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Disentangling the riddle of systemic lupus erythematosus with antiphospholipid syndrome: blood transcriptome analysis reveals a less-pronounced IFN-signature and distinct molecular profiles in venous versus arterial events

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ABSTRACT

Introduction Systemic lupus erythematosus with antiphospholipid syndrome (SLE-APS) represents a challenging SLE endotype whose molecular basis remains unknown.

Methods We analysed whole-blood RNA-sequencing data from 299 patients with SLE (108 SLE-antiphospholipid antibodies (aPL)-positive, including 67 SLE-APS; 191 SLE-aPL-negative) and 72 matched healthy controls (HC). Pathway enrichment analysis, unsupervised weighted gene coexpression network analysis and machine learning were applied to distinguish disease endotypes.

Results Patients with SLE-APS demonstrated upregulated type I and II interferon (IFN) pathways compared with HC. Using a 100-gene random forests model, we achieved a cross-validated accuracy of 75.6% in distinguishing these two states. Additionally, the comparison between SLE-APS and SLE-aPL-negative revealed 227 differentially expressed genes, indicating downregulation of IFN-α and IFN-γ signatures, coupled with dysregulation of the complement cascade, B-cell activation and neutrophil degranulation. Unsupervised analysis of SLE transcriptome identified 21 gene modules, with SLE-APS strongly linked to upregulation of the 'neutrophilic/myeloid' module. Within SLE-APS, venous thromboses positively correlated with 'neutrophilic/myeloid' and 'B cell' modules, while arterial thromboses were associated with dysregulation of 'DNA damage response (DDR)' and 'metabolism' modules. Anticardiolipin and anti-β2GPI positivity—irrespective of APS status—were associated with the 'neutrophilic/myeloid' and 'protein-binding' module, respectively.

Conclusions There is a hierarchical upregulation and—likely—dependence on IFN in SLE with the highest IFN signature observed in SLE-aPL-negative patients. Venous thrombotic events are associated with neutrophils and B cells while arterial events with DDR and impaired metabolism. This may account for their differential requirements for anticoagulation and provide rationale for the potential use of mTOR inhibitors such as sirolimus and the direct fIIa inhibitor dabigatran in SLE-APS.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Gene expression studies in systemic lupus erythematosus with antiphospholipid syndrome (SLE-APS) have suggested a plasmacytoid dendritic and neutrophil signature but these studies have been limited by small sample size.
⇒ Direct oral anticoagulants, particularly factor Xa inhibitor (Xai) rivaroxaban, may not be protective against arterial thrombosis in APS. Direct factor IIa inhibitor (fIIai) dabigatran—but not fXai—can completely repair the double-stranded DNA breaks and a post-hoc analysis of three randomised clinical trials (RCTs) has demonstrated equivalence between warfarin and dabigatran in APS.

WHAT THIS STUDY ADDS

⇒ APS in SLE is characterised by enhanced type I and II interferon (IFN) signatures which however are less prominent compared with their antiphospholipid antibodies-negative counterparts.
⇒ Venous thrombotic events in SLE-APS are associated by an enhanced neutrophilic and B cell response.
⇒ DNA damage response aberrancies and altered metabolic pathways are linked to arterial thromboses in SLE-APS.

INTRODUCTION

Approximately 30% of patients with systemic lupus erythematosus (SLE) harbour antiphospholipid antibodies (aPL) such as anticardiolipin (anti-CL), anti-beta2 glycoprotein I (anti-β2GPI) and lupus

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ Patients with SLE-APS might be less dependent to type I IFN but the clinical implications of this finding need further study.

⇒ Inhibitors of fIIai such as dabigatran and mTOR inhibitors such as sirolimus—that reduce DNA damage and metabolic aberrancies—have been used and tested for the treatment of arterial events in SLE-APS and could be further explored.

anticoagulant (LA).[1] Among these, one-third will eventually develop thrombotic events or maternal morbidity, collectively termed SLE-antiphospholipid syndrome (SLE-APS).[2] In addition to their association with severe disease manifestations, aPL contribute to the progressive accrual of organ damage and morbidity.[3]

SLE-APS represents one of the most challenging lupus endotypes, with both inflammatory and thrombotic mechanisms implicated.[4] Experimental studies suggest an aPL-induced activation of monocytes, platelets and endothelial cells leading to release of proinflammatory molecules.[5] Current management involves prevention of thrombotic events, with the use of low-dose aspirin for individuals carrying aPL and vitamin-K antagonists (VKA) for the treatment of patients with thrombotic APS.[6] However, certain primary APS phenotypes, like refractory or catastrophic APS, are treated with anti-inflammatory therapy (ie, anticomplement therapy), suggesting an aPL-mediated inflammatory effect. Thus, delineating the molecular underpinnings of SLE-APS is essential for novel therapeutic interventions.

Genome-wide expression analyses offer an unbiased and comprehensive approach in the study of complex diseases like SLE or APS, providing valuable insights into molecular characteristics and aberrancies.[7] To date, RNA-sequencing studies have been conducted in patients with APS focusing on plasmacytoid dendritic cells (pDCs) and neutrophils and revealed P-selectin glycoprotein ligand-1 as key adhesion molecule overexpressed in APS neutrophils, but these studies have been limited by their small sample size.[8 9] In a recent whole blood RNA-seq analysis in 62 patients with thrombotic primary APS, interferon (IFN)-regulated genes emerged as key drivers.[10]

Herein, we report a comprehensive RNA-sequencing analysis to profile the blood transcriptome of a large cohort of patients with aPL-positive SLE, with or without APS. We compared their transcriptomic profiles to those of patients with aPL-negative SLE and healthy individuals and analysed gene expression patterns associated with SLE-APS. Our data suggest distinct transcriptomic signatures of patients with SLE-APS between venous and arterial thrombosis. These data may have implications for the pathogenesis and management of SLE-APS.

MATERIALS AND METHODS

Patients and healthy individuals

Patients were recruited from the Departments of Rheumatology at 'Attikon' University Hospital, Athens,[11] and University Hospital of Crete.[12] We included 299 patients with SLE according to the American College of Rheumatology 1997 criteria and/or the Systemic Lupus Erythematosus International Collaborating Clinics 2012 criteria, and 72 age and sex-matched healthy controls (HC). Among patients with SLE, 108 were aPL-positive (SLE-aPL-positive), of whom 67 were diagnosed with thrombotic APS (SLE-APS), according to Sydney classification criteria and 41 were aPL carriers without clinical APS; the remaining 191 patients with SLE were negative for all aPL (SLE-aPL-negative).

