Universidad de Oviedo

Programa de Doctorado en Biomedicina y Oncología Molecular

BIOMARCADORES DE DAÑO ENDOTELIAL Y RIESGO CARDIOVASCULAR EN PACIENTES DE ARTRITIS REUMATOIDE

Tesis Doctoral

Javier Rodríguez Carrio

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RESUMEN DEL CONTENIDO DE TESIS DOCTORAL

1.- Título de la Tesis	
Español/Otro Idioma: *Biomarcadores de daño endotelial y riesgo cardiovascular en pacientes de Artritis Reumatoide*	Inglés: *Biomarkers of endothelial damage and cardiovascular risk in Rheumatoid Arthritis patients*

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RESUMEN (en español)

La artritis reumatoide (AR) es una enfermedad inflamatoria crónica asociada con elevada mortalidad y morbilidad cardiovascular (CV). Aunque los factores clásicos son relevantes, éstos no explican la totalidad del riesgo incrementado, por lo que se sospecha que la inflamación crónica y la disregulación inmunitaria son determinantes, aunque los mecanismos exactos no se conocen. En esta Tesis Doctoral se aborda el análisis de esta situación a diferentes niveles, con el objetivo de identificar mediadores que puedan jugar un papel en los procesos de daño y reparación endotelial, así como potenciales biomarcadores de interés para el ámbito clínico.

Se observó que el IFNα, incrementado en un grupo de pacientes de AR, se asoció con una maduración prematura de las células progenitoras endoteliales (EPC), mayores niveles séricos de citocinas proinflamatorias, parámetros clínicos de severidad así como con una mayor prevalencia de eventos CV. Además, se observó una depleción de células T angiogénicas (Tang) asociada a la actividad de la enfermedad y a los niveles séricos de IFNα, que era más pronunciada en pacientes con enfermedad CV. En conjunto, estos resultados sugieren un fallo a distintos niveles de los mecanismos de reparación endotelial en AR.

Por otro lado, el análisis de distintas subpoblaciones de MP circulantes permitió observar que los pacientes de AR exhibían un perfil de MP alterado cuantitativa y cualitativamente, asociado a factores tanto clásicos como específicos de la enfermedad, y que refleja una situación de daño endotelial y de fallo de reparación vascular. Estudios in vitro demostraron que las MP aisladas de pacientes de AR, pero no de individuos controles o con factores clásicos de riesgo, desencadenaban efectos deletéreos sobre células endoteliales, presumiblemente debido a un fenómeno de activación endotelial.

Por otra parte, se estudió la utilidad de la amplitud de distribución eritrocitaria (RDW), un parámetro clínico clásico del hemograma, como biomarcador pronóstico de enfermedad CV. Se observó que tanto el valor de RDW al diagnóstico como el acumulado durante el primer año de evolución son predictores de enfermedad CV. Además, un estudio transversal mostró que el

RDW se asociaba de forma independiente con una baja frecuencia de EPC, así como niveles elevados de IL-8 en pacientes con una duración de la enfermedad mayor de un año, pero no en pacientes al inicio. El hecho de que el RDW permita predecir el desarrollo de eventos CV aun cuando no se detectan alteraciones en los mediadores implicados en procesos de remodelado vascular, lleva a pensar que éste puede ser considerado un biomarcador temprano de riesgo CV en AR. Asimismo, el análisis de los anticuerpos anti-HDL IgG avaló su potencial como biomarcador de riesgo CV. Los anticuerpos anti-HDL, incrementados en AR en comparación con la población control y de forma independiente a los factores clásicos, se asociaron con un perfil lipídico alterado y un ambiente sérico proinflamatorio, en línea con la mayor frecuencia de enfermedad CV.

Finalmente, se realizó un estudio prospectivo para analizar el efecto de la terapia con bloqueantes del TNFα sobre estos biomarcadores. La respuesta clínica a este tratamiento se asoció con una reducción de la población $CD4^{+}CD28^{null}$ y una recuperación de células Tang, además de con una menor liberación de MP derivadas de Tang. Igualmente, los anticuerpos anti-HDL se asociaron de forma independiente a un perfil lipídico más favorable tras el tratamiento.

En conjunto, los resultados de esta Tesis Doctoral proponen varios biomarcadores con posible utilidad clínica para evaluar tanto el daño exacerbado como la reparación endotelial defectiva que subyacen a la patología CV en AR, y que además explican su asociación con la actividad y la duración de la enfermedad. Asimismo, apoyan la existencia de un riesgo CV incrementado al diagnóstico que puede ser aminorado, en cierto grado, cuando hay una buena respuesta al tratamiento.

RESUMEN (en Inglés)

Rheumatoid arthritis (RA) is an inflammatory chronic disease associated with increased cardiovascular (CV) mortality and morbidity. Although traditional CV risk factors are important, they cannot account for the increased CV risk, so chronic inflammation and immune dysregulation are thought to have a pivotal role, but the actual mechanisms are still unclear. This situation is addressed in the present thesis, with the main goal of the identification of both mediators which can play a role in the endothelial damage and repair mechanisms, as well as potential biomarkers for the clinical setting.

First, IFNα was found increased in a subset of RA patients, related to an endothelial progenitor cells (EPC) imbalance, increased proinflammatory cytokine serum levels, clinical parameters of severity and increased prevalence of CV disease (CVD). In addition, a depletion of angiogenic T cells (Tang) was observed in RA in association with disease activity and also IFNα serum levels. Interestingly, Tang subset was decreased to a higher degree in patients with CVD. Overall, these findings point to generalized vascular repair impairment in RA.

Analysis of circulating microparticles (MP) showed that RA patients exhibited a quantitative and qualitative altered MP profile which is the result of both disease-specific and traditional CV risk factors and suggests increased endothelial damage and impaired Tang-mediated endothelial repair. Moreover, RA-derived MP, but not those from healthy controls or individuals with marked traditional CV risk factors exhibited detrimental effects on endothelial cells in vitro, presumably linked to the promotion of endothelial activation.

On the other hand, the prognostic value of RDW, a classical parameter of anisocytosis, as CV risk biomarker was analyzed. Both RDW at diagnosis and 1-year cumulative RDW predicted the occurrence of CV events in RA patients. An increase in RDW during the first year also predicted reduced CV-free survival during the follow up. Further analyses in a cross-sectional cohort of RA patents revealed that RDW was independently associated with an EPC depletion and IL-8 serum levels in patients with a disease duration longer than 1 year, but not in their early counterparts. Therefore, the fact that RDW can predict CVD occurrence even before than any alteration in mediators involved in vascular remodeling became apparent, lead us to think that RDW could be considered as a very early biomarker of CV risk in RA. Similarly, the findings concerning IgG anti-HDL antibodies supported its promising use as biomarkers. Anti-HDL antibodies were increased in RA patients compared to control populations, regardless of traditional CV risk factors, and they exhibited interesting associations with blood lipid profiles and proinflammatory mediators, which could underlie the increased rate of CVD in patients with the highest anti-HDL levels.

Finally, the effect of TNFα blockade on these biomarkers was assessed in a prospective group of TNFα-naïve RA patients. Clinical response upon TNFα blockade was related to decreased $CD4^{+}CD28^{null}$, Tang recovery and lower Tang-MP shedding. Furthermore, decreasing IgG anti-HDL levels could mediate, at least in part, the beneficial effect of TNFα blockade on serum lipid profile.

Overall, the results herein presented provide insight on new biomarkers with potential interest for the clinical setting not only to assess the increased endothelial damage as well as the impaired endothelial repair underlying CVD in RA, but also because they can be the missing link between disease activity and duration and CVD development. Moreover, these results support the increased CV risk at RA onset linked to an immune dysregulation which could be counteracted, to certain degree, provided that a satisfactory clinical response is achieved.

SR. PRESIDENTE DE LA COMISIÓN ACADÉMICA DEL PROGRAMA DE DOCTORADO EN BIOMEDICINA Y ONCOLOGÍA MOLECULAR

Resumen

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Abstract

Rheumatoid arthritis (RA) is an inflammatory chronic disease associated with increased cardiovascular (CV) mortality and morbidity. Although traditional CV risk factors are important, they cannot account for the increased CV risk, so chronic inflammation and immune dysregulation are thought to have a pivotal role, but the actual mechanisms are still unclear. This situation is addressed in the present thesis, with the main goal of the identification of both mediators which can play a role in the endothelial damage and repair mechanisms, as well as potential biomarkers for the clinical setting.

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On the other hand, the prognostic value of RDW, a classical parameter of anisocytosis, as CV risk biomarker was analyzed. Both RDW at diagnosis and 1-year cumulative RDW predicted the occurrence of CV events in RA patients. An increase in RDW during the first year also predicted reduced CV-free survival during the follow up. Further analyses in a cross-sectional cohort of RA patents revealed that RDW was independently associated with an EPC depletion and IL-8 serum levels in patients with a disease duration longer than 1 year, but not in their early counterparts. Therefore, the fact that RDW can predict CVD occurrence even before than any alteration in mediators involved in vascular remodeling became apparent, lead us to think that RDW could be considered as a very early biomarker of CV risk in RA. Similarly, the findings concerning IgG anti-HDL antibodies supported its promising use as biomarkers. Anti-HDL antibodies were increased in RA patients compared to control populations, regardless of traditional CV risk factors, and they exhibited interesting associations with blood lipid profiles and

proinflammatory mediators, which could underlie the increased rate of CVD in patients with the highest anti-HDL levels.

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Índice

Abreviaturas

ACL: anticuerpo anticardiolipina
ACR: *American College of Rheumatology* (Colegio Americano de Reumatología)
ADN: ácido desoxirribonucleico
AL: anticoagulante lúpico
ANA: anticuerpos antinucleares
Apo: apolipoproteína
AR: artritis reumatoide
ARN: ácido ribonucleico
α-CCP: *anti-cyclic cittrullinated peptide antibody* (anticuerpo anti-péptido cíclico citrulinado)

BMI: *body mass index* (índice de masa corporal)
BSA: *bovine serum albumin* (albúmina sérica bovina)

CBA: *cytometric bead array* (ensayo citométrico de partículas)
CD: *cluster of differentation* (clúster de diferenciación)
CE: célula endotelial
CTLA4: *cytotoxic T lymphocyte associated antigen 4* (antígeno 4 asociado a linfocitos T citotóxicos)
CV: cardiovascular
CXCR4: *C-X-C chemokine receptor type 4* (receptor 4 de quimiocinas tipo C-X-C)

DAS: *disease activity index* (índice de actividad de la enfermedad)
DM: diabetes mellitus

EPC: *endotelial progenitor cell* (célula progenitora endotelial)
ELISA: *enzyme-linked immunoassay* (inmunoensayo ligado a enzima)
EULAR: *European League Against Rheumatism* (liga europea contra los reumatismos)

FAME: fármacos modificadores de la enfermedad
FCS: *fetal calf serum* (suero de ternera fetal)
Foxp3: forkhead box protein 3
FR: factor reumatoide

GC: glucocorticoides
GM-CSF: *granulocyte-macrophage colony-stimulating factor* (factor estimulante de colonias de granulocitos y monocitos)

HDL: *high density lipoprotein* (lipoproteína de alta densidad)
HTA: hipertensión

IFN: interferón
Ig: inmunoglobulina
IL: interleucina

LDL: *low density lipoprotein* (lipoproteína de baja densidad)
LES: lupus eritematoso sistémico

MCP1: *monocyte chemoattractant protein 1* (proteína quimioatrayente de monocitos 1)
mEPC: *mature EPC* (EPC madura)
MFI: *mean fluorescence intensity* (intensidad de fluorescencia media)
MHC: *major histocompatiblity complex* (complejo principal de histocompatibilidad)
MIP1α: *macrophage inflammatory protein 1 alpha* (proteína inflamatoria derivada de macrófagos 1 alfa)
MP: micropartícula
MTX: metotrexato

NK: célula natural killer
NO: *nitric oxide* (óxido nitrico)

PBS: *phosphate buffer saline* (solución tampón fosfato)
PCR: proteína C reactiva

RDW: *red cell distribution width* (amplitud de distribución eritrocitaria)

SNP: *single nucleotide polymorphism*

Tang: célula T angiogénica
TBS: *tris buffer saline* (solución tampón tris)
TCR: *T cell receptor* (receptor de células T)
TGFβ: *transforming growth factor β* (factor de crecimiento transformante β)
Th: linfocito T helper
TLR: *toll like receptor* (receptor tipo Toll)
TNFα: *tumour necrosis factor alpha* (factor de necrosis tumoral alfa)
Treg: células T reguladoras

VEGF: *vascular endotelial growth factor* (factor de crecimiento del endotelio vascular)
VEGFR: *vascular endotelial growth factor receptor* (receptor del factor de crecimiento del endotelio vascular)
VSG: velocidad de sedimentación globular

NOTA: las abreviaturas de algunas expresiones se han mantenido en su forma inglesa por ser ésta la más habitual y conocida. Se incluyen otras abreviaturas más específicas en cada uno de los artículos que componen la presente memoria.

Introducción

1. Introducción

1.1. Artritis reumatoide

La artritis reumatoide (AR) es una enfermedad autoinmune inflamatoria crónica, cuya etiología no está totalmente clara en la actualidad. A nivel articular, se caracteriza por una sinovitis persistente acompañada de hiperplasia sinovial con invasión hacia los tejidos adyacentes (hueso, cartílago y ligamentos). A nivel sistémico, la AR se caracteriza por la presencia de niveles elevados de reactantes de fase aguda (proteína C reactiva y velocidad de sedimentación globular), producción de autoanticuerpos y desarrollo de algunas manifestaciones extraarticulares (Klareskog *et al*, 2009;McInnes & Schett, 2011).

La AR es la enfermedad reumática más frecuente, oscilando su prevalencia mundial entre el 0,5 y el 1,0% (Spector, 1990), situándose en un 0,5 en España (Carmona *et al*, 2002), con una incidencia de 20 – 50 individuos por cada 100.000 casos al año (Carmona *et al*, 2010). Es más habitual en el género femenino que en el masculino, con una razón 3:1, y suele aparecer en la cuarta o quinta década de la vida.

1.1.1. Etiopatogénesis

La AR es una enfermedad multifactorial, de etiología muy compleja en la que intervienen una combinación de factores genéticos, hormonales y ambientales, que desencadenan la alteración de la respuesta inmunitaria, caracterizada por una pérdida de la tolerancia frente a lo propio. Sin embargo, los mecanismos exactos que juegan un papel en este proceso, no están del todo claros.

Diferentes estudios han puesto de manifiesto el carácter poligénico de la AR, con un componente genético que confiere una heredabilidad del 60% (Viatte *et al*, 2013). Los loci más fuertemente asociados a la susceptibilidad a AR se encuentran en la región del Complejo Principal de Histocompatibilidad (MHC), y explican hasta el 30% del total de la susceptibilidad genética a la AR, siendo el locus *HLA-DRB1* el más estudiado (Viatte *et al*, 2015). Sin embargo, un buen número de loci fuera de la región MHC, como son *PTPN22*, *STAT4*, *TRAF1/C5* y *CTLA4* entre otros, han sido ampliamente documentados por su asociación con la susceptibilidad a la AR, así como a su pronóstico y diferentes manifestaciones clínicas de la enfermedad.

Sin embargo, el sustrato genético de la enfermedad no es una condición suficiente para el desarrollo de las manifestaciones clínicas, sino que se requiere una interacción entre los factores genéticos y factores ambientales de diversa índole, como el tabaquismo, agentes infecciosos o la obesidad, que actúan como desencadenantes de las respuestas de tipo autoinmune (Klareskog *et al*, 2009).

Resulta interesante que muchos autores han reportado la presencia de niveles elevados de autoanticuerpos (Brink *et al*, 2015;Rantapaa-Dahlqvist *et al*, 2003), reactantes de fase aguda e incluso citocinas proinflamatorias (Deane *et al*, 2010;Jorgensen *et al*, 2008;Kokkonen *et al*, 2010;Rantapaa-Dahlqvist *et al*, 2003) en el suero varios años antes del inicio de los síntomas de la AR, si bien la composición celular del tejido diana de la enfermedad, esto es, la membrana sinovial, no presenta cambios drásticos en su composición incluso en las primeras fases de la enfermedad. De hecho, un artículo reciente muestra únicamente una ligera infiltración de células T en la membrana sinovial en pacientes con AR de reciente comienzo (de Hair *et al*, 2014). Por tanto, parece claro que la autoinmunidad sistémica precede en el tiempo a la aparición de la inflamación sinovial durante el desarrollo de AR (van de Sande *et al*, 2011).

En este escenario, recientemente se ha sugerido que existen alteraciones tempranas en los ganglios linfáticos que pueden tener un papel crucial en la iniciación y promoción de las reacciones autoinmunes y, por tanto, en el desarrollo de la AR (van Baarsen *et al*, 2013). De hecho, modelos animales avalan el papel de los ganglios linfáticos como escenario iniciador de la enfermedad (Li *et al*, 2010;Rodriguez-Palmero *et al*, 1999).

Si bien los estímulos que llevan al desarrollo de las respuestas autoinmunes y su localización no están del todo claros, menos conocidos aún resultan los mecanismos que conducen desde el desarrollo de autoinmunidad a nivel sistémico hasta la aparición de inflamación local a nivel articular. En este aspecto, se hipotetiza que pueden ser determinantes la formación o exposición de nuevos autoantígenos, debido a cambios en el metabolismo celular o en la microvasculatura, factores biomecánicos o traumas (McInnes & Schett, 2011).

En la fase clínica de la enfermedad, la sinovitis es iniciada y mantenida debido a la concurrencia de mecanismos patológicos de retroalimentación positiva que coexisten con fallos en los mecanismos de regulación, involucrando a diferentes agentes de la inmunidad innata y adaptativa así como a tipos celulares locales, como osteoclastos, condrocitos, fibroblastos y células endoteliales (Figura 1).

La sinovitis se desencadena inicialmente por la migración y acumulación de diferentes poblaciones leucocitarias en la membrana sinovial, en un proceso mediado por la activación endotelial de las células que componen la microvasculatura local (Szekanecz *et al*, 2009). La infiltración leucocitaria se ve favorecida por la neoangiogénesis, desencadenada por las condiciones hipóxicas y la producción de factores de crecimiento, así como por una linfoangiogénesis insuficiente, que limita el egreso celular (Polzer *et al*, 2008).

Los linfocitos T son una de las poblaciones celulares más abundantes en la membrana sinovial durante la AR, llegando a suponer el 30 – 50% de las células del tejido, siendo mayoritaria la subpoblación $CD4^+$ (Zvaifler *et al*, 1994). Históricamente, la AR fue considerada una enfermedad de tipo Th1. Sin embargo, se sabe que en las etapas tempranas la respuesta Th2 es crucial para la producción de autoanticuerpos (Raza *et al*, 2005). Asimismo, en los últimos años, se ha establecido y reforzado la contribución del papel de la respuesta Th17, especialmente en las fases más tardías de la enfermedad y en asociación a los procesos de erosión y daño articular (Chabaud *et al*, 1998;Miossec *et al*, 2009). Por otro lado, existe una fuerte infiltración de células T reguladoras en la membrana sinovial, si bien su funcionalidad parece estar comprometida en pacientes (Behrens *et al*, 2007), posiblemente debido a la acumulación de TNFα (Nie *et al*, 2013).

De forma global, los trabajos publicados hasta la fecha parecen sugerir un compromiso funcional de las células T, ligada a una excesiva actividad proliferativa, que tiene como consecuencia la reducción del repertorio del receptor de células T (TCR) y la aparición, como consecuencia, de un fenotipo inmunosenescente (Thewissen *et al*, 2005;Weyand *et al*, 2003).

Los linfocitos B juegan un papel importante en la patogénesis de la AR. No en vano, la presencia de autoanticuerpos es un marcador de la enfermedad. Las células B en la membrana sinovial se localizan en agregados de linfocitos T y B, y en estructuras linfoides ectópicas en las fases más tardías de la enfermedad; mientras que los plasmablastos y las células plasmáticas tienen una distribución más ubicua (McInnes & Schett, 2011). Prueba de la contribución de las respuestas mediadas por células B a la patogénesis de la enfermedad es el beneficio clínico del tratamiento con rituximab (anticuerpo monoclonal anti-CD20) en pacientes con AR (Edwards *et al*, 2004). Sin embargo, el hecho de que las células plasmáticas no sean atacadas por el rituximab, unido a que los niveles de autoanticuerpos resulten afectados en un grado variable tras el tratamiento, hace pensar que la contribución de las células B en la enfermedad va más allá de la producción de autoanticuerpos,

otorgando un papel igualmente importante a la presentación antigénica y a la producción de citocinas y otros factores solubles (Seyler *et al*, 2005).

Del mismo modo, otras poblaciones celulares de la respuesta inmune innata juegan un papel importante en la patogénesis de la AR, siendo especialmente relevante el de los macrófagos y los neutrófilos. En particular, los neutrófilos contribuyen a la sinovitis mediante la producción de prostaglandinas, proteasas, enzimas, especies reactivas de oxígeno y algunos tipos de citocinas (Cascao *et al*, 2010). Algunas de estas moléculas, junto con la señalización a través de los receptores tipo Toll (TLR), la presencia de inmunocomplejos y el contacto con células T, llevan a la activación de los macrófagos. Estas células son unos agentes centrales en el desarrollo de la sinovitis debido a su producción de citocinas (TNFα, IL-1, IL-6, IL-12, IL-15, IL18 e IL-23), especies reactivas de oxígeno, derivados prostanoides y enzimas proteolíticas, así como a su capacidad para llevar a cabo la fagocitosis y presentación antigénica (Liew & McInnes, 2002).

La producción descontrolada y autoperpetuada de citocinas proinflamatorias en la membrana sinovial desencadena la activación de macrófagos, condrocitos y fibroblastos (tipo sinoviocitos)(Lefevre *et al*, 2009), con la consiguiente producción de enzimas que provocan la degradación del colágeno y la matriz extracelular, como las metaloproteasas de matriz (MMP) y las proteasas de la familia ADAMTS (*a disintegrin and metalloproteinase with thrombospondin motifs*), iniciándose así el daño articular (Karouzakis *et al*, 2006).

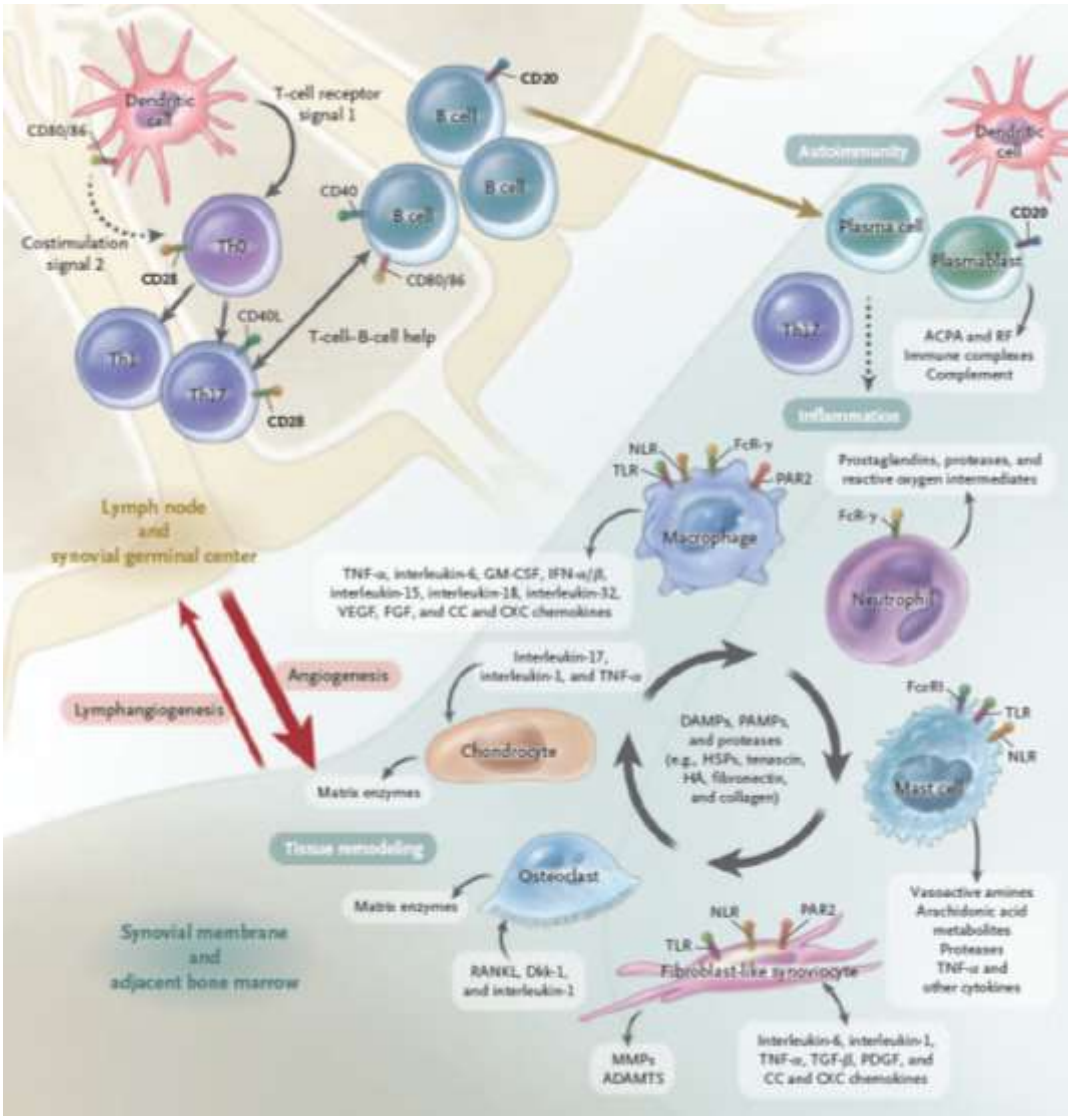

Figura 1 | Modelo integrativo de iniciación y perpetuación de la patogénesis de la AR. La activación de clones autorreactivos en el ganglio linfático precede al egreso de éstos al tejido sinovial, donde la interacción entre diferentes procesos inmunitarios de tipo innato y adaptativo da lugar a fenómenos de remodelado tisular y daño. La activación de bucles de retroalimentación positiva entre diferentes poblaciones leucocitarias, fibroblastos locales, osteoclastos, condrocitos y células endoteliales, da lugar a la perpetuación del daño y, con ello, la cronicidad de la enfermedad. Tomado de (McInnes & Schett, 2011).

