INSTITUTO MAIMÓNIDES DE INVESTIGACIÓN BIOMÉDICA DE CÓRDOBA

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PAPEL DE LOS MICROARNS Y LA INFLAMACIÓN EN LA PATOGÉNESIS DE ENFERMEDADES AUTOINMUNES

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ROLE OF MICRORNAS AND INFLAMMATION IN THE PATHOGENESIS OF AUTOIMMUNE DISEASES

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TITULO: *Papel de los microARNs y la inflamación en la patogénesis de enfermedades autoinmunes*

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PAPEL DE LOS MICROARNS Y LA INFLAMACIÓN EN LA PATOGÉNESIS DE ENFERMEDADES AUTOINMUNES

Memoria presentada por

Iván Arias de la Rosa

Graduado en Biología y especializado en Investigación Biomédica Traslacional, para optar al grado de **Doctor en Biomedicina**

Tesis doctoral realizada bajo la dirección de la doctora Nuria Barbarroja Puerto, la doctora Rosario López Pedrera y el doctor Carlos Pérez Sánchez en el Instituto Maimónides de Investigación Biomédica de Córdoba (IMIBIC).

Programa de Doctorado en Biomedicina

El doctorando

Iván Arias de la Rosa

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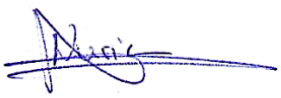	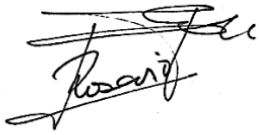	
Nuria Barbarroja Puerto	Rosario López Pedrera	Carlos Pérez Sánchez

TÍTULO DE LA TESIS: Papel de los microARNs y la inflamación en la patogénesis de enfermedades autoinmunes

DOCTORANDO: Iván Arias de la Rosa

INFORME RAZONADO DE LOS DIRECTORES DE LA TESIS

(se hará mención a la evolución y desarrollo de la tesis, así como a trabajos y publicaciones derivados de la misma).

Don Iván Arias de la Rosa presenta un trabajo original en el que se ha analizado el papel de la inflamación y los miRs en la patogénesis del Síndrome Antifosfolípido Primario y Artritis Reumatoide mediante la integración de análisis inmunológicos, celulares, moleculares y epigenéticos.

Los resultados obtenidos de este trabajo se han presentado en numerosos congresos nacionales e internacionales y han sido publicados en tres artículos en revistas científicas de reconocido prestigio internacional en el campo de la investigación en biomedicina: Dos en *Haematologica* (Decil 1) y uno en *Journal of Internal Medicine* (Decil 1).

Además el doctorando realizó una estancia predoctoral de tres meses en el "*Department of Clinical and Biological Sciences", "Center of Research of Immunopathology and Rare Diseases-Coordinating Center of Piemonte and Valle d'Aosta Network for Rare Diseases", "Saint Giovanni Bosco Hospital", "University of Turin*", Italia, bajo la dirección de los Doctores Savino Sciascia y Dario Rocatello, ambos investigadores de reconocido prestigio en el ámbito del Síndrome Antifosfolípido.

La tesis doctoral presentada se enmarca dentro de diversos proyectos de investigación financiados por el Fondo de Investigación Sanitaria (CP15/0158, PI17/01316, PI15/01333 y PI18/00837).

Finalmente, cabe destacar la formación técnica y científica alcanzada por el doctorando. El desarrollo de la tesis le ha permitido adquirir conocimientos teóricos y metodológicos que lo capacitan para desarrollar nuevas hipótesis y participar activamente en la redacción y coordinación de nuevos artículos científicos y proyectos de investigación.

Por todo ello, se autoriza la presentación de la tesis doctoral.

Córdoba, 6 de Junio de 2020

Firma del/de los director/es

Nuria Barbarroja Puerto	Rosario López Pedrera	Carlos Pérez Sánchez
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TÍTULO DE LA TESIS: Papel de los microARNs y la inflamación en la patogénesis de enfermedades autoinmunes

DOCTORANDO/A: Iván Arias de la Rosa

INFORME RAZONADO DEL TUTOR

(Ratificando el informe favorable del director. Sólo cuando el director no pertenezca a la Universidad de Córdoba).

El estudio que se presenta en esta tesis doctoral integra diferentes análisis inmunológicos, celulares y moleculares para dar respuesta al papel que presentan los microARNs y la inflamación en la patogénesis de enfermedades autoinmunes sistémicas como el Síndrome Antifosfolípido y la Artritis Reumatoide.

El desarrollo de este trabajo ha dado lugar a la publicación de artículos de alto impacto englobados en revistas de primer decil. Asimismo, los resultados de estos estudios han sido presentados en congresos nacionales como el Congreso Nacional de la Sociedad Española de Reumatología (SER) e internacionales como el congreso de la "*European League against Rehumatism, EULAR*" y el "*American College of Rheumatology (ACR) Congress*". Además, hay que resaltar que estos artículos han sido objeto de varios premios como "el premio SER de Ciencia Básica 2019 al mejor trabajo presentado sobre cualquier aspecto de ciencia básica publicado en el 2018 a "Defective glucose and lipid metabolism in rheumatoid arthritis is determined by chronic inflammation in metabolic tissues", recibiendo también el XVII premio Nacional de Investigación Ilustre Colegio de Médicos de Córdoba patrocinado por CaixaBank: el III premio. Asimismo, el trabajo titulado "Circulating microRNAs as biomarkers of disease and typification of the atherothrombotic status in antiphospholipid syndrome" ha recibido el premio SER de otras enfermedades autoinmunies sistémicas 2019 al mejor trabajo presentado sobre cualquier aspecto de otras enfermedades autoinmunes sistémicas publicado en el año 2018. Finalmente, el trabajo titulado "Impaired microRNA processing in neutrophils from rheumatoid arthritis patients confers their pathogenic profile. Modulation by biological therapies" ha recibido el premio a la mejor comunicación oral de la sección "Multidisciplinar" de las IX Jornadas de Jóvenes investigadores del IMIBIC (2018).

Por lo tanto, el trabajo presentado reúne, a mi juicio, los méritos suficientes para optar al grado de Doctor. Por todo ello, se autoriza la presentación de la tesis doctoral.

Córdoba, 6 de Junio de 2020
Firma del responsable de línea de investigación

Fdo.: Eduardo Collantes Estévez

AGRADECIMIENTOS

En primer lugar, me gustaría agradecer a todas las personas que han formado parte de mi vida durante el desarrollo de esta tesis y que han contribuido a que evolucionara de manera personal y profesional.

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Dr. Carlos Pérez ("Maaaaaano"), el mejor, gracias por ver en mí lo que podía llegar a ser hoy, enseñarme que la autocrítica es buena en su justa medida y dejarme salvaje en el laboratorio haciendo que me involucre de lleno… "*Dime y lo olvido, enséñame y lo recuerdo, involúcrame y lo aprendo, Benjamin Franklin*". Nos veremos en los bares o en el mercado victoria con un plato de arroz.

Prof. Eduardo Collantes, gracias por transmitir esa inquietud por la investigación que te representa y buscarle el sentido a lo que hacemos, por reunirnos, reunirnos y reunirnos con un único sentido común de hacer ciencia de calidad y aplicable para mejorar la calidad de vida de los pacientes.

Señorita **Alejandra Patiño**, compañera de mesa, de máster, de memorias de seguimiento, de congresos, de cotilleos, de dudas existenciales, de cenas en restaurante japonés riendo más que comiendo, "recuerda la bolsa bajo la cama". La Alejandra de las anéctodas, inteligente y competente, de valorar a la familia por encima de todas las cosas, de esta calidad humana me he rodeado y me siento afortunado. Me alegro mucho de haber podido compartir esta etapa maravillosa contigo. Sólo me queda destacar ese "apoyo" tuyo en los peores momentos, porque cuando "peor estás" siempre has sabido como mejorarlo, he notado ese "support" que hacía falta para continuar, tú si que sabes animar amiga. Te quiero. Posdata: siento ocupar toda la mesa siempre y golpearte sin querer porque soy un completo búfalo.

Señorita **María Luque**, compañera "snap", "it girl", "tumblr", eres la alegría del laboratorio, un torbellino comprometido con su causa, fresca, con ganas de aprender, ideas y muchas inquietudes que harán que llegues muy lejos, estoy completamente seguro. Ahora sabemos que todo esto no es solo curro, qué entre pipetas, placas de cultivo y compromiso por lo que hacemos se forjan amistades de verdad que van a perdurar para siempre, por que las cosas de verdad se mantienen en el tiempo y estemos donde estemos siempre vamos a recordar el momento de "Venga Iván, snap, corre" o tu "aaaaaaaaaah de cantante ópera". Te quiero.

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Y por último, gracias a él, lo que mas quiero en este mundo, mi pequeño, porque siempre intentaré ser el mejor ejemplo que puedas tener y logres todo lo que te propongas.

ACAs: anticuerpos anticardiolipina.

ACPAs: “antibodies to citrullinated proteins antigens”.

ADN: ácido desoxirriboucleico.

AGO: “argonaute”.

Anti-β2GPI: anticuerpos anti-beta 2 glicoproteína I.

Anti-ccps: anticuerpos antipéptidos cíclicos citrulinados.

Anx A2: “annexin A2”.

APLs: anticuerpos antifosfolípidos.

AR: artritis reumatoide.

ARN: ácido ribonucleico.

BLT1: “leukotriene B4 receptor”.

BLyS: “B lymphocyte stimulator”.

CAMs: “cell adhesion molecules”.

C5a: “complement 5a”.

C5aR: “complement C5a receptor”.

CD: “cluster differentiation”.

CCR1: “C-C motif chemokine receptor 1”.

CCR2: “C-C motif chemokine receptor 2”.

COX-2: ciclooxigenasa 2.

CV: cardiovascular.

CXCL5: “C-X-C motif chemokine ligand 5”.

CXCR2: “C-X-C motif chemokine receptor 2”.

Cyr61: “cysteine-rich angiogenic inducer 61”.

DM2: “diabetes mellitus 2”.

DROSHA: “double-stranded RNA-specific endoribonuclease”.

ECV: enfermedad cardiovascular.

Fc: fracción constante.

Flt-1: “fms related receptor tyrosine kinase 1”.

FR: factor reumatoide.

FT: factor tisular.

G-CSF: “granulocyte colony-stimulating factor”.

GLUT-4: “glucose transporter 4”.

GM-CSF: “granulocyte macrophage-colony stimulating factor”.

GPIIb/IIIa: “glycoprotein IIb/IIIa”.

HDL: “high density lipoprotein”.

ICAM: “intracellular adhesion molecule 1”.

IFN-γ: interferon gamma.

Ig: inmunoglobulina.

IgM: inmunoglobulina M.

IL-1β: interleuquina 1-beta.

IL-6: interleuquina 6.

IL-8: interleuquina 8.

IL-15: interleuquina 15.

IL-17b: interleuquina 17b.

IL-20: interleuquina 20.

IL-22: interleuquina 22.

IL-23: interluquina 23.

IR: “insulin resistance”.

IRS: “insulin receptor substrate”.

IS: “insulin sensitivity”.

LDGs: “low density granulocytes”.

LDLox: “low density lipoprotein-oxidized”.

LES: lupus eritematoso sistémico.

LTB4: “leukotriene 4”.

MAP: “mitogen activated protein kinase”.

MCL-1: “myeloid cell leukemia 1”.

MCP-1: “monocyte chemotactic protein 1”.

MetS: “metabolic syndrome”.

MPO: “myeloperoxidase”.

NADPH: “nicotinamide adenine dinucleotide phosphate”.

NE: “neutrophil elastase”.

NETs: “neutrophil extracellular traps”.

NFkB: “nuclear factor kappa B”.

MIP-1α: “macrophage inflammatory protein 1-alpha”.

PAD: peptidil arginina deaminasa.

PARs: “protease activated receptors”.

PDI: “protein disulfide isomerase”.

PF4: “platelet factor 4”.

PI3K: “phosphoinositide 3-kinase”.

P38-MAPK: “p38 mitogen-activated protein kinases”.

RhoA: “ras homolog family member A”.

RISC: “RNA-induced silencing complex”.

ROS: “reactive oxygen species”.

SAF: síndrome antifosfolípido.

Ser: “serin”.

SLEDAI: “systemic lupus erythematosus disease activity index”.

Th: “T helper”.

Thr: “threonine”.

TLR4: “toll like receptor 4”.

TLR8: “toll like receptor 8”.

TNF-α: “tumor necrosis factor alpha”.

TRBP: “trans-activator responsive RNA-binding protein”.

TXB2: tromboxano B2.

Tyr: “tyrosine”.

VCAM: “vascular cell adhesion protein 1”.

VEGF: “vascular endothelial growth factor”.

VEGF-R1: “vascular endothelial growth factor receptor 1”.

XPO-5: “exportin 5”.

ÍNDICE

RESUMEN

Las enfermedades autoinmunes sistémicas se desarrollan como consecuencia de un conjunto de interacciones entre factores genéticos predisponentes, la perturbación del sistema inmune y diversos factores ambientales. El Síndrome Antifosfolípido (SAF) se caracteriza por la presencia de trombosis y complicaciones obstétricas, con especial implicación del sistema hemostático y mecanismos inflamatorios asociados. Por otro lado, la Artritis Reumatoide (AR), también asociada a un proceso inflamatorio crónico, se caracteriza por el deterioro progresivo de las articulaciones periféricas. Además, aunque el daño principal de la AR es la sinovitis articular, presenta también alteraciones metabólicas a nivel sistémico, tales como la resistencia a la insulina. De hecho, la enfermedad cardiovascular (ECV) es la principal causa de mortalidad en pacientes SAF y AR. La inflamación y los elementos autoinmunes presentes en estas patologías parecen ser los principales factores desencadenantes de la ECV.

Estudios recientes muestran que los microARNs (miRNAs) juegan un papel relevante y novedoso en la regulación epigénetica de la expresión de genes y proteínas claves en la patogénesis de estas enfermedades. Por ello, la identificación y caracterización de los miRNAs asociados a estos desórdenes autoinmunes podría favorecer la identificación de nuevos biomarcadores que permitan la tipificación clínica de la enfermedad y faciliten su manejo terapeútico.

El **objetivo principal** de esta tesis es investigar el papel de la inflamación y los miRNAs en la patogénesis del SAF y la AR, mediante la integración de aproximaciones inmunológicas, celulares, moleculares y epigenéticas.

Principales resultados obtenidos:

1. Hemos identificado una firma molecular de miRNAs circulantes como potenciales biomarcadores de enfermedad en el SAF. Esta firma se asoció a la ocurrencia de pérdidas fetales, aterosclerosis y trombosis y correlacionó con parámetros de inflamación, trombosis y autoinmunidad. Además, dicha firma permaneció estable en el tiempo, hecho demostrado por análisis realizados en un intervalo de tiempo de 3 meses. Asimismo, se demostró la especificidad de la

firma de miRNAs en pacientes SAF, mediante el análisis paralelo de dos cohortes de pacientes con trombosis pero sin enfermedad autoinmune y pacientes con Lupus Eritematoso Sistémico sin anticuerpos antifosfolípido, los cuales presentaban perfiles diferenciales. De modo complementario, el análisis mediante clústeres permitió establecer grupos de pacientes con diferente riesgo trombótico y expresión de miRNAs.

Estudios mecanísticos *in vitro* revelaron que los anticuerpos antifosfolípidos promovían la desregulación del perfil de miRNAs analizado y sus potenciales proteínas diana en monocitos y células endoteliales.

En suma, se ha identificado una firma de miRNAs diferencialmente expresados en pacientes con SAF como potenciales biomarcadores de la enfermedad que permiten tipificar el estado aterotrombótico de estos pacientes, constituyendo una posible herramienta en la práctica clínica habitual.

2. El estudio del perfil de expresión de miRNAs en neutrófilos de pacientes con AR demostró una reducción global de su expresión, asociado al descenso de genes involucrados en su biogénesis (DICER), así como una sobre-expresión de sus potenciales genes diana involucrados en inflamación y migración celular. Además, dichos niveles reducidos de miRNAs y DICER correlacionaron con mediadores de autoinmunidad, inflamación y el índice de actividad de la enfermedad.

   Los estudios *in vitro* revelaron que los anticuerpos antipéptidos cíclico citrulinados (ACPAs) y el factor de necrosis tumoral alfa (TNF-α) promovían una reducción tanto de la expresión de estos miRNAs como de los genes involucrados en su biogénesis, en paralelo al incremento de sus potenciales genes diana. El tratamiento concomitante con fármacos inhibidores de TNF-α revirtió dichos efectos.

   Transfecciones con agonistas de varios de estos miRNAs mostraron también la especificidad de regulación de sus genes diana asociados con inflamación, supervivencia y migración celular. Adicionalmente, el silenciamiento del gen clave en el proceso de biogénesis de miRNAs, DICER, influenció de manera significativa el perfil inflamatorio de los neutrófilos.

En suma, los neutrófilos en el contexto de AR exhiben una reducida expresión de miRNAs, promovida por los principales inductores de la activación patogénica de este tipo celular: autoanticuerpos y mediadores inflamatorios. Asimismo, la maquinaria de biogénesis de miRNAs se encuentra significativamente alterada en los neutrófilos de estos pacientes y fuertemente asociada con una desregulación de los miRNAs relacionados con la migración e inflamación en neutrófilos sinoviales. Finalmente, terapias dirigidas contra TNF-α podrían modular estos niveles de expresión de miRNAs, minimizando de este modo el perfil inflamatorio.

3. El estudio de la actividad inflamatoria en pacientes con AR y su implicación en la alteración del metabolismo glucídico y lipídico demostró, en primer lugar, que los pacientes con AR mostraron una fuerte asociación entre el grado de inflamación sistémica y el desarrollo de resistencia a insulina. Estos resultados fueron corroborados mediante el desarrollo de un modelo murino de artritis inducida por colágeno que resultó en un estado de inflamación global caracterizado por un metabolismo defectuoso de la glucosa y de los lípidos en diferentes tejidos, siendo el tejido adiposo el principal tejido afectado y el más susceptible a las alteraciones inducidas por la AR. Estos resultados fueron confirmados asimismo mediante estudios *in vitro*, tras tratamiento de adipocitos con el suero de pacientes con AR.

   En su conjunto, los datos obtenidos en este estudio han mostrado cómo las alteraciones metabólicas observadas en pacientes AR dependen del grado de inflamación e identifica al tejido adiposo como el principal tejido diana para el desarrollo de resistencia a insulina. Por tanto, estrategias terapéuticas dirigidas a controlar el estatus inflamatorio podrían permitir normalizar o prevenir las alteraciones metabólicas asociadas con la AR.

En suma, los resultados globales obtenidos en esta tesis doctoral han permitido identificar potenciales biomarcadores del estatus de la enfermedad y sus comorbilidades asociadas en pacientes con Síndrome Antifosfolípido y Artritis Reumatoide, así como diversos mecanismos moleculares asociados a procesos patológicos clave en estas enfermedades, tales como la ECV. Dichos resultados podrían sentar las bases para la realización de estudios futuros, cuyo fin sea desarrollar una medicina

personalizada dirigida a optimizar el cuidado de los pacientes con enfermedades autoinmunes sistémicas.

ABSTRACT

Systemic autoimmune diseases (including antiphospholipid syndrome and rheumatoid arthritis) are the result of a complex interaction between predisposing genetic factors, disturbance of the immune system, and several environmental factors. Antiphospholipid Syndrome (APS) is characterized by the presence of thrombosis and obstetric complications, with special involvement of the hemostatic system and associated inflammatory mechanisms. On the other hand, Rheumatoid Arthritis (RA), also associated with a chronic inflammatory process, is characterized by the progressive deterioration of the peripheral joints. Furthermore, although the main focus of RA is joint synovitis, it also presents extra-articular manifestations such as metabolic disorders and cardiovascular disease. In fact, cardiovascular disease (CVD) is the main cause of mortality in both, APS and RA patients. Inflammation and the autoimmune elements seem to be the main triggers for CVD in these pathologies.

Recent studies showed that microRNAs (miRNAs) are involved in crucial cellular processes and their dysregulation has been described in many cell types and fluids in a broad range of diseases. Therefore, the characterization of the miRNAs associated with the pathogenesis of systemic autoimmune disorders, such as APS and RA, could identify new biomarkers that might facilitate its diagnosis, outcome and therapeutic management.

The **main objective** of this thesis is to investigate the role of inflammation and miRNAs in the pathogenesis of APS and RA, through the integration of immunological, cellular, molecular and epigenetic approaches.

Main results obtained:

1. We have identified a molecular signature of circulating miRNAs as potential disease biomarkers in APS. This signature was associated with the occurrence of fetal loss, atherosclerosis, and thrombosis and correlated with parameters of inflammation, thrombosis, and autoimmunity. Furthermore, this signature remained stable over time, a fact demonstrated by analysis carried out in a time interval of 3 months. Likewise, the specificity of the signature of miRNAs in APS patients was demonstrated, through the parallel analysis of two cohorts of patients with thrombosis but without autoimmune disease and patients with Systemic Lupus Erythematosus without

antiphospholipid antibodies, which displayed differential profiles. In addition, hard clustering analysis established groups of patients with different thrombotic risk and expression of miRNAs.

In vitro mechanistic studies revealed that antiphospholipid antibodies promoted the dysregulation of the analyzed miRNA profile and its potential target proteins in monocytes and endothelial cells.

In sum, differentially expressed circulating miRNAs in APS patients, modulated at least partially by antiphospholipid antibodies of IgG isotype, might have the potential to serve as novel biomarkers of disease features and to typify patients' atherothrombotic status, thus constituting a useful tool in the management of the disease.

2. The study of the expression profile of miRNAs in neutrophils of RA patients showed an overall reduction in their expression, associated with the decrease in genes involved in their biogenesis (DICER), as well as an over-expression of their potential target genes involved in inflammation and cell migration. Furthermore, these reduced levels of miRNAs and DICER correlated with mediators of autoimmunity, inflammation and disease activity.

   In vitro studies revealed that anti-citrullinated protein antibodies (ACPAs) and tumor necrosis factor alpha (TNF-α) promoted a reduction in both the expression of these miRNAs and the genes involved in their biogenesis, in parallel with the increase in their potential target genes. Concomitant treatment with TNF-inhibitors could reverse these effects.

   Transfections with agonists of several of these miRNAs also showed the regulatory specificity of their target genes associated with inflammation, survival, and cell migration. Additionally, the silencing of the key gene in the miRNA biogenesis process, DICER, significantly influenced the inflammatory profile of neutrophils.

   In sum, neutrophils in the context of RA exhibit reduced expression of miRNAs, promoted by the main inducers of pathogenic activation of this cell type: autoantibodies and inflammatory mediators. Likewise, the biogenesis machinery of miRNAs is significantly altered in the neutrophils of these patients and strongly associated with a dysregulation of miRNAs related to migration and inflammation in synovial neutrophils. Finally, therapies directed against TNF-α could

modulate these expression levels of miRNAs, thus minimizing the inflammatory profile.

3. The study of the inflammatory activity in patients with RA and its implication in the alteration of the glucose and lipid metabolism showed, first of all, that there was a strong association between the degree of systemic inflammation and the development of insulin resistance. These results were corroborated through the development of a murine model of collagen-induced arthritis that resulted in a state of global inflammation characterized by a defective metabolism of glucose and lipids in different tissues, being adipose tissue the main affected tissue and the more susceptible to the alterations induced by RA. These results were also confirmed by *in vitro* studies, after treatment of adipocytes with the serum of RA patients. Taken together, the data obtained in this study indicates how the metabolic alterations observed in RA patients depend on the degree of inflammation and identify adipose tissue as the main target tissue for the development of insulin resistance. Therefore, therapeutic strategies focused on tightly controlling the inflammatory status could reduce or prevent the metabolic disorders associated with RA.

In sum, the global results obtained in this doctoral thesis allowed the identification of potential biomarkers of the status of the disease and its associated comorbidities in patients with Antiphospholipid Syndrome and Rheumatoid Arthritis, as well as various molecular mechanisms related to key pathological processes in these diseases, such as CVD. These results could pave the way for future studies, based on the development of personalized medicine aimed at optimizing the care of patients with systemic autoimmune diseases.

INTRODUCCIÓN

Los problemas que resuelve el sistema inmunitario de los mamíferos no se limitan a los animales superiores: se enfrentan a todas las formas de vida y ninguno las ignora. La presión que ejerce la selección natural es inagotable e interminable. De tal manera que es de vital importancia comprender cómo maximizar el potencial del sistema inmune al servicio de la salud humana. Es una propiedad fundamental de la inmunidad que ninguna parte de nuestro cuerpo esté aislada de su vigilancia. Por esta razón, aunque el sistema inmune parece algo menos sustancial que un órgano como el corazón o el hígado, en conjunto la inmunidad consume enormes recursos, produciendo una gran cantidad de células de las que depende el cuerpo humano para conseguir un funcionamiento exitoso.

1. El sistema inmune. Tolerancia inmunológica. Autoinmunidad.

La función principal del sistema inmune es la de proteger al huésped frente a agentes infecciosos. Sin embargo, este sistema es pleiotrópico por lo que bajo ciertas circunstancias podría contribuir al desarrollo de una determinada enfermedad. Entre dichas circunstancias destacan, en primer lugar, los síndromes inmunes deficientes, en los que el sistema inmune es incapaz de responder ante la presencia de patógenos y, en segundo lugar, las enfermedades autoinmunes, en las que ocurre una respuesta inmune exacerbada, reaccionando contra las propias células y tejidos (1, 2).

La tolerancia inmune puede definirse como la ausencia de respuesta del sistema inmune frente a un antígeno, ya sea propio o extraño, inducida por el contacto previo con ese antígeno. No se trata solo de la ausencia de respuesta, si no que está dotada de especificidad y memoria. Los principales órganos implicados en la tolerancia inmune son el timo y la médula ósea, los cuales juegan un papel esencial en la configuración de la homeostasis del sistema inmune. En el timo, los linfocitos en desarrollo experimentan una selección positiva antes de entrar en circulación. Así, en un huésped sano, los linfocitos con potencial reactividad contra autopéptidos se seleccionan negativamente y se eliminan en la médula tímica. Una vez los linfocitos T maduros salen del timo son sometidos a una selección secundaria (tolerancia periférica) mediante la cual la mayoría de células T autorreactivas se eliminan o se vuelven anérgicas. Por otro lado, las células B inmaduras que expresan IgM de superficie que reconoce los antígenos ubicuos de la superficie celular, son eliminadas por un proceso de selección clonal. Las células B autorreactivas puede también ser eliminadas mediante edición del receptor. Asimismo, las células B maduras están bajo control de la tolerancia periférica. A pesar del estricto control que el sistema inmune ejerce sobre la tolerancia inmunológica central y periférica, un pequeño número de células potencialmente reactivas escapan de éste. No obstante, la existencia de estas células potencialmente reactivas, como linfocitos T y B, y la habilidad de éstas últimas para producir autoanticuerpos no necesariamente desemboca en el desarrollo de una patología (3).

En base a esta premisa, la autoinmunidad puede a veces ser clasificada como "fisiológica" o "patológica" (4,5). La autoinmunidad fisiológica es usualmente un punto intermedio sin evidencia de enfermedad a nivel clínico. Esto se ejemplifica con la presencia de los denominados autoanticuerpos naturales (6), que permiten eliminar antígenos propios y extraños degradados para mantener la homeostasis. Los autoanticuerpos naturales más comunes son los anticuerpos antinucleares y el factor reumatoide, que se observan a menudo en individuos sanos y cuya prevalencia aumenta con la edad. Sin embargo, cuando la tolerancia inmune se rompe y los autoanticuerpos y linfocitos autorreactivos actúan, se desarrolla autoinmunidad patológica implicando procesos inflamatorios que desembocan en daño tisular (**Figura 1**).

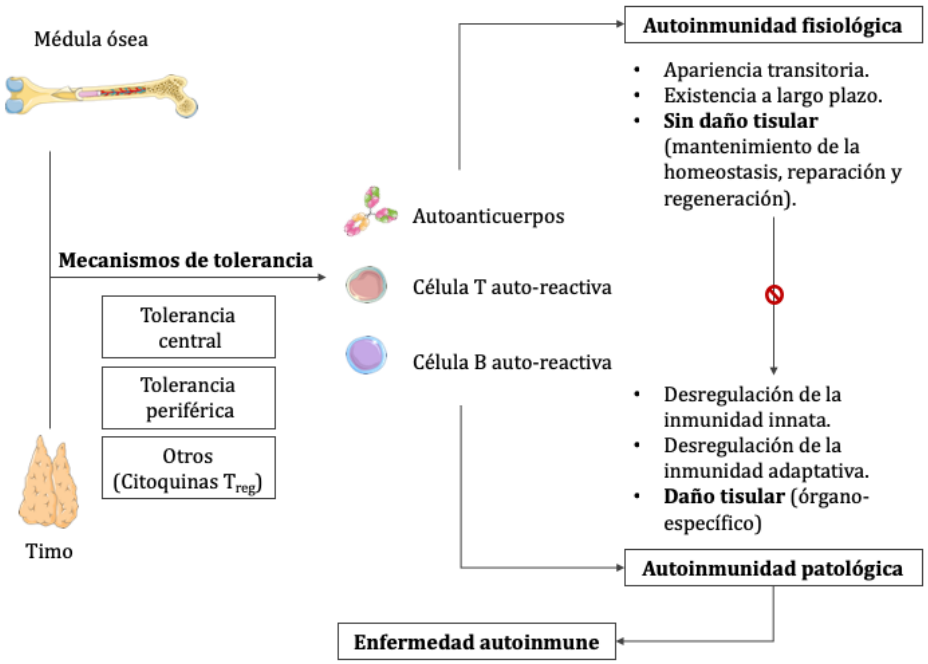

Figura 1. Desarrollo de autoinmunidad. Incluso bajo condiciones restrictivas de control de la tolerancia central y periférica, un pequeño número de células auto-reactivas pueden filtrarse hacia la periferia en individuos sanos. Sin embargo, estas células serán inofensivas a menos que exista una predisposición genética o se rompa la tolerancia debido a algún agente desencadenante. *Modificado a partir de (3).*

2. Síndrome Antifosfolípido (SAF).

El SAF es una enfermedad autoinmune sistémica caracterizada por la presencia de trombosis arteriales y/o venosas, así como por complicaciones obstétricas (desde pérdidas fetales recurrentes o partos prematuros secundarios hasta insuficiencia placentaria o preeclampsia severa). Se estima que la incidencia de SAF es de alrededor de 5 casos por 100.000 personas al año. Además, está asociado a la presencia persistente de anticuerpos antifosfolípidos (aPLs), incluyendo anticuerpos anticardiolipina (ACA), anti-β2 glicoproteína-I (anti-β2GPI) y/o anticoagulante lúpico y factores del complemento. Muchos de estos anticuerpos están dirigidos contra proteínas plasmáticas y proteínas expresadas o unidas a la superficie celular de células endoteliales o plaquetas. Los aPLs están presentes en aproximadamente un 13% de los pacientes con accidente cerebrovascular, un 11% con infarto de miocardio, un 9,5% en los pacientes con trombosis venosa profunda y un 6% de pacientes con morbilidad de embarazo (7, 8). Asimismo, se han propuesto diversos mecanismos que podrían explicar la tendencia trombótica existente en los pacientes con SAF, sin embargo, la patogénesis de esta enfermedad parece ser multifactorial.

2.1. Patogénesis del SAF.

Diversos estudios han evaluado los mecanismos de transducción de señales inducidas por aPLs así como su efecto sobre las manifestaciones patológicas asociadas a esta condición autoinmune. Así, se ha demostrado que los aPLs, secretados por células plasmáticas, dan lugar a la activación de diversos tipos celulares, tales como células endoteliales, plaquetas, neutrófilos y monocitos promoviendo un estado inflamatorio. En células endoteliales se potencia la expresión de TLR4 y FT en la superficie celular, así como la liberación de citoquinas pro-inflamatorias y el aumento de la adhesión celular de los leucocitos a través de moléculas de adhesión como ICAM, VCAM y E-selectina. En plaquetas se produce la liberación del factor plaquetario IV y tromboxano B2, que promueve la expresión del receptor principal del fibrinógeno. En neutrófilos parece tener un papel importante el sistema del complemento y finalmente en monocitos, la interacción de los aPLs promueve el incremento de ROS y la disfunción mitocondrial además del aumento de expresión de FT, VEGF y otras moléculas pro-inflamatorias (9).

Asimismo, la unión a la superficie celular de monocitos y células endoteliales induce el aumento de los niveles y la activación de un receptor celular pro-coagulante, conocido como factor tisular (TF). La señalización intracelular asociada a dicha activación está mediada por los receptores activados por proteasas (PARs, mediadores de respuestas críticas para la trombosis, hemostasia y procesos inflamatorios, cuya expresión se encuentra incrementada en pacientes con SAF) (10, 11). Dicha señalización intracelular, inducida por los aPLs, implica la activación constitutiva de MAP kinasas y NFkB (12) y el incremento de la expresión de la citoquina pro-inflamatoria VEGF (factor de crecimiento endotelial vascular) y de su receptor Flt-1 (VEGFR-1) en monocitos de pacientes con anticuerpos aPLs (13). La imbricación de todas estas proteínas y mecanismos intracelulares resulta en la inducción de un estado pro-coagulante y pro-inflamatorio en estos pacientes (**Figura 2**).

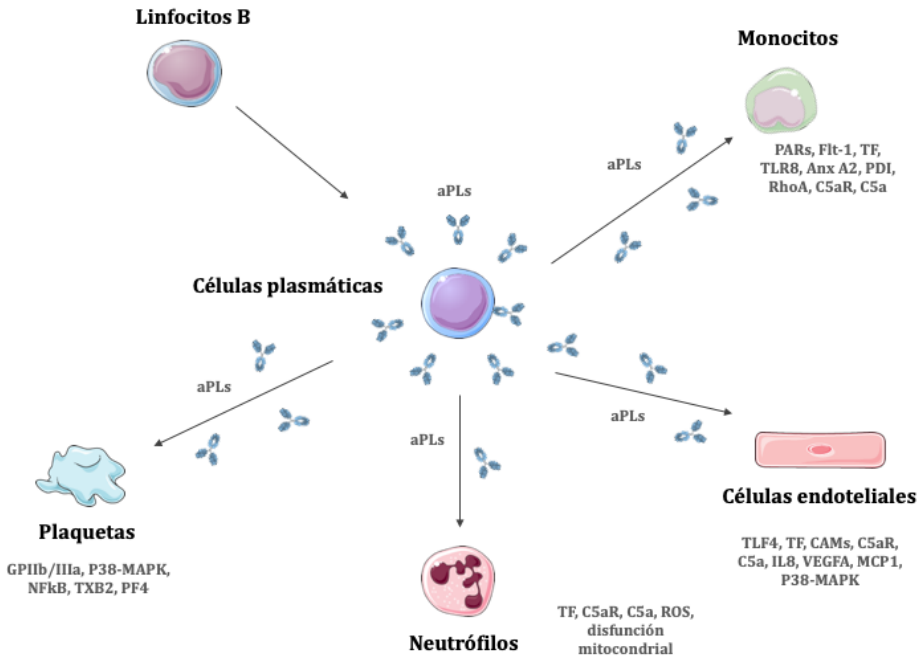

Figura 2. Activación celular mediada por aPLs. Los aPLs secretados por células plasmáticas interaccionan con moléculas asociadas a distintos tipos celulares, dando lugar a su activación.

Además, tanto los aPLs como otros anticuerpos presentes en el SAF [i.e. los anti-lipoproteínas de alta densidad (HDLs) y anti- apolipoproteína A-I], están asociados tanto al desarrollo de aterosclerosis como de enfermedad arterial periférica y cerebrovascular (14, 15) a través de otros mecanismos complementarios, que incluyen la formación de complejos anti-LDLox/anti-ß2GPI, lo que facilita o acelera la captación de LDLox por los macrófagos y su consecuente conversión en células espumosas. Además, estos anticuerpos activan a estas células promoviendo la adhesión de los macrófagos al endotelio mediada por moléculas de adhesión (ICAM-1, VCAM-1, E-Selectina). De hecho, se ha demostrado una correlación positiva entre los títulos de anticuerpos anti-cardiolipina y anti-ß2GPI y la incidencia y severidad del síndrome coronario agudo, el infarto de miocardio o el accidente cerebrovascular (16-18).

Asimismo, en patologías autoinmunes como el SAF y el LES, se ha demostrado que los aPLs parecen jugar un papel relevante en la promoción de un estatus prooxidativo, a través de la inducción de la producción de óxido nítrico y anión superóxido, con la consecuente generación de peroxinitritos, potentes sustancias prooxidantes asociadas con disfunción vascular y aterogénesis (19). Más concretamente, en el ámbito del SAF, se ha demostrado que existe una alteración del estado redox intracelular en leucocitos, mediado por los aPLs, que regula el estado inflamatorio y aterotrombótico, y se halla directamente asociado a la dinámica y el metabolismo mitocondrial (20-23).

Se trata pues de una patología compleja, que probablemente requiera de un sistema integrado de análisis de factores clínicos, celulares y moleculares de riesgo cardiovascular y la identificación de biomarcadores que ayuden a reconocer y tratar pacientes con alto riesgo. En los últimos años, los mecanismos fisiopatológicos que interrelacionan la aterotrombosis con las enfermedades autoinmunes sistémicas están siendo mejor caracterizadas gracias al uso de tecnologías genómicas y epigenéticas.

3. Artritis Reumatoide (AR).

La AR es una enfermedad autoinmune de carácter inflamatorio crónico, que se caracteriza por la destrucción progresiva de las articulaciones, afectando en gran medida a manos y pies, causando múltiples deformidades. Además, con frecuencia también pueden presentarse manifestaciones extra-articulares (24). La prevalencia de esta enfermedad está en torno al 1-2% de la población mundial. Asimismo, diversos estudios han mostrado que las mujeres presentan entre 2 y 3 veces más esta enfermedad que los hombres, probablemente debido a la carga hormonal. La enfermedad suele aparecer en edades entre los 50 y 75 años, por lo que, en mujeres mayores de 65, la prevalencia aumenta un 5% (25, 26)

Aunque es de etiología desconocida, existen claras evidencias de su naturaleza autoinmune, debido a la presencia de autoanticuerpos como el factor reumatoide (FR, IgM que interacciona con el fragmento Fc de la IgG) o los anticuerpos anti-CCPs (anticuerpos antipéptidos cíclicos citrulinados), también llamados ACPAs (anticuerpos anti-proteínas citrulinadas) (27). El FR no es específico y puede ser hallado en otros desórdenes reumáticos, infecciones o en individuos sanos. Sin embargo, los ACPAs son potentes marcadores serológicos para el diagnóstico temprano de la AR y el pronóstico de la destrucción de la articulación.

3.1. Patogénesis de la AR.

La patogénesis de la AR conlleva la activación del sistema inmune innato (por causas aún desconocidas) en un paciente con una predisposición previa, que promovería la generación de autoanticuerpos por el sistema inmune adaptativo, desencadenando de este modo la enfermedad (28). Desde la aparición de autoanticuerpos hasta que las manifestaciones clínicas aparecen, puede transcurrir un largo periodo de tiempo, aunque se sabe que determinados factores ambientales pueden hacer que su desarrollo se acelere. Un mecanismo clave en este proceso es la activación de la enzima peptidil arginina deaminasa (PAD), cuya función es desaminar péptidos que contienen arginina a péptidos cíclicos citrulinados (29). En pacientes AR tiene lugar un aumento en la actividad de dicha enzima, provocando así un aumento de proteínas citrulinadas y la inducción de la respuesta inmune

contra los epítopos generados por esta citrulinación proteica, con la consecuente producción de ACPAs (30).

Por otra parte, se han identificado diversos antígenos reumatoideos como potenciales responsables de la generación de autoanticuerpos y, de este modo, inductores del proceso inflamatorio presente en estos pacientes, tales como vimentina, enolasa, fibrinógeno o colágeno tipo II (31-33).

Los ACPAs representan un marcador inmunológico sensible y específico para el diagnóstico de la AR, especialmente en etapas iniciales de la enfermedad. Además, distintas observaciones indican que la presencia de estos autoanticuerpos se asocia al desarrollo de una enfermedad más agresiva (34). Una manifestación muy precoz de la AR es la formación de nuevos vasos sinoviales, lo que desencadena una acumulación de líquido sinovial en la articulación afectada con la posterior infiltración de los distintos leucocitos. A medida que se van formando nuevos vasos sanguíneos, el TNF-α, secretado por diversos tipos leucocitarios, promueve a su vez la secreción de otras citoquinas que inducen en las células del endotelio la producción de moléculas de adhesión, facilitando así la diapédesis. Además, la producción de cascadas de citoquinas pro-inflamatorias es crucial en el desarrollo de la AR, al provocar la proliferación de las células sinoviales (35).

3.2. Papel de los neutrófilos en la patogénesis de la AR.

El papel patogénico de los neutrófilos en la AR involucra una alteración coordinada de varios procesos, incluyendo un aumento de la capacidad migratoria, una actividad inflamatoria anormal, así como un incremento del estrés oxidativo y la presencia incrementada de NETosis, un tipo de muerte celular de los neutrófilos caracterizada por la extrusión de una malla de cromatina unida a péptidos granulares denominados trampas extracelulares de neutrófilos (36, 37). En un contexto de AR, los neutrófilos expresan diversos receptores de citoquinas (BLT1, CXCR2, CCR1 y CCR2), liberan una gran cantidad de mediadores inflamatorios (TNF-α, IL-1β, IL-6, IL-8, IL-17b, IL-20, IL-22, LTB4 y CXCL5) y expresan proteínas anti-apoptóticas (MCL-1, G-CSF, GM-CSF, IFN-γ e IL15). Estas moléculas actúan incrementando la capacidad migratoria de los neutrófilos, produciendo una infiltración masiva al sinovio y prologando la supervivencia celular en la articulación. Como consecuencia de la activación por TNF-α, los neutrófilos

en la AR pueden también expresar BLyS, un potente activador de los linfocitos B. De este modo, diversas citoquinas inflamatorias, junto con los autoanticuerpos pueden inducir NETosis en los neutrófilos y LDGs ("Low density granulocytes") a nivel local y sistémico. Las NETs contienen numerosas enzimas (MPO, NE y NADPH oxidasa) y proteínas citrulinadas, así como PADs, lo que representa una fuente de generación de nuevos autoanticuerpos que activarán a las células B para producir nuevos autoanticuerpos. Además, las NETs pueden inducir la expresión de IL-8 e IL-6 en fibroblastos. Por otro lado, los fibroblastos activados en las articulaciones de pacientes AR pueden producir Cyr61, que media la expresión de IL-8. Estas citoquinas, a su vez, pueden inducir la infiltración de neutrófilos al sinovio y la formación de NETs, promoviendo daño óseo (**Figura 3**) (36).