Table 1 Demographic and clinical characteristics of patients with SLE and healthy individuals included in the study

	Patients with SLE (n=299)	Patients with SLE with APS (n=67)	Patients with SLE without APS (n=232)	Healthy individuals (n=72)
Gender				
Female	261 (87.2%)	56 (83.6%)	205 (88.4%)	67 (93.1%)
Male	38 (13.8%)	11 (16.4%)	27 (11.6%)	5 (6.9%)
Race				
Caucasian	297 (99.3%)	67 (100%)	230 (99.1%)	72 (100%)
Other	2 (0.7%)	0 (2.4%)	2 (0.9%)	0 (0%)
Age (years)				
Mean±SD	47±13.2	49.2±13.1	46.4±13.3	43.4±12
Disease manifestations				
Venous thrombosis	57 (18.7%)	45 (68.2%)	12 (5.2%)	
Pulmonary embolism	8 (2.7%)	7 (10.4%)	1 (0.4%)	
Arterial thrombosis	58 (19.4%)	22 (32.8%)	36 (15.5%)	
Pregnancy complications	–	10 (14.9%)	–	
Antibodies				
aPL negative	191 (63.9%%)	0 (0%)	191 (82%)	
aPL positive	108 (36.1%)	67 (100%)	41 (17.6%)	
High aPL profile	62 (57.4%)	51 (76.1%)	11 (26.8%)	
Low aPL profile	46 (42.6%)	16 (23.9%)	30 (73.2%)	
Anti-CL	90 (30.1%)	55 (82.1%)	35 (15%)	
Anti-β2GPI	56 (18.2%)	39 (58.2%)	17 (7.3%)	
LA	39 (13%)	35 (52.2%)	4 (1.7%)	

anti-β2GPI, anti-beta2 glycoprotein I; aPL, antiphospholipid antibodies; APS, antiphospholipid syndrome; CL, cardiolipin; LA, lupus anticoagulant; SLE, systemic lupus erythematosus.

For subgroup analysis, aPL profile was categorised as high risk (aPL^{high}) and low risk (aPL^{low}) according to the European Alliance of Associations for Rheumatology (EULAR) recommendations. A high-risk aPL profile was defined as the presence of (1) LA, or (2) a double positivity of LA, anti-CL or anti-β2GPI, or (3) triple aPL positivity, or (4) persistently high aPL titres, while a low-risk profile was defined as isolated anti-CL or anti-β2GPI positivity at low-medium titres.[13] Anti-CL (both IgG and IgM) and anti-β2GPI (both IgG and IgM) were tested/quantified with ELISA. Patients with SLE with SLEDAI-2K >8 were not included in the study to minimise transcriptome signatures driven by SLE disease activity. Demographic, clinical and serological characteristics were recorded for each patient at the time of sampling and are summarised in table 1, online supplemental figure 1 and online supplemental table 1. Patient subgroups did not differ with respect to disease activity, disease manifestations at the sampling, medications and autoantibodies profiles.

Written informed consent was obtained from all participants, after which whole blood samples were collected, and RNA was extracted for subsequent analysis.[14]

RNA-sequencing

Total whole-blood RNA was extracted using Paxgene Blood miRNA kit (PreAnalytiX) and libraries were prepared using the Illumina stranded Truseq mRNA protocol. Paired-end 67 bp or 100bp mRNA sequencing was performed on Illumina HiSeq2000 or HiSeq4000 at the Department of Genetic Medicine and Development, University of Geneva Medical School. Library preparation and sequencing were performed as previously described.[7 14] Quality of sequencing was assessed using FastQC software.[15] Samtools[16] was used to sort bam files, and HTSeq[17] was used to extract gene expression counts.

Differential expression analysis

Raw counts were normalised and analysed with edgeR package[18] (V.3.40.2, RRID: SCR_012802) in R (V.4.2.0, RRID: SCR_001905), to identify differentially expressed genes (DEGs) between (1) patients with SLE-APS and HC, (2) SLE-aPL-positive patients and HC, (3) SLE-APS and SLE-aPL-negative patients, and (4) SLE-aPL-positive and SLE-aPL-negative patients. DEGs were defined as genes with $|FC|>1.5$ and p value <0.05. Gene ontology (GO) analysis was performed using gProfiler,[19] as previously described.[14]

Gene set enrichment analysis

Preranked gene set enrichment analysis (GSEA) was performed against the gene sets of MsigDB database[20] for the following terms: (1) GO: (a) biological process (GO:BP;c5.go.bp.v2023.1.Hs.symbols.gmt), (b) hallmark (h.all.v2023.1.Hs.symbols.m), (c) canonical pathways: KEGG (c2.cp.kegg.v2023.1.Hs.symbols.gmt), and (d) canonical pathways: reactome (c2.cp.reactome.v2023.1.Hs.symbols.gmt). GSEA was performed using the software (V.4.3.2) applying $\log_2$FC and FDR values from each DE analysis.[21] Genes were ranked based on the product of $-\log_{10}$(p value) multiplied by $\log_2$FC in descending order, as previously described.[22]

Deconvolution of whole-blood transcriptomic data

CIBERSORTx[23] deconvolution tool was used, through its web portal, to estimate the abundance of immune cell subsets in our whole-blood samples. Transcript-per-million expression values and the LM22 signature matrix were used as input to quantify 22 infiltrating immune cell types, namely 3 macrophage subsets (M0, M1 and M2), natural killer (NK) cells (activated and resting NK cells), T cells (memory resting CD4+, CD8+, naïve CD4+, regulatory T cells, memory activated CD4+, gamma delta T cells and T follicular helper cells), B cells (memory and naïve B cells), mast cells (activated and resting mast cells), monocytes, dendritic cells (resting and activated dendritic cells), neutrophils, plasma cells and eosinophils. Differences in cell type proportions among sample groups were tested by the Wilcoxon rank sum test.

Weighted gene coexpression network analysis

Weighted gene coexpression network analysis (WGCNA) R package[24] (V.1.72-1, RRID: SCR_003302) was used to perform unsupervised cluster analysis to identify molecular groups (modules) of coexpressed genes, using the gene expression data of the 299 patients with SLE. Genes with a central role (Hub genes) in each of the significant modules were determined using the connectivity measure of module membership (>0.8), while significant modules (p <0.05) were tested for functional enrichment using g:Profiler,[19] in order to associate modules and genes with specific biological processes or functions. Identified modules were correlated with patients' clinical manifestations and autoantibody status.

Protein–protein interaction networks

Networks of interactions between genes associated with SLE-APS, arterial thrombosis and venous thrombosis were created through StringDB[25] using the STRINGdb R package.[26] Genes included in the significantly correlated modules (APS—*salmon*, arterial thrombosis—*turquoise and green*, venous thrombosis—*salmon and lightyellow*) were used as input. Version 12.0 of the database was selected and query was limited to the physical subnetwork of interactions identified in *Homo sapiens* having a score threshold of 500. Networks were exported in tsv format and visualised in R using packages ggraph[27] and igraph.[28]

Machine learning

The RNA-sequencing dataset was randomly split into training (70%) and validation (test) (30%) sets. The training set was used to develop a prediction model and the test to validate the results using *caret*[29] package in R. Using the training set, differential expression using Deseq and recursive feature elimination (RFE) (random forest model) using 5 repeats of 10-fold cross-validation (CV) were used as a feature selection step (random forest model) to remove noise and keep the smallest set of genes which best predicted coexistence of APS based on accuracy. Next, six different prediction models (rf, glm, glmnet, svmRadial, svmPoly, nnet from caret package) were fit to identify which performs best using the genes based on feature selection step and the best model was selected based on accuracy, sensitivity and specificity. The final model was validated in the validation set. Receiver operating characteristic curves (ROC) were generated using the pROC package[30] in R and PCA plots using FactoMineR.[31] The importance of each feature in the final predictive models was calculated using varImp function from caret package.[32]

RESULTS

Patients with SLE with APS demonstrate extensive aberrancies in blood transcriptome

