Arteriosclerosis, Thrombosis, and Vascular Biology

TRANSLATIONAL SCIENCES

Characterization of Antiphospholipid Syndrome Atherothrombotic Risk by Unsupervised Integrated Transcriptomic Analyses

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OBJECTIVE: Our aim was to characterize distinctive clinical antiphospholipid syndrome phenotypes and identify novel microRNA (miRNA)-mRNA-intracellular signaling regulatory networks in monocytes linked to cardiovascular disease.

APPROACH AND RESULTS: Microarray analysis in antiphospholipid syndrome monocytes revealed 547 differentially expressed genes, mainly involved in inflammatory, cardiovascular, and reproductive disorders. Besides, this approach identified several genes related to inflammatory, renal, and dermatologic diseases. Functional analyses further demonstrated phosphorylation of intracellular kinases related to thrombosis and immune-mediated chronic inflammation. miRNA profiling showed altered expression of 22 miRNAs, enriched in pathways related to immune functions, cardiovascular disease, and autoimmune-associated pathologies. Unbiased integrated mRNA-miRNA analysis identified a signature of 9 miRNAs as potential modulators of 17 interconnected genes related to cardiovascular disease. The altered expression of that miRNA-mRNA signature was proven to be stable along time and distinctive of nonautoimmune thrombotic patients. Transfection studies and luciferase assays established the relationship between specific miRNAs and their identified target genes and proteins, along with their involvement in the regulation of monocytes procoagulant activity and cell adhesion. Correlation analyses showed relationship among altered miRNAs and their interconnected genes with aPL (antiphospholipid antibodies)-titers, along with microvascular endothelial dysfunction. In vitro studies demonstrated modulation in healthy monocytes by IgG-aPLs of several genes/miRNAs, which further intermediated downstream effects on endothelial function. The identified transcriptomic signature allowed the unsupervised division of three clusters of patients with antiphospholipid syndrome showing distinctive clinical profiles, mainly associated with their prothrombotic risk (thrombosis, autoantibody profile, cardiovascular risk factors, and atherosclerosis).

CONCLUSIONS: Extensive molecular profiling of monocytes in patients with primary antiphospholipid syndrome might help to identify distinctive clinical phenotypes, thus enabling new patients' tailored treatments.

GRAPHIC ABSTRACT: A graphic abstract is available for this article.

Key Words: antiphospholipid syndrome ■ cardiovascular disease ■ microRNA ■ monocytes ■ thrombosis

Antiphospholipid syndrome (APS) is an autoimmune disease characterized by pregnancy morbidity or hypercoagulability, involving the venous or arterial vasculature, accompanied by persistent positivity for aPLs (antiphospholipid antibodies) comprising aCL (anticardiolipin antibodies), anti-β2GPI (anti–beta2-glycoprotein I), and LA (lupus anticoagulant). Patients with APS have a higher incidence of cardiovascular disease (CVD), which involves vascular damage, mainly including thrombosis, accelerated atherosclerosis, stroke, or myocardial infarction.[1,2] It has been speculated that multiple mechanisms can lead to the development of thrombosis in patients with APS, including the synergistic

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The Data Supplement is available with this article at https://www.ahajournals.org/doi/suppl/10.1161/ATVBAHA.120.315346.

For Sources of Funding and Disclosures, see page 876.

Arterioscler Thromb Vasc Biol is available at www.ahajournals.org/journal/atvb

Nonstandard Abbreviations and Acronyms

aCL	anticardiolipin antibodies
aGAPSS	adjusted global antiphospholipid syndrome score
anti-b2GPI	anti–beta2-glycoprotein I
aPL	antiphospholipid antibodies
APS	antiphospholipid syndrome
CVD	cardiovascular disease
FX	factor X
HD	healthy donors
LA	lupus anticoagulant
miRNA	microRNA
PF-RF	peak flow-rest flow
TF	tissue factor
TGF	transforming growth factor
VCAM	vascular cell adhesion molecule
VEGF	vascular endothelial growth factor

Highlights

- The present study has developed an integrative analytical approach in patients with antiphospholipid syndrome that combines high-throughput genomic datasets with clinical data to build models that inform the underlying disease process.
- The integrated transcriptome and protein profile allowed the identification of central genes, microRNAs, and altered pathways in monocytes of patients with antiphospholipid syndrome, which are particularly relevant to the unique characteristics of the disease and are at least partially regulated by aPL (antiphospholipid antibodies).
- Extensive molecular profiling of monocytes and plasma in patients with primary antiphospholipid syndrome might help to identify distinct clinical phenotypes, which, in turn, might facilitate a patient-tailored treatment.

effect of autoantibodies and prothrombotic molecules, adhesion receptors, inflammatory mediators, oxidative stress, NETosis, and various intracellular signaling molecules.[3]

The pathogenesis of both atherosclerosis and thrombosis involves many immune system cell types, among which monocytes have been demonstrated to play a key role. Monocytes exert different functions in atherogenesis, thrombosis, immunity, and reparative processes.[4,5] In the setting of APS and systemic lupus erythematosus, several studies have demonstrated multiple alterations in the activation status and secretory functions of circulating and tissue monocytes, further associated with the induction of an enhanced procoagulant activity—mainly related to the overexpression of tissue factor—the dysregulation of T-cell functionality, and the production of autoantibodies.[3] Likewise, their phagocytic capacity and activity as antigen-presenting cells are altered in these patients.[6] Therefore, the analysis of molecular profiles that typify APS monocytes may provide an effective way to identify heterogeneous subsets of patients for new targeted therapies.

Numerous studies have shown the relevance of gene expression profiles to identify subtle distinctions that delineate clinical phenotypes. The limited number of microarray studies conducted so far in APS have reported differences in gene expression profiles of peripheral blood mononuclear cells among patients with APS and healthy donors (HD).[7] A previous microarrays study by our group allowed the comparative characterization of APS versus systemic lupus erythematosus specific signatures that explained the proatherosclerotic, prothrombotic, and inflammatory gene profiles in these autoimmune diseases.[8] Newly, microarray analyses comparing, in vitro, the effects of IgG-APS treatment—purified from patients with distinctive manifestations of APS—on healthy monocytes, identified different genes and functional categories altered in treated monocytes, suggesting that IgG from patients with pregnancy morbidities and vascular thrombosis trigger distinctive physiological pathways.[9]

Yet, to date, no study has performed comparative in vivo studies in monocytes purified from a wide population of healthy donors and patients with primary APS with distinctive clinical and immunologic profiles.

Whole-genome studies have shown that the human genome is widely transcribed and produces thousands of regulatory noncoding RNAs. Among them, microRNA (miRNA) are universally expressed small noncoding RNAs, which have a profound influence on the regulation of almost all the cell processes studied and whose expression changes have been observed in many human pathologies.[10]

In a previous study, we characterized several miRNAs associated with cardiovascular disease present in patients with APS and systemic lupus erythematosus. These miRNAs are considered to be regulators of targets involved in clinical features of APS (such as atherosclerosis, thrombosis, immune response, and oxidative stress).[11]

In addition, it has been shown that due to cell death (by either necrosis or apoptosis) or active release, miRNAs are present in almost all body fluids (including blood, plasma, serum, saliva, urine, and pleural effusion). More and more evidence highlights the role of miRNAs in cell-to-cell communication. We recently analyzed the circulating miRNA signature of APS and identified a signature that showed potential mRNA targets closely related to the physiopathology of APS.[12]

Therefore, in the last years, there have been many advances in the understanding of the molecular basis of thrombosis development in APS, but further research is needed in many fields, especially the relationship among changes in transcriptomic profiles in monocytes with autoantibodies and clinical manifestations.

In this study, an integrated approach was employed to combine data obtained by multiple gene and miRNA expression profiling methods in monocytes and plasma from patients with primary APS, to characterize distinctive clinical APS phenotypes and identify novel miRNA-mRNA-intracellular signaling regulatory networks related to CVD.

METHODS

Patients

The authors declare that all supporting data are available within the article (and its Data Supplement).