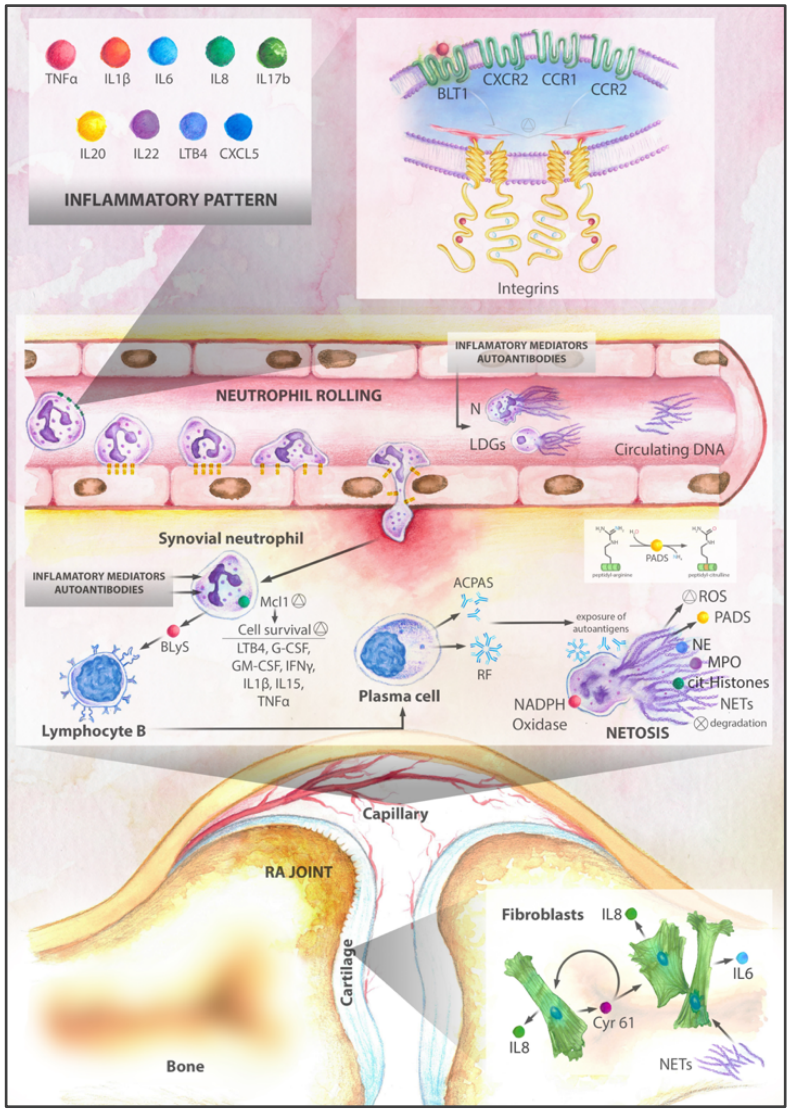

Figura 3. Mecanismos patogénicos de los neutrófilos en la AR. En un contexto de AR, los neutrófilos pueden expresar multitud de receptores de citoquinas, liberar una gran cantidad de mediadores inflamatorios y expresar proteínas anti-apoptóticas, incrementando así la capacidad migratoria de estas células. Asimismo, tanto la acción de citoquinas como la de los autoanticuerpos pueden favorecer la producción de NETosis, liberando multitud de enzimas y proteínas citrulinadas e induciendo la generación de nuevos autoanticuerpos (36).

3.2. Comorbilidades asociadas a la AR: enfermedad cardiovascular y alteraciones metabólicas.

Aunque el foco principal es la sinovitis, la AR presenta manifestaciones extra-articulares o comorbilidades asociadas, tales como enfermedad cardiovascular, síndrome metabólico, depresión, y osteoroposis entre otras (38), siendo la enfermedad cardiovascular la principal causa de mortalidad en estos pacientes (39). La comorbilidad cardiovascular en la AR deriva de varios procesos patogénicos presentes en estos pacientes: disfunción microvascular, arritmias, desregulación cardíaca, inflamación y anomalías inmunológicas, entre otras, así como del efecto de los tratamientos farmacológicos. Adicionalmente, en la AR se ha observado un incremento de aterosclerosis temprana comparado con la población general. Paradójicamente, los factores de riesgo cardiovascular tradicionales (edad, sexo, tabaco, diabetes, hipertensión e hiperlipidemia) no explican completamente el incremento de aterosclerosis en estos pacientes. Esto sugiere la existencia de mecanismos adicionales que podrían potenciar el desarrollo de enfermedad CV en pacientes AR, considerándose así a la AR como un factor de riesgo cardiovascular independiente (40, 41).

En este sentido, se ha demostrado que la inflamación juega un papel muy importante en el aumento del riesgo CV presente en estos pacientes, ya que la magnitud y cronicidad de la respuesta inflamatoria favorece el desarrollo de aterosclerosis prematura (42). Asimismo, un estudio reciente llevado a cabo por nuestro grupo de investigación ha descrito que los autoanticuerpos específicos de la enfermedad, ACPAs, participan de modo directo en la patogenia de la enfermedad cardiovascular, a través de la inducción de inflamación y estrés oxidativo en los leucocitos de pacientes con AR (**Figura 4**) (43).

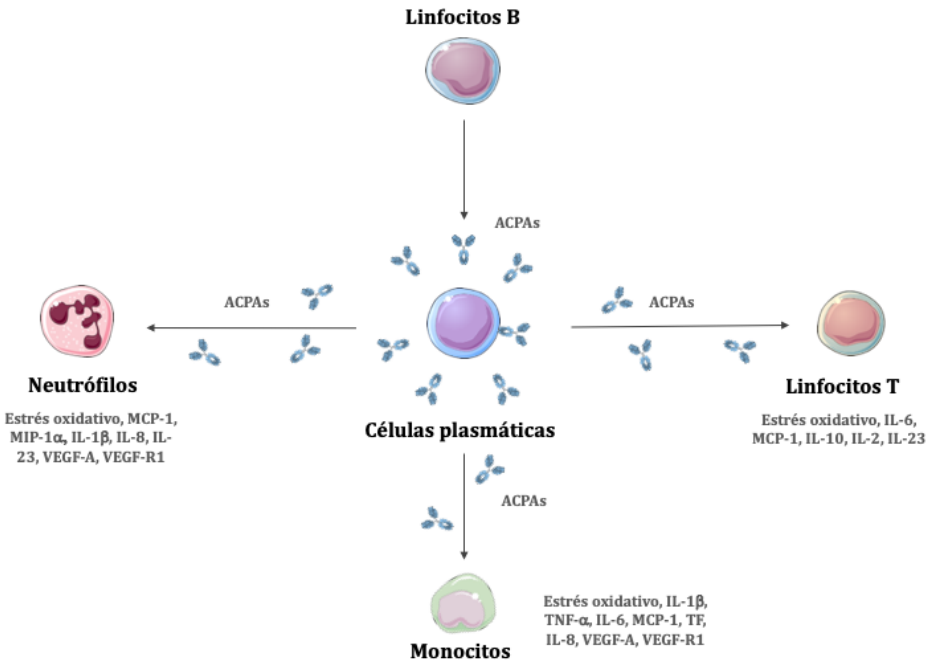

Figura 4. Activación celular mediada por ACPAs. Los ACPAs secretados por células plasmáticas interaccionan con moléculas asociadas a distintos tipos celulares dando lugar a su activación, promoviendo estrés oxidativo y el aumento de la expresión de moléculas asociadas con procesos inflamatorios (43).

Por otro lado, se ha observado un aumento de las alteraciones metabólicas en la AR, siendo las más relevantes y prevalentes la diabetes mellitus tipo 2 (DM2), el síndrome metabólico, la obesidad y la resistencia a insulina (IR). Así, existe una estrecha relación entre la AR y la diabetes, hallándose una doble relación entre ambas: 1) existe una alta prevalencia de diabetes tipo 2 en pacientes con AR y 2) la AR es un factor de riesgo para el desarrollo de diabetes (44). Del mismo modo, los pacientes con AR presentan un incremento en la prevalencia de síndrome metabólico (MetS) comparado con la población general (45).

Asimismo, diversos estudios han sugerido que existe una estrecha relación entre la obesidad y la AR, pues ambas comparten la sobreexpresión de diversas citoquinas con actividad pro-inflamatoria (46).

Entre las citoquinas pro-inflamatorias liberadas por el tejido adiposo en condiciones de sobrepeso u obesidad que intervienen también en los mecanismos patogénicos de la inflamación en AR, destacan el TNF-α, IL-6 e IL-1β (47, 48).

3.3. Tejido adiposo: Obesidad, Resistencia a Insulina y Artritis Reumatoide.

El tejido adiposo es un órgano conformado principalmente por adipocitos y células inmunitarias rodeadas de capilares e inervación. La principal función que presenta es la de almacenamiento de energía en forma de lípidos, pero además también tiene funciones endocrinas, ya que secreta gran variedad de hormonas y citoquinas con funciones autocrinas y paracrinas, denominadas, adipoquinas (49).

La obesidad y/o sobrepeso suponen un importante peligro para la salud. Se caracterizan por la acumulación excesiva de grasa y por un estado inflamatorio de bajo grado (47) que contribuye significativamente al desarrollo de ECV a través de diferentes vías fisiopatológicas, entre las que incluyen la IR, DM2, hipertensión, dislipidemia y la causa subyacente del MetS (50, 51). En condiciones de obesidad, los adipocitos sufren estrés homeostático, debido a un aumento en su capacidad de almacenamiento de energía, dando lugar a la liberación de un amplio espectro de mediadores pro-inflamatorios y adipoquinas al torrente sanguíneo (**Figura 5**). Las adipoquinas regulan numerosas funciones fisiológicas como la homeostasis, el metabolismo glucídico y lipídico, e intervienen en la sensibilidad a la insulina (IS) (52, 53). Asimismo, diversos estudios han atribuido a las adipoquinas un papel clave como mediadores de la inflamación y la respuesta inmune, estando implicadas en la fisiopatología de enfermedades reumáticas como la AR (47).

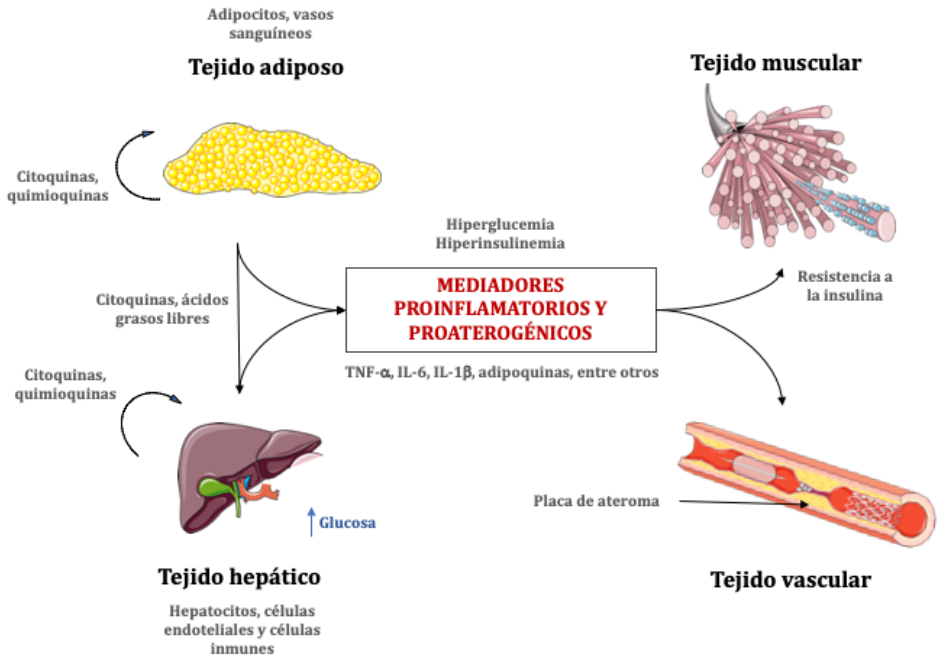

Figura 5. El incremento de la adiposidad activa respuestas inflamatorias en el tejido adiposo y hepático y como consecuencia se incrementa la producción de citoquinas y quimioquinas. Las células inmunes, incluyendo monocitos y macrófagos, son reclutados y activados, ocasionando insulino-resistencia local. La liberación de citoquinas y lípidos al flujo sanguíneo por parte de la grasa abdominal contribuye a la inflamación hepática y la resistencia a insulina. Los mediadores pro-inflamatorios y pro-aterogénicos son producidos por el hígado, el tejido adiposo y las células inmunes. Todo esto genera una inflamación sistémica que promueve el desarrollo de resistencia a insulina en el músculo esquelético y otros tejidos y aterogénesis a nivel vascular (52).

De manera general, la IR se manifiesta por una disminución en el transporte de glucosa inducido por la insulina en adipocitos y músculo esquelético, un aumento de la producción de glucosa hepática y alteraciones en el metabolismo de lípidos en tejido adiposo y hepático (**Figura 5**) (50-52). La IR no afecta de la misma forma a los tejidos. En cada uno de ellos, la insulina ejerce un papel diferente y, por tanto, una alteración en la interacción con ella también afecta de manera diferente. El músculo, responsable del 20% del metabolismo de la glucosa, cuando es estimulado

por la insulina, puede captar hasta un 80% de la glucosa sanguínea para su transformación en glucógeno. En casos de pacientes con resistencia a insulina, solo se capta entre un 30-40% de la glucosa que captaría una persona sana y la mayor parte de este descenso se debe al músculo (52).

A nivel molecular, los mecanismos por los que se genera la IR pueden ser múltiples y variar de un individuo a otro. Sin embargo, la IR es la consecuencia de una deficiente señalización de la insulina causada por mutaciones o modificaciones post-traduccionales del receptor de insulina o de moléculas efectoras del mismo. En algunos casos la resistencia a la insulina se debe a un defecto en la unión de la insulina a su receptor, pero más a menudo se atribuye a alteraciones posteriores a la unión de la insulina, que alteran desde la funcionalidad de su receptor hasta la actividad de otras proteínas que desempeñan funciones importantes en dicha señalización de la insulina (53). Entre las alteraciones más comunes se encuentran: la disminución en el número de receptores y de su actividad kinasa; un aumento en el estado de fosforilación en residuos de Ser/Thr de proteínas clave como el receptor y su sustrato; la disminución de la actividad de las cinasas PI3K y Akt y defectos en la expresión y función del transportador GLUT-4 (58). De entre estas alteraciones el aumento en la fosforilación en residuos de Ser/Thr a nivel del IR y de sustrato receptor de insulina (IRS), ha sido considerado como uno de los mecanismos clave en el desarrollo de la resistencia a la insulina. Un aumento en el estado de fosforilación de ambas proteínas puede alterar su asociación a otras proteínas, bloquear sitios de fosforilación en Tyr, disminuir su activación e inducir su degradación (53-55).

Por otro lado, el TNF-α contribuye a la aparición de IR, ya que es capaz de afectar a su receptor disminuyendo la actividad enzimática de éste y por consiguiente reduciendo la actividad de la insulina (47, 56, 57). Concretamente, el TNF-α disminuye la actividad tirosin quinasa del receptor de insulina, mediante la inhibición de la fosforilación en treonina e induciendo la fosforilación de serina, la cual inactiva al receptor. Además, se ha demostrado que el TNF-α disminuye la expresión del transportador de glucosa GLUT-4, conduciendo a una reducción de captación de glucosa por parte de las células (57).

Así podemos decir que la inflamación juega un papel fundamental en el desarrollo de resistencia a la insulina, de forma que los niveles séricos de citoquinas inflamatorias son importantes para el metabolismo lipídico y

glucídico, ya que tanto su exceso como su déficit se puede relacionar con diferentes estadios de resistencia a insulina (58).

Asimismo, diversos estudios indican que existe un incremento de la prevalencia de IR en pacientes con AR, indicando una asociación entre el desarrollo y severidad de la AR con la alteración del metabolismo de la glucosa (40, 41). Según esto, podemos decir que la AR y la resistencia a la insulina son estados de inflamación crónica que poseen elementos comunes en su patogénesis. De manera que es concebible pensar que los pacientes AR, en los cuales la respuesta inmune se encuentra exacerbada con la consiguiente amplificación de la cascada inflamatoria, sean más susceptibles a desarrollar IR. Sin embargo, aunque la relación entre AR e IR parece evidente, aún se desconocen los mecanismos moleculares específicos involucrados en el desarrollo de resistencia a la insulina en esta enfermedad.

4. Mecanismos epigenéticos involucrados en la patogénesis de las enfermedades autoinmunes: microARNs (miRNAs).

El término "epigénetica" se refiere a aquellas alteraciones heredables que no son debidas a los cambios en la secuencia de ADN. Las modificaciones epigenéticas pueden estar mediadas por diferentes mecanismos, tales como la metilación del ADN, modificación de histonas y la regulación por ARNs no codificantes. Estos alteran la accesibilidad al ADN y la estructura de la cromatina, regulando patrones de expresión génica. Estos procesos son esenciales para el desarrollo y diferenciación normal de los diferentes linajes celulares en organismos adultos y su alteración se ha asociado al desarrollo de diferentes condiciones patológicas (59, 60).

Los miRNAs son ARNs monocatenarios no codificantes de unos 18-25 nucleótidos que se unen por complementariedad en su secuencia a las regiones no traducidas 3´UTR de los ARN mensajeros (ARNm) dianas para suprimir la traducción de proteínas o bien promover la degradación del ARNm (61). Recientemente, también ha sido descrita la función inversa en la que los miRNAs pueden aumentar la traducción de ARNm específicos, aunque es menos frecuente. El mecanismo subyacente a esta función inversa es poco conocido, pero se sabe que el miRNA se une a la región 5´UTR del ARNm diana (61, 62). La mayor parte de los autores han clasificado a los miRNAs como moduladores epigenéticos ya que afecta a los niveles de proteínas de los ARNm dianas sin modificar la secuencia de ADN (63).

Además, los miRNAs pueden también ser el objetivo de modificaciones epigenéticas que modulan sus niveles de expresión (61).

En las últimas décadas, los miRNAs se han postulado como importantes moléculas reguladoras de numerosas vías celulares y de desarrollo (64, 65). Numerosos estudios se han centrado en la evaluación de los niveles de expresión de miRNAs y han mostrado cambios considerables en sus perfiles de expresión en diversas enfermedades (66, 67). Un aspecto relevante a nivel clínico es que los resultados de estos estudios apuntan al perfil de la expresión de miRNAs como una herramienta potencialmente útil para el pronóstico, diagnóstico y tratamiento de enfermedades (66, 68, 69).

4.1. Biogénesis de miRNAs.

Durante el proceso de biogénesis, los miRNAs se transcriben en primer lugar a miRNAs primarios (pri-miRNAs) de varias kilobases de longitud. Estos pri-miRNAs son procesados por una ribonucleasa, Drosha, en unos miRNAs precursores (pre-miRNAs) de alrededor de 70 nucleótidos. Estos pre-miRNAs son transportados fuera del núcleo a por la exportina 5 (XPO-5) y posteriormente procesado por DICER para generar un miRNA de doble cadena maduro de aproximadamente 22 nucleótidos. DICER se considera como la proteína crucial en el procesamiento de los miRNAs. Finalmente, una de las dos cadenas se incorpora al complejo RISC [de las siglas en inglés "RNA-induced silencing complex", el cuál está compuesto por un complejo de proteínas entre las que destacan la proteína de unión a ARN sensible a la transactivación (TRBP) y la familia de proteínas argonauta (AGO)]. Una vez funcionales, los miRNAs maduros en el complejo RISC se unen a regiones 3´UTR de sus ARNm dianas para llevar a cabo su función (**Figura 6**) (70, 71).

De una u otra forma, generalmente el resultado final es una bajada en la expresión de su proteína diana. De este modo, un aumento en los niveles de un determinado miRNA, podría implicar un descenso en los niveles de expresión de su proteína diana. De igual forma, un descenso en los niveles de un determinado miRNA, podría promover un aumento en la expresión de su proteína diana (72).

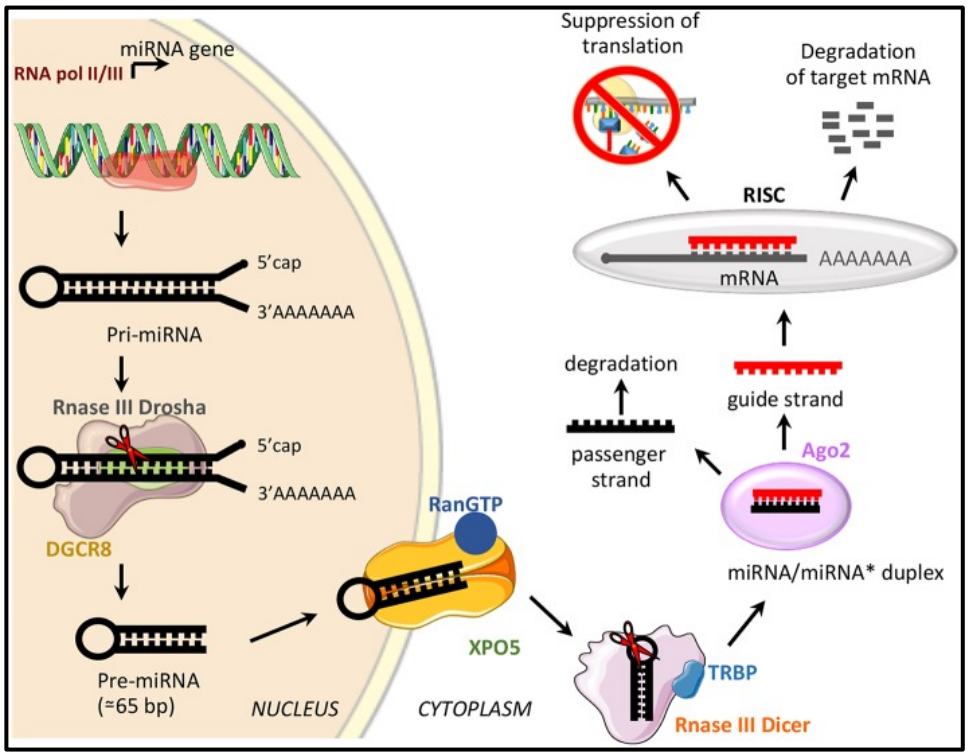

Figura 6. Biogénesis y función de los miRNAs. Representación esquemática de la ruta de biogénesis de miRNAs y la función biológica. "Di George syndrome critical región 8 (DGCR8)", exportina 5 (XPO5), "GTP-binding nuclear protein Ran (RanGTP)", "trans-activator RNA binding protein (TRBP)", argonauta 2 (AGO2) y "RNA-induced silencing complex (RISC)" (73).

4.2. Inflamación y miRNAs.

La inflamación crónica está involucrada en la aparición y desarrollo de muchas enfermedades, incluyendo obesidad, aterosclerosis, diabetes mellitus tipo 2, osteoartritis, enfermedades autoinmunes y degenerativas, asma, periodontitis y cirrosis. Avances recientes confieren un papel a los ARN no codificantes, especialmente los miRNAs, en la progresión y el mantenimiento de la respuesta inflamatoria. Varios miRNAs, tales como miR-126, miR-132, miR-146, miR-155 y miR-221 han surgido como importantes reguladores de algunos mediadores inflamatorios importantes (74).

Las redes moleculares que controlan la aparición y resolución de la inflamación deben regularse adecuadamente para un mantenimiento óptimo de la salud (75). Las respuestas inmunes innatas y adaptativas se encuentran altamente controladas y recientemente se ha identificado una regulación epigenética subyacente que controla el sistema de defensa (76, 77). Los miRNAs han sido ampliamente implicados como reguladores negativos de procesos inflamatorios a nivel post-transcripcional (78). Entre otros, los miR-146, miR-155 y miR-223 son probablemente los más estudiados. Estos se han identificado como miRNAs de respuesta inflamatoria que se sobreexpresan por NFkB. Diferentes estudios indican que miR-146 y miR-155 en conjunto juegan un papel esencial en la regulación de rutas involucradas en la respuesta inmune innata y procesos inflamatorios crónicos de diferentes tejidos humanos (79-81).

Cuando se produce el proceso inflamatorio ocurre una sobreexpresión de varias citoquinas pro-inflamatorias así como la expresión de numerosos miRNAs que regulan proteínas específicas (82, 83). Por otro lado, la expresión de numerosos miRNAs está reducida durante la activación de las células inmunes. Cuando la expresión de los miRNAs anti-inflamatorios se encuentra reducida o los miRNAs pro-inflamatorios se expresan a niveles elevados, el sistema inmune se sobreactiva o viceversa (84). Por tanto, una desregulación de la expresión de miRNAs puede conducir una deficiente respuesta inmune, autoinmunidad o a una infección crónica (85-88).

La expresión de citoquinas y factores pro-inflamatorios, incluyendo interleuquina-1 (IL-1), IL-4, IL-6, IL-8, IL-10, GM-CSF ("granulocyte-macrophage colony-stimulating factor"), VEGF ("Vascular Endothelial Growth Factor"), prostaglandina E2, ciclooxigenasa 2 (COX-2), metaloproteinasas de la matriz y factores de crecimiento fibroblástico están fuertemente regulados a diferentes niveles, incluyendo la transcripción génica, la traducción de ARNm y la degradación de éste (89). Por otro lado, aunque prácticamente se pasa por alto, la relación reguladora entre citoquinas y miRNAs parece ser recíproca: no solo los miRNAs presentan como dianas determinados ARNm y consecuentemente regulan su expresión, sino que también las citoquinas tienen impacto sobre la expresión de los miRNAs (90). Por ejemplo, la IL-1β y el TNF-α son potentes estimuladores de la expresión de miR-146a y miR-155 en una variedad de tipos celulares (91). Sorprendentemente, el miR-23b es regulado negativamente por IL-17,

una citoquina que juega un importante papel en la regulación de la activación de la inmunidad innata (92).

4.2. MiRNAs en enfermedades autoinmunes (AR y SAF).

Los miRNAs muestran una expresión alterada en varios tipos celulares en pacientes con enfermedades autoinmunes (93-98). Esta expresión alterada ha sido asociada con rutas de señalización inflamatorias que promueven la secreción de citoquinas pro-inflamatorias y otros procesos que mantienen el ciclo de la autoinmunidad (99).

En este contexto, adquieren una importancia relevante aquellas proteínas involucradas con la biogénesis de miRNAs, destacando la proteína DICER como la principal proteína reguladora de este proceso. El impacto de la maquinaría de biogénesis en el desarrollo de autoinmunidad y enfermedad cardiovascular ha sido previamente demostrado a través de varios modelos animales que presentan delecciones de DICER. En 2008, Zhou X y *col.,* mostraron que ratones "knockout" para DICER en células T específicas desarrollaron una autoinmunidad incontrolada a través de una desregulación en el desarrollo y función de células T reguladoras. Estas células mostraron una reducida expresión del factor de transcripción FoxP3 en paralelo a una alteración en la expresión de múltiples genes y proteínas reguladoras de la función de las células T, lo que se tradujo en la pérdida de su actividad supresora (100). Otro estudio observó que la delección de DICER en células T promovió una diferenciación defectuosa de células T "helper", las cuales presentaban un fenotipo preferentemente "Th-1" con una importante producción de IFN. Estas células proliferaron pobremente bajo estimulación y presentaron un incremento en el ratio de apoptosis (101). En cuanto a las células B, un estudio demostró que una delección de DICER en ratones mutantes producía una sobrexpresión de proteínas proapoptóticas y genes inhibidores del ciclo celular, resultando en un defecto de la supervivencia celular y la proliferación de células B. Asimismo, estas células presentaron un defecto en la producción de anticuerpos (102).

Con respecto a la AR, una amplia variedad de miRNAs están diferencialmente expresados y desregulados y modulan negativamente genes diana que codifican para proteínas involucradas en procesos inflamatorios que participan en su patogénesis (103).

Así, en células mononucleares de sangre periférica de pacientes con AR han sido identificados numerosos cambios en la expresión de miRNAs que resultan en un aumento en la secreción de citoquinas y un desequilibrio del balance de células T reguladoras (Th17) (la expresión de miR-16, miR-103a, miR-132, miR-145, miR-146a, miR-155, miR-221, miR-222 y miR-301a se encontró elevada, mientras que la expresión de miR-21, miR-125b y miR-548a reducida) (103-116). En cuanto a las células T de sangre periférica, la expresión de miR-146a se encontró incrementada en células Th17 periféricas (117) y reducida en células T reguladoras de sangre periférica (118) contribuyendo a un incremento en la secreción de citoquinas pro-inflamatorias. Además, el miR-223 está sobreexpresado en células T CD4+ naïve periféricas y regulado a la baja en células Th17 (119). Asimismo, las alteraciones en la expresión de miRNAs son también responsables de muchos aspectos de la actividad de fibroblastos sinoviales en la AR (120). Se ha demostrado que la alteración de los niveles de expresión de determinados miRNAs, como miR-124a, miR-126, miR-146a, miR-52, miR-155 y miR-221 conduce a la secreción de citoquinas pro-inflamatorias o metaloproteinasas e incrementa la supervivencia y proliferación de fibroblastos sinoviales en AR. En el tejido sinovial, los niveles alterados de miR-27a, miR-30a, miR708 y miR-206 han sido implicados principalmente en la invasión del cartílago por los fibroblastos y macrófagos, en el aumento de la supervivencia, migración de fibroblastos y la neovascularización (121-124). Además, la expresión alterada de let-7b, miR-155 y miR-223 en los macrófagos que invaden el tejido sinovial influye en el desarrollo y perpetuación de la inflamación sinovial. No obstante, no existen evidencias acerca de los niveles de expresión de miRNAs en neutrófilos de pacientes con AR y su potencial papel patogénico.

Por otro lado, en el SAF los primeros miRNAs identificados e involucrados en la patogénesis de la trombosis en estos pacientes fueron el miR-19b y el miR-20a. Sus niveles fueron encontrados significativamente reducidos en monocitos de pacientes con SAF en comparación con donantes sanos y negativamente correlacionados con la sobre-expressión de FT en estas células (125). Posteriormente, fueron caracterizados un número de miRNAs relacionados con la presencia de enfermedad cardiovascular en pacientes con SAF, los cuales estaban relacionados con características de la enfermedad como la respuesta inmune, estrés oxidativo, aterosclerosis y trombosis: miR-125a, miR-125b, miR-124a, miR-146a, miR-155 y miR-222

(126). Los niveles de estos miRNAs se encontraron reducidos en neutrófilos de pacientes con SAF mientras en monocitos dos de estos miRNAs evaluados, miR-155 y miR-146a, se encontraron incrementados. Por otro lado, estudios mecanísticos han revelado cómo los aPLs inducen la expresión de miR-146a-5p, miR-146-3p, miR-144 y miR-210 en los trofoblastos (127). Todo esto indica que las alteraciones en la expresión de los miRNAs están estrechamente relacionadas con la inmunopatología de los pacientes SAF.

4.1. MiRNAS circulantes.

Los miRNAs han demostrado una alta estabilidad en tejidos y plasma humano. De hecho, la mayoría de los perfiles de expresión de miRNAs, realizados en fluidos corporales de diversas patologías, indican que los miRNAs circulantes están protegidos frente a la actividad RNAsa endógena al ser transportados en el interior de exosomas o microvesiculas. Así, se ha sugerido que los miRNAs podrían actuar como moléculas de comunicación intercelular. Los exosomas son vesículas endocíticas derivadas de membranas celulares, con un tamaño de 30-120nm, que participan en la comunicación célula-célula y trasvase de proteína y ARN. Los miRNAs transportados en exosomas pueden ser absorbidos por células contiguas o distantes y modular de este modo la fisiología de determinadas células receptoras (128, 129).

Por otra parte, en diversas patologías se ha demostrado la presencia de algunos miRNAs sobre-expresados en un tejido concreto, mientras que en circulación han mostrado niveles reducidos. Por ejemplo, un estudio reciente ha demostrado que el miR-145, derivado de células del músculo liso vascular, muestra niveles de expresión reducidos en plasma y sobre-expresión en placas ateroscleróticas inestables. Los autores de este estudio sugieren que es posible que el tejido enfermo absorba preferentemente los exosomas que contienen miRNAs específicos, mostrando un descenso de los niveles de expresión de éstos en sangre, un incremento en su degradación en circulación, o que se deba al silenciamiento epigenético (130).

Los miRNAs circulantes fueron los primeros en ser utilizados como biomarcadores en suero de pacientes con linfoma difuso de células B (131). Posteriormente, multitud de estudios han informado acerca de una desregulación de miRNAs circulantes en diferentes enfermedades. De este modo, entre otros miRNAs relacionados con la patología cardiovascular, los

miR-1 y miR-208a se encuentran cuantitativamente alterados en enfermedades como el infarto de miocardio, aterosclerosis, enfermedad arterial coronaria, fallo cardíaco, hipertrofia, fibrosis y fibrilación auricular, de manera que han sido propuestos como biomarcadores de enfermedad cardiovascular (132, 133).

Algunos estudios que han evaluado el perfil de miRNAs circulantes en lupus eritematoso sistémico, han encontrado que el índice de actividad de la enfermedad (SLEDAI) correlaciona inversamente con los niveles de miR-200a. Asimismo, un trabajo reciente que ha analizado complicaciones vasculares en esta patología ha demostrado una correlación significativa entre la expresión de un perfil de miRNAs específico, la ocurrencia de trombosis venosas y la presencia de autoanticuerpos anti-ß2GPI (129).

En suma, aunque la utilidad diagnóstica de los miRNAs en enfermedades autoinmunes está por explorar, los estudios desarrollados en el ámbito de estas patologías sugieren que el estudio del perfil de expresión de miRNAs circulantes podría permitir la identificación de biomarcadores potenciales de diagnóstico, pronóstico o actividad de enfermedad.

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HIPÓTESIS

La patogénesis de las enfermedades autoinmunes como el Síndrome Antifosfolípido (SAF) o la Artritis Reumatoide (AR) es extremadamente compleja y multifactorial, donde están involucrados numerosos mecanismos genéticos, inmunológicos e inflamatorios.

Recientemente, **los miRNAs** han sido descritos como importantes reguladores de la expresión de genes y proteínas claves en procesos inflamatorios y autoinmunes. Por tanto, para la identificación de los mecanismos moleculares subyacentes a las manifestaciones patológicas asociadas a estas enfermedades autoinmunes, es precisa una caracterización detallada de los miRNAs implicados en estos procesos. Además, los miRNAs muestran una importante estabilidad en tejidos y biofluidos humanos, aspecto que potencia su utilidad diagnóstica como biomarcadores. Así, el análisis del perfil de expresión de estos miRNAs en APS y AR y su modulación por elementos intrínsecos de la enfermedad como los autoanticuerpos o moléculas inflamatorias podría facilitar el establecimiento de nuevos modelos para el diagnóstico, pronóstico y manejo de la enfermedad.

Se ha demostrado que **la inflamación** juega un papel clave en el desarrollo de enfermedad cardiovascular asociada a la artritis reumatoide. De hecho, se ha observado un claro paralelismo entre el estatus inflamatorio presente en pacientes AR y el asociado a estados de obesidad y resistencia a la insulina, aunque los mecanismos que lo desencadenan aún son desconocidos. Se ha sugerido asimismo la importancia de las interacciones recíprocas entre el sistema metabólico y las células inmunes, lo que explica que enfermedades donde el sistema inmune se encuentra alterado pueden dar lugar a desórdenes metabólicos asociados. Por todo ello creemos que los mediadores inflamatorios presentes de forma crónica en la AR podrían ser los responsables del desarrollo de resistencia a la insulina en los distintos tejidos metabólicos y que seria de gran relevancia caracterizar las rutas intracelulares involucradas como procesos clave en el desarrollo de enfermedad cardiovascular.

En suma, la integración de análisis inmunológicos, celulares, moleculares y epigéneticos permitirían caracterizar los mecanismos patogénicos asociados al SAF y la AR, así como identificar potenciales biomarcadores asociados a las distintas manifestaciones clínicas en estas patologías autoinmunes.

OBJETIVOS

El **objetivo principal** de esta Tesis doctoral ha sido investigar el papel de la inflamación y los miRNAs en la patogénesis del Síndrome Antifosfolípido y la Artritis Reumatoide, mediante la integración de análisis inmunológicos, celulares, moleculares y epigenéticos.

Objetivos específicos:

1. Identificar el perfil de miRNAs presente en el plasma de pacientes con SAF, su modulación por efecto de los anticuerpos antifosfolípido y su potencial papel como biomarcadores no invasivos para la tipificación clínica de la enfermedad.

   Artículo: "*Circulating microRNAs as biomarkers of disease and typification of the atherothrombotic status in antiphospholipid syndrome*". Haematologica (2018).

2. Analizar la alteración del perfil de miRNAs y de las proteínas implicadas en su biogénesis en neutrófilos de sangre periférica y sinovio de pacientes AR, así como evaluar el efecto de autoanticuerpos, componentes inflamatorios y tratamientos biológicos en su regulación con el fin de definir la función de los miRNAs en la activación anómala de este tipo celular asociada a la artritis reumatoide.

   Artículo: "*Impaired microRNA processing in neutrophils from Rheumatoid Arthritis patients confers their pathogenic profile. Modulation by biological therapies*". Haematologica (2020).

3. Investigar si los componentes inflamatorios presentes en la AR pueden contribuir al desarrollo de resistencia a insulina en estos pacientes, y estudiar los mecanismos moleculares que gobiernan la disfunción de los procesos homeostáticos que controlan el metabolismo de la glucosa y los lípidos en la AR.

   Artículo: "*Defective glucose and lipid metabolism in rheumatoid arthritis is determined by chronic inflammation in metabolic tissues*". Journal of Internal Medicine (2018).

CAPÍTULO I

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

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

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Running Heads: MicroRNAs as Antiphospholipid Syndrome biomarkers.

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1. ABSTRACT

The present study aimed at identifying the plasma miRNA profile of antiphospholipid syndrome patients and to investigate the potential role of specific circulating miRNAs as non-invasive disease biomarkers. Ninety antiphospholipid syndrome patients and 42 healthy donors were recruited. Profiling of miRNAs by PCR-array in plasma of antiphospholipid syndrome patients allowed identifying a set of miRNAs differentially expressed and collectively involved in clinical features. Logistic Regression and ROC-curve analyses identified a signature of 10 miRNA ratios as biomarkers of disease. Besides, miRNA signature was related to foetal loss, atherosclerosis, and type of thrombosis, and correlated with parameters linked to inflammation, thrombosis, and autoimmunity. Hard clustering analysis differentiated 3 clusters representing different thrombotic risk profile groups. Significant differences between groups for several miRNA ratios were found. Moreover, miRNA signature remained stable along time, demonstrated by their analysis three months after the first sample collection. Parallel analysis in two additional cohorts of patients including either, thrombosis without autoimmune disease, and Systemic Lupus Erythematosus without antiphospholipid antibodies, displayed each specific miRNA profiles, distinct from those of antiphospholipid syndrome patients. In vitro, antiphospholipid antibodies of IgG isotype promoted deregulation in selected miRNAs and their potential atherothrombotic protein targets in monocytes and endothelial cells. Taken together, differentially expressed circulating miRNAs in antiphospholipid syndrome patients, modulated at least partially by antiphospholipid antibodies of IgG isotype, might have potential to serve as novel biomarkers of disease features and for typifying the atherothrombotic status of patients, thus constituting a useful tool in the management of the disease.

2. INTRODUCTION

The Antiphospholipid syndrome (APS) is a clinical disorder characterized by the occurrence of thrombosis and/or pregnancy morbidity associated with the persistent presence of antiphospholipid antibodies (aPL), including anti-cardiolipin antibodies (aCL), anti-β2-glycoprotein 1 antibodies (anti-β2GPI) and/or lupus anticoagulant (LA).

Cardiac, cerebral and vascular strokes in these patients are responsible for a significant reduction in life expectancy.[1] The course of cardiovascular disease (CVD) in APS patients may rapidly change from asymptomatic to severe life-threatening manifestations difficult to deal with. Timely diagnosis and accurate monitoring of APS course are essential to improve the quality of therapy, avoiding approaches based on medical empiric protocols. In the same way, as many other autoimmune diseases, APS is characterized by a heterogeneous nature that has a dramatic impact on diagnosis and treatment.[2]

Pathophysiological mechanisms explaining how atherosclerosis and CVD are associated to APS have been greatly broadened with the application of genomic technologies.[3] One emerging and important mechanism controlling gene expression is epigenetics, which regulates gene packaging and independent expression of alterations in the DNA sequence. Epigenetics, which comprises DNA methylation, histone modifications, and microRNAs (miRNAs) activity, is providing new directions linking genomics and environmental factors.[4] miRNAs are small, non-coding RNAs that, depending upon base pairing to messenger RNA (mRNA) mediate mRNA cleavage, translational repression or mRNA destabilization. miRNAs are known to be involved in crucial cellular processes and their dysregulation has been described in many cell types and fluids in a broad range of diseases.[5-7]

In the setting of APS, a previous study by our group recognized that aPL modulate the expression of 2 miRNAs in monocytes (miR-19b and miR-20a) that control the expression of key proteins involved in the pathology of the disease, such as tissue factor (TF).[8] Moreover, we recently demonstrated that both, aPL and the anti-double stranded DNA antibodies (anti-dsDNA) promote specific changes in the expression of proteins related to the biogenesis of miRNAs in leukocytes of APS and systemic lupus erythematosus (SLE) patients, which are translated in the altered expression of the miRNAs profile and that of their protein targets, related to CVD, in these disorders.[9]

Extensive analyses have shown that miRNAs are released into the circulation, where they are present in concentration levels that differ between healthy subjects and patients. Although little is known about the origin and function of such circulating miRNAs, these molecules are increasingly recognized as non-invasive and readily accessible biomarkers for risk stratification, diagnosis and prognosis of multiple forms of CVD.[10]

Specific profiles of circulating miRNAs are also associated to the pathophysiology of different systemic autoimmune diseases, including SLE, systemic sclerosis, and rheumatoid arthritis (RA), and some of them appear to be of diagnostic and possibly prognostic value.[11] To date, in the context of APS, no study has analyzed the potential role of the circulating miRNAs as biomarkers of the disease. Therefore, the present study was designed to determine the plasma miRNA specific profile of APS patients, their modulation by autoantibodies, and their potential role as non-invasive biomarkers of disease features.