We first asked what distinguishes the SLE-APS transcriptome both from the SLE-aPL-negative transcriptome and the healthy transcriptome. Unsupervised principal component analysis of SLE-aPL-negative patients, patients with SLE-APS and HC is illustrated in figure 1A. A total of 995 upregulated and 343 downregulated DEGs were found in the SLE-APS group compared with HC (figure 1B). A further comparison between SLE-aPL-positive and HC demonstrated 1346 DEGs (987 upregulated and 359 downregulated; figure 1C), which significantly overlapped (86.9%) with the DEGs identified in the SLE-APS group (online supplemental figure 2A). GO analysis between SLE-APS and HC revealed dysregulation of multiple pathways, including collagen degranulation, apoptotic signalling, angiogenesis and cell surface interaction at the vascular wall (figure 1D). GSEA identified the most enriched upregulated pathways as IFN-α, IFN-γ, complement, coagulation, oxidative stress and neutrophil degranulation (figure 1E). Some of the key dysregulated genes associated with each pathway are highlighted in figure 1F. Similar dysregulated pathways were also observed in SLE-aPL-positive patients compared with HC (online supplemental figures 2B–D). Taken together, these data suggest that similar to the SLE transcriptome, the SLE-APS transcriptome demonstrates extensive aberrancies with quantitative and qualitative differences characterised mainly by neutrophilic, apoptotic, complement, coagulation and type-I/II IFN signatures.

Machine learning discriminates patients with SLE with APS from HC and uncovers genes implicated in disease pathogenesis

We next examined whether an ML-based approach could also discriminate patients with SLE-APS from HC based on specific gene expression patterns. To this end, the complete mRNA-sequencing dataset was randomly split into training and validation sets, followed by feature selection using differential expression analysis (p <0.05) followed RFE with a random forest model under a fivefold CV. Based on model accuracy, a set of 100 genes was selected and tested to determine which

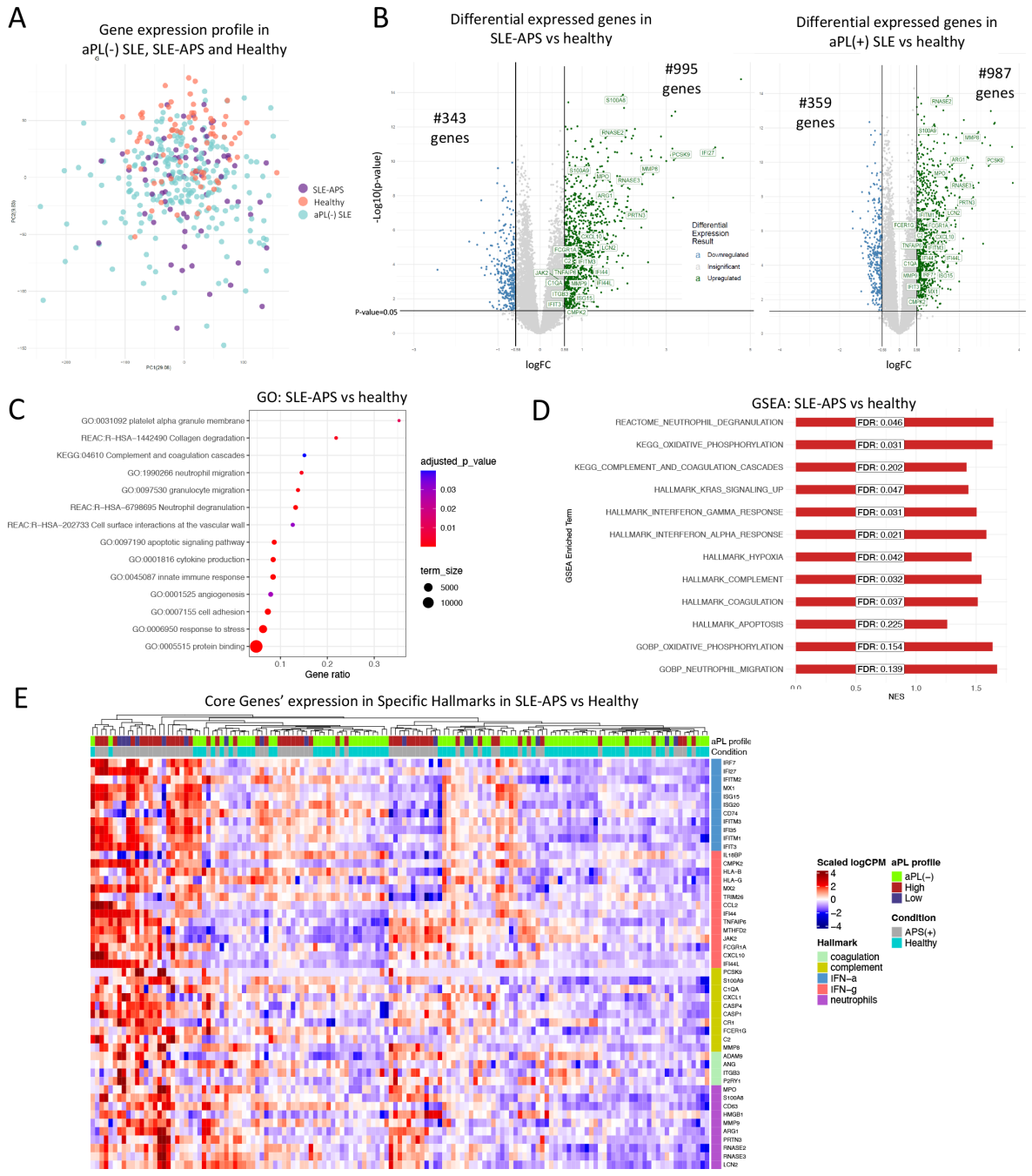

Figure 1 Transcriptomic signature in patients with SLE with APS versus healthy individuals demonstrates extensive aberrancies in blood transcriptome. A total of 1338 dysregulated DEGs in SLE-APS compared with HC revealed upregulation of pathways related to interferon (IFN)-α, INF-γ, complement, oxidative stress and neutrophil degranulation. (A) PCA of blood gene expression profiles from patients with SLE with APS (SLE-APS; n=67), SLE-aPL-negative patients (n=191) and HC (n=72). (B) Volcano plot highlighting the DEGs in patients with SLE-APS versus HC (left) and SLE-aPL-positive patients versus HC (right). Upregulated DEGs are coloured green, and downregulated DEGs are coloured blue. Genes not reaching our significance thresholds (|log2FC| >0.58 and p value <0.05) are shown in grey. (C) Dot plot showing the results of GO analysis representing biological pathways that are deregulated in patients with SLE-APS versus HC. The size of the dots represents the number of genes included in each enriched term and the colour represents the adjusted p value. (D) Bar plot showing the results of GSEA analysis representing biological pathways associated with the Hallmark V.7.5 database. The figure shows the positively enriched pathways (false discovery rate (FDR) <0.25) in patients with SLE-APS versus HC. (E) Heatmap showing the expression profile of the 49 genes belonging to specific Hallmarks (IFN-a, IFN-g, neutrophils, complement and coagulation) found as DEGs between SLE-APS and HC. Expression values were z-score normalised. Top annotation row shows the condition of each sample, coloured grey for patients with SLE-APS, turquoise for HC and lightgreen for aPL-negative patients, red for aPL-positive patients with aPL^{high} profile and blue for aPL-positive patients with aPL^{low} profile. aPL, antiphospholipid antibodies; APS, antiphospholipid syndrome; DEGs, differentially expressed genes; FC, fold change; GO, gene ontology; GSEA, gene set enrichment analysis. HC, healthy controls; PCA, principal component analysis; SLE, systemic lupus erythematosus.