Forty consecutive patients fulfilling the classification criteria for primary APS and 40 HD were recruited to this study, during a period of 24 months. Experimental protocols were approved by the ethics committee of the Reina Sofia Hospital in Cordoba (Spain) and written informed consent was obtained. Subjects were selected from patients with stable disease for more than 6 months, without infections, miscarriage, thrombosis, or changes in treatment regimen. The adjusted global APS score (aGAPSS) was evaluated in all patients with APS as earlier reported.[13] Clinical and laboratory profiles of patients with APS and HDs are shown in the Table.

An additional cohort of patients was analyzed as disease control, including 20 patients with thrombosis in the absence of an associated autoimmune disease (12 nonpregnant women and 8 men; mean age 48 [range, 25–72 years], including patients with objectively verified thrombotic events: 9 deep venous thrombosis and 9 thrombosis in intracerebral vessels). The study was conducted according to the principles of the Declaration of Helsinki. The workflow diagram is presented in the Figure I in the Data Supplement.

Blood Sample Collection, Assessment of Biological Parameters, B-Mode Ultrasound Intima-Media Thickness Measurements, and Evaluation of Microvascular Endothelial Function

See Data Supplement for details.

PathScan intracellular Signaling Protein Array and Bio-Plex Assay of the Inflammatory Profile in Plasma of Patients With APS

See Data Supplement for details.

Microarray Analysis, NanoString nCounter Assay, and Quantitative Real-Time Polymerase Chain Reaction

Microarray studies were performed using an Agilent G4112F platform (Whole Human Genome Microarray 44k) through the One-Color gene expression system.

The NanoString human v2 array was used for miRNA expression data generation. This array was conducted on pools obtained from the same monocyte RNA purified from the 3 patients with APS and the 3 HD from an exploratory cohort on which the microarray analysis was performed (See Data Supplement for further details).

Table. Clinical and Laboratory Parameters of the Patients With APS and the Controls.

	HDs (n=40)	APS (n=40)	*P* value
Clinical parameters			
Females/males, n	26/14	27/13	n.s.
Age, y (mean±SD)	47±10	52±10	n.s.
Thrombosis, n	0/40	30/40	
Arterial thrombosis, n	0/40	24/40	
Venous thrombosis, n	0/40	7/40	
Recurrences, n	0/40	18/40	
Pregnancy morbidity, n	1/40	16/40	
Pathological CIMT,* n	2/40	20/40	
LA positivity, n	0/40	37/40	
aCL positivity, n	0/40	16/40	
Anti-β2GPI positivity, n	0/40	13/40	
Arterial hypertension, n	3/40	11/40	
Hyperlipidemia, n	18/40	18/40	
aGAPSS (mean±SD)	NA	8.73±3.82	
Endothelial dysfunction			
Hyperemia area	3462,27±412,47	2077,78±288,58	*
PF-RF	58,70±5,12	41,40±4,30	*
Treatment			
Antiplatelet agents, n	0/40	18/40	
Anticoagulant agents, n	0/40	13/40	
Prednisone, n	0/40	5/40	
Statins, n	0/40	18/40	
Hydroxychloroquine	0/40	3/40	
Laboratory parameters			
Total cholesterol level, mg/dL	197±25	191±32	n.s.
Cholesterol HDL level, mg/dL	52±14	52±14	n.s.
Cholesterol LDL level, mg/dL	126±18	113±32	*
Triglycerides level, mg/dL	91±57	130±88	*
CRP, mg/dL	0.9±1.3	3.5±4.7	*
ESR, mm/h	7.6±4.8	12.4±11.9	*

PF-RF: maximum perfusion value after release of occlusion. aCL indicates anticardiolipin antibodies; aGAPSS, adjusted global antiphospholipid syndrome score; anti-β2GPI, anti–beta2-glycoprotein I; APS, antiphospholipid syndrome; CIMT, carotid intima-media thickness; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; HD, healthy donors; HDL, high-density lipoprotein; LA, lupus anticoagulant; LDL, low-density lipoprotein; NA, not applicable; n.s., not significant; and PF-RF, peak flow-rest flow.

*$P<0.05$.

Characterization of Biological Functions, Pathways, and Networks of Altered Gene and miRNA Profiles. Integrated miRNA-mRNA Analysis

Using the web-based bioinformatics tool QIAGEN's Ingenuity Pathway Analysis (IPA), differentially expressed genes in monocytes were classified and used for computational analysis to identify potentially enriched functional pathways and networks

(Ingenuity Systems, http://www.INGENUITY.com) and the Gene Ontology enrichment analysis (http://www.geneontology.org/).

By using the miRNA enrichment analysis tool TAM 2.0, the altered miRNAs were further analyzed to obtain information about biological functions, pathways, and networks (http://www.lirmed.com/tam2/).

Differentially expressed mRNA and miRNA data sets were introduced into IPA to identify relationships, either predicted or validated. mRNA-miRNA interactions were documented using the miRNA Target Filter function.

Pathway enrichment analysis of genes was performed by using the STRING platform (https://string-db.org/).

miRNA Mimics Transfection, Luciferase Assay, Analysis of Procoagulant Activity, Cell Adhesion, and In Vitro Studies

Isolated monocytes from 4 patients with APS, selected among that suffering thrombosis and positive for aPLs were transfected with 100 nmol/L of the miRNA mimics hsa (human)-miR-30b-5p, hsa-miR-145-5p, miR146b-5p, or hsa-miR-199a-5p (Life Technologies, Madrid, Spain) or a nonspecific control (scrambled) by using the transfection agent siPORT NeoFX (Life Technologies, Madrid, Spain).

After 48 hours of transfection, the mRNA and protein levels of their potential targets (STAT-1 [signal transducer and activator of transcription 1], IL [interleukin]-1A, TGF [transforming growth factor]-beta and VCAM [vascular cell adhesion molecule]-1) were assessed by real-time polymerase chain reaction, Western blot, and flow cytometry.

The direct interaction between potential targets and miRNA regulators was analyzed by using the LightSwith Luciferase Assay reporter system (SwithGear Genomics) following the manufacturer's instructions. Shortly, 250000 HEK293 cells were seeded in 96 white well plates 24 hours before transfection. Then, cells were cotransfected with SwitchGear GoClone reporter constructs of 3′UTR (untranslated region) region for each target and with 100 nmol/L of either miRNA mimic or negative scrambled control. As negative controls, one Empty 3′UTR vector and a housekeeping gene 3′UTR vector (GAPDH) with no binding sites for those miRNAs were included. Finally, luciferase activity was detected by a luminometer 24 hours after cotransfection.

The effect of transfections on the procoagulant activity of monocytes was evaluated in cell lysates by using the TF (tissue factor) human chromogenic activity assay kit—which measures the ability of lipoprotein TF/FVIIa to activate FX (factor X) to factor Xa—following the manufacturer's recommendations (Abcam, Cambridge, United Kingdom).

The Vybrant Cell adhesion assay kit, which utilizes the fluorogenic dye calcein acetoxymethyl ester (calcein-AM) to measure cell adhesion, was used to evaluate cell adhesion of monocytes after transfections (Molecular Probes Inc, Leiden, the Netherlands).

IgG purification, in vitro treatment of monocytes and endothelial cells with aPL antibodies, and detailed information for statistical analysis are available in the Data Supplement.[14–17]

Statistical Analysis

All data were expressed as mean±SD. Statistical analyses were performed using SSPS 25.0 (SPSS, Inc, Chicago, IL). After testing for normality and equality of variance, the clinical features were compared using paired Student *t* test or nonparametric test (Mann-Whitney rank-sum test). Paired samples in the same subjects were compared by Wilcoxon rank-sum test. Correlations were evaluated by Spearman rank correlation. The differences were considered significant at $P<0.05$. A Bonferroni correction was applied for multiple testing in correlation studies.

Next, to stratify patients with APS according to their molecular profile, classification analyses, including partial least-squares discriminant analysis and unsupervised k-means clustering were conducted with Metaboanalyst.[13]

RESULTS

Monocytes From Patients With Primary APS Display Gene and miRNA Expression Profiles Related to Inflammatory and Cardiovascular Diseases

Gene expression array recognized 547 altered genes in monocytes from patients with primary APS in comparison with HD (fold change>2), comprising 374 upregulated and 173 downregulated (Figure 1A and Table IV in the Data Supplement).

