Circulating microRNAs as biomarkers of disease and typification of the atherothrombotic status in antiphospholipid syndrome

Carlos Pérez-Sánchez,[1*] Iván Arias-de la Rosa,[1*] María Ángeles Aguirre,[1,2] María Luque-Tévar,[1] Patricia Ruiz-Limón,[1] Nuria Barbarroja,[1] Yolanda Jiménez-Gómez,[1] María Carmen Ábalos-Aguilera,[1] Eduardo Collantes-Estévez,[1,2,3] Pedro Segui,[1,4] Francisco Velasco,[5] María Teresa Herranz,[6] Jesús Lozano-Herrero,[6] María Julia Hernandez-Vidal,[6] Constantino Martínez,[7] Rocío González-Conejero,[7] Massimo Radin,[8] Savino Sciascia,[8] Irene Cecchi,[8] María José Cuadrado[9] and Chary López-Pedrera[1,2**]**

[1]Instituto Maimónides de Investigación Biomédica de Córdoba (IMIBIC), Spain; [2]Unidad de Gestión Clínica Reumatología, Hospital Universitario Reina Sofía, Córdoba, Spain; [3]Departamento de Medicina (Medicina, Dermatología y Otorrinolaringología), Universidad de Córdoba, Spain; [4]Unidad de Gestión Clínica Radiología, Hospital Universitario Reina Sofía, Córdoba, Spain; [5]Unidad de Gestión Clínica Hematología, Hospital Universitario Reina Sofía, Córdoba, Spain; [6]Servicio de Medicina Interna, Hospital Morales Meseguer, Murcia, Spain; [7]Centro Regional de Hemodonación, Universidad de Murcia, IMIB-Arrixaca, Spain; [8]Department of Clinical and Biological Sciences, Center of Research of Immunopathology and Rare Diseases, Torino, Italy and [9]Lupus Research Unit and St. Thomas' Hospital, London, UK

**CP-S and IA-R shared first authorship and contributed equally to this work. **MJC and CL-P shared last authorship and contributed equally to this work.*

 doi:10.3324/haematol.2017.184416

Received: November 10, 2017.

Accepted: February 22, 2018.

Pre-published: March 15, 2018.

Correspondence: rosario.lopez.exts@juntadeandalucia.es

SUPPLEMENTAL MATERIAL

Circulating microRNAs as biomarkers of disease and typification of the atherothrombotic status in Antiphospholipid Syndrome

Carlos Perez-Sánchez,[1*] Iván Arias-de la Rosa,[1*] María Ángeles Aguirre,[1,2] María Luque-Tévar,[1] Patricia Ruiz-Limón,[1] Nuria Barbarroja[1], Yolanda Jiménez-Gómez,[1] María Carmen Ábalos-Aguilera,[1] Eduardo Collantes-Estévez,[1-3] Pedro Segui,[1,4] Francisco Velasco,[5] María Teresa Herranz,[6] Jesús Lozano-Herrero,[6] María Julia Hernandez-Vidal,[6] Constantino Martínez,[7] Rocío González-Conejero,[7] Massimo Radin,[8] Savino Sciascia,[8] Irene Cecchi,[8] María José Cuadrado[9#] and Chary López-Pedrera[1,2#]

[1]Instituto Maimónides de Investigación Biomédica de Córdoba (IMIBIC), España.
[2]Unidad de Gestión Clínica Reumatología. Hospital Universitario Reina Sofía, Córdoba, España.
[3]Departamento de Medicina (Medicina, Dermatología y Otorrinolaringología), Universidad de Córdoba, España.
[4]Unidad de Gestión Clínica Radiología, Hospital Universitario Reina Sofía, Córdoba, España.
[5]Unidad de Gestión Clínica Hematología, Hospital Universitario Reina Sofía, Córdoba, España.
[6]Servicio de Medicina Interna, Hospital Morales Meseguer, Murcia, España.
[7]Centro Regional de Hemodonación, Universidad de Murcia, IMIB-Arrixaca, España;
[8]Department of Clinical and Biological Sciences, Center of Research of Immunopathology and Rare Diseases, Torino, Italy.
[9]Lupus Research Unit and St. Thomas Hospital, London, United Kingdom

*CP-S and IA-R shared first authorship and contributed equally to this work.
[#]MJ C and CL-P shared last authorship and contributed equally to this work.