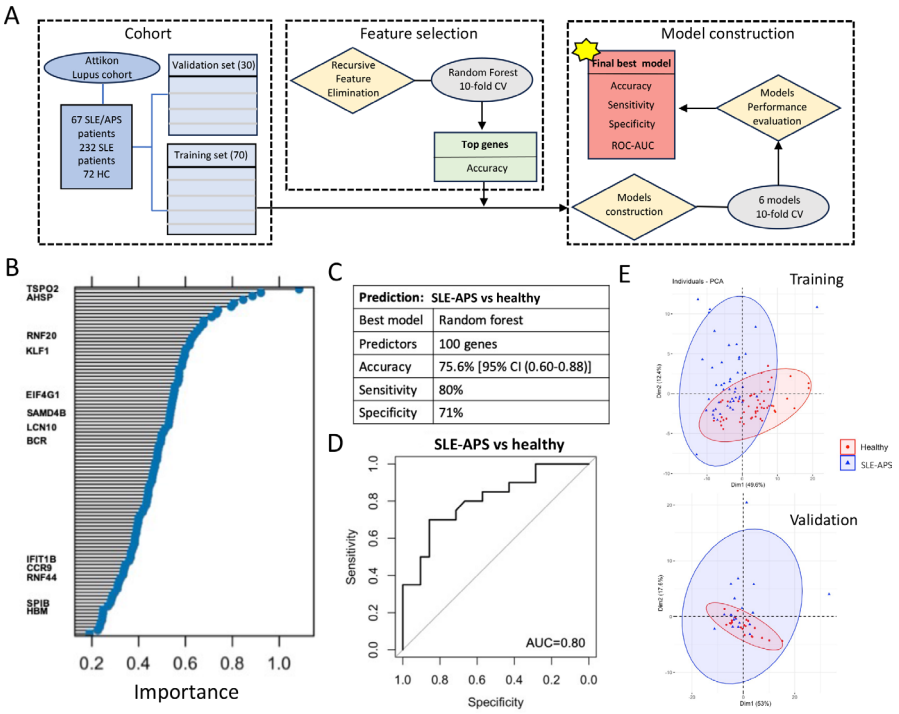

Figure 2 Machine-learning algorithm of whole-blood RNA-sequencing data distinguishes patients with systemic lupus erythematosus (SLE) with antiphospholipid syndrome (APS) from healthy individuals. A group of 100 genes discriminates SLE-APS from healthy controls (HC) (specificity=71%, sensitivity=80%, area under the curve (AUC)=0.80) including the key transcription factors *SPIB* and *KLF1* that regulate the expression of *IFIT1B, BCR, MPO* and *MMP9.* (A) Schematic overview of the machine-learning approach; RNA-sequencing were split in training and test sets at 70:30 ratio. (B) The 100 gene predictors of the random forest model distinguishing patients with SLE with APS from HC based on their importance, as evidenced by their absolute coefficient. Gene predictors potentially implicated in the pathogenesis in the SLE-APS are highlighted. (C) Characteristics of the prediction model of patients with SLE-APS from HC. (D) Receiver operating characteristic curve (ROC) analysis of the random forest model in the validation set reveals an AUC of 0.80. (E) Principal component analysis (PCA) in training and validation sets using the 100 genes.

prediction model performs best with the selected genes, using a 2-fold cross-validation approach (figure 2A). The random forest model using these 100 genes (figure 2B), best distinguished patients with SLE-APS from HC with a 10-fold CV, calculated accuracy of 75.6% (95% CI 60% to 88%), sensitivity of 80% and specificity of 71% (0.80 area under the curve (AUC) of the ROC analysis) in the validation set (figure 2C and D), demonstrating good model efficiency to discriminate true positive (patients with SLE-APS) from false positive (HC) cases. Thus, using the training and validation set, PCA showed that the 100 selected genes could accurately distinguish SLE-APS from HC (figure 2E).

Among these 100 genes derived from ML, we identified 13 keys genes potentially implicated in the pathogenesis of APS (highlighted in figure 2B). To uncover underlying mechanisms and possible crosstalk among these genes at molecular level, we next performed in silico analysis to identify potential transcription factors regulating the expression of these 13 genes. *SPIB* and *KLF1* were found as key transcription factors associated with SLE-APS based on the ML model, while their expression may be regulated by inflammation-related genes, such as *IRF1, IRF3* and *STAT5a/b* (online supplemental figure 3A). Genes with the highest importance in the final model included *TSPO2*, implicated in the redistribution of cholesterol, and *AHSP* and *HBM*, which encode alpha haemoglobin stabilising protein and haemoglobulin subunit Mu, respectively; both may be regulated by *KLF1* and *SPIB* (online supplemental figure 3B). More importantly, *SPIB* and/or *KLF1* may also regulate the expression of IFN-related genes (*IFIT1*; Online supplemental figure 3C), B cell-related genes (*BCR*; Online supplemental figure 3D) and neutrophil-related genes (*MMP9*, *MPO*; Online supplemental figure 3E). These data underscore the potential role of *SPIB* and *KLF1* in SLE-APS pathogenesis.

IFN signature is less pronounced in SLE-APS versus SLE-aPL-negative patients

We next analysed transcriptomic perturbations between SLE-APS and SLE-aPL-negative after controlling for the effects of disease activity. We found 136 upregulated and 91 downregulated DEGs in SLE-APS versus SLE-aPL-negative groups, mainly involving pathways related to complement cascade, B cell activation and neutrophil degranulation (figure 3A and B). GSEA revealed a robust downregulation of IFN-α and IFN-γ signatures in patients with SLE-APS with key deregulated genes contributing to that signature (figure 3C). To further explore this finding, we performed deconvolution analysis using CIBERSORTx to estimate the proportion of circulating immune cells. We observed a tendency towards reduced T cell frequency and significantly increased macrophage proportions in patients with SLE-APS (figure 3D). Patients with SLE-APS exhibited lower frequency of CD4 memory T cells and higher frequency of resting (non-activated) M0 macrophages, suggesting that altered myeloid populations towards an undifferentiated stage (M0 stage) may account for the downregulation of IFN signature (online supplemental figure 4A).[33] We also compared SLE-aPL-positive to SLE-aPL-negative patients, unravelling 126 DEGs (76 upregulated and 50 downregulated; figure 3E, online supplemental figure 4B) with GSEA demonstrating deregulation of several pathways including IFN-α, IFN-γ, complement cascade and oxidative