Gene Enrichment analyses showed that the most relevant diseases associated with these genetic changes were cardiovascular and reproductive diseases. In addition, some genes significantly altered in APS patient's monocytes were found involved in inflammatory, renal, and dermatologic diseases. The main biological processes integrated by these genes were cytokine/chemokine signaling, intracellular signaling pathways, such as TLR (Toll-like receptors), JAK (Janus kinase), p38 (p38 MAP kinase), Erk (extracellular signal-regulated kinases), STAT and NF (nuclear factor) κB, cell adhesion, angiogenesis, and pathways involved in reproduction, among others (Figure 1B).

Twenty-two miRNAs were found differentially expressed in monocytes from patients with APS, including 3 upregulated and 19 downregulated (Figure 1C and Table III in the Data Supplement). Functional classification of those miRNAs showed an enrichment in functions, such as inflammatory response, cell proliferation, apoptosis, and angiogenesis, among others, linked to the development of cardiovascular and connective tissue disorders (Figure 1D).

Intracellular Kinases Involved In Thrombosis and Immune-Mediated Chronic Inflammation Are Phosphorylated on Monocytes From Patients With APS

To evaluate the activation of intracellular signaling pathways regulating the expression of several altered genes involved in the pathophysiology of the syndrome, we analyzed their phosphorylation status by using an intracellular signaling protein array.

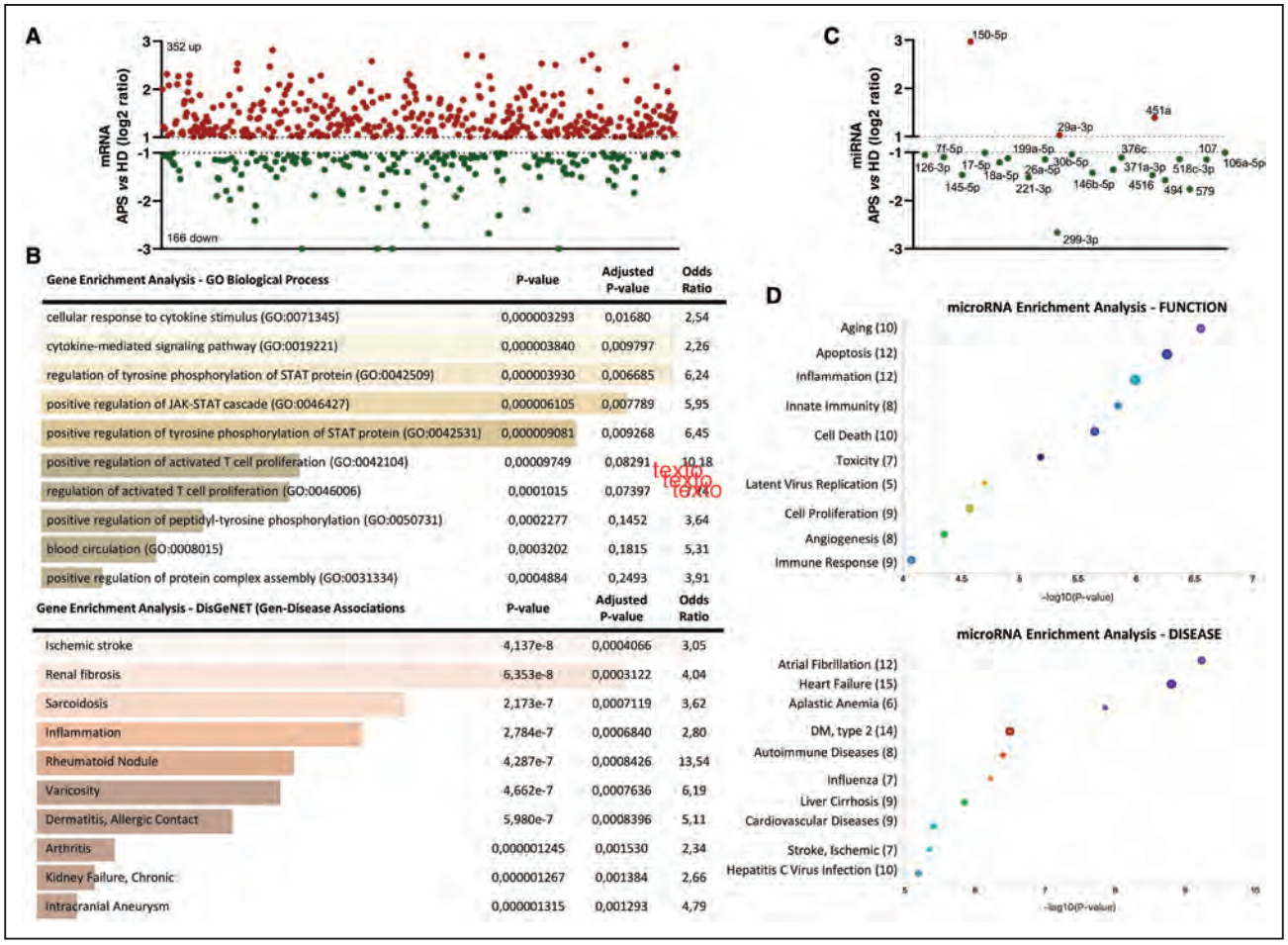

Figure 1. Gene and microRNA (miRNA) expression profiles in monocytes from primary antiphospholipid syndrome (APS) patients and functional categorization.

A, Scatter plot of gene expression profiles in monocytes of pools obtained from the 3 patients with APS vs 3 healthy donors (HD). Expression levels of 352 genes were found upregulated in APS (FC [fold change] >2), whereas 166 were found downregulated (FC <2). **B**, Functional categorization of gene expression signatures in patients with APS monocytes by gene enrichment analyses, including the top-ten gene ontology (GO) biological processes and gene-disease associations. **C**, Scatter plot of miRNA expression in monocytes of pools obtained from the 3 patients with APS vs 3 HD. Expression levels of 3 miRNAs were found upregulated in APS (FC >2), whereas 19 were found downregulated (FC <2). **D**, The main enriched biological functions and diseases in which these miRNAs are involved, by using the software TAM 2.0, are displayed, including the number of miRNAs involved in each process. DM indicates diabetes mellitus; JAK-STAT, Janus kinase-signal transducer and activator of transcription; and STAT, signal transducer and activator of transcription.

This array showed a significant activation of 18 intracellular kinases, all of them reported to be involved in immune-mediated chronic inflammation as well as on the thrombotic status present in patients with primary APS (Figure II in the Data Supplement).

Integrated mRNA-miRNA Analysis Identified a Signature of Interconnected CVD-Related Genes Modulated by a Specific Set of Altered miRNAs

By using the miRNA Target Filter function within IPA, we first recognized that 147 genes could be potential targets of 20 miRNAs. Then, focusing on miRNA-mRNA interactions that exhibited a reciprocal relationship, we identified predicted or validated interactions between 19 miRNAs and 115 mRNAs (Figure 2A and Figure II in the Data Supplement).