DETAILED METHODS

Patients
Ninety patients with primary APS and 42 healthy donors (HDs) were included in this study, during a period of 24 months. All experimental protocols were approved by the ethics committee of the Reina Sofia Hospital in Cordoba (Spain) and written informed consent was obtained. All methods were carried out in accordance with approved guidelines. Subjects were selected among patients with stable disease for more than 6 months, without infections, abortions, thrombosis, or changes in their treatment protocol. None of the HDs had a history of autoimmune disease, bleeding disorders, thrombosis, or pregnancy losses

Blood sample collection and assessment of biological parameters
Whole blood from subjects was collected by direct venous puncture either, into tubes with ethylenediaminetetraacetic acid as an anticoagulant, or into specific tubes for obtaining serum. All the blood was processed for the isolation of plasma -within 4 hours of collection- by spinning at 2,000 × g for 10 minutes at room temperature. Then, plasma and serum were transferred to a fresh RNase-free tube and stored at −80°C. Plasma levels of monocyte chemotactic protein (MCP-1), plasminogen activator inhibitor-1 (PAI-1), vascular endothelial growth factor A (VEGF-A) and VEGF-receptor-1 (Flt-1), were quantified using ProcartaPlex multiplex immunoassay, following the manufacturer's recommendations (AffymetrixeBioscience, Vienna, Austria). Plasma levels of TF were determined by ELISA [Human Tissue factor (CD142) ELISA Abcam, Cambridge, MA, US].

B-Mode Ultrasound IMT and Ankle Brachial Index measurements
B-mode ultrasound imaging of the carotid arteries was performed in patients and HDs as described previously[1,2] using Toshiba equipment (Aplio platform) equipped with 7- to 10-MHz broadband linear array transducers. Plaque was defined as a focal structure that encroached into the arterial lumen of at least 50% of the surrounding CIMT value or demonstrated a thickness more than 1.5 mm as measured from the media-adventicia interface to the intima-lumen interface (pathologic CIMT).
The ankle brachial index (ABI) was measured using a blood pressure cuff and a doppler ultrasound sensor. The method used was in accordance with a recent consensus statement on measuring the ABI[3] . The cuff was applied to both arms and ankles. The doppler probe was used to determine systolic blood pressure in both brachial arteries in the antecubital fossa, in the right and left posterior tibial arteries and the right and left dorsalis pedis arteries. A 12-cm cuff was inflated to 20 mm Hg above the systolic arterial pressure and slowly deflated. With an 8 MHz doppler probe (mod MD200) we obtained the systolic arterial pressure when the first doppler signal was heard. The ankle brachial index for each leg was calculated as the ratio of the higher of the two systolic pressures (posterior tibial or dorsalis pedis) in the leg and the higher systolic pressure of either the left or right arm.

Isolation of miRNAs
A total of 200 µl of plasma or supernatant from *in vitro* studies were thawed on ice and lysed in 1 mL QIAzol Lysis Reagent (Qiagen). Samples in QIAzol were incubated at room temperature for 5 minutes to inactivate RNases. To adjust for variations in RNA extraction and/or copurification of inhibitors, 5 fmol of spike-in control non-human miRNA (C. elegans miR-39 miRNA mimic: 5′-UCACCGGGUGUAAAUCAGCUUG-3′) were added to the samples after the initial denaturation. The remaining extraction protocol was performed according to the manufacturer's instruction. Total RNA was eluted in 14 µl of RNase-free water and stored at −80°C.

miRNAs Expression Profiling

In a reverse-transcription reaction using miScript HiSpec Buffer from the miScript II RT kit (Qiagen), mature microRNAs (miRNAs) were polyadenylated by poly(A) polymerase and subsequently converted into cDNA by reverse transcriptase with oligo-dT priming. The formulation of miScript HiSpec Buffer facilitated the selective conversion of mature miRNAs into cDNA, while the conversion of long RNAs, such as mRNAs was suppressed. As a result, background signals potentially contributed by long RNA were non-existent. The cDNA prepared in a reverse-transcription reaction was used as a template for real-time PCR analysis using miScript miRNA PCR array (which contains miRNA-specific miScript Primer Assays) and the miScript SYBR Green kit, (which contains the miScript Universal Primer, reverse primer, and QuantiTect SYBR Green PCR Master Mix). To profile the mature miRNA expression, a premix of cDNA, miScript Universal Primer, QuantiTect SYBR Green PCR Master MIX, and RNAse-free water, was added to a miScript miRNA PCR array. That array was provided in a 96-well plate format and included replicates of a miRNA reverse transcription control assay (miRTC) and a positive PCR control (PPC). Those were the quality control assays used to determine the presence of reverse transcription and real-time PCR inhibitors.