1.1.2. Manifestaciones clínicas

La AR se puede definir como una poliartritis crónica, simétrica, erosiva y destructiva. Afecta predominantemente a las articulaciones diartrodiales pequeñas de manos (metacarpofalángicas y carpos, principalmente) y pies (metatarsofalángicas), si bien con la evolución de la enfermedad se pueden ir afectando grandes articulaciones (rodillas, hombros, codos y caderas) y articulaciones cervicales y temporo-mandibulares, siempre de forma simétrica. No obstante, las articulaciones interfalángicas distales, y las de la columna dorsal y lumbar suelen mantenerse intactas. En un pequeño número de pacientes puede

tener lugar un debut clínico con presentación oligoarticular y asimétrica (Gomez-Reino, 2008).

Por otro lado, se conoce que la AR puede cursar con manifestaciones extraarticulares, si bien en la mayor parte de los casos no tienen una gran importancia clínica (Gomez-Reino, 2008). Las más comunes son:

- Nódulos reumatoides: son la manifestación extraarticular más común (presentes en el 10 – 30% de los pacientes). Pueden aparecer en cualquier órgano, si bien se localizan preferentemente en zonas periarticulares y expuestas a presiones mecánicas.
- Manifestaciones oculares
- Manifestaciones pleuropulmonares
- Manifestaciones cardiacas
- Vasculitis reumatoide
- Amiloidosis
- Síndrome de Felty

1.1.3. Diagnóstico

El diagnóstico de AR es esencialmente clínico. Durante las últimas décadas, este diagnóstico se realizaba en base a los criterios propuestos en 1987 por el American College of Rheumatology (Tabla 1) (Arnett *et al*, 1988), que mostraban unos valores aceptables de especificidad y sensibilidad (Arnett *et al*, 1988;Hakala *et al*, 1993;Levin *et al*, 1996), especialmente en fases establecidas de la enfermedad .

Tabla 1: Criterios del ACR 1987 para la clasificación de la AR

- Rigidez articular prolongada tras la inactividad
  Rigidez matutina en y alrededor de las articulaciones, durante al menos una hora antes de la mejoría máxima.
- Artritis en tres o más articulaciones
  Afectación poliarticular en, al menos, tres áreas de forma simultánea, con hinchazón de tejidos blandos o líquido sinovial. Las posibles áreas articulares contempladas son interfalángicas proximales, metacarpofalángicas, metatarsofalángicas, muñecas, codos, rodillas y tobillos.
- Afectación de las articulaciones de las manos
  Inflamación en, al menos, un área en articulaciones de muñeca, interfalángicas proximales, metacarpofalángicas o muñecas.
- Artritis simétrica
  Implicación simultánea de las mismas áreas en ambos lados del cuerpo.
- Nódulos reumatoideos
  Presencia de nódulos subcutáneos, sobre prominencias óseas, en zonas de extensores o en regiones yuxtaarticulares.
- Factor reumatoide positivo
  Niveles elevados de Factor Reumatoide (FR) en suero.
- Cambios radiológicos
  Alteraciones radiológicas típicas de la AR (erosiones y osteoporosis yuxtaarticular) en radiografías posteroanteriores de mano y muñeca.

Según los criterios anteriores, se considera un diagnóstico positivo de AR cuando se cumplen, al menos, 4 de los 7 criterios. Sin embargo, aunque esta clasificación fue bien aceptada como un punto de partida para la definición de la enfermedad y para discriminar pacientes de AR establecida de aquellos que padecían otras enfermedades reumáticas, estos criterios no resultaban útiles para el diagnóstico en las fases tempranas de la enfermedad (Banal *et al*, 2009;Harrison *et al*, 1998). En estudios longitudinales de pacientes con artritis de reciente comienzo, se ha demostrado que el número de criterios que se cumplen aumenta con la duración del seguimiento y que no todos ellos se comportan igual (Saraux *et al*, 2001). Asimismo, estos criterios no contemplaban la presencia de anticuerpos anti-péptido cíclico citrulinado (anti-CCP), cuya positividad confiere una aceptable sensibilidad y elevada especificidad al diagnóstico de AR (Nishimura *et al*, 2007;Zendman *et al*, 2006), ni tenían en cuenta la elevación de reactantes de fase aguda como marcadores de la enfermedad. Por todo ello, el ACR publicó en 2010 los nuevos criterios de clasificación de AR (Tabla 2), que proporcionan mayor sensibilidad para las fases precoces de la AR (Aletaha *et al*, 2010).

Estos criterios contemplan 4 dominios con distintas variables clínicas y de laboratorio, con diferente importancia relativa en cada una de las categorías que los integran.

Tabla 2: Criterios de ACR/EULAR 2010 para la clasificación de la AR

		Puntuación
A.	**Afectación articular**	
	1 articulación grande	0
	2 – 10 articulaciones grandes	1
	1 – 3 articulaciones pequeñas (con/sin afectación de articulaciones grandes)	2
	4 – 10 articulaciones pequeñas (con/sin afectación de articulaciones grandes)	3
	> 10 articulaciones (al menos una articulación pequeña)	5
B.	**Serología**	
	FR y anti-CCP negativos	0
	FR o anti-CCP positivos a niveles bajos	2
	FR o anti-CCP positivos a niveles altos	3
C.	**Reactantes de fase aguda**	
	Proteína C reactiva y velocidad de sedimentación normales	0
	Proteína C reactiva o velocidad de sedimentación alterados	1
D.	**Duración de los síntomas**	
	< 6 semanas	0
	≥ 6 semanas	1

A partir de estos criterios, se clasifica una enfermedad como AR definida si se presenta sinovitis en, al menos, una articulación en ausencia de un diagnóstico que lo justifique y una puntuación de 6, sobre un total de 10, en los cuatro dominios contemplados.

Factor Reumatoide

El Factor Reumatoide (FR) es un autoanticuerpo de isotipo IgM (aunque también se han descrito isotipos IgG e IgA) dirigido contra la fracción Fc de las moléculas de IgG. El FR IgM proporciona una sensibilidad diagnóstica del 40 – 80%, dependiendo de la población cribada (Greiner *et al*, 2005;Renaudineau *et al*, 2005). En pacientes con artritis del ámbito hospitalario, proporciona un valor predictivo positivo del 70 – 80% y negativo de más del 95% (Wolfe *et al*, 1991;Wolfe, 1998). Asimismo, algunos autores sugieren un valor pronóstico para el FR, ya que se asocia a enfermedad más grave, con más extensión del compromiso articular, con mayores tasas de destrucción y discapacidad (Scott, 2000).

El FR puede aparecer en el suero varios años antes de que se presenten los síntomas de la artritis (Aho *et al*, 1991;Rantapaa-Dahlqvist *et al*, 2003).

Anticuerpos anti-péptidos cíclicos citrulinados

Los anticuerpos anti-péptidos cíclicos citrulinados (anti-CCP) o anti-proteínas citrulinadas (ACPA, siguiendo la terminología actual), reconocen proteínas con residuos de citrulina, que constituye una modificación postraduccional de la arginina, llevada a cabo por el enzima peptidoarginil deaminasa (van Venrooij *et al*, 2004) en diferentes situaciones patológicas.

La sensibilidad de los anticuerpos anti-CCP oscila entre el 12 – 93%, proporcionando una especificidad aceptablemente alta (63 – 100%), comparada con la proporcionada por el FR (Nishimura *et al*, 2007;Whiting *et al*, 2010). Además, el hecho de que alrededor del 40% de los pacientes con AR y FR negativo, exhiban positividad para los anti-CCP, unida a su baja prevalencia en la población sana, hace que su valor diagnóstico sea muy importante (Quinn *et al*, 2006;Zendman *et al*, 2006), avalando su uso como herramienta indispensable en el diagnóstico de AR en la actualidad.

Al igual que el FR, los anti-CCP pueden estar presentes en suero varios años antes del debut clínico de la enfermedad (Nielen *et al*, 2004) y sus niveles parecen asociarse con el pronóstico de la enfermedad (Berglin *et al*, 2006), si bien la evidencia de este último punto es moderada (Liao *et al*, 2011).

1.1.4. Curso clínico: actividad y remisión

El comienzo de la enfermedad suele caracterizarse por un debut clínico poco específico, con astenia, anorexia, fatiga, cansancio vespertino y debilidad muscular. Este cuadro clínico puede preceder a la AR en semanas e incluso meses, hasta que el paciente desarrolla un cuadro poliartrítico simétrico, que se acompaña de dolor e inflamación articular. La rigidez matutina prolongada es el síntoma de comienzo más frecuente en la AR (Gomez-Reino, 2008).

La evolución de la AR es variable, pero la gran mayoría de los pacientes muestran un curso fluctuante o bien agresivo de forma mantenida, con grados variables de deformidad articular a medio plazo. A la vista de esta situación, se hace imprescindible contar con medidas objetivas para evaluar el grado de afectación articular e inflamatoria de forma consistente y sistematizada. En por ello por lo que se recomienda monitorizar el estado del paciente de AR mediante el uso de recuentos articulares (distinguiendo articulaciones dolorosas y tumefactas), un parámetro de evaluación global de la enfermedad por parte del

paciente y del médico (mediante una escala visual analógica) y la determinación de reactantes de fase aguda (PCR y VSG). La síntesis de todos estos parámetros puede realizarse con los denominados índices de actividad compuestos, que actualmente constituyen el paradigma de la monitorización clínica de los pacientes de AR.

El índice de actividad de la enfermedad más ampliamente utilizado es el *Disease Activity Index 28-joints*, recomendado por la *European League Against Rheumatism* (EULAR), que se calcula en base a recuentos articulares de 28 articulaciones (dolorosas y tumefactas), el valor de VSG y la medida del estado global del paciente (EGP) (Prevoo *et al*, 1995). Los valores de actividad de la enfermedad según el índice DAS28 pueden tomar valores entre 0 y 10 y permiten la clasificación del paciente según su grado de actividad (Tabla 3) (van Riel & van Gestel, 2000), que puede depender de la historia natural de la enfermedad o de la respuesta al tratamiento.

Tabla 3: Actividad de la enfermedad según el índice DAS28

Grado de actividad	DAS28
Remisión	$< 2,6$
Actividad baja	$< 3,2$
Actividad moderada	3,2 – 5,1
Actividad alta	$> 5,1$

La clasificación de la actividad de la enfermedad en base a índices compuestos conlleva la formulación del concepto de remisión, que puede ser definido como la ausencia de signos y síntomas acompañada de niveles normales de reactantes de fase aguda (Gomez-Reino, 2008). El concepto de remisión es crucial en la estrategia terapéutica de la AR, como se verá más adelante.

Importancia del diagnóstico precoz

Las características clínicas más importantes de la AR son la cronicidad y la destrucción articular, necesitando ambas cierto tiempo para presentarse. Sin embargo, diferentes estudios han demostrado que la mayoría de los pacientes tienen un daño radiológico significativo en los dos primeros años de la enfermedad, siendo este periodo en el que más rápido progresa la patología (Boers, 2003;Scott, 2000). Asimismo, se ha comprobado que cuanto antes se comienza el tratamiento, mayor es la probabilidad de controlar el proceso inflamatorio y reducir el daño estructural (Raza *et al*, 2006;Raza, 2010). Este periodo ha sido denominado la ventana terapéutica de oportunidad y justifica el abordaje clínico basado en el diagnóstico de artritis de reciente comienzo como una prioridad diagnóstica.

1.1.5. Tratamiento

El tratamiento en la AR tiene un enfoque global y persigue esencialmente el control del dolor y de la inflamación articular a corto plazo, para así conseguir evitar la aparición de deformidades articulares e incapacidad funcional en el medio y largo plazo. En la mayoría de los casos, debe de aplicarse un tratamiento fundamentalmente farmacológico, apoyado de fisioterapia y rehabilitación si así lo requiere el estado del paciente, reservando la cirugía únicamente para casos precisos de alguna articulación en la que no cabe esperar mejoría clínica.

La estrategia terapéutica en la AR ha cambiado significativamente en los últimos años. El compromiso articular y la incapacidad tienen lugar de forma precoz en el curso de la enfermedad, por lo que las estrategias más actuales van enfocadas a controlar rápidamente la actividad de la enfermedad y evitar con ello la progresión del daño. Estas estrategias incluyen el control precoz y estricto de la actividad mediante objetivos (estrategias Treat-to-target, T2T) (Smolen *et al*, 2010), el uso de fármacos biológicos para el tratamiento de la enfermedad moderada y la inducción de la remisión con el uso temprano de fármacos biológicos. No en vano, diferentes estudios apuntan a que la instauración de forma precoz del tratamiento se acompaña de mayores probabilidades de remisión (Anderson *et al*, 2000;Landewe *et al*, 2002;van der Heide *et al*, 1996), y existe cierta evidencia de obtención de mejores resultados (mayor reducción de actividad clínica, menor progresión radiológica y menor discapacidad) en pacientes tratados con fármacos más potentes y más rápidos (van Jaarsveld *et al*, 2000).

Las estrategias T2T tienen como objetivo principal en el tratamiento de la AR la inducción de remisión clínica o, en su defecto, de un estado de baja actividad de la enfermedad, especialmente en enfermedad establecida (Smolen *et al*, 2010). Hasta alcanzar esta meta, el tratamiento farmacológico debe ajustarse cada 3 meses, aproximadamente.

Como consecuencia de la implementación del uso de índices de actividad y de la remisión como objetivo terapéutico, se han establecido asimismo los criterios de respuesta al tratamiento. La utilización de criterios de respuesta objetivos junto con los cambios ágiles en la pauta terapéutica para conseguir una respuesta objetiva predefinida, mejora el pronóstico clínico y radiológico de la AR (Grigor *et al*, 2004). Los criterios de respuesta más empleados en la actualidad son los criterios de respuesta EULAR, que tienen en cuenta tanto el cambio en la actividad de la enfermedad como el grado de actividad actual (van Gestel *et al*, 1999), permitiendo clasificar la respuesta en buena, moderada o nula. No obstante, a partir de los criterios originales, algunos autores proponen una clasificación en dos únicas

categorías: respuesta satisfactoria (remisión completa de la enfermedad o, al menos, una respuesta "suficiente" sin alcanzar la remisión completa) y respuesta insatisfactoria (ausencia completa o casi completa de respuesta).

Agentes farmacológicos

- **Antiinflamatorios no esteroideos (AINEs):** son útiles para reducir los signos y síntomas de la AR, pero no modifican la enfermedad ni evitan la progresión radiológica, por lo que sólo son recomendados como una terapia concomitante para el control del dolor y la inflamación, siempre que sean precisos (O'Dell, 2004).
- **Glucocorticoides (GC):** mejoran los signos y síntomas de la AR y además disminuyen la progresión del daño radiológico (O'Dell, 2004). Se aconseja su uso, siempre a dosis bajas (<15 mg prednisona/día), durante los meses iniciales de la enfermedad en combinación con un fármaco modificador de la enfermedad como terapia "puente", y en los periodos de reactivación de la misma, por su rápido efecto antiinflamatorio (van Jaarsveld *et al*, 2000;Wassenberg *et al*, 2005). Sin embargo, su uso prolongado, incluso a dosis bajas, puede asociarse con un incremento de mortalidad y la aparición de importantes comorbilidades (Del, I *et al*, 2014;Listing *et al*, 2015;Panoulas *et al*, 2008).
- **Fármacos modificadores de la enfermedad (FAMEs) tradicionales:** se asocian con un beneficio clínico importante puesto que son capaces de retrasar la progresión radiológica, mejorando con ello la calidad de vida del paciente a medio plazo. Sin embargo, su comienzo de acción es lento (1 a 6 meses) (Gomez-Reino, 2008).
    - Metotrexato (MTX): actúa inhibiendo enzimas que intervienen en la síntesis de purinas y pirimidinas, interfiriendo así con los procesos de síntesis de ADN y, por tanto, de replicación celular. Se ha descrito que el principal mecanismo de acción del MTX es el bloqueo de la proliferación y activación de linfocitos T, si bien éste no es el único mecanismo que parece operar in vivo (Gomez-Reino, 2008;O'Dell, 2004).

      Los efectos del MTX no aparecen hasta 3 – 4 semanas desde el inicio del tratamiento, mostrando una respuesta máxima a los 2 – 4 meses. Su excelente perfil de eficacia y seguridad hace que el MTX sea considerado como el fármaco de elección para el tratamiento de AR de inicio, ya sea en monoterapia o en terapia combinada (Gomez-Reino, 2008;O'Dell, 2004).
    - Leflunomida (LEF): es un derivado isoxazol, que interfiere con los procesos de replicación y activación de linfocitos T. Al igual que el MTX, es eficaz

controlando los signos y síntomas de la enfermedad, así como disminuyendo la progresión del daño articular (Gomez-Reino, 2008;O'Dell, 2004).

- Otros: la sulfasalacina muestra un buen perfil de seguridad y es eficaz disminuyendo la progresión radiológica. En AR de inicio es eficaz en combinación con otros FAME (Gomez-Reino, 2008). Por otro lado, cloroquina e hidroxicloroquina, si bien son eficaces disminuyendo signos y síntomas de la AR, no muestran un efecto significativo en la progresión radiológica. Sin embargo, se ha visto que pueden actuar a diferentes niveles: interferencia en la presentación antigénica, inhibición en la liberación de citocinas proinflamatorias *in vitro* e in vivo, así como de prostagandinas, y son además capaces de reducir la producción de inmunoglobulinas (Fox, 1993).

o **Fármacos modificadores de la enfermedad biológicos:** estos tratamientos permiten abordar el tratamiento de la AR mediante un enfoque específico hacia alguna de las dianas terapéuticas de la enfermedad. Debido a su especificidad, su considerable eficacia y la capacidad que brindan para la optimización del uso de FAME tradicionales, han supuesto una auténtica revolución en el tratamiento de la AR (y de otras enfermedades autoinmunes).

- Bloqueantes del TNFα: hasta el momento existen 5 fármacos bloqueantes del TNFα, que difieren en su mecanismo de acción y propiedades farmacocinéticas (O'Dell, 2004;Olsen & Stein, 2004;Smolen *et al*, 2007):
  - Infliximab: se trata de un anticuerpo monoclonal quimérico de isotipo IgG1.
  - Adalimumab: se trata de un anticuerpo monoclonal humano de isotipo IgG1, que presenta una mayor vida media que el infliximab; si bien su capacidad para neutralizar moléculas de TNFα es similar.
  - Golimumab: se trata de un anticuerpo monoclonal humano de isotipo IgG1.
  - Etanercept: es una proteína recombinante humana, formada por dos recetores solubles humanos para el TNFα unidos a la porción Fc de una IgG1. Su afinidad por el TNFα soluble es inferior que la mostrada por los anticuerpos anti-TNFα.
  - Certolizumab: está formado por el fragmento Fab' de un anticuerpo monoclonal murino humanizado unido a dos moléculas de polietilenglicol. Su estructura pegilada le confiere una mayor vida media plasmática y le permite una mejor distribución en tejidos

blandos. Su capacidad para neutralizar moléculas de TNFα parece ser superior a la de los anticuerpos monoclonales. Debido a que carece de la fracción Fc, no induce citotoxicidad mediada por anticuerpos ni por complemento.

- Bloqueante de la IL-6: recientemente, se desarrolló un anticuerpo monoclonal humanizado dirigido contra el receptor, tanto soluble como de membrana, de la IL-6 (tocilizumab) (O'Dell, 2004;Smolen *et al*, 2007).
- Otros: existen otros FAME biológicos para el tratamiento de la AR, como son el rituximab (anticuerpo monoclonal anti-CD20), abatacept (proteína de fusión formada por una molécula de CTLA4 unida al fragmento Fc de una IgG1) o anakinra (antagonista del receptor de la IL-1) (O'Dell, 2004;Smolen *et al*, 2007).

1.1.6. Comorbilidad

El impacto de la AR se refleja en términos de disminución de la capacidad física, pérdida de la calidad de vida y acortamiento de la supervivencia, siendo la enfermedad reumática que produce el mayor grado de incapacidad. Diferentes estudios sugieren que existe destrucción de las articulaciones en el 70% de los pacientes de AR dos años después del diagnóstico de la enfermedad (Eberhardt & Fex, 1995;Scott *et al*, 2000). Más del 50% de los pacientes sufre discapacidad grave a los 10 años y, únicamente el 40% puede trabajar tras 15 años de la aparición de la enfermedad (Blumberg & Fox, 2001). No obstante, estos datos son consecuencia de épocas con estrategias de tratamiento ya en desuso. En relación a esto, se ha sugerido que en las últimas décadas la enfermedad es más benigna (Welsing *et al*, 2005;Pincus *et al*, 2005), probablemente debido al impacto de las nuevas pautas de diagnóstico precoz y tratamiento.

La AR supone en consecuencia un elevado coste socioeconómico, cifrándose en 2.250 millones de Euros en el año 2001, siendo un coste anual por paciente de 10.700 Euros (Lajas *et al*, 2003). De forma relativa, se estima que el coste de tratar a un individuo con AR equivale al triple necesario para un individuo de la misma edad y sexo (Lajas *et al*, 2003). Además, se calcula que en España hasta un 5% de todas las incapacidades laborales y permanentes se deben a la AR (Carmona *et al*, 2001).

La mortalidad asociada a la AR es superior a la de la población general y está directamente relacionada con la gravedad de la misma (Gabriel *et al*, 2003;Pincus & Sokka,

2001). La esperanza de vida en un paciente con AR puede verse reducida entre 3 y 7 años, llegando a ser hasta 10 – 15 años menor en pacientes con enfermedad más severa (afectación de múltiples articulaciones o elevación muy marcada de los reactantes de fase aguda) (Myasoedova *et al*, 2010b).

No se observan en la literatura cambios que sugieran una menor mortalidad debida a la AR en los últimos años (Gonzalez *et al*, 2008a;Radovits *et al*, 2010), algo que se ha observado en la población general. Estos hallazgos sustentan la aparición de una brecha de mortalidad creciente entre los pacientes de AR y la población general (Gonzalez *et al*, 2007).

1.2. Enfermedad cardiovascular en artritis reumatoide

La causa más importante del exceso de mortalidad en AR es la que tiene un origen cardiovascular, seguida por las infecciones y los tumores. Esta situación resulta paradójica teniendo en cuenta los avances producidos en los últimos años en el manejo clínico de esta patología, que se han traducido en un mejor control de la enfermedad así como un curso más benigno en términos poblacionales (Pincus *et al*, 2005;Welsing *et al*, 2005), lo que confiere una mayor relevancia el estudio de la enfermedad cardiovascular en la AR.

1.2.1. Artritis reumatoide y riesgo cardiovascular

Si bien es conocido desde hace tiempo, en los últimos años se han publicado un gran número de estudios epidemiológicos y registros que confirman la elevada morbilidad y mortalidad de enfermedad CV en pacientes de AR (Avina-Zubieta *et al*, 2008;Avina-Zubieta *et al*, 2012;del Rincon *et al*, 2001a;Symmons & Gabriel, 2011;Wolfe *et al*, 2003;Wolfe & Michaud, 2008;del Rincon *et al*, 2001a;Solomon *et al*, 2003;Maradit-Kremers *et al*, 2005).

Los pacientes de AR tienen un riesgo 1,5 – 2 veces superior de padecer infarto de miocardio (Solomon *et al*, 2003;Wolfe & Michaud, 2008), 1,4 – 2,7 veces superior de sufrir un accidente cerebrovascular (Nadareishvili *et al*, 2008;Watson *et al*, 2003;Wolfe *et al*, 2003) y 1,3 – 1,7 veces superior de padecer fallo cardiaco (Nicola *et al*, 2005;Wolfe *et al*, 2003;Wolfe & Michaud, 2004), respecto a la población normal. De hecho, el riesgo CV absoluto para los pacientes de AR es equivalente al que tendrían los sujetos 10 – 15 años mayores de la misma población que no padecen AR, o bien, aquellos individuos de la misma población y clase etaria pero que padecen diabetes mellitus de tipo 2 (Peters *et al*, 2009;Symmons & Gabriel, 2011). Por otro lado, la enfermedad CV en pacientes de AR difiere

de aquella hallada en la población normal no sólo en su edad de presentación, sino también en su forma de presentación clínica y su fatalidad (Davis, III *et al*, 2008;Myasoedova & Gabriel, 2010). De hecho, la muerte súbita por enfermedad coronaria es dos veces más frecuente en pacientes de AR que en la población general (Maradit-Kremers *et al*, 2005).

A nivel clínico, el aumento de morbi-mortalidad CV en pacientes de AR es debido al desarrollo precoz de aterosclerosis. De hecho, se ha encontrado una elevada prevalencia de aterosclerosis subclínica en pacientes de AR (Gonzalez-Juanatey *et al*, 2003;Roman *et al*, 2006). Asimismo, se ha observado la presencia de aterosclerosis subclínica (Hannawi *et al*, 2007) y disfunción endotelial (Bergholm *et al*, 2002) en pacientes de AR durante el primer año tras el diagnóstico, incluso en pacientes jóvenes y sin factores clásicos de riesgo (Georgiadis *et al*, 2008).

1.2.2. Factores de riesgo cardiovascular en artritis reumatoide

En este escenario, resulta interesante valorar si este incremento en el riesgo CV se debe a un incremento en prevalencia o severidad de los factores clásicos de riesgo CV (hipertensión, dislipemia, diabetes, obesidad y tabaquismo) en la población de pacientes de AR, o bien, si existen otros mecanismos implicados.