Functional analysis of genes modulated by miRNAs showed that a set of them was interconnected, including several cytokines, adhesion proteins, proteins of the serpin family, intracellular regulators such as STAT-1, and genes involved in oxidative stress processes. These connected genes were identified to be associated with pathways closely related to the development of cardiovascular and kidney diseases in APS (Figure III in the Data Supplement). The specific network integrated by those genes and their potential miRNAs regulators is highlighted in Figure 2B. This network included several mRNAs potentially modulated by a set of miRNAs whose alteration might have an overall impact in the development of the cardiovascular pathology of patients with

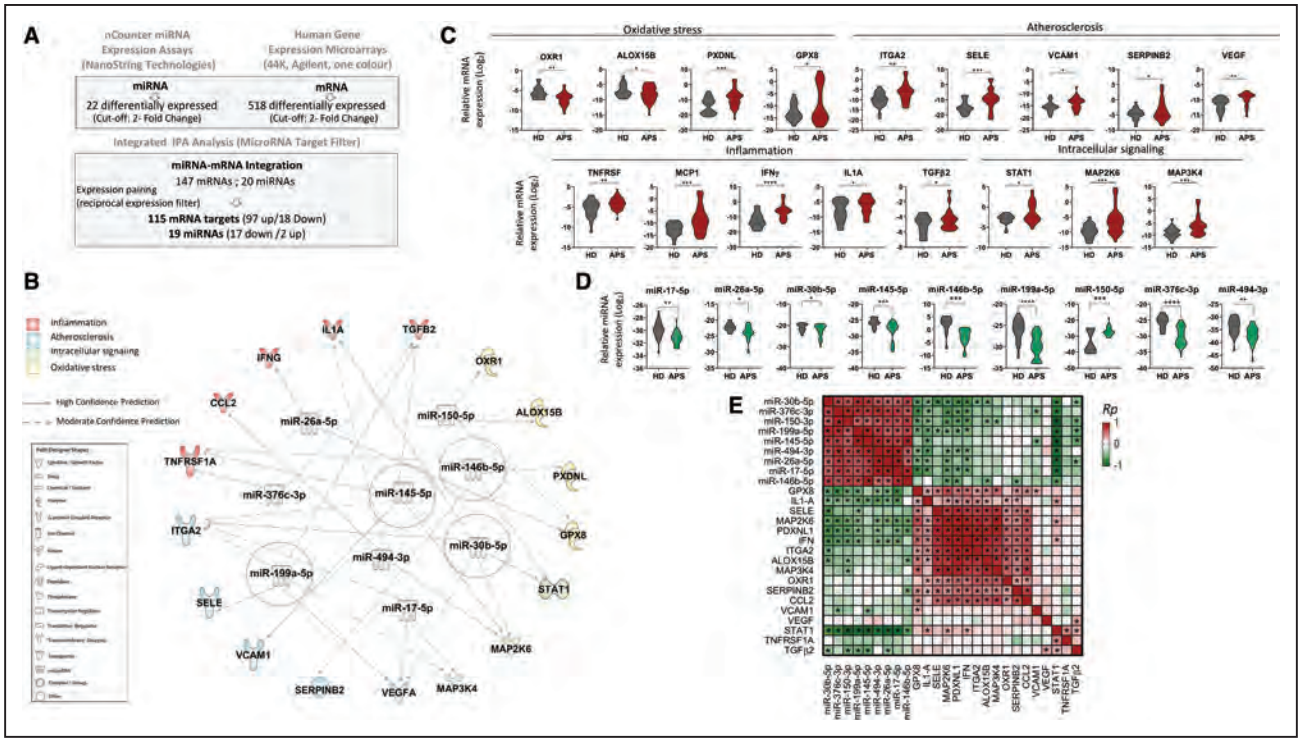

Figure 2. Integrated mRNA-microRNA (miRNA) analysis in monocytes from antiphospholipid syndrome (APS) patients. **A**, Integrated analysis pipeline. Altered mRNA and miRNAs were identified in monocytes of pools obtained from the 3 patients with APS and 3 healthy donors (HD). Then, potential biological interactions were identified by using Ingenuity Pathway Analysis (IPA) Software (miRNA target filter tool) followed by an analysis of reciprocal expression filter. **B**, Interaction network of key interconnected genes associated with pathways and biological functions related to the pathogenesis of APS and their potential miRNA regulators. High and moderate interactions are based on the probability of conserved targeting in mammals (TargetScan algorithms). **C**, mRNA validation analysis where violin plots show individual expression of those interconnected genes in monocytes from all patients with APS (n=40) and HD (n=40) included in the study. **D**, miRNA validation analysis where violin plots show individual expression of the potential miRNAs modulators of the gene signature in monocytes from all patients with APS and HD included in the study. *Significant differences vs HD at $P<0.05$; **significant differences vs HD at $P<0.01$; ***significant differences vs HD at $P<0.001$. The P value was obtained in the Student t test results. **E**, Heatmap showing the correlation analysis between the relative expression of the genes and miRNAs validated in APS monocytes. Red or green colors in each square represent positive or negative Spearman correlation coefficients respectively. Color scales are defined for the ranges delimited by the correlation coefficients where darker color represents higher correlation coefficients and lighter color indicates lower correlation coefficients. *Statistically significant correlations in each square ($P<0.05$).

APS. We could identify several genes that are simultaneously targeted by various miRNAs, and similarly, a number of miRNAs that simultaneously target various genes. Thus, for example, miR30b-5p and miR146b-5p simultaneously targeted *STAT-1*, and *TGFβ* was the predicted target of miR-145-5p and miR199a-5p.

Then, to validate the data obtained in the array, we analyzed in the entire cohorts of patients with APS and HD, the expression of this specific signature of interconnected genes along with the miRNAs that were predicted to modulate their expression. These analyses confirmed the data of mRNA and miRNA arrays (Figure 2C and 2D). Besides, a strong positive correlation between levels of validated mRNAs was identified, supporting the interconnection between them in the regulation of shared pathways. Accordingly, positive correlations between all miRNAs supported the synergic relationship between them in the control of these particular targets and pathways (Figure 2E).

Moreover, levels of several validated miRNAs showed an inverse correlation with those of several validated genes, thus supporting their reciprocal relationship.

Transfection Assays in APS Patients' Monocytes Recognized the Interaction Between Several miRNAs and Their Potential Molecular Targets, Along With Their Role on Monocytes Activity

To better delineate the functional relevance of the in silico identified interactions among several miRNAs and their potential molecular targets, we developed several experimental approaches (Figure 3A).

First, transfections on monocytes from patients with APS with mimics of various miRNAs were performed. For this purpose, those miRNAs (miR-30b-5p, miR-145-5p, miR-146b-5p and miR-199a-5p) with a higher number of potential target genes. Then, we evaluated the influence of their overexpression on the modulation of the genes and proteins identified as potential targets with