Raw data were analysed with the data analysis software for miScript miRNA PCR arrays. The expression levels of miRNAs were normalized to the mean of spiked-in miRNA Cel-miR-39 and were calculated using the 2-$\Delta\Delta$Ct method.

The reaction was conducted in a GeneAmp PCR System 9700 (Life Technologies) at 16°C for 30 minutes, 42°C for 30 minutes and 85°C for 5 minutes. A preamplification step was performed at 95°C for 10 minutes, 20 cycles of 95°C for 15 seconds, and 60°C for 4 minutes. Real-time PCR was carried out on a Roche LightCycler 480 (Roche Applied Science, Penzberg, Germany) at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute using the TaqMan microRNA assay along with TaqMan Universal PCR Master Mix, No AmpErase UNG (Applied Biosystems, San Francisco, CA, USA).

Target gene prediction and integrated analysis by ingenuity pathway analysis

The altered miRNAs were further analysed to obtain information about biological functions, pathways and networks by using the web-based bioinformatics tool QIAGEN's Ingenuity Pathway Analysis (IPA; Ingenuity Systems, http://www.INGENUITY.com). For this purpose, all differentially regulated miRNAs and fold changes were imported into IPA.[4] The right-tailed Fisher's exact test was used to calculate the p-value determining the statistical probability that the association between a set of molecules and a pathway or function might be due to chance alone.

Purification of IgG and in vitro exposure of monocytes and endothelial cells to aPL antibodies

Total IgG from the pooled sera of 7 APS patients, characterized by high titres of aCL and anti-β2GPI antibodies, were purified by protein G-Sepharose high-affinity chromatography (MAbTrap kit; Amersham Biosciences) following the manufacturer's recommendations. Anti-β2GPI and IgG-aCL activities of purified IgG were confirmed by enzyme-linked immunosorbent assays (QUANTA Lite® ß2GPI-IgG and QUANTA Lite® ACA IgG III kits, Inova Diagnostics; San Diego, CA, USA). For in vitro studies, monocytes isolated from HDs were incubated with human IgG (500 µg/mL) (Jackson InmunoResearch Laboratories, Inc, Newmarket, Suffolk, UK) or purified APS patient-IgG (500 µg/mL) for 6 hours at 37 °C in RMPI medium without FBS, which could contain exogenous miRNAs. Primary human umbilical vein endothelial cells (HUVECs) were purchased from Lonza Group Ltd (Basel, Switzerland) and cultured in Endothelial Basal Medium (EBM, Lonza, Walkersville, MD USA) supplemented with 10% fetal bovine serum (FBS, Lonza), 0.1% human epidermal growth factor (hEGF, Lonza), 0.1% hydrocortisone (Lonza), 0.1% Gentamicin-Amphotericin-B (GA-1000,

Lonza), 0.4% bovine brain extract (BBE, Lonza), and 1% Zell Shield (Minerva Biolabs, GmbH, Berlin, Germany) at 37 °C and 5% CO_2. Confluent cell monolayers were treated for 24 hours at 37 °C with aPL-IgG and control-IgG, as described above in the absence of FBS. Four independent experiments –performed in triplicate- were carried out on passage 4. Supernatants were collected to evaluate the expression levels of the miRNAs and potential target proteins released.