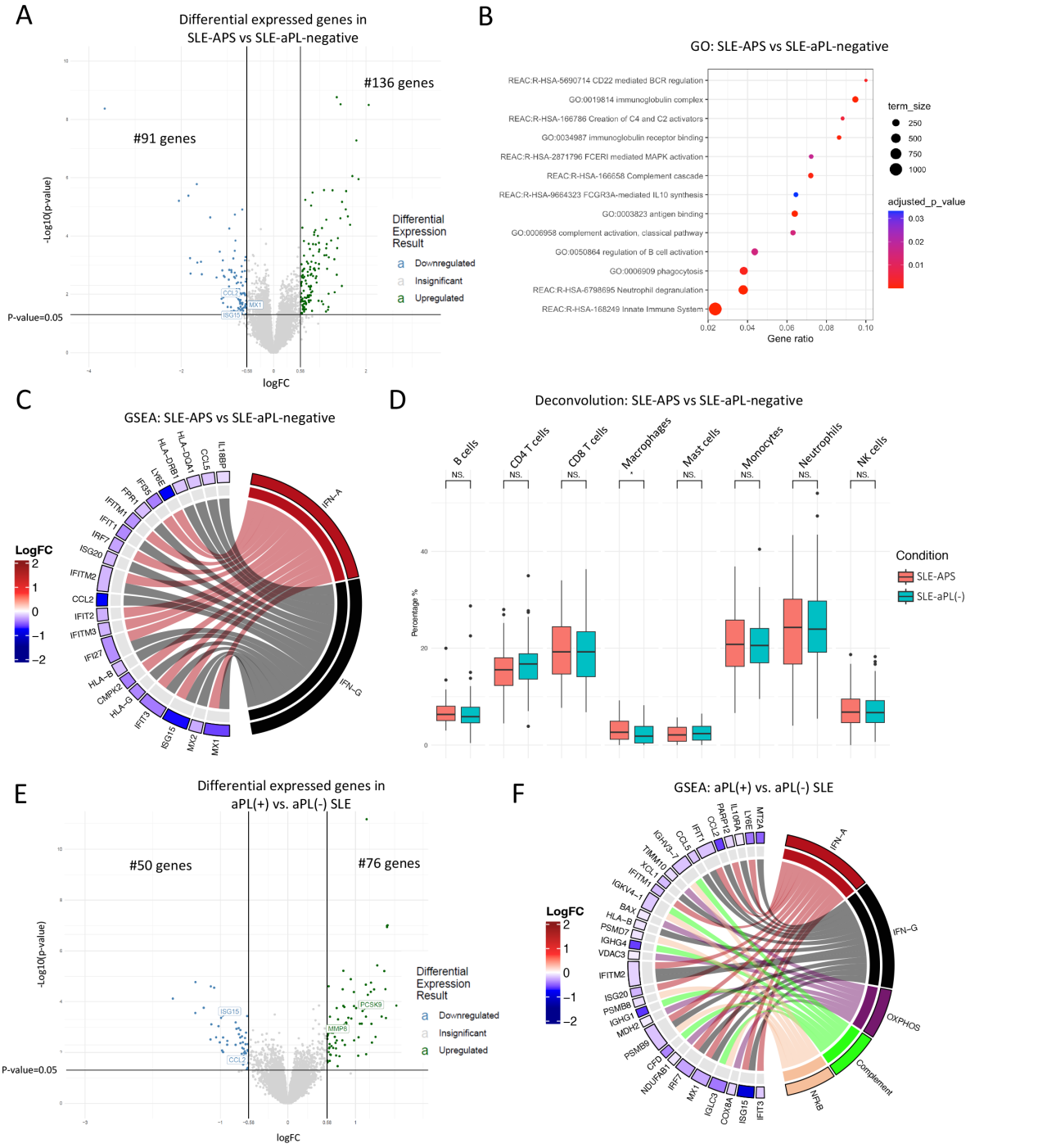

Figure 3 Transcriptomic signature in patients with SLE with APS (SLE-APS) versus SLE-aPL-negative: Interferon signature is less profound in patients with SLE-APS versus SLE-aPL-negative. Dysregulation of a group of 227 DEGs underlines the SLE-APS phenotype in SLE characterised by downregulation of type-I and type-II IFN signatures along with dysregulation of complement cascade, B-cell activation, and neutrophil degranulation. (A) Volcano plot highlighting the DEGs in SLE-APS versus SLE-aPL-negative. Upregulated DEGs are coloured green, and downregulated DEGs are coloured blue. DEGs not reaching our significance thresholds (|log2FC| >0.58 and p value <0.05) are shown in grey. (B) Dot plot showing the results of GO analysis representing biological pathways that are deregulated in patients with SLE-APS versus SLE-aPL-negative. The size of the dots represents the number of genes included in each enriched term and the colour represents the adjected p value. (C) Chord plot showing the results of GSEA analysis representing biological pathways associated with the Hallmark v2023.1.Hs database. The figure shows the significantly enriched pathways (FDR <0.25) in patients with SLE-APS versus SLE-aPL-negative (right) and key deregulated genes of each pathway found to DE between the two groups (left). (D) Deconvolution analysis using CIBERSORTx shows the estimated proportions of different immune cell subsets in SLE-APS versus SLE-aPL-negative. (E) Volcano plot highlighting the DEGs in SLE-aPL-positive versus SLE-aPL-negative patients. Upregulated DEGs are coloured green, and downregulated DEGs are coloured blue. DEGs not reaching our significance thresholds (|log2FC| >0.58 and p value <0.05) are shown in grey. (F) Chord plot showing the results of GSEA analysis representing biological pathways associated with the Hallmark v2023.1.Hs database. The figure shows the significantly enriched pathways (FDR <0.25) in SLE-aPL-positive versus SLE-aPL-negative patients (right) and key deregulated genes of each pathway found to DE between the two groups (left). aPL, anti-phospholipid antibodies; APS, antiphospholipid syndrome; DEGs, differentially expressed genes; FC, fold change; GO, gene ontology; GSEA, gene set enrichment analysis; IFN, interferon; SLE, systemic lupus erythematosus.

phosphorylation between these groups (figure 3F, online supplemental figure 4C). To further explore the impact of aPL on SLE transcriptome, we compared aPL^{high} to aPL^{low} patients with SLE, which revealed 231 DEGs mainly involved in adaptive immune response and complement (online supplemental figure 5A, B). GSEA showed downregulation of IFN-α, IFN-γ along with cholesterol homeostasis and angiogenesis in patients with SLE-aPL^{high} (online supplemental figure 5C), further supporting the association of aPL positivity with less profound IFN signature in SLE.

Unsupervised analysis differentiates pathogenetic mechanisms in venous from arterial thrombosis

Arterial and venous thromboses are distinct clinical entities with regards to pathogenesis (vascular wall injury vs stasis/hypercoagulation, respectively), location and therapies (antiplatelets vs anticoagulants).[34] To explore the transcriptomic landscape related to APS-specific thrombotic manifestations, we selected a WGCNA using transcriptomic data from the entire SLE cohort. WGCNA analysis identified 21 modules of coexpressed genes. Within the SLE cohort, APS was associated with the *salmon* module. Six gene modules significantly correlated with arterial thrombosis in patients with SLE-APS ('arterial thrombosis modules'). Venous thrombosis in SLE-APS was associated with two gene modules ('venous thrombosis modules'), one of which also correlated with pulmonary embolism (figure 4A).

Next, we performed enrichment analysis to identify deregulated pathways in each module. *Salmon* module was annotated to neutrophilic/myeloid signature, which further supports that secondary APS is characterised by a robust myeloid response, primarily mediated by neutrophils (figure 4B). The 'arterial thrombosis modules' (*greenyellow, turquoise, brown, magenta and green*) were associated with oxidative phosphorylation, DNA damage response (DDR), neutrophilic/myeloid response, immune response and metabolism, respectively (figure 4B), suggesting that arterial thrombosis in APS may be mediated by neutrophils through deregulation of pathways related to DDR and metabolism. To further evaluate these findings, we constructed a protein–protein interaction network based on deregulated coding genes in *green* (metabolism) and *turquoise* (DDR) modules, and we found key orchestrators of arterial thrombosis including *TP53, ATM, ATR* and *HIF1A* (figure 4C, online supplemental figure 6A). The 'venous thrombosis modules' (*salmon* and *lightyellow*) were associated with neutrophilic/myeloid response and adaptive immunity, respectively, suggesting that both neutrophils and B cells are important regulators of venous thromboses in patients with SLE-APS (figure 4B). The 'venous thrombosis' protein–protein interaction network revealed *CD79A, GATA1, TRAF4, STPA1* as significant genes that are associated with venous thromboses (figure 4D, (online supplemental figure 6B).