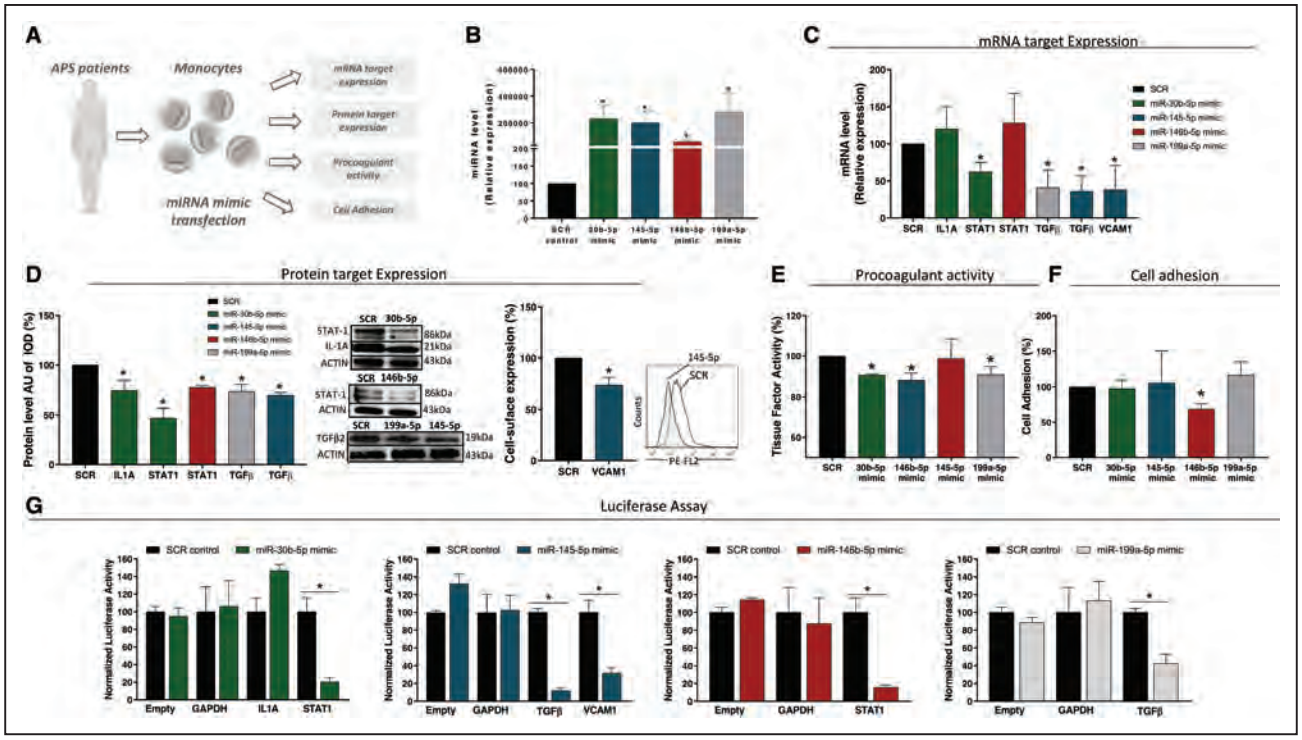

Figure 3. Transfection assays in monocytes from patients with antiphospholipid syndrome (APS).
A, Diagram showing the experimental design of the microRNA (miRNA) mimic transfections. Monocytes isolated from 4 patients with APS were transfected with 100 nmol/L of miR-30b-5p, miR-145, and miR-199a-5p and miR-146b-5p mimics, and a nonspecific control (scrambled) by using siPORTTM NeoFXTM transfection agent, following manufacturer's protocols. Forty-two hours after transfection potential targets (STAT-1, IL [interleukin]-1A, TGF [transforming growth factor] β2 and VCAM [vascular cell adhesion molecule]-1) were analyzed at mRNA and protein level. Moreover, functional assays related to the monocyte function, including procoagulant activity and cell adhesion were also carried out. **B**, miRNA levels of APS monocytes after mimic transfection. **C**, Real-time polymerase chain reaction analysis showing the effect of the miRNA mimic transfection on potential targets at mRNA level. **D**, Effect of the miRNA mimic transfections on potential targets at protein level using Flow cytometry (VCAM-1) and Western blot (STAT-1, IL-1A, and TGFβ2). **E**, Tissue factor activity in APS monocytes transfected with miRNA mimic (**left**) following manufacturer´s instructions. **F**, Cell adhesion of APS monocytes transfected with miRNA mimic (right panel) following manufacturer´s instructions. **G**, Luciferase activity of HEK293 cells after 24 h of cotransfection with 3′UTR (untranslated region)-luciferase reporter clones of potential targets and miRNA mimic or scrambled control. Negative controls included both 3′UTR empty vector and 3′UTR GAPDH vector. Data, obtained from 4 independent transfection experiments, were expressed as changes relative to the values of the cells transfected with a scrambled control (SCR) and set as 100%. *Significant differences (at $P<0.05$) vs cells transfected with SCR. AU indicates arbitrary units; HEK293, human embryonic kidney 293; IOD, integrated optical density; PE-FL2, phycoerythrin-filter channel 2; and STAT, signal transducer and activator of transcription.

high confidence in in silico analyses. These targets included genes involved in inflammation, atherosclerosis, intracellular signaling, and oxidative stress, recognized as relevant players in the development of thrombosis and CVD in the setting of APS (Figure 3B).

The overexpression of miR-30b promoted a significant reduction on both, the mRNA and the protein levels of STAT-1, whereas IL-1A was reduced at protein levels. Likewise, transfection with the mimic of miR-146b-5p reduced the protein expression of STAT-1. The overexpression of miR-145-5p reduced both, gene and protein expression of TGFβ2 and VCAM-1. Lastly, the mimic of miR199a downregulated both, gene and protein levels of TGFβ2 (Figure 3C and 3D).

To assess the impact of the altered miRNA expression on the monocyte activity, the effects of the overexpression of selected miRNAs on monocyte procoagulant activity and cell adhesion were analyzed. Monocyte incubation with the mimics of miRNAs 30b-5p, 145-5p, and 199a-5p promoted a significant reduction in TF-dependent procoagulant activity (Figure 3E and 3F). Analysis of cell adhesion showed that overexpression of miR-146b-5p reduced the adhesive capacity of monocytes, while other miRNA mimics had no effects.

Finally, to evaluate the direct interaction between the seed sequence of selected miRNAs and the 3′UTR region of their selected predicted targets, luciferase assays were performed. These assays confirmed the direct interaction between several mRNA-miRNA pairs including *STAT-1* with both, miR-30b and miR-146b; *TGFβ2* with miR-145; and miR-199a with both, *VCAM-1* and miR-145. The regulation of *IL-1A* by miR-30b previously identified seems to be mediated by a nondirect interaction as the luciferase activity did not change. No differences were observed in the luciferase activity of the negative control vectors transfected with mimic and scrambled control (Figure 3G).

APS Integrated mRNA-miRNA Signature Is Stable Over Time and Dissimilar From Nonautoimmune Patients With Previous Thrombotic Events

Three months after the first blood sample was collected, monocytes from various patients with APS comprised in the study (n=21 APS) were evaluated again to analyze the stability of signatures composed of interconnected genes.

Results demonstrated that gene expression remained stable in the second sample. Moreover, the expression levels of this signature across the 2× evaluated were found significantly correlated (Figure IVA in the Data Supplement). Therefore, our data support the theory that APS monocytes display a specific prothrombotic signature that remains stable over time.

To assess the specificity of mRNA signature found in monocytes of patients with APS, and to analyze whether the changes in mRNA expression measured were related to their thrombosis status, another disease group, including 20 subjects with thrombosis but without autoimmune-related diseases, was analyzed. In these thrombotic patients, the expression levels of the selected mRNAs were significantly different from the expression levels described in patients with APS (Figure IVB in the Data Supplement).

In parallel, the altered expression of the miRNAs validated in our APS cohort, both in monocytes and plasma, was evaluated in the cohort of patients with thrombosis but without associated autoimmune disorder. Again, both the monocytes and plasma miRNA profiles exhibited by those patients were distinct from those found in patients with primary APS (Figure IVC in the Data Supplement).

Potential Influence of Standard Therapy on the APS Integrated mRNA-miRNA Signature of Monocytes

According to the main treatment received, patients with APS were divided into two groups, including 14 patients with primary APS receiving antiplatelet drugs and 20 patients with primary APS receiving anticoagulant drugs. Statistical comparisons between patients treated with antiplatelet and anticoagulant drugs showed no significant differences in mRNA and miRNA signatures, except for genes *VEGF* (vascular endothelial growth factor), *TGFβ2*, *STAT-1*, and miR-17-5p, all of them closely interrelated and playing a key role in the assembly and preservation of the vasculature[18–20] (Figure IVD and IVE in the Data Supplement).

Antiphospholipid antibodies Modulate the Molecular Profile of Monocytes and Promote Endothelial Dysfunction

Correlation studies demonstrated a significant relationship among various deregulated genes and miRNAs with the levels of aPL-IgG antibodies. Thus, high titers of either aCL or anti-β2GPI were positively correlated with high levels of several genes involved in oxidative stress, intracellular signaling, thrombosis, and inflammation and negatively with low levels of miR-26a-5p and miR-17-5p.

Besides, several parameters related to the microvascular endothelial dysfunction, such as reduced hyperemic area and peak flow minus rest flow after temporary occlusion of blood flow, were negatively linked to increased expression of some genes involved in inflammation and atherosclerosis and positively associated with reduced levels of several microRNAs (Figure 4A).

In vitro, APS-IgG antibodies promoted in HDs monocytes the upregulation of a number of the interconnected genes, along with the downregulation of their miRNA regulators (Figure 4B through 4D).

Moreover, the addition of supernatants obtained from monocytes treated with APS-IgG to cultured endothelial cells promoted a substantial rise in the levels of several genes related to endothelial activation (Figure 4E).