Los trabajos que han abordado esta compleja situación sugieren que, en términos generales, no existen diferencias claras en la prevalencia de factores tradicionales de riesgo CV entre pacientes de AR y la población sana (del Rincon *et al*, 2001b;Solomon *et al*, 2004). No obstante, existen ciertos resultados discordantes en el caso de la diabetes (Boyer *et al*, 2011) y las dislipemias (Steiner & Urowitz, 2009), si bien ambas están íntimamente ligadas al curso de la enfermedad, pudiendo ser éste un factor de confusión que explique esta controversia (Choy & Sattar, 2009;Sattar *et al*, 2003).

Un caso particular dentro de los factores clásicos es el tabaquismo. Diferentes estudios epidemiológicos parecen concluir que el hábito tabáquico es más frecuente en pacientes de AR que en la población sana (Boyer *et al*, 2011). Sin embargo, el tabaquismo no puede ser considerado solamente como un factor tradicional de riesgo CV, puesto que se ha visto implicado directamente en la iniciación de los mecanismos patogénicos de la AR en asociación con la producción de autoanticuerpos anti-CCP (Klareskog *et al*, 2009). De hecho, los pacientes de AR fumadores presentan una enfermedad más severa, caracterizada por mayores niveles de autoanticuerpos, mayor daño articular y peor respuesta al tratamiento (Masdottir *et al*, 2000).

Por otro lado, se ha comprobado que los factores clásicos de riesgo CV no pueden explicar por sí solos el aumento del riesgo CV en pacientes de AR (del Rincon *et al*, 2001a), si bien se asocian de forma independiente con la aparición de enfermedad CV (Del, I *et al*, 2005;Dessein *et al*, 2005b;Gonzalez *et al*, 2008b). A este respecto, son varios los estudios que señalan que ciertos marcadores clínicos de la enfermedad, como son el índice de actividad (Banerjee *et al*, 2008), marcadores de severidad (Farragher *et al*, 2007) y la presencia de manifestaciones extraarticulares (Turesson *et al*, 2007), se asocian de forma independiente al desarrollo de eventos cardiovasculares. A la vista de estas observaciones, algunos autores proponen que la AR podría ser considerada un factor de riesgo per se (Peters *et al*, 2009;van Halm *et al*, 2009).

De forma global, los parámetros clínicos asociados al desarrollo de enfermedad CV en AR pueden considerarse ligados, en mayor o menor grado, a la carga inflamatoria presente en esta patología. De hecho, niveles elevados de reactantes de fase aguda (Maradit-Kremers *et al*, 2005;Wallberg-Jonsson *et al*, 1999), especialmente durante las primeras fases de la enfermedad (Goodson *et al*, 2005), permiten predecir el desarrollo de estas complicaciones en el curso de la AR. Por otro lado, varios grupos han observado que la aterosclerosis subclínica y la disfunción endotelial en pacientes de AR se asocian con marcadores de inflamación sistémica (Chung *et al*, 2005;Hannawi *et al*, 2007;Kerekes *et al*, 2008;Maki-Petaja *et al*, 2006). Estudios en modelos animales parecen confirmar esta asociación (Branen *et al*, 2004). Finalmente, se observa que la prevalencia de la enfermedad CV se presenta igualmente incrementada en otras enfermedades inflamatorias sistémicas, como el lupus eritematoso sistémico (LES) (Esdaile *et al*, 2001;Ward, 1999), pero no así en otras enfermedades reumáticas caracterizadas por una menor carga inflamatoria, como la osteoartritis (OA) (Wolfe *et al*, 2003). En resumen, todas estas evidencias dan cuenta de la relevancia de la disregulación inmunitaria subyacente como consecuencia de la autoinmunidad, en el inicio y progresión de la enfermedad CV en la AR, así como en otras enfermedades inflamatorias crónicas.

Estos resultados parecen indicar un origen multifactorial del riesgo CV en la AR en el que, si bien los factores clásicos tienen un papel, su contribución relativa es menor que en la población general (Gonzalez *et al*, 2008b;Symmons & Gabriel, 2011), debido a la presencia de factores de riesgo "no clásicos" o asociados a la enfermedad. Esta idea implica la existencia de un fenómeno de "competencia" entre los factores clásicos y los factores no clásicos en la determinación del riesgo CV total en la AR (Figura 2). De este modo, y en tanto que los factores tradicionales no parecen diferir entre la población sana y los pacientes de AR, cabe esperar que el incremento de riesgo CV en estos últimos sea debido a los factores

no clásicos o asociados a la enfermedad. Sin embargo, los factores asociados a la enfermedad pueden a su vez interaccionar con los factores clásicos. Así por ejemplo, las respuestas inflamatorias pueden provocar un estado de resistencia a la acción de la insulina (Chung *et al*, 2008), o bien alterar el perfil lipídico de forma cuantitativa y cualitativa (Park *et al*, 1999). Del mismo modo, el tabaquismo puede provocar alteraciones en algunas respuestas inflamatorias (Klareskog *et al*, 2009), al igual que algunas partículas lipoproteicas (Hyka *et al*, 2001). Además, la compartimentación del riesgo CV según diferentes factores clásicos y asociados a la enfermedad no es un escenario fijo, sino que puede variar a lo largo del curso de la enfermedad (Del, I *et al*, 2005), añadiendo un nivel más de complejidad al origen del riesgo cardiovascular en la AR.

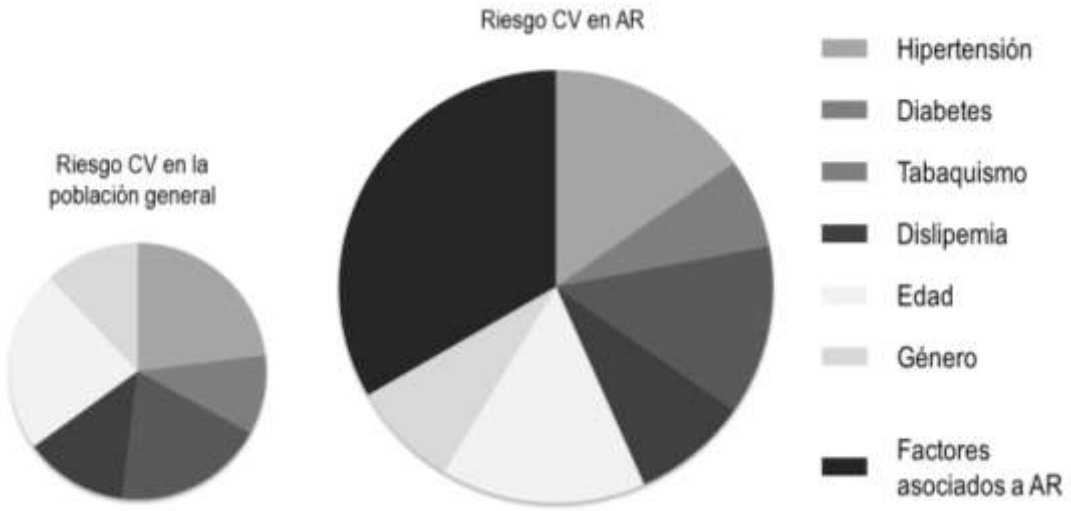

Figura 2 | Distribución hipotética de los factores de riesgo CV en la población general y en pacientes de AR. Pese a que el riesgo CV total en pacientes de AR es superior al hallado en la población general, los factores clásicos explican una proporción menor de éste, debido a la "competencia" con los factores asociados a la AR, como resultado de la inflamación crónica y la disregulación inmunitaria. Modificado de (Symmons & Gabriel, 2011).

A la vista de la creciente importancia de la morbi-mortalidad CV en pacientes de AR, se han realizado importantes esfuerzos dirigidos a identificar y cuantificar el riesgo CV en estos pacientes. Sin embargo, una consecuencia inmediata de lo anteriormente expuesto es que los algoritmos tradicionalmente empleados para la estratificación del riesgo CV en la población general no son útiles en AR, debido a que subestiman el mismo (del Rincon *et al*, 2001a;Gomez-Vaquero *et al*, 2013), al no tener en cuenta los factores no clásicos o asociados a la enfermedad.

Esta situación pone de manifiesto la apremiante necesidad de identificar nuevos marcadores de riesgo CV en AR. Estos nuevos marcadores no sólo permitirían una mejora en

el manejo clínico de estos pacientes, sino que también podrían proporcionar evidencias acerca de los mecanismos implicados en el riesgo CV en esta patología.

En los últimos años, se han identificado diferentes factores que pueden ser considerados como biomarcadores de riesgo CV en AR (Figura 3). Sin embargo, aspectos como el papel que juegan en la patogénesis de la enfermedad CV, sus posibles vínculos con los parámetros clínicos de la AR y su asociación con el resto de factores de riesgo, no están del todo claros. En general, varios autores coinciden en señalar que estos factores pueden interferir en dos procesos clave para el riesgo CV, como son el daño y la reparación endotelial.

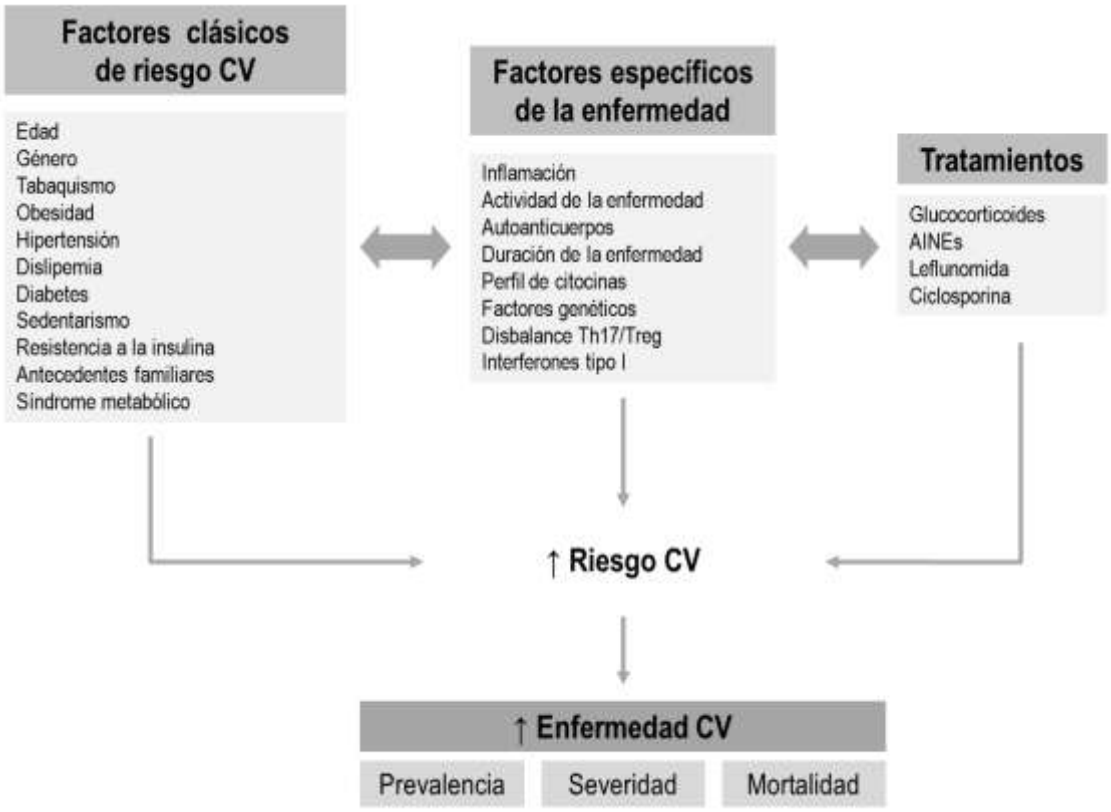

Figura 3 | Modelo de participación de los factores de riesgo CV en AR. Además de los factores clásicos, factores relacionados con la enfermedad, así como factores derivados del uso de diferentes tratamientos contribuyen a explicar el incremento de riesgo CV en pacientes de AR, tanto a nivel de las elevadas tasas de enfermedad CV, como a nivel de su peor pronóstico. La implicación de otros factores de riesgo diferentes a los clásicos no ha de ser considerada como un efecto aditivo solamente, sino que la interacción entre ellos juega un papel relevante en este campo de estudio. Modificado de (Symmons & Gabriel, 2011).

1.3. Mecanismos de daño y reparación endotelial

El endotelio supone la capa más interna (o capa íntima) de los vasos sanguíneos del organismo. En los últimos años, la concepción clásica del endotelio como una mera barrera física que permite contener, en primera línea, el torrente circulatorio, ha dado paso a un

concepto del endotelio como un verdadero órgano, con capacidad para llevar a cabo funciones fisiológicas altamente complejas y relevantes para la homeostasis del organismo, como son (i) proporcionar una barrera de permeabilidad selectiva regulable, (ii) controlar el tono vascular, (iii) modular la homeostasia tanto con acciones procoagulantes como mediante mecanismos anticoagulantes, (iv) modular algunas etapas de la respuesta inmunitaria y (v) llevar a cabo procesos locales de remodelado y expansión tisular (Michiels, 2003;Verma & Anderson, 2002).

Bajo diferentes condiciones patológicas, las células endoteliales modifican sus funciones de permeabilidad selectiva y capacidades biosintéticas como una estrategia adaptativa transitoria para hacer frente al cambio en el microambiente (Simionescu, 2007), dando lugar a un estado de activación o disfunción endotelial (hipótesis de "respuesta al daño") (Newby, 2000;Ross, 1999). Existe cierta controversia en cuanto a si los términos "disfunción" y "activación" endotelial han de ser considerados equivalentes o no. Algunos autores proponen referirse con "disfunción endotelial" a la situación patológica caracterizada por la incapacidad de las células endoteliales para llevar a cabo las funciones homeostáticas de forma adecuada; mientras que con "activación endotelial" se haría referencia a los cambios en las células endoteliales que les permiten llevar a cabo nuevas funciones (Pober *et al*, 2009). No obstante, un estado crónico de activación endotelial puede desencadenar a su vez el desarrollo de disfunción endotelial.

La disfunción endotelial se caracteriza funcionalmente por una disregulación de la producción de óxido nítrico (NO) y endotelina-1 (ET-1) (Kawashima & Yokoyama, 2004;Sudano *et al*, 2007), que resulta en la incapacidad de las células endoteliales para producir una respuesta vasodilatadora correcta, así como por un aumento de la expresión de moléculas de adhesión y quimiotaxis y una alteración de la permeabilidad (Holubarsch, 2000). De forma progresiva, este estado de disfunción endotelial conduce a una fase de daño endotelial, desprendiéndose de la capa íntima las células dañadas y originando un "hueco desnudo", que expone al exterior el espacio subendotelial rico en colágeno y proteínas fibrilares, cuya consecuencia a largo plazo es el establecimiento de una lesión aterosclerótica (Ross & Glomset, 1973;Ross, 1999). Una revisión más reciente de la hipótesis de respuesta al daño sugiere que, de forma alternativa, la lesión aterosclerótica puede producirse por la acumulación y modificación de partículas lipoproteicas sin que se produzca denudación (Newby, 2000). En cualquier caso, en ausencia de mecanismos homeostáticos de reparación que permitan contrarrestar estos procesos patológicos, tiene lugar la progresión de la lesión aterosclerótica.

Existen varios mecanismos de respuesta al daño endotelial que permiten revertir la integridad estructural y funcional del endotelio, y que son los encargados de mantener el balance entre daño y reparación endotelial, evitando así la progresión de lesiones ateroscleróticas y con ello, el desarrollo de enfermedad CV (Figura 4). Sin embargo, estos mecanismos pueden verse desbalanceados en diferentes situaciones patológicas. En AR, al igual que en otras enfermedades autoinmunes, se sabe que existe un desbalance entre ambos procesos, ocurriendo así un daño endotelial exacerbado, unido a una deficiente capacidad de reparación.

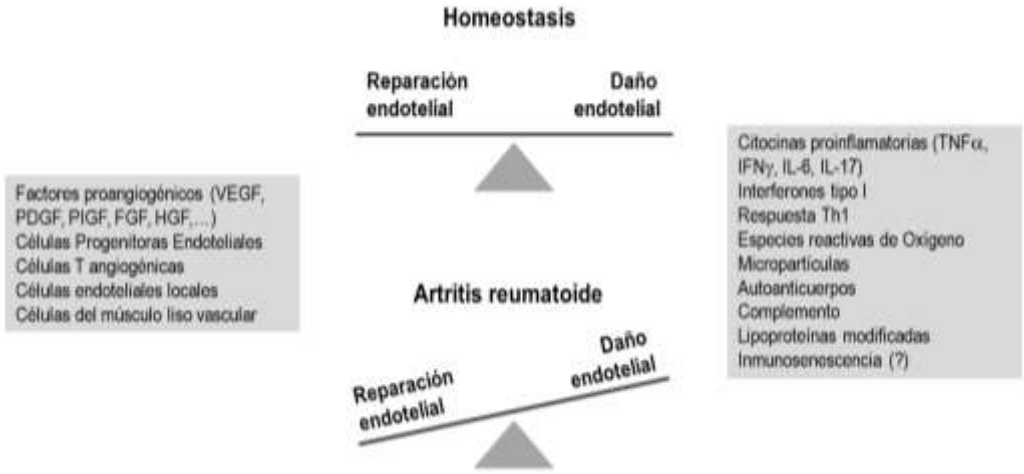

Figura 4 |Balance entre procesos de daño y reparación endotelial. En condiciones homeostáticas, el daño endotelial puede ser contrarrestado de forma eficiente y proporcionada por la actuación de mecanismos de reparación. En AR, existe una descompensación entre el daño endotelial, que se torna excesivo, y la afectación de los mecanismos de reparación, que no son capaces de llevar a cabo adecuadamente su función.

1.3.1. Células Progenitoras Endoteliales

Las Células Progenitoras Endoteliales (*Endothelial Progenitor Cells*, EPC) fueron descritas por primera vez en 1997 por T. Asahara y colaboradores tras observar que una población de células mononucleares aisladas de sangre periférica de individuos sanos adquirían un fenotipo endotelial in vitro y se incorporaban en capilares *in vivo* (Asahara *et al*, 1997). Posteriormente fueron identificadas en sangre de cordón umbilical, médula ósea e hígado fetal (Murohara *et al*, 2000a;Reyes *et al*, 2002).

Fenotipo y caracterización

Las EPC comparten un antecesor común (hemangioblasto) con las células madre hematopoyéticas (Murasawa, 2004) y pueden enmarcarse dentro del grupo de células pluripotenciales adultas. Su principal característica definitoria es la expresión simultánea de

marcadores de linaje endotelial y marcadores característicos de células progenitoras. Si bien inicialmente sólo fueron caracterizadas como células que expresaban los marcadores CD34 y VEGFR2 (Asahara *et al*, 1997;Khakoo & Finkel, 2005), estos marcadores también pueden estar presentes en células endoteliales maduras, por lo que la identificación de las EPC como células $CD34^{+}VEGFR2^{+}$ no resulta precisa. Estudios posteriores permitieron encontrar que las EPC también expresaban la molécula CD133, un marcador característico de células pluripotentes inmaduras. De hecho, trabajos subsecuentes permitieron confirmar que la expresión de CD133 identificaba la subpoblación que era capaz de diferenciarse hacia un fenotipo endotelial adulto *in vitro* (Gehling *et al*, 2000;Peichev *et al*, 2000;Salven *et al*, 2003), así como que la expresión de este marcador se perdía con la diferenciación de esta población celular. La pérdida de expresión de CD133 parece coincidir con la ganancia de marcadores característicos de células endoteliales maduras (CD146, CD105, CD106, factor de von Willebrand, factor tisular,…), si bien esto parece ocurrir de forma progresiva (Figura) (Khakoo & Finkel, 2005;Woywodt *et al*, 2004;Hristov *et al*, 2003). Estudios aún más recientes permitieron establecer que incluso puede hallarse una población de EPC más inmadura, caracterizada por la ausencia de expresión de CD34 (Figura 5), pero que posee las mismas capacidades funcionales *in vitro* (Friedrich *et al*, 2006).

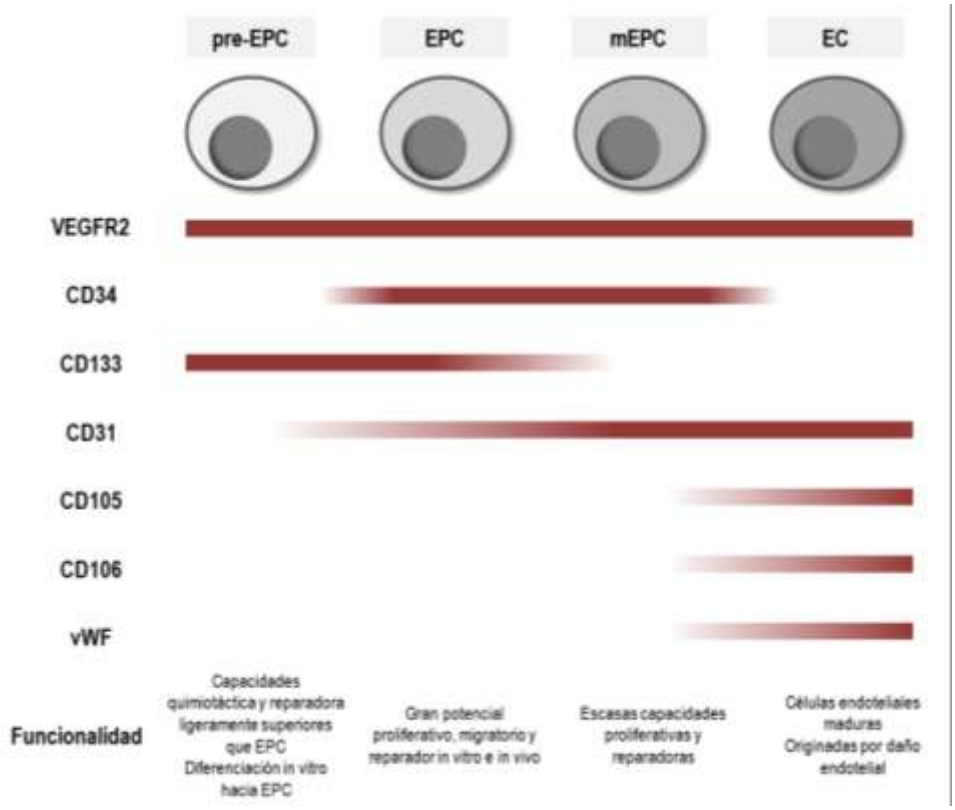

Figura 5 | Expresión de diferentes marcadores y características funcionales en las poblaciones EPC y *EPC-like*. La expresión de los diferentes marcadores extracelulares en base a la literatura reciente se representa según la intensidad de las barras horizontales. EC: *endotelial cell* (célula endotelial madura).

Funciones

Funcionalmente, se sabe que las EPC son capaces de proliferar y diferenciarse hacia varias estirpes celulares *in vitro* (Reyes *et al*, 2002), en línea con su carácter pluripotencial. La principal función fisiológica que se les atribuyó a las EPC es la de dirigir el proceso de vasculogénesis postnatal, esto es, son capaces de llevar a cabo el proceso de neovascularización tras su proliferación y migración desde el reservorio medular hacia el tejido diana, donde se diferencian a células endoteliales maduras (Asahara *et al*, 1997;Khakoo & Finkel, 2005;Schaper & Scholz, 2003). Además de ser el principal sustrato del proceso vasculogénico, las EPC pueden ejercer una acción paracrina simultánea, promoviendo la angiogénesis a nivel local de forma indirecta (Li & Asahara, 2008). La participación de las EPC en la formación de nuevos vasos sanguíneos fue demostrada mediante estudios experimentales en los que se observó la formación de estructuras similares a capilares, a partir de EPC en diferentes modelos experimentales (Li & Asahara, 2008).

La vasculogénesis constituye además un eficaz mecanismo de reendotelización ante situaciones de daño endotelial. Diferentes estudios muestran que las EPC pueden migrar y colonizar implantes aórticos, tanto en modelos animales (Shi *et al*, 1998) como en pacientes (Peichev *et al*, 2000). Asimismo, se observa que en modelos animales de isquemia, la neovascularización promovida por la administración local o sistémica de EPC media en la recuperación de la funcionalidad de aquellos tejidos isquémicamente comprometidos (Kamihata *et al*, 2002;Li & Asahara, 2008;Murohara *et al*, 2000b). Por tanto, actualmente se acepta que las EPC tienen un papel central en el mantenimiento de la homeostasis vascular mediante su capacidad para llevar a cabo mecanismos de reparación del endotelio.

Las EPC en sangre periférica en adultos sanos representan alrededor del 0,01 – 0,1%, dependiendo de los estudios consultados. En todo caso, se trata de una población minoritaria, siendo su frecuencia superior en la médula ósea, que supone su reservorio natural. La movilización de las EPC desde la médula ósea hacia los tejidos periféricos es un proceso complejo en el que están involucrados diferentes mediadores (factores de crecimiento, citocinas, proteasas, moléculas de adhesión,…), cuyos mecanismos de acción interaccionan con la vía de señalización intracelular Pi3K/Akt/eNOS, que juega un papel central en este proceso (Everaert *et al*, 2010) (Figura 6). Las EPC quiescentes permanecen ancladas a las células estromales de la médula ósea (Luttun *et al*, 2002). El primer paso para su movilización parece ser la activación de la metaloproteasa de matriz MMP-9, como así concluyen algunos estudios *in vitro* y en modelos animales (Everaert *et al*, 2010;Heissig *et*

al, 2002;Rafii *et al*, 2002), que actúa a nivel de las uniones entre c-kitL en su forma soluble y el receptor c-kit expresado en las EPC, pero además es capaz de desencadenar la secreción de factores de crecimiento que parecen promover la proliferación y maduración de EPC en el reservorio medular (Heissig *et al*, 2002).