Unsupervised analysis differentiates pathogenetic mechanisms in patients with SLE with distinct aPL profiles

To further illustrate the transcriptomic landscape in SLE-APS, we performed WGCNA in relation autoantibody-positivity irrespective of APS status. Anti-CL positivity was associated with the *salmon* module, while anti-β2GPI was associated with *yellow* and *black* modules (figure 5A). Enrichment analysis demonstrated that *salmon* module is linked to a neutrophilic/myeloid signature, while *yellow* and *black* were associated with generic transcription and protein-binding pathways, respectively (figure 5B). These data indicate that distinct mechanisms may be implicated in patients with different autoantibody profiles.

DISCUSSION

Our study provides a comprehensive characterisation of gene signatures in patients with SLE with either APS or aPL positivity. Herein, we report the transcriptomics aberrancies associated with specific APS-related manifestations and autoantibodies, providing novel insights into SLE-APS. SLE-APS is associated with a less profound IFN signature within the SLE population. Furthermore, we showed that venous thrombotic events are linked to an enhanced myeloid/neutrophilic response, while arterial thromboses are associated with aberrant DDR and impaired metabolism (figure 6). Finally, we have developed a machine learning-based blood-gene model capable of identifying patients with SLE with APS.

The diagnosis of APS, currently dependent on the presence of aPL, poses challenges, as some patients may develop thrombotic events and non-criteria APS-related manifestations without aPL, giving rise to the suspicion of the 'seronegative APS'. The new American College of Rheumatology (ACR)/EULAR classification criteria for APS, published recently,[35] include manifestations previously considered as 'non-criteria' and thus these criteria may capture patients with possible APS. The initial performance of the ACR/EULAR criteria demonstrated very high specificity (almost 100%), yet with a sensitivity of 84% indicating that approximatively 15% of patients with clinical APS may not be classified by the new criteria. ML has emerged as a useful tool to construct diagnostic and prognostic models in rheumatology; to this end, we suggest that new features (eg, gene expression patterns) could be incorporated into existing classification systems to further improve their performance. Herein, we developed an ML model based on the expression of 100 genes that distinguished patients with SLE-APS from healthy individuals. The potential diagnostic value of these gene predictors in clinical setting can only be established through rigorous validation studies in independent cohorts, particularly among patients with thrombotic events.

Further on the ML-uncovered genes implicated in pathogenesis of APS, we found that *SPIB* and *KLF1* might be critical transcription factors of IFN (*IFIT1b*), adaptive immunity (*BCR*) and neutrophilic (*MMP9, MPO*) associated pathways, based on in silico analysis. These findings suggest a shared molecular regulation underlying traditional pathogenetic mechanisms involved in disease pathogenesis. In addition to the inflammation-related genes, ML identified haemoglobin-related genes (*AHSP*, *HBM*) as significant contributors to the algorithm, implicating a pathogenetic role of haemoglobin in thrombosis. Indeed, red blood cells crosstalk with platelets regulating their physical location in vessels and promoting platelet margination, which further enhances platelet–wall interactions and deposition on thrombi.[36] To this end, the gene with the most important contribution to the algorithm was the *TSPO2*, which encodes a protein that binds to cholesterol and plays a role in transferring cholesterol from lipid droplets to the endoplasmic reticulum.[37] Dyslipidaemia is frequently present in patients with aPL, serving as an additional risk factor for cardiovascular events. Our data indicate that aberrant gene regulation may play a role in the development of dyslipidaemia in these patients, thereby amplifying the risk of thrombosis. These findings also raise the possibility for shared mechanisms underlying immunothrombosis and dyslipidaemia/atherosclerosis.[38]