3. METHODS

Patients

Ninety patients with primary APS and 42 healthy donors (HDs) were included in this study, during a period of 24 months (see Supplemental Material). All experimental protocols were approved by the ethics committee of the Reina Sofia Hospital in Cordoba (Spain) and written informed consent was obtained. The characteristics of patients and HDs are shown in Table 1.

For each APS patient, the adjusted global anti-phospholipid syndrome score (aGAPSS) was calculated as previously described.[12] Briefly, aGAPSS was calculated by adding the points corresponding to both, the cardiovascular and thrombotic risk factors, based on a linear transformation derived from the β regression coefficient as follows: 3 for hyperlipidaemia, 1 for arterial hypertension, 5 for aCL IgG/IgM, 4 for anti-β2GPI IgG/IgM and 4 for LA.
Two additional cohorts of patients -as disease control-, were further analyzed, including 23 patients with thrombosis in the absence of an associated autoimmune disease [12 non-pregnant women and 11 men, mean age 44 (range: 21–73 years), including patients with objectively verified thrombotic events: 14 deep venous thrombosis and 9 thrombosis in intra-cerebral vessels], and 25 SLE patients without aPLs (Supplemental Table S1).

Blood sample collection and assessment of biological parameters
(Supplemental Material).

B-Mode Ultrasound IMT and Ankle Brachial Index measurements
(Supplemental Material).

Isolation of miRNAs and analysis of miRNAs expression profiling
Total RNA, including the miRNA fraction, was extracted from both, plasma and supernatants obtained from in vitro studies by using the QIAzol miRNeasy kit (Qiagen, Valencia, CA, USA) following manufacturer's instructions[13] (Supplemental Material).
To identify the changes that occurred in the expression levels of miRNAs in plasma from APS patients and HDs, a Human Serum & Plasma miRNA PCR-array (Qiagen) was performed (Supplemental Material) on an exploratory cohort (Supplemental Table S2).

Quantitative real-time PCR
A fixed volume of 3 µl of RNA solution from the 14 µl-eluate from RNA isolation of 200 µl plasma sample was used as input into the reverse transcription. Input RNA was reverse transcribed using the TaqMan miRNA Reverse Transcription kit and miRNA-specific stem-loop primers (Life Technologies, Madrid, Spain) (Supplemental Material). The expression levels of miRNAs were calculated by using 2-Ct and reciprocal ratios were performed [Ratio miR-A/miR-B = log2 (2-Ct miR-A/2-Ct miR-B)] as previously described.[14-19] Reciprocal ratios analysis is an approach that allowed to bypass the controversial issue of data normalization of miRNAs in plasma (self-normalization). Furthermore, miRNAs whose concentrations are changed due to a pathology in opposite directions, can be effective in differentiating investigated populations.

Target gene prediction and integrated analysis by ingenuity pathway analysis

The altered miRNAs were further analyzed 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.20 (Supplemental Material).

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

(Supplemental material).

Statistical analysis

(Supplemental material).

4. RESULTS

4.1. Clinical and analytical details of APS patients

Table I. Clinical and laboratory parameters of the APS patients and the healthy donors.

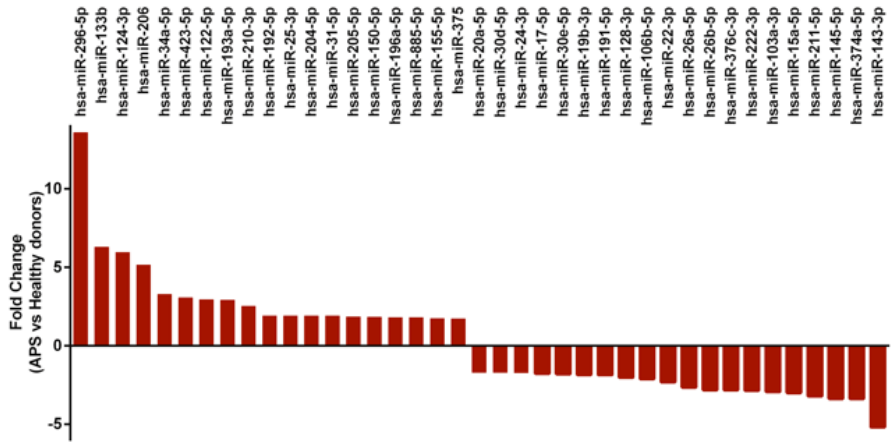
CLINICAL AND LABORATORY PARAMETERS	APS (total no. 90)	HDs (total no. 42)	P
Females/males, no.	48/42	22/20	
Age, years	51.2 ± 13.1	46.2 ± 13.4	NS
Arterial thrombosis, no.	35/90	0/42	
Venous thrombosis, no.	55/90	0/42	
Recurrences, no.	37/90	0/42	
Pregnancy morbidity, no.	23/90	0/42	
Pathologic CIMT, no.	24/90	6/42	0.00
ABI – left*	1.3 ± 0.11	1.2 ± 0.09	0.02
ABI – right*	1.27 ± 0.11	1.2 ± 0.09	0.02
LA positivity, no.	85/90	0/42	0.00
aCL-IgG,# GPL	23.4 (0.5-448)	1.3 (0.5-5)	0.00
aCL-IgM,# MPL	21.8 (0-354)	4.9 (0.8-17)	0.00
Anti-β2GPI IgG,# SGU	23.9 (0-361)	1 (1-2)	0.02
Anti-β2GPI IgM,# SMU	14.8 (0-289)	1.2 (1-2.6)	0.02
Antiplatelet agents,† no.	30/90	0/42	
Anticoagulant agents,‡ no.	62/90	0/42	
Total cholesterol level,* mg/dL	191.7 ± 32.06	190 ± 41.8	NS
Cholesterol HDL level,* mg/dL	51.09 ± 12.4	55.4 ± 13.1	NS
Cholesterol LDL level,* mg/dL	112.1 ± 33.2	118.4 ± 26.9	NS
Triglycerides level,* mg/dL	155.1 ± 163.2	88.3 ± 50.07	NS
ESR,* mm/h	13.5 ± 13.6	6.6 ± 4	0.05

APS indicates 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; SMU, standard IgM units; HDL, high-density lipoprotein; LDL, low-density lipoprotein; and ESR, erythrocyte sedimentation rate;
*Results are expressed as mean ± SD; #Results are expressed as mean and values range. †antiplatelet agents include acetylsalicylic acid and Clopidogrel; ‡anticoagulant agents indicate vitamin K antagonists, including warfarin and acenocumarol.

4.2. Differentially expressed miRNAs in the plasma of APS patients and HDs

In the discovery phase (exploratory cohort), we identified 39 miRNAs that were differentially expressed between APS patients and HDs (Cut off: 1.7-Fold Change), including 19 up-regulated and 20 down-regulated (Figure 1A). The functional analysis of the altered miRNAs in APS patients, by using the IPA software, showed that a large number of them had validated and putative target mRNAs mainly involved in connective tissue disorders, inflammatory response, reproductive system disease, CVD or skeletal and muscular disorders (Figure 1B).

A



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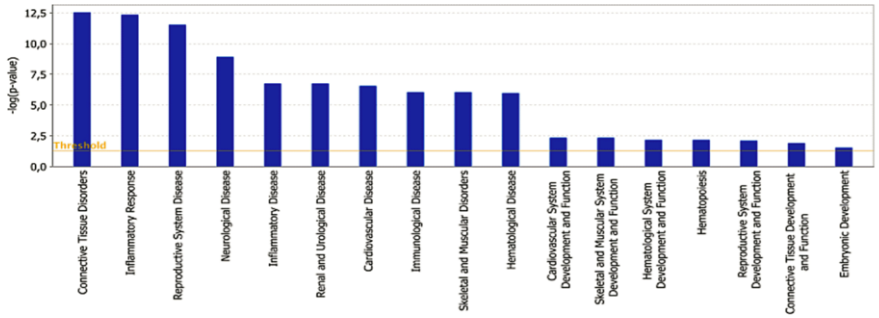

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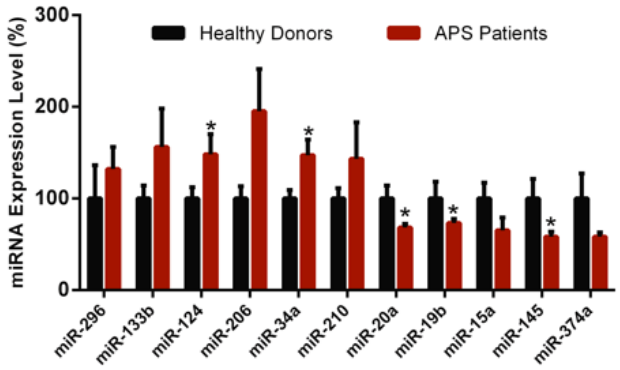


Figure 1. APS patients showed a specific circulating miRNAs profile related to clinical features of this autoimmune disorder. (A) To identify the changes that occurred in the expression levels of microRNAs (miR) in plasma from antiphospholipid syndrome vs controls, Human Serum & Plasma miRNA PCR-array (Qiagen) was performed in the exploratory cohort. In this profile, the expression levels of 19 miRNAs were found up-regulated in antiphospholipid syndrome, while 20 miRNAs were down-regulated. (B) Ingenuity Pathway Analysis (IPA) uncovered the main enriched biological functions and pathways on which these microRNAs are involved. The analysis included only the functions and pathways with average IPA score >2 [indicated as -log (*P* value)]. (C) Validation of selected miRNAs by RT-PCR in the whole cohort of APS patients and Healthy donors. (*) indicates P-value<0.05.

4.3. *Bioinformatic identification and analysis of deregulated miRNAs related to the pathophysiology of APS and analysis of potential protein targets*

In-silico studies were performed to identify the altered miRNAs that might have as potential targets a number of genes/proteins involved in the development of clinical manifestations related to APS, such as coronary artery disease, thrombosis, abortion and cerebrovascular dysfunction. Eleven altered miRNAs were identified by IPA as the main regulators of proteins involved in the pathology of APS, including miRNA 34a-5p, 15a-5p, 145a-5p, 133b-3p, 124-3p, 206, 20a-5p, 19b-3p, 210-3p, 296-5p and 374a-5p. This set of 11 miRNAs included, among others, the top 5 up-regulated miRNAs and 3 out of the top 5 down-regulated miRNAs in the PCR-array. The expression levels of the 11 selected miRNAs were analyzed in all the subjects recruited to the study by RT-PCR (Figure 1C). MiR-124 and miR-34a were found increased in APS patients in relation to healthy donors, while miR-20a, miR-19b and miR145a were found reduced. The remaining microRNAs were found also altered, showing a trend to either, increase or reduction as observed in the discovery phase, thus validating the data obtained by PCR-array.

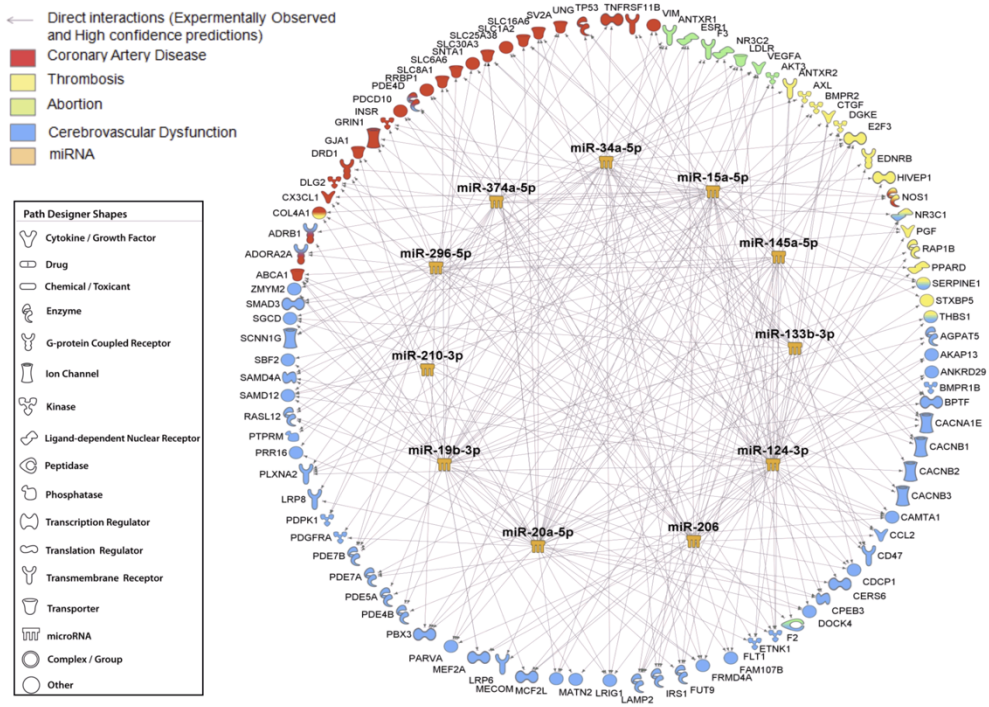


Figure 2. Interaction Network of microRNAs identified potential mRNA targets involved in clinical features of APS. By using the tool microRNA Target Filter of QIAGEN's Ingenuity Pathway Analysis (IPA, QIAGEN Redwood City, www.qiagen.com/ingenuity), the software generated a network including the selected microRNAs (miRNAs or miR) and their mRNA targets, filtered by coronary artery disease, thrombosis, abortion and cerebrovascular dysfunction. Only targets experimentally observed and predicted with high confidence are shown and related by direct interactions with their specific miRNA regulators.

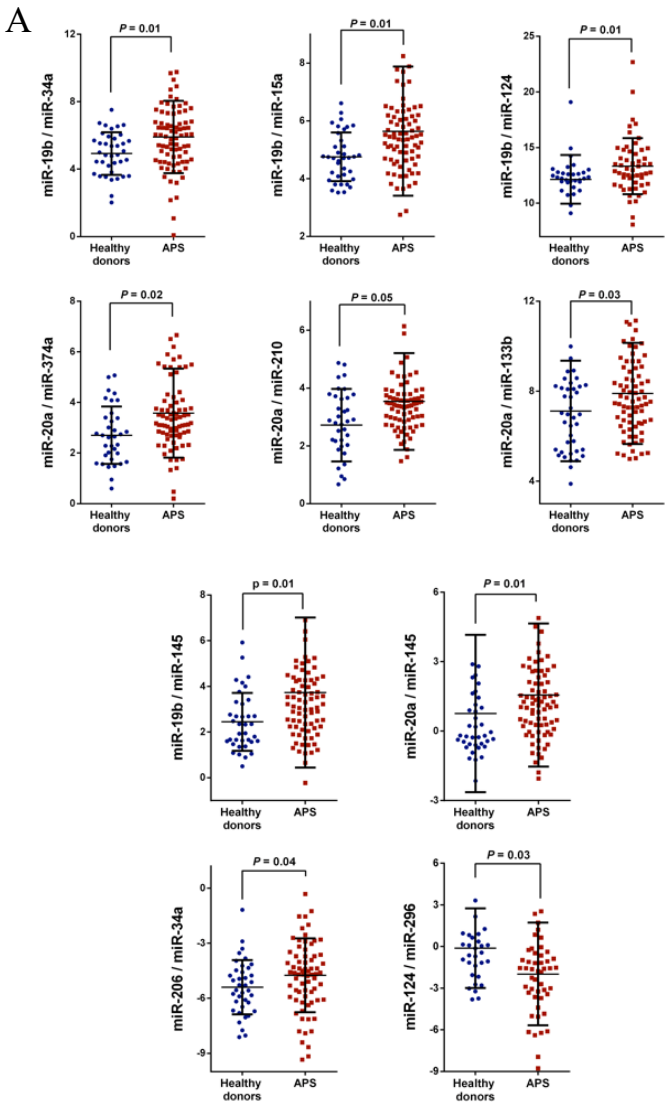
We further developed a network that defined the interaction miRNA-mRNA targets (Figure 2). Key proteins involved in the pathophysiology of APS, and identified as potential mRNA targets of those miRNAs, were quantified in the plasma of APS patients and HDs. As previously reported,[20-23] APS patients showed significantly increased plasma levels of TF, PAI-1, MCP-1, VEGF-A and VEGFR-1 (Supplemental Figure S1).

4.4. Circulating miRNA signature as potential biomarkers of disease in APS

It has been shown that the combination of miRNAs improves their predictive potential to differentiate 2 pathological conditions.[14-19] Thus, to assess the potential of specific circulating miRNAs in APS patients as biomarkers of disease features, reciprocal ratios of the miRNAs analyzed were performed by using statistical tools. By this approach, we identified 10 miRNA ratios,

integrated by the 11 selected miRNAs, and differentially expressed in plasma of APS patients in comparison with HDs, including miR-19b/miR-34a, miR-19b/miR-15a, miR19b/miR-124, miR-19b/miR-145, miR-20a/miR-145, miR-20a/miR-374a, miR-20a/miR-210, miR-20a/miR-133b, miR-206/miR-34a and miR-124/miR-296 (Figure 3A).

To further explore the efficiency of these biomarkers to identify APS patients, a combination of the 10 miRNA ratios as a panel was carried out by using a logistic regression on the data set as previously described.[24] Thus, all miRNA-ratios were integrated in a single model or equation, which provided a single 'score' that allowed us to perform the ROC-curve analysis and stablish the cut off for prediction. The ROC curve for the 10 miRNA ratios signature revealed a marked accuracy, evidenced by an AUC of 0.81. At the optimal cut-off value of 0.6, the sensitivity and specificity of the combined miRNA panel for APS identification were of 78% and 80%, respectively (Figure 3B).



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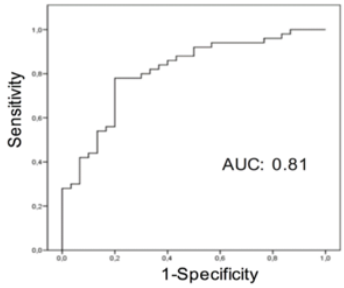


Combined miRNA Panel-ROC Analysis	
Area under the curve (AUC)	0.81
Sensitivity, %	78
Specificity, %	80
Cut-off	0.6

Figure 3. A circulating miRNA signature in APS might have potential value as biomarkers of disease. (A) Selected microRNAs (miRNAs or miR) were analysed in the whole cohort, including 90 antiphospholipid syndrome (APS) patients and 42 healthy donors, and reciprocal ratios were performed. Beeswarm plot of each differentially expressed miR ratio is shown, along with mean, standard deviation, and p-value. For statistical analysis, after normality and equality of variance tests, comparisons were made by paired Student t test or alternatively using a nonparametric test (Mann-Whitney rank sum test) (B) A combination of the 10 miRNA ratios as a panel was carried out by using logistic regression on the data set. ROC curve of miRNA panel and cut-off were generated based on the predicted probability (P) for each subject as a single score.
The equation used in our model was: Combined miRNA-ratio panel [Logit(p)] = -0,64+ 0,034x(miR-19b/miR-34a) + 1,061x(miR-19b/miR-15a) + 0,248x(miR-19b/miR-124) – 1,704x(miR-19b/miR-145) + 2,34x(miR-20a/miR-145) – 0,729x(miR-20a/miR-374a) – 0,624x(miR-20a/miR-210) + 0,088x(miR-20a/miR-133b) + 0,166x(miR-206/miR-34a) + 0,056x(mir-124/miR-296)". The area under the curve (AUC), sensitivity and specificity are displayed, and a cut-off value with higher specificity was selected.

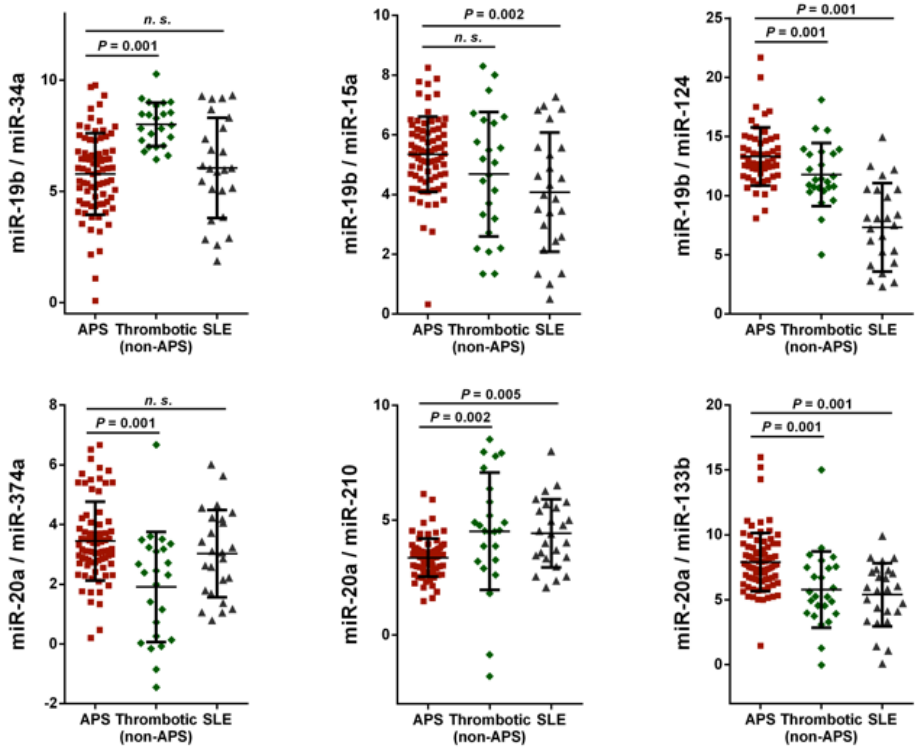
4.5. Stability of miRNA expression profile over time in APS

Plasma from 21 APS patients included in the study was evaluated again three months after the first blood sample collection, to analyze the stability of the circulating miRNA profile.
Results demonstrated that miRNA expression in the second sample collection did not change in relation to the first analysis (Supplemental Figure S2A). Moreover, the levels of miRNA ratios at baseline correlated significantly with the levels of these ratios three months later (Supplemental Figure S2B). Thus, our data support the theory that there is a specific circulating miRNA signature in APS, which remains stable along time.

4.6. APS patients show a specific miRNA profile different from both, non-autoimmune patients with previous thrombotic events, and aPL-negative SLE patients

To assess the specificity of the miRNA signature found in APS patients and in order to analyse whether the altered expression of the circulating miRNAs evaluated was linked to their thrombophilic status, an additional disease

group, including 23 patients with thrombosis in the absence of an associated autoimmune disease was evaluated. In these patients, the ratios formed by the expression levels of the 11 selected miRNAs were significantly different from those described in APS patients, except for the ratios miR-19b/miR-15a and miR-19b/miR-145 which *exhibited non*-significant *differences* (Figure 4). To evaluate if the altered expression of the miRNA signature was a sign of an autoimmune status, an additional disease group, including 25 SLE patients negative for aPL, was analyzed. In this SLE cohort the ratios produced by the selected circulating miRNAs were significantly different from those found in APS patients, except for the ratios miR-19b/miR-34a, miR-20a/miR-374a and miR-124/miR-296 which *exhibited non*-significant *differences* (Figure 4).



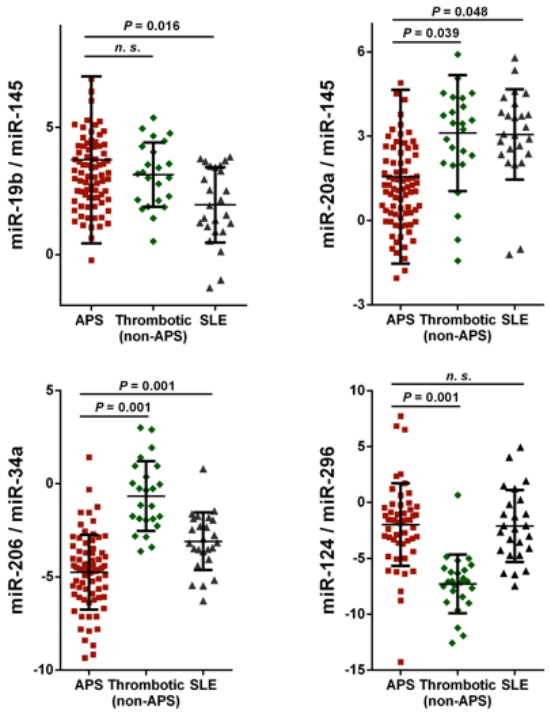


Figure 4. APS patients show a specific miRNA profile distinct from both, non-autoimmune patients with previous thrombotic events, and aPL-negative SLE patients. Twenty-three thrombotic non-antiphospholipid syndrome patients (non-APS) and twenty-five aPL-negative SLE patients were included and the circulating microRNA (miRNA or miR) signature of APS were compared. One-way ANOVA was used for statistical comparisons. A Bonferroni correction was applied for multiple testing. $p < 0.05$ was considered statistically significant. Beeswarm plot of each differentially expressed miRNA ratio is shown along with mean, standard deviation and P value. n.s. indicates *no significant statistical differences.*

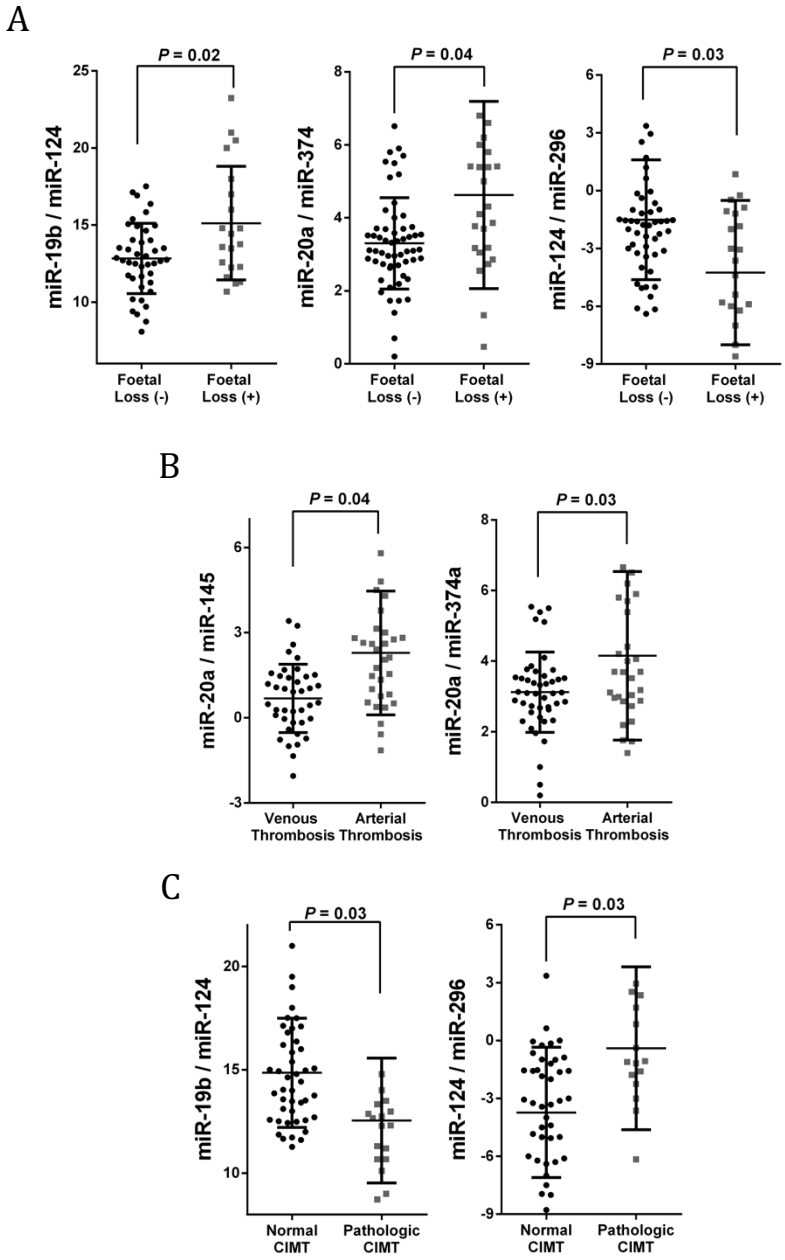
4.7. Potential influence of standard therapy on the profile of circulating miRNAs in APS

APS patients were classified in two groups, based on the treatment received, including 30 primary APS patients treated with antiplatelet agents and 62 primary APS patients treated with anticoagulant drugs. The statistical comparison between patients treated with antiplatelet and anticoagulant agents showed no significant differences in miRNA signature, except for the ratio miR-20a/374 (Supplemental Figure S3).

4.8. Circulating miRNAs are associated with clinical features of APS and show potential as biomarkers for the development of atherosclerosis

The levels of some circulating miRNA ratios that integrate the signature in APS were associated with the occurrence of foetal losses in these patients, including elevated levels of miR-19b/miR-124 and miR-20a/miR-374, and reduced levels of miR-124/miR-296 (Figure 5A). Associations between

miRNA ratios and the type of thrombosis suffered by APS patients were also identified. Thus, elevated levels of ratios miR-20a/miR-145 and miR-20a/miR-374 were significantly associated with the occurrence of arterial thrombosis in APS patients (Figure 5B). Furthermore, the ratios composed by miR-19b/miR-124 and miR-124/miR-296 were also found associated with the presence of a pathological CIMT in these patients (Figure 5C). To accurately evaluate their relevance as biomarkers of early atherosclerosis, we conducted combined ROC analyses of these miRNA ratios. The combination of both circulating miRNA ratios as a panel showed an evident accuracy, with an AUC of 0.76 at a sensitivity of 67% and specificity of 78% from a cut-off value of 0.41 (Figure 5D).



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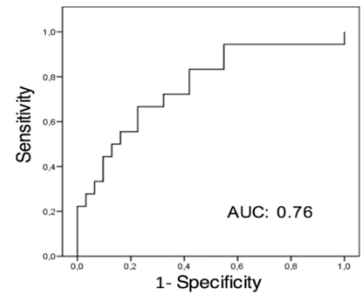


ROC Analysis Combined miRNA Panel	
Area under the curve (AUC)	0.76
Sensitivity, %	67
Specificity, %	78
Cut-off	0.41

Figure 5. Circulating miRNAs are related to clinical features of APS and show potential as biomarkers for the development of atherosclerosis. Associations studies of altered circulating microRNA (miRNA or miR) ratios and the occurrence of previous foetal loss (A), the type of thrombosis suffered (B) and the presence of a pathologic carotid intima-media thickness (CIMT) (C). Beeswarm plot of each miR ratio is shown, along with mean, standard deviation, and p-value. (D) A combination as a panel of the 2 miRNA ratios associated to the pathologic CIMT was carried out by using logistic regression on the data set and receiver-operator characteristics (ROC) curve analyses were performed. ROC curve of miRNA panel and cut-off were generated based on the predicted probability (P) for each patient as a single score. The equation used was: "Combined miRNA-ratio panel [Logit(p)] = 0,599 – 0,133x(miR-19b/miR-124) + 0,007x(miR-124/miR-296)". The area under the curve (AUC), sensitivity and specificity are displayed, and a cut-off value with higher specificity was selected.

4.9. Cluster analysis

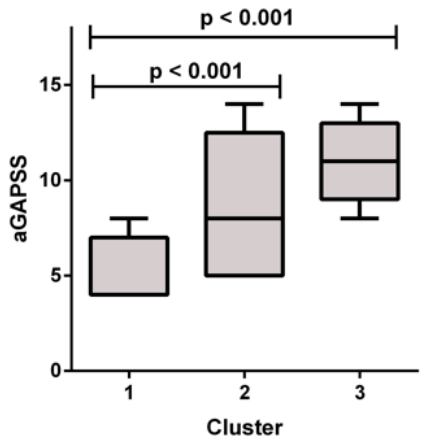
Hard clustering analysis in the APS cohort differentiated 3 clusters representing different thrombotic risk profile groups. Clinical and laboratory parameters of each cluster are resumed (Figure 6A). Briefly, cluster 1 (50% of the clustered cohort) was characterized by lower prevalence of cardiovascular risk factors and aPL multiple positivity. Conversely, cluster 1 shows a higher rate of venous thrombotic event when compared to the other clusters. Cluster 2 (17.6% of the clustered cohort) was characterized by a higher rate of cardiovascular risk factors, arterial thrombotic events, recurrences and a low prevalence of multiple aPL positivity. Cluster 3 (32.4% of the clustered cohort) was represented by a higher rate of multiple aPL positivity, arterial thrombotic events, and lower rate of cardiovascular risk factors. When evaluating different miRNA ratios expression among clusters, we found a statistically significant difference between groups for the following miRNA ratios: miR-19b/miR-124 (ANOVA $P < 0.001$), miR-20a/miR-374 (ANOVA $P < 0.05$), miR-20a/miR-210 (ANOVA $P < 0.001$) and miR-124/miR-296 (ANOVA $P < 0.05$). miRNA ratios expression in the different clusters are summarized (Figure 6B).

When comparing the aGAPSS values among the different clusters, we found a significant difference (t-test $P = 0.008$) between cluster 1 (mean aGAPSS 5.38; SD ±1.628) and Cluster 2 (mean aGAPSS 8,67; SD ±3.67). Similarly, we found a significant difference (t-test $P < 0.001$) between cluster 1 and cluster 3 (mean aGAPSS 10.82; SD ±2.316). aGAPSS values stratifying for clusters are represented (Figure 6C).

A

	CLUSTER 1 (50%)	CLUSTER 2 (17,6%)	CLUSTER 3 (32,4%)
HTA, %	0	100	9,10
Dislipidemia, %	35,30	50	36,40
Diabetes, %	0	33,30	0
Smoking, %	47,10	0	18,20
LA, %	100	100	9,10
aCL, %	0	16,70	63,60
anti-β-2GPI, %	0	0	72,70
Arterial thrombosis, %	33	50	50
Venous thrombosis, %	67	50	50
Recurrences, %	44	67	56

B



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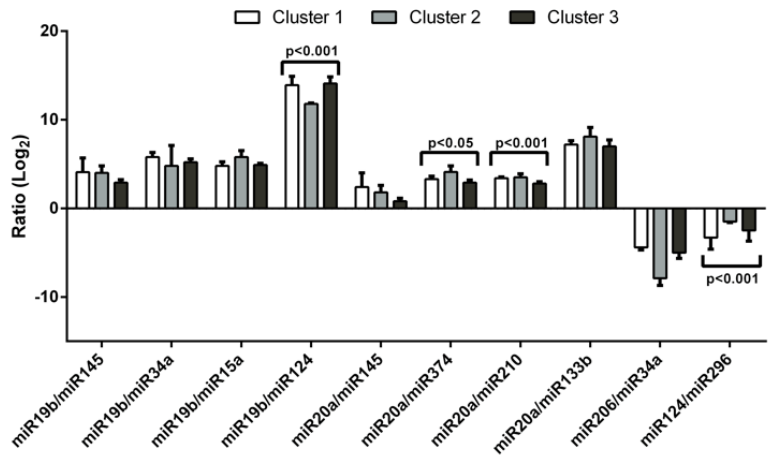


Figure 6. Specific miRNA signatures might identify subgroups of APS patients showing different thrombotic risk profiles: cluster analysis. (A) Clinical and laboratory parameters of the 3 clusters. (B) Comparison of the adjusted global anti-phospholipid syndrome score (aGAPSS) values among the different clusters. (C) Evaluation of different microRNA (miR) ratios expression among clusters. HTA indicates arterial hypertension; LA, lupus anticoagulant; aCL, anti-cardiolipin IgG/IgM; anti-β2GPI, anti-β2 glycoprotein 1 IgG/IgM.

4.10. Circulating miRNAs correlate with clinical and serological parameters in APS

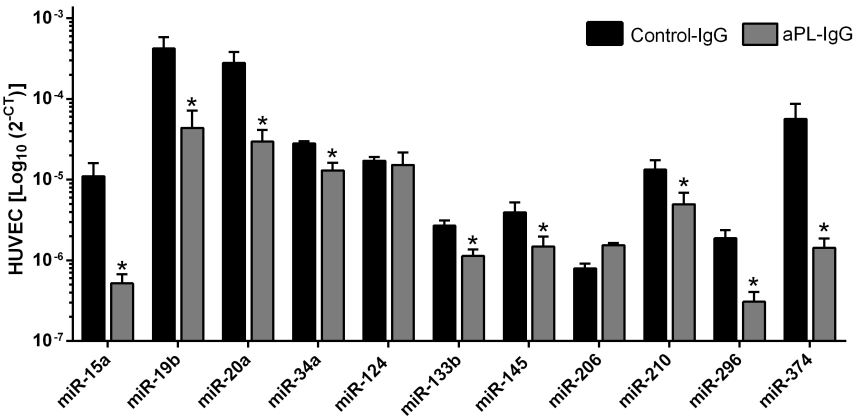
Correlation studies showed that the levels of some circulating miRNA ratios that integrate the signature in APS, were linked with clinical parameters such as the ABI, the presence of elevated titers of aPL, particularly aCL and anti-β2GPI antibodies, and the erythrocyte sedimentation rate (Supplemental Table S3). Correlation analyses with serological markers related to atherothrombosis further showed significant positive correlations with the expression levels of various miRNA ratios and with levels of TF, PAI-1, VEGF-A, VEGF-R1 and MCP-1 (Supplemental Table S3). Some of these correlations were also found among various miRNA ratios in plasma of APS patients.

4.11. Antiphospholipid antibodies modulate the expression of both, the circulating miRNAs that integrate the signature in APS and their potential protein targets

The expression of the 11 selected miRNAs was significantly altered in the supernatant of HUVECs treated with aPL-IgG in relation to those treated with a non-immune-IgG (Figure 7A) except for the miR-124 and miR-206. Accordingly, this treatment promoted in HUVECs the secretion of atherothrombotic proteins, such as TF, PAI-1 and VEGF-R1 (Figure 7B),

potential targets of the miRNAs analysed. On the other hand, the expression levels of several miRNAs were deregulated in the supernatant of monocytes treated with aPL-IgG, including miR-19b, miR-20a, miR-145, miR-210 and miR-296 (Figure 7C). Concomitantly, aPL-IgG treatment promoted in monocytes an increase in the secretion of TF, PAI-1 and MCP-1 (Figure 7D)

A



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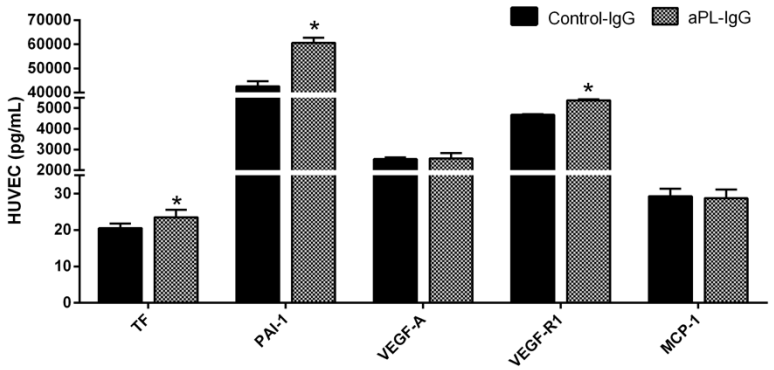

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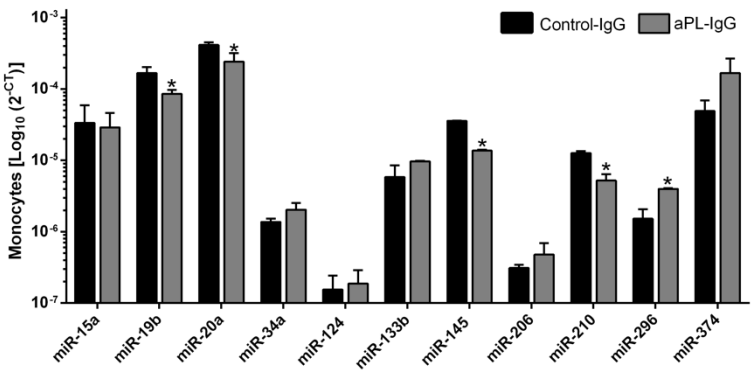

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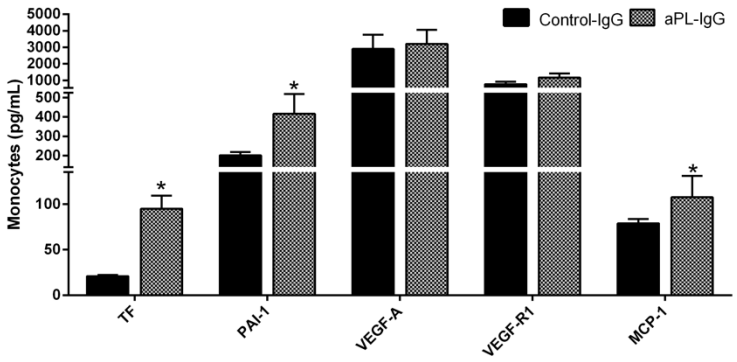

Figure 7. Antiphospholipid antibodies modulate the expression of both, the circulating miRNAs that integrate the signature in APS, and their putative protein targets. Human umbilical vein endothelial cells (HUVECs) were treated with antiphospholipid antibodies and secreted selected microRNAs (miRNAs) (A) and putative target proteins (B) levels were determined in the supernatant. Monocytes were also treated with antiphospholipid antibodies and secreted selected miRNAs (C) and putative target proteins (D) levels were evaluated in the supernatant of culture. Differences were analyzed by means of Student's t-test. Values are the means and SEM of 4 independent experiments performed in triplicate. Statistical significance was taken as $P < .05$. TF indicates tissue factor; PAI-1, plasminogen activator inhibitor-1; VEGF-A, vascular endothelial growth factor A; VEGF-R1, VEGF-Receptor-1; MCP-1, monocyte chemotactic protein.