Numerosos estudios han demostrado que el factor de crecimiento factor de crecimiento del endotelio vascular (*vascular endotelial growth factor*, VEGF) es el principal mediador implicado en la movilización e incorporación de EPC a las áreas de daño endotelial. VEGF es secretado por células endoteliales y estromales como consecuencia de las condiciones hipóxicas generadas tras estímulos de daño endotelial de diversa índole, como traumas físicos, daño isquémico estímulos tóxicos (Khakoo & Finkel, 2005;Li & Asahara, 2008). Estudios con pacientes de infarto de miocardio revelaron que el incremento de EPC circulantes que tiene lugar tras el daño isquémico, se correlaciona con los niveles plasmáticos de este mediador (Shintani *et al*, 2001). Asimismo, en modelos animales se observa que la administración exógena de VEGF incrementa la movilización, proliferación y capacidad migratoria de las EPC (Asahara *et al*, 1999), un efecto similar al descrito en estudios con humanos (Kalka *et al*, 2000). Por otro lado, el VEGF también puede desencadenar la liberación de otros factores de crecimiento, tanto por las EPC como por células estromales de la médula ósea o células endoteliales maduras (Asahara *et al*, 1999;Khakoo & Finkel, 2005). En resumen, estos resultados apoyan el papel central del VEGF en la movilización y funcionalidad de las EPC en diferentes situaciones.

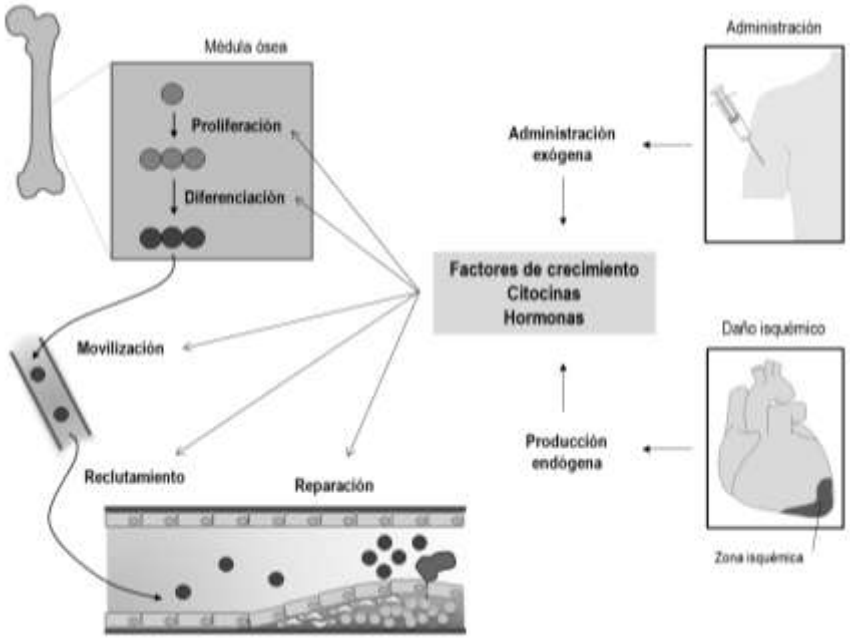

Figura 6 | Movilización de las EPC desde el reservorio medular hacia la circulación periférica en respuesta a diferentes estímulos. Modificado de (Murasawa & Asahara, 2005).

Del mismo modo, se conocen otros factores de crecimiento que pueden igualmente tener un papel en estos procesos (Everaert *et al*, 2010). Por otro lado, se sabe que la regulación de los procesos de vasculogénesis es compleja y, en cierto modo, redundante (Carmeliet, 2003). Esto permitiría que, en el caso de que la vía del VEGF no fuese funcional, los procesos homeostáticos de reparación del endotelio pudiesen tener lugar, al menos en cierto grado. Esta hipótesis se ve reforzada por la observación de que algunos pacientes que han sido tratados con bloqueantes específicos del VEGF muestran igualmente fenómenos de neovascularización (Bartolotti *et al*, 2014). Finalmente, si bien se ha sugerido que algunos mediadores de la inflamación podrían alterar los mecanismos vasculogénicos, qué mediadores concretos y cuál es su alcance, no están del todo claros.

EPC y riesgo cardiovascular

A la vista de su papel como mecanismo de reparación endotelial, ya sea incorporándose directamente a zonas desnudas en el endotelio, o bien reemplazando células endoteliales disfuncionales, el estudio de las EPC cobró gran relevancia en el campo de las enfermedades cardiovasculares y el riesgo cardiovascular.

Las primeras evidencias de una asociación entre EPC y riesgo cardiovascular fueron publicadas por Vasa y colaboradores, que observaron una correlación negativa entre el número de factores de riesgo CV y el número de EPC circulantes y su funcionalidad (Vasa *et al*, 2001). Estudios posteriores permitieron confirmar la asociación entre el número de EPC y la función endotelial y el riesgo CV (Hill *et al*, 2003) en población sana. En este caso, Hill y colaboradores, en lugar de llevar a cabo una cuantificación del número de EPC circulantes en sangre periférica, establecieron un protocolo de cultivo que permite la observación del número de colonias de EPC in vitro. El número de colonias de EPC podría ser un marcador indirecto de funcionalidad de las EPC, lo cual viene apoyado por su asociación con marcadores de riesgo cardiovascular clásico y de disfunción endotelial (Hill *et al*, 2003;Murphy *et al*, 2007). De hecho, el número de colonias de EPC parece ser mejor predictor de la función endotelial que los factores clásicos (Hill *et al*, 2003), sugiriendo que existen otros parámetros además de los factores clásicos, que han de ser tenidos en cuenta. Un buen número de estudios han proporcionado conclusiones similares empleando diferentes enfoques experimentales en diferentes grupos de población (Tabla 4). Finalmente, Werner y colaboradores observaron una asociación negativa entre el número de EPC circulantes y el pronóstico de pacientes con enfermedad coronaria, observándose una asociación dosis-dependiente tras ajustar para factores clásicos de riesgo cardiovascular (Werner *et al*, 2005).

Tabla 4: Alteraciones en EPC según factores clásicos de riesgo CV

Factor de riesgo CV	Efecto observado en el número y función de EPC	Metodología	Referencia
Dislipemia	Frecuencia de EPC reducida Migración defectiva	EPC circulantes (CD45, CD34, CD133) Colonias de EPC	(Chen *et al*, 2004)
Diabetes	Frecuencia de EPC reducida Migración defectiva	EPC circulantes (CD31, CD34, VEGFR2) Colonias EPC	(Tepper *et al*, 2002)
Hipertensión	Correlación negativa entre el número de EPC y presión arterial sistólica	EPC circulantes (CD34, VEGFR2, CD133)	(Vasa *et al*, 2001)
Tabaquismo	Correlación negativa entre el número de EPC y el hábito tabáquico	EPC circulantes (CD34, VEGFR2, CD133)	(Kondo *et al*, 2004;Vasa *et al*, 2001)
Edad	Migración y proliferación defectivas	EPC circulantes (CD45, CD34, CD133) Colonias de EPC	(Hoetzer *et al*, 2007)
Ejercicio físico	Incremento en frecuencia y funcionalidad de EPC	EPC circulantes (CD45, CD34, CD133) Colonias de EPC	(Hoetzer *et al*, 2007)

En consonancia con lo anterior, diferentes estudios longitudinales con pacientes de enfermedad coronaria (George *et al*, 2004), diabetes (Tepper *et al*, 2002) e ictus (Ghani *et al*, 2005) concluyen que existe una reducción en la frecuencia de EPC circulantes en estas patologías. Otra evidencia que avala la conexión entre los niveles circulantes de EPC y el riesgo CV es que se ha observado que, al menos en parte, el beneficio clínico de un buen número de agentes farmacológicos dirigidos a la prevención, primaria o secundaria, de enfermedades CV puede ser atribuido a cambios en la movilización o funcionalidad de las EPC circulantes (Siddique *et al*, 2010).

A la vista de los resultados hallados hasta la fecha, parece lógico concluir que la capacidad de las EPC circulantes como marcador de función endotelial y de riesgo cardiovascular, subyacen a su función como mecanismo de reparación endotelial. De este modo, los factores de riesgo CV podrían desencadenar una movilización insuficiente, una migración defectiva, un agotamiento del reservorio medular o una apoptosis acelerada en las EPC circulantes, si bien los mecanismos exactos que actúan no están del todo claros.

EPC y artritis reumatoide

Como se ha mencionado, la asociación entre los niveles de EPC y los factores de riesgo CV, dieron paso al estudio de las EPC en diferentes estados patológicos asociados a

enfermedad CV. En este aspecto, muchos grupos pusieron su atención en las enfermedades autoinmunes sistémicas, como la AR y el LES, debido al incrementado riesgo CV concomitante en estas patologías. Asimismo, otras evidencias como la disregulación de algunos de los factores implicados en la movilización de EPC, o la observación de que estos pacientes exhibían un daño endotelial exacerbado, que en parte podría ser consecuencia de unos mecanismos de reparación defectivos, sugerían una alteración de las EPC en número o función en estas patologías.

La primera evidencia de una alteración de las EPC en pacientes de AR fue publicada por Grisar y colaboradores, que encontraron una depleción de EPC circulantes en pacientes de AR, en asociación con la actividad de la enfermedad (Grisar *et al*, 2005). Asimismo, estos autores observaron que la aparición de colonias de EPC en cultivo estaba reducida. Por último, se observaba una asociación entre niveles bajos de EPC circulantes y niveles séricos elevados de TNFα. Posteriormente, Herbrig y colaboradores confirmaron que tanto el número como la función in vitro de las EPC estaban reducidas en pacientes de AR y que estos hallazgos se relacionaban con una función endotelial asimismo alterada (Herbrig *et al*, 2006). No obstante, existen diferencias relevantes en cuanto al fenotipo de las EPC, así como al método de identificación empleado y los resultados relativos a los tratamientos seguidos por estos pacientes. Además, las poblaciones de pacientes analizadas en ambos estudios no son totalmente comparables a nivel clínico y presentan como criterio de exclusión la presencia de factores clásicos de riesgo CV, lo que puede suponer un sesgo respecto a la población de pacientes de AR general.

Un estudio posterior, en el que no se aplicaron estos criterios de exclusión, aportó resultados controvertidos: aunque los niveles de EPC circulantes no eran significativamente diferentes a los hallados en individuos control, el número de colonias de EPC estaba reducido en pacientes. Sin embargo, si bien la frecuencia de EPC circulantes se asociaba negativamente con parámetros clínicos de la enfermedad (VSG y presencia de FR), el número de colonias se correlacionaba negativamente con los factores de riesgo CV (Egan *et al*, 2008). En este caso, además de existir diferencias importantes en parámetros clínicos respecto a los trabajos anteriores, el grupo estudiado era relativamente menor pese a ser más heterogéneo, lo cual podría explicar, al menos en parte, estas controversias. En cualquier caso, las diferencias entre los niveles de EPC circulantes y las colonias de EPC, dan cuenta de la complejidad de este campo.

Finalmente, Jodon y colaboradores analizaron la frecuencia de EPC mediante citometría de flujo empleando un protocolo de separación previo, y observaron que la

frecuencia de EPC circulantes, así como la formación de colonias in vitro, estaban incrementadas en pacientes de AR en relación a la actividad de la enfermedad (Jodon, V *et al*, 2010).

De forma general, existen diferencias en los grupos de pacientes analizados, los criterios y la metodología experimental empleados para la identificación de las EPC, lo que dificulta sobremanera la comparación entre estudios y puede ser una explicación para la variabilidad de los resultados hallados. Asimismo, estos trabajos se han realizado en grupos relativamente reducidos de pacientes y no siempre se han tenido en cuenta los factores clásicos de riesgo CV, por lo que resulta difícil sacar conclusiones acerca de una alteración a nivel de las EPC en pacientes de AR con los datos publicados hasta el momento.

1.3.2. Células T angiogénicas

Si bien las EPC cobraron mucho interés como posibles biomarcadores de riesgo CV, un buen número de dificultades metodológicas y conceptuales dan cuenta de los controvertidos resultados obtenidos hasta la fecha (Fadini *et al*, 2008).

A pesar de que el método de cultivo de colonias de EPC según el protocolo de Hill y colaboradores fue rápidamente aplicado en diversos trabajos, el estudio de las colonias de EPC no atrajo menos atención. Las colonias de EPC in vitro se componen de un núcleo de células redondeadas desde el que irradian células ahusadas hacia la periferia (Hill *et al*, 2003) (Figura 7). Dos grupos independientes (Neumuller *et al*, 2006;Yoder *et al*, 2007) concluyeron que las colonias de EPC estaban compuestas por células funcional y fenotípicamente diferentes a células endoteliales maduras, que expresaban diferentes marcadores leucocitarios. Un estudio posterior (Medina *et al*, 2010) concluyó que tanto el perfil de expresión global como el proteoma de las colonias de EPC era diferente al de células endoteliales, estando caracterizando el de las primeras por la expresión de genes relacionados con inmunidad, inflamación y hematopoyesis.

La caracterización fina de las colonias de EPC por el grupo de Hur y colaboradores (Hur *et al*, 2007) permitió concluir que el núcleo central de las colonias estaba formado por células T ($CD3^+$) que expresaban el marcador CD31. La presencia de estas células dobles positivas era imprescindible para la formación de colonias de EPC *in vitro*. Además, se observó que estas células expresaban el marcador CXCR4, de gran relevancia en la respuesta frente a hipoxia, y además producían niveles de VEGF, IL-8 e IL-17, así como de MMP-9, significativamente superiores a los hallados en la subpoblación $CD3^+CD31^-$. A nivel

funcional, estas células eran capaces de incorporarse a capilares *in vitro*, promoviendo así la formación de éstos. Asimismo, estas células participaban en la formación de nuevos vasos in vivo y permitían la restauración del flujo sanguíneo en un modelo murino de isquemia. En base a estos hallazgos, los autores acuñaron el término de células T angiogénicas (Tang) (Hur *et al*, 2007) para referirse a esta población, que parece ser un mediador indispensable para los procesos de reparación endotelial dirigidos por las EPC, aunque su papel reparador puede ir más allá dadas sus capacidades para integrarse en los capilares en formación y para la producción de los mediadores proangiogénicos VEGF e IL-8.

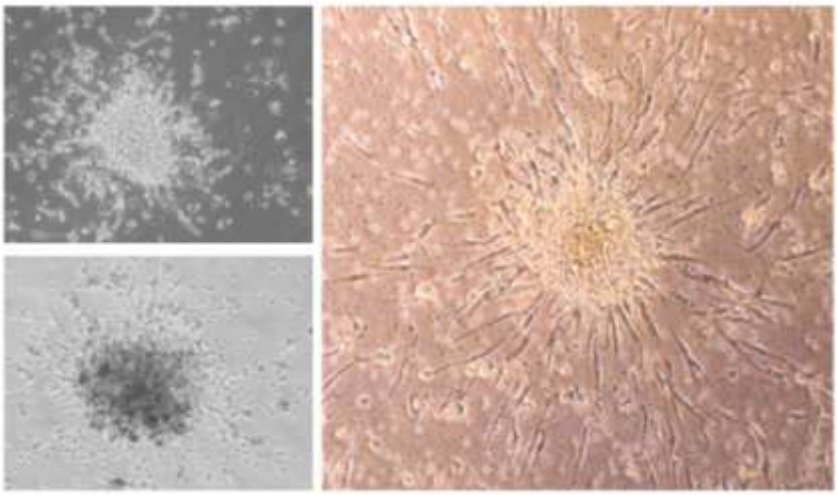

Figura 7 | Microfotografías de las colonias formadas por EPC. Se observa la arquitectura de las colonias de EPC, formadas por un núcleo central de células redondeadas con un halo exterior de células ahusadas que irradian hacia la periferia. Imágenes de microscopía óptica de contraste de fases tomadas de (Hill *et al*, 2003;Hur *et al*, 2007;Yoder *et al*, 2007).

La caracterización de las células Tang podría tener un gran impacto en el terreno clínico, no sólo por ser potenciales biomarcadores de riesgo CV, sino también por su presumible papel como diana terapéutica. Sin embargo, el estudio de las células Tang en pacientes con enfermedad CV sólo ha sido abordado en un trabajo reciente.

Rouhl y colaboradores analizaron la frecuencia de células Tang, así como de EPC, en un pequeño grupo de pacientes con enfermedad cerebral de pequeño vaso, cuya patogénesis está ligada a un proceso de disfunción endotelial a nivel de la barrera hemato-encefálica (Farrall & Wardlaw, 2009). Estos autores concluyeron que tanto las células Tang como las EPC estaban disminuidas en sangre periférica en estos pacientes en comparación con pacientes hipertensos sin dicha complicación cerebral (Rouhl *et al*, 2012). Además, la disminución de células Tang no parecía estar influida por factores como género, edad o

presión arterial, sugiriendo así un papel de las células Tang como marcadores independientes de enfermedad vascular.

Los resultados publicados por Rouhl y colaboradores suponen la prueba de concepto que permite relacionar las células Tang con la patología cardiovascular en humanos, sustentada sobre los mecanismos identificados en el artículo de Hur y colaboradores sobre la participación de éstas en los procesos de formación y reparación de vasos.

1.3.3. Micropartículas

El término micropartícula (MP) hace referencia a pequeñas vesículas membranosas que se originan por procesos de evaginación de la membrana plasmática y que son liberadas hacia el exterior. Las MP tienen un diámetro entre 0,1 y 1 micras y contienen tanto una porción del citosol como de la membrana plasmática de la célula a partir de la cual se originan (Burger *et al*, 2013;Hugel *et al*, 2005) (Figura 8).

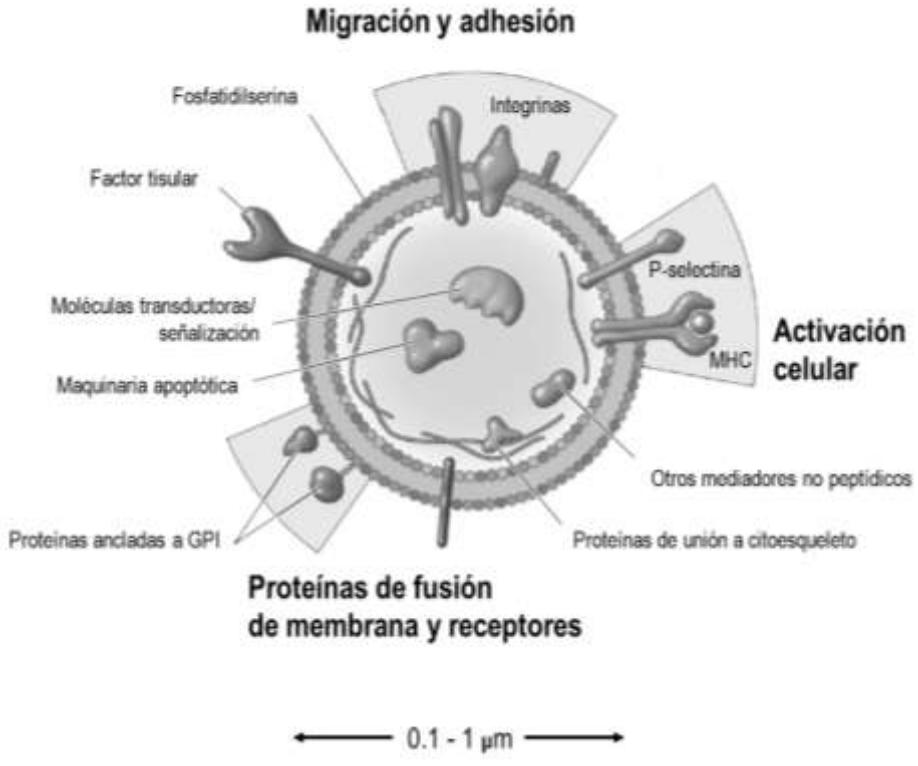

Figura 8 | Estructura general de una micropartícula. Las MP contienen diferentes componentes citoplasmáticos y proteínas extracelulares procedentes de la célula a partir de la cual se originan. Estos componentes son los responsables de su función como mediadores de la comunicación intercelular. Modificado de (Hugel *et al*, 2005).

A pesar de que durante mucho tiempo fueron consideradas como desechos celulares, originadas por tanto de forma pasiva ante estímulos de daño celular, actualmente se acepta que las MP pueden ser generadas no sólo en respuesta a daño sino también de una forma activa, y que poseen una importante función como mediadores de la comunicación intercelular por su capacidad para transportar enzimas y otras proteínas de señalización, fragmentos de ARN y ADN, así como moléculas de miARN (Beyer & Pisetsky, 2010;Burger *et al*, 2013). Concretamente, se ha descrito que las MP pueden tener un papel en procesos como la expresión de citocinas y quimiocinas o de moléculas de adhesión y migración, la modulación de receptores celulares, la activación de diferentes vías de señalización así como de la cascada de la coagulación o de respuesta al estrés oxidativo, entre otros (Beyer & Pisetsky, 2010;Burger *et al*, 2013). En líneas generales, que las MP desencadenen mecanismos homeostáticos o deletéreos sobre otras poblaciones diana dependerá tanto de su contenido interno, que será consecuencia de los estímulos que hayan llevado a su formación, como del número de MP generadas (Hugel *et al*, 2005).

Las MP, también denominadas microvesículas por algunos autores, son diferentes de otras vesículas extracelulares, como los exosomas o los cuerpos apoptóticos (Figura 9). Los exosomas presentan un diámetro menor (40 – 100 nm) y se forman vía cuerpos multivesiculares; mientras que los cuerpos apoptóticos son notablemente mayores (1 – 4 micras), pueden contener orgánulos y material nuclear, y su membrana siempre contiene residuos de fosfatidilserina, además de presentar otras alteraciones en su topología como consecuencia de su origen apoptótico (Burger *et al*, 2013).

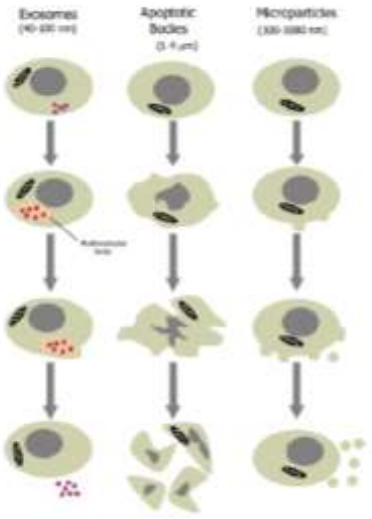

Figura 9 | Imagen comparativa de la formación de exosomas, cuerpos apoptóticos y micropartículas. En la imagen se ponen de manifiesto las diferencias en origen, tamaño y composición de estos tres tipos de vesículas celulares. Tomado de (Burger *et al*, 2013).

La formación de las MP tiene lugar mediante una evaginación de la membrana plasmática hacia el exterior, mediado por una reorganización del citoesqueleto local. Recientemente se ha observado que la formación de MP tiene lugar, de forma preferente, en microdominios especializados de la membrana plasmática, como las regiones ricas en balsas lipídicas (*lipid rafts*) o las caveolas (Morel *et al*, 2011). Si bien los mecanismos estructurales que orquestan la formación de MP parecen ser similares en todos los tipos celulares, los estímulos que desencadenan este proceso no lo son tanto, observándose una marcada heterogeneidad según el tipo celular estudiado (Burger *et al*, 2013) (Figura 10).

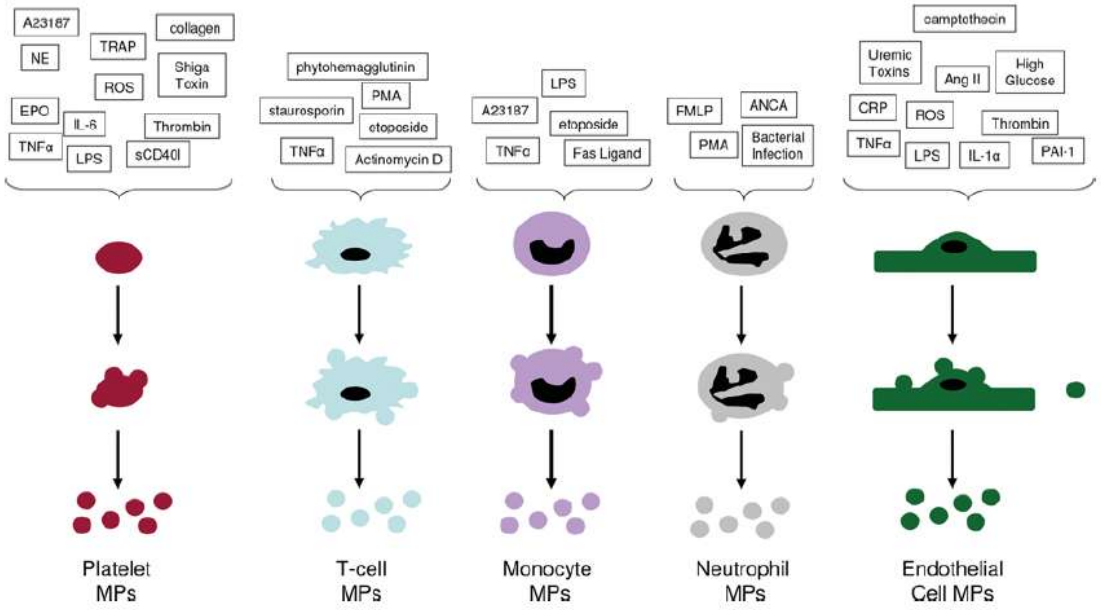

Figura 10 | Estímulos que desencadenan la liberación de MP en diferentes tipos celulares. La liberación de MP es un proceso activo que depende de la biología de cada población celular, si bien existen algunos estímulos que desencadenan la liberación de MP en la mayor parte de los tipos celulares analizados. Tomado de (Burger *et al*, 2013).

Las MP pueden generarse a partir de cualquier tipo celular. Si bien las primeras MP observadas fueron las derivadas de plaquetas, se ha descrito la formación de MP a partir de células endoteliales, eritrocitos, células musculares, células T, monocitos, neutrófilos o fibroblastos (Anfossi *et al*, 2010;Barteneva *et al*, 2013;Burger *et al*, 2013). Debido a su proceso de formación, las MP expresan en su membrana plasmática aquellos marcadores que estaban presentes en la célula a partir de la que se originaron, lo cual permite su identificación mediante el uso de anticuerpos utilizando de diferentes técnicas (Lacroix *et al*, 2010) (Tabla 5).