Unsupervised Cluster Analysis of the Integrated miRNA-mRNA Signature Stratified Patients With APS According to Their Thrombotic Risk

By using hard k-means clustering analysis in the APS cohort 3 clusters were distinguished representing different molecular profile groups with respect to the monocyte expression levels of the signature integrated by genes and miRNAs (Figure 4A and 4B).

Partial least-squares discriminant analysis confirmed a clear separation between these molecular clusters (Figure 5C). Besides, to determine which genes and miRNAs contribute to this discrimination, we conducted the variable importance in projection analysis (Figure 5D). This analysis identified *E-selectin*, miR-494-3p, *PXDNL* (peroxidasin-like protein), and *GPX8* (glutathione peroxidase 8) topping this list with a variable importance in projection score over 1.5.

The clinical and laboratory profiles of each cluster were then evaluated (Figure 5E). Concisely, cluster 1 (16% of the clustered cohort) was described by patients that had suffered thrombotic episodes—either thrombotic or venous—with low incidence of obstetric morbidity (20%), high positivity for aPL antibodies (either aCL or β2GPI), and high prevalence of cardiovascular risk factors (ie, arterial hypertension, dyslipidemia, and smoking habits).

On the contrary, cluster 3 (24% of the clustered cohort) comprised the youngest patients, with a high incidence of thrombosis and obstetric complications, but low positivity for aPL antibodies and the lowest prevalence of cardiovascular risk factors.

Cluster 2 (60% of the clustered cohort) represented an intermediate clinical phenotype with thrombotic

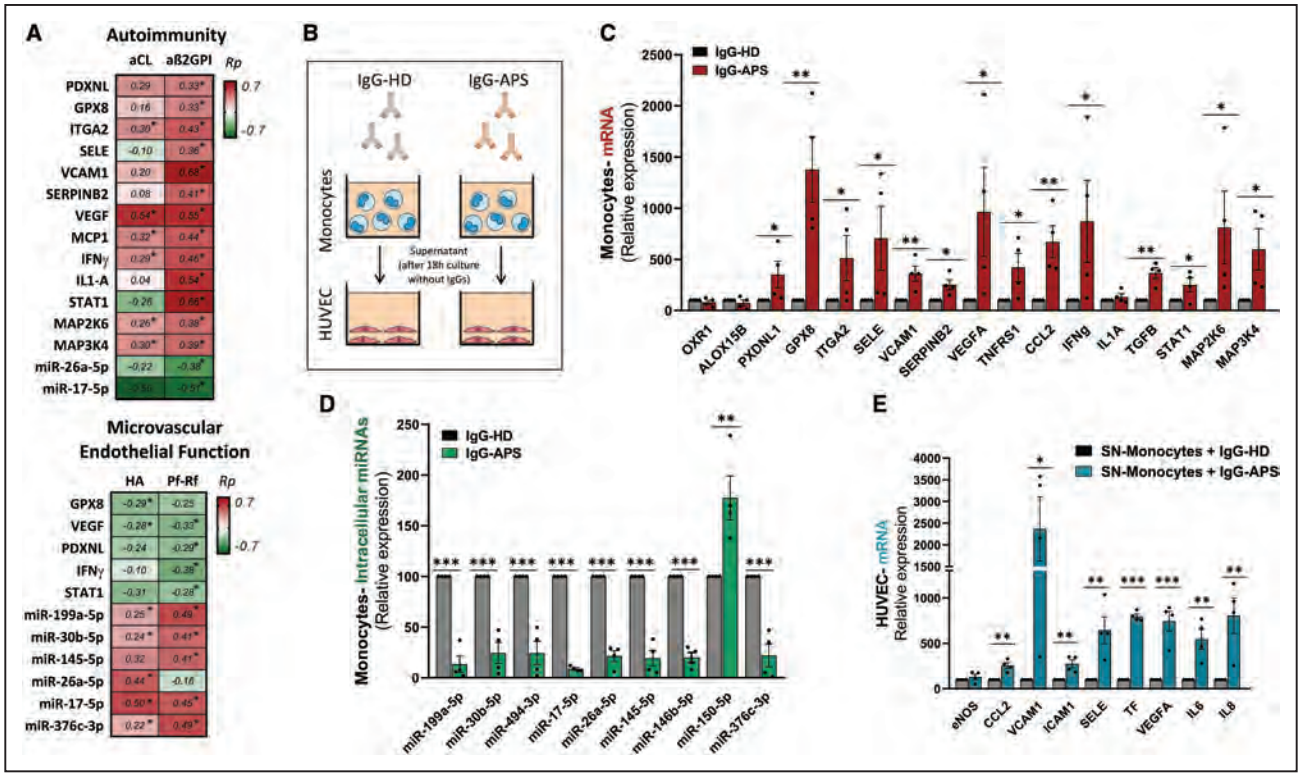

Figure 4. aPL (antiphospholipid antibodies) modulate the molecular profile of monocytes and promote endothelial dysfunction. **A**, Heatmap showing the correlation analysis between genes/microRNAs (miRNAs) validated and parameters related to either, autoimmunity or microvascular endothelial dysfunction. Each square represents the pairwise Spearman correlation coefficient for the listed genes/miRNAs, whereas every column is associated with a specific parameter related to autoantibodies titers in patients with antiphospholipid syndrome (APS; aCL [anticardiolipin antibodies] or anti-β2GPI [anti–beta2-glycoprotein I]) and endothelial dysfunction (hyperemic area [HA] or peak flow minus rest flow [Pf–Rf] after released blood flow occlusion). Red or green colors in each square represent positive or negative Spearman correlation coefficients respectively. Color scales are defined for the ranges delimited by the correlation coefficients where darker color represents higher correlation coefficients and lighter color indicates lower correlation coefficients. *Statistically significant correlations in each square ($P<0.05$). **B**, Schematic experimental diagram: primarily, healthy monocytes purified from healthy donors (HDs) were treated for 24 h with IgG antibodies isolated from the serum of either, 7 HD or 7 patients with APS with high aPL (antiphospholipid antibody) titers; thereafter, the supernatants obtained from the first experimental approach were added to human endothelial cells and incubated for 24 h. **C** and **D**, IgG-APS antibodies promoted the upregulation of several genes related to the pathogenesis of APS, along with the downregulation of their miRNA regulators. **E**, The addition of supernatants obtained from monocytes treated with aPL-IgG to cultured endothelial cells promoted a significant increase in the levels of several genes related to endothelial activation. Differences were analyzed by the Student *t* test. Values are the mean and SEM of 4 independent experiments performed in triplicate. *Significant differences (at $P<0.05$) vs HD; **significant differences (at $P<0.01$) vs HD; ***significant differences (at $P<0.01$) vs monocytes treated with IgG from HD. HUVEC indicates human umbilical vein endothelial cells; and SN, supernatant.

events and obstetric morbidity and also intermediate positivity for aPLs and medium prevalence of cardiovascular risk factors.

Therefore, when comparing aGAPSS between different clusters, we found the prevalence of patients with high or moderate aGAPSS in cluster 1, while in clusters 2 and 3 more than 70% of patients displayed an intermediate or low aGAPSS score. Moreover, those patients with the highest aGAPSS further displayed the highest incidence of atheroma plaques (Figure 5G).

Furthermore, Bio-Plex analyses recognized an inflammatory profile in APS patients' plasma, on which patients belonging to each cluster displayed specific and distinctive expression patterns of interleukins, chemokines, and growth factors involved in vascular damage and hematopoiesis (Figure 5H).

DISCUSSION

The present study has developed an integrative analytical approach that combines high-throughput genomic datasets with clinical data to build models that inform the underlying disease process. Hence, a specific mRNA-miRNA signature was identified in monocytes from patients with primary APS that allowed to stratify their thrombotic risk.