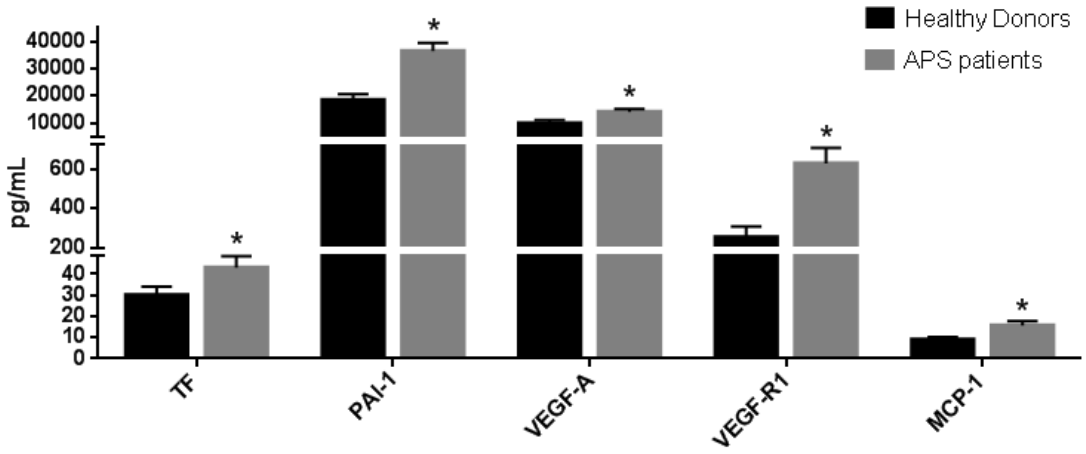
Statistical analysis

All data were expressed as mean ± SD. Statistical analyses were performed with SSPS 17.0 (SPSS Inc, Chicago, IL, USA). Following normality and equality of variance tests, clinical characteristics were compared using paired Student's t-test or alternatively by a non-parametric test (Mann-Whitney rank sum test). Paired samples within the same subjects were compared by Wilcoxon signed-rank test. Differences among groups of treatments were analysed by repeated measures ANOVA. Correlations were assessed by Spearman's rank correlation. Differences were considered significant at $P < 0.05$. A Bonferroni correction was applied for multiple testing in both, one-way Anova analysis and in correlation studies. Receiver-operator characteristics (ROC) curves, plotting the true positive rate (sensitivity) versus the false positive rate (1-specificity) at various threshold settings, and the areas under the curve (AUC) analysis were used to determine the sensitivity, specificity and corresponding cut-off values for each plasma miRNA using SPSS. Logistic regression was used to develop composite panels of biomarkers to identify signatures that could distinguish APS from control and pathologic CIMT with the greatest sensitivity and specificity. ROC analysis and arithmetic mean of level expression for miRNA-combined was calculated. $P < 0.05$ was considered statistically significant. Subsequently, in order to stratify APS patients according to their relative thrombotic risk, we performed a cluster analysis with hard clustering method. Variables included in the cluster analysis were cardiovascular risk factors (dyslipidemia, arterial hypertension, smoking, diabetes) and aPL profile (LA, aCL IgG/IgM, anti-β2GPI IgG/IgM). Evaluation fields included arterial or venous thrombosis, recurrences and miRNAs ratio profile. One-way ANOVA and t-test were used to assess significant differences between clusters regarding the evaluation fields.

References

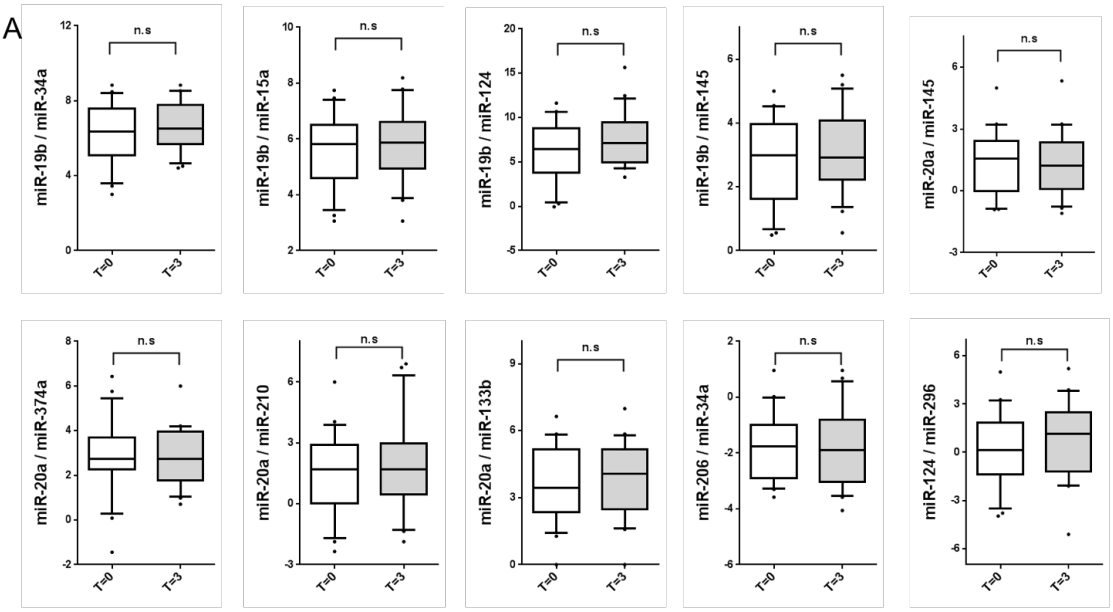
1. Ames PR, Antinolfi I, Scenna G, Gaeta G, Margaglione M, Margarita A. Atherosclerosis in thrombotic primary antiphospholipid syndrome. J Thromb Haemost. 2009;7(4):537-542.
2. Touboul PJ, Hennerici MG, Meairs S, et al. Mannheim carotid intima-media thickness consensus (2004-2006). An update on behalf of the Advisory Board of the 3rd and 4th Watching the Risk Symposium, 13th and 15th European Stroke Conferences, Mannheim, Germany, 2004, and Brussels, Belgium, 2006. Cerebrovasc Dis. 2007;23(1):75-80.
3. Sacks D, Bakal CW, Beatty PT, et al. Position statement on the use of the ankle-brachial index in the evaluation of patients with peripheral vascular disease. A consensus statement developed by the standards division of the Society of Cardiovascular and Interventional Radiology. J Vasc Interv Radiol. 2002;13(4):353.
4. Mayburd AL, Martlínez A, Sackett D, et al. Ingenuity network-assisted transcription profiling: Identification of a new pharmacologic mechanism for MK886. Clin Cancer Res. 2006;12(6):1820-1827.