Tabla 5: Marcadores extracelulares comúnmente empleados para la identificación de MP

Población celular de origen	Marcadores
Plaquetas	CD41, CD42
Células endoteliales	CD146, CD144, CD31, CD62E
Leucocitos	CD45
Células T	CD3
Células B	CD19, CD20
Monocitos	CD14
Granulocitos	CD66b
Eritrocitos	CD235

La formación de MP tiene lugar en condiciones fisiológicas, si bien ésta se incrementa notablemente en condiciones de activación, daño o muerte celular (Beyer & Pisetsky, 2010;Burger *et al*, 2013). Por tanto, la cuantificación de MP a partir de una muestra biológica nos permite conocer qué procesos están activos en ese momento y en qué grado. De este modo, las MP son unos interesantes biomarcadores que cuentan con importantes ventajas (Tabla 6)

Tabla 6: Ventajas de las MP como biomarcadores

Obtención por procedimientos no invasivos
Información de órganos/tejidos no accesibles (endotelio, músculo liso, …)
Información de poblaciones minoritarias
Información simultánea y completa de procesos complejos en los que intervienen diferentes poblaciones celulares

En términos generales, a la vista de las poblaciones celulares principalmente implicadas y los estímulos que conducen a la liberación de MP, los niveles de MP en plasma sanguíneo se asocian con patologías que implican daño endotelial en diferente grado (Anfossi *et al*, 2010;Barteneva *et al*, 2013;Beyer & Pisetsky, 2010;Burger *et al*, 2013). Así, se han encontrado niveles elevados de MP de diverso origen en pacientes de diabetes, hipertensión, algunos tipos de nefropatía, síndrome coronario agudo y enfermedad coronaria, entre otros (Barteneva *et al*, 2013). Asimismo, la tensión tangencial es un potente inductor de la formación de MP (Nomura *et al*, 2001).

A nivel funcional, se ha descrito que niveles elevados de MP de diferente origen se asocian negativamente a la función endotelial *in vivo* (Essayagh *et al*, 2005;Pirro *et al*, 2008) y positivamente al desarrollo de aterosclerosis subclínica (Bernard *et al*, 2009). Estudios posteriores en modelos animales permitieron determinar que las MP derivadas de linfocitos

T y células endoteliales son capaces de alterar la producción de NO y de prostaciclinas (Martin *et al*, 2004), explicando así la disfunción endotelial observada *in vivo*, y apoyando el papel de las MP como agentes implicados en la comunicación intercelular. Por otro lado, existe cierta evidencia de que una elevación de MP puede tener carácter pronóstico en enfermedad CV (Boulanger *et al*, 2006). Finalmente, cabe destacar que el tratamiento farmacológico con agentes cardioprotectores, como las estatinas, provoca un descenso en los niveles plasmáticos de algunos tipos de MP (Suades *et al*, 2013). Estas evidencias sugieren por tanto un papel relevante de las MP como potenciales biomarcadores de daño endotelial y riesgo CV.

MP y artritis reumatoide

Diversos trabajos han puesto de manifiesto la participación de las MP en procesos implicados en la patogenia de las enfermedades autoinmunes, como la modulación de las respuestas inflamatorias, la activación endotelial, la activación de fibroblastos, la activación de la cascada de la coagulación y la formación y presentación de inmunocomplejos (Beyer & Pisetsky, 2010), aunque los resultados son relativamente heterogéneos y poco concluyentes.

Se ha observado que, además de estar incrementadas en número, las MP derivadas de pacientes de AR son capaces de activar el sistema del complemento, la cascada de la coagulación, la producción de quimiocinas y moléculas de adhesión, la activación de células B, así como la liberación de metaloproteasas de matriz (Pisetsky *et al*, 2012;Berckmans *et al*, 2005). Además, las MP procedentes de pacientes de AR contienen moléculas de inmunoglobulinas de isotipos G y M, que se cree pueden jugar un papel en la formación de inmunocomplejos y la presentación de autoantígenos (Pisetsky *et al*, 2012;Ullal *et al*, 2010).

Por otra parte, las MP también se encuentran presentes en el líquido sinovial (Berckmans *et al*, 2002), lo cual, teniendo en cuenta los procesos en los que se han visto implicadas, sugiere que pueden jugar un papel en la amplificación de las respuestas inflamatorias a nivel local.

Las enfermedades autoinmunes sistémicas constituyen un interesante modelo de estudio para las MP, puesto que no sólo presentan una activación inmunitaria, como consecuencia de las reacciones autoinmunes, sino que también presentan un estado de activación endotelial como consecuencia de la exposición acumulada a moléculas proinflamatorias y otros mediadores. De este modo, además de desencadenar o promover mecanismos patogénicos en relación al contexto autoinmune, la formación de MP en AR podría contribuir al daño y disfunción endotelial.

1.3.4. Interferones de tipo I

Debido a que la inflamación crónica y la disregulación inmunitaria juegan un papel en el desarrollo y progreso de la aterosclerosis en la patología autoinmune (Sattar *et al*, 2003), resulta evidente que los mediadores de las respuestas autoinmunes puedan inducir o promover mecanismos de daño o alterar los mecanismos de reparación endotelial. Así, resulta interesante el caso de los interferones (IFN) de tipo I.

Los IFN de tipo I, y concretamente el IFNα, son unas citocinas producidas como respuesta a una infección de tipo vírico por parte de células dendríticas, mayoritariamente células dendríticas plasmacitoides, si bien un buen número de tipos celulares son capaces de producir IFN de tipo I (Siegal *et al*, 1999). La producción de IFN de tipo I permite inhibir la replicación viral, constituyendo así uno de los principales mecanismos antivirales del sistema inmunitario (Ivashkiv & Donlin, 2014). Los IFN de tipo I, tras ser reconocidos por su receptor específico, inducen la expresión coordinada de un conjunto de genes denominados genes de respuesta a IFN (*Interferon-responding genes,* IRF) que son los responsables, entre otras funciones, de la actividad antiviral de estas citocinas (Ivashkiv & Donlin, 2014). La expresión conjunta de estos genes se ha denominado "firma de IFN de tipo I" (Ronnblom, 2011;Ronnblom & Eloranta, 2013), siendo éste un concepto ampliamente referido en la literatura debido a los problemas que presenta la cuantificación directa de los niveles de IFNα.

Además, estas citocinas son capaces de llevar a cabo un buen número de acciones activadoras de la respuesta inmunitaria, como el incremento de la expresión de moléculas de MHC-I y MHC-II, moléculas coestimuladoras, activación de células NK, expresión de quimiocinas y sus receptores, así como la estimulación de la diferenciación de células B, la producción de anticuerpos y el cambio de clase de inmunoglobulinas. Además, la producción de estas citocinas se asocia a una polarización de la respuesta inmunitaria hacia un fenotipo Th1 (Ivashkiv & Donlin, 2014;Kalliolias & Ivashkiv, 2010).

Dadas sus funciones, los IFN de tipo I pueden ser claves en la iniciación y promoción de reacciones de tipo autoinmune, por lo que han sido objeto de estudio en estas patologías desde hace más de 50 años. Es interesante destacar que se ha observado una producción aberrante de IFN de tipo I en diferentes patologías autoinmunes sistémicas, lo cual ha llevado a acuñar el término de "Interferonopatías de tipo I" para hacer referencia colectivamente a estas enfermedades (Crow, 2011;Ronnblom, 2013).

Las evidencias más consistentes de la participación del IFNα en la patogénesis de las enfermedades autoinmunes provienen de los estudios en LES, donde los niveles séricos tanto en pacientes (Preble *et al*, 1982;Willis *et al*, 2012) como en modelos animales están incrementados respecto a la población sana. Del mismo modo, se ha encontrado una expresión más marcada de genes de respuesta a IFN, esto es, de la firma del IFN, en relación con algunos parámetros clínicos de severidad (Baechler *et al*, 2003;Bengtsson *et al*, 2000;Higgs *et al*, 2011). Por todo ello, la neutralización del IFNα podría proporcionar un beneficio clínico a estos pacientes, y actualmente existen fármacos en ensayos clínicos con este objetivo (McBride *et al*, 2012;Merrill *et al*, 2011;Yao *et al*, 2009;Yao *et al*, 2010).

Además de su contribución a los mecanismos patogénicos de la enfermedad, existen evidencias recientes de que el IFNα podría además tener un papel en el daño endotelial en esta patología. Estudios con modelos animales de lupus (Thacker *et al*, 2010b) así como con pacientes (Lee *et al*, 2007) sugirieron una relación entre el daño endotelial y los niveles de expresión de diferentes genes relacionados con la firma del IFN (Denny *et al*, 2007). Curiosamente, el daño y la disfunción endotelial asociados al IFNα en el contexto del LES, tienen como nexo de unión una alteración a nivel de las EPC. Se ha descrito que el IFNα altera los procesos de proliferación, migración y funcionalidad de las EPC *in vitro* y *ex vivo* (Thacker *et al*, 2010a). Además, el IFNα puede reducir la producción de factores proangiogénicos in vitro, promoviendo un fenotipo disfuncional o "no angiogénico" de las EPC (Denny *et al*, 2007;Thacker *et al*, 2010a). Por otro lado, la neutralización del IFNα restaura el fenotipo y la funcionalidad de las EPC aisladas de pacientes de LES (Denny *et al*, 2007), avalando el papel patogénico de esta citocina en el daño endotelial en LES.

Estudios posteriores con modelos animales de lupus, permitieron caracterizar otros mecanismos por los cuales el IFNα puede promover el desarrollo de aterosclerosis en LES (Kaplan & Salmon, 2011). Por último, Somers y colaboradores observaron que la expresión de genes relacionados con la firma del IFN se asociaba con la presencia de disfunción endotelial y aterosclerosis subclínica en pacientes de LES tras ajustar para factores clásicos (Somers *et al*, 2012), confirmando así a nivel clínico la importancia de este mediador en la enfermedad CV en pacientes de LES.

A diferencia del LES, el papel del IFNα en la AR ha sido menos estudiado (Tabla 7). Sin embargo, existen indicios de que éste pueda tener un papel en la patogénesis de la enfermedad. Por un lado, la expresión incrementada de genes de respuesta a IFN en pacientes de LES se ha visto que se asocia a determinados polimorfismos genéticos que están asociados con la susceptibilidad a la enfermedad (Feng *et al*, 2010;Remmers *et al*,

2007;Sigurdsson *et al*, 2008) y que también se ha determinado que confieren susceptibilidad a AR (Kim *et al*, 2013;Lee *et al*, 2013;Remmers *et al*, 2007;Sigurdsson *et al*, 2007). Por otro lado, se ha observado la presencia de firma del IFN en pacientes de AR, tanto en sangre periférica (Bokarewa *et al*, 2008) como en tejidos locales (Higgs *et al*, 2011), si bien en este caso, sólo una fracción de los pacientes muestran esta característica, a diferencia del LES, donde está presente en la práctica totalidad.

Tabla 7: Comparación de la evidencia actual del papel del IFNα en LES y AR

	LES	AR
Asociación de genes relacionados con la firma del IFN con la susceptibilidad a la enfermedad	✓✓✓ (Cunninghame Graham *et al*, 2007;Cunninghame Graham *et al*, 2008;Feng *et al*, 2010;Remmers *et al*, 2007;Sigurdsson *et al*, 2008)	✓✓ (Kim *et al*, 2013;Lee *et al*, 2013;Remmers *et al*, 2007;Sigurdsson *et al*, 2007)
Aparición de síntomas o autoanticuerpos característicos de la enfermedad tras la administración de IFNα	✓✓✓ (;Ioannou & Isenberg, 2000;Niewold & Swedler, 2005)	✓ (Cacopardo *et al*, 2013;Ionescu *et al*, 2008;Johnson *et al*, 1999;Kotter *et al*, 1999;Nesher & Ruchlemer, 1998)
Inducción de la enfermedad en modelos animales por IFNα	✓ (Mathian *et al*, 2005)	✓ (Magnusson *et al*, 2006)
Presencia de firma del IFN en sangre periférica en pacientes (% pacientes)	✓✓✓ (90%) (Baechler *et al*, 2003;Bengtsson *et al*, 2000;Higgs *et al*, 2011)	✓ (25-65%) (Higgs *et al*, 2011;van der Pouw Kraan TC *et al*, 2007;van der Pouw Kraan TC *et al*, 2008)
Niveles séricos de IFNα incrementados	✓✓ (Preble *et al*, 1982;Willis *et al*, 2012)	✓ (Bokarewa *et al*, 2008)
Asociación con parámetros clínicos e inmunológicos	✓✓ (Baechler *et al*, 2003;Bauer *et al*, 2006;Bengtsson *et al*, 2000) (Baechler *et al*, 2004)	✓ (Bokarewa *et al*, 2008;van der Pouw Kraan TC *et al*, 2008)
IFNα como diana terapéutica (fármacos en diseño o ensayos clínicos)	✓ (McBride *et al*, 2012;Merrill *et al*, 2011;Yao *et al*, 2009;Yao *et al*, 2010)	×

En línea con lo anterior, no existen evidencias en la literatura actual de una implicación del IFNα en el daño endotelial en pacientes de AR. Sin embargo, el hecho de que las EPC se encuentren presumiblemente alteradas en número y función en esta patología como se ha comentado, de forma, en cierto modo similar, a lo reportado en LES, podría implicar igualmente a esta citocina en el daño endotelial en RA.

1.3.5. Autoanticuerpos

Habida cuenta del papel de la disregulación inmunitaria en la iniciación y progreso de la enfermedad CV en las patologías reumáticas (Bartoloni *et al*, 2011), y de la etiología inflamatoria de la aterosclerosis (Ross, 1999) cabe pensar en un papel de la respuesta humoral en este contexto. Así, el estudio de este fenómeno ha conducido en los últimos años a sugerir que algunos autoanticuerpos puedan jugar un papel esencial en el daño endotelial y el desarrollo de enfermedades CV (Carbone *et al*, 2013).

En AR, la primera evidencia que permitió relacionar la presencia de autoanticuerpos con el riesgo CV, provenía de estudios epidemiológicos que relacionaron la positividad para FR (Tomasson *et al*, 2010) o anti-CCP (Lopez-Longo *et al*, 2009) con una mayor tasa de eventos CV, así como a una mayor fatalidad de los mismos (Arnab *et al*, 2013;Liang *et al*, 2009). Sin embargo, no se ha reportado en la literatura ningún mecanismo directo mediante el cual estos autoanticuerpos puedan jugar un papel en el desarrollo de estas complicaciones, por lo que la hipótesis más plausible es que la positividad para estos autoanticuerpos refleje una mayor actividad o severidad de la enfermedad (van der Helm-van Mil AH *et al*, 2005), y con ello una mayor carga inflamatoria. De hecho, estudios más recientes parecen no confirmar los resultados anteriores (Arts *et al*, 2015a;Arts *et al*, 2015b), lo cual podría ser debido a que las mejoras en el abordaje terapéutico de esta patología se hayan traducido en un curso más benigno y, por tanto, en una carga inflamatoria de menor orden.

Por otro lado, se ha observado la presencia de otros autoanticuerpos en pacientes de AR que sí pueden estar implicados de una forma más directa en el desarrollo y progresión del daño endotelial (Figura 11).

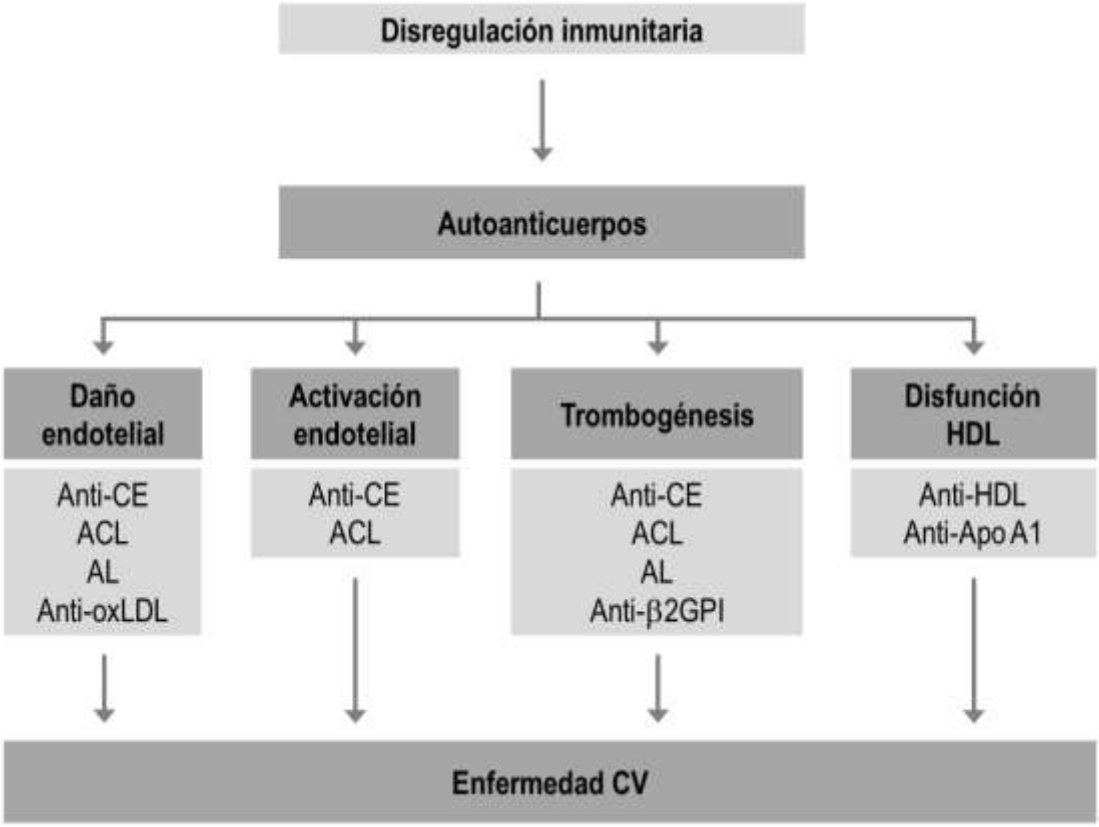

Figura 11 | Mecanismos de acción que relacionan la presencia de autoanticuerpos con el desarrollo de enfermedad CV. Se resumen diferentes mecanismos que pueden ser llevados a cabo por diversos grupos de autoanticuerpos.

Un ejemplo clásico de autoanticuerpo asociado a complicaciones de tipo CV son los anticuerpos anti-fosfolípidos, que incluyen anticuerpos anti-cardiolipina (ACL), anticoagulante lúpico (AL) y anti-β2glicoproteína I (anti-β2GPI). Estos autoanticuerpos pueden provocar un estado de hipercoagulabilidad caracterizado por la aparición de trombosis espontáneas, y además pueden desencadenar daño y activación endotelial de forma directa (Frostegard, 2005). Se ha descrito la presencia de ACL y anti-β2GPI en pacientes de AR en un tercio de los pacientes, aproximadamente (Gladd & Olech, 2009;Olech & Merrill, 2006). La presencia de estos autoanticuerpos se asocia a niveles elevados de homocisteína (Seriolo *et al*, 2001), lo cual está en línea con su asociación con marcadores de aterosclerosis (Sherer *et al*, 2007). No obstante, su contribución como factores independientes no se ha confirmado en estudios posteriores (Holc *et al*, 2011).

Otro tipo de autoanticuerpos relevantes en este escenario son los anticuerpos anti-células endoteliales (anti-CE). Estos anticuerpos son característicos de vasculitis, aunque se han descrito en pacientes de AR con vasculitis secundaria en el 60% de los casos y en un 20% de pacientes de AR sin manifestaciones de vasculitis (Heurkens *et al*, 1989;Westphal *et*

al, 1994). Los autoanticuerpos anti-CE reconocen antígenos presentes en la membrana de las células endoteliales y producen su activación o bien su muerte por apoptosis (Belizna *et al*, 2006;Domiciano *et al*, 2009). El antígeno exacto reconocido por los anti-CE es aún una incógnita, debido a la diversidad de métodos que se emplean para su detección. No obstante, parece ser que no se trata de un solo antígeno, sino que estos anticuerpos podrían reconocer múltiples antígenos e incluso que éstos fueran específicos de cada patología (Praprotnik *et al*, 2001).

Más recientemente, el estudio de autoanticuerpos dirigidos hacia antígenos presentes en las lipoproteínas circulantes ha cobrado cierta relevancia. Las lipoproteínas de alta densidad (*High Density Lipoproteins,* HDL) desempeñan funciones ateroprotectivas que van más allá de su papel en el transporte reverso del colesterol, puesto que protegen a las lipoproteínas LDL de la oxidación mediante la acción del enzima paraoxonasa, reducen la expresión de marcadores de activación endotelial en células endoteliales y son capaces de inhibir la producción de citocinas proinflamatorias (Calabresi *et al*, 2003;Hyka *et al*, 2001), por lo que constituyen una importante defensa frente al daño endotelial y sus consecuencias. Los niveles de HDL están relacionados con la dieta y el estilo de vida en la población general, aunque evidencias recientes parecen indicar que sus niveles, y más particularmente su función, pueden estar alterados en enfermedades sistémicas, aunque los mecanismos exactos no parecen del todo claros en la actualidad (Choy & Sattar, 2009;Robertson *et al*, 2013).

En el año 2002, Delgado y colaboradores observaron que la actividad paraoxonasa en suero estaba reducida en pacientes de LES y que existía una correlación negativa entre la actividad de este enzima y la presencia de anticuerpos anti-HDL de isotipo IgG (Delgado *et al*, 2002). Además, estos autoanticuerpos estaban incrementados en pacientes de LES en comparación con controles sanos y pacientes con síndrome antifosfolípido primario. Un estudio posterior analizó simultáneamente la presencia de autoanticuerpos anti-HDL así como anti-Apolipoproteína A-1, que constituye el principal componente proteico de aquéllas. A pesar de que los resultados estaban de acuerdo con lo esperado, observándose que ambos autoanticuerpos se asociaban con marcadores de estrés oxidativo e inflamación, así como la actividad de la enfermedad, llama la atención que la correlación entre ambos autoanticuerpos (r=0,640) no era tan alta como cabría esperar (Batuca *et al*, 2009). Un artículo posterior confirmó estos hallazgos, observándose en este caso una concordancia del 62% entre ambos autoanticuerpos (O'Neill *et al*, 2010). Asimismo, la presencia de niveles elevados de estos autoanticuerpos se asociaba a enfermedad más severa, si bien no se

encontró una asociación con la presencia de enfermedad CV en estos pacientes (Croca *et al*, 2015).

En el caso de la AR, se había descrito que los niveles elevados de autoanticuerpos anti-Apo A-1 de isotipo IgG se asociaban de forma independiente al desarrollo de eventos CV en el curso de la enfermedad (Vuilleumier *et al*, 2010a). Asimismo, se encontró que los niveles de estos anticuerpos se asociaban a niveles de IL-8 y de metaloproteasa de matriz 9 en suero, los cuales podrían explicar el efecto patogénico de estos autoanticuerpos. Es interesante destacar que, tanto en pacientes de AR como en pacientes con historia de infarto de miocardio, los autoanticuerpos anti-Apo A-1 resultaron los mejores predictores de entre todos los estudiados (Keller *et al*, 2012;Vuilleumier *et al*, 2010a;Vuilleumier *et al*, 2010b).

Sin embargo, no se tienen evidencias en la literatura actual acerca de los autoanticuerpos anti-HDL de isotipo IgG en pacientes de AR, pese a que se ha sugerido que los niveles circulantes de HDL en estos pacientes se encuentran alterados en el curso de la enfermedad, así como en respuesta a algunos tratamientos farmacológicos (Choy & Sattar, 2009;Robertson *et al*, 2013). De hecho, la alteración de los niveles séricos de las lipoproteínas en pacientes de AR en asociación a la actividad de la enfermedad ha llevado a algunos autores a proponer el término *"lipid paradox"*, para hacer referencia a la situación caracterizada por bajos niveles de lipoproteínas circulantes asociados a un contexto de elevado riesgo CV (Myasoedova & Gabriel, 2010), como es la AR. El estudio de este fenómeno ha sido abordado por diferentes autores, con resultados discordantes acerca de su origen y grado (Choy & Sattar, 2009;Gonzalez-Gay & Gonzalez-Juanatey, 2014;Robertson *et al*, 2013). Asimismo, no se conoce el impacto que este perfil lipídico pueda tener sobre los niveles de citocinas proinflamatorias en pacientes de AR.

Objetivos

2. Objetivos

Existen evidencias claras de la contribución de la inflamación crónica y la disregulación inmunitaria al riesgo cardiovascular en AR, aunque los mediadores exactos que subyacen a este fenómeno, no están claros. Teniendo en cuenta los antecedentes previamente mencionados, el objetivo general de la presente Tesis Doctoral es el estudio de nuevos biomarcadores de daño endotelial y riesgo cardiovascular en pacientes de AR.

Para ello, se plantearon los siguientes objetivos concretos:

1. Identificar biomarcadores de daño vascular en AR analizando diferentes mediadores celulares y moleculares asociados con daño o reparación endotelial (EPC, subpoblaciones linfocitarias, micropartículas circulantes citocinas).

2. Determinar posibles biomarcadores pronósticos de riesgo CV que puedan ser fácilmente implementables en la práctica clínica.