5. DISCUSSION

The present study identifies, for the first time, a specific signature of circulating miRNAs in APS patients that might serve as potential biomarkers of clinical features of this autoimmune disorder. Moreover, this signature could represent a useful tool to typify and stratify patients based on their thrombotic status and cardiovascular risk profile (Supplemental Figure S4).

Circulating miRNAs were firstly described in peripheral blood as promising specific biomarkers for a wide range of diseases, such as cancer and other inflammatory pathologies.[25,26] Thereafter, several studies revealed the altered expression of numerous miRNAs in plasma, blood cells, and tissues of systemic autoimmune conditions, such as RA and SLE, which were directly associated to disease activity, making them potential useful biomarkers for clinical features and follow-up.[9,26-29] However, to date, the specific profile of circulating miRNAs in APS patients has not been evaluated. In the present study, the profiling of miRNAs by PCR-array in plasma of APS patients has allowed to identify a set of miRNAs differentially expressed and collectively associated to clinical features of the disease, such as inflammatory response, reproductive system disease, and CVD, among others. We further developed a model, by using logistic regression, that identified 10 miRNA ratios, differentially expressed, that showed great potential as biomarkers of disease of APS patients.

Recent studies support the evidence that a miRNAs signature has a higher diagnostic value than individual miRNAs.14-19 The use of ratios is a feasible approach that overlooks the controversial question of normalizing plasma levels of miRNAs, given the lack of a reliable normalizer for circulating miRNAs. In addition, the establishment of these ratios allows the identification of a combination of expression profiles closer to reality in vivo in patients, where the interactions between miRNAs and their specific potential targets never occur in a unique or individualized way. In fact, it is likely that, in some cases, various miRNAs, whose concentrations are shifted in opposite directions in a particular pathology, contribute together and specifically to certain clinical profiles.

The signatures of circulating miRNAs identified in APS patients integrated miRNAs previously described to be altered in other autoimmune and CVD. Thus, miR-19b and miR-20a have been shown to be essential modulators of TF expression in APS and SLE patients,[8] so that reduced expression of such miRNAs contributes to the overexpression of TF in monocytes, which is directly associated with the occurrence of thrombotic events in APS.[21] On the other hand, miR-124, found altered in APS, SLE and RA patients at both cellular and plasma levels, modulates the overexpression of MCP-1, a key chemokine directly involved in CVD associated to these autoimmune conditions.[30-33] Likewise, miR-133b and miR-145 have been identified as the most promising biomarkers of the pathogenesis of CVD. Both miRNAs participate in the differentiation of vascular smooth muscle cells. In addition, miR-133b regulates angiogenesis and endothelial function, while miR-145 participates in the stabilization of atheromatous plaque.[34] The miR-34a is

highly expressed in endothelial cells and elevated circulating levels of this miRNA have been associated to myocardial infarction.35 Moreover, the main target of miR-34a is VEGF-A, a key inflammatory protein involved in numerous cardiovascular and autoimmune pathologies, including APS.[23,36] In the same way, miR-374 has been described as regulator of maintenance of vascular integrity.[37] The remaining miRNAs members of the signature, including miR-296, miR-210, miR-206 and miRNA-15 have been found altered in severe preeclampsia, one of the leading causes of maternal mortality and neonatal morbidity worldwide.[38-40] Thus, all the processes regulated by these miRNAs seem to orchestrate distinct aspects of APS pathogenesis.

To assess the specificity of the circulating miRNA signature in APS we evaluated the miRNA profile in an additional cohort of patients characterized by the presence of previous thrombotic events in the absence of an associated autoimmune disease. The miRNAs analysis revealed a differential pattern of expression between these 2 cohorts. Those results substantiate previous studies that evidenced the presence of a distinct miRNA profile in monocytes and neutrophils of thrombotic non-autoimmune patients compared to APS patients.[9] This fact could reflect a differential mode of regulation and activity of miRNAs in thrombotic patients compared to APS patients, on which the role of autoantibodies might be crucial. Moreover, the analysis of a parallel autoimmune population –SLE patients- negative for aPL, also identified a miRNA signature distinct from that of APS, thus underlying the potential role of aPLs as regulators of thrombosis-related miRNAs in APS, and pointing at the presence of a specific miRNA profile relative to the pathogenesis of each disease.

APS patients recruited in this study were mainly treated with anticoagulant and/or antiplatelet agents. All of them have been shown to influence the miRNAs expression, an epigenetic process that might help to delineate the mechanisms underlying their effects.[9,41,42] Thus, we evaluated the potential effect of these treatments on the circulating miRNA expression profile. No significant differences were observed in our cohort of APS patients between those who received antiplatelet and those treated with anticoagulant agents, suggesting that the prothrombotic status induced by effects of aPLs, and the consequently deregulated miRNAs, were not differentially modulated among these drugs.

In order to understand the clinical relevance of the altered circulating miRNA signature, association and correlation studies were performed. Altered expression of various miRNA ratios was associated with the presence of previous foetal losses. In line with these findings, several studies have shown that the misregulation of circulating placental miRNAs in maternal blood, might lead to pregnancy complications, thus acting as non-invasive diagnostic and prognostic biomarkers for pregnancy monitoring.[42-44] Association studies further established a significantly increased expression of 2 miRNA ratios in APS patients that had suffered arterial thrombosis in comparison with those who experienced venous thrombotic events. Interestingly, both miRNA ratios were integrated by the miR-20a, previously

reported to be the main regulator of TF, whose expression levels has been found to be related to the development of arterial thrombosis in the setting of APS.[8,45] Finally, we identified 2 miRNA ratios as clinical relevant biomarkers related to early atherosclerosis development in APS patients, which were integrated by the miR-19b and miR-124, both of them critical players in the expression of proteins related to inflammation and thrombosis in APS and SLE.[8,9]

Correlation studies revealed that the altered circulating miRNA signature in APS is linked to parameters related to increased risk of peripheral artery disease such as the ABI. Moreover, correlations between circulating miRNA levels and numerous altered parameters related to inflammation and thrombosis were also identified. These correlations support the relationship observed in the in-silico study between the selected miRNAs and potential target proteins involved in various clinical features of APS. The influence of the autoimmunity in the circulating profile of miRNAs in APS was also revealed by the significant correlation between high titers of aPL-IgG and the altered expression of several miRNAs integrating the signature. These relationships further sustain the ones previously identified among the altered profile of miRNAs in APS and SLE at cellular level and the autoimmune and inflammatory profile of both autoimmune conditions.9 Therefore, our data suggest that the altered plasma profile of miRNAs is an important mechanisin that might contribute to the regulation of the pro-atherothrombotic status of APS patients, on which aPL seem to play a key role.

Our *in vitro* studies further confirmed this hyphotesis, demonstrating that aPL-IgG antibodies promoted a significant deregulation in the expression levels of both, the selected miRNAs and their potential protein targets in the supernatant of cultured monocytes and HUVECs, the main drivers of the CVD in the setting of APS. These results also confirm and complement previous studies which showed the in vitro effects of aPL-IgG in the induction of prothrombotic/inflammatory mediators[3,31] and the modulation of specific cellular miRNAs involved in their modulation.[8,9]

Nevertheless, although our data show specific effects of aPL-IgG on the secretion of several circulating microRNAs related to CVD, the contribution of other components of the vascular and immune system to the altered profile of circulating miRNAs remains to be defined. In addition, since we did not perform a complete plasma human microarray analysis, we cannot exclude the complementary role of other circulating miRNAs in the physiopathology of APS.

Interestingly, our analysis supports a clinical role for the use miRNA ratios when stratifying patients for their thrombotic risk. While studying the miRNA expression profile have offered enlightement in the understanding of APS pathogenesis,9 its clinical utility is still on debate. Our data support that specific miRNA signatures could identify subgroups of APS patients showing different clinical profiles (in term of site of thrombosis and risk of recurrences), potentially paving the way for their use as useful biomarkers that will increase specificity and sensitivity of thrombotic risk assessment.

Altogether, our data suggest that differentially expressed miRNAs in the plasma of APS patients, modulated at least partially by aPL-IgG antibodies, might have potential to serve as novel biomarkers of disease features and for typifying the atherothrombotic status of patients, thus constituting a useful tool in the management of the disease.

Author Contributions

P.R-L, I.A-R, N.B, ML-T, and MC. A-A developed the *in vivo* assays, performed the experiments and solved technical problems. MA.A, MJ.C, E.C-E, MT.H, J.L-H, and MJ.H-V followed up with patients and contributed useful discussion and suggestions. C.P-S, C.M, R.G-C, S.S, and C.L-P formed the hypothesis, directed and coordinated the project, designed the experiments, analyzed the data, and wrote the manuscript. F.V and P.S performed clinical analysis and contributed useful suggestions. Y.J-G, M.R, S.S, I.C, performed statistical analysis and discussed results.

Conflict-of-interest disclosure

The authors declare no competing financial interests.

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7. SUPPLEMENTARY INFORMATION.

7.1. DETAILED METHODS

7.1.1. 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

7.1.2. 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].

7.1.3. 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 ABI3. 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.

7.1.4. 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.

7.1.5. 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-ΔΔCt 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).

7.1.6. 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.

7.1.7. 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% CO2. 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.

7.1.8. 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.

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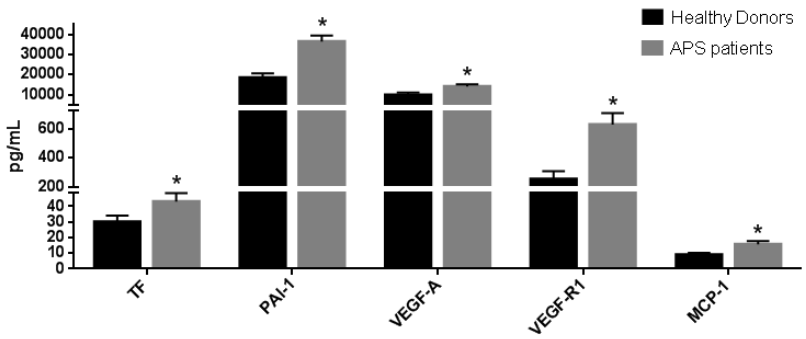
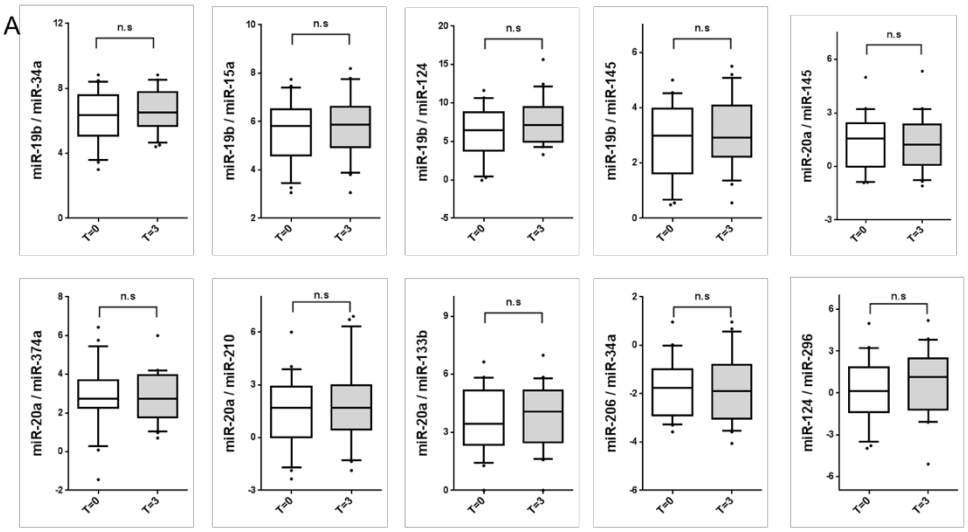
7.2. SUPPLEMENTAL FIGURES



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

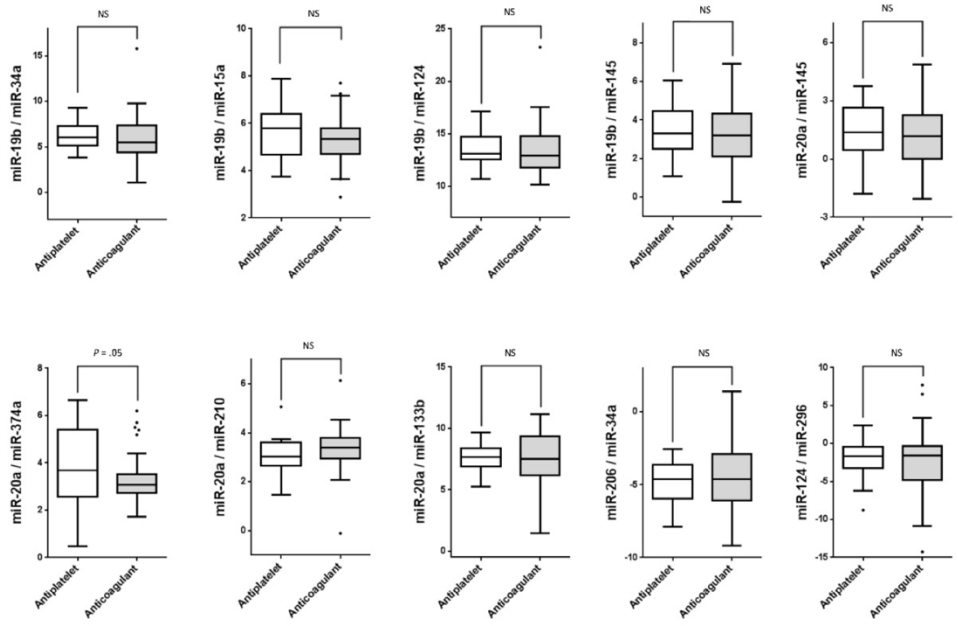


B

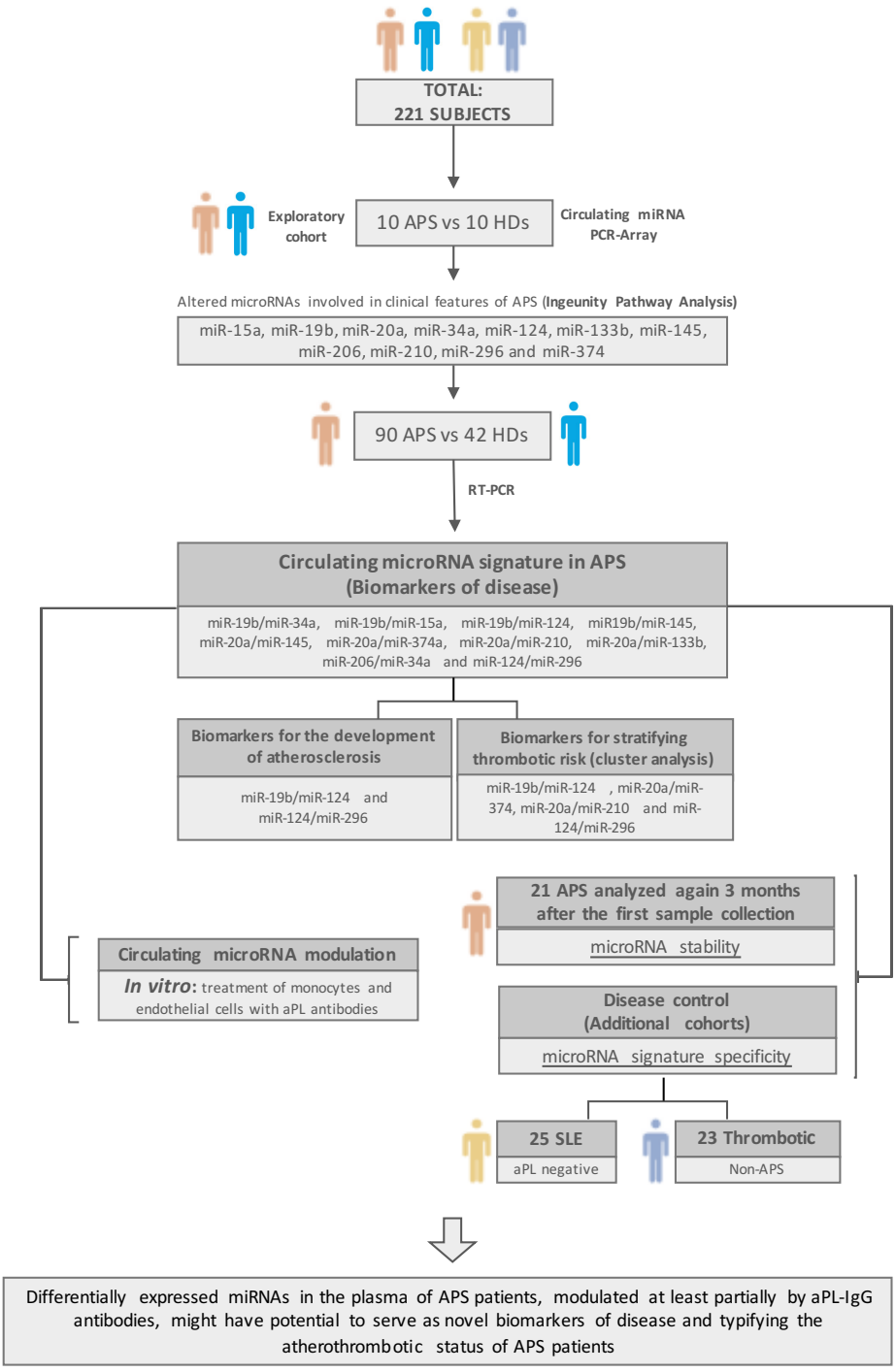
miR/miR	19b/34a	19b/15a	19b/124	19b/145	20a/145	20a/374a	20a/210	20a/133b	206/34a	124/296
	TIME 0	TIME 0	TIME 0	TIME 0	TIME 0	TIME 0	TIME 0	TIME 0	TIME 0	TIME 0
TIME 3	r: 0.44 p: 0.05	r: 0.46 p: 0.03	r: 0.55 p: 0.01	r: 0.50 p: 0.02	r: 0.57 p: 0.01	r: 0.53 p: 0.02	r: 0.52 p: 0.01	r: 0.52 p: 0.01	r: 0.60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

7.3. SUPPLEMENTAL TABLES

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
Nephropathy, 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 levels,* mg/dL	173.1 ± 29.5
Cholesterol HDL levels,* mg/dL	58.4 ± 26.5
Cholesterol LDL levels,* mg/dL	100.4 ± 25.1
Triglycerides levels,* 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

Supplemental Table S1. Clinical and laboratory parameters of the SLE patients. 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 as mean ± SD.

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 levels,* mg/dL	196 ± 37	197 ± 32	NS
Cholesterol HDL levels,* mg/dL	54 ± 14	61 ± 9.6	NS
Cholesterol LDL levels,* mg/dL	123 ± 28	124 ± 27	NS
Triglycerides levels,* mg/dL	80 ± 28	65 ± 16	NS
ESR,* mm/h	13 ± 6	5.1 ± 5	.05

Supplemental Table S2. Clinical and laboratory parameters of the exploratory cohort. APS indicates the antiphospholipid syndrome; HDs, healthy 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 as mean and values range. †antiplatelet agents include acetylsalicylic acid and clopidogrel; ‡anticoagulant agents indicate vitamin K antagonists, including warfarin and acenocumarol.

	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

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

CAPÍTULO II

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Impaired MicroRNA Processing In Neutrophils From Rheumatoid Arthritis Patients Confers Their Pathogenic Profile. Modulation By Biological Therapies

Ivan Arias de la Rosa, Carlos Perez-Sanchez, Patricia Ruiz-Limon, Alejandra Patiño-Trives, Carmen Torres-Granados, Yolanda Jimenez-Gomez, Maria del Carmen Abalos-Aguilera, Irene Cecchi, Rafaela Ortega, Miguel Angel Caracuel, Jerusalem Calvo-Gutierrez, Alejandro Escudero-Contreras, Eduardo Collantes-Estevez, Chary Lopez-Pedrera, Nuria Barbarroja

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Impaired microRNA processing in neutrophils from Rheumatoid Arthritis patients confers their pathogenic profile. Modulation by biological therapies

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*IAR, CPS and PRL shared first authorship and contributed equally to this work.
#ECE, CLP and NB shared last authorship and contributed equally to this work.

Running heads: Impaired microRNA processing in RA neutrophils.

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Number of tables: 1. Number of figures: 6.

1. ABSTRACT

The aim of this study was to investigate the microRNA expression pattern in neutrophils from rheumatoid arthritis patients and its contribution to their pathogenic profile and to analyze the effect of specific autoantibodies or inflammatory components in the regulation of microRNAs in rheumatoid arthritis neutrophils and its modulation by biological therapies.
Neutrophils were isolated from paired peripheral blood and synovial fluid samples of 40 patients with rheumatoid arthritis and from peripheral blood of 40 healthy donors. A microRNA array was performed using nCounter technology. Neutrophils from healthy donors were treated in vitro with antibodies to citrullinated protein antigens isolated from rheumatoid arthritis patients and tumor necrosis factor-α or interleukin-6. A number of cytokines and chemokines were analyzed. In vitro treatments of rheumatoid arthritis-neutrophils with tocilizumab or infliximab were carried out. Transfections with pre-microRNAs and DICER downregulation were further performed.
Rheumatoid arthritis-neutrophils showed a global downregulation of microRNAs and genes involved their biogenesis, alongside with an upregulation of various potential mRNA targets related to migration and inflammation. Decreased levels of microRNAs and DICER correlated with autoimmunity, inflammation and disease activity. Citrullinated protein antigens and tumor necrosis factor-□ decreased the expression of numerous microRNAs and their biogenesis-related genes, increasing their potential mRNA targets. Infliximab reversed those effects. Transfections with pre-miRNAs-223, -126 and -148a specifically modulated genes regulating inflammation, survival and migration. DICER depletion influenced the neutrophils inflammatory profile.
Taking together rheumatoid arthritis neutrophils exhibit a global low abundance of microRNAs induced by autoantibodies and inflammatory markers, which might contribute to their pathogenic activation. microRNA biogenesis is significantly impaired in rheumatoid arthritis-neutrophils and further associated with a greater downregulation of microRNAs mainly related to migration and inflammation in synovial neutrophils. Finally, anti-tumor necrosis factor-α and anti-interleukin-6 receptor treatments can modulate microRNA levels in the neutrophils, minimizing their inflammatory profile.

2. INTRODUCTION

Several immune cells including T and B lymphocytes, macrophages, synovial fibroblast and neutrophils are known to be relevant in the Rheumatoid Arthritis (RA) pathogenesis.[1] Among them, RA neutrophils are activated cells, characterized by a prolonged lifespam, increased migratory capacity and production of inflammatory molecules and reactive oxygen species (ROS). In severe acute inflammation, synovium accumulates a great number of these

cells in a more activated state, promoting cartilage destruction and joint damage. [2]

Antibodies to citrullinated protein antigens (ACPAs) are currently considered the most specific autoantibodies in RA, being related to the activity of the disease and poorer prognosis.[3] ACPAs have been shown able to induce neutrophils to produce high levels of inflammatory mediators, ROS and to generate NETosis. [2,4]

Epigenetic modifications contribute to the development of RA, affecting disease susceptibility and severity. [5, 6] Among them, several microRNAs have been linked to the chronic inflammation in RA. [5] MicroRNAs (miRNAs) are short noncoding RNAs present in all multi-cellular organisms involved in a broad range of cellular processes. They cause posttranscriptional and posttranslational gene silencing, by recognizing a specific sequence of mRNA, binding to it and inhibiting its translation to protein. [7] The miRNA is first transcribed to long primary miRNA of several kb in length (pri-miRNA). This pri-miRNA is then processed by Drosha in a precusor miRNA (pre-miRNA) of 70-nucleotide approximately. The pre-miRNA transported out of the nucleus by exportin 5 (XPO-5) and then processed by DICER to a mature double stranded miRNA of approximately 22 nucleotides. RNA-induced silencing complex (RISC) (composed by the transactivation-responsive RNA-binding protein (TRBP) and Argonaute (AGO)) removes the complementary strand. DICER binds to RISC, forming the core of RISC-loading complex. DICER is considered a crucial factor in the miRNA processing since its presence is necessary to stimulate RNA processing by AGO. [8,9]. Functional miRNA is able to bind to the 3'-untranslated region (UTR) of the target mRNA, causing mRNA cleavage or translational repression.[10]

Several studies, mainly been conducted on lymphocytes, monocytes, macrophages and synovial fibroblast, have reported the role of various miRNAs in the pathogenesis of RA, being critical for the increased expression of inflammatory cytokines and prolonged cell survival.[5, 11]

We undertook this study to evaluate the miRNA profile and the proteins involved in miRNA processing in circulating and synovial neutrophils from RA patients, in order to gain insight about its role in the different activation states of these cells. The effects of ACPAs or inflammatory components and biological therapies in the expression of miRNAs in neutrophils was further assessed.

3. METHODS

3.1. RA patients and healthy donors

Forty RA patients and 40 healthy donors (HD) were included in this study. RA patients fulfilled at least four 1987 American College of Rheumatology (ACR) and achieved a total score of 6 or greater according to 2010 criteria. The patients were taking the following treatments: corticosteroids (50.0%), leflunomide (42.5%), hydroxychloroquine (45.0%), NSAIDs (80.0%) and methotrexate (65%). All patients were tested for the presence of ACPAs and

rheumatoid factor (RF) by clinical laboratory routine analysis. All participants enrolled were Caucasian, recruited at the department of Rheumatology, and gave their written informed consent approved by the ethical committee of the Reina Sofia Hospital (Cordoba, Spain).
Clinical details of RA patients and HD are shown in table 1. Peripheral blood was withdrawn from all the RA patients and the HD. Synovial fluid (SF) from RA patients was obtained through arthrocentesis. Study design is displayed on a flow chart (Supplementary figure 1).

3.2. Isolation of neutrophils from PB and SF

Neutrophils from peripheral blood of HD and paired synovial fluid and peripheral blood samples of RA patients were isolated (after centrifugation to obtain buffy coat and osmotic lysis of the pellet) by immunomagnetic positive selection with human anti-CD15 microbeads (Miltenyi Biotec S.L, Bergisch Gladback, Germany) using AUTOMACs (Miltenyi Biotec). [12]

3.3. miRNA expression profiling

The nCounter miRNA Assay (NanoString Technology, Seattle, WA, USA) detects simultaneously 800 human miRNAs in each sample. 100 ng of RNA, pooled samples of neutrophils from PB of 10 HD, neutrophils from PB of 10 RA patients and neutrophils from SF of 10 RA patients were prepared by ligating a specific DNA tag (miR-tag) onto the 3´end of each mature miRNA followed by 16-20h hybridization (65ºC) to nCounter Reporter and Capture probes. The rest of the protocol was performed following the manufacturer's recommendations (NanoString Technologies; Seattle, WA). Data were normalized by the geometric mean of top 100 miRNAs detected using the nSolver software. This microRNA array was performed in pooled samples of the 10 RA patients that best represented the mean values of age, gender, disease activity, evolution time and autoimmunity of the clinical validation cohort.

3.4. IgG-ACPAs isolation from RA patients

IgGs from serum of 5 different RA patients with high titers of ACPAs and negative for RF (enriched IgG-ACPAs) and 5 HD ((IgG- normal human serum (NHS)) were isolated using HiTrap protein G HP columns (GE Healthcare).

3.5. In vitro treatments of neutrophils

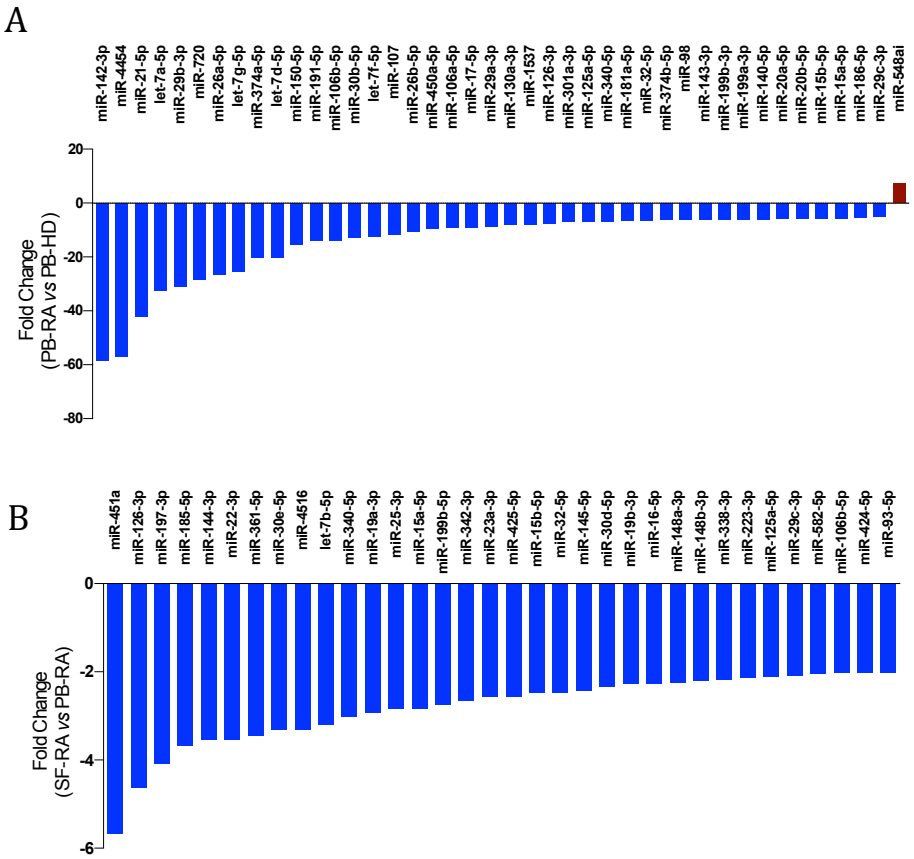
Neutrophils purified from 5 RA patients (having DMARDs and not taking any biological therapies) were pre-treated with FCRII blocking Reagent (Miltenyi Biotec) for 15 min and subsequently incubated with IFX (100 μg/ml) or TCZ (20 μg/ml) for 6 hours. That selection of these patients allowed to isolate neutrophils that assumingly were activated, which meant increased expression of inflammatory cytokines, so that the effects of miRNAs

transfection could be demonstrated by proving the reduction in the levels of those molecules.
Neutrophils purified from 5 HD were treated in vitro with IgG-NHS or enriched IgGs-ACPAs (500 ug/ml), TNF-α and IL-6 (10 ng/ml) for 6 hours. Samples were processed for RT-PCR analyses.

4. RESULTS

4.1. Global decrease in miRNA levels of neutrophils from RA patients.

Among the 800 miRNAs analyzed, levels of 133 miRNAs were detected in neutrophils. Using a fold change cut-off of >2, 94 miRNAs were reduced in PB-RA neutrophils comparing to PB-HD, and 3 of them were elevated (Supplementary table 1, Figure 1A). Besides, synovial neutrophils showed 34 miRNAs even more reduced compared to its paired PB sample (fold change cut-off of >2) (Figure 1B). IPA software uncovered the main enriched biological functions and pathways on which those miRNAs are involved, including immune disease, inflammatory response and connective disorders (Figure 1D).



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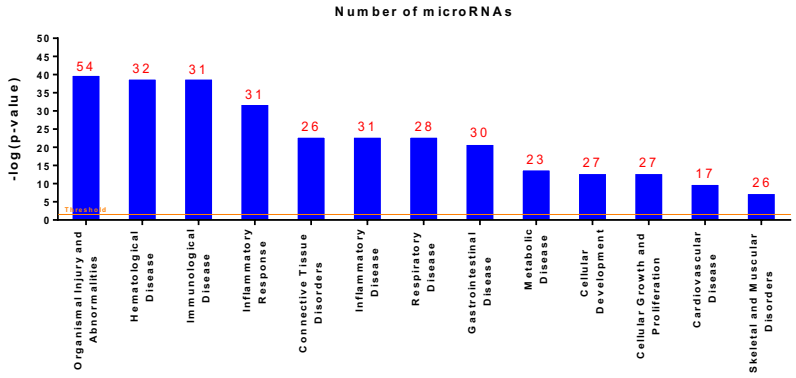

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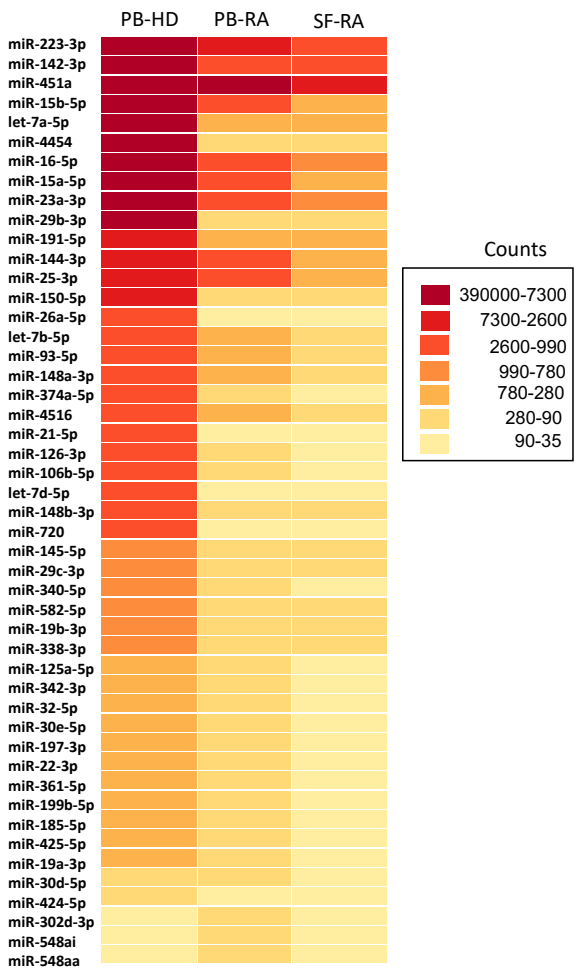


Figure 1. Global downregulation of miRNA expression profile in RA neutrophils. (A) Forty-one miRNAs altered in neutrophils from PB-RA patients compared to neutrophils from PB-HD using a fold change cut-off >5. (B) Thirty-four miRNAs more reduced in RA synovial neutrophils compared to its paired PB sample (fold change cut-off> 2). (C) Heat-map of the differentially expressed miRNA profile in PB-HD, PB-RA and SF-RA. (D) Functional classification of the altered miRNAs using Ingenuity Pathway Analysis (IPA, QIAGEN Redwood City, *https://analysis.ingenuity.com*). The

analysis included only the functions and pathways with average IPA score >2 [indicated as –log (p value)]. Threshold bar indicates cut-off point of significance (p >0.05), using Fisher's exact test, miRNAs. MicroRNA, miR; Rheumatoid Arthritis, RA; Peripheral Blood, PB; Healthy donor, HD; Synovial fluid, SF.

4.2. Low abundance of miRNA levels in RA neutrophils might be due to a defect in the miRNA processing.

Eight altered miRNAs were identified by IPA as the main regulators of proteins involved in the abnormal activation of neutrophils in RA, including miRNA -126, -148a, -29c, let- 7b, -30c, -17, -21 and 223 (Figure 2). The expression of these miRNAs was validated in all the samples separately. A technical validation was performed separately in the 10 samples previously used for the pool. In addition, a clinical validation was carried out separately in the 30 remaining samples (Supplementary figure 2). Thus, levels of most of the selected miRNAs were significantly reduced in PB-RA neutrophils compared to PB-HD neutrophils. A greater reduction in the expression of miR-148a, miR-29c and let-7b in the SF paired samples was observed (Figure 3A). In addition, there was not significant differences in the reduced levels of miRNAs among patients treated or not treated with methotrexate (Supplementary figure 3). There was a significant reduction in the expression of genes involved in the miRNA processing (DICER and AGO-1) in neutrophils from PB-RA patients compared to PB- HD. Of note, DICER, AGO-1, AGO-2 and XPO-5 were diminished in neutrophils from synovial fluid of RA patients (Figure 3B).

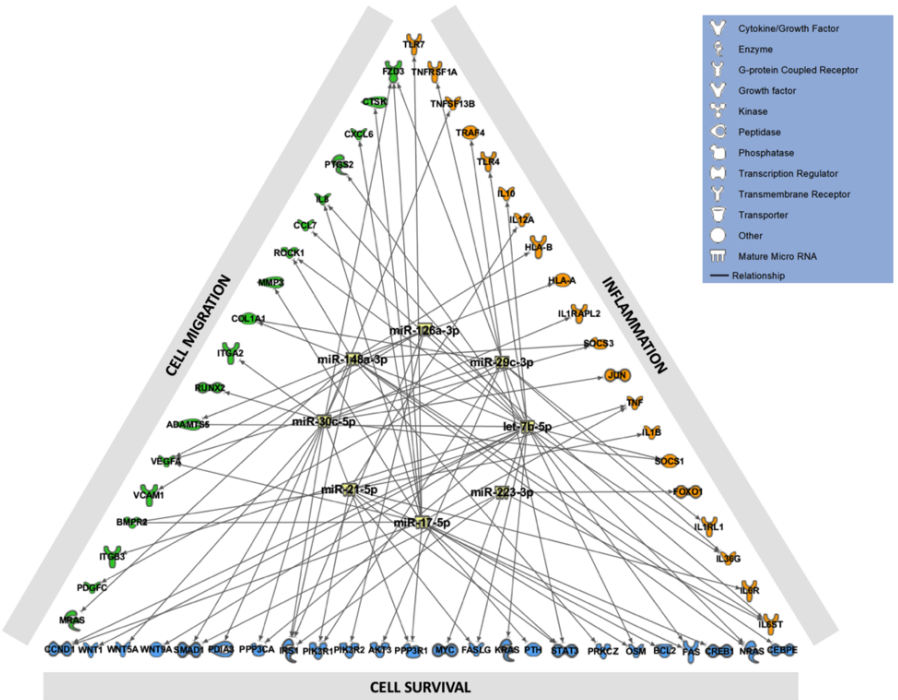
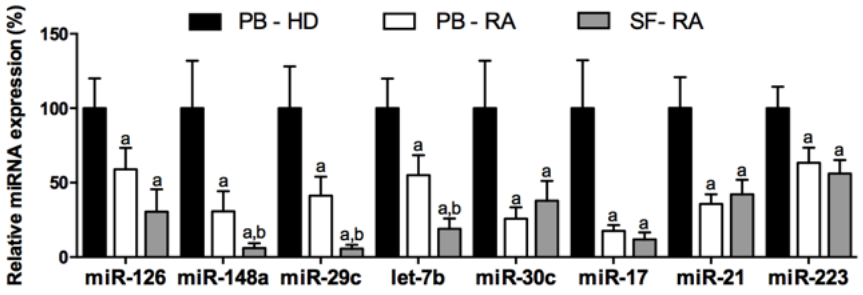


Figure 2. Bioinformatic identification of deregulated miRNAs and protein targets related to the pathogenic profile of neutrophils in RA. In-silico studies were performed to identify eight altered miRNAs using QIAGEN's Ingeunity Pathway Analysis (IPA, QIAGEN Redwood City, https://analysis.ingenuity.com) as the main regulators of proteins involved in the abnormal activation of neutrophils in RA: inflammation, migration and cell survival.
By using the tool miRNA target filter of IPA, a network including the selected miRNAs and mRNA targets experimentally observed and predicted with high confidence, was generated. MicroRNA, miR; Rheumatoid Arthritis, RA.

A



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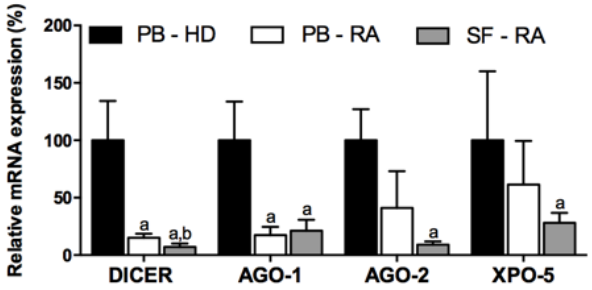

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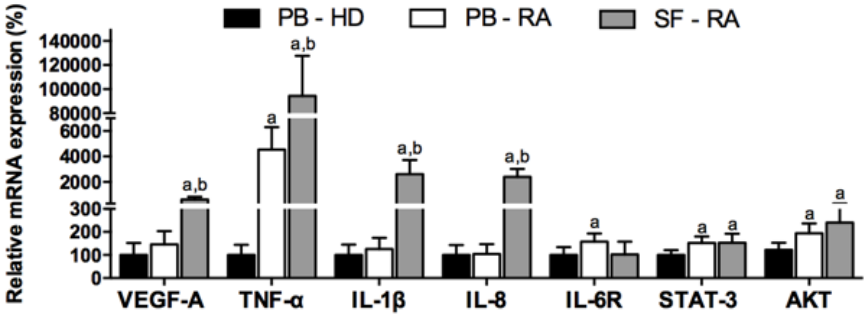

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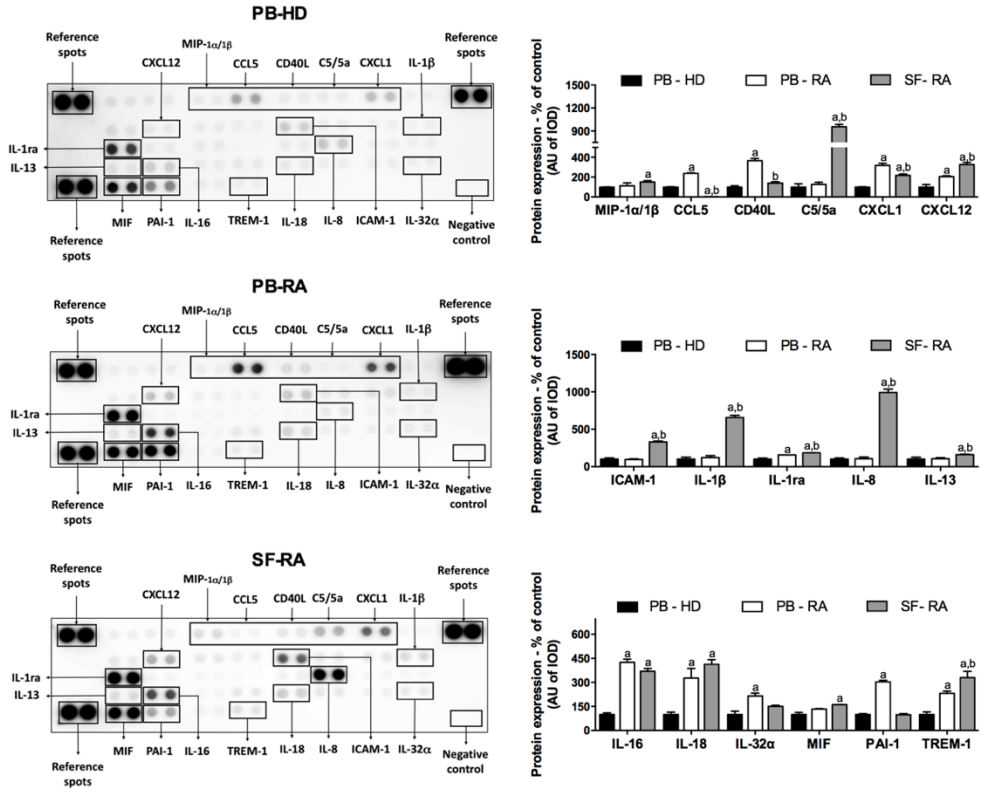


Figure 3. Low abundance of miRNA levels in RA neutrophils might be due to a defect in the miRNA processing. (A) Validation of the miRNA array in PB-HD neutrophils (n=40), PB-RA neutrophils (n=40) and SF-RA neutrophils (n=40). (B) Expression of genes related to miRNA biogenesis machinery in PB-HD (n=40), PB-RA (n=40) and SF-RA neutrophils (n=40). (C) Gene expression of putative mRNA targets of the selected miRNAs in PB-HD (n=40), PB-RA (n=40) and SF-RA neutrophils (n=40). (D) Proteome profile of chemokines and cytokines in neutrophils from PB-HD (n=10), PB-RA (n=10) and SF-RA neutrophils (n=10).