First, delineation of the gene profiling data allowed to identify specific gene networks involved in the inflammatory response, cell adhesion, or angiogenesis, among

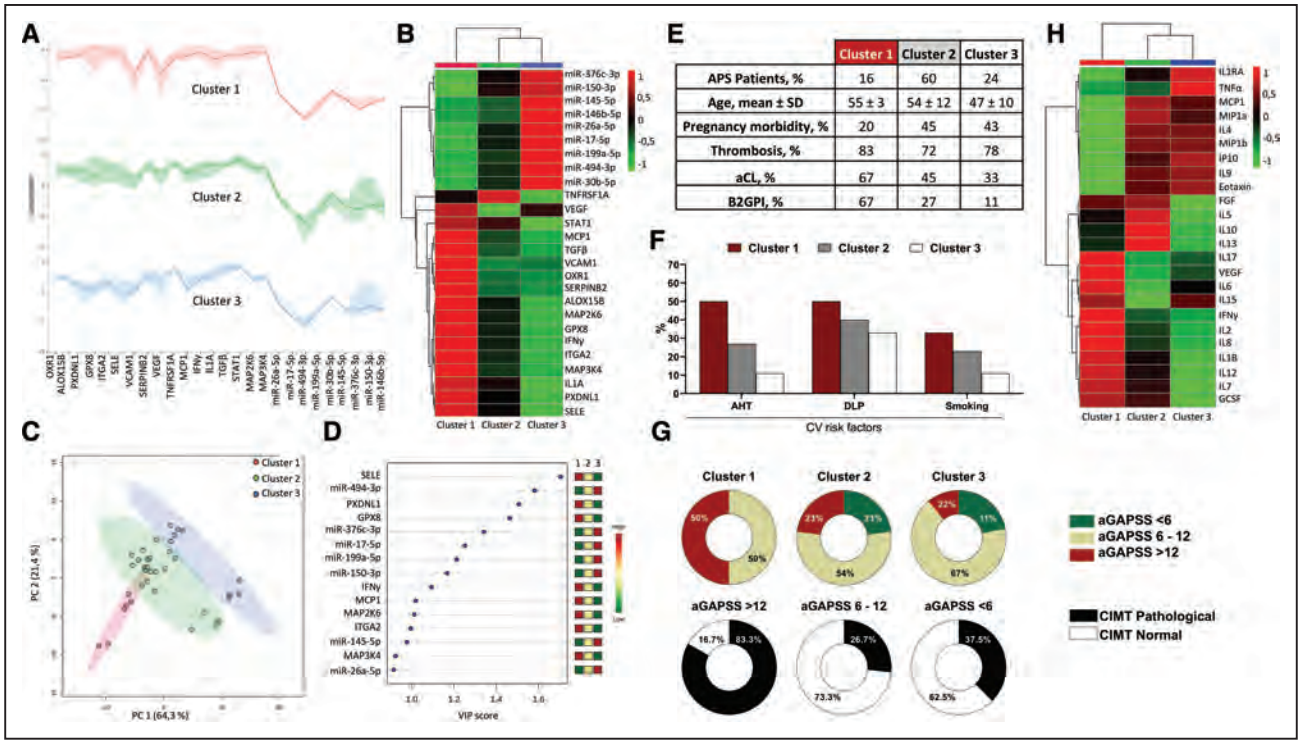

Figure 5. Integrated mRNA-microRNA (miRNA) signature of monocytes is intimately associated with clinical features of antiphospholipid syndrome (APS): Clustering analyses.

A, Molecular signatures identified by clustering of validated genes and miRNAs differentially expressed in monocytes of 40 patients with primary APS, using MetaboAnalyst 4.0. The *y* axis indicates z-transformed quantities of the signature of genes and miRNAs validated. The median values of patients in each cluster are connected by blue lines. **B**, Heatmap obtained from the hierarchical clustering of the 40 patients with APS in 3 clusters–cluster 1, 2, and 3–according to the expression profiles of genes and miRNAs across groups. **C**, Partial least-squares discriminant analysis (PLS-DA) analysis to differentiate 40 patients with APS using the signature of genes and miRNAs measurements in monocytes. Lack of overlap between the 3 groups of different genes and miRNAs profiles means well-defined molecular separation of patients with APS. **D**, Variable importance in projection plot (VIP): genes and miRNAs identified by PLS-DA in descending order of importance. The graph represents the relative contribution of these genes and miRNAs to the variance between the 3 clusters. The green and red boxes on the **right** indicate whether the gene and miRNA expression are increased (red) or decreased (green) in the monocytes of 40 patients with APS across groups. **E**, Clinical and laboratory parameters of patients with APS belonging to the 3 clusters. **F**, Bar graph shows % of cardiovascular (CV) risk factors in patients with APS belonging to the 3 clusters. **G**, Pie chart shows the distribution of adjusted global antiphospholipid syndrome score (aGAPSS) among the three clusters of patients with APS and the distribution of patients with APS with pathological carotid intima-media thickness (CIMT) among these three groups of patients with APS with high, medium, and low levels of aGAPSS. **H**, Heatmap obtained from the hierarchical clustering of the 40 patients with APS in three clusters, according to the similar and differential expression levels of 27 cytokines/chemokines/adhesion molecules in the plasma of these patients using MetaboAnalyst 4.0. aCL indicates anticardiolipin antibodies; AHT, arterial hypertension; anti-β2GPI, anti -beta2-glycoprotein I; DLP, dyslipidemia; FGF, fibroblast growth factor; GCSF, granulocyte colony-stimulating factor; IFN, interferon; IL, interleukin; IL-1RA, interleukin-1 receptor antagonist; IP10, interferon-inducible protein 10; MCP-1, monocyte chemoattractant protein-1; MIP1b, macrophage inflammatory protein 1beta; PC, principal component analysis; TNF, tumor necrosis factor; and VEGF, vascular endothelial growth factor.

others. These molecular alterations have been previously reported to be directly associated with atherosclerosis and thrombosis development.[21,22] In addition, deranged genes related to intracellular pathways implicated in cellular growth, inflammation, and immune reactions, were also recognized, implying a central role in the pathogenesis of APS. Accordingly, a cell-signaling activation array demonstrated that a number of those intracellular mediators, which have been demonstrated to control the expression of greater part of the prothrombotic and inflammatory molecules found deregulated in patients with APS[23]—and even being activated by aPLs[24–26]— displayed a phosphorylated status, underlying their key role as molecular pathways involved in the disease activity of this autoimmune condition.

In parallel, a miRNA array in monocytes identified numerous miRNAs differentially expressed in patients with APS which, interestingly, were associated with pathways involved in the regulation of the immune system, inflammation, and CVD.

Bioinformatic integrative approaches allowed the characterization of an interaction network among deregulated genes and miRNAs, intimately connected and associated with both, the autoimmune profile of patients with APS, and the development of atherosclerosis, thrombosis, and renal disorders. Moreover, we could demonstrate in our cohort of patients with APS a close relationship among those miRNAs and their potential targets in monocytes, both in terms of simultaneous and

inverse altered in vivo expression and by the observed specific inhibition of several target genes after inducing the overexpression of their regulating miRNAs in monocytes purified from patients with APS.

Among the deregulated miRNAs, those selected for transfection, which showed the highest number of gene targets on in silico analyses, were closely associated with the development of thrombosis, atherosclerosis, and obstetric complications. Thus, miR-145 has been demonstrated to participate in the stabilization of atheromatous plaque.[27] In addition, a recent study has demonstrated that this microRNA is a key molecule for regulating thrombus formation in venous thrombosis so that it has as a main target the procoagulant factor tissue factor.

Likewise, miR30b has been implicated in diverse cardiac remodeling[28] and ischemic heart disease.[29]

Equally, miR146b-5p is a key regulator of inflammation, endothelial activation, and atherosclerosis[30] and has been involved in several cardiovascular disorders, such as type 2 diabetes[31] and myocardial infarction.[32] Lastly, miR199a-5p has been found to be upregulated in placental tissues of patients with preeclampsia[33] and downregulated in patients with venous thromboembolism.[34] Interestingly, in the present study, the induced overexpression of three of these miRNAs (miR30b-5p, miR-145-5p, and miR199a-5p) promoted the inhibition of the procoagulant activity in APS monocytes, identified by a reduction of the TF activity. TF is the main initiator of the coagulation cascade and elements that upregulate its expression might promote thrombotic events. Moreover, APS is characterized by a high monocyte TF expression, intimately associated with the development of thrombosis.[24] Thus, the present study suggests that endogenous levels of these miRNAs may be critical for the aberrant activity of monocytes in APS and thus involved in the development of thrombosis.