3. Investigar el efecto de la terapia con bloqueantes del TNFα sobre los biomarcadores anteriores.

Resultados

3. Resultados

Capítulo I: Estudio del IFNα como biomarcador de daño endotelial

Se ha descrito que el IFNα puede ser un factor determinante en el daño endotelial y el riesgo CV en LES, siendo una alteración en el número y función de las EPC uno de los mecanismos responsables. Sin embargo, no existían evidencias que avalaran este hecho en AR al inicio de esta Tesis Doctoral. Curiosamente, en los últimos años, la hipótesis del IFNα como un mediador patogénico en AR había empezado a tomar fuerza. Asimismo, si bien algunos autores apuntaban a una alteración en el número y función de las EPC en AR, el origen de este proceso no estaba claro. Dada la posible implicación del IFNα en diferentes enfermedades autoinmunes sistémicas, cabe pensar que la alteración de las EPC mediada por el IFNα pueda ser un mecanismo compartido entre diferentes patologías, y no específico del LES.

En un primer estudio, abordamos un análisis comparativo de las distintas poblaciones celulares relacionadas con las EPC en controles sanos y pacientes de LES y AR y analizamos su relación con parámetros clínicos, inmunológicos y niveles de citocinas séricas, con el fin de obtener una idea general de la alteración de las EPC en ambas patologías y analizar sus similitudes y diferencias. A la vista de los resultados, analizamos en profundidad el papel de los niveles séricos de IFNα en relación a las poblaciones de EPC, parámetros clínicos, citocinas circulantes y factores clásicos de riesgo CV en una muestra mayor de pacientes de AR. Por último, llevamos a cabo una revisión bibliográfica que permitió definir los diferentes mecanismos que relacionan al IFNα con la patogenia de la AR, con especial énfasis en su posible papel en el daño vascular, así como en su potencial uso como biomarcador para el manejo clínico de esta patología, de una forma integradora partiendo de la evidencia científica actual.

Los resultados obtenidos en este capítulo han dado lugar a las siguientes publicaciones científicas:

Artículo 1: Rodríguez-Carrio J, Prado C, de Paz B, López P, Gómez J, Alperi-López M, Ballina-García FJ, Suárez A (2012); *Circulating endothelial cells and their progenitors in Systemic Lupus Erythematosus and early Rheumatoid Arthritis patients;* Rheumatology (Oxford) 51(10):1775-84.

Aportación personal al trabajo: me incorporé a este proyecto tras mi llegada al laboratorio, centrándome inicialmente en la finalización del reclutamiento de pacientes de LES y AR y la realización de los inmunoensayos. Asimismo, llevé a cabo el análisis, interpretación y discusión de los resultados en colaboración con el resto de coautores. Por último, trabajé en la redacción del manuscrito y la confección de las figuras que lo acompañan bajo la supervisión de la Dra. Ana Suárez Díaz.

Artículo 2: Rodríguez-Carrio J, de Paz B, López P, Prado C, Alperi-López M, Ballina-García FJ, Suárez A (2013); *IFNα serum levels are associated with Endothelial Progenitor Cells imbalance and disease features in Rheumatoid Arthritis patients;* PLOS One 9(1):e86069.

Aportación personal al trabajo: mi aportación a este trabajo se centró en la realización de las determinaciones por citometría de flujo y los inmunoensayos, así como el análisis e interpretación de los resultados. Finalmente, llevé a cabo la preparación del manuscrito y sus figuras bajo la supervisión de la Dra. Ana Suárez Díaz.

Artículo 3: Rodríguez-Carrio J, López P, Suárez A (2014); *Type I IFNs as biomarkers in Rheumatoid Arthritis: towards personalized medicine and disease profiling;* Clinical Science 128:449-464.

Aportación personal al trabajo: en lo referente a este trabajo, llevé a cabo la búsqueda bibliográfica, su posterior organización y la identificación de las ideas claves a destacar en el trabajo, en colaboración con las coautoras del mismo. Asimismo, llevé a cabo la redacción del manuscrito y preparación de sus figuras bajo la supervisión de la Dra. Ana Suárez Díaz.

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Circulating endothelial cells and their progenitors in systemic lupus erythematosus and early rheumatoid arthritis patients

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Abstract

Objective. The aim of this study was to investigate the endothelial progenitor cell population in SLE and early RA patients and its potential relationships with disease features and cytokine serum levels.

Methods. Endothelial progenitor cells (EPCs), mature EPCs (mEPCs) and endothelial cells (ECs) were measured in peripheral blood samples from 83 SLE and 85 early RA patients and 39 healthy controls by flow cytometry on the basis of CD34, VEGF receptor 2 and CD133 expression. Serum levels of IL-1β, IL-6, IL-8, IL-17, VEGF-A, IFN-α, TGF-β and GM-CSF were quantified by immunoassays. Clinical and immunological data were obtained by reviewing clinical histories.

Results. Circulating EPCs were increased in SLE but not in early RA patients associated with an enhanced $CD34^+$ bone marrow-progenitor cell release but unrelated to disease features. The amount of mEPCs, however, was significantly higher in SLE patients presenting anti-SSA/SSB antibodies and/or malar rash, whereas the presence of specific autoantibodies was associated with EC counts in early RA and SLE patients. As expected, most cytokines tested were altered in both diseases but, interestingly, IFN-α levels, and to a lesser extent IL-6 and IL-1β, were associated with CD133 loss and increased mEPC number, whereas VEGF and TGF-β seem to exert an opposite effect.

Conclusion. Our results show that high IFN-α levels and/or the presence of disease-specific antibodies may identify a group of SLE patients with increased mEPC and EC counts, and consequently probably defective endothelial repair, thus supporting their use as surrogate biomarkers of endothelial damage and high cardiovascular risk.

Key words: systemic lupus erythematosus, rheumatoid arthritis, endothelial progenitor cells, vascular damage, autoantibodies, interferon-α.

BASIC SCIENCE

Introduction

Several studies have demonstrated that most autoimmune patients have an increased cardiovascular risk, leading to high cardiovascular morbidity and mortality rates. However, the accelerated development of atherosclerosis observed in patients with SLE and RA cannot be fully accounted for by traditional Framingham risk factors [1], and it has been proposed that systemic inflammation and immune-mediated disease-related mechanisms could play a pivotal role in the increased cardiovascular risk [2], probably through adhesion molecules and pro-inflammatory cytokine overexpression [3]. New cardiovascular damage biomarkers are needed to improve the cardiovascular health of autoimmune patients.

Vascular endothelial injury, increased in autoinflammatory conditions, is the primary event in atherosclerosis and its regeneration involves endothelial progenitor cell (EPC) mobilization from the bone marrow and their recruitment

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into damaged tissue. EPCs are a heterogeneous population whose physiological role is to carry out vasculogenesis and vascular repair [4]. EPCs express endothelial [VEGF receptor-2 (VEGFR2)] and haematopoietic (CD34 and CD133) cell markers. Nevertheless, there is no consensus on the precise definition of EPC. During differentiation to mature EPCs (mEPCs), CD133 expression is lost and begins to express vascular endothelial (VE)-cadherin and von Willebrand factor [5]. Because of their potential role in vascular repair, the amount of circulating EPCs is considered a surrogate marker for vascular dysfunction and cardiovascular risk, and thus a promising tool in cell therapy for cardiovascular diseases, especially in connective tissue diseases [6].

Variations in the level of circulating EPCs were reported in different conditions affecting the vascular system. Several reports have shown that reduced levels or impaired EPC function correlated inversely with cardiovascular risk factors and cardiovascular outcomes [7, 8]. In the field of autoimmune diseases, contradictory results have been reported. Indeed, some studies suggested a lower EPC number in both SLE [9–13] and RA [14, 15], but conversely, others reported higher values [16, 17], no differences [18] or impaired function [19, 20]. Lack of standardized procedures is probably the main source of variability between studies [21].

Pro-angiogenic factors, such as VEGF, can mobilize EPCs and may potentiate their recruitment to the site of endothelial injury, whereas anti-angiogenic molecules exert an opposite effect. In fact, several pro-inflammatory and immunosupressor cytokines, including IL-1β, IL-6, IL-8, IL-17A, TGF-β, GM-CSF and IFN-α, are involved in EPC mobilization, survival, recruitment or function. Given that most of them have been reported to be deregulated in autoimmune patients, these alterations could be a relevant immune-mediated mechanism involved in vascular damage and EPC dysfunction. In addition, it has been suggested that disease-specific autoantibodies also affect endothelial cells (ECs), causing inflammatory vascular damage and EC apoptosis [2, 22]. Because of the contradictory results reported with regard to circulating EPCs in autoimmune diseases, in this work we determined circulating ECs and their progenitors in SLE and RA patients, and evaluated the potential associations with disease features and serum levels of cytokines relevant for these autoimmune disorders.

Materials and methods

Patients and controls

The study group consisted of 83 SLE patients selected from the Asturian Register of Lupus [23], 85 patients with early RA consecutively recruited from the Early Arthritis Diagnosis outpatient clinic of the Hospital Central de Asturias and 39 sex- and age-matched healthy controls (HCs, 32 women and 7 men; mean age (S.D.) 42.6 (11.3)], all of whom were of Caucasian origin. The ACR criteria were used for the diagnosis of RA and SLE. Information on clinical and immunological manifestations and therapies received was obtained by reviewing clinical histories. SLEDAI and DAS-28 were used to evaluate disease activity in SLE and RA patients. Informed consent was obtained from all participants, and the study was approved by the Regional Ethics Committee for Clinical Investigation from Hospital Universitario Central de Asturias (Oviedo), in compliance with the Declaration of Helsinki. None of the patients or controls had a history of coronary artery disease, myocardial infarction, cardiac insufficiency, statin therapy, cancer, diabetes or smoking habit.

EPC quantification

EPCs were analysed by FACS as described previously, following European League Against Rheumatism (EULAR) Scleroderma Trials and Research (EUSTAR) recommendations on EPC measurement [24]. Briefly, 100 μl of peripheral blood was pre-incubated with 10 μl of FcR blocking reagent (Miltenyi Biotech, Bergisch Gladbach, Germany). Then the cells were incubated with anti-VEGFR2-phycoerythrin (R&D Systems, Minneapolis, MN, USA), anti-CD34-FITC (BD Pharmingen, San José, CA, USA), and anti-CD133-allophycocyanin (Miltenyi Biotech) or with isotype-matched antibodies (BD Pharmingen). Labelled cells were analysed in a BD FACSCanto II flow cytometer with FACSDiva Software acquiring at least 100 000 events per sample. EPCs were identified as triple-positive cells for CD34/VEGFR2/CD133 in the lymphocyte gate. $CD34^+VEGFR2^+CD133^-$ and $CD34^-VEGFR2^+CD133^-$ were identified as mEPCs and ECs, respectively. Cell counts were expressed as the number of positive cells per 100 000 events in the lymphocyte gate.

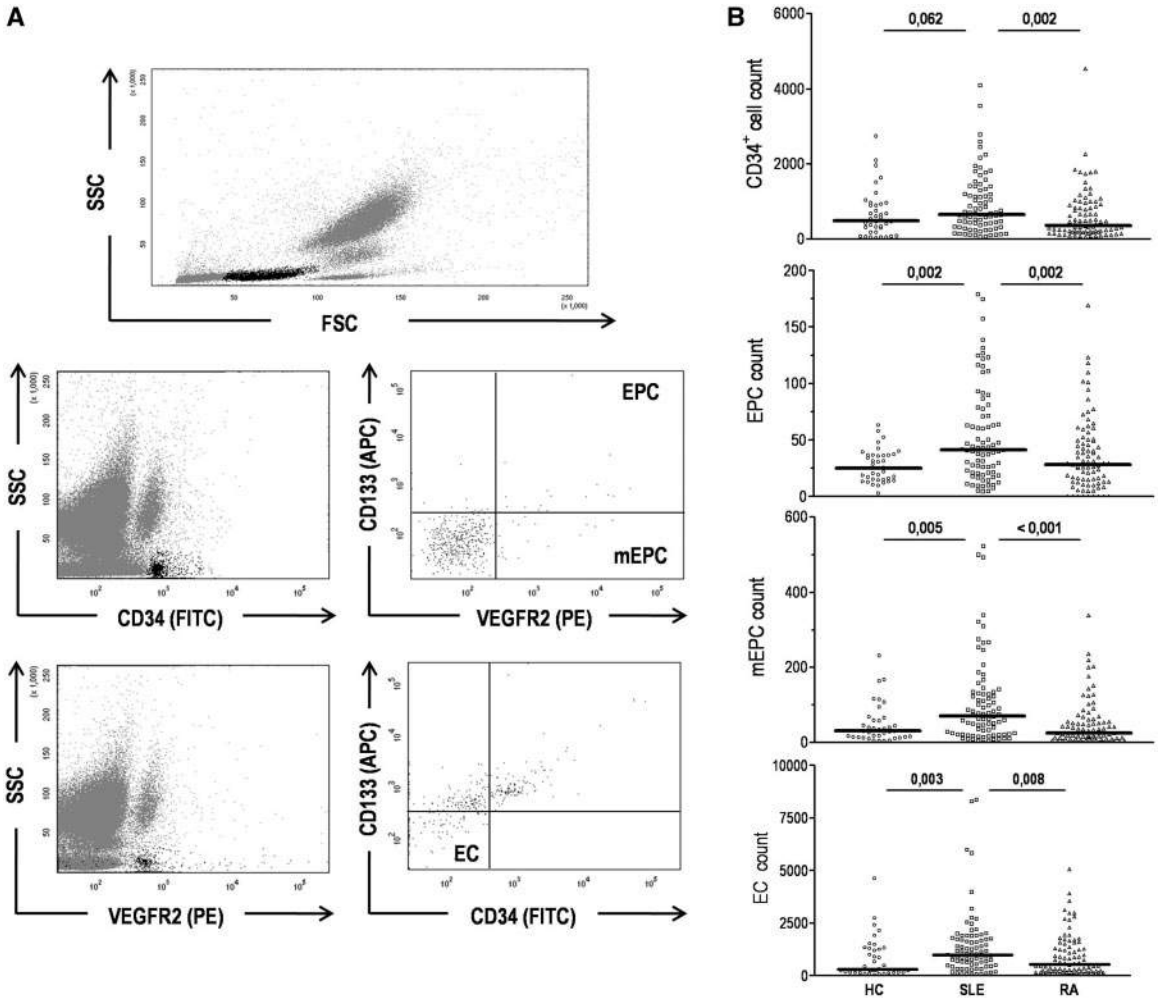
Quantification of cytokine serum levels

Serum samples were stored at −80°C until cytokine measurements. VEGF-A_{165}, IFN-α, GM-CSF, IL-1β, IL-6, IL-8 and IL-17A were quantified using a Cytometric Bead Array Flex Set (BD Biosciences, San José, CA, USA) and analysed in a BD FACS Canto II flow cytometer using FCAP Array software (BD Biosciences). For IL-1β, IL-6 and IL-17A, an Enhanced Sensitivity Flex Set was used. The assay sensitivity was 4.0 pg/ml for VEGF-A_{165}, 1.5 pg/ml for IFN-α, 0.2 pg/ml for GM-CSF, 48.4 fg/ml for IL-1β, 68.4 fg/ml for IL-6, 1.2 pg/ml for IL-8 and 26.1 fg/ml for IL-17A. TGF-β1 was quantified using an ELISA kit (eBioscience, San Diego, CA, USA), according to the manufacturer's instructions, with 5 ng/ml of detection limit.

Statistical analyses

All data were analysed with SPSS software (v.15.0) (IBM, Armonk, NY, USA). The Kolmogorov–Smirnov test was used to evaluate normality. The Mann–Whitney U-test was used to compare differences between groups, and Spearman's rank test was used to evaluate correlations. Data were expressed as median (interquartile range) unless stated otherwise. A P-value of <0.05 was considered statistically significant.

FIG. 1 Quantification of EPC population by flow cytometry.



(**A**) Gating strategy followed for determination of EPCs ($CD34^+VEGFR2^+CD133^+$), mEPCs ($CD34^+VEGFR2^+CD133^-$) and ECs ($CD34^-VEGFR2^+CD133^-$) in patients and controls. Representative flow cytometry analysis based on the expression of CD34, CD133 and VEGFR2 markers in an SLE patient. (**B**) Comparison of total $CD34^+$ progenitor cells, EPC, mEPC and EC counts in HCs (open circles), SLE (open squares) and early RA (open triangles) present in peripheral blood. Cell counts were expressed as number of positive cells per 10^5 events in the lymphocyte gate. Differences were evaluated by the Mann–Whitney U-test.

Results

Circulating $CD34^+$, EPC and EC populations in SLE and RA patients

The presence of ECs and their progenitors in the peripheral circulation of 39 HC, 83 SLE and 85 early RA patients (23 recruited at diagnosis) was analysed by flow cytometry following the gating strategy shown in Fig. 1A. After selecting the lymphocyte population, $CD34^+$ cells were gated and assessed for CD133 and VEGFR2 expression. Triple-positive cells were considered EPCs and $CD34^+VEGFR2^+CD133^-$ cells mEPCs. On the other hand, $VEGFR2^+$ cells on the lymphocyte gate were selected and those lacking CD34 and CD133 expression were considered ECs detached from the endothelial wall.

Tables 1 and 2 show demographic characteristics and disease parameters of SLE and RA patients, respectively. Total $CD34^+$ cells and levels of circulating EPCs, mEPCs and ECs are shown in Fig. 1B. No statistical differences between patients and HCs were detected in the $CD34^+$ population; however, the SLE group tended to have an increased proportion of these cells ($P = 0.062$), being statistically significant when compared with RA ($P = 0.002$). Interestingly, a trend to reduce the $CD34^+$ population was observed in RA related to disease progression, as patients at onset ($n = 23$) had significantly higher amounts than those recruited after diagnosis [674 (574)/10^5 *vs* 313 (725)/10^5, $P = 0.020$], despite the short duration of the disease [26 (23.75) months].

On the other hand, SLE patients exhibit higher EPC and mEPC absolute counts than HCs, whereas no significant

Table 1 Characteristics and disease parameters of SLE patients

Total SLE patients	83
Sex (female/male), *n*	79/4
Age at diagnosis, mean (S.D.), years	35.7 (14.5)
Disease duration, mean (S.D.), years	12.3 (8.9)
Clinical features, *n* (%)	
Malar rash	36 (43.4)
Discoid lesions	9 (10.8)
Photosensitivity	41 (49.4)
Oral ulcers	30 (36.1)
Arthritis	51 (61.4)
Serositis	12 (14.5)
Nephritis	24 (28.9)
Neurological disorders	6 (7.2)
Haematological disorders	48 (57.8)
SLEDAI, mean (S.D.)	4.02 (4.11)
Positivity of anti-dsDNA	51 (61.4)
Anti-dsDNA levels, mean (S.D.), U/ml	19.34 (30.69)
Positivity of anti-Sm	7 (8.4)
Positivity of anti-SSA	27 (32.5)
Positivity of anti-SSB	13 (15.7)
Positivity of anti-RNP	10 (12.0)
Treatment, *n* (%)	
None or NSAIDs	15 (18.1)
Anti-malarials	54 (65.1)
Glucocorticoids	28 (33.7)
Immunosupressive drugs[a]	13 (15.7)

[a]AZA, CSA, MMS or CYC.

Table 2 Characteristics and disease parameters of RA patients

Total RA patients	85
Sex (female/male), *n*	74/11
Age at diagnosis, mean (S.D.), years	52.9 (15.1)
Disease duration, mean (S.D.), years	2.2 (1.7)
Clinical features, mean (S.D.).	
Number of tender joints	5.29 (5.38)
Number of swollen joints	2.63 (3.42)
Patient global assessment (0–100)	33.21 (22.87)
DAS-28	3.83 (1.67)
HAQ	6.06 (5.53)
CRP, md/dl	0.43 (0.64)
ESR, mm/h	24.11 (20.49)
RF positivity, *n* (%)	41 (48.2)
Anti-CCP positivity, *n* (%)	43 (50.6)
Treatments, *n* (%)	
None or NSAIDs	19 (22.4)
Glucocorticoids	36 (42.4)
MTX	51 (61.5)
LEF	15 (18.0)
TNF-α blockers	7 (8.4)

differences were detected in RA. However, both populations decreased after RA diagnosis [EPC 47.5 (42.9) *vs* 24.9 (35.3), $P = 0.020$; mEPC 52.9 (65.9) *vs* 19.8 (25.2), $P = 0.010$]. Interestingly, $CD34^+$ cells exhibited a positive correlation with EPC levels (SLE: $r = 0.487$, $P < 0.001$; RA: $r = 0.573$, $P < 0.001$; HC: $r = 0.278$, $P = 0.079$), so there were no significant differences in the percentage of circulating EPCs out of $CD34^+$ cells between the patients and the controls [SLE: 6.20 (10.40), RA: 6.31 (8.77); HC: 4.92 (7.02)]. However, the frequency of mEPCs out of $CD34^+$ cells was slightly increased in SLE, being statistically significant when compared with RA [SLE: 10.31 (14.24); RA: 6.49 (6.16); HC: 7.38 (13.57); SLE *vs* RA $P < 0.001$]. Finally, SLE patients exhibited significantly higher EC counts than HCs, whereas no significant differences were detected in RA (Fig. 1B), although, once again, patients at diagnosis presented increased levels [1220 (1362) *vs* 444 (1293), $P = 0.002$].

All these data suggest an enhanced bone marrow-progenitor cell release, which include EPCs, in patients with SLE, whereas these populations seem to be influenced by disease duration in RA. Additionally, the noticeably enhanced absolute levels of circulating mEPCs and ECs in SLE support the existence of systemic vascular damage.

Autoantibody status was associated to mEPC and EC levels

Next, we wanted to determine whether alterations in the size of the $CD34^+$, EPC or EC population was associated with a specific disease phenotype or treatment followed. Regarding $CD34^+$ cells, no associations were detected with clinical or immunological features, but SLE patient users of anti-malarial treatment (alone or in combination) presented higher levels compared with non-users [802 (975) *vs* 353 (124), $P = 0.018$].

On the other hand, despite the increased EPC numbers detected in SLE, they were unrelated to disease characteristics or treatments. In early RA, a trend to increase this population was observed in patients with glucocorticoids ($n = 36$, $P = 0.068$) and TNF-α blockers ($n = 7$, $P = 0.08$).

An interesting association was observed in the case of mEPCs in SLE because this population was higher in anti-SSA/B-positive patients as well as in those presenting malar rash when compared with their negative counterparts or with HCs (Fig. 2A). Moreover, we observed a trend towards increased $CD34^+$ release but not EPC number, indicating that bone marrow precursors could be prematurely differentiated towards mEPCs. The analysis of early RA patients did not show any significant relationship with the amount of mEPCs.

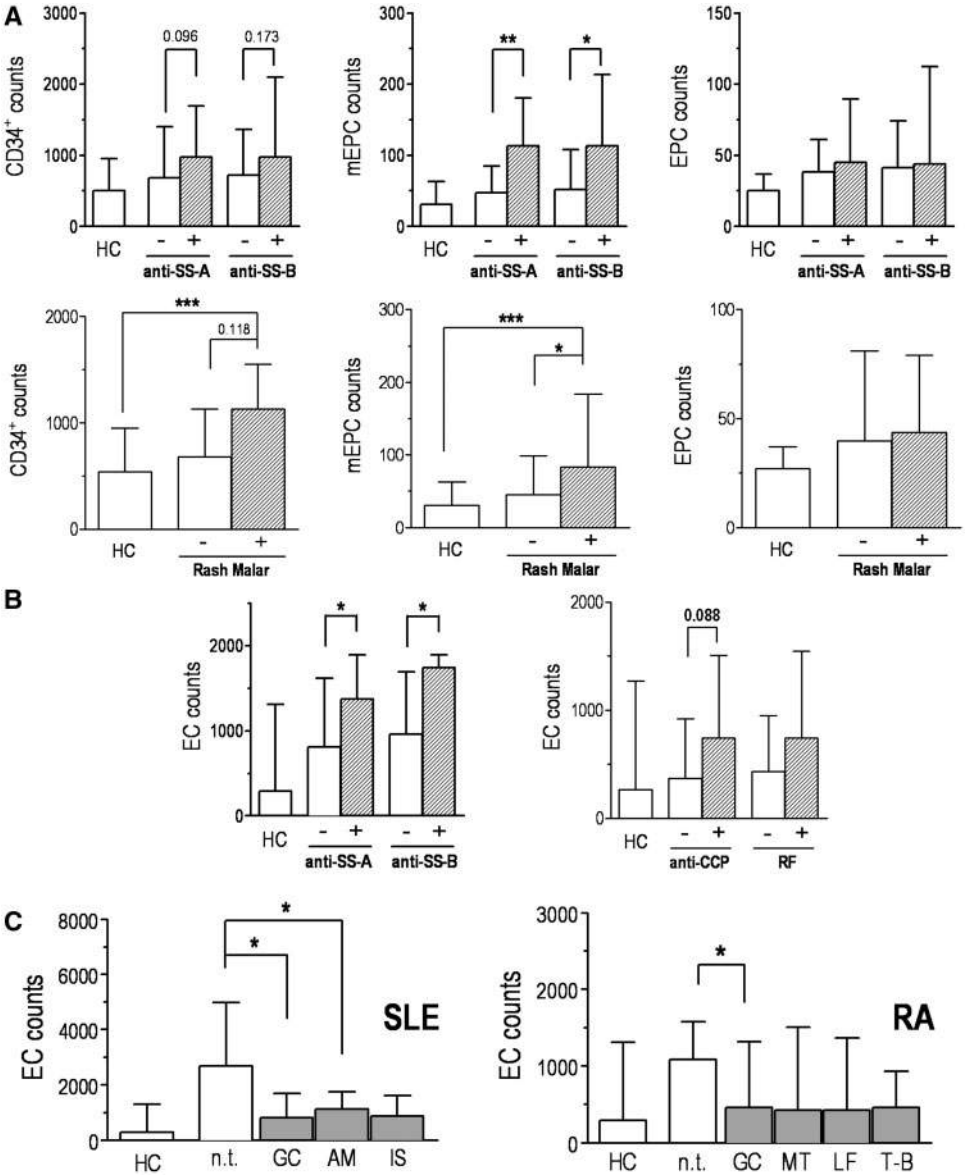
Study of the EC population supports their relevance as a biomarker of endothelial damage (Fig. 2B). The amount of circulating ECs was higher in anti-SSA/B-positive SLE patients, and there was a trend in early RA patients with anti-CCP antibodies ($P = 0.080$). Looking at the effects of pharmacological treatments, both SLE and early RA untreated patients showed the highest EC levels (Fig. 2C), but no significant differences were detected between treatments. Of note, the EC number exhibited a positive correlation with disease activity in early RA (DAS-28: $r = 0.259$, $P = 0.040$) and a clear trend with anti-DNA titre in SLE ($r = 0.243$, $P = 0.057$) patients. Other autoantibodies failed to display similar associations with the studied populations.

