) (2011) 669–673.
[48] A. Mekinian, N. Costedoat-Chalumeau, A. Masseau, et al., Obstetrical APS: is there a place for hydroxychloroquine to improve the pregnancy outcome? Autoimmun. Rev. 14 (1) (2015) 23–29.
[49] C. Pericleous, L.A. Clarke, P.A. Brogan, et al., Endothelial microparticle release is stimulated in vitro by purified IgG from patients with the antiphospholipid syndrome, Thromb. Haemostasis 109 (1) (2013) 72–78.
[50] B.M. Velásquez, G.Á.M. Álvarez, J.Á.P. Cadavid, Cuantificación sistematizada de la remodelación vascular in vitro en la morbilidad gestacional asociada al síndrome antifosfolípido, Rev. Chil. Obstet. Ginecolog. 81 (2016) 455–464.
[51] M. Agelopoulos, D. Thanos, Epigenetic determination of a cell-specific gene expression program by ATF-2 and the histone variant macroH2A, EMBO J. 25 (20) (2006) 4843–4853.
[52] P.G. Vlachoyiannopoulos, P.G. Kanellopoulos, J.P. Ioannidis, M.G. Tektonidou, I. Mastorakou, H.M. Moutsopoulos, Atherosclerosis in premenopausal women with antiphospholipid syndrome and systemic lupus erythematosus: a controlled study, Rheumatology (Oxford) 42 (5) (2003 May) 645–651, https://doi.org/10.1093/rheumatology/keg182.
[53] C. D'Angelo, O. Franch, L. Fernández-Paredes, C. Oreja-Guevara, M. Núñez-Beltrán, A. Comins-Boo, M. Reale, S. Sánchez-Ramón, Antiphospholipid antibodies overlapping in isolated neurological syndrome and multiple sclerosis: neurobiological insights and diagnostic challenges, Front. Cell. Neurosci. 1 3 (2019 Mar 19) 107, https://doi.org/10.3389/fncel.2019.00107, eCollection 2019.
[54] R. Cartin-Ceba, T. Peikert, A. Ashrani, K. Keock, M.E. Wylam, S. Yttenberg, U. Specks, Primary antiphospholipid syndrome-associated diffuse alveolar hemorrhage, Arthritis Care Res. 66 (2) (2014) 301–310.
[55] C.M. Rodríguez, M. Velásquez-Berrío, C. Rúa, M. Viana, V.M. Abrahams, A. P. Cadavid, A.M. Alvarez, Antiphospholipid antibodies from women with pregnancy morbidity and vascular thrombosis induce endothelial mitochondrial dysfunction, mTOR activation, and autophagy, Front. Physiol. 12 (2021), 706743, https://doi.org/10.3389/fphys.2021.706743.
[56] Guillaume Canaud, Bienaimé Frank, Fanny Tabarin, Guillaume Bataillon, Danielle Seilhean, Laure-Hélène Noël, Marie-Agnès Dragon-Durey, Snanoudj Renaud, Gérard Friedlander, Lise Halbwachs-Mecarelli, Christophe legendre, and fabiola terzi, N. Engl. J. Med. 371 (2014) 303–312, https://doi.org/10.1056/NEJMoa1312890.
[57] A. Martirosyan, M. Petrek, Z. Navratilova, A. Blbulyan, A. Boyajyan, G. Manukyan, Differential regulation of proinflammatory mediators following LPS- and ATP-induced activation of monocytes from patients with antiphospholipid syndrome, BioMed Res. Int. 2015 (2015), 292851, https://doi.org/10.1155/2015/292851.