MicroRNA, miR; Rheumatoid Arthritis, RA; Peripheral Blood, PB; Healthy donor, HD; Synovial fluid, SF; Argonaute-1, AGO-1; Argonaute-2, AGO-2; Exportin-5, XPO-5; Vascular endothelial growth factor A, VEGF-A; Tumor necrosis factor-alpha, TNF-α; Interleukin-1β, IL-1β; Interleukin-8, IL-8; Interleukin-6 receptor, IL-6R; Signal transducer and activator of transcription 3, STAT-3; protein kinase B, AKT; Macrophage inflammatory protein 1 alpha/1 beta, MIP-1α/1β; Chemokine (C-C motif) ligand 5, CCL5; Cluster differentiation 40 ligand, CD40 ligand; Complement component C5/5a, C5/5a; Chemokine (C-X-C motif) ligand 1, CXCL-1; Chemokine (C-X-C motif) ligand 12, CXCL12; Intercellular adhesion molecule 1, ICAM-1; Interleukin-1 receptor antagonist, IL-1ra; Interleukin 13, IL-13; Interleukin 16, IL-16; Interleukin 18, IL-18; Interleukin 32α, IL-32α; Macrophage migration inhibitory factor, MIF; Plasminogen activator inhibitor-type 1, PAI-1; Triggering receptor expressed on myeloid cells 1, TREM-1. Data are presented as mean ± SEM; aSignificant differences vs PB-HD $p < 0.05$. bSignificant differences vs PB-RA $p < 0.05$.

4.3. Bioinformatic identification and expression of the putative targets of reduced miRNAs in RA neutrophils.

Seven putative mRNA targets were chosen in based on their recognized role in the pathogenesis of RA, being key factors in inflammation (TNF-α, IL-1β, IL-6R), cell adhesion (VEGF-A), migration (IL-8) and survival (STAT3 and AKT). These targets were significantly upregulated in PB-RA neutrophils (Figure 3C). A greater alteration was observed in SF neutrophils. Using enrichment analysis of those selected targets, enriched pathways mainly related to inflammatory processes were revealed. This included a broad range of secondary chemokines and cytokines which are indirectly connected with the eight selected mRNA targets, amplifying the inflammatory cascade (Supplementary figure 4). Thus, a human cytokine array was performed in neutrophils from RA patients (PB and SF) and HD (PB). Neutrophils from PB of RA patients showed increased protein expression of CCL5, CD40L, CXCL1, CXCL2, IL-1ra, IL-16, IL-18, IL-32a, PAI-1 and TREM-1 compared to HD (Figure 3D). A differential proteome profile was observed in neutrophils from synovial fluid of RA patients compared to HD and PB paired samples (MIP-1α/1β, CCL5, CD40L, C5/5a, CXCL1, CXCL12, ICAM-1, IL-1β, IL-1ra, IL-8, IL-13, IL-16, IL-18, MIF and TREM-1) (Figure 3D).

4.4 Reduced levels of miRNAs in RA neutrophils are related to autoimmunity, clinical and serological parameters.

Decreased levels of both, miRNAs and DICER significantly correlated with the activity of the disease, levels of ACPAs and clinical inflammatory markers. Elevated serum levels of TNFα correlated with low levels of DICER. However, there was not association between the levels of miRNAs and serum TNF-α (Figure 4A).

A

	miR-126	miR-148a	miR-29c	let-7c	miR-30c	miR-17	miR-21	miR-223	mRNA DICER
ACPAs	**r=-0.632 p=0.000**	**r=-0.566 p=0.000**	**r=-0.575 p=0.000**	**r=-0.656 p=0.000**	**r=-0.372 p=0.023**	r=-0.263 p=0.071	**r=-0.369 p=0.032**	**r=-0.344 p=0.050**	**r=-0.537 p=0.005**
DAS28	**r=-0.467 p=0.005**	**r=-0.455 p=0.004**	**r=-0.446 p=0.006**	**r=-0.512 p=0.002**	**r=-0.348 p=0.028**	r=-0.136 p=0.346	**r=-0.326 p=0.046**	**r=-0.385 p=0.050**	r=-0.538 p=0.071
CRP	**r=-0.486 p=0.004**	**r=-0.404 p=0.015**	**r=-0.438 p=0.008**	**r=-0.480 p=0.005**	r=-0.141 p=0.344	r=-0.177 p=0.225	**r=-0.512 p=0.002**	r=-0.388 p=0.067	r=-0.552 p=0.063
Serum TNF-α	r=-0.518 p=0.125	r=0.035 p=0.440	r=0.005 p=0.973	r=0.023 p=0.440	r=0.178 p=0.440	r=0.178 p=0.440	r=-0.530 p=0.115	r=0.178 p=0.440	**r=-0.496 p=0.048**
Serum IL-6	r=-0.117 p=0.439	r=0.035 p=0.815	r=0.005 p=0.973	r=0.023 p=0.880	r=-0.006 p=0.964	r=-0.082 p=0.569	r=-0.042 p=0.779	r=-0.182 p=0.335	r=-0.266 p=0.148

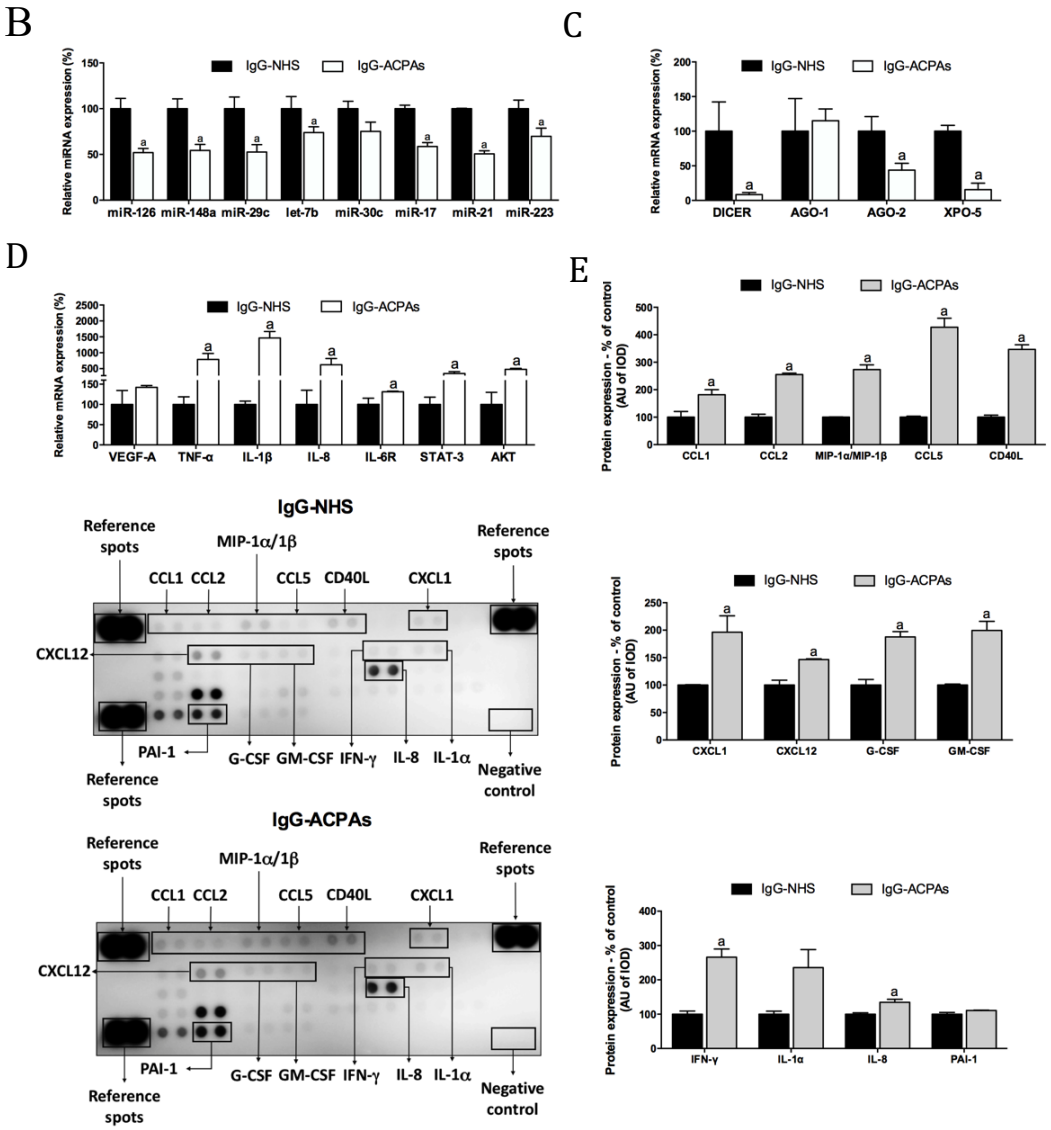


Figure 4. Reduced levels of miRNAs in RA neutrophils are related to ACPAs. (A) Correlation studies of reduced miRNA and DICER expression levels in neutrophils from peripheral blood with clinical parameters such as, ACPAs levels, DAS28, CRP and inflammatory markers including TNF-α and IL-6 serum cytokine levels. (B) In vitro effect of enriched IgG-ACPAs in miRNAs expression in healthy neutrophils. (C) In vitro effect of enriched IgG-ACPAs in the expression of genes related to the miRNA biogenesis in healthy neutrophils. (D) In vitro effect of enriched IgG-ACPAs in the mRNA expression of potential mRNA targets of the validated miRNAs. (E) Proteome profile of chemokines and cytokines in neutrophils treated with IgG-NHS and enriched IgG-ACPAs.

Antibodies to citrullinated protein antigens, ACPAs; IgGs from normal human serum, IgG-NHS; MicroRNA, miR; Healthy donor, HD; Disease activity score 28, DAS28; C-reactive protein, CRP; Tumor necrosis factor-alpha, TNF-α; Interleukin-6, IL-6;

Argonaute-1, AGO-1; Argonaute-2, AGO-2; Exportin-5, XPO-5; Vascular endothelial growth factor A, VEGF-A; Interleukin-1β, IL-1β; Interleukin-8, IL-8; Interleukin-6 receptor, IL-6R; Signal transducer and activator of transcription 3, STAT-3; protein kinase B, AKT. Chemokine (C-C motif) ligand 1, CCL1; Chemokine (C-C motif) ligand 2, CCL2; Macrophage inflammatory protein 1 alpha/1 beta, MIP-1α/1β; Chemokine (C-C motif) ligand 5, CCL5; Cluster differentiation 40 ligand, CD40 ligand; Chemokine (C-X-C motif) ligand 1, CXCL-1; Chemokine (C-X-C motif) ligand 12, CXCL12; Granulocyte colony-stimulating factor, G-CSF; Granulocyte-macrophage colony-stimulating factor, GM-CSF; Interferon gamma, IFN-γ; Interleukin-1α, IL-1α; Plasminogen activator inhibitor-type 1, PAI-1. Data are presented as mean ± SEM (n= 5), [a]Significant differences $p < 0.05$.

4.5. ACPAs reduces miRNA levels in healthy neutrophils

Enriched IgG-ACPAs downregulated the expression of the 8 selected miRNAs in healthy neutrophils (Figure 4B). Accordingly, a significant reduction of genes involved in the miRNA biogenesis was observed (Figure 4C). Enriched IgG-ACPAs also increased the expression of the selected mRNA targets (Figure 4D). Finally, enriched IgG-ACPAs promoted a significant upregulation of secondary chemokines and cytokines indirectly related with the eight selected mRNA targets (CCL1, CCL2, MIP-1α/β, CCL5, CD40L, CXCL1, CXCL12, G-CSF, GM-CSF, IFN-γ and IL-8) (Figure 4E).

4.6. Inflammatory mediators decrease the expression of miRNAs in neutrophils, which might be restored by IFX or TCZ.

TNF-α and IL-6 levels were significant elevated in serum from RA patients; a further increase was observed in SF from those RA patients (Figure 5A). In vitro, TNF-α downregulated the levels of the 8 selected miRNAs alongside with a decrease in the expression of DICER and AGO-2 in healthy neutrophils (Figure 5B). Treatment with IL-6 reduced the levels of miR-126, let-7b, miR-17, AGO1 and AGO2 (Figure 5C). We observed that fresh neutrophils from RA patients had significantly higher levels of TNF-α and IL-6 mRNA compared to freshly isolated neutrophils from healthy donors (Supplementary figure 5A). In addition, after 6 hours of in vitro culture, levels of TNFα and IL6 were elevated in the culture media of RA neutrophils (Supplementary figure 5B). In vitro treatment of active neutrophils purified from RA patients with IFX restored the low levels of the 8 selected miRNAs while TCZ only up-regulated the expression of miR-148a (Figure 5D). Accordingly, IFX upregulated the expression levels of AGO1. Regarding mRNA targets, IFX reduced the mRNA expression of VEGF-A, TNF-α, IL- 1β, IL-8 and STAT3. Treatment with TCZ also diminished the expression of various of these mRNA targets such as IL-1β, IL-8, IL-6R and STAT3 (Figure 5E). In addition, protein release of TNF-α, IL-8 and IL-1β was reversed in RA neutrophils after treatments with both, IFX and TCZ (Figure 5F).

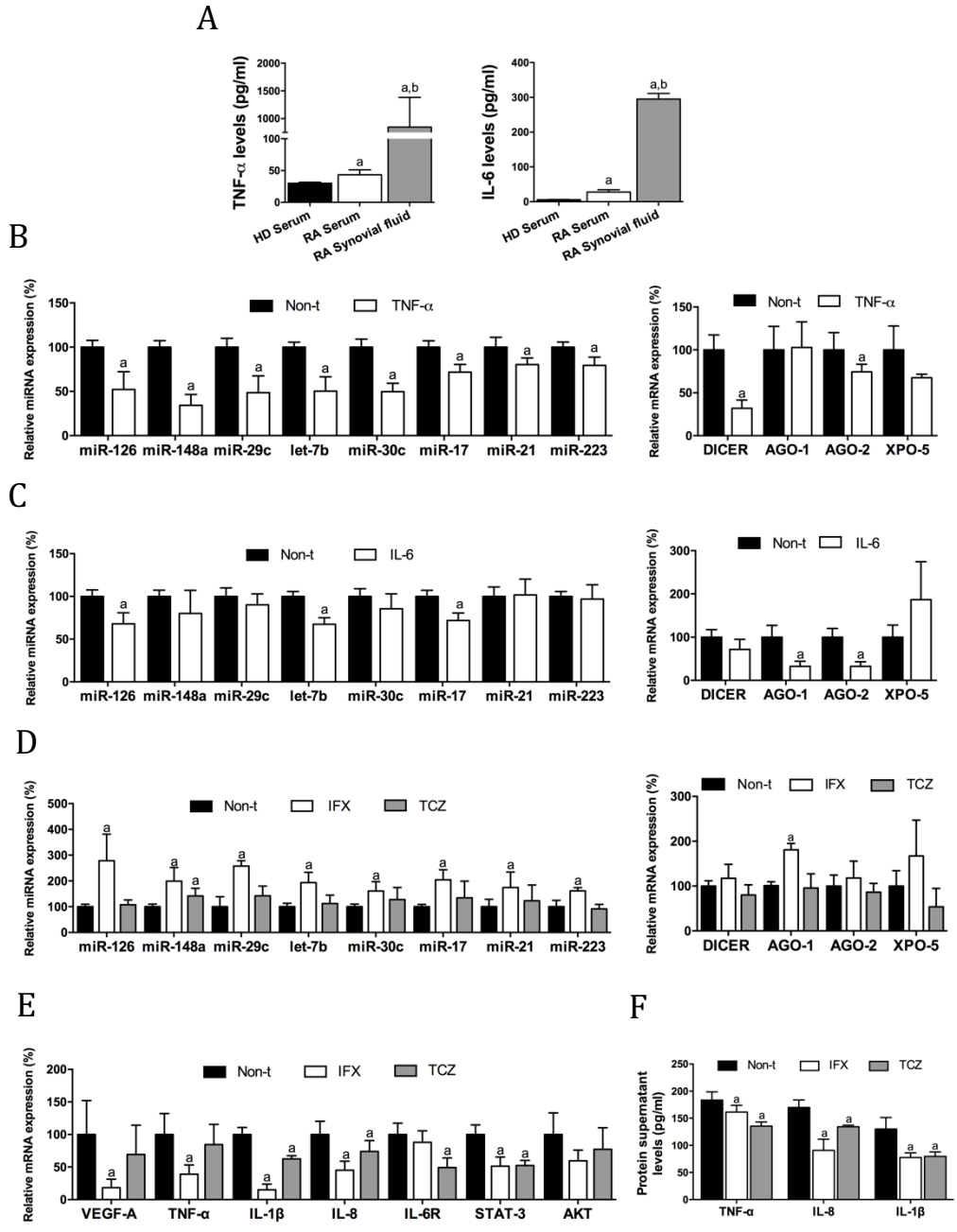


Figure 5. Modulation of the expression of miRNAs and genes involved in their processing in HD neutrophils by inflammatory components. In vitro effects of TCZ and IFX in RA active neutrophils. (A) Serum and synovial levels of TNF-α and IL-6 (40 HD-serum, 40 RA-serum and 40 RA-SF). aSignificant differences vs HD serum $p < 0.05$; bSignificant differences vs RA serum $p < 0.05$. (B) Expression of miRNAs and genes involved in their biogenesis machinery in HD neutrophils treated with TNF-α. (C) Expression of miRNAs and genes involved in their biogenesis machinery in HD neutrophils treated with IL-6. (D) Expression of miRNAs and genes involved in their processing in RA active neutrophils treated in vitro with TCZ or IFX. (E) Gene

expression of putative mRNA targets of the validated miRNAs in RA active neutrophils treated in vitro with TCZ or IFX. (F) Protein levels of putative mRNA targets of the validated miRNAs in RA active neutrophils treated in vitro with TCZ or IFX. MicroRNA, miR; Healthy donor, HD; Tocilizumab, TCZ; Infliximab, IFX; Rheumatoid Arthritis, RA; Argonaute-1, AGO-1; Argonaute-2, AGO-2; Exportin-5, XPO-5; Vascular endothelial growth factor A, VEGF-A; Tumor necrosis factor-alpha, TNF-α; Interleukin-1β, IL-1β; Interleukin-8, IL-8; Interleukin-6 receptor, IL-6R; Signal transducer and activator of transcription 3, STAT-3; protein kinase B, AKT. Non-treated, Non-t. Data are presented as mean ± SEM of five independent experiments; [a]Significant differences respective non-treated $p < 0.05$.

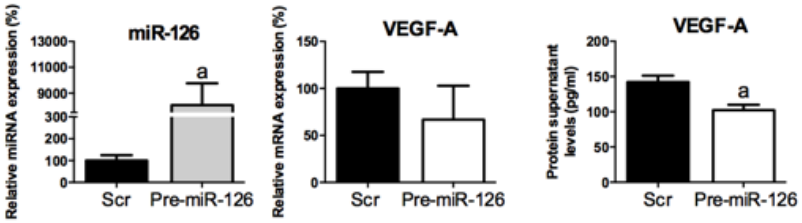
4.7. Overexpression of miR-126, miR-148a and miR-223 in RA neutrophils decreases specific targets involved in inflammation, migration and cell survival.

We selected three downregulated miRNAs to evaluate their role in migration, proinflammatory profile and cell survival of RA neutrophils: miR-223 was the most abundant in neutrophils, miR-148a and miR-126 have several potential and demonstrated mRNA targets involved in inflammation. As seen in figure 6, miR-126, miR-148a and miR-223 overexpression led to a downregulation of their specific mRNA targets: miR-126 overexpression induced a significant downregulation of VEGF-A protein expression (Figure 6A), and miR-223 overexpression promoted a significant decrease in the protein expression of IL-8 and IL-1β. Interestingly, miR-223 transfection induced a significant increase of VEGF mRNA and protein (Figure 6C). On the otherhand, miR-148a overexpression reduced gene and protein expression levels of TNF-α (Figure 6B).

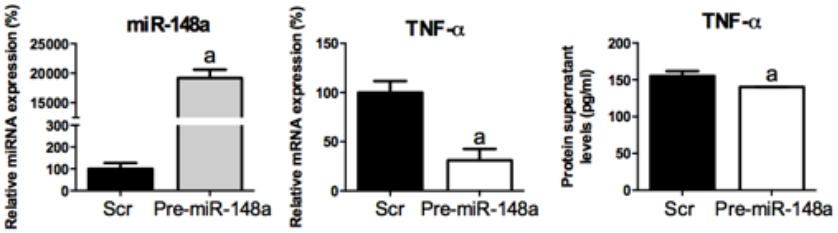
4.8. DICER downregulation in neutrophils might exacerbate their inflammatory profile.

Using a low number of lentiviral particles, 25% of DICER expression was inhibited in HL-60 neutrophil-like cells (Figure 6D). This reduction promoted a significant decrease in all the selected miRNAs (Figure 6E). Protein levels of a number of cytokines and chemokines were significantly upregulated in neutrophils after DICER downregulation (Figure 6F).

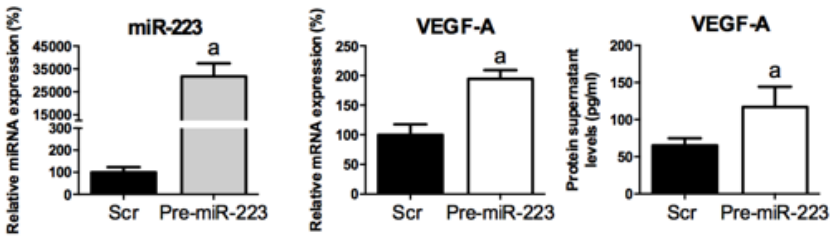
A

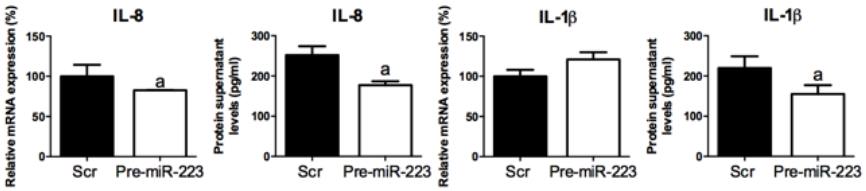


B

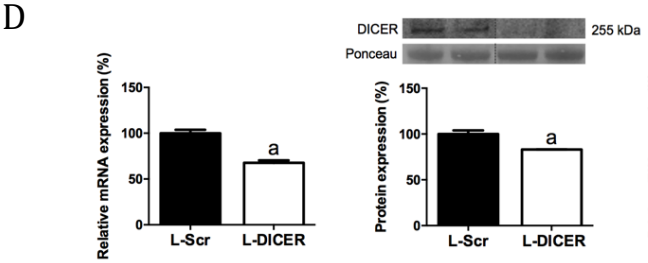

miR-148a
Relative miRNA expression (%)
25000
20000
15000
300
200
100
0
a
Scr
Pre-miR-148a
TNF-α
Relative mRNA expression (%)
150
100
50
0
a
Scr
Pre-miR-148a
TNF-α
Protein supernatant levels (pg/ml)
200
150
100
50
0
a
Scr
Pre-miR-148a

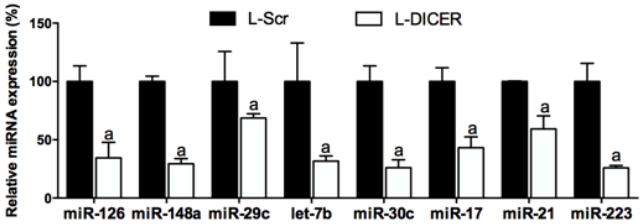
C


miR-223
Relative miRNA expression (%)
45000
35000
25000
15000
300
200
100
0
a
Scr
Pre-miR-223
VEGF-A
Relative mRNA expression (%)
250
200
150
100
50
0
a
Scr
Pre-miR-223
VEGF-A
Protein supernatant levels (pg/ml)
200
150
100
50
0
a
Scr
Pre-miR-223


IL-8
Relative mRNA expression (%)
150
100
50
0
a
Scr
Pre-miR-223
IL-8
Protein supernatant levels (pg/ml)
300
200
100
0
a
Scr
Pre-miR-223
IL-1β
Relative mRNA expression (%)
150
100
50
0
a
Scr
Pre-miR-223
IL-1β
Protein supernatant levels (pg/ml)
300
200
100
0
a
Scr
Pre-miR-223

D


DICER
255 kDa
Ponceau
Relative mRNA expression (%)
150
100
50
0
a
L-Scr
L-DICER
Protein expression (%)
150
100
50
0
a
L-Scr
L-DICER


L-Scr
L-DICER
Relative miRNA expression (%)
150
100
50
0
a
miR-126
miR-148a
miR-29c
let-7b
miR-30c
miR-17
miR-21
miR-223

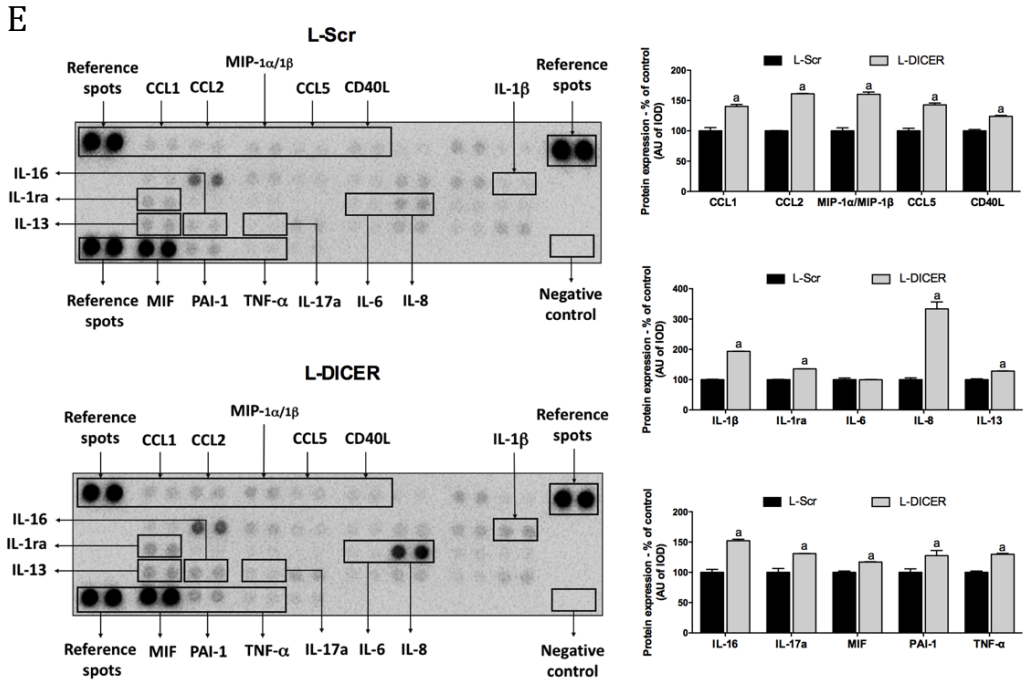


Figure 6. miR-126, miR-148a and miR-223 regulate the expression of specific mRNA targets involved in inflammation, migration and cell survival on neutrophils. DICER downregulation induces an inflammatory profile in neutrophils. (A) Overexpression of miR-126 decreased the expression of VEGF in RA active neutrophils. (B) Overexpression of miR-148a decreased the expression of TNF-α in RA active neutrophils. (C) Overexpression of miR-223 decreased the expression of IL-8 and Il-1β and increased VEGF in RA active neutrophils. (D) DICER expression levels after lentivirus transfection by PCR and western blot. (E) Expression levels of miRNAs in DICER downregulated neutrophils. (G) Proteome array of cytokines and chemokines in HL60 neutrophil-like cells after downregulation of DICER by lentiviral transfection. MicroRNA, miR; Rheumatoid Arthritis, RA; Vascular endothelial growth factor A, VEGF-A; Tumor necrosis factor-alpha, TNF-α; Interleukin-8, IL-8; Interleukin-1β, IL-1β; Chemokine (C-C motif) ligand 1, CCL1; Chemokine (C-C motif) ligand 2, CCL2; Macrophage Inflammatory Proteins Alpha and Beta, MIP-1α/1β; Chemokine (C-C motif) ligand 5, CCL5; Interleukin 1 receptor antagonist, IL-1ra; Interleukin-6, IL-6; Interleukin-13, IL-13; Interleukin-16, IL-16; Interleukin-17a, IL-17a; Macrophage migration inhibitory factor, MIP; Plasminogen activator inhibitor-1, PAI-1. Scrambled, Scr; Lentivirus-Scrambled, L-Scr; Lentivirus-DICER, L-DICER. Data are presented as mean ± SEM of two independent experiments; [a]Significant differences $p < 0.05$.

5. DISCUSSION

This study reports for the first time on the altered miRNA expression profile in neutrophils from RA patients, describing a defect in miRNAs processing machinery responsible for a global low abundance of miRNAs, mediated by ACPAs and inflammatory mediators, promoting the high inflammatory profile of these cells in RA. Several miRNAs have been shown increased in PB and inflamed joints in RA patients, correlating with disease activity and promoting the production of inflammatory mediators involved in the synovitis. [13, 14] Likewise, various single nucleotide polymorphism (SNPs) have been studied in miRNAs in RA .[15] Our study shows a global downregulation of the miRNAs expression in RA neutrophils, more marked in synovial neutrophils, suggesting that it might contribute to the abnormal activated profile of these cells in the synovium. In this sense, global downregulation of miRNAs has been shown in human alveolar macrophages induced by cigarettes smoking, responsible for the changes in gene expression associated with the disease.[16] In autoimmune disorders, we recently described a global downregulation of the miRNA levels in neutrophils from patients with systemic lupus erythematosus and antiphospholipid syndrome, which may indicate that chronic inflammation and/or autoimmunity is associated to a reduction of miRNAs in neutrophils.[17] In the present study, we demonstrate that either ACPAs or inflammatory mediators, especially TNF-α can modulate the miRNA expression profile, through a reduction of several proteins involved in its processing, which might be translated into an increase of genes that might be involved in inflammation, cell survival and migration. Up to date, no study has reported the effect of ACPAs in the expression of miRNAs. In our hands, the reduced levels of miRNAs and DICER in RA neutrophils correlated with elevated levels of ACPAs. Accordingly, our in vitro studies demonstrated a direct involvement of these autoantibodies in the deregulation of various miRNAs -and their specific protein targets- globally related to the pathogenesis of RA. We further demonstrated that the global downregulation of the miRNAs expression in RA neutrophils was associated, at least partially, to the reduced levels of DICER. A recent study suggested the role of DICER in neutrophils differentiation, where the DICER inhibition attenuated the activation of autophagy, a process that is needed for proper neutrophil differentiation.[12] DICER plays a crucial role in miRNA biogenesis. Thus, it has been suggested that mRNA and protein levels of DICER must be strictly controlled since small changes can initiate various pathological processes. [18] Here, we prove a novel role for DICER in neutrophils, showing that little reductions can induce a proinflammatory profile in neutrophils by downregulating several miRNAs and, hence, a number of putative targeted cytokines and chemokines. Currently, little is known about the miRNAs regulating the neutrophil function. Several miRNAs have been recently involved in the development and function of the neutrophils and in various pathological states, including miRNA-155, miRNA-34a, miRNA-223, miRNA-142, miRNA-452 and miRNA-466L.[19] Overexpression of miRNA-155 and

miRNA-34a in neutrophils from patients with myelodysplastic syndrome has been shown to contribute to an alteration of the migration.[20] In addition, decreased levels of both, the miRNA-145 and the miRNA-143 have been shown in AML, which were responsible for the blockade of the differentiation process of the neutrophils.[21] Alongside with previous evidence, here we show that miRNA-223 is one of the most abundant miRNA on neutrophils.[22] It has recently been demonstrated that the miRNA- 223 is an important regulator blocking the infiltration of neutrophils in alcoholic hepatic disease.[22] Supporting a role for this miRNA in the infiltration capacity of the neutrophils, in the present work we demonstrated how the overexpression of the miRNA-223 in the neutrophils of RA patients reduced specifically the expression of IL-1β and IL-8, molecules involved in inflammation and migration. The role of the miRNA-126 in the vascular integrity has been also evidenced.[23] We observed reduced levels of the miRNA-126 in RA neutrophils, while its induced overexpression in RA neutrophils reduced significantly the levels of VEGF, pointing out the role of this miRNA in the neutrophil adhesion and migration. Multiple functions have been attributed to miRNA-148a in several diseases. Thus, low levels of miRNA-148 were related to less survival time and increased recurrence risk in bladder cancer.[24] In addition, miRNA-148 has been related to innate and adaptive immune responses.[25] Our data is in agreement with such studies, since we found reduced levels of the miRNA-148 in RA neutrophils, associated to increased levels of TNF-α, a key inflammatory protein driving the RA disease. Others miRNAs found decreased in RA neutrophils, such as miRNA-21, Let-7 and miRNA-30, have previously been reported to be altered in different types of tumors, thus playing a relevant role in tumorigenesis, invasion and metastasis of cancer cells.[26- 29] In addition, Let-7 and miRNA-17 regulate the response of the T cells.[27, 30] Finally, a recent study demonstrated that the levels of all the members of the miRNA 29 family were decreased in PBMC and CB34+ cells of bone marrow of AML patients. The normalization of their levels partially inhibited the abnormal proliferation of the blasts, blocked the myeloid differentiation and repressed the apoptosis.[31] TNF-α and IL-6 are key inflammatory effectors in RA, whose levels are elevated in RA serum and even more increased in RA SF. We found a marked effect of TNF-α on neutrophils, reducing genes related to miRNA processing (including DICER and AGO-1) and downregulating the 8 miRNAs selected. By contrast, IL-6 had not that stronger effect but was able to reduce the levels of miRNA-126, let-7b and miRNA-17 alongside with the expression of AGO-1 and AGO-2. Treatment of active RA neutrophils with IFX or TCZ reduced the inflammatory profile, downregulating gene expression of VEGFA, TNF-α, IL-1β, IL8, IL6R and STAT3. However, only IFX was able to restore the global levels of selected miRNAs, alongside with genes involved in their processing in RA neutrophils, an effect that might be expected after the stronger effect of TNF-α observed in reducing the miRNAs levels, thus suggesting that IFX might specifically minimize the abnormal profile of the RA neutrophils through the inhibition of TNFα, which directly acts reducing the expression of miRNAs. In agreement with these results, we recently demonstrated that

in vivo treatment with anti-TNFα drugs during 6 months regulated the levels of several miRNAs in plasma of RA patients. Moreover, miRNA-23 and miRNA-223 were identified as potential biomarkers of therapy effectiveness.[32] Altogether, our study shows that neutrophils from RA patients have a defect in the miRNA biogenesis machinery, more marked in synovial neutrophils, and induced by ACPAs and inflammatory mediators. This defect might be directly associated with the abnormal neutrophil activation, increasing their proinflammatory profile, observed by the higher expression of a number of chemokines and cytokines.

Among the miRNAs altered in RA neutrophils, we demonstrate that the miRNA-223, miRNA-126 and miRNA-148 are involved in the modulation of genes involved in processes such as migration, inflammation and cell survival in neutrophils. Finally, biological therapies would be able to improve miRNAs processing, upregulating the levels of miRNAs, which might reduce the activation of the neutrophil. Beyond the regulation of miRNAs in RA neutrophils, there should be other epigenetic mechanisms that might contribute to the abnormal activation of these cells in RA context, such as chromatin modification.

Limitation of the study: this is a cross-sectional study where consecutive patients from standard clinical practice were recruited. Those patients were being treated with standard therapy, including immunosuppressants, by the time of the samples and clinical details collection. Thus, the effects of specific treatments in the expression levels of miRNAs or the molecules involved in their biogenesis in neutrophils could not be analyzed. The isolation of neutrophils with anti-CD15 microbeads could be considered as a potential limitation of this work. Up to date there is not a worldwide accepted method, so that neutrophil isolation techniques have shown either some activation or functional impairment of the cells and presence of small amounts of contaminating cells. Choosing an adequate method to isolate neutrophils from synovial fluid is challenging. In our hands, after testing potential priming/activation and percentage of contaminating cells, isolation of neutrophils with anti-CD15 microbeads was proven to be a suitable approach to obtain enough number of inactivated neutrophils. Nevertheless, the consensus in the selection of the right isolation method that allows to compare neutrophil paired samples from synovium and peripheral blood is still needed.

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7. SUPPLEMENTARY INFORMATION

METHODS

Purity and priming of isolated granulocytes

Purity of the neutrophil fraction was evaluated via flow cytometry (FACSCalibur cytometer), by analysing the size and complexity of the population (forward and size scatters) and using specific antibodies anti-human CD15, anti-human CD14 and anti-human CD66b (Immunostep, Salamanca, Spain) and staining with Wright-Giemsa. By these methods, 98.5 ± 2.5 viable neutrophils were obtained (Supplementary figure 6).
In addition, as neutrophils could be activated or primed by the isolation method, ROS production induced by fMLP (1μM for 15 min) (Sigma-Aldrich) and CD66b expression were evaluated before and after isolation of CD15+ cells with anti-CD15 microbeads from peripheral blood of healthy donors and RA patients and synovial fluid of RA patients. Thus, ROS production and CD66b expression (measured by flow cytometry) were not different in neutrophils from buffy coat and neutrophils isolated with anti-CD15 microbeads (Supplementary figure 7 and 8).

RNA isolation.

Total RNA from neutrophils was extracted using TRI Reagent (Sigma, St Louis, Missouri, USA) following the manufacturer's recommendations.

Target gene prediction and integrated analysis by IPA.

Pathway analysis was performed using Ingenuity Pathway Analysis (IPA, Ingenuity Systems, Redwood City, CA, USA; https://analysis.ingenuity.com).
In silico analysis software reveals enrichment for molecular networks and signaling pathways. For this purpose, all differentially regulated miRNAs and fold changes were imported into IPA. The right-tailed Fisher's exact test was used to calculate a p-value determining the statistical probability that association between a set of molecules and a pathway or function might be due to chance alone. Additionally, specific targets (experimentally observed and predicted with high bioinformatics confidence) regulated by the differentially expressed miRNAs were also identified by using the different database integrated in IPA software.
Enrichment analysis of the potential targets of the altered miRNAs was performed using the Enrich web-server tool (http://amp.pharm.mssm.edu/Enrichr/).

Gene expression and validation miRNA arrays through quantitative real-time reverse transcriptase PCR.

Gene expression was assessed by real time PCR using a LightCycler Thermal Cycler System (Roche Diagnostics, Indianapolis, Indiana, USA).
For the validation of the miRNA array, RNA was reverse transcribed using the TaqMan miRNA Reverse Transcription kit and miRNA-specific stem-loop primers (Life Technologies, Madrid, Spain). The reaction was conducted in a GeneAmp PCR System 9700 (Life Technologies) at 16°C for 30 min, 42°C for 30 min and 85°C for 5 min. The RT product was combined with 5 µl of Taqman 2x Universal PCR Master Mix, No AmpErase UNG, 0,5 µl of specific 20X Taqman miRNA Assay and 0,5 µl of water to generate a PCR of 10 µl of total volume. Real-time PCR was carried out on a LightCycler Thermal Cycler System (Roche Diagnostics) at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Data were normalized to the mean of miRNA U6. Fold changes compared with the endogenous control were then determined by calculating 2-ΔΔCt. Samples were analysed in triplicate and negative controls were included in all the reactions.

Protein extraction, western blot and human cytokine array

Neutrophils were lysed on ice for 15 minutes in NP-40 lysis buffer, containing 10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM Na2-ethylene glycol tetraacetic acid, 0.1 mM Na2−ethylene diamine tetraacetic acid, 1 mM sodium orthovanadate, 1% NP-40, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and phosphate and protease inhibitor cocktails (Sigma-Aldrich, St. Louis, Missouri, USA). Cytoplasmic lysates were pelleted by centrifugation at 15,000g for 5 minutes at 4°C. The supernatant was recovered and frozen at −80°C.
Forty micrograms of cytoplasmic extracts were resolved on a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and then transferred to nitrocellulose membranes. Membranes were stained with human monoclonal anti-DICER (Abcam, Cambridge, UK). Visualization of immune complexes was performed using secondary antibodies conjugated to HRP and the Luminol Reagent detection system (Santa Cruz Biotechnology Inc. Data are presented as integrated optical density (IOD) and expressed in arbitrary units (AU).
250 µg of neutrophil cytoplasmic lysates was subjected to human cytokine array to detect 36 human cytokines, chemokines, and acute phase proteins simultaneously (R&D Systems, Minneapolis, USA), following the manufacturer's recommendations. Data are presented as integrated optical density (IOD) and expressed in arbitrary units (AU) (a map displaying the coordinate and the protein of each spot is shown in supplementary figure 9).