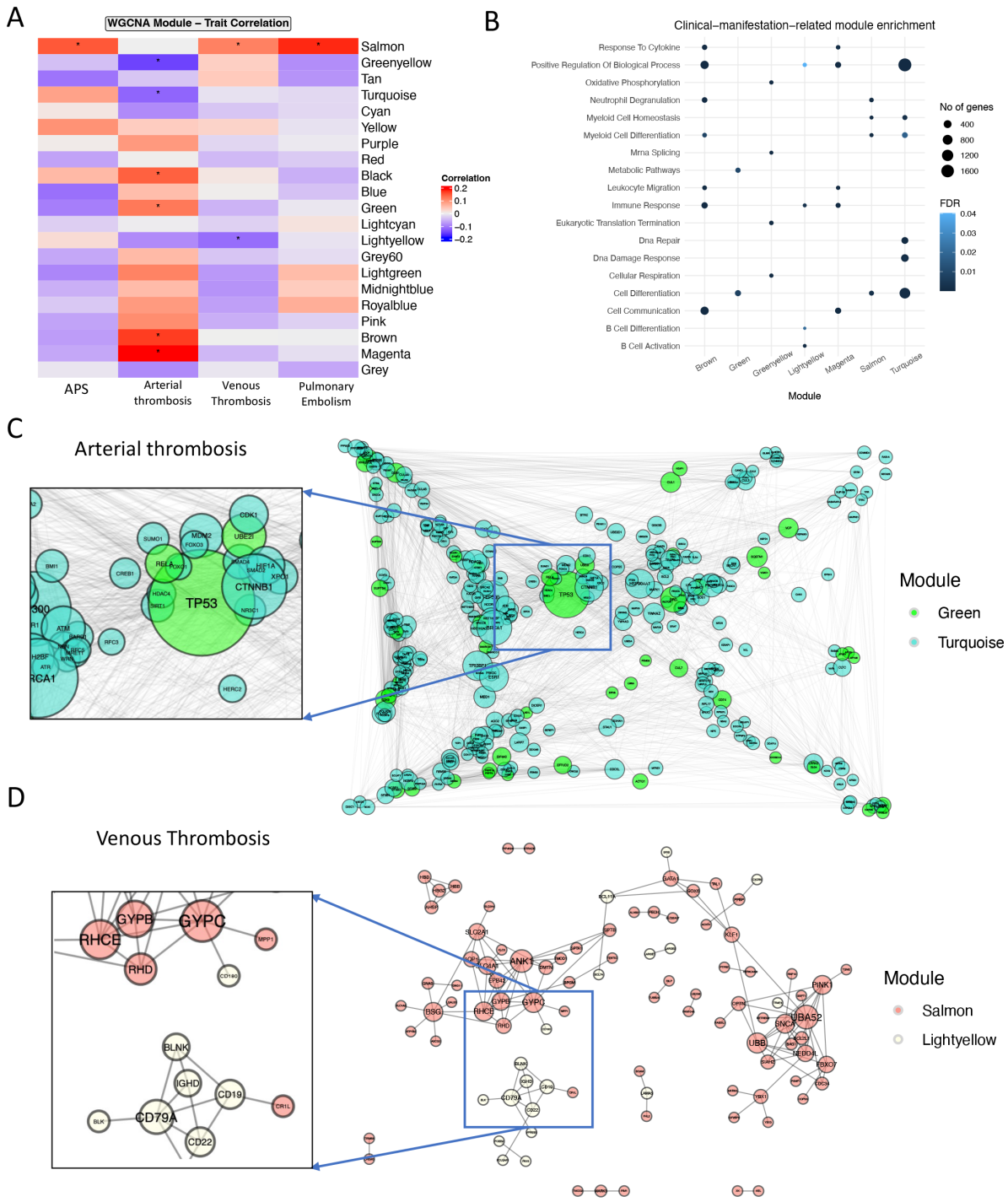

Figure 4 Unsupervised cluster analysis reveals distinct pathogenetic mechanisms implicated in venous and arterial thromboses. Venous thromboses are positively correlated with 'neutrophilic/myeloid' and 'B cell' modules, while the arterial thromboses were associated with dysregulation of 'DNA damage response (DDR)' and 'metabolism' modules. (A) Heatmap showing gene modules derived from WGCNA using transcriptomic data of the entire SLE cohort. Asterisks indicate statistically significant correlations between modules (rows) and APS-related clinical manifestations (columns). (B) Bubble plot of gene ontology terms found as significantly enriched in the correlated modules (salmon, greenyellow, turquoise, lightyellow, brown, magenta, green, black). Colour represents adjusted FDR, and size represents the number of genes related to a term found in each module. (C) Network from protein–protein interactions of proteins derived from *green* (green) and *turquoise* (turquoise) modules. Node fill colour corresponds to the module. The network layout was created using *Davidson and Harels* simulated annealing algorithm from the package *igraph*. (D) Network from protein–protein interactions of proteins derived from *salmon* (salmon) and *lightyellow* (lightyellow) modules. Node fill colour corresponds to the module. The network layout was created using *Davidson and Harels* simulated annealing algorithm from the package *igraph*. WGCNA, weighted gene coexpression network analysis; SLE, systemic lupus erythematosus; APS, antiphospholipid syndrome; DEGs, differentially expressed genes; aPL, antiphospholipid antibodies; FDR, false discovery rate; FC, fold change.

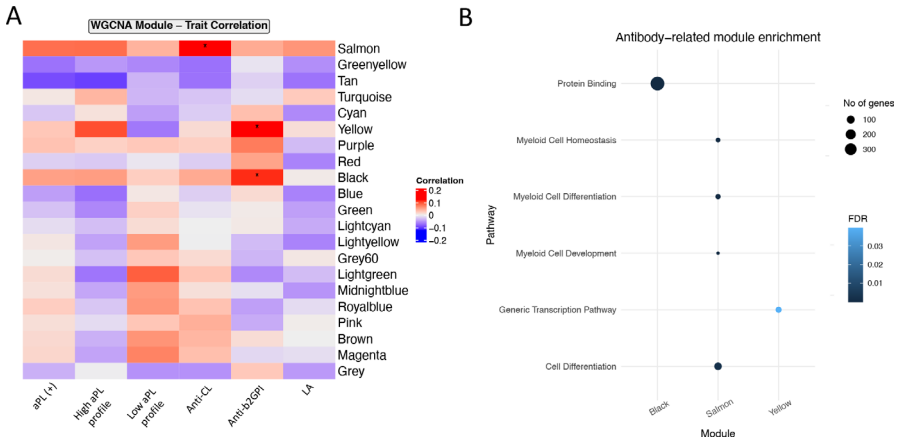

Figure 5 Unsupervised cluster analysis reveals distinct pathogenetic mechanisms implicated in patients with specific autoantibody profile. Anticardiolipin and anti-beta2 glycoprotein I (anti-β2GPI) positivity without clinical APS is associated with 'neutrophilic/myeloid response' and 'protein-binding' modules, respectively. (A) Heatmap showing gene modules derived from WGCNA using transcriptomic data of the entire SLE cohort. Asterisks indicate statistically significant correlations between modules (rows) and APS-related autoantibodies (columns). (B) Bubble plot of gene ontology terms found as significantly enriched in the correlated modules (black, salmon, yellow). Colour represents adjusted FDR, and size represents the number of genes related to a term found in each module. APS, antiphospholipid syndrome; FDR, false discovery rate; SLE, systemic lupus erythematosus; WGCNA, weighted gene coexpression network analysis.

While numerous studies have dwelled into APS pathogenesis, there has been limited investigation into the molecular-level pathogenetic distinctions between patients with SLE with and without APS. In our SLE cohort, we observed 91 significantly downregulated genes mainly involving in type-I and type-II IFN pathways in patients with SLE with APS. Although an upregulated IFN signature is typically associated with primary APS,[39] our observations using a large SLE cohort indicate downregulation of critical IFN genes in SLE-APS, including IFN-stimulated genes (*ISG15*, *ISG20*), IFIT protein-encoding genes (*IFIT1*, *IFIT2*, *IFIT3*), IFN-inducible genes (*IFI27*, *IFI35*), IFN regulatory factors genes (*IRF7*) and IFN-induced GTP-binding genes (*MX1*, *MX2*). Of note, type-I IFN pathway was enhanced in our SLE-APS population when compared with healthy individuals, although not to the level of SLE-aPL-negative. Patients with SLE-APS had more myeloid cells, particularly macrophages, suggesting a skewing towards myeloid lineage.[40] More specifically, patients with SLE-APS had significantly higher proportions of M0 macrophages, which may be driven by the lower IFN levels, as IFN-α promotes myeloid cell differentiation towards M1 macrophages.[33] Of note, CIBERSORTx has limitations in capturing populations with a very low percentage within the overall immune cell populations, such as pDCs, which are considered the main source of IFN-α.[41] In another study, RNA-sequencing in pDCs from patients with SLE, SLE-APS and primary APS showed that pDCs from SLE with and without APS

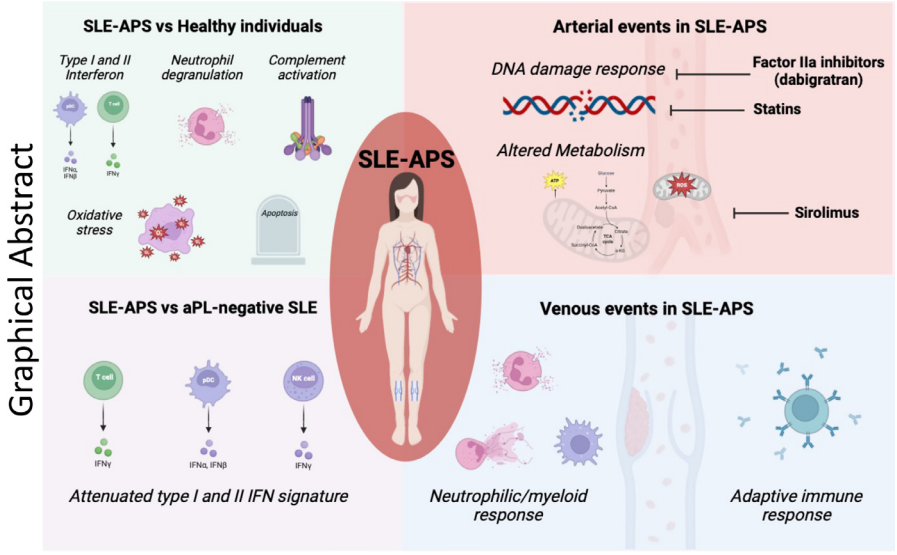

Figure 6 Schematic overview of deregulated mechanisms in SLE-APS. The SLE-APS transcriptome demonstrates extensive aberrancies with quantitative and qualitative differences characterised mainly by neutrophilic, apoptotic, complement, coagulation and type-I/II IFN signatures. APS in SLE is characterised by enhanced type I and II interferon (IFN) signatures which however are less prominent compared with their aPL-negative counterparts. Venous thrombotic events are predominantly driven by an enhanced neutrophilic and B cell response. DNA damage response aberrancies and altered metabolic pathways underlie arterial thromboses. Inhibitors of fIIai such as dabigatran, statins and mTOR inhibitors such as sirolimus—that attenuate DNA damage and metabolic aberrancies—have been used tested for the treatment of arterial events in SLE-APS and could be further explored. APS, antiphospholipid syndrome; aPL, antiphospholipids antibodies; SLE, systemic lupus erythematosus.