SUPPLEMENTAL FIGURES



Supplemental Figure S1. Protein Levels of potential targets of selected microRNA in APS. Differential expression of potential proteins targets of the microRNAs selected between antiphospholipid syndrome patients (APS patients) and healthy donors at plasma level. Differences were analyzed by means of Student's t-test. Statistical significance was taken as $p<0.05$. TF indicates tissue factor; PAI-1, plasminogen activator inhibitor-1; VEGF-A, vascular endothelial growth factor A; VEGF-R1, VEGF-Receptor-1; and MCP-1, monocyte chemotactic protein.

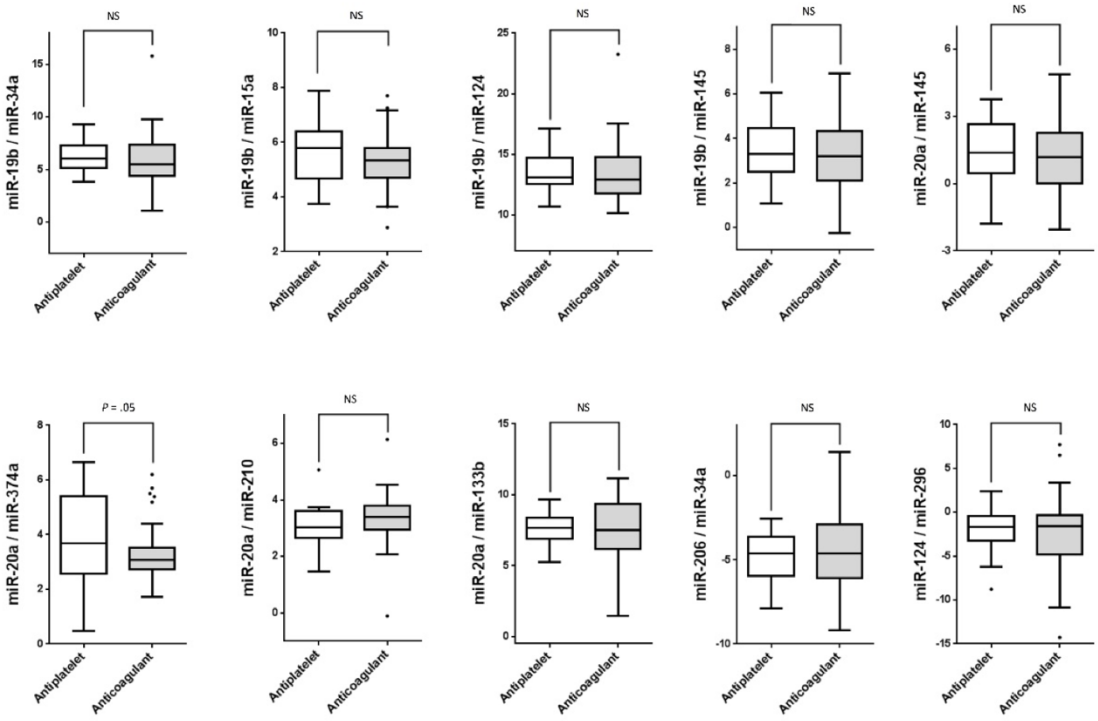
A



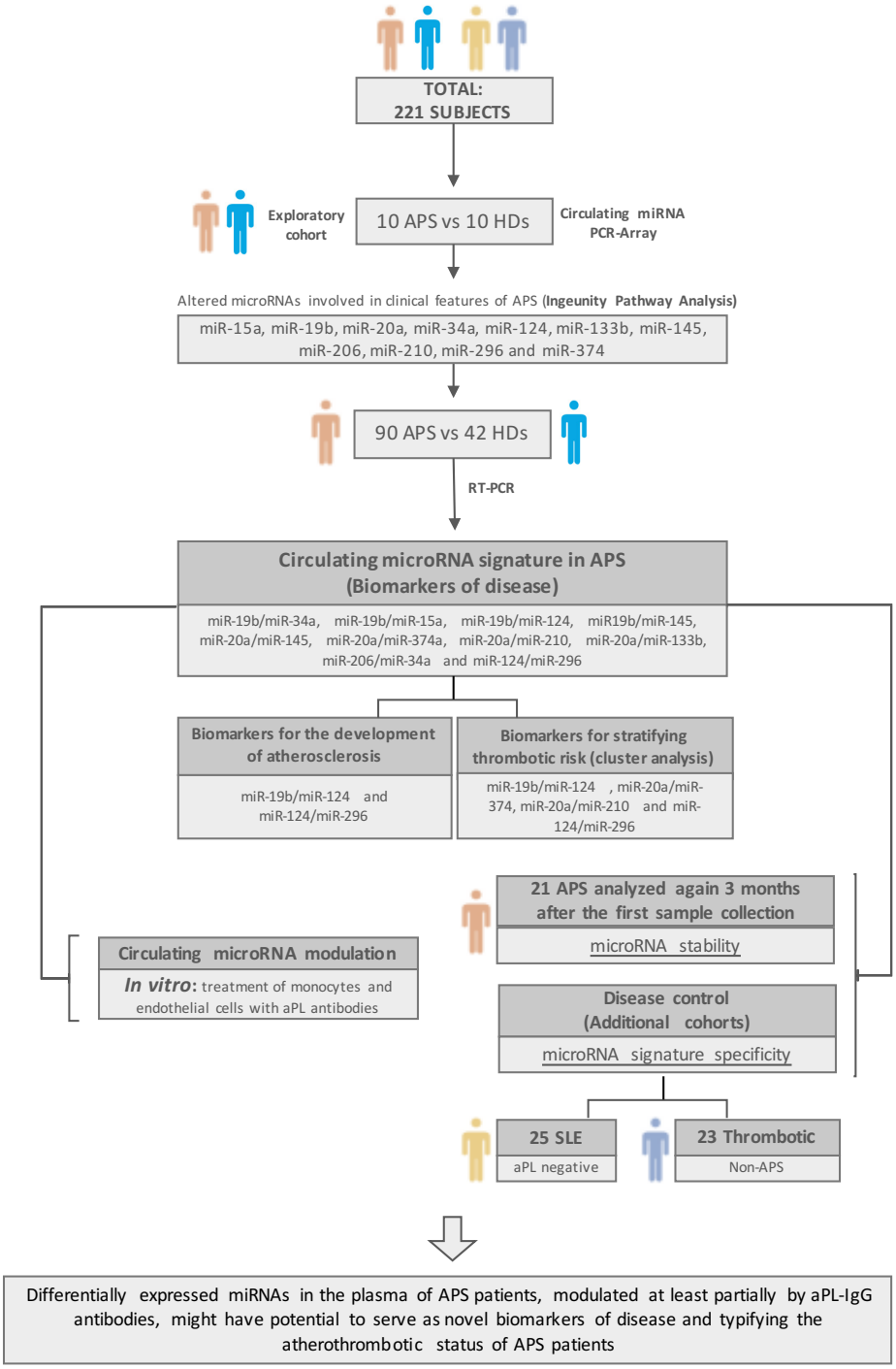
B

miR/miR	19b/34a	19b/15a	19b/124	19b/145	20a/145	20a/374a	20a/210	20a/133b	206/34a	124/296
	TIME 0	TIME 0	TIME 0	TIME 0	TIME 0	TIME 0	TIME 0	TIME 0	TIME 0	TIME 0
TIME 3	r: 0.44 p: 0.05	r: 0.46 p: 0.03	r: 0.55 p: 0.01	r: 0.50 p: 0.02	r: 0.57 p: 0.01	r: 0.53 p: 0.02	r: 0.52 p: 0.01	r: 0.52 p: 0.01	r: 0.60 p: 0.00	r: 0.45 p: 0.05

Supplemental Figure S2. Stability of miRNA expression profile over time in APS patients. Levels of miRNA ratios profile among 21 APS patients at baseline and three months later. A) Boxes indicate the interval between the 25th and 75th percentiles and horizontal bars inside boxes indicate median. Whiskers indicate the interval of data within 1.5 × interquartile ranges (IQR). Closed circles indicate data