Inflammatory protein levels

Levels of protein in culture supernatants, serum and synovial fluid (TNF-α, IL-8, IL-6, VEGF-A and IL1β) were analyzed through ELISA (Bionova, Madrid, Spain) following the manufacturer's recommendations. Some modifications regarding time of incubation, washing steps and sample dilution were included in order to avoid non-specific binding.[1]

Apoptosis

Rates of apoptosis in neutrophils after *in vitro* treatments were analysed using annexin V or Draq7-propidium iodide (ThermoFisher, Waltham, MA, USA; Biostatus, Shepshed, UK) following the manufacturer's recommendations by flow cytometry (Supplementary figure 10). No significant apoptosis was observed.

Transfection of RA neutrophils with pre-miRNAs

Transient transfection of primary neutrophils purified from RA patients was performed with 100 nM miRNA mimics (Life Technologies) (miR-126, miR-148a and miR-223 separately and a non-specific control (scrambled)), using 2ul of siPORTTMNeoFXTM transfection agent (Life Technologies) for 106 cells/500ul medium (RPMI1640 supplemented with 10% fetal bovine serum). Transfection efficiency was controlled by flow cytometry using a Cy3™ Dye-Labeled Pre-miR scrambled control (ThermoFisher scientific) and was estimated to be between 96-100 %. Apoptosis was also tested after transfection using Annexin V/Propidium iodide by flow cytometry (Supplementary figure 11). Transfections had no significant effect in neutrophil apoptosis. After 12 hours, cells were collected to analyze potential targets of these miRNAs. Data were expressed as relative changes to the values of the cells transfected with scrambled control.

Downregulation of DICER in neutrophils

Since human neutrophil lifespan is short, downregulation of DICER was performed on human neutrophils derived from a cell line, HL60. HL-60 cells were differentiated to neutrophils with DMSO (1.25%) during 6 days. Neutrophil differentiation was evaluated through flow cytometry using human anti-CD11b antibody (BD Bioscience, New Jersey, USA). After 6 days of treatment the 65% of HL-60 cells were differentiated to neutrophils. Knockdown of DICER was performed as previously described.12 pLKO.1 lentiviral vectors, expressing shRNAs targeting DICER1 (shDICER1_ 3155 NM_030621 TRCN0000290426) or non-targeting shRNA were purchased from Sigma-Aldrich. Cells (2x105) were seeded in 1 ml of medium with low number of viral particles (6x105) and polybrene (8 μg/ml) on a 6-well plate. Plate was centrifuged at 1000g, 32ºC for 1h and incubated overnight. Thereafter, the medium was replaced with fresh medium and cells

were incubated at 37ºC, 5% CO2 for 48h. Transfection was carried out for 48 hours and with lower number of lentiviral particles in order to avoid apoptosis. Transduction efficiency was evaluated with a green fluorescence protein lentivirus positive control by flow cytometry (Sigma-Aldrich). Eighty percent of the cells were infected.

Statistical analysis

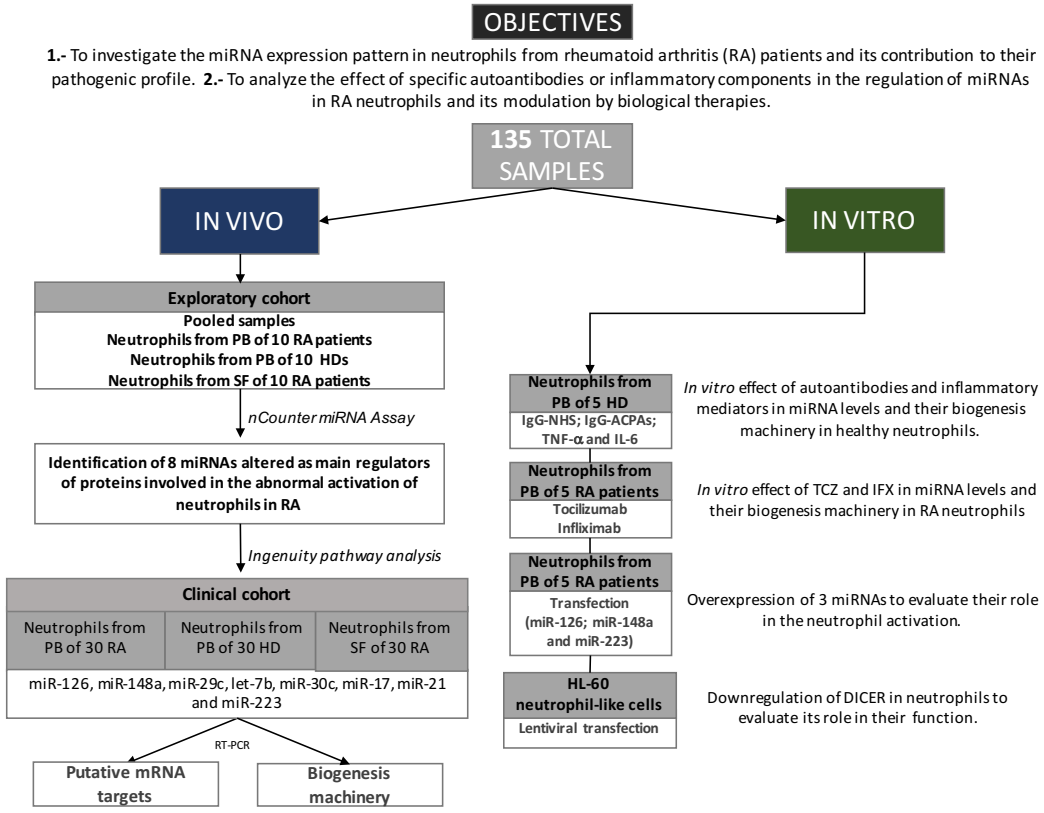
Statistical analyses were performed with SSPS 17.0 (SPSS Inc., Chicago, IL, USA). Following normality and equality of variance tests, variables were compared using paired Student's t test or alternatively by a non-parametric test (Mann–Whitney rank sum test). Paired samples within the same subjects were compared by Wilcoxon signed-rank test. Spearman correlation was calculated to estimate the linear correlations between variables. P-values <0.05 were considered statistically significant

Supplementary table 1. Expression levels of miRNAs in neutrophils from peripheral blood of RA patients and healthy donors.

	Normalized counts		Fold change
miRNA	**RA**	**HD**	
hsa-miR-142-3p	2043,03	119134,67	-58,31
hsa-miR-4454	174,56	9965,90	-57,09
hsa-miR-21-5p	35,00	1481,20	-42,32
hsa-let-7a-5p	332,96	10813,64	-32,48
hsa-miR-29b-3p	250,53	7767,26	-31,00
hsa-miR-720	35,00	993,26	-28,38
hsa-miR-26a-5p	88,90	2363,70	-26,59
hsa-let-7g-5p	329,73	8346,42	-25,31
hsa-miR-374a-5p	105,06	2119,00	-20,17
hsa-let-7d-5p	61,42	1234,33	-20,10
hsa-miR-150-5p	210,12	3203,48	-15,25
hsa-miR-191-5p	471,97	6659,62	-14,11
hsa-miR-106b-5p	93,75	1308,18	-13,95
hsa-miR-30b-5p	35,00	450,30	-12,87
hsa-let-7f-5p	40,41	498,80	-12,34
hsa-miR-107	48,49	579,16	-11,94
hsa-miR-26b-5p	202,04	2102,35	-10,41
hsa-miR-450a-5p	109,91	1042,49	-9,48
hsa-miR-106a	114,76	1046,83	-9,12
hsa-miR-17-5p	114,76	1046,83	-9,12
hsa-miR-29a-3p	143,85	1290,08	-8,97
hsa-miR-130a-3p	38,79	317,09	-8,17
hsa-miR-1537	43,64	340,98	-7,81
hsa-miR-126-3p	174,56	1350,17	-7,73
hsa-miR-301a-3p	35,00	246,87	-7,05
hsa-miR-125a-5p	100,21	693,54	-6,92
hsa-miR-340-5p	139,00	949,82	-6,83
hsa-miR-181a-5p	140,62	935,34	-6,65
hsa-miR-32-5p	101,83	666,76	-6,55
hsa-miR-374b-5p	35,00	220,08	-6,29
hsa-miR-98	35,00	215,01	-6,14
hsa-miR-143-3p	35,00	214,29	-6,12
hsa-miR-199a-3p	64,65	393,83	-6,09
hsa-miR-199b-3p	64,65	393,83	-6,09
hsa-miR-140-5p	150,32	904,94	-6,02
hsa-miR-20a-5p	179,41	1075,07	-5,99
hsa-miR-20b-5p	179,41	1075,07	-5,99
hsa-miR-15b-5p	1928,27	11179,96	-5,80
hsa-miR-15a-5p	1550,05	8802,51	-5,68
hsa-miR-186-5p	58,19	317,09	-5,45
hsa-miR-29c-3p	189,11	959,23	-5,07
hsa-let-7i-5p	161,63	805,03	-4,98
hsa-miR-28-5p	46,87	231,66	-4,94
hsa-miR-423-5p	56,57	273,65	-4,84
hsa-miR-582-5p	193,96	916,52	-4,73
hsa-miR-223-3p	82619,83	385315,19	-4,66
hsa-miR-144-3p	1124,96	5190,00	-4,61
hsa-miR-148b-3p	221,44	1001,95	-4,52
hsa-miR-148a-3p	520,46	2263,79	-4,35
hsa-miR-145-5p	224,67	974,44	-4,34
hsa-miR-454-3p	35,00	150,58	-4,30
hsa-miR-93-5p	538,23	2315,19	-4,30
hsa-miR-24-3p	53,34	224,42	-4,21
hsa-miR-342-3p	168,10	691,37	-4,11
hsa-miR-16-5p	2240,22	9019,69	-4,03
hsa-miR-338-3p	214,97	853,54	-3,97
hsa-miR-199b-5p	109,91	425,68	-3,87
hsa-let-7e-5p	35,00	134,65	-3,85
hsa-miR-27b-3p	30,71	113,66	-3,70
hsa-miR-197-3p	153,55	568,30	-3,70
hsa-miR-30e-5p	164,86	602,33	-3,65
hsa-miR-320e	42,02	153,48	-3,65
hsa-miR-19b-3p	242,45	881,77	-3,64
hsa-miR-30c-5p	63,04	221,53	-3,51
hsa-miR-22-3p	163,25	563,23	-3,45
hsa-miR-363-3p	35,00	120,18	-3,43

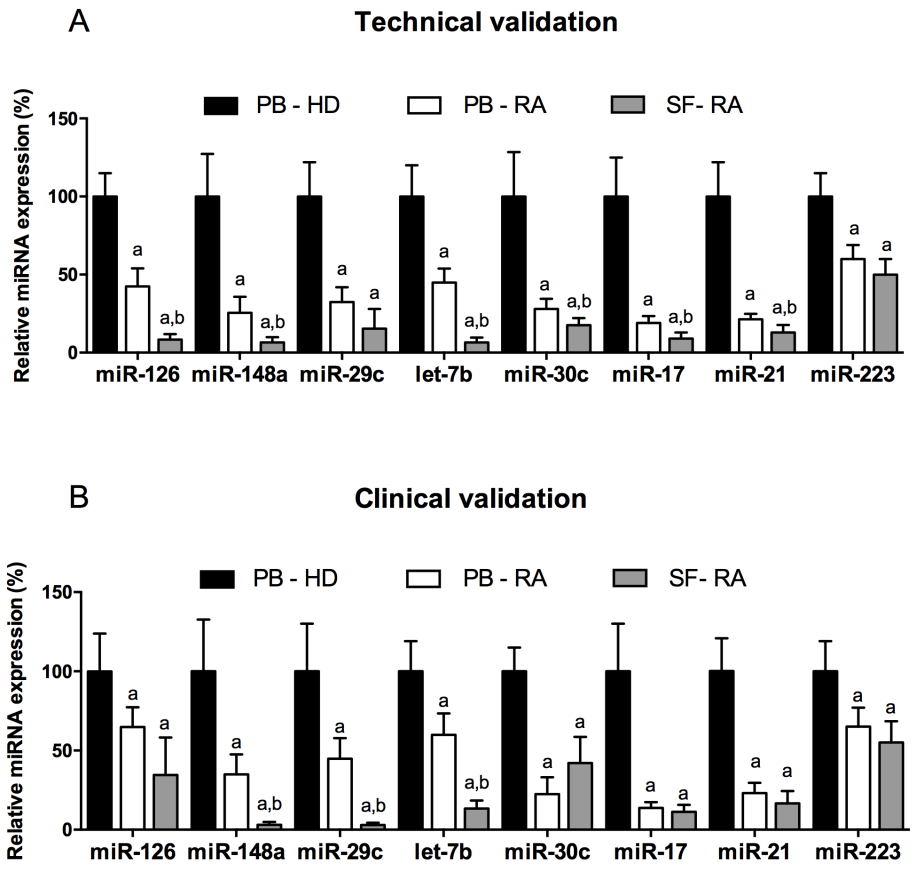
	Normalized counts		Fold change
miRNA	**RA**	**HD**	
hsa-miR-590-5p	40,41	136,83	-3,39
hsa-miR-361-3p	63,04	209,22	-3,32
hsa-miR-361-5p	147,09	487,94	-3,32
hsa-miR-92a-3p	53,34	176,64	-3,31
hsa-miR-346	45,26	148,41	-3,28
hsa-let-7b-5p	720,88	2330,40	-3,23
hsa-miR-23a-3p	2511,76	7869,34	-3,13
hsa-miR-185-5p	129,31	398,17	-3,08
hsa-miR-423-3p	30,71	93,39	-3,04
hsa-miR-664-3p	35,00	103,52	-2,96
hsa-miR-23b-3p	61,42	180,26	-2,93
hsa-miR-132-3p	35,56	102,08	-2,87
hsa-miR-193a-5p	35,56	99,18	-2,79
hsa-miR-505-3p	40,41	110,76	-2,74
hsa-miR-25-3p	1205,77	3300,49	-2,74
hsa-miR-222-3p	185,88	508,21	-2,73
hsa-miR-4516	665,92	1818,56	-2,73
hsa-miR-142-5p	35,00	91,94	-2,63
hsa-miR-221-3p	37,18	97,01	-2,61
hsa-miR-425-5p	127,69	332,29	-2,60
hsa-miR-194-5p	35,00	90,49	-2,59
hsa-miR-30d-5p	96,98	239,63	-2,47
hsa-miR-200c-3p	35,56	86,87	-2,44
hsa-miR-199a-5p	35,00	83,98	-2,40
hsa-miR-181c-5p	35,00	83,25	-2,38
hsa-miR-424-5p	71,12	167,23	-2,35
hsa-miR-19a-3p	145,47	325,78	-2,24
hsa-miR-99b-5p	40,41	81,81	-2,02
hsa-miR-1260a	35,00	68,05	-1,94
hsa-miR-324-5p	35,00	66,60	-1,90
hsa-miR-451a	41408,51	67873,22	-1,64
hsa-miR-4443	35,00	57,19	-1,63
hsa-miR-27a-3p	35,00	56,47	-1,61
hsa-miR-146a-5p	35,00	53,57	-1,53
hsa-let-7c	38,79	59,36	-1,53
hsa-miR-34c-5p	37,18	54,30	-1,46
hsa-miR-365a-3p	35,00	50,68	-1,45
hsa-miR-128	35,00	47,78	-1,37
hsa-miR-769-5p	35,00	47,06	-1,34
hsa-miR-190a	35,00	46,33	-1,32
hsa-miR-30a-5p	35,00	46,33	-1,32
hsa-miR-378a-3p	35,00	45,61	-1,30
hsa-miR-378i	35,00	45,61	-1,30
hsa-miR-421	35,00	44,88	-1,28
hsa-miR-216b	42,02	51,40	-1,22
hsa-miR-140-3p	30,71	36,92	-1,20
hsa-miR-494	35,00	41,27	-1,18
hsa-miR-28-3p	35,00	39,82	-1,14
hsa-miR-7-5p	35,00	39,82	-1,14
hsa-miR-151a-3p	35,00	39,09	-1,12
hsa-miR-33a-5p	35,00	38,37	-1,10
hsa-miR-299-3p	35,00	37,65	-1,08
hsa-miR-495	35,00	37,65	-1,08
hsa-miR-147b	35,00	36,92	-1,05
hsa-miR-4286	77,58	76,01	1,02
hsa-miR-192-5p	50,11	48,50	1,03
hsa-miR-378e	116,38	94,11	1,24
hsa-miR-515-5p	37,18	35,00	1,24
hsa-miR-215	37,18	35,00	1,24
hsa-miR-216a	37,18	35,00	1,24
hsa-miR-411-5p	38,79	35,00	1,29
hsa-miR-4531	40,41	35,00	1,35
hsa-miR-188-5p	42,02	35,00	1,40
hsa-miR-579	69,50	35,00	1,88
hsa-miR-302d-3p	113,14	43,44	2,60
hsa-miR-548aa	145,47	35,00	4,85
hsa-miR-548ai	214,97	35,00	7,17

The list of the miRNAs detected after the nCounter miRNA Assay in neutrophils from peripheral blood of healthy donors (HD) and rheumatoid arthritis (RA) patients are shown in the 1st and 5th columns of the table. The normalized NanoString counts for the different miRNAs in RA patients and HD are displayed in the 2nd, 3rd, 6th and 7th columns of the table, respectively. miRNAs are sort depending on the fold change expression value between RA patients and HD as indicated in the 4th and 5th columns of the table.

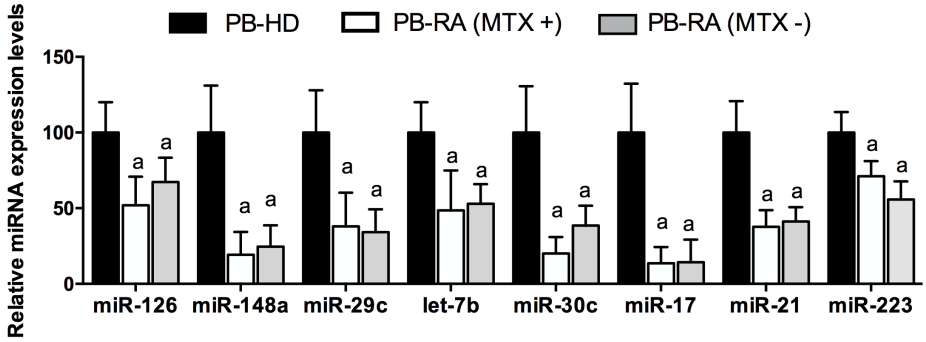


Supplementary figure 1. Flowchart of the study design. One hundred thirty-five human samples were included in this study. Firstly, nCounter miRNA assay was performed on pooled samples of neutrophils from PB of 10 HD, neutrophils from PB of 10 RA patients and neutrophils from SF of 10 RA patients. Eight altered miRNAs were identified by IPA as the main regulators of proteins involved in the abnormal activation of neutrophils in RA, the expression of these 8 miRNAs, genes involved in miRNA biogenesis and putative mRNA targets of those miRNAs were analyzed individually in neutrophils from 40 PB-RA samples, 40 PB-HD and 40 SF-RA. In addition, neutrophils isolated from PB of 5 HD were treated in vitro with ACPAs isolated from RA patients, TNF-α or IL-6 for 6 hours. Neutrophils isolated from PB of 5 RA patients with high disease activity and no taking any biological therapies were treated in vitro with Tocilizumab or Infliximab. Additionally, overexpression of miR-126, miR-148a and miR-223 was performed in neutrophils from PB of 5 RA patients with high disease activity. HL-60 cell line was differentiated to neutrophils using DMSO 1.25% during 6 days. DICER1 expression was

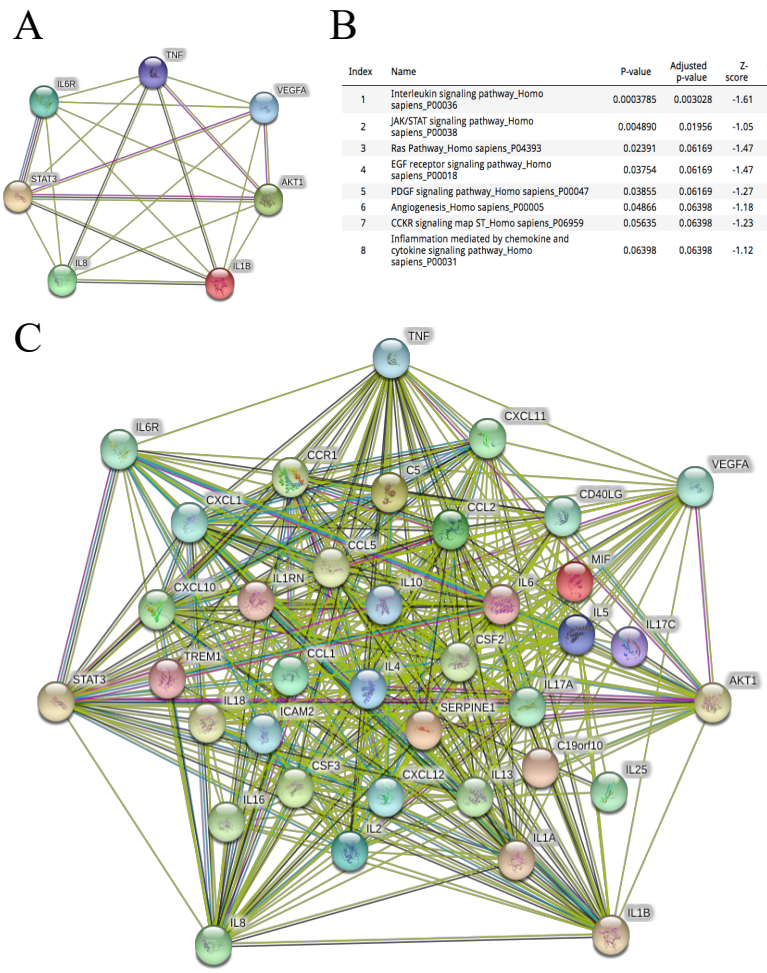
reduced on HL-60 neutrophil-like cells through lentiviral transfection. MicroRNA, miR; Peripheral Blood, PB; Rheumatoid Arthritis, RA; Healthy donor, HD; Synovial fluid, SF; Antibodies to citrullinated protein antigens, ACPAs; Tumor necrosis factor alpha, TNF-α; Infliximab, IFX; Tocilizumab: TCZ.



Supplementary figure 2. (A) Technical validation: relative miRNA expression levels in neutrophils of paired peripheral blood (PB) and synovial fluid samples of 10 patients with RA and from PB of 10 healthy donors previously used pooled in the miRNA array. (B) Clinical validation: relative miRNA expression levels in neutrophils of paired peripheral blood (PB) and synovial fluid samples of 30 patients with RA and from PB of 30 healthy donors by RT-PCR. [a]$p<0.05$ vs PB-HD.

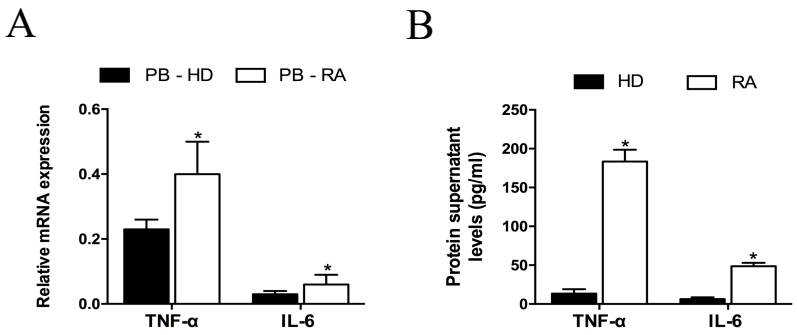


Supplementary figure 3. Relative miRNA expression levels in neutrophils from peripheral blood (PB) of healthy donors (HD) (n=40) and PB of rheumatoid arthritis (RA) patients with methotrexate (MTX) (n=28) or without MTX (n=12). [a]p<0.05 vs PB-HD.

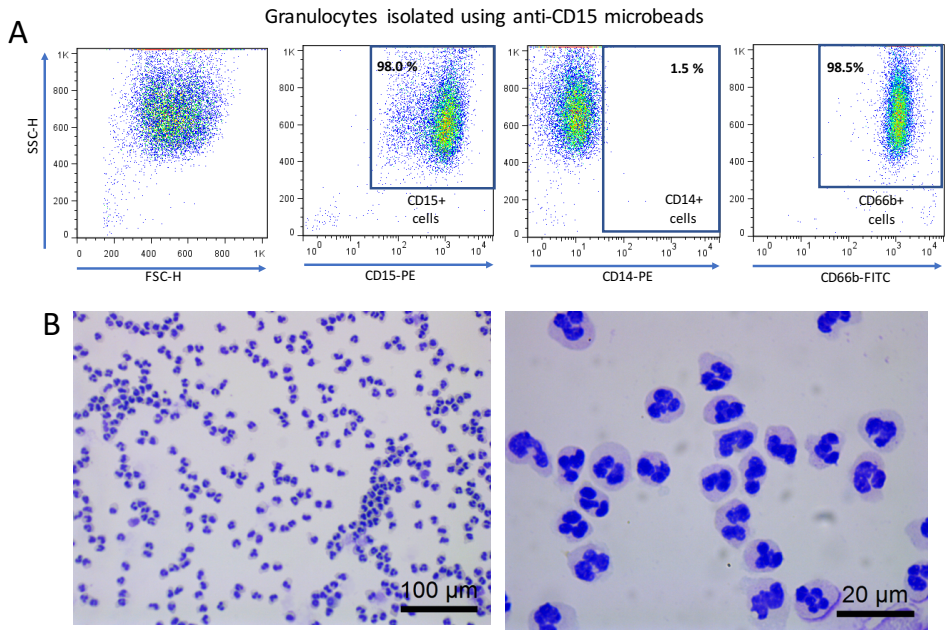


Index	Name	P-value	Adjusted p-value	Z-score	Combined score
1	Interleukin signaling pathway_Homo sapiens_P00036	0.0003785	0.003028	-1.61	12.69
2	JAK/STAT signaling pathway_Homo sapiens_P00038	0.004890	0.01956	-1.05	5.60
3	Ras Pathway_Homo sapiens_P04393	0.02391	0.06169	-1.47	5.48
4	EGF receptor signaling pathway_Homo sapiens_P00018	0.03754	0.06169	-1.47	4.84
5	PDGF signaling pathway_Homo sapiens_P00047	0.03855	0.06169	-1.27	4.13
6	Angiogenesis_Homo sapiens_P00005	0.04866	0.06398	-1.18	3.58
7	CCKR signaling map ST_Homo sapiens_P06959	0.05635	0.06398	-1.23	3.55
8	Inflammation mediated by chemokine and cytokine signaling pathway_Homo sapiens_P00031	0.06398	0.06398	-1.12	3.09

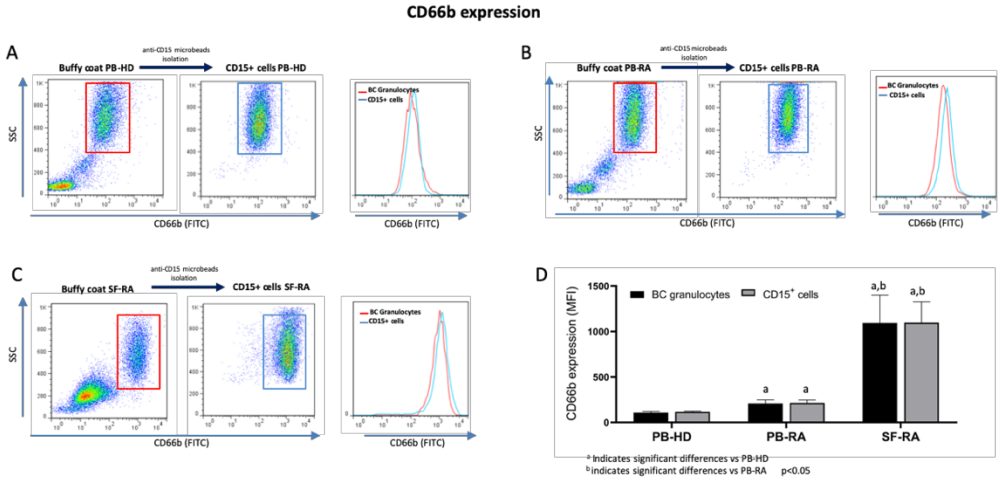
Supplementary figure 4. Functional analysis of the selected potential targets associated with the altered miRNAs of RA neutrophils. (A) Protein-protein interaction network among the selected putative targets of the 8 altered miRNAs in RA neutrophils. The network was generated through the database STRING (http://string-db.org/), which includes known and predicted functional associations between proteins. (B) Enrichment analysis of the selected potential targets of the altered miRNAs using the Enrich web-server tool (http://amp.pharm.mssm.edu/Enrichr/). The results displayed in the table show that the enriched pathways are mainly related to inflammatory processes. (C) Protein-protein interaction network between the selected potential targets of the 8 altered miRNAs in RA neutrophils and the proteins included in the human cytokine array. The network shows that the selected potential targets of the altered miRNAs are directly connected with a broad range of secondary molecules involved in the amplification of the inflammatory cascade.



Supplementary figure 5. (A) Relative mRNA expression levels of TNF-α and IL-6 in neutrophils isolated from peripheral blood of RA patients and healthy donors. (B) Supernatant levels of TNF-α and IL-6 in the culture media of neutrophils from peripheral blood of RA patients and healthy donors.

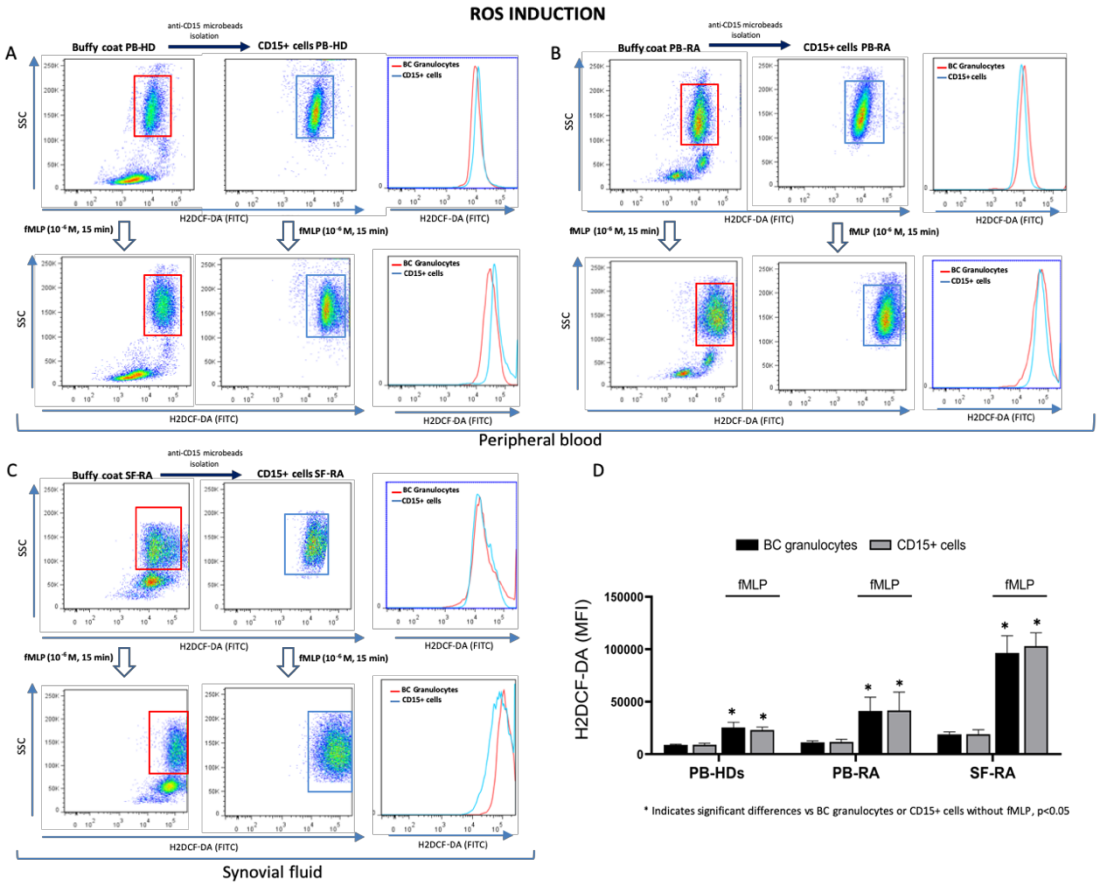


Supplementary figure 6. (A) Percentage of granulocytes isolated from peripheral blood of a RA patient using anti-CD15 microbeads (AUTOMACs) by flow cytometry. (B) Wright-Giemsa in granulocytes isolated from peripheral blood using anti-CD15 microbeads (AUTOMACs).

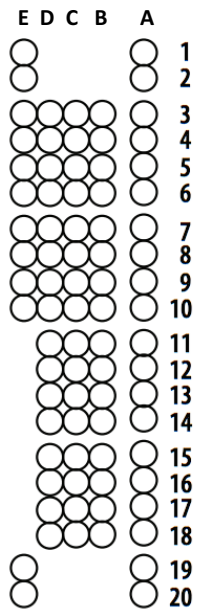


Supplementary figure 7: Analysis of CD66b expression on granulocytes before and after anti-CD15 isolation procedure in peripheral blood of healthy donors (n=10) and RA patients (n=10) and in synovial fluid of RA patients (n=10). (A) Representative dot plot and histograms showing the expression of CD66b in granulocytes from buffy coat (before isolation) and CD15+ cells (after isolation with anti-CD15 microbeads) from peripheral blood of a healthy donor. (B) Dot plot and histograms showing the expression of CD66b in granulocytes from buffy coat (before isolation) and CD15+ cells (after isolation with anti-CD15 microbeads) from peripheral blood of RA patients. (C) Dot plot

and histograms showing the expression of CD66b in granulocytes from buffy coat (before isolation) and CD15+ cells (after isolation with anti-CD15 microbeads) from synovial fluid of RA patients. (D) CD66b expression measured as median fluorescence intensity. a indicates significant differences vs PB-HD, b indicates significant differences vs PB-RA, p <0.05.



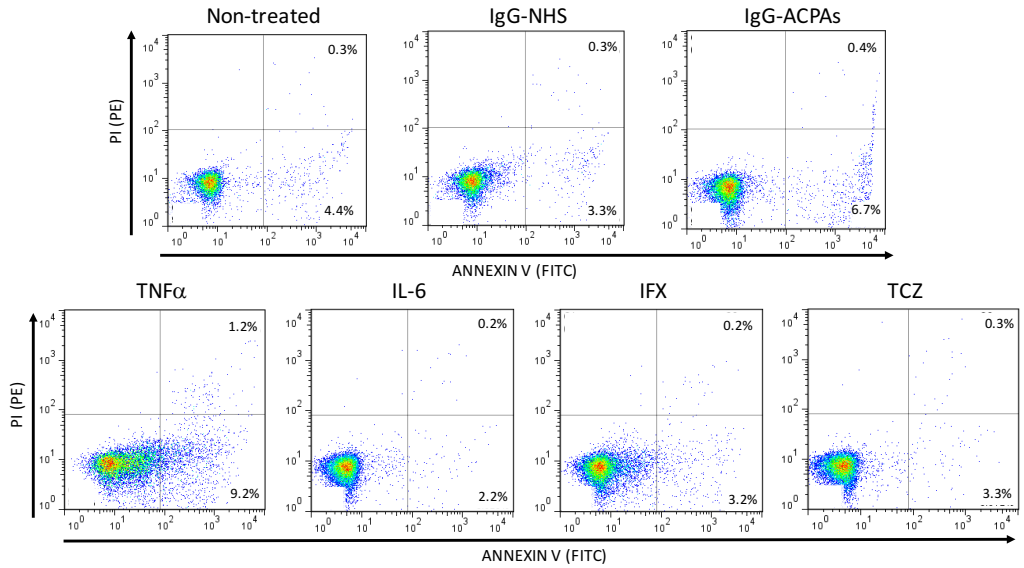
Supplementary figure 8: Analysis of ROS production before and after anti-CD15 isolation procedure in peripheral blood of healthy donors (n=10) and RA patients (n=10) and in synovial fluid of RA patients (n=10). Cells were treated without or with fMLP 1μM for 15 min. ROS production was measured using H2DCF-DA by flow cytometry. (A) Representative dot plot and histograms showing the ROS production in granulocytes from buffy coat (before isolation) and CD15+ cells (after isolation with anti-CD15 microbeads) from peripheral blood of a healthy donor treated with or without fMLP. (B) Representative dot plot and histograms showing the ROS production in granulocytes from buffy coat (before isolation) and CD15+ cells (after isolation with anti-CD15 microbeads) from peripheral blood of a RA patient treated with or without fMLP. (C) Representative dot plot and histograms showing the ROS production in granulocytes from buffy coat (before isolation) and CD15+ cells (after isolation with anti-CD15 microbeads) from synovial fluid of a RA patient treated with or without fMLP. (D) ROS production measured as median fluorescence intensity. * indicates significant differences vs non-treated cells (without fMLP), p < 0.05.



Coordinate	Target/Control
A1, A2	Reference Spots
A3, A4	CCL1/I-309
A5, A6	CCL2/MCP-1
A7, A8	MIP-1a/MIP-1b
A9, A10	CCL5/RANTES
A11, A12	CD40 Ligand/TNFSF5
A13, A14	Complement Component C5/C5a
A17, A18	CXCL10/IP-10
A19, A20	Reference Spots
B3, B4	CXCL11/I-TAC
B5, B6	CXCL12/SDF-1
B7, B8	G-CSF
B9, B10	GM-CSF
B11, B12	ICAM-1/CD54
B13, B14	IFN-g
B15, B16	IL-1a/IL-1F1
B17, B18	IL-1b/IL-1F2
C3, C4	IL-1ra/IL-1F3
C5, C6	IL-2
C7, C8	IL-4

Coordinate	Target/Control
C9, C10	IL-5
C11, C12	IL-6
C13, C14	IL-8
C15, C16	IL-10
C17, C18	IL-12 p70
D3, D4	IL-13
D5, D6	IL-16
D7, D8	IL-17A
D9, D10	IL-17E
D11, D12	IL-18/IL-1F4
D13, D14	IL-21
D15, D16	IL-27
D17, D18	IL-32a
E1, E2	Reference Spots
E3, E4	MIF
E5, E6	Serpin E1/PAI-1
E7, E8	TNF-a
E9, E10	TREM-1
E19, E20	Negative Control

Supplementary figure 9. Human cytokine array map. Figure displaying the coordinate and the target protein of each spot of the array.



Supplementary figure 10. (A) Levels of apoptosis in neutrophils isolated from healthy donors treated with IgGs purified from healthy donors (IgG-NHS) and RA patients (enriched IgG-ACPAs) for 6 hours measured by Annexin V/Propidium iodide (PI) through flow cytometry. (B) Levels of apoptosis in neutrophils isolated from healthy donors treated with TNF-α or IL-6 and neutrophils from RA patients treated with IFX or TCZ for 6 hours, measured by Annexin V/Propidium iodide (PI) through flow cytometry.

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1. Güven, E., Duus, K., Lydolph, M.C., Jørgensen, C.S., Laursen, I., Houen, G. Non-specific binding in solid phase immunoassays for autoantibodies correlates with inflammation markers. J Immunol Methods. 2014; 403 (1-2): 26-36

CAPÍTULO III

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Defective glucose and lipid metabolism in rheumatoid arthritis is determined by chronic inflammation in metabolic tissues

I. Arias de la Rosa[1,*], A. Escudero-Contreras[1,*], S. Rodríguez-Cuenca[2,*], M. Ruiz-Ponce[1], Y. Jiménez-Gómez[1], P. Ruiz-Limón[1], C. Pérez-Sánchez[1], M. C. Abalos-Aguilera[1], I. Cecchi[1,3], R. Ortega[1], J. Calvo[1], R. Guzmán-Ruiz[4,5], M. M. Malagón[4,5], E. Collantes-Estevez[1], A. Vidal-Puig[2], Ch. López-Pedrera[1,†] & N. Barbarroja[1,5,†]

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Total number of figures and tables: 5 figures and 4 tables.

1. ABSTRACT

Background. Rheumatoid arthritis (RA) patients are at increased risk of insulin resistance (IR); however, the specific mechanisms mediating this associa- tion are currently unknown. Objective. To investigate whether the inflammatory activity associated with RA accounts for the observed defective glucose metabolism and lipid metabolism in these patients.
Methods. We followed two main strategies: (i) extensive metabolic profiling of a RA cohort of 100 patients and 50 healthy control subjects and (ii) mechanistic studies carried out in both a collagen-induced arthritis mouse model and 3T3-L1 adipocytes treated with conditioned serum from RA patients.
Results. Following the exclusion of obese and dia- betic subjects, data from RA patients demonstrated a strong link between the degree of systemic inflammation and the development of IR. These results were strengthened by the observation that induction of arthritis in mice resulted in a global inflammatory state characterized by defective car- bohydrate and lipid metabolism in different tis- sues. Adipose tissue was most susceptible to the RA-induced metabolic alterations. These metabolic effects were confirmed in adipocytes treated with serum from RA patients.
Conclusions. Our results show that the metabolic disturbances associated with RA depend on the degree of inflammation and identify inflammation of adipose tissue as the initial target leading to IR and the associated molecular disorders of carbohydrate and lipid homeostasis. Thus, we anticipate that therapeutic strategies based on tighter control of inflammation and flares could provide promising approaches to normalize and/or prevent metabolic alterations associated with RA.

Keywords: adipose tissue, inflammation, insulin resistance, molecular pathways, rheumatoid arthritis.