IFN-α levels were related to an increased mEPC population

To evaluate the possible relationship between EPC populations and cytokines involved in neovascularization and usually altered in autoinflammatory disorders, we quantified serum levels of VEGF-A_{165}, IFN-α, TGF-β, IL-1β, IL-6, IL-8, IL-17 and GM-CSF in patients and controls at the time of EPC determination (Fig. 3). IFN-α was undetectable in most HCs, and IL-17 was only detected in 5 HC, 15 SLE and 35 early RA samples.

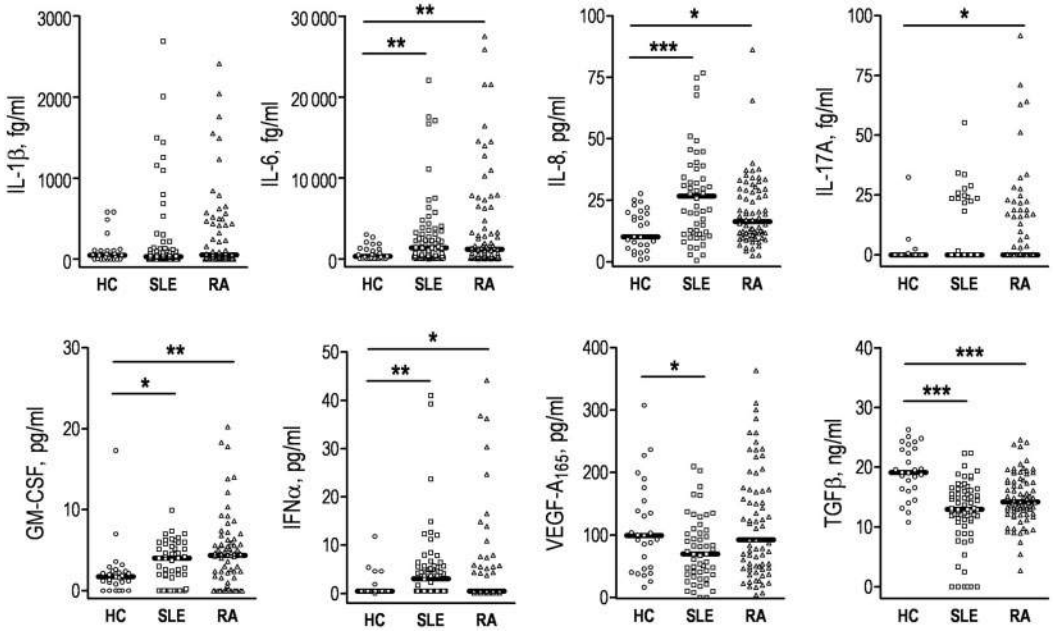
Serum levels of VEGF were decreased in SLE but not in early RA patients compared with HCs. Despite its vasculogenic potential, no association with EPC counts was found in any disease condition. Similarly, TGF-β levels, decreased in both diseases, did not associate with the number of EPCs. In contrast, serum levels of both molecules were negatively correlated with mEPCs in early RA patients (VEGF: $r = -0.364$, $P = 0.002$; TGFβ: $r = -0.432$, $P < 0.001$), thus suggesting a protective role. Curiously, IL-17 levels, increased in early RA, were strongly

Fig. 2 Association between mEPC and EC populations and disease features.



(**A**) CD34+, mEPC and EPC levels in SLE patients presenting anti-SSA/B autoantibodies and malar rash. (**B**) EC levels in SLE and early RA patients with or without disease-specific autoantibodies. (**C**) Circulating ECs in SLE and early RA patients without treatment (n.t.) or after at least 3 months of treatment with glucocorticoids (GC), anti-malarials (AM), MTX (MT), immunosuppressive drugs (IS), LEF (LF) or TNF-α blockers (T-B) alone or in combination. Differences were evaluated by the Mann–Whitney U-test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.0001$.

Fig. 3 Cytokine serum levels in SLE and early RA patients.



VEGF-A_{165}, IFN-α, GM-CSF, IL-1β, IL-6, IL-8 and IL-17A serum levels were quantified using a Cytometric Bead Array Flex Set. For IL-1β, IL-6 and IL-17A, an Enhanced Sensitivity Flex Set was used. TGF-β serum levels were quantified using ELISA techniques. Levels of the cytokines tested were compared between HCs (open circles) and patients with SLE (open squares) and early RA (open triangles) by the Mann–Whitney U-test. *$P < 0.05$, **$P < 0.001$, ***$P < 0.0001$.

associated with VEGF in all individuals (HC: $r = 0.520$, $P = 0.005$; SLE: $r = 0.561$, $P < 0.001$; RA: $r = 0.472$, $P < 0.001$).

The most noticeable result was obtained by analysing IFN-α, a cytokine playing a central role in SLE and strongly up-regulated in these patients. However, early RA patients also showed increased levels of IFN-α, which were associated with disease activity (DAS-28: $r = 0.282$, $P = 0.028$). Interestingly, as shown in Fig. 4A, serum levels of IFN-α correlated positively with $CD34^+$ and mEPC populations in both diseases, but not with EPC, suggesting a specific role on mEPC premature differentiation. In fact, IFN-α increases in parallel with the mEPC/EPC ratio in early RA ($r = 0.234$, $P = 0.048$) and SLE ($r = 0.228$, $P = 0.061$), a balance indicative of vasculogenic EPC capability, suggesting the involvement of this cytokine in CD133 loss and EPC maturation. In addition, IFN-α levels were associated with EC number in both diseases, thus supporting the pathogenic role of this cytokine.

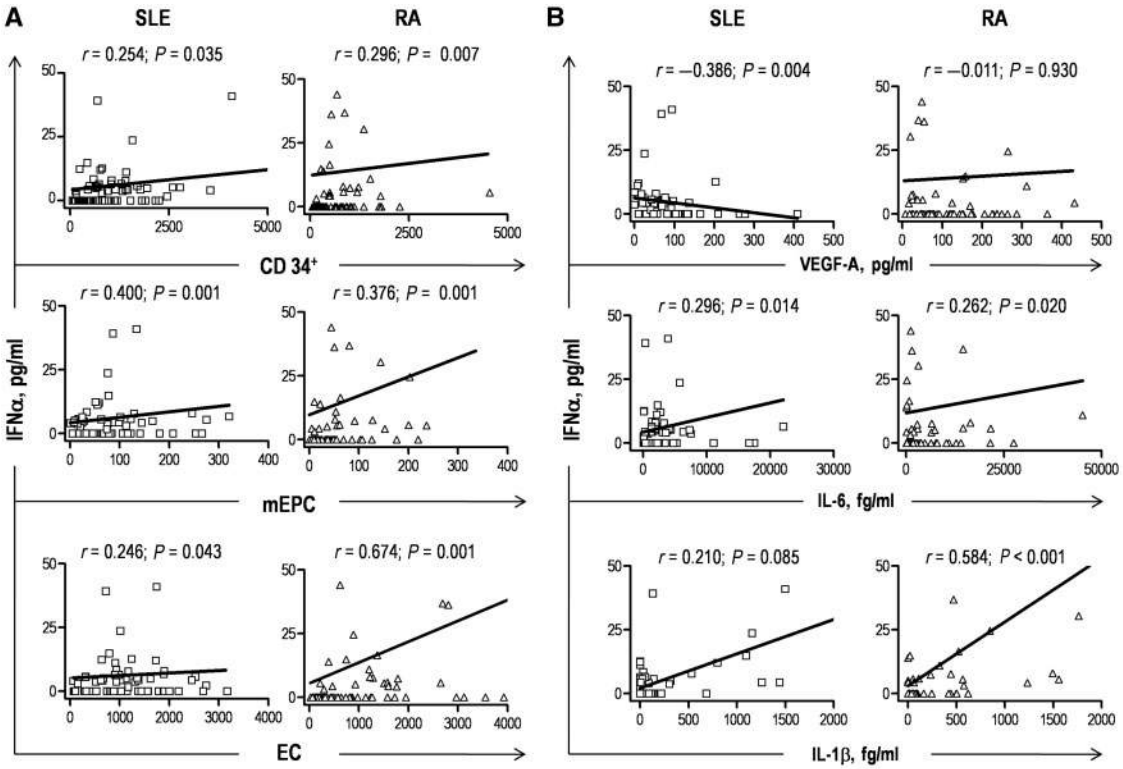
It was remarkable that in SLE patients, IFN-α increased at the same time as VEGF decreased ($r = -0.386$, $P = 0.004$), whereas no correlation was detected in HCs or early RA patients, in which VEGF was found at normal levels (Fig. 4B). On the contrary, IFN-α correlates positively with IL-6, a cytokine significantly augmented in both diseases. Furthermore, IL-6 correlated positively with $CD34^+$cells in all individuals (HC: $r = 0.533$, $P = 0.004$; SLE: $r = 0.263$, $P = 0.029$; early RA: $r = 0.235$, $P = 0.036$) and seemed to exhibit a similar ability to IFN-α in increasing the mEPC/EPC ratio in SLE ($r = 0.303$, $P = 0.012$). In the same way, IFN-α correlated directly with IL-1β in early RA and showed a trend in SLE patients. This cytokine, not significantly altered in patients, was also related to increased mEPC counts in both SLE ($r = 0.233$, $P = 0.05$) and early RA patients ($r = 0.301$, $P = 0.007$) and increased in parallel with the mEPC/EPC ratio in SLE ($r = 0.264$, $P = 0.03$).

Finally, IL-8 and GM-CSF levels were mutually correlated in SLE ($r = 0.313$, $P = 0.022$) and early RA ($r = 0.392$, $P = 0.001$) and significantly augmented in both diseases, but did not exhibit any significant relationship with EPC populations. Of note, both cytokines showed a strong positive correlation with IL-17 levels in SLE (GM-CSF: $r = 0.470$, $P < 0.001$; IL-8: $r = 0.559$, $P < 0.001$) and early RA (GM-CSF: $r = 0.655$, $P < 0.001$; IL-8: $r = 0.584$, $P < 0.001$), whereas IL-8 was directly correlated with VEGF in all groups (HC: $r = 0.741$, $P < 0.001$; SLE: $r = 0.483$, $P < 0.001$ and RA: $r = 0.562$, $P < 0.001$). All these results confirmed significant alterations in cytokine levels in both the diseases and suggest that IFN-α, and to a lesser extent IL-6 and IL-1β, are associated with increased EPC maturation and mEPC/EPC ratio, whereas VEGF and TGF-β seem to exert the opposite effect.

Discussion

Despite the current advances in EPC biology and its relevance in vascular homeostasis [5, 21], the most adequate

FIG. 4 IFN-α associated with circulating mEPC and EC counts and with VEGF and cytokine levels.



Correlation between the amount of IFN-α in the serum of SLE and early RA patients with (**A**) mEPC and EC counts and (**B**) serum levels of VEGF, IL-6 and IL-1 β. Statistical analyses were performed by Spearman's rank correlation test.

immunophenotyping technique remains unclear. In the present study, we included CD34 and CD133 as haematopoietic progenitor cell markers, and VEGFR2 as a specific endothelial-lineage marker, expressed by ECs in all differentiation stages [5]. We considered EPCs as triple-positive for these markers, in accordance with other authors [18, 25], whereas we contemplated the loss of CD133 as a sign of cell differentiation and maturation [5], giving rise to mEPCs. The use of these markers after pre-staining with a blocking reagent and a correct quantification by FACS, as performed in our study, are the most important recommendations of EUSTAR on EPC measurements [24].

This study showed an increased absolute number of circulating EPCs in patients with SLE, which was directly correlated with total $CD34^+$ counts, suggesting an enhanced release of precursor cells from the bone marrow, probably, in some way, because of the secretion of soluble factors in response to systemic inflammation. In fact, serum levels of IL-6 and IFN-α were associated with an increase in the amount of circulating $CD34^+$ precursors. It has been reported that the elevated levels of nitric oxide (NO) present in the serum of autoimmune patients, which could be enhanced by IFN-α [26], can act on integrins, reducing their affinity to different ligands and leading to cell detachment from the bone marrow [27, 28]. Accordingly, reduced NO availability correlated with decreased numbers of EPCs in RA patients [15]. In addition, enhanced EPC release could be a repair response to the disseminated endothelial damage present in SLE patients [27].

Because different protocols have been used for EPC quantification in the studies performed with SLE patients, published results are heterogeneous and contradictory [21]. Only Grisar *et al.* [18] analysed $CD34^+VEGFR2^+$ $CD133^+$ cells, in a smaller SLE group ($n = 31$), showing no differences in EPC counts between HCs and SLE patients. In agreement with our results, other works reported high EPC levels, although they have not used the VEGFR2 marker [18, 29]. In contrast, some authors found reduced levels of $CD34^+VEGFR2^+$ [11, 13, 32] or $CD133^+VEGFR2^+$ cells [10]. Data about EPCs in RA seem to be less conflictive, as most authors reported normal or reduced levels [14, 15, 32]. Although our RA patients presented significantly less $CD34^+$, EPCs and mEPCs than those with SLE, results did not reflect significant differences with controls. The relatively short disease duration of our RA cohort, as compared with other studies, could be an important source of discrepancies. In fact, we showed that all these populations tend to decrease after diagnosis. Of note, $CD34^-VEGFR2^+CD133^+$ cells, considered as pre-EPC [33], were significantly reduced in RA patients

compared with both controls ($P = 0.049$) and SLE patients ($P = 0.017$).

In addition to SLE, increased EPC counts have been detected in other rheumatic diseases [34–36]. It could seem paradoxical that in autoimmune diseases, characterized by a high risk of cardiovascular events, a cell population involved in maintaining vascular homeostasis was elevated rather than diminished. However, a defective functionality has been reported in EPCs from SLE patients, exhibiting accelerated senescence [13, 20], impaired migration or adhesive properties [18, 19] and reduced cluster formation [9]. The elevated IFN-α serum levels and/or the pro-inflammatory environment usually present in these patients could act on the EPC population, modulating their differentiation or impairing their functionality. Consequently, a deficient vascular repair could enhance progenitor cell release from bone marrow.

The quantification of CD133 EPCs (mEPCs) as a separate EPC population performed in this work allows us to achieve relevant results and supports the relevance of CD133 labelling for determining the true EPC population. Accordingly, a poor correlation has been reported between $CD34^+VEGFR2^+CD133^+$ and $CD34^+VEGFR2^+$ cells [37]. It has been proposed that mEPCs represent a mature subset of EPCs with little or no vasculogenic and/or repair capability [5]. In our work, this subset was significantly increased in SLE patients depending on their autoantibody status, as only anti-SSA and/or anti-SSB-positive patients presented increased levels of these cells. Furthermore, malar rash, associated with the presence of anti-SSA/B [38, 39], was the unique clinical feature associated with high mEPC counts. Accordingly, anti-SSA antibodies may isolate a subset of patients at higher risk of multiorgan vasculopathy [38] and were associated with increased intima–media thickness [31]. In line with this, a recent study by Kahlenberg *et al.* [40] showed that anti-SSA-positive SLE patients displayed an enhanced endothelial progenitor dysfunction due to high IL-18 levels and was associated with an inflammasome activation linked to IFN-α. All these results support the use of these autoantibodies as biomarkers of defective vascular repair in SLE patients.

An interesting result of this work was the close relationship between IFN-α levels and mEPC counts, observed not only in SLE, but also in early RA patients, presenting a slight increase in IFN-α as well. Therefore our data suggest that IFN-α may play a role in bone marrow progenitor release and premature EPC differentiation towards the mEPC subpopulation and the consequent failure in endothelial repair. It has been reported that IFN-α could contribute to vascular damage and development of atherosclerosis in SLE by acting on EC apoptosis [20], platelet function [41], foam cell and monocyte recruitment [42] and, according to our results, the EPC population, impairing function, promoting apoptosis and leading to an 'antiangiogenic signature' [10, 11, 40, 43]. On the other hand, Thacker *et al.* [43], using a genomic approach, proposed that IFN-α could impair vascular repair on damaged endothelium by down-regulating VEGF expression, thus explaining the strong negative correlation observed in our SLE patients between IFN-α and VEGF levels.

Our results suggest that cytokines other than IFN-α, for instance, IL-6 and IL-1β, could also promote EPC maturation, whereas TGF-β and VEGF, probably involved in the maintenance and function of EPCs, may exert an opposite effect. Accordingly, Thacker *et al.* [43] showed that addition of IL-1β to EPC cultures from SLE patients increased the number of mature ECs. However, our results suggest that the repressive effects of IFN-α on IL-1β in EC cultures reported by Thacker *et al.* could be masked *in vivo* by the pro-inflammatory environment of autoimmune patients, specially the cytokine background, as a negative correlation between IL-1β and IFN-α serum levels was not found. IL-6, linked to endothelial dysfunction in SLE [44] and RA [45], could disturb vascular repair by impairing EPC differentiation. On the other hand, despite the reported angiogenic role of IL-8 [46], GM-CSF [47] and IL-17A [48], we have not found any relationship between these cytokines and EPC populations. However, the strong positive correlation observed with VEGF leads us to hypothesize that they could be acting indirectly on EPC maintenance, up-regulating this growth factor.

Finally, EC results support their previously proposed use as a systemic endothelial damage biomarker. Actually these cells were increased in SLE patients with anti-SSA/B as well as in early RA patients presenting anti-CCP antibodies, in accordance with the reported association of these autoantibodies with subclinical atherosclerosis [37, 39, 49]. Thus the autoantibody profile and IFN-α levels, also associated with ECs, could serve in clinical practice to determine patients with elevated cardiovascular risk. It should be noted that, although ECs are usually identified by positive labelling for CD31 or CD146 markers, it has been shown that the majority of $CD133^-$ cells are $CD146^+$ [50]; thus we think that endothelial mature-specific labelling could be obviated, as the negative labelling for progenitor cells performed ($CD34^-VEGFR2^+CD133^-$) an adequate estimation of ECs detached from the endothelial wall.

In conclusion, this work shows that high IFN-α levels and/or the presence of anti-SSA/B antibodies may identify a group of SLE patients with increased mEPCs and, consequently, defective endothelial repair. The observed relationship between this population and IFN-α supports the use of therapeutic intervention blocking this cytokine in patients at high cardiovascular risk, thus proposing circulating IFN-α levels as a potential biomarker of endothelial repair status and responsiveness to anti-IFN-α therapy. Although the main limitation of this work is the lack of functional assays on patient EPCs to verify our hypothesis, this is the first study in which EPC, mEPC and EC populations were quantified and associated with disease features just as with cytokines involved in neovascularization or pathogenesis of SLE and RA, analysing >80 patients with each disease, being one of the largest EPC quantification studies in autoimmune diseases.

Rheumatology key messages

- Circulating mEPCs are increased in SLE patients with anti-SSA/B antibodies.
- IFN-α could promote EPC maturation in SLE and early RA patients.
- Disease-specific autoantibodies are associated with increased ECs in SLE and early RA.

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IFNα Serum Levels Are Associated with Endothelial Progenitor Cells Imbalance and Disease Features in Rheumatoid Arthritis Patients

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Abstract

Introduction: IFNα has been largely implicated in the ethiopathogenesis of autoimmune diseases but only recently it has been linked to endothelial damage and accelerated atherosclerosis in autoimmunity. In addition, proinflammatory conditions are supposed to be implicated in the cardiovascular status of these patients. Since a role for IFNα in endothelial damage and impaired Endothelial Progenitor Cell (EPC) number and function has been reported in other diseases, we aimed to evaluate the potential associations of IFNα serum levels on EPC populations and cytokine profiles in Rheumatoid Arthritis (RA) patients.

Methods: pre-EPC, EPC and mature EPC (mEPC) populations were quantified by flow cytometry analyzing their differential CD34, CD133 and VEGFR2 expression in blood samples from 120 RA patients, 52 healthy controls (HC), and 83 systemic lupus erythematosus (SLE) patients as disease control. Cytokine serum levels were measured by immunoassays and clinical and immunological data, including cardiovascular (CV) events and CV risk factors, were retrospectively obtained by reviewing clinical records.

Results: Long-standing, but not recent onset RA patients displayed a significant depletion of all endothelial progenitor populations, unless high IFNα levels were present. In fact, the IFN^{high} RA patient group (n = 40, 33%), showed increased EPC levels, comparable to SLE patients. In addition, high IFNα serum levels were associated with higher disease activity (DAS28), presence of autoantibodies, higher levels of IL-1β, IL-6, IL-10 and MIP-1α, lower amounts of TGF-β, and increased mEPC/EPC ratio, thus suggesting higher rates of endothelial damage and an endothelial repair failure. Finally, the relationship between high IFNα levels and occurrence of CV events observed in RA patients seems to support this hypothesis.

Conclusions: IFNα serum marker could be used to identify a group of RA patients with increased disease activity, EPC imbalance, enhanced proinflammatory profile and higher cardiovascular risk, probably due, at least in part, to an impaired endothelial repair.

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Introduction

Rheumatoid Arthritis (RA) is associated with increased cardiovascular (CV) disease morbidity and mortality rates that cannot be explained by traditional risk factors [1,2]. Moreover, endothelial dysfunction, the main cause of premature atherosclerosis, has been found even in young RA patients without traditional CV risk factors [3], thus suggesting the involvement of disease-related pathways.

Endothelial damage leads to denuded sites at the endothelial wall that must be repaired. In this sense, bone marrow-derived Endothelial Progenitor Cells (EPCs) carry out vasculogenesis and endothelial repair functions, contributing to vascular homeostasis [4]. Although there is no consensus on their precise phenotypic definition, functional EPC are characterized by the expression of Vascular Endothelial Growth Factor Receptor-2 (VEGFR-2 or CD309), CD34 and CD133 [5,6]; whereas those lacking CD34 expression are considered a pre-EPC subpopulation [7]. During EPC differentiation, CD133 expression is lost and they begin to express mature endothelial-specific markers, becoming mature EPC (mEPC) with lower vasculogenic functionality [6]. As endothelial status depends on injury and repair, the balance between EPC populations could be a surrogate marker which may be used as a potential CV risk factor. In fact, some studies have shown that circulating EPC could serve as a predictor of CV

events in several conditions [8,9]. EPC studies in RA patients, however, are contradictory.

On the other hand, disease-related risk factors have been identified [10,11], suggesting that immune dysregulation could play a role in RA endothelial damage. Although the specific pathways remains unclear, a number of inflammatory and immune mediators seem to have a role, including C-reactive protein (CRP), cytokines, chemokines and growth factors [12,13], most of them dysregulated in RA patients and implicated in the pathogenesis of autoimmune diseases. Among these mediators, it is worth noting the case of IFNα, since type-I interferons play a role in the pathogenesis of SLE and probably other autoimmune diseases [14], and recent evidence suggests their involvement in endothelial damage and EPC dysfunction. It has been reported that IFNα impair EPC function *in vitro* as well as *in vivo* and, as a consequence, endothelial repair [15–18]. Moreover, type I IFNs have been linked to atherothrombosis by acting on platelets and foam cells [19]. In addition, IFNα-signature has been linked to vasculopathy in systemic sclerosis patients [20].

Since previous studies suggest that circulating EPC populations and type I IFNs could be involved in increasing cardiovascular risk in autoimmune diseases, the main aim of this study is to determine EPCs frequency in RA patients' peripheral blood and evaluate the potential associations with IFNα serum levels and clinical and immunological features.

Patients and Methods

Patients and Controls

Our study involved 120 RA patients fulfilling the 1987 revised criteria of the American College of Rheumatology, recruited from the Rheumatology outpatient clinic of the Hospital Universitario Central de Asturias, and 52 sex- and age-matched unrelated healthy controls (47 women, age (mean±SD): 44.74±11.04 years). Eighty-three SLE patients (79 women, age: 48.28±16.30 years, disease duration: 12.3±8.9 years, SLEDAI: 4.02±4.11) were included as disease controls. Routine clinical examination, information on clinical and immunological manifestations, therapies received in the previous three months and 28-joint disease activity score (DAS28) were obtained at the time of sampling. Clinical response to anti-TNFα therapy, in a six-month period, was analyzed using EULAR response criteria [21]. Patients were classified on having a "good" "moderate" or "no response" according to DAS28 change from baseline (6-month previous clinical visit). Patients' clinical records were exhaustively revised in order to register the history of CV events and traditional CV risk factors (diabetes mellitus, hypercholesterolemia, hypertension and smoking habits). A CV event was considered if the patient suffered from heart failure, ischemic heart disease, cerebrovascular accident or peripheral arteriopathy from their RA diagnosis. Clinical definition of CV events and risk factors was performed as previously stated [22,23].

Ethics Statement

Approval for the study was obtained from the Regional Ethics Committee for Clinical Investigation (Servicio de Salud del Principado de Asturias, Hospital Universitario Central de Asturias), according to the Declaration of Helsinki. All procedures were performed with an informed written consent from all individuals.