points outside 1.5 x IQR. Ns, indicates no significant statistical difference. B) Correlation study of miRNA ratios profile between time 0 and time 3. r, Spearman's rank correlation coefficient; p denotes p value (calculated probability).



Supplemental Figure S3. Evaluation of altered circulating microRNA ratios in APS in relation to the treatments received. Comparison of circulating microRNA (miR) ratio levels between APS patients taking antiplatelet or anticoagulant agents. Boxes indicate the interval between the 25th and 75th percentiles and horizontal bars inside boxes indicate median. Whiskers indicate the interval of data within 1.5 × interquartile ranges (IQR). Closed circles indicate data points outside 1.5 x IQR. * Statistical significance was taken as $P < .05$. NS indicates no significant statistical difference.



Supplemental Figure S4. Flow chart of the study. The total number of subjects included in the study were 221. In a first step a circulating miRNA PCR Array was performed in the exploratory cohort. Then, 11 altered miRNAs involved in clinical features of APS were selected to determine their expression in the whole cohort of APS patients and HDs. A signature of 10 miRNA ratios was identified as biomarkers for diagnosis, along with the development of atherosclerosis and typifying the thrombotic risk in APS patients. This signature was stable over time and distinct from two additional disease controls (thrombotic non-APS and aPL-negative SLE). In vitro studies with aPLs and in vivo supplementation with ubiquinol modulated the expression of the altered miRNA signature in APS. miR, microRNA; APS, antiphospholipid syndrome; HDs, healthy donors; SLE, systemic lupus erythematosus; aPLs, antiphospholipid antibodies; Qred, ubiquinol.

SUPPLEMENTAL TABLES

Supplemental Table S1. Clinical and laboratory parameters of the SLE patients

CLINICAL AND LABORATORY PARAMETERS	SLE (total no. 25)
Females/males, no.	22/3
Age, years	36 ± 12
SLEDAI	2.4 ± 1.7
Thrombosis, no.	2/25
Neprhopathy, no.	7/25
Obesity, no.	4/25
Hypertension, no.	4/25
Pathologic CIMT, no.	2/25
Anti-ds-DNA positivity, no	11/25
aPL positivity, no.	0/25
Corticosteroids, no.	4/25
Antimalarials, no.	25/25
Antiplatelets, no.	11/25
Total cholesterol level,* mg/dL	173.1 ± 29.5
Cholesterol HDL level,* mg/dL	58.4 ± 26.5
Cholesterol LDL level,* mg/dL	100.4 ± 25.1
Triglycerides level,* mg/dL	85.4 ± 32.4
C reactive protein,* mg/dL	3.9 ± 6.6
C3,* mg/dL	103.8 ± 27
C4,* mg/dL	14.9 ± 6

SLE indicates the systemic lupus erythematosus; SLEDAI, Systemic Lupus Erythematosus Disease Activity Index; CIMT, carotid intima-media thickness; aPL, antiphospholipid antibodies; aPL, antiphospholipid antibodies; HDL, high-density lipoprotein; and LDL, low-density lipoprotein; *All results are expressed in mean ± SD.