2. INTRODUCTION

Patients with rheumatoid arthritis (RA) have an increased risk of developing cardiovascular disease (CVD), and CVD is the leading cause of morbidity and mortality in these patients [1, 2]. The traditional risk factors do not fully account for the increased CVD risk in RA patients, suggesting that additional mechanisms may be pathogeni- cally more relevant in these patients. Thus, the disease itself could constitute an independent risk factor for the development of CVD in patients with RA [3, 4].
Rheumatoid arthritis patients exhibit a cluster of CVD risk factors [5] including insulin resistance (IR), type 2 diabetes mellitus and dyslipidaemia, with an increased prevalence of metabolic syndrome (up to 40%) [6, 7]. The reverse rela- tionship has also been observed: patients diagnosed with metabolic syndrome seem to have an increased risk of RA [6]. Furthermore, the prevalence of IR is increased in patients with RA in comparison with the general population. Recent studies indicate an association between IR and

either increased body mass index (BMI) (probably due to inadequate physical activity) or prolonged glucocorticoid therapy in RA patients [7, 8].
Insulin resistance is associated with metabolic factors dysregulated in the context of overnutrition as well as with lipotoxicity (i.e. ectopic lipid accumulation in peripheral organs other than adipose tissue) and in many cases with an inflammatory component. Excessive and/or inappropriate accumulation of lipids can trigger inflammatory responses that contribute to the development of IR [9]. Conversely, it is conceivable that inflammation-induced IR may be exacerbated in individuals whose immune cells exhibit a relatively low threshold to respond to inflam- matory triggers and/or a robust amplification of the inflammatory cascades [10, 11]. Thus, we hypothesized that inflammatory pathogenic mediators involved in RA may also contribute to facilitate the development of IR in these patients. The specific molecular mechanisms governing the dysfunction of the homeostatic processes controlling glucose metabolism and lipid metabolism in RA have not yet been elucidated. This is the first study in which the pathogenic effect of systemic inflammation to disturb insulin sensitivity and lipid metabolism in RA patients has been investigated. We explored this pathogenic process using multiple approaches *in vivo*, *ex vivo* and *in vitro*, combining the characterization of a cohort of RA patients, a mouse model of collagen-induced arthritis (CIA) and studies in murine 3T3-L1 adipocytes.

3. METHODS

3.1. Patients

In total, 100 RA patients and 50 healthy control subjects matched for age, gender and BMI were included in this study. RA patients fulfilled at least four 1987 American College of Rheumatology (ACR) disease criteria and achieved a total score of ≥6 according to 2010 ACR classification [12, 13]. To avoid the effects of increased BMI and diabetes on IR, obese (BMI > 30 kg m2) and diabetic subjects (fasting blood glucose levels >126 mg dL1, haemoglobin A1c level >6.5% or antidiabetic medication) were excluded. Patients were receiving the following treatments: corticosteroids [low doses (5.0–7.5 mg), 94.5% deflazacort and 5.5% prednisone], antimalarials, nonsteroidal anti-inflammatory drugs (NSAIDs) and methotrexate. Tests were performed in all patients to determine the presence of anticyclic citrullinated protein antibodies (ACPAs) and rheumatoid factor (RF). Disease activity score in 28 joints (DAS28) was determined following the guidelines of the ACR. Moderate–high disease activity was defined as DAS28 > 3.2 [14]. None of the healthy controls had a history of other autoimmune diseases, atherothrombosis or thrombosis. All participants enrolled were Caucasian and recruited at the Department of Rheumatology, Reina Sofia University Hospital, Cordoba, Spain.
Metabolic features (lipid profile, BMI, glucose and insulin), disease activity and disease-modifying antirheumatic drug (DMARD) and glucocorticoid

therapy were recorded (Table 1). DAS28 variables comprised erythrocyte sedimentation rate (ESR), swollen joint count (in 28 joints), tender joint count (in 28 joints) and patient assessment of disease activity (measured on a 0- to 100-mm visual analogue scale).
Blood samples collected from patients following fasting for 8 h were used for laboratory tests. The homeostasis model assessment (HOMA)-IR index was used to measure IR: [blood insulin concentration (mU L1) 9 blood glucose concentration (mg dL1)]/405. HOMA-IR values >2.5 indicated IR [15, 16].

3.2. CIA mouse model

All animal experiments were carried out in accordance with the ARRIVE guidelines and with the UK Animals (Scientific Procedures) Act, 1986, and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978).
Twenty-five DBA1/J male mice (7–8 weeks old) were used in this study. Five mice were used as healthy controls, and 20 mice were injected subcutaneously with collagen/complete Freund's adjuvant emulsion (100 lg per mouse); on day 21, mice were boosted with a mixture of collagen solution and incomplete Freund's adjuvant emul- sion (100 lg per mouse). Between days 22 and 42, macroscopic signs of arthritis were scored three times weekly, where each paw received a score: 0 = no visible effects of arthritis; 1 = edema and/or erythema of one digit; 2 = edema and/or erythema of two digits; 3 = edema and/or erythema of more than two digits; and 4 = severe arthritis of entire paw and digits. The arthritic index (AI) was calculated by addition of individual paw scores (up to maxi- mum of 16). Diseased mice were classified into two groups according to the AI score: low disease, 1–4; and moderate–severe disease, 5–16. Mice were weighed daily. The CIA mouse model was generated by Washington Biotechnology Inc. (Baltimore, MD, USA). Next, mice were killed, and gonadal adipose tissue, skeletal muscle, buffy coat and plasma were isolated and frozen at 80 °C and shipped to our laboratory in Spain for gene and protein analyses.

3.3. Culture, differentiation and treatment of 3T3-L1 pre-adipocytes

3T3-L1 cells were purchased from ATCC (Manassas, VA, USA). Cells were cultured, tested for mycoplasma contamination and differentiated into adipocytes according to the protocol described by Guzman-Ruiz et al. [17]. Differentiated cells were used only when at least 90% showed an adipocyte phenotype by accumulation of lipid droplets by day 8. On day 8 of differentiation, 3T3-L1 adipocytes were treated for 24 h with medium containing 10% inactivated serum (incubated at 56 °C for 30 min) from 12 healthy donors [C-reactive protein (CRP) 0.58 ± 0.48 mg x mL^{-1}] and 12 nonobese and non- diabetic RA patients with moderate–high disease activity (DAS28 > 3.2 and CRP >5 mg x mL^{-1}). The clinical characteristics of this second cohort of participants are shown in Table 2. Subsequently, cells were collected for protein and mRNA analyses.

All participants enrolled were Caucasian and recruited at the Department of Rheumatology, Reina Sofia University Hospital, and gave their informed consent.

3.4. Serum levels of TNF-a and IL6

Serum levels of tumour necrosis factor alpha (TNFa) and interleukin (IL)-6 in RA patients and healthy donors (for in vitro studies) were quantified by enzyme-linked immunosorbent assay, following the manufacturer's instructions (Bionova, Diaclone, Madrid, Spain).

3.5. Western blotting

Total protein from mice tissues and buffy coat or 3T3-L1 adipocytes was extracted using radioimmunoprecipitation assay buffer (0.1% SDS, 0.5% sodium deoxycholate, 1% Nonidet P-40, 150 mmol x L^{-1}, NaCl and 50 mmol x L^{-1}, Tris–HCl; pH 8.0) supplemented with protease inhibitors.
Proteins (25 μg) were subjected to Western blotting. Immunoblots were incubated with the following antibodies: AKT, phospho-AKT Ser473, IL1b, GAPDH, b-actin, ERK, phospho-ERK, STAT3, phos- pho-STAT3 and NFkB (Santa Cruz Biotechnology, Madrid, Spain), phospho-IRS Ser636/639, phos- pho-HSL Ser563 mTOR, Rictor, GbL and Raptor (Cell Signaling Technology, Inc., MA, USA), IRS, phospho-IRS Tyr 608 and HSL (Abcam, Cambridge, UK) and JNK and phospho-JNK (RD System, Minneapolis, MN).

3.6. RT-PCR

RNA was extracted using TRI Reagent (Sigma- Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions and reverse-transcribed into cDNA. Real-time PCR using SYBR green or TaqMan was performed according to the manufacturer's instructions (Thermo Fisher Scientific, Madrid, Spain). Expression of genes of interest was corrected by the geometrical average of 18s, b2m, b-actin and 36b4 using the BestKeeper tool [18].
The expression levels of genes involved in lipid metabolism [DGAT1/DGAT2 (diacylglycerol O-acyltransferase 1/2), PLIN1/PLIN2 (perilipin 1/ 2), SREBP1a (sterol regulatory element-binding transcription factor 1), INSIG1 (insulin-induced gene 1), ACC (acetyl-CoA carboxylase), ATGL (adi- pose triglyceride lipase), HSL (hormone-sensitive lipase), PPARa (peroxisome proliferator-activated receptor alpha), MCAD (medium-chain acyl-CoA dehydrogenase), PGC1a/PGC1b (peroxisome proliferator-activated receptor gamma coactivator 1- alpha/1-beta), CD36 (cluster differentiation 36) and LPL (lipoprotein lipase)] and insulin sig- nalling [GLUT4 (glucose transporter type 4) and IRS1/IRS2 (insulin receptor substrate ")] were analysed.

3.7. Adipocyte size

Histological sections of white adipose tissue stained with haematoxylin and eosin were pre- pared as described previously [19]. Adipocyte sizes were measured using Cell P (Olympus Soft Imaging Solutions GmbH, Munster, Germany). Between 1000 and 3000 adipocytes per tissue section from each mouse were used to determine the mean cell area.

3.8. Statistical analysis

Student's unpaired t-test, ANOVA and Duncan's test were used for the statistical analysis. Spearman's correlation was calculated to estimate the linear correlations between variables ($P < 0.01$). Multiple linear regression analysis was performed to exclude the influence of potential confounding variables on the levels of IR. HOMA-IR was selected as the dependent variable. Different treatments (methotrexate, leflunomide, hydroxy-chloroquine, corticosteroids and NSAIDs) were selected as independent variables. As a positive control, IR was included as an independent variable. Statistical significance was set at $P < 0.05$.

4. RESULTS

4.1. Comorbidities associated with RA: relationships between inflammation, disease activity and degree of IR

Our cohort of 100 nonobese, nondiabetic RA patients had an increased prevalence of IR com- pared to the age- and gender-matched control group, with significantly elevated levels of fasting blood glucose and insulin (Table 1). After classify- ing RA patients based on their degree of IR (insulin-resistant group: HOMA-IR >2.5; normoglycaemic group: HOMA-IR < 2.5), we found significant dif- ferences in parameters related to inflammation and disease activity. Thus, RA patients with IR had higher levels of CRP, ESR and DAS28 (Fig. 1a). However, we did not find any association between IR and levels of autoantibodies (ACPAs and RF) or disease duration (Fig. 1b). We observed a strong correlation between levels of DAS28 or CRP and HOMA-IR values in RA patients (Spearman's $q = 0.223$, $P = 0.011$; Spearman's $q = 0.367$, $P = 0.000$, respectively). Thus, RA patients with moderate–high disease activity (DAS28 > 3.2) had significantly elevated levels of HOMA-IR compared to patients in the low disease activity group (DAS28 < 3.2) (Fig. 1c). Additionally, patients with high levels of systemic inflammation (CRP > 5 mg L1) had higher levels of insulin and HOMA-IR compared to those with low levels of systemic inflammation (CRP < 5 mg L1) (Fig. 1d). In addition, low-dose cor- ticosteroid therapy was not associated with high levels of fasting blood glucose and insulin (Fig. 1e). In multiple linear regression analysis in our cohort of RA patients, no treatment was a statistically signifi- cant confounding variable for HOMA-IR levels: methotrexate ($b = 0.201$, $P =$

0.340), hydroxychloro- quine (b = 0.251, P = 0.232), leflunomide (b = 0.151, P = 0.477) and NSAIDs (b = 0.239, P = 0.240). Thus, corticosteroid therapy had no effect in HOMA-IR levels (b = 0.246, P = 0.272) (Fig. 1e and Table 3).

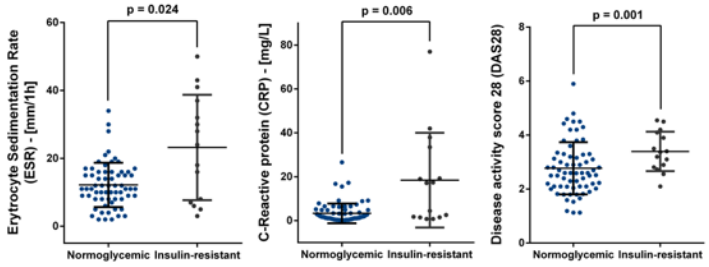
Table 1. Clinical details of the Rheumatoid Arthritis patients and the healthy donors

	RA patients	Healthy donors
Clinical parameters		
Female/Male (%/%) (n/n)	75 / 25 (n=100)	38 / 12 (n=50)
Age (years)	54.93 ± 13.94	46.06 ± 10.08
Disease duration (years)	6.30 ± 5.75	--------
RF positive (%)	55	--------
Anti-CCPs antibodies (%)	66	--------
Tender joints (n)	2.5 ± 2.16	--------
Swollen joints (n)	4.2 ± 7.9	--------
DAS28	2.86 ± 0.96	--------
Smoker (%)	25	22
BMI (kg/m^2)	23.00 ± 2.93	24.36 ± 2.32
Comorbidities		
Hypertension (%)	18.00[a]	2.00
Insulin resistance (%)	15.00[a]	6.00
Metabolic syndrome (%)	7.00	5.00
Laboratory parameters		
Glucose (mg/dl)	90.54 ± 19.96 [a]	83.25 ± 9.40
Insulin (mg/dl)	7.71 ± 3.91 [a]	6.20 ± 3.39
Cholesterol (mg/dl)	196.44 ± 31.16	125.53 ± 32.04
HDL-Cholesterol, mg/dl	59.75 ± 15.38	56.98 ± 14.41
LDL-Cholesterol, mg/dl	119.38 ± 23.54	125.53 ± 32.04
Triglycerides, mg/dl	90.92 ± 38.67	84.64 ± 44.64
ESR	14.90 ± 9.83 [a]	7.75 ± 4.33
CRP, mg/dl	12.33 ± 2.70[a]	1.30 ± 1.60
Treatments		
Corticosteroids (%)	37.00	------
Antimalarial (%)	41.00	------
NSAIDS (%)	68.00	------
Methotrexate (%)	60.00	------
Leflunomide (%)	32.00	------

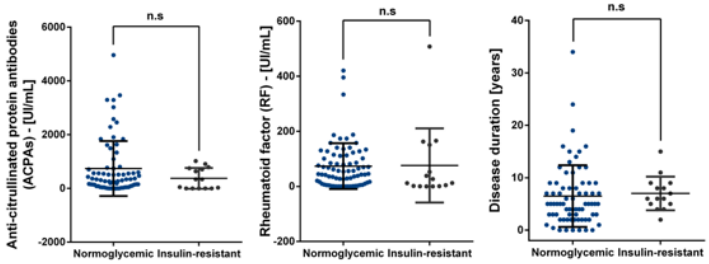
Values are mean (± SD). HDL= High density lipoprotein; LDL= Low density lipoprotein; DAS= Disease activity score; anti-CCPs = Anti-cyclic citrullinated proteins; ESR= Erythrocyte sedimentation rate; CRP= C reactive protein; BMI= Body Mass Index; NSAIDS= Non-steroideal anti-inflammatory drugs; RF= Rheumatoid factor.

[a]Significant differences vs controls ($p < 0.01$)

A


p = 0.024
Erytrocyte Sedimentation Rate (ESR) - [mm/1h]
Normoglycemic
Insulin-resistant
p = 0.006
C-Reactive protein (CRP) - [mg/L]
Normoglycemic
Insulin-resistant
p = 0.001
Disease activity score 28 (DAS28)
Normoglycemic
Insulin-resistant

B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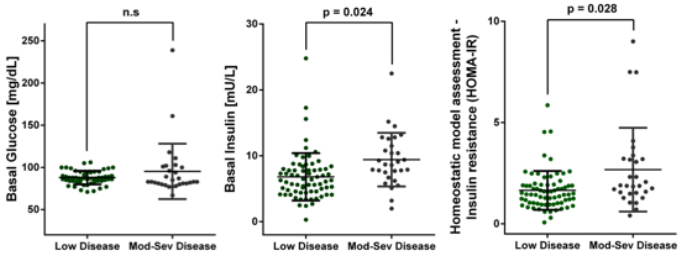
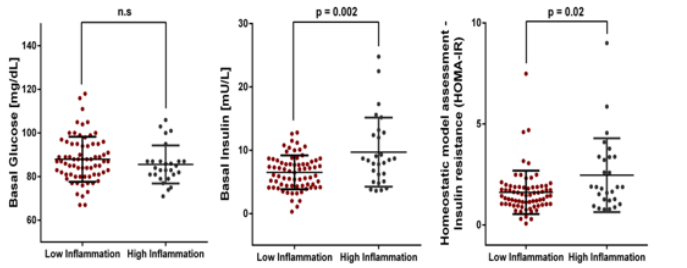
Anti-citrullinated protein antibodies (ACPAs) - [UI/mL]
Normoglycemic
Insulin-resistant
n.s
Rheumatoid factor (RF) - [UI/mL]
Normoglycemic
Insulin-resistant
n.s
Disease duration [years]
Normoglycemic
Insulin-resistant

C


n.s
Basal Glucose [mg/dL]
Low Disease
Mod-Sev Disease
p = 0.024
Basal Insulin [mU/L]
Low Disease
Mod-Sev Disease
p = 0.028
Homeostatic model assessment - Insulin resistance (HOMA-IR)
Low Disease
Mod-Sev Disease

D


n.s
Basal Glucose [mg/dL]
Low Inflammation
High Inflammation
p = 0.002
Basal Insulin [mU/L]
Low Inflammation
High Inflammation
p = 0.02
Homeostatic model assessment - Insulin resistance (HOMA-IR)
Low Inflammation
High Inflammation

E

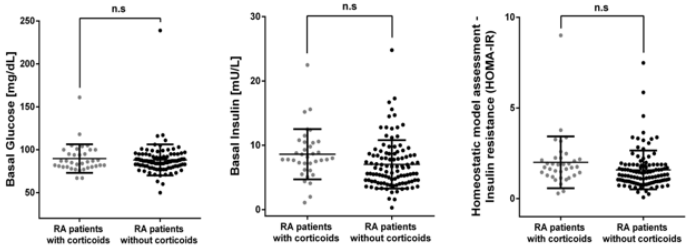
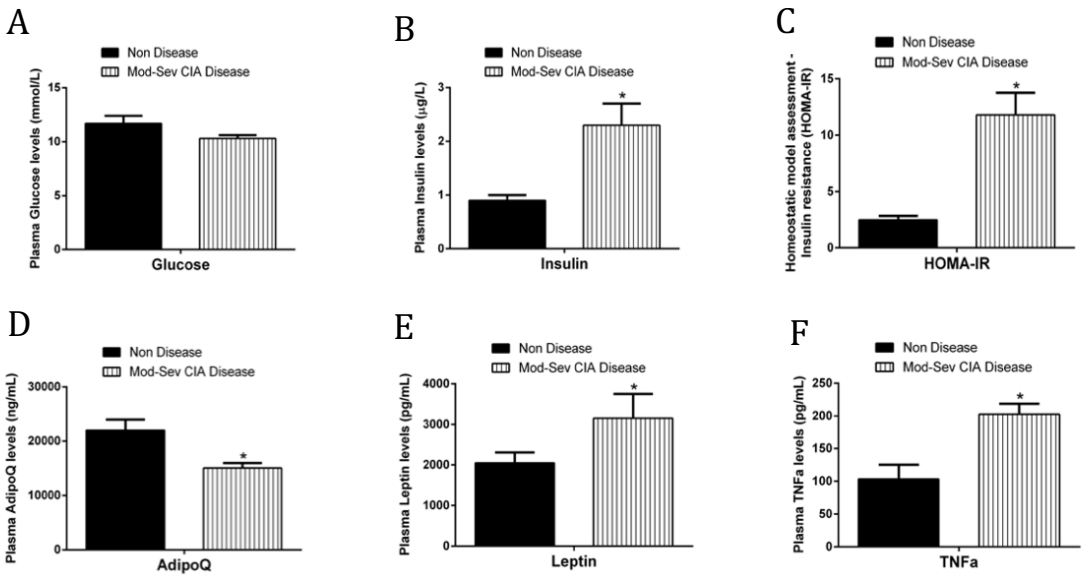

n.s
Basal Glucose [mg/dL]
RA patients with corticoids
RA patients without corticoids
n.s
Basal Insulin [mU/L]
RA patients with corticoids
RA patients without corticoids
n.s
Homeostatic model assessment - Insulin resistance (HOMA-IR)
RA patients with corticoids
RA patients without corticoids

Figure 1: Association between inflammation markers and disease activity with the IR state. (A-B) Relationship between IR state and inflammatory parameters such as erythrocyte sedimentation rate (ESR), C-reactive protein (CRP) and disease activity score 28 (DAS28), and levels of anti-citrullinated protein antibodies (a-CCP), rheumatoid factor (RF) and disease duration. (C) Association between moderate-severe activity of the disease and glucose, insulin and HOMA-IR levels. (D) Relationship between high levels of inflammation in RA patients, insulin and HOMA-IR. (E) No association between corticoids therapy and blood glucose, insulin levels and HOMA-IR. RA patients data. Paired t-test were performed *p<0.05

4.2. Effects of the arthritis development on inflammation and lipid and glucose metabolism in peripheral blood and metabolic tissues of CIA mice.

4.2.1. Systemic level: plasma and leukocytes

Arthritis development did not change the levels of fasting glucose on plasma of CIA mice (Figure 2A), however this group had significant increased levels of insulin (Figure 2B) which was translated into an elevation of the HOMA-IR values (Figure 2C). Plasma levels of adiponectin (AdipoQ) were significant decreased, alongside with an increase of the plasma levels of leptin in CIA mice compared to non arthritic mice (Figure 2D and 2E). As expected, TNF-α levels were elevated in plasma of CIA mice compared to non-diseased control group (Figure 2F). In contrast, a significant drop in the non-esterified fatty acids (NEFA) plasma levels was detected in CIA disease mice (Figure 2G). Accordingly, leukocytes from CIA mice showed an upregulation of IL-1β protein expression levels (Figure 2H). Phosphorylation and expression levels of AKT were constitutively increased in CIA mice compared to the healthy group (Figure 2H).



G

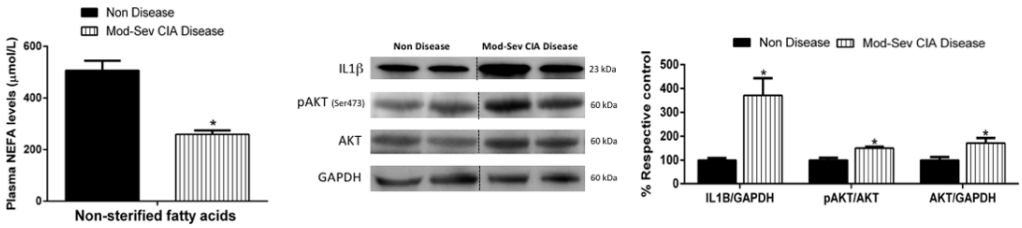
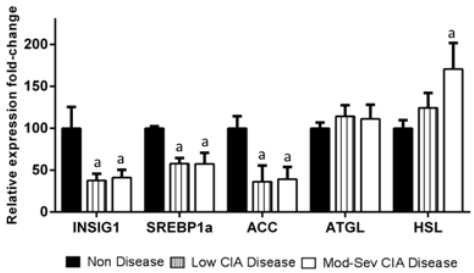


Figure 2: Effect of the disease development in CIA mice at systemic level: plasma and leukocytes. (A) Plasma fasting glucose levels (B) Plasma fasting insulin levels (C) HOMA-IR values (D) Plasma levels of adiponectin (AdipoQ) (E) Plasma levels of leptin (F) Plasma levels of TNF (G) Plasma NEFA levels. (H) Phosphorylation and protein expression levels of AKT and protein expression of IL1β; Tumor necrosis factor-alpha, TNFα; Protein kinase B, PKB, AKT; Interleukin 1 beta, IL1β; Glyceraldehyde-3-Phosphate Dehydrogenase, GAPDH; non-esterified fatty acids, NEFA. Mice data. *indicates significant differences vs non-disease mice ($p<0.05$).

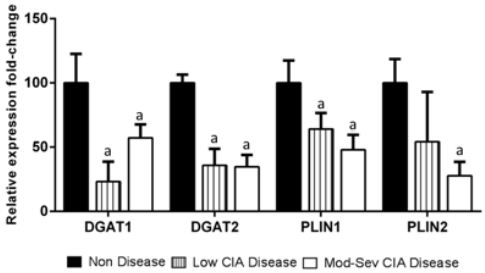
4.2.2. Effects on Adipose tissue (AT)

Disease progression in CIA mice was associated with a significant reduction in genes involved in lipogenesis (INSIG1, SREBP1a and ACC) (Figure 3A) and lipid accumulation (DGAT1, DGAT2, PLIN1 and PLIN2) (Figure 3B) which was evident in gonadal AT from initial stages. We also observed a significant downregulation of genes involved in insulin signalling such as GLUT4, IRS1 and IRS2 (Figure 3C). In addition, a significant increase in the expression and phosphorylation of HSL, a key lipolytic enzyme, was observed in gonadal AT of CIA mice compared to non-diseased mice (Figure 3A and 3D). A reduction in the size of the adipocytes was also noticed in the diseased mice (Figure 3E). Despite these changes, no significant effect on the body weight was detected (data no shown). The CIA mice AT had increased protein phosphorylation and expression levels of AKT in comparison to their controls (Figure 3E). We next tested the inflammation levels in gonadal AT, and observed an upregulation of IL1-β, mTOR and Rictor (mTORC2) protein expression levels in moderate-severe CIA disease (Figure 3E).

A



B



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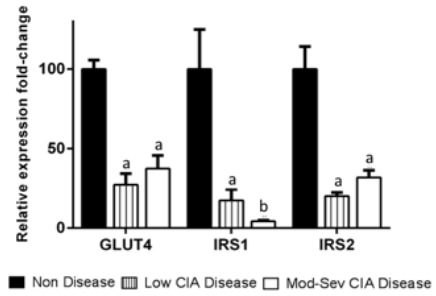
Relative expression fold-change
100
50
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a
a
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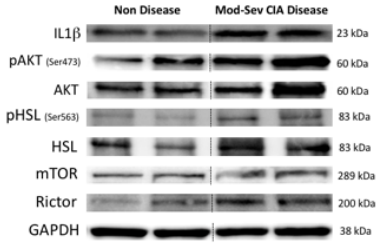
a
b
a
a
GLUT4
IRS1
IRS2
Non Disease
Low CIA Disease
Mod-Sev CIA Disease

D


Non Disease
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IL1β
23 kDa
pAKT (Ser473)
60 kDa
AKT
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mTOR
289 kDa
Rictor
200 kDa
GAPDH
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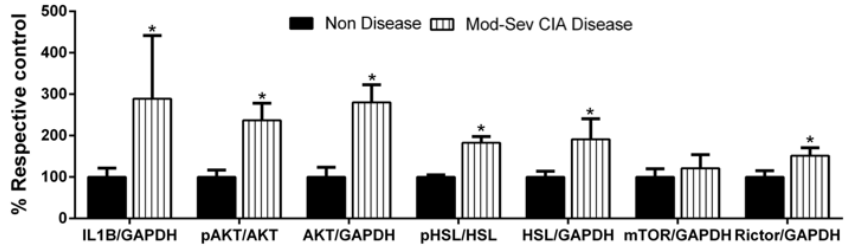
% Respective control
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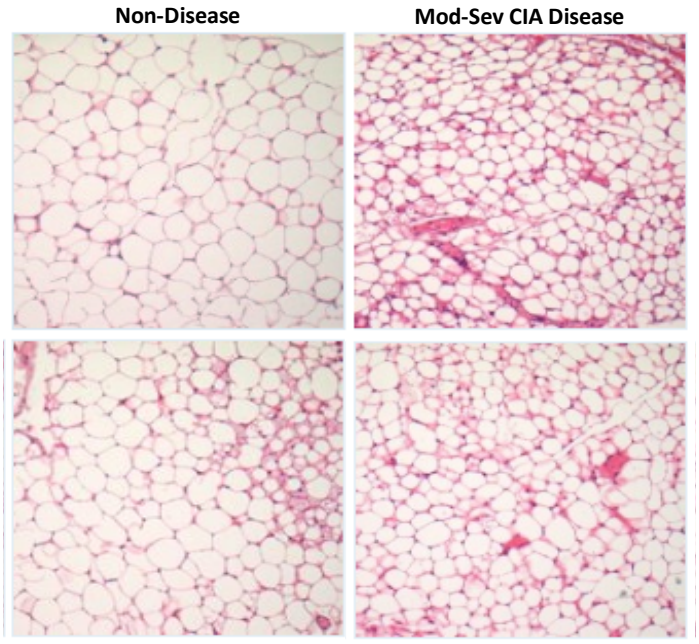
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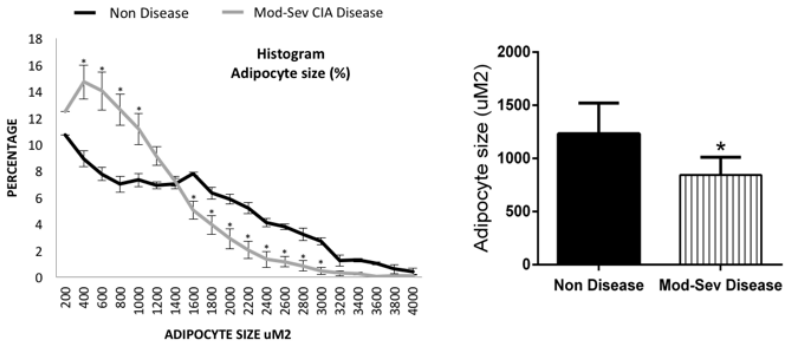

Figure 3: Effect of the disease development on adipose tissue (AT) of CIA mice. (A) mRNA relative expression of genes involved in lipogenesis and lipolysis (B) mRNA relative expression of genes involved in lipid accumulation (C). mRNA relative expression of genes involved in glucose and insulin signaling (D) Protein expression of IL1β, mTOR and Rictor. Phosphorylation and protein expression of AKT and HSL (E) Adipocytes size in CIA mice compared to non-disease mice. Diacylglycerol O-Acyltransferase 1, DGAT1; Diacylglycerol O-Acyltransferase 2, DGAT2; Perilipin 1, PLIN1; Perilipin 2, PLIN2; Glucose transporter type 4, GLUT4; Insulin receptor substrate 1, IRS1; Insulin receptor substrate 2, IRS2; Insulin Induced Gene 1 INSIG1; Sterol regulatory element-binding transcription factor 1, SREBP1a; Acetyl-CoA carboxylase, ACC; adipose triglyceride lipase, ATGL; Hormone-Sensitive Lipase, HSL; Protein kinase B, PKB, AKT; Interleukin 1 beta (IL1β); mammalian target of rapamycin, mTOR; Rapamycin-insensitive companion of mTOR, Rictor; Glyceraldehyde-3-Phosphate Dehydrogenase, GAPDH. Mice data. Paired t test was performed, a,* indicates significant differences vs non-disease mice ($p<0.05$), b indicates significant differences vs low CIA disease ($p<0.05$).

4.2.3. Effects on Skeletal muscle (SKM)

The mRNA expression of several genes involved in fatty acid oxidation (CPT1B, PGC1α and MCAD) was significantly reduced in skeletal muscle of CIA mice at moderate-severe state compared to non-diseased mice (Figure 4A). Similarly, genes involved in fatty acid uptake and lipid accumulation (PPARα, DGAT1, DGAT2, PLIN2, CD36 and LPL) were also found significantly reduced in skeletal muscle of the CIA mice in a more severe state (Figure 4B). Regarding insulin signalling, a significant reduction of mRNA expression of GLUT4, IRS1 and IRS2 was detected in moderate-severe CIA mice compared to non-diseased group (Figure 4C). Phosphorylation and protein expression levels of AKT were found increased in SKM of moderate-severe CIA mice compared to control group (Figure 4D). We further observed a significant elevation of IL1β, mTOR and Rictor (mTORC2) protein expression levels in moderate-severe CIA disease (Figure 4D).

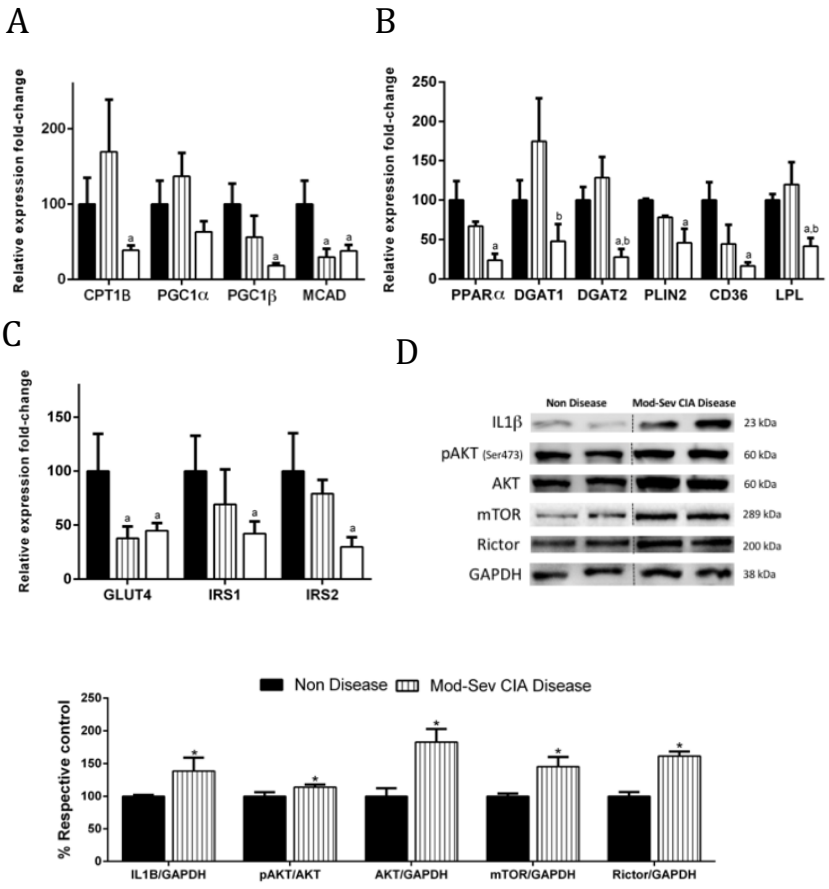

Figure 4: Effect of the disease development on skeletal muscle (SKM) of CIA mice. (A) mRNA relative expression levels of genes involved in fatty acid oxidation. (B) mRNA relative expression levels of genes involved in lipid accumulation. (C) mRNA relative expression levels of genes involved in glucose and insulin signaling. (D) Protein expression levels of IL1beta (IL-1β), mTOR and Rictor and phosphorylation and protein expression levels of AKT. Carnitine Palmitoyltransferase 1B, CPT1B; Peroxisome proliferator-activated receptor gamma coactivator 1-alpha, PGC1α; Peroxisome proliferator-activated receptor gamma coactivator 1-beta, PGC1 α; Medium-chain acyl-CoA dehydrogenase , MCAD; Peroxisome Proliferator Activated Receptor Alpha, PPAR; Diacylglycerol O-Acyltransferase 1, DGAT1; Diacylglycerol O-Acyltransferase 2, DGAT2; Perilipin 2, PLIN2; Cluster differentiation 36, CD36; Lipoprotein lipase, LPL; Glucose transporter type 4, GLUT4; Insulin receptor substrate 1, IRS1; Insulin receptor substrate 2, IRS2; Protein kinase B, PKB, AKT; Interleukin 1beta (IL1β) mammalian target of rapamycin, mTOR; Rapamycin-insensitive companion of mTOR, Rictor; Glyceraldehyde-3-Phosphate Dehydrogenase, GAPDH. Paired t test was performed a,* indicates significant differences vs non-disease mice ($p<0.05$), b indicates significant differences vs low CIA disease ($p<0.05$).

4.2.4. Effect of in vitro treatment with serum of RA patients in 3T3-L1 adipocytes

To evaluate the direct effect of the inflammatory mediators, present in the serum of RA patients on the metabolic changes observed in the AT of CIA mice, we performed in vitro studies in which 3T3-L1 adipocytes were exposed to serum from RA patients (exhibiting enhanced circulating IL6 and TNFα levels as compared to healthy donors (Figure 5A). We evaluated the effect of the RA serum in inflammation, lipogenesis, lipolysis and insulin signalling. RA serum promoted a significant reduction in genes involved in lipogenesis and lipid accumulation (SREBP1a, INSIG1, DGAT2, PLIN1 and PLIN2) compared with the serum from healthy donors (Figure 5B and 5C). In contrast, genes involved in lipolysis showed a significant upregulation in adipocytes treated with serum from RA patients (HSL) (Figure 5C). At protein levels, after treatment with serum of RA patients, phosphorylation and protein expression of HSL were significantly upregulated in adipocytes compared with 3T3-L1 adipocytes exposed to serum of healthy donors (Figure 5E). Insulin signalling was also affected by RA serum through a significant reduction in GLUT4, IRS1 and IRS2 mRNA levels (Figure 5D) and an increase of the Ser636/639 phosphorylated IRS alongside with a drop in the phosphorylation of IRS on Tyr608 (Figure 5E). In addition, in the same way to what was observed in AT and SKM of CIA mice, phosphorylation of AKT and the expression of both, mTOR and Rictor was upregulated in adipocytes treated with serum of RA patients (Figure 5E). The levels of diverse inflammatory mediators were also elevated in adipocytes treated with RA serum, represented by increased levels of IL1β and NFκB and the elevated phosphorylation of JNK, ERK and STAT3 (Figure 5E). Correlation studies showed that in 3T3-L1 adipocytes treated with serum of RA patients, the expression of several genes and proteins, involved in inflammation, lipolysis and insulin signalling was correlated with some clinical parameters of RA patients. Thus, CRP and DAS28 strongly correlated with HSL expression levels in RA serum-treated adipocytes. These two clinical parameters also correlated with inflammatory mediators such as IL1β and mTOR, moreover CRP correlated with the expression of NFκB, Rictor and the phosphorylation of IRS on serine 636/639, AKT, ERK and JNK (Table 4). Of note, IL6 and TNFα serum levels strongly correlated with the activation and the expression of HSL, AKT phosphorylation and the expression levels of IL1β and mTOR. In addition, phosphorylation of IRS (ser636/639) and ERK and expression of Rictor in 3T3-L1 adipocytes correlated with the levels of IL6 present on the serum of RA patients. Both inflammatory markers, IL6 and TNFα, negatively correlated with levels of genes involved in lipid accumulation and insulin signalling (Table 4). These data further support that inflammatory mediators present in the serum of RA patients are closely linked to the metabolic and inflammatory changes observed in adipocytes including the activation of inflammatory pathways, promotion of IR, lipolysis and a reduction in lipogenesis.

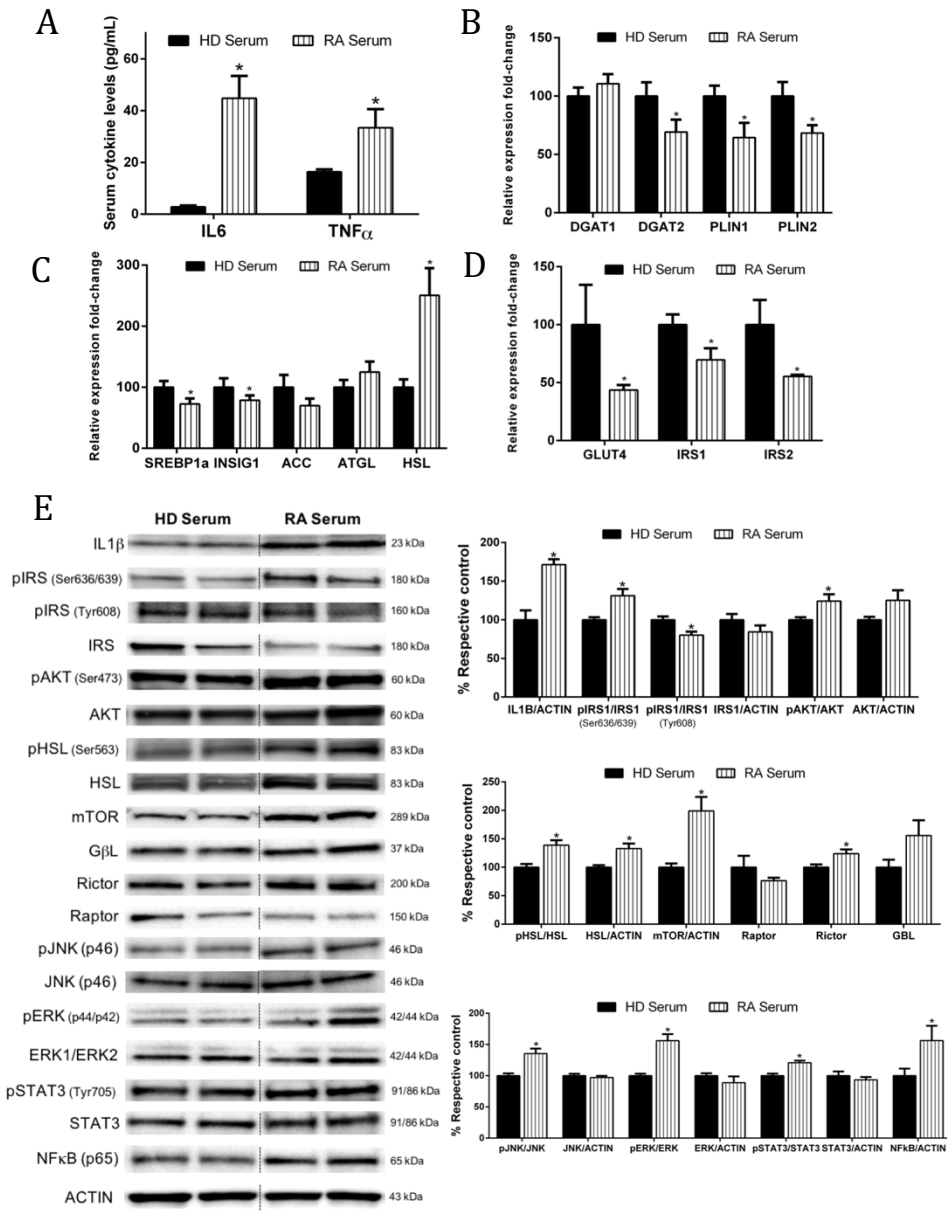

Figure 5: Effect of in vitro treatment with RA serum in 3T3-L1 adipocytes. (A) IL6 and TNFα cytokine levels in serum from healthy donors and RA patients. (B) mRNA relative expression levels of genes involved in lipid accumulation. (C) mRNA relative expression levels of genes involved in lipogenesis and lipolysis. (D) mRNA relative expression levels of genes involved in glucose and insulin signaling. (E) Protein expression levels of IL1β, IRS1, AKT, HSL, mTOR, GβL, Rictor, Raptor, JNK, ERK, STAT3 and NFκB. Phosphorylation of IRS, AKT, HSL, JNK, ERK and STAT3. Diacylglycerol O- Acyltransferase 1, DGAT1; Diacylglycerol O-Acyltransferase 2, DGAT2; Perilipin 1, PLIN1; Perilipin 2, PLIN2; Glucose transporter type 4, Sterol regulatory element-binding transcription factor 1, SREBP1a; Insulin Induced Gene 1, INSIG1; Acetyl-CoA carboxylase, ACC; adipose triglyceride lipase, ATGL; Hormone-

Sensitive Lipase, HSL; GLUT4; Insulin receptor substrate 1, IRS1; Insulin receptor substrate 2 IRS2;; Protein kinase B, PKB, AKT; Interleukin 1 beta, IL1β; mammalian target of rapamycin, mTOR; Rapamycin-insensitive companion of mTOR, Rictor; Regulatory-associated protein of mTOR, Raptor; c-JUN N-terminal kinases, JNK; extracellular signal-regulated kinase, ERK; signal transducer and activator of transcription 3, STAT3; nuclear factor kappa-light-chain-enhancer of activated B cells, NFκB; ACTIN. Paired t test was performed, *indicates significant differences vs 3T3-L1 adipocytes treated with serum from HD ($p<0.05$).