express comparable levels of IFN, while pDCs from primary APS might express slightly lower IFN levels.[8] From a clinical standpoint, an alternative plausible explanation for these findings is that aPL positivity in SLE is linked to haematological and neuropsychiatric manifestations but does not exhibit an association with nephritis or cutaneous manifestations, which are traditionally tied to IFN-α.[1]

The molecular heterogenicity of APS prompted us to examine whether specific APS-related manifestations or autoantibody profiles are associated with unique molecular patterns. Importantly, we uncovered significant differences between venous and arterial thromboses at molecular level. As expected, given that APS is commonly manifested with venous thrombosis and anti-CL positivity, venous thrombosis and pulmonary embolism were positively correlated with the 'neutrophilic/myeloid' module. This may explain the beneficial effects of immunomodulators that interfere with a neutrophil function such as glucocorticoids and immunosuppressive agents in thrombotic events associated with generalised inflammatory activity in SLE without APS. Moreover, venous thrombosis was associated with pathways related to adaptive immune response. Arterial thrombosis was significantly associated aberrant DDR and impaired metabolism. Although aberrant DDR and altered metabolism are primarily associated with tumourigenesis, their involvement in vascular function and autoimmunity has also been recognized.[42 43] Different pathogenetic roles of p53 have been identified across various immune cells; both upstream ATM and ATR-related pathways enhance inflammatory response via distinct mechanisms in autoimmune diseases,[44] yet its role in APS remains to be elucidated. Aberrant DDR has been linked to cardiovascular diseases, which are characterised by atherosclerosis and arterial thrombosis. Increased DDR-mediated senescence and apoptosis are evident in atherosclerotic lesions, while accumulation of DNA damage is positively associated with the severity of atherosclerosis.[42 45] Aberrant DDR in endothelial cells may result in prothrombotic and antiangiogenic phenotype.[46] Statins are recommended in patients with APS with recurrent arterial thrombosis for primary prevention; statins mitigate DNA damage induced by oxidative stress and impede downstream signalling associated with aberrant DDR, thus exerting beneficial effects in patients with cardiovascular diseases.[46 47] Specifically, atorvastatin suppresses both ATM and ATR-related pathways decreasing senescence and apoptosis in atherosclerotic lesions.[48] These findings support that aberrant DDR may be involved in arterial-related events in APS. aPL are considered pathogenetic, yet additional pathogenetic 'hits' (the so-called '2 hit' concept) are required for the thrombus formation, and thus different 'second hit' mechanisms may drive the diverse clinical manifestations of APS. Herein, we demonstrated that DDR aberrancies and metabolic pathways may orchestrate arterial thromboses in APS. Again, our data raise the possibility for shared pathogenetic mechanisms between atherosclerosis/immune thrombosis and SLE-APS.[38]

Our findings have possible therapeutic implications. The mTORC pathway is activated in intrarenal arteries from patients with APS in a PI3K-AKT-dependent manner.[49] Treatment with sirolimus in kidney-transplant recipients with aPL showed downregulation of mTROC pathway in intrarenal vessels which was associated with more favourable outcomes. Sirolimus is a mTOR inhibitor with extensive effects on metabolism,[50] which further supports the critical role of altered metabolism on thrombus formation. Together, our data encourage further assessment of metabolic-targeting drugs such as sirolimus in clinical trials, to mitigate the altered 'metabolism' signature seen in patients with APS and arterial events.

Currently, direct oral anticoagulants (DOACs) are not recommended in APS due to higher incidence of arterial—but not venous—events observed in the DOACs arm during clinical trials, comparing their efficacy to warfarin.[51] Commonly used DOACs include two direct factor Xa inhibitors (fXAi; rivaroxaban, apixaban) and one direct fIIa inhibitor (fIIai; dabigatran). Of note, clinical trials and observational studies in APS have been conducted with fXAi rivaroxaban or apixaban.[51–53] Although all DOACs are considered equal in terms of anticoagulation, fXAi but not fIIai, suppress inflammatory response by reducing the expression of proinflammatory mediators (interleukin (IL)-1, IL-6, TNF-a, NF-kb) and inflammasome activation.[54] These findings may explain the efficacy of fXAi in patients with APS with respect to venous events, since venous thromboses are mediated by a potent inflammatory/myeloid signature based on our findings. Another study demonstrated that these two DOACs have different mechanisms of action with respect to DNA damage repair. Although both drugs are able to reduce ROS levels in vascular-damaged vessels, only dabigatran can completely repair the double-stranded DNA breaks suggesting that fIIai but not fXai enhance DNA damage repair mechanisms,[55] which are involved in APS arterial immunothrombosis. The data suggest that fIIai might be more effective than fXai in treating APS, especially arterial APS, providing a rationale for evaluating fIIai (eg, dabigatran) in clinical trials. Of note, a post-hoc analysis of three randomised clinical trials of dabigatran versus warfarin in patients with thrombophilia including patients APS (20% of population) showed no differences with respect to thrombotic events between dabigatran and VKA groups.[56]

In summary, we show that SLE-APS is associated with a less profound IFN signature within the SLE population suggesting that in contrast to renal and cutaneous expressions, SLE-APS may be less driven by IFN-α mediated pathways. Furthermore, we showed that venous thrombotic events are predominantly driven by an enhanced myeloid/neutrophilic response, while arterial thromboses are mediated by aberrant DDR and impaired metabolism suggesting that distinct pathogenetic mechanisms may account for specific APS-related manifestations. These data could account for the differential response of the various anticoagulants in arterial versus venous thromboses and they provide a basis to study the effectiveness of such interventions in SLE-APS.

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Contributors DN and DTB conceived the project and act as guarantors for the overall content. DN recruited and took care of patients, collected blood samples, extracted clinical data, and drafted the manuscript, with contributions from all authors. CL, GS, PG and AFilia performed analysis. AFilia supervised the analysis. PG and TM perform data interpretation and visualisation. NK, MN, AP and SF contributed to recruitment of patients and healthy individuals and extracted clinical data from the medical charts. IP: data interpretation and manuscript editing. GB, AFanouriakis and DTB took care of the patients, supervised the study and the writing. All authors read and approved the final manuscript for publication.

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Patient consent for publication Consent obtained directly from patient(s)

Ethics approval The study was approved by the Ethics Committee of 'Attikon' University Hospital, Athens (protocol number 103/06-03-2014). Participants gave informed consent to participate in the study before taking part.

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