Supplemental Table S2. Clinical and laboratory parameters of the exploratory cohort

CLINICAL AND LABORATORY PARAMETERS	APS (total no. 10)	HDs (total no. 10)	*P*
Females/males, no.	8/2	7/3	
Age,* years	49 ± 13	39 ± 7.6	NS
Arterial thrombosis, no.	6/10	0/10	
Venous thrombosis, no.	4/10	0/10	
Recurrences, no.	5/10	0/10	
Pregnancy morbidity, no.	4/10	0/10	
Obesity, no.	2/10	0/10	
Pathologic CIMT, no.	6/10	0/10	
LA positivity, no.	7/10	0/10	
aCL IgG,# GPL	86 (0-462)	1 (0-2)	.00
aCL IgM,# MPL	41 (0.5-321)	4.1 (0.5-9)	.00
Anti-β2GPI,# SGU	109.4 (0-387)	2 (0-2.7)	.00
Antiplatelet agents,† no.	6/10	0/10	
Anticoagulant agents,‡ no.	4/10	0/10	
Total cholesterol level,* mg/dL	196 ± 37	197 ± 32	NS
Cholesterol HDL level,* mg/dL	54 ± 14	61 ± 9.6	NS
Cholesterol LDL level,* mg/dL	123 ± 28	124 ± 27	NS
Triglycerides level,* mg/dL	80 ± 28	65 ± 16	NS
ESR,* mm/h	13 ± 6	5.1 ± 5	.05

APS indicates the antiphospholipid syndrome; HDs, healty donors; NS, not significant; CIMT, carotid intima-media thickness; ABI, ankle brachial index; aPL, antiphospholipid antibodies; LA, lupus anticoagulant; aCL, anti-cardiolipin antibodies; GPL, IgG phospholipid units; MPL, IgM phospholipid units; anti-β2GPI, anti-β2 glycoprotein 1 antibodies; SGU, stantard IgG units; HDL, high-density lipoprotein; LDL, low-density lipoprotein; and ESR, erythrocyte sedimentation rate;
*All results are expressed in mean ± SD; #Results are expressed in mean and values range. †antiplatelet agents include acetylsalicylic acid and clopidogrel; ‡anticoagulant agents indicate vitamin K antagonists, including warfarin and acenocumarol.

Supplemental Table S3. Correlation studies between circulating miRNAs and clinical and serological parameters in APS patients

	miR RATIO (miR/miR)	CORRELATION COEFFICIENT (r) AND p-VALUE (p)
CLINICAL PARAMETERS		
ABI-left	20a/374	r= 0.47 p=0.022
	20a/210	r= 0.56 **p=0.002**
aPL	20a/374	r= 0.36 p=0.007
	20a/210	r= 0.45 p=0.028
	20a/133b	r= 0.43 **p=0.001**
	20a/145	r= 0.33 p=0.019
ESR	19b/124	r= 0.35 p=0.031
	124/296	r= 0.33 p=0.049
SEROLOGICAL PARAMETERS		
TF	19b/34a	r= 0.33 p=0.042
	20a/133b	r= 0.32 p=0.044
PAI-1	19b/34a	r= 0.29 p=0.048
	19b/15a	r= 0.31 p=0.029
	19b/124	r= 0.37 p=0.024
	20a/133b	r= 0.35 **p=0.006**
VEGF-A	19b/145	r= 0.39 p=0.038
	19b/34a	r= 0.38 p=0.043
	19b/15a	r= 0.40 p=0.034
	206/34a	r= 0.39 p=0.039
VEGF-R1/Flt-1	19b/145	r= 0.43 **p=0.006**
	19b/34a	r= 0.65 **p=0.003**
	19b/15a	r= 0.34 p=0.042
	19b/124	r= 0.38 p=0.029
	20a/145	r= 0.33 p=0.048
	20a/374	r= 0.37 p=0.024
	206/34a	r= 0.44 **p=0.006**
MCP-1	19b/34a	r= 0.28 p=0.046
	19b/15a	r= 0.27 p=0.047
	20a/210	r= 0.34 **p=0.006**

Spearman´s rank correlations between clinical and serological parameters and miRNA ratios showing a p<0,05 are indicated. According to Bonferroni correction a number of correlations, indicated in bold, were found significant. ABI indicates ankle brachial index; APS, antiphospholipid syndrome; ESR, erythrocyte sedimentation rate; TF, tissue factor; PAI-1, plasminogen activator inhibitor-1; VEGF-A, vascular endothelial growth factor A; VEGF-R1/Flt-1, vascular endothelial growth factor receptor-1; and MCP-1, monocyte chemoattractant protein-1.