To assess the specificity of mRNA and miRNA signatures in APS, we analyzed the expression of validated mRNA/miRNA in a new cohort of patients, characterized by previous thrombotic events without associated autoimmune diseases.

These analyses showed differential expression patterns between these 2 cohorts. Our results confirm previous studies that showed the unique mRNA/miRNA profiles of monocytes and neutrophils in thrombotic non-autoimmune patients.[11]

Compared with patients with APS, this may reflect the differential pattern of gene and miRNA regulation and activity in patients with thrombosis and support the idea that the role of autoantibodies in APS may be crucial. The in vitro effects of APS-IgG on the monocyte expression of mRNAs and miRNAs found in our experimental setting further supported this hypothesis.

In primary APS, aPL-IgG has been shown to be an independent predictor of both, thrombosis and increased intima-media thickness, the main marker of atherosclerotic vascular disease.[24,35] In this study, we provided further proof that aPL may be an additional risk for thrombosis in APS, as demonstrated, in vivo, by the relationship between aPL-IgG titers and the levels of several monocytes mRNAs/miRNAs that codify for proteins associated to inflammation and thrombosis development and, in vitro, by the modulated expression of several of those molecules after treatment of healthy monocytes with APS-IgG antibodies isolated from patients with thrombotic APS. In addition, our data show effects of aPL-IgG on the secretion by monocytes of some circulating miRNAs related to CVD.

Vascular inflammation is an early step in the development of atherosclerosis, and many genes and miRNAs induce EC (endothelial cells) inflammation.[36] Accordingly, we could demonstrate, in vivo, a direct relationship among several deranged genes and miRNAs in monocytes and the presence of an endothelial microvascular dysfunction in patients with APS. Moreover, we shown that monocytes activated by APS-IgG autoantibodies secrete several genes/miRNAs that promote aberrant endothelial cell activation. In addition, transfection studies showed that the induced overexpression of miR-146b-5p reduced the cell adhesion capacity of monocytes. Monocyte adhesion is considered an initial step in the development of the atherosclerotic plaque, which is influenced by a range of signal molecules and by the state of endothelial cells.[37] Thereby, our study adds relevant information to this still unknown process, by showing the potential involvement of reduced monocyte miR146b-5p expression on the development of atherosclerosis in the setting of APS.

Clustering analyses allowed to identify subgroups of patients with APS showing specific molecular shapes that further displayed distinctive clinical profiles. Thus, patients with the highest incidence of thrombosis, prevalence of autoantibodies and cardiovascular risk factors, and presence of atheroma plaques, while suffering scarce obstetric complications (cluster 1), demonstrated to have on their monocytes the most significant number of deregulated genes and miRNAs, involved in the triggering of the coagulation cascade as well as on the proliferation, adhesion, and migration of cells to inflammatory sites. On the contrary, the youngest patients, with high incidence of thrombosis and obstetric complications, but low positivity for aPL antibodies and the lowest prevalence of cardiovascular risk factors (cluster 3), showed no significant alterations on the expression of these genes/miRNAs. Furthermore, a specific inflammatory profile in APS patients' plasma was identified, on which patients belonging to each cluster displayed specific and distinctive expression patterns of interleukins, chemokines, and growth factors involved in vascular damage and hematopoiesis. Those results allowed the identification of specific signatures of genes, miRNA, and proteins that might constitute potential biomarkers susceptible to being therapeutically modified to control the cardiovascular pathology of patients with APS.

Our overall data essentially agree with that obtained recently by Ripoll and coworkers[9] with reference to the genes that are essential in thrombotic and obstetric APS, based on the differential expression and variability of genes found in their study in monocytes exposed, in vitro, to IgG from patients with APS with thrombosis or pregnancy morbidity. Yet, as also stated by these authors, patients with APS are highly complex and heterogeneous. Thus, in our hands, a more multifaceted picture derived from the categorization of patients with APS according to their molecular profiles (clustering), and the subsequent documentation of their clinical profiles, so that, over and above the influence of aPLs, the simultaneous occurrence of obstetric complications and thrombotic events in a high proportion of patients, the concomitance of cardiovascular risk factors, and the presence of atheroma plaques in a significant proportion of them, might turn far more numerous and complex the factors that promote the altered molecular profile displayed by the monocytes in these patients. Consequently, this molecular profile appeared largely altered and complex in patients with simultaneous disease traits or increased risk of suffering those pathologies.

The main limitation of this study would be the relatively small number of patients recruited so that clustering analysis allowed the classification of a reduced number of subjects on each group. However, the groups identified were homogeneous on their clinical profiles, in terms of incidence and recurrences of thrombotic events, treatments received, or time of disease evolution, among others. Moreover, the molecular profile on each group demonstrated also homogeneity in parameters such as prothrombotic/proinflammatory profile or alterations of mRNA or miRNA profiles. In this sense, a recent study, developed in a large cohort of aPL-positive patients belonging to the APS action consortium, performed a clustering analysis that allowed the identification of well-defined clinical phenotypes among these patients.[38] Although that study also involved other autoimmune diseases (ie, lupus) and was only restricted to clinical profiles, its results demonstrated the suitability of this approach to stratify patients with APS, thus supporting that integrated clinical and molecular analyses might allow a deeper understanding of APS disease mechanisms.

Although a number of previous studies emphasized the relevance of monocytes on the development of thrombosis in APS, more recently, the significance of other innate and adaptive immune cells in APS pathology has been established. Thus, it has been demonstrated that aPLs have the potential to activate monocytes, neutrophils, and plasmacytoid dendritic cells. Disturbances in T-cell homeostasis reported in patients with APS further contribute to the immunopathology of APS.[39] Therefore, to delineate the role of immune response in the physiopathology of APS, and to reveal good prognostic biomarkers, it would be necessary to develop an integrated immune cell profiling approach. New transcriptomic analyses in patient blood with single-cell RNA sequencing would add new and interesting information in this aspect.

Taken together, our whole data suggest that (1) specific miRNA-mRNA regulatory networks in monocytes seem to regulate the biological processes related to the pathology in APS. (2) The integrated transcriptome and protein profile allowed the identification of central genes, miRNAs, and altered pathways in monocytes and plasma of patients with APS, which are particularly relevant to the unique characteristics of the disease and are at least partially regulated by aPL. Based on these premises, new studies involving larger cohorts, on which integrated clinical and molecular analyses were developed, might allow a deeper understanding of APS disease mechanisms.

Overall, our study demonstrated that the extensive molecular profiling of monocytes and plasma in patients with primary APS might help to identify distinct clinical phenotypes, which, in turn, might facilitate a patient-tailored treatment.

ARTICLE INFORMATION

Received August 6, 2020; accepted December 11, 2020.

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Acknowledgments

L. Pérez-Sánchez, A.M. Patiño-Trives, and M. Luque-Tévar developed the in vivo assays and in vitro experiments and resolved technical problems; M.A. Aguirre-Zamorano, F. Velasco-Gimena, P. Seguí, and E. Collantes-Estévez followed up with patients and gave suggestions and useful discussion; N. Barbarroja made statistical analysis and discussed results; M.C. Ábalos-Aguilera and I. Arias-de la Rosa performed some experiments and analyzed the data. C. Pérez-Sánchez and C. López-Pedrera formed the hypothesis, directed and coordinated the project, designed the experiments, analyzed the data, and wrote the article. We thank all patients and healthy subjects for their participation in this study.

Sources of Funding

This study was supported by grants from the Instituto de Salud Carlos III (PI18/00837), cofinanciado por el Fondo Europeo de Desarrollo Regional de la Unión Europea "Una manera de hacer Europa," Spain, and the Spanish Inflammatory and Rheumatic Diseases Network (RIER), Instituto de Salud Carlos III (RD16/0012/0015). C. López-Pedrera was supported by a contract from the Spanish Junta de Andalucía ("Nicolas Monardes" program).

Disclosures

None.

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