Table 3. Multiple linear regression analysis.

	Independent Variables	**B**	***P***	**95% confidence intervals for B**	
				Lower boundary	**Upper boundary**
HOMA-IR	**Model 1**				
	Methotrexate	-0,201	0,340	-0,620	0,217
	Hydroxychloroquine	0,251	0,232	-0,164	0,665
	Leflunomide	0,151	0,477	-0,270	0,571
	AINEs	-0,239	0,240	-0,640	0,163
	Corticosteroids	0,246	0,272	-0,197	0,689
	IR	2,789	0,000	2,218	3,361
	Model 2				
	Methotrexate	-0,143	0,482	-0,548	0,261
	Hydroxychloroquine	0,288	0,155	-0,112	0,688
	AINEs	-0,259	0,185	-0,645	0,126
	Corticosteroids	0,272	0,213	-0,159	0,703
	IR	2,712	0,000	2,166	3,258
	Model 3				
	Hydroxichloroquine	0,300	0,166	-0,097	0,697
	AINEs	-0,272	0,162	-0,655	0,111
	Corticosteroids	0,307	0,159	-0,112	0,725
	IR	2,731	0,000	2,190	3,272
	Model 4				
	Hydroxychloroquine	0,273	0,173	-0,122	0,668
	Corticosteroids	0,274	0,194	-0,142	0,689
	IR	2,729	0,000	2,188	3,271
	Model 5				
	Hydroxychlroquine	0,216	0,270	-0,171	0,603
	IR	2,787	0,000	2,250	3,323

5. DISCUSSION

This is, to the best of our knowledge, the first study that evaluates the molecular mechanism underlying the linkage between IR and RA by using simultaneously human, animal and cellular models, unravelling the effects of inflammatory mediators present in RA on the physiology of metabolic tissues such as adipose tissue and skeletal muscle. Thus, our data shows the close association between systemic inflammation, the activity of the disease and the development of IR in a cohort of 100 non-obese RA patients. Up to now, a number of articles have already reported a higher prevalence of IR in RA patients, which, independently on the disease duration and the therapy received, demonstrated a strong relationship between the increased BMI and the development of IR (7, 8, 10, 20, 21). Of note, we investigated in this study a cohort of patients characterised by similar BMI (<30) between the RA patients and healthy donors and where obese and diabetic subjects were excluded from the study. Despite this exclusion criteria, our cohort of RA patients confirmed and validated a higher prevalence of IR (15%) even after correcting any potential bias due to treatment with DMARDs and corticoids, short-medium history of the disease. It is well recognized that the prolonged used of corticoids therapy affects glucose metabolism inducing IR. However, this effect might depend on the dosage and type of corticoid administered (22, 23). Thus, in our cohort of RA patients, the dose of corticoids was low (< 7.5 mg) and the type of corticoid used was mainly deflazacort, which has been shown to be less prone to cause hyperglycaemia compared to other types of corticoids (23).

Despite the large heterogeneity found on the different cohorts of RA patients published until now, it seems that there are some consensus regarding the association of systemic inflammation and IR (24). In the same line of evidence, we have found a strong correlation between IR and both, the activity and the inflammatory profile of the disease, with no influence of either, the treatment administered or the BMI, thus suggesting that inflammation per se may be the main determinant of IR in patients with RA.

Several studies have suggested that inflammatory mediators involved in RA such as TNFα and IL6 are associated with the onset and development of IR and type 2 diabetes mellitus (25). Beyond these associations, there are no previous studies that directly explore the effects of RA development on glucose and lipid metabolism at systemic and tissue-specific levels. Our research shows that the development of RA affects lipid metabolism and insulin signalling in adipose tissue and skeletal muscle in a CIA mouse model. At systemic level, we observed a significant increase in TNFα plasma levels and IL1β upregulation in leukocytes, demonstrating the inflammatory status induced by the disease development. In addition, plasma insulin levels and HOMA-IR values were elevated in CIA mice. Adiponectin levels have been inversely related to insulin resistance (26), while high leptin levels have been associated with IR independently on the BMI (27). In CIA mice, lower

adiponectin plasma levels and higher levels of leptin alongside higher HOMA-IR values suggest that arthritis induced a state of insulin resistance in those mice.

At tissue level, we also observed a significant increase of IL1β in SKM and AT. Several studies demonstrated how inflammatory mediators such as TNFα and IL1β upregulated in immune cells and adipocytes in obesity, induce the development of IR through mechanisms including the reduction of the expression of GLUT4 and IRS1 genes (28, 29). Accordingly, in our study, RA-induced inflammation was accompanied by a significant reduction in the expression of IRS1, IRS2 and GLUT4 genes in AT and SKM. Of note, it has been demonstrated that the inhibition of these genes in animal models lead to insulin resistance states (30-32). Moreover, it has been shown that normoglycemic subjects with low levels of IRS1 and GLUT4 in adipose tissue developed IR later in life (33). Our results also identified an increased phosphorylation of AKT in leukocytes, AT and SKM of CIA mice. Thus, it could be speculated that prolonged activation of AKT may lead to a negative feedback of insulin signalling. The acute effect of AKT activation would cause Thr308 phosphorylation, whereas the prolonged stimulation would lead to Ser473 phosphorylation. It has been demonstrated that AKT phosphorylation in Ser473 acts as a negative regulator through phosphorylation of the insulin receptor β-subunit at threonine, thus causing decreased autophosphorylation of the receptor (34). In addition, this activation might be caused by inflammatory stimulus since AKT dysfunction has been reported in diverse pathological settings such as cancer or cardiovascular disease (35, 36). This is in accordance with our results, where the development of arthritis induced the prolonged Ser473 phosphorylation of AKT in leukocytes, SKM and AT, accompanied by increased IL1β levels and a reduction in the IRS1 and 2 expression, thus promoting a defect in insulin response which might lead to insulin resistance in these cells and tissues.

In addition, the development of arthritis promoted a reduction in lipid accumulation markers in AT of mice. This reduction was accompanied by an increase in the phosphorylation and expression of HSL, alongside the reduction of the adipocyte size compatible with an increase in lipolysis. In obese states, it has been reported increased basal lipolysis in adipocytes, closely related to the development of IR (37). In this sense, several in vitro studies have demonstrated that TNFα can induce lipolysis in mouse and human adipocytes through the upregulation of HSL (38, 39). According to our results, AT of CIA mice had increased lipolysis, probably mediated by the inflammation-induced IR caused by the disease. Whereas the effects on insulin signalling and lipid metabolism in AT were evident from early stages of the disease, similar effects in skeletal muscle were observed upon reaching a more severe condition reflected in significant changes in fatty acid metabolism. Thus, at moderate-severe stages a marked reduction of genes involved in lipid accumulation, fatty acid oxidation and insulin signalling was also noticed in skeletal muscle of CIA mice.

The reduction of lipid accumulation in SKM and AT and levels of NEFA in plasma might suggest that lipids could be either oxidised and/or accumulated in either bloodstream or other tissues such as liver, contributing to the development of IR.

Our results indicate that among all metabolic tissues affected by RA, inflammation of the AT appears to be an early target, being more susceptible to the metabolic changes caused by the arthritis (observed from the initial stages of the disease). In more common metabolic disorders, it has also been suggested that IR is initiated in AT, playing a pivotal role in the subsequent induction of IR in other organs such as muscle and liver (39).

Interestingly, we could demonstrate that inflammatory mediators present in the serum of RA patients (TNFα and IL6 among others) also induce the alterations observed in AT. Treatment of 3T3-L1 adipocytes with RA serum containing high levels of these inflammatory mediators recapitulated the results observed in AT of CIA mice, inducing lipolysis (increased phosphorylation and expression of HSL) and reducing lipid accumulation (decreased genes involved in lipid accumulation). RA serum induced a high inflammatory state on adipocytes through the activation of several intracellular kinases such as JNK, ERK and STAT3, and the elevated expression of IL1β, NFκB and mTORC2 (mTOR and Rictor). These kinases have been shown to be involved in the inactivation of IRS. IRS proteins are activated by tyrosine phosphorylation and inhibited by serine phosphorylation (40). Thus, this inflammation was associated with an impaired insulin signalling, evidenced by the decreased mRNA expression of IRS1 and 2 and GLUT4 and increased levels of phosphorylation on ser IRS, and a reduction in the phosphorylation of tyr IRS. Paradoxically, we also noticed high levels of phosphorylated AKT at ser 473. AKT is a complex metabolic hub that can be modulated by a large number of proteins in response to a variety of nutritional and cellular stressors. One of those is the mTOR complex 2. The mTOR is a serine/threonine kinase that controls a wide spectrum of cellular processes, including cell growth, differentiation, inflammation and metabolism. mTOR exists in two functionally complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). mTORC1 consists of mTOR, regulatory-associated protein of mTOR (raptor), mammalian lethal with Sec13 protein 8 (mLST8), and two inhibitory subunits, proline-rich AKT substrate of 40 kDa (PRAS40) and DEP domain-containing mTOR-interacting protein (DEPTOR). mTORC2 contains mTOR, the rapamycin insensitive companion of the mammalian target of rapamycin (rictor), stress-activated map kinase (SAPK)-interacting 1 (SIN1), mLST8, proline-rich protein 5-like (PRR5L) and DEPTOR. These two complexes are involved in the activation of AKT in response to various stimuli including growth factors such as insulin, fatty acids and cytokines (41).

It has been reported that active mTORC2 is able to phosphorylate AKT and the loss of mTORC2 abrogate the activation of AKT (42). In addition, mTORC2

is involved in the IRS degradation and IR tyrosine phosphorylation (41). There is also evidence that engagement of AKT/mTOR pathway may account of TNFα-mediated insulin resistance. Activation of PI3K/AKT/mTOR cascade by TNFα regulates the phosphorylation of IRS1 on serines 636/639 antagonizing its phosphorylation on tyrosine by insulin receptor (43).

In RA context, we showed an increase of mTORC2 (represented by high levels of mTOR and Rictor) and AKT in adipose tissue and skeletal muscle of CIA mice, as well as in adipocytes treated with RA serum, suggesting a priori that activation of mTORC2 may be responsible for the activation of AKT and impaired activation of IRS in our CIA mice model.

The relevance of these observations was further supported by our correlation studies between clinical parameters and the metabolic and inflammatory changes induced in the adipocytes by exposure to RA serum. This further supports that inflammatory mediators present in that sera may be the responsible for these alterations.

Thus, we anticipate that inflammatory mediators such as IL6 and TNFα would activate a complex repertoire of pathways that include mTORC2 and other intracellular kinases such as ERK, JNK, STAT3 that would inactivate IRS-through its phosphorylation on serines 636/639. mTORC2 complex containing mTOR, GBL and Rictor would also activate AKT, which alongside JNK and ERK will be involved in the activation of NFκB, promoting the expression of inflammatory genes further contributing to the inactivation of IRS and aggravating insulin resistance on peripheral tissues such as adipose tissue and skeletal muscle (Figure 6).

Our results suggest that RA promotes a global inflammatory state (including systemic and tissue levels) that affects the AT where it promotes insulin resistance, stimulated lipolysis and reduction of lipid accumulation. This is followed at more advanced stages by a reduction in lipid content and exacerbated insulin resistance in SKM. According to those results, the adipose tissue is an early target in this process.

Altogether, our results show a direct effect of RA-induced chronic inflammation mediating the alterations occurred in glucose and lipid metabolism associated with this disorder. Thus, therapeutic strategies aimed at inhibiting inflammation, by targeting proinflammatory cytokines, might be an excellent option to normalize the metabolic alterations associated with RA.

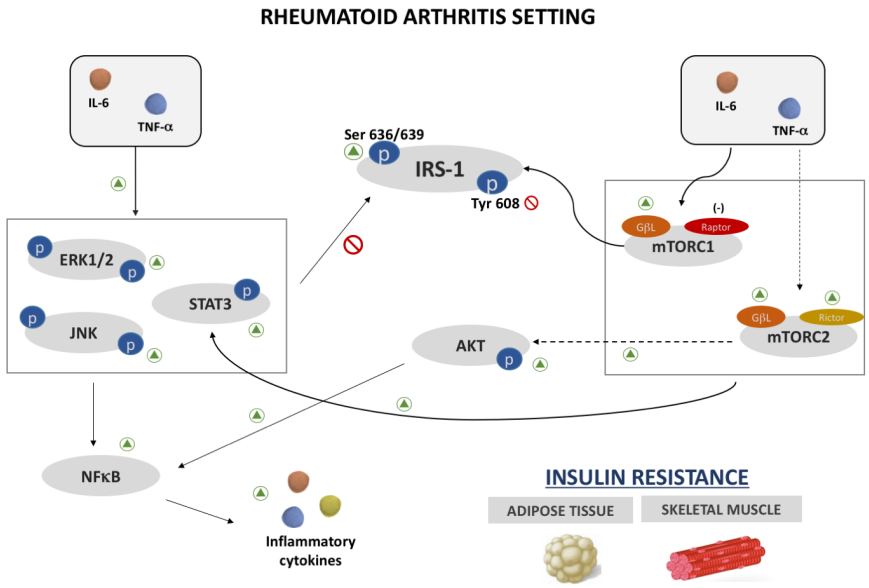

Figure 6: Suggested model explaining the molecular pathways involved in the development of IR in rheumatoid arthritis. In the setting of rheumatoid arthritis, inflammatory mediators such as IL6 and TNFα would activate a complex repertoire of pathways that include mTORC2 and other intracellular kinases such as ERK, JNK, STAT3 that would inactivate IRS-through its phosphorylation on serines 636/639. mTORC2 complex containing mTOR, GBL and Rictor would also activate AKT, which alongside JNK and ERK will be involved in the activation of NFκB, promoting the expression of inflammatory genes further contributing to the inactivation of IRS and aggravating insulin resistance on peripheral tissues such as adipose tissue and skeletal muscle.

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CONCLUSIONES

1- En el plasma de los pacientes con síndrome antifosfolípido existe una firma de microRNAs (miRNAs) diferencialmente expresados, modulados por los autoanticuerpos antifosfolípidos y asociados con el estatus aterotrombótico presente en esta patología autoinmune. Dichos miRNAs podrían por tanto ser considerados como nuevos biomarcadores de enfermedad, constituyendo una herramienta útil para el manejo clínico de estos pacientes.

Artículo: "*Circulating microRNAs as biomarkers of disease and typification of the atherothrombotic status in antiphospholipid syndrome*". Haematologica (2018).

2- Los neutrófilos de sangre periférica y líquido sinovial de pacientes con artritis reumatoide exhiben una reducción generalizada de los niveles de miRNAs, promovida tanto por anticuerpos anti-péptidos cíclicos critrulinados como por mediadores inflamatorios, que podría contribuir a su perfil patogénico. En consonancia con dicha expresión reducida, la biogénesis de miRNAs está significativamente alterada en los neutrófilos de estos pacientes, y se asocia con una regulación negativa más acentuada de los miRNAs relacionados con la migración y la inflamación en los neutrófilos sinoviales. Los tratamientos con fármacos dirigidos contra el TNF-α y el receptor de la IL-6 modulan los niveles de miRNAs en los neutrófilos, minimizando su perfil inflamatorio.

Artículo: "*Impaired microRNA processing in neutrophils from Rheumatoid Arthritis patients confers their pathogenic profile. Modulation by biological therapies*". Haematologica (2020).

3- La inflamación crónica presente en la artritis reumatoide puede inducir alteraciones en el metabolismo de la glucosa y los lípidos en los tejidos metabólicos, afectando principalmente al tejido adiposo, favoreciendo así el desarrollo de resistencia a la insulina. Por tanto, la aplicación de estrategias terapéuticas basadas en un control mas estricto de la inflamación podría contribuir a normalizar y prevenir las alteraciones metabólicas asociadas a la AR.

Artículo: "*Defective glucose and lipid metabolism in rheumatoid arthritis is determined by chronic inflammation in metabolic tissues*". Journal of Internal Medicina (2018).

En suma, los resultados globales obtenidos en esta tesis doctoral han permitido identificar potenciales biomarcadores del status de la enfermedad y sus comorbilidades en pacientes con Sindrome Antifosfolípido y Artritis Reumatoide, así como diversos mecanismos moleculares asociados a procesos patológicos clave en estas enfermedades, tales como la enfermedad

cardiovascular. Dichos resultados podrían sentar las bases para la realización de estudios futuros, cuyo fin sea desarrollar una medicina personalizada dirigida a optimizar el cuidado de los pacientes con enfermedades autoinmunes sistémicas.

CONCLUSIONS

1- In the plasma of patients with antiphospholipid syndrome there is a signature of differentially expressed miRNAs, modulated by antiphospholipid autoantibodies and associated with the atherothrombotic status present in this autoimmune pathology. These miRNAs could be new disease biomarkers, constituting a useful tool for the clinical management of these patients.

Article: "Circulating microRNAs as disease biomarkers and atherothrombotic state typing in antiphospholipid syndrome". Haematologica (2018).

2- Peripheral blood and synovial fluid neutrophils from patients with rheumatoid arthritis exhibit a global reduction in miRNA levels, promoted by both antibodies to citrullinated proteins antigens and inflammatory mediators, which could contribute to their pathogenic profile. Consistent with such reduced expression, the biogenesis of miRNAs is significantly altered in the neutrophils of these patients, and is associated with a more pronounced down-regulation of miRNAs related to migration and inflammation in synovial neutrophils. Drug treatments directed against TNF-a and the IL-6 receptor could modulate the levels of miRNAs in neutrophils, minimizing their inflammatory profile.

Article: “The processing of impaired microRNA in neutrophils from patients with rheumatoid arthritis confers its pathogenic profile. Modulation by biological therapies ". Haematologica (2020).

3- The chronic inflammation present in rheumatoid arthritis can induce alterations in the metabolism of glucose and lipids in metabolic tissues, mainly affecting adipose tissue, thus favoring the development of insulin resistance. Therefore, the application of therapeutic strategies based on a tighter control of inflammation could contribute to normalize and prevent the metabolic alterations associated with RA.

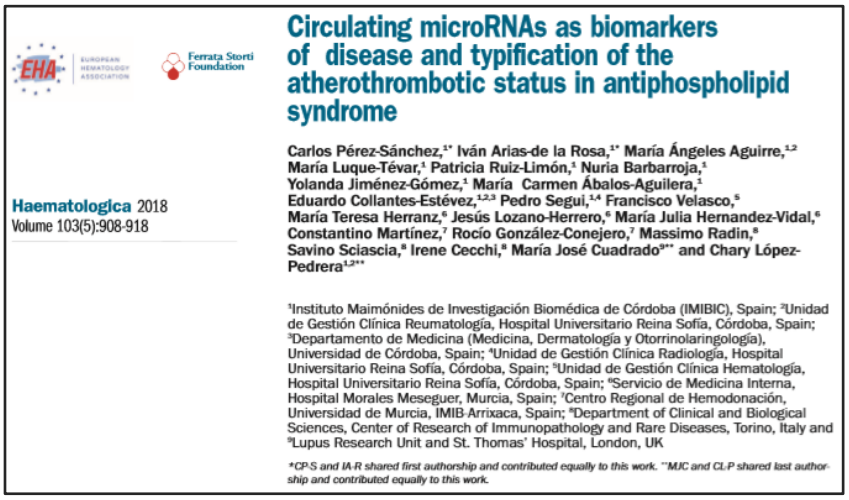
Article: "Defective glucose and lipid metabolism in rheumatoid arthritis is determined by chronic inflammation in metabolic tissues." Journal of Internal Medicine (2018).

In sum, these results have allowed the identification of potential biomarkers of the disease state and its comorbidities in patients with antiphospholipid syndrome and rheumatoid arthritis, as well as various molecular mechanisms associated with key pathological processes in these diseases, such as cardiovascular disease. These findings could lay the bases for future clinical studies, which purpose is to develop personalized medicine aimed to optimize the care of patients with systemic autoimmune diseases.

ANEXO

I. Informe del factor de impacto y cuartil de "Journal of Citation Reports (SCI y/o SSCI)" o de las bases de datos de referencias del área en el que se encuentran las publicaciones presentadas.

Primer artículo:
Haematologica. 2018 May;103(5):908-918.
Doi: 10.3324/haematol.2017.184416



Haematologica 2018
Volume 103(5):908-918

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**]

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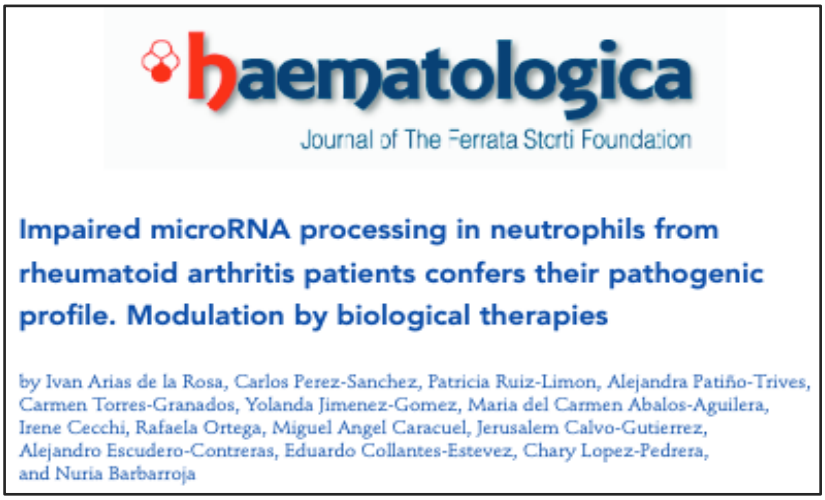
JCR Year	HEMATOLOGY		
	Rank	Quartile	JIF Percentile
2018	7/73	Q1	91.096

Segundo artículo

Haematologica 2020 Jan 16: haematol.2018.205047.
Doi: 10.3324/haematol.2018.205047



JCR Year	HEMATOLOGY		
	Rank	Quartile	JIF Percentile
2019	7/76	Q1	91.447

Tercer artículo:
J Intern Med. 2018 Jul;284(1):61-77.
doi: 10.1111/joim.12743.

JIM Original Article

doi: 10.1111/joim.12743

Defective glucose and lipid metabolism in rheumatoid arthritis is determined by chronic inflammation in metabolic tissues

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JCR Year	MEDICINE, GENERAL & INTERNAL		
	Rank	Quartile	JIF Percentile
2018	14/160	Q1	91.563

II. Producción científica.

1. Aranda-Valera IC*, **Arias-de la Rosa I***, Roldán R, Ábalos-Aguilera MC, Torres-Granados C, Patiño-Trives A, Luque-Tevar M, Ibáñez-Costa A, Guzmán-Ruiz R, Malagón MM, Escudero-Contreras A, López-Pedrera Ch, Collantes-Estévez E, Barbarroja N. Subclinical cardiovascular risk signs in adults with juvenile idiopathic arthritis in sustained remission. Pediatric Rheumatology 2020 Jul 14;18(1):59. doi: 10.1186/s12969-020-00448-3. **IF: 2.59. Q1.**
2. Cecchi I, Pérez-Sánchez C, Sciascia S, Radin M, **Arias-de la Rosa I**, Barbarroja N, Scudeler L, Pérez-Sánchez L, Patiño-Trives AM, Aguirre-Zamorano MÁ, Menegatti E, Roccatello D, López-Pedrera C. Circulating microRNAs as potential biomarkers for monitoring the response to in vivo treatment with Rituximab in systemic lupus erytehmatosus patients. Autoimmun Rev. 2020 Apr;19(4):102488. doi: 10.1016/j.autrev.2020.102488. **IF: 7.76. Q1.**
3. **Arias-de la Rosa I***, Pérez-Sánchez C*, Ruiz-Limón P*, Patiño-Trives A, Torres-Granados C, Jiménez-Gómez Y, Ábalos-Aguilera MC, Cecchi I, Ortega R, Caracuel MA, Calvo-Gutiérrez J, Escudero-Contreras A, Collantes-Estévez E, López-Pedrera Ch, Barbarroja N. Impaired microRNA processing in neutrophils from Rheumatoid Arthritis patients confers their pathogenic profile. Modulation by biological therapies. Haematologica 2020 Jan 16. **IF: 7.57. Q1 (D1).**
4. Barbarroja N*, **Arias-de la Rosa I***, López-Medina C, Camacho-Sánchez MR, Gómez-García I, Vélez-García AJ, Escudero-Contreras A, López-Pedrera Ch, López-Montilla MD, Collantes-Estévez E. Cardiovascular risk factors in psoriatic disease: psoriasis vs psoriatic arthritis. Ther Adv Musculoskelet Dis 2019 Oct 16;11:1759720X19880742. **IF: 5.043. Q1.**
5. Pérez-Sánchez C*, Cecchi I*, Barbarroja N, Patiño-Trives AM, Luque-Tévar M, Pérez-Sánchez L, Ibáñez-Costa A, **Arias de la Rosa I**, Ortega R, Escudero A, Castro MC, Radin M, Roccatello D, Sciascia S, Aguirre MÁ, Collantes E, López-Pedrera C; BIOSAR Study Group. Early restoration of immune and vascular phenotypes in systemic lupus erythematosus and rheumatoid arthritis patients after B cell depletion. J Cell Mol Med. 2019 Sep;23(9):6308-6318. doi: 10.1111/jcmm.14517. **IF: 4.302. Q2.**
6. Ruiz-Limón P*, Ortega-Castro R*, Barbarroja N, Pérez-Sánchez C, Jamin C, Patiño-Trives A, Luque-Tévar M, Ibáñez-Costa A, Pérez-Sánchez L, **Arias-de la Rosa I**, Ábalos-Aguilera MC, Jiménez-Gómez Y, Calvo-Gutiérrez J, Font P, Escudero-Contreras A, Alarcón-Riquelme ME, Collantes-Estévez E, López-Pedrera Ch, PRECISESADS Clinical Consortium and Flow Cytometry Study Group. Front Immunol 2019 May 21;10:1111. doi: 10.3389/fimmu.2019.01111. **IF: 5.085. Q1.**
7. **Arias-de la Rosa I***, Radin M*, Cecchi I, Rubini E, Pérez-Sánchez C, Aguirre MÁ, Menegatti E, Barbarroja N, Collantes E, Sciascia S, Roccatello D, López-Pedrera Ch. Translational validation of the Global

Antiphospholipid Syndrome Score in patients with thrombotic APS. Rheumatology 2019 Oct 1;58(10):1870-1872. doi: 10.1093/rheumatology/kez166. **IF: 5.146. Q1.**

8. Cecchi I*, **Arias-de la Rosa I***, Menegatti E, Roccatello D, Collantes-Estévez E, López-Pedrera Ch, Barbarroja N. Neutrophils: novel key players in Rheumatoid Arthritis. Current and future therapeutic targets. Autoimmun Rev 2018 Nov;17(11):1138-1149. doi: 10.1016/j.autrev.2018.06.006. **IF: 7.716. Q1 (D1).**
9. **Arias-de la Rosa I***, Escudero-Contreras A*, Rodríguez-Cuenca S*, Ruiz-Ponce M, Jiménez-Gómez Y, Ruiz-Limón P, Pérez-Sánchez C, Ábalos MC, Cecchi I, Ortega R, Calvo J, Guzmán-Ruiz R, Malagón MM , Collantes E, Vidal-Puig A, López-Pedrera C, Barbarroja N. Defective glucose and lipid metabolism in rheumatoid arthritis is determined by chronic inflammation in metabolic tissues. J Intern Med 2018 Jul;284(1):61-77. doi: 10.1111/joim.12743. **IF: 6.051. Q1 (D1).**
10. Pérez-Sánchez C*, **Arias-de la Rosa I***, Aguirre MÁ, Luque-Tévar M, Ruiz-Limón P, Barbarroja N, Jiménez-Gómez Y, Ábalos-Aguilera MC, Collantes-Estévez E, Seguí P, Velasco F, Teresa-Herranz M, Hernández-Vidal MJ, Martínez C, González-Conejero R, Radin M, Siascia S, Cecchi I, Cuadrado MJ, López-Pedrera C. Circulating microRNAs as biomarkers of disease and typification of the atherothrombotic status in Antiphospholipid Syndrome Patients. Haematologica 2018 May;103(5):908-918. doi: 10.3324/haematol.2017.184416. **IF: 7.57. Q1 (D1).**
11. Pérez-Sánchez C*, Font-Ugalde P*, Ruiz-Limón P, López-Pedrera C, Castro-Villegas MC, Ábalos-Aguilera MC. Barbarroja N, **Arias-de la Rosa I**, López-Montilla MD, Escudero-Contreras A, López-Medina C, Collantes-Estévez E, Jiménez-Gómez Y. Circulating microRNAs as Potential Biomarkers of Disease Activity and Structural Damage in Ankylosing Spondylitis Patients. Hum Mol Genet. 2018 Mar 1;27(5):875-890. doi: 10.1093/hmg/ddy008. **IF: 4.544. Q1.**
12. Pérez-Sánchez C*, Aguirre MÁ*, Ruiz-Limón P, Ábalos-Aguilera MC, Jiménez-Gómez Y, **Arias-de la Rosa I**, Rodríguez-Ariza A, Fernández-del Río L, González-Reyes JA, Segui P, Collantes-Estévez E, Barbarroja N, Velasco F, Sciascia S, Cecchi I, Cuadrado MJ, Villalba JM, López-Pedrera C. Ubiquinol Effects on Antiphospholipid Syndrome Prothrombotic Profile: A Randomized, Placebo-Controlled Trial Arterioscler Thromb Vasc Biol. 2017 Oct;37(10):1923-1932. doi: 10.1161/ATVBAHA.117.309225. **IF: 6.086. Q1 (D1).**
13. Pérez-Sánchez C*, Ruiz-Limón P*, Aguirre MÁ, Jiménez-Gómez Y, **Arias-de la Rosa I**, Ábalos-Aguilera MC, Rodríguez-Ariza A, Castro-Villegas MC, Ortega R, Segui P, Martínez C, González-Conejero R, Rodríguez-López S, González-Reyes JA, Villalba JM, Collantes Estévez, E, Escudero A, Barbarroja N, López Pedrera, Ch. Diagnostic potential of NETosis-derived products for disease activity, atherosclerosis and therapeutic effectiveness in Rheumatoid Arthritis patients J

Autoimmun. 2017 Aug;82:31-40. doi: 10.1016/j.jaut.2017.04.007. **IF: 7.607. Q1 (D1).**

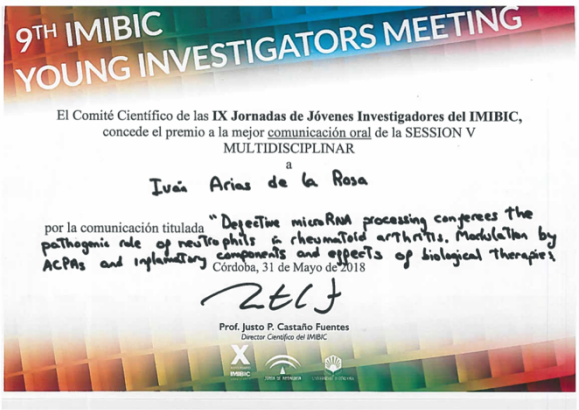
14. Ruiz-Limón P*, Ortega R*, **Arias-de la Rosa I**, Ábalos-Aguilera MC, Pérez-Sánchez C, Jiménez-Gómez Y, Peralbo-Santaella E, Font P, Ruiz-Vilches D, Ferrín G, Collantes-Estévez E, Escudero-Contreras A, López-Pedrera Ch, Barbarroja N. Tocilizumab improves the proatherothrombotic profile of rheumatoid arthritis patients modulating endothelial dysfunction, NETosis, and inflammation. Transl Res. 2017 May;183:87-103. doi: 10.1016/j.trsl.2016.12.003. **IF: 4.880. Q1 (D1).**
15. Pérez-Sánchez C*,Aguirre MÁ*, Ruiz-Limón P, Barbarroja N, Jiménez-Gómez Y, **Arias-de la Rosa I**, Rodríguez-Ariza A, Collantes-Estévez E, Segui P, Velasco F, Cuadrado MJ, Teruel R, González-Conejero R, Martínez C, López-Pedrera Ch. Atherothrombosis-associated microRNAs in Antiphospholipid syndrome and Systemic Lupus Erythematosus patients. Sci Rep. 2016 Aug 9;6:31375. doi: 10.1038/srep31375.**IF: 4.259. Q1.**

*Los autores comparten la primera posición.

III. Premios.

1. Premio a la mejor comunicación oral de la sección "Enfermedades crónicas e inflamatorias". VII Jornadas de Jóvenes Investigadores del IMIBIC (11 de Mayo de 2016). Comunicación titulada: "Potential impact of in vivo Ubiquinol supplementation in the prevention of atherothrombobis in Antiphospholipid Syndrome Patients. Preliminary results of a clinical trial".

2. Premio a la mejor comunicación oral de la sección "Multidisciplinar". IX Jornadas de Jóvenes Investigadores del IMIBIC (31 de Mayo de 2018). Comunicación titulada: "Defective microRNA procesing conferees the pathogenic role of neutrophils in rheumatoid arthritis. Modulation by ACPAs and inflammatory components and effects of biological therapies"



3. Premio de la Sociedad Española de Reumatología (SER) de Artritis Reumatoide 2019: premio al mejor trabajo presentado sobre cualquier aspecto de la artritis reumatoide publicado o aceptado para su publicación en el año 2018 a: "Neutrophils: Novel key players in rheumatoid arthritis. Current and future therapeutic targets".

Sociedad Española de Reumatología

PREMIO SER
DE ARTRITIS REUMATOIDE

2019

El Comité Evaluador del Premio
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el premio al mejor trabajo presentado sobre cualquier aspecto de la ***artritis reumatoide*** **publicado o aceptado para su publicación en el año 2018 a:**

"Neutrophils: Novel key players in rheumatoid arthritis. Current and future therapeutic targets"

Cuyos autores son: Irene Cecchia, Iván Arias de la Rosab, Elisa Menegattia, Dario Roccatelloa, Eduardo Collantes-Estevez, Chary López-Pedrera y Nuria Barbarroja

Sociedad Española de Reumatología

4. Premio de la Sociedad Española de Reumatología (SER) de Ciencia Básica 2019: premio al mejor trabajo presentado sobre cualquier aspecto de ciencia básica publicado en el año 2018 a: "Defective glucose and lipid metabolism in rheumatoid arthritis is determined by chronic inflammation in metabolic tissues".

Sociedad Española de Reumatología

PREMIO SER
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el premio al mejor trabajo presentado sobre cualquier aspecto de ***ciencia básica*** **publicado o aceptado para su publicación en el año 2018 a:**

"Defective glucose and lipid metabolism in rheumatoid arthritis is determined by chronic inflammation in metabolic tissues"

Cuyos autores son: I. Arias de la Rosa, A. Escudero-Contreras, S. Rodríguez-Cuenca, M. Ruiz-Ponce, Y. Jiménez-Gómez, P. Ruiz-Limón, C. Pérez-Sánchez, M. C. Ábalos-Aguilera, I. Cecchi, R. Ortega, J. Calvo, R. Guzmán-Ruiz, M. M. Malagón, E. Collantes-Estevez, A. Vidal-Puig, Ch. López-Pedrera y N. Barbarroja

Sociedad Española de Reumatología

5. Premio de la Sociedad Española de Reumatología (SER) de otras enfermedades autoinmunes sistémicas 2019: premio al mejor trabajo presentado sobre cualquier aspecto de otras Enfermedades Autoinmunes Sistémicas publicado en el año 2018 a: "Circulating microRNAs as biomarkers of disease and typification of the atherothrombotic status in antiphospholipid syndrome".

Sociedad Española de Reumatología

PREMIO SER
OTRAS ENFERMEDADES AUTOINMUNES SISTÉMICAS

2019

El Comité Evaluador del Premio SER 2019 de otras Enfermedades Autoinmunes Sistémicas (EAS)

otorga

el premio al mejor trabajo presentado sobre cualquier aspecto de ***otras Enfermedades Autoinmunes Sistémicas*** **publicado** o **aceptado para su publicación en el año 2018 a:**

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

Cuyos autores son: Carlos Pérez-Sánchez, Iván Arias-de la Rosa, María Ángeles Aguirre, María Luque-Tévar, Patricia Ruiz-Limón, Nuria Barbarroja, Yolanda Jiménez-Gómez, María Carmen Ábalos-Aguilera, Eduardo Collantes-Estévez, Pedro Seguí, Francisco Velasco, María Teresa Herranz, Jesús Lozano-Herrero, María Julia Hernandez-Vidal, Constantino Martínez, Rocío González-Conejero, Massimo Radin, Savino Sciascia, Irene Cecchi, María José Cuadrado y Chary López-Pedrera

Sociedad Española de Reumatología

6. Premio de la Sociedad Española de Reumatología (SER) de espondiloartritis 2019: premio al mejor trabajo presentado sobre cualquier aspecto de la espondiloartritis publicado en el año 2018 a: "Circulating microRNAs as potential biomarkers of disease activity and structural damage in ankylosing spondylitis patients".

Sociedad Española de Reumatología

PREMIO SER
DE ESPONDILOARTRITIS

2019

El Comité Evaluador del Premio SER 2019 de espondiloartritis

otorga

el premio al mejor trabajo presentado sobre cualquier aspecto de la ***espondiloartritis*** **publicado** o **aceptado para su publicación en el año 2018 a:**

"Circulating microRNAs as potential biomarkers of disease activity and structural damage in ankylosing spondylitis patients"

Cuyos autores son: Carlos Pérez-Sánchez, Pilar Font-Ugalde, Patricia Ruiz-Limon, Chary López-Pedrera, María C. Castro-Villegas, María C. Ábalos-Aguilera, Nuria Barbarroja, Iván Arias-de la Rosa, María D. López-Montilla, Alejandro Escudero-Contreras, Clementina López-Medina, Eduardo Collantes-Estévez y Yolanda Jiménez-Gómez

Sociedad Española de Reumatología

6. XVII Premio Nacional de Investigación Ilustre Colegio de Médicos de Córdoba, patrocinado por CaixaBank: el III Premio al trabajo: "Defective glucose and lipid metabolism in rheumatoid arthritis is determined by chronic inflammation in metabolic tissues"


ILUSTRE COLEGIO OFICIAL DE MÉDICOS DE CÓRDOBA

CaixaBank

EL TRIBUNAL CALIFICADOR DEL XVII PREMIO NACIONAL DE INVESTIGACIÓN ILUSTRE COLEGIO DE MÉDICOS DE CÓRDOBA, PATROCINADO POR CAIXABANK

Acuerda por unanimidad, conceder el III PREMIO al trabajo

Defective glucose and lipid metabolism in rheumatoid arthritis is determined by chronic inflammation in metabolic tissues

Siendo sus autores

I. Arias de la Rosa, A. Escudero -Contreras, S. Rodriguez-Cuenca, M. Ruiz-Ponce, Y. Jiménez-Gómez, P. Ruiz-Limón, C. Pérez-Sánchez, M.C. Abalos-Aguilera, I. Cecchi, R. Ortega, J. Calvo, R. Guzmán-Ruiz, M.M. Malagón, E. Collantes-Estévez, A. Vidal-Puig, Ch. López-Pedrera, N. Barbarroja

Publicado en

Journal of Internal Medicine, 2018, 284; 61-77

Córdoba, 28 de noviembre de 2019

El Presidente del Colegio de Médicos de Córdoba

Fdo: Dr. Bernabé Galán Sánchez

El Presidente del Jurado

Fdo: Dr. Pablo Pérez Martínez

7. XVIII Premio de Investigación "Gonzalo Miño Fugarolas" por el trabajo titulado: "Neutrophils: Novel key players in rheumatoid arthritis. Current and future therapuetic targets".


UNIVERSIDAD D CORDOBA

El Jurado del XVIII Premio de Investigación *"Gonzalo Miño Fugarolas"* reunido en Córdoba el día 19 de diciembre de 2019, ha acordado su concesión ex "aequo" a

D. Iván Arias de la Rosa

por el trabajo titulado

"Neutrophils: Novel key players in rheumatoid arthitis. Current and future therapeutic targets".

EL RECTOR

José Carlos Gómez Villamandos

Córdoba, 3 de febrero de 2019