Flow Cytometry EPCs Quantification

Blood samples were immediately transported to the laboratory and processed. EPC were analyzed by FACS as described previously [24], following EUSTAR recommendations [25] with few modifications. Briefly, 100 µl of peripheral blood were preincubated with 10 µl of FcR Blocking Reagent (Miltenyi Biotech) for 20 minutes, followed by 30-minutes triple-labelling with anti-VEGFR2-phycoerythrin (PE, R&D Systems), anti-CD34-fluorescein isothiocyanate (FITC, BD Pharmigen) and anti-CD133-allophycocyanin (APC, Miltenyi Biotech) or with identical isotype antibodies (BD Pharmigen). Labelled cells were lysed with 2 ml BD Lysing Solution (BD Biosciences) for 5 minutes and washed twice with PBS. Finally, samples were analyzed in a BD FACSCanto II flow cytometer. After gating the lymphocyte population, CD34-positive events were selected and analyzed in a CD133 *vs.* VEGFR2 dot plot, thus considering CD34/VEGFR2/CD133 triple-positive cells as EPCs while $CD34^+VEGFR2^+CD133^-$ cells were identified as mature EPCs (mEPCs) [24] (Figure 1A). On the other hand, VEGFR2-positive events within the lymphocyte gate were analyzed for CD34/CD133 expression and $CD34^-VEGFR2^+CD133^+$ cells were considered as pre-EPCs. At least 100,000 events in the lymphocyte gate and more than 100 $CD34^+$ cells were acquired per sample. Cell counts were expressed as the number of positive cells per 100,000 events in the lymphocyte gate.

Cytokine Serum Levels Quantifications

Serum aliquots were stored at −80°C until cytokine immunoassay measurement. Levels of IL-1β, IL-6, IL-8, IL-10, IFNα, MIP-1α (CCL3) and VEGF-A_{165}were quantified using a Cytometric Bead Array Flex Set (BD) and analyzed in a BD FACS Canto II flow cytometer using FCAP Array v.1.0.1. For IL-1β, IL-6 and IL-10, an Enhanced Sensitivity Flex Set was needed. Technical detection limits were 48.4 fg/ml for IL-1β, 68.4 fg/ml for IL-6, 1.2 pg/ml for IL-8, 13.7 fg/ml for IL-10, 1.5 pg/ml for IFNα, 0.2 pg/ml for MIP-1α and 4.0 pg/ml for VEGF-A_{165}. TGF-β1 and TNFα serum levels were quantified using ELISA kits (OptEIA, BD Bioscience), in accordance with the manufacturer's instructions. Detection limits for these cytokines were 5 ng/ml and 0.48 pg/ml, respectively.

Statistical Analysis

All data are presented as median (Interquartile Range) unless otherwise stated. Comparisons were performed by non-parametric tests (Mann-Whitney U, Kruskal-Wallis tests and Spearman's rank) as data were not normally distributed. Categorical variables were compared with a chi-squared test. The association between categorical variables and the CV events was assessed and adjusted for other factors (sex, age, traditional CV risk factors and disease activity) using multiple logistic regression analysis. Adjusted odds ratios (OR) and 95% confidence intervals (95% CI) were calculated so as to evaluate the strength of the associations. A p-value <0.05 was considered statistically significant. All data were analyzed with SPSS v.15.0 software.

Results

Circulating EPC Populations and IFNα Serum Levels in RA Patients

We aimed to investigate the possible relationship between EPC populations and IFNα levels in RA patients. To this end, IFNα serum levels and circulating pre-EPC, EPC and mEPC populations were quantified in 52 healthy controls (HC) and 120 RA patients with different disease duration (range 0–219 months) (Table 1). No significant differences in any endothelial progenitor population were found between patients and HC. However, disease duration was negatively correlated with EPC ($r=-0.316$, $p<0.001$) and mEPC ($r=-0.342$, $p<0.001$), suggesting an EPC

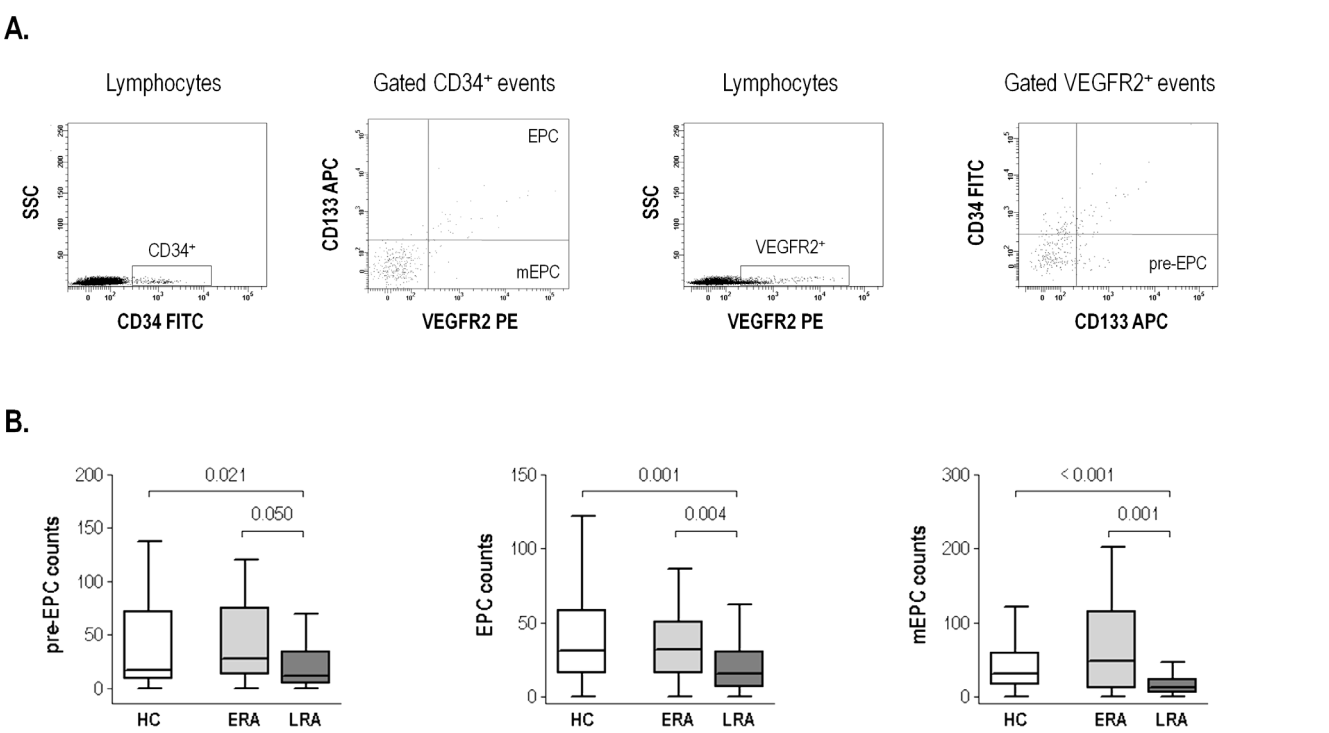

Figure 1. EPC analyses in RA patients by flow cytometry. (A) Gating strategy of EPC, mEPC and pre-EPC analysis by flow cytometry in peripheral blood samples. Representative dot plots of a HC are shown. (B) The size of endothelial precursor populations is influenced by disease duration. Pre-EPC, EPC and mEPC counts in early (ERA, disease duration <1 year, n = 36) and long-standing (LRA, n = 84) Rheumatoid Arthritis patients and healthy controls (HC, n = 52). Differences were measured by Mann-Withney U-test.
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depletion associated with disease progression. In fact, patients at recent onset (less than one year, n = 36, early RA, ERA) showed similar levels of these populations than HC, whereas those with longer disease duration (n = 84, long-standing RA, LRA) exhibited a significant depletion of all EPC populations (Figure 1B). Neither associations with age at sampling, age at diagnosis, nor autoantibodies status were found.

On the other hand, IFNα serum levels were increased in RA patients compared with HC (20.25±47.61 vs. 1.76±3.08 pg/ml, p = 0.001), and positively correlated with all endothelial progenitor populations in patients (EPC: r = 0.294, p<0.001; mEPC r = 0.265, p<0.001, pre-EPC r = 0.367, p<0.001) but not in controls. Of note, this cytokine was associated with DAS-28 score (r = 0.253, p = 0.023) but unrelated to disease duration (r = 0.055, p = 0.547). In fact, ERA and LRA patients showed similar IFNα levels (14.43±31.16 vs. 22.74±53.10, p = 0.718).

In spite of the IFNα increase in RA, Figure 2 evidences that only a fraction of patients showed high levels of this molecule, whereas the other group presented low levels, similar to HC. Thus, we classified RA patients in IFN^{low} and IFN^{high} using the HC 90^{th} percentile ($P90^{th}$ = 4.092 pg/ml) as cut off. As shown in Table 1, these RA groups did not differ in age, disease duration or treatment followed, but IFN^{high} patients (n = 40, 33%) exhibited higher disease activity (DAS28) and ESR as well as increased positivity for autoantibodies. Other clinical markers such as Tender Joint Count, Patient Global Assessment or CRP were slightly augmented.

EPC Populations Differ According to IFNα Levels

In view of these results, we analyzed EPC populations in RA patients according to IFNα levels and disease duration, using as controls healthy donors (HC) and patients with SLE, a disease presenting altered levels of IFNα and EPCs [24]. Figure 3 shows that among ERA patients, those with normal IFNα levels (IFN^{low}) displayed similar pre-EPC, EPC and mEPC counts to HC, whereas IFN^{high} ERA patients exhibited higher levels of these populations compared with both HC and IFN^{low}, but similar to SLE patients. However, the most remarkable results were detected in LRA patients, since those with normal IFNα levels showed significantly lower pre-EPC and EPC counts than HC, thus highlighting a significant depletion that was missing in IFN^{high} patients. Therefore, EPC depletion seems to be a characteristic of RA patients unless the presence of high IFNα levels hides this effect. In fact, no significant differences in EPC populations were present between SLE and IFN^{high} RA patients, independently of disease duration. In any case, it is important to note that although IFN^{high} RA patients displayed enhanced EPC populations, the mEPC/EPC ratio, indicative of the endothelial repair capability [5,6], was increased in this group compared with their IFN^{low} counterparts (1.22(1.40) vs. 0.56(1.71), p = 0.013), thus suggesting an endothelial repair failure in these patients. Interestingly, no significant differences in mEPC/EPC ratio were found between ERA and LRA patients (p = 0.090), neither by treatments (all p>0.050), so there is no evidence that disease duration or longer exposure to treatment drugs could modify the mEPC/EPC ratio in RA patients.

IFNα is Associated with a Higher Rate of Cardiovascular Events

Taking into account the reported role of IFNα in endothelial damage and vascular repair, we aimed to evaluate the relevance of IFNα serum levels as a CV risk factor for RA patients. To this end,

Table 1. Demographic, immunological and clinical parameters of the RA patients.

	RA patients	IFNα	
	(n = 120)	IFN^{low} (n = 80)	IFN^{high} (n = 40)
Sex (female/male)	101/19	70/10	31/9
Age at sampling, years	55.33±15.23	55.24±15.04	55.56±16.00
Age at diagnosis, years	53.09±18.00	52.78±14.97	53.77±16.23
Disease duration, months	21.02±20.50	23.33±20.83	16.00±19.26
Clinical features			
Number of tender joints	5.24±5.25	4.29±5.59	6.86±7.00
Number of swollen joints	2.50±3.31	1.83±3.15	3.64±3.38
Patient global assessment (0–100)	32.05±23.63	28.13±25.20	38.79±19.69
Pain of patient's assessment (0–10)	3.21±2.42	2.71±2.40	4.07±2.30
Duration of morning stiffness, min	48.42±70.93	46.25±78.30	52.14±58.72
DAS28	3.81±1.61	3.35±1.52	4.59±1.49*
HAQ	0.77±0.70	0.68±0.70	0.95±0.67
CRP, mg/dl	0.39±0.63	0.28±0.45	0.59±0.83
ESR, mm/h	23.47±19.94	16.87±10.64	34.78±26.71*
RF positivity, n (%)	68 (56.7)	36 (45.0)	32 (80.0)***
Anti-CCP positivity, n (%)	69 (57.5)	36 (45.0)	33 (82.5)***
ANA positivity, n (%)	46 (38.3)	25 (31.2)	21 (52.5)*
Smoking habit, n (%)	49 (40.8)	32 (40.0)	17 (42.5)
Hypertension, n (%)	30 (25.0)	19 (23.7)	11 (27.5)
Hypercholesterolemia, n (%)	11 (9.1)	8 (6.6)	3 (2.5)
Diabetes mellitus, n (%)	15 (12.5)	8 (10.0)	7 (17.5)
***Treatments** n (%)*			
None or NSAIDs	19 (15.8)	10 (10.1)	9 (22.5)
Glucocorticoids	58 (48.3)	40 (50.0)	18 (45.0)
Methotrexate	79 (65.8)	57 (71.3)	22 (55.0)
Leflunomide	16 (13.3)	11 (13.7)	5 (12.5)
TNF-α blockers	26 (21.6)	14 (17.5)	12 (30.0)
***Cardiovascular events**, n(%)*			
Cardiovascular events	27 (22.5)	12 (15.0)	15 (37.5)**
Ischemic heart disease	10 (8.33)	4 (5.0)	6 (15.0)
Cerebrovascular accidents	4 (3.33)	2 (2.5)	2 (5.0)
Heart failure	12 (10.0)	6 (7.5)	6 (15.0)
Peripheral arteriopathy	1 (0.83)	0 (0.0)	1 (2.5)

Data of the whole RA patients group and classified according to IFNα serum levels. Data are expressed as (mean ± SD) unless otherwise was stated. Differences between categorical variables were evaluated by chi-square test, whereas Mann-Withney U test was used for continuous ones. *p<0.05, **p<0.01, ***p<0.001. IFN^{low}: serum levels $<90^{th}$ percentile in HC (4.092 pg/ml); IFN^{high}: serum levels $\geq 90^{th}$ percentile in HC.

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we analyzed the CV events suffered by RA patients in relation to both IFNα groups, demographic and clinical variables. The frequency of RA patients who had suffered CV events was higher in the IFN^{high} than in the IFN^{low} group (37.5 vs 15.0%, p = 0.005), although no significant differences between groups were detected in traditional CV risk factors (Table 1), thus supporting the role of IFNα as an independent CV risk factor. Univariate logistic regression analysis (Table 2) revealed that high IFNα levels, male sex, age at diagnosis, hypertension and diabetes were associated with the risk of CV events. After multivariate analysis by logistic regression adjusted by age at diagnosis, sex, disease activity (DAS28) and traditional CV risk factors, only the association with IFNα and age at diagnosis remained significant.

On the other hand, patients who had suffered CV events showed an increased mEPC/EPC ratio compared to those who had not experienced such complications (1.27(3.21) vs. 1.00(2.04), p = 0.010), as well as lower VEGF levels (55.84(90.69) vs. 122.34(150.39) pg/ml, p = 0.044), thus supporting the relevance of angiogenic cytokines and EPC balance in the endothelial repair maintenance. All these results support that high IFNα serum levels in RA patients could be associated with a higher rate of CV events, maybe by increasing the mEPC/EPC ratio and impairing endothelial repair.

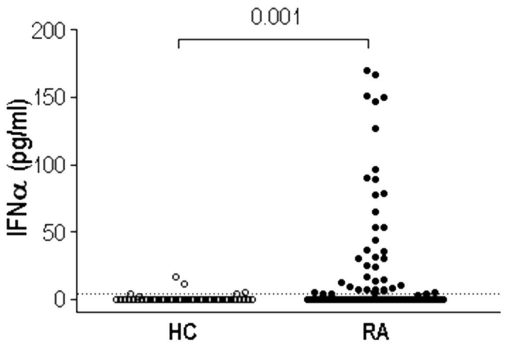

Figure 2. IFNα serum levels are increased in a subgroup of RA patients. IFNα was quantified in 52 HC and 120 RA patients by CBA immunoassay. Dotted line represents HC 90th Percentile (4.092 pg/ml), used to classify RA patients in IFN^{low} (n = 80, 66%) or IFN^{high} (n = 40, 33%). Differences were measured by Mann-Withney U-test.
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IFN$^{low/high}$ Groups Differ in their Cytokine Profiles

Finally, to analyze whether IFNα serum marker may influence cytokine profiles in RA patients, we studied IL-1β, IL-6, IL-8, IL-10, MIP-1α, VEGF-A$_{165}$, TNFα and TGF-β levels in patients and controls. The whole RA population was characterized by increased levels of IL-6 (1.05(3.41) vs. 0.32(1.15) pg/ml, p = 0.004), IL-8 (17.12(18.08) vs. 10.18(14.06) pg/ml, p = 0.008), IL-10 (0.37(0.68) vs. 0.10(0.18) pg/ml), and TNFα (5.76(4.01) vs. 3.26(1.93) pg/ml, p = 0.015), whereas TGF-β was decreased (14.47(4.60) vs. 19.42(6.71) ng/ml, p<0.001). Regarding to disease duration, we observed that LRA patients showed lower amounts of IL-1β (p = 0.002), IL-6 (p = 0.001) and IL-10 (p = 0.028) and slightly higher of TGF-β (p = 0.060) than ERA, whereas TNFα levels were strikingly higher in the LRA group (p<0.001). Restoration of cytokine levels in LRA could probably be due to a successful response to the therapy, since almost all LRA patients were under treatment while half of the ERA patients were untreated. In fact, striking differences were observed between treated and untreated patients in IL-1β (p<0.001), IL-6 (p = 0.002) and IL-10 (p = 0.004), but not in TNFα.

Interestingly, it is remarkable that IFN^{high} patients showed a cytokine profile more similar to SLE patients than those IFN^{low}, except for IL-1β (Figure 4A). In fact, IFN^{high} RA patients displayed higher levels of IL-1β (0.47(1.48) vs. 0.13(0.06) pg/ml, p<0.001), IL-6 (1.93(13.76) vs. 0.75(2.27) pg/ml, p = 0.004), IL-10 (0.65(1.15) vs. 0.26(0.46) pg/ml, p<0.001), MIP-1α (3.29(4.47) vs. 0.00(4.28) pg/ml, p = 0.001) and lower of TGF-β (12.86(4.01) vs. (14.98(5.45) ng/ml, p = 0.025) than IFN^{low} patients. In addition, although no association between IFNα and TNFα levels was detected in the whole RA group (r = −0.028, p = 0.766), a positive correlation was found in the IFN^{high} group (r = 0.407, p = 0.011). All these results indicate that both IFNα and treatment influence cytokine levels. In fact, Figure 4B shows that treatments seem to restore IL-1β, IL-6, IL-8, IL-10 and TGF-β levels to a greater extent in IFN^{low} patients than in IFN^{high} ones. Moreover, several clinical markers suggest a better outcome of IFN^{low}-treated patients than in their IFN^{high} counterparts (Figure 4C), thus suggesting a potential IFNα role in therapy outcomes.

Finally, we analyzed the clinical response to anti-TNF therapy among a 6-month period, since it has been proposed a role of the IFN/TNF cross-regulation in the response to this treatment [26–27]. Of note, within the IFN^{low} group (n = 14), 35% of the patients (n = 5) reached a good response, and a similar percentage was found for those with a moderate response. In contrast, any patient of the IFN^{high} group (n = 12) reached a good response, whereas only 41.6% (n = 5) fulfilled the criteria for a moderate one and more than a half (n = 7) exhibited no response to anti-TNF treatment.

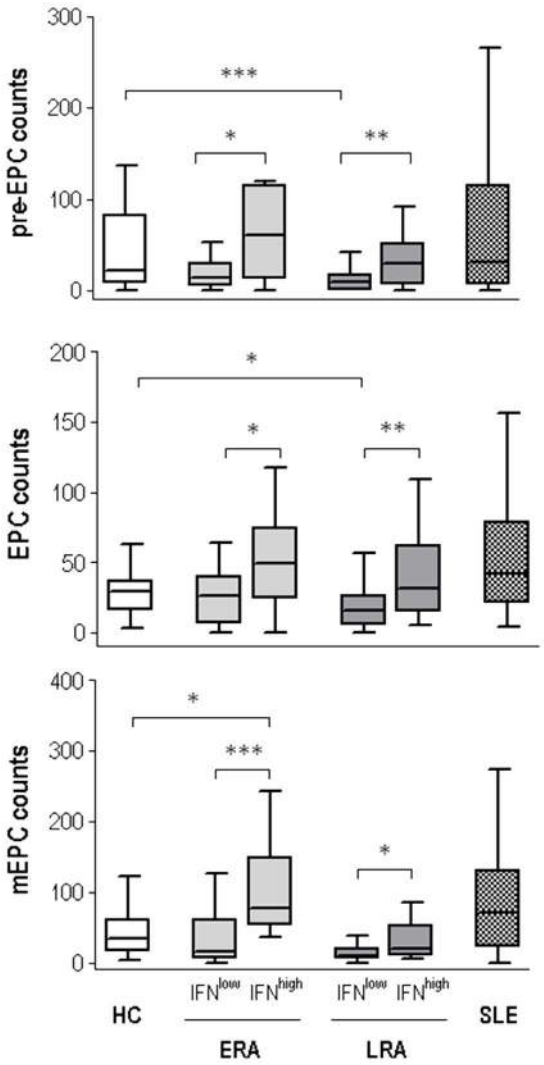

Figure 3. Long-standing RA patients with low IFNα levels exhibited a depletion of all endothelial progenitor populations. Pre-EPC, EPC and mEPC counts in early (ERA) and long-standing (LRA) Rheumatoid Arthritis patients were analyzed according to their IFNα serum levels. Healthy donors (HC) and SLE patients were included as both healthy and disease controls. Differences were assessed by Kruskal-Wallis test and Dunn's multiple comparisons post hoc test. *p<0.05, **p<0.01.
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Discussion

Recent evidence suggests a role of type I IFNs in vascular damage and EPC disbalance, mainly in SLE patients [15–18,24], probably due to the central involvement of IFNα in the SLE pathogenesis [14,28–30]. However, whether IFNα levels could play a major role in the clinical outcome and/or vascular damage in RA patients remains unknown.

Although most of the previous works reported an EPC depletion in RA patients that could be associated with disease activity [31–33], recent studies show contradictory results [34–36]. Our data

Table 2. Association between presence of IFNα serum marker and CV events in RA patients.

	Cardiovascular events		Univariate Analysis		Multivariate Analysis*	
	Absent (n = 93)	Present (n = 27)	OR [95% CI]	*p*	OR [95% CI]	*p*
IFNα						
IFN^{low}	68 (73.1)	12 (44.4)	**1**		**1**	
IFN^{high}	25 (26.9)	15 (55.6)	**3.400 [1.401–8.253]**	**0.007**	**4.816 [1.254–18.488]**	**0.022**
Sex						
Women	83 (89.2)	18 (66.6)	**1**			
Men	10 (10.8)	9 (33.6)	**4.150 [1.475–11.680]**	**0.007**		
Age at diagnosis	51.00 (17.00)	56.00 (23.00)	**1.050 [1.008–1.093]**	**0.019**	**1.038 [1.015–1.084]**	**0.021**
DAS28 score	3.94 (2.66)	3.26 (2.09)	0.814 [0.580–1.143]	0.235		
HTA						
Normotensive	72 (77.4)	15 (55.5)	**1**			
Hypertensive	21 (22.6)	12 (44.4)	**3.032 [1.218–7.547]**	**0.017**		
DM						
Non-diabetic	84 (91.3)	20 (74.1)	**1**			
Diabetic	9 (8.7)	7 (25.9)	**3.675 [1.192–11.326]**	**0.023**		
Smoking habit						
Non-smoker	50 (53.7)	19 (70.3)	1			
Smoker	43 (46.3)	8 (29.6)	1.947 [0.773–4.904]	0.157		
Hypercholesterolemia						
Normocholesterolemic	83 (89.2)	26 (96.2)	1			
Hypercholesterolemic	10 (10.7)	1 (3.70)	0.311 [0.038–2.559]	0.278		
RF						
Negative	34 (36.5)	8 (30.8)	1			
Positive	60 (64.5)	19 (69.2)	1.530 [0.598–3.916]	0.375		
Anti-CCP						
Negative	33 (35.4)	7 (25.9)	1			
Positive	50 (53.7)	20 (74.1)	1.791 [0.678–4.734]	0.415		
ANA						
Negative	56 (60.2)	18 (66.6)	1			
Positive	37 (47.3)	9 (33.3)	0.730 [0.296–1.800]	0.499		

Associations were evaluated by logistic regression analysis using the presence of CV events (ischemic heart disease, n = 10; cerebrovascular accidents, n = 4; heart failure, n = 14; peripheral arteriopathy, n = 1) as dependent variable. Associations that reached statistic significance in multivariate analyses are highlighted in bold.
*Multivariate analysis adjusted by sex, age at diagnosis, disease activity, smoking habits and presence or absence of HTA, DM and hypercholesterolemia. Accuracy of prediction of the final model was 76.7%.
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may explain these conflicting data, since we demonstrated that only a group of RA patients exhibited a significant EPC depletion. We have previously confirmed that EPC population decreases with disease duration, whereas at disease onset it was similar to healthy subjects [24]. This finding is in line with previous studies where CV risk in RA patients has been reported to be associated with disease duration, probably due to disease-specific factors [37]. In addition, we reported for the first time, that EPC and pre-EPC populations were significantly reduced in patients with low IFNα serum levels, whereas higher levels of this cytokine were associated with higher counts of EPC populations, which leads to an increase in the mEPC/EPC ratio, in a similar way to the results observed in SLE [24]. Moreover, IFN^{high} patients displayed higher disease activity and an elevated prevalence of autoantibodies, as was reported in IFN^{high} SLE patients [38]. Thus, we think that IFNα serum levels could be an important bias in EPC studies in autoimmune diseases and it could be taken into account in future works.

Recent genomic studies have reported the presence of type I IFN signature in around 25–50% of RA patients [28–30,39], which is according to our IFN^{high} subset size (30%) and using similar criteria as cut off (90^{th} percentile), but no correlations had been detected between IFN signature and clinical or immunological disease parameters. However, in this study, we showed that serum IFNα is correlated with clinical parameters, in the same way that has been previously reported in SLE patients [40], thus supporting the feasibility of IFNα serum marker in autoimmunity.

Although EPC depletion has been linked to higher rates of CV disease, our data show that IFN^{high} patients, with increased EPC counts, exhibit a higher occurrence of CV events, thus highlighting the role of IFNα levels as an independent CV risk biomarker. In fact, these results are in accordance with the reported role of IFNα in vascular damage and EPC dysfunction [15,17,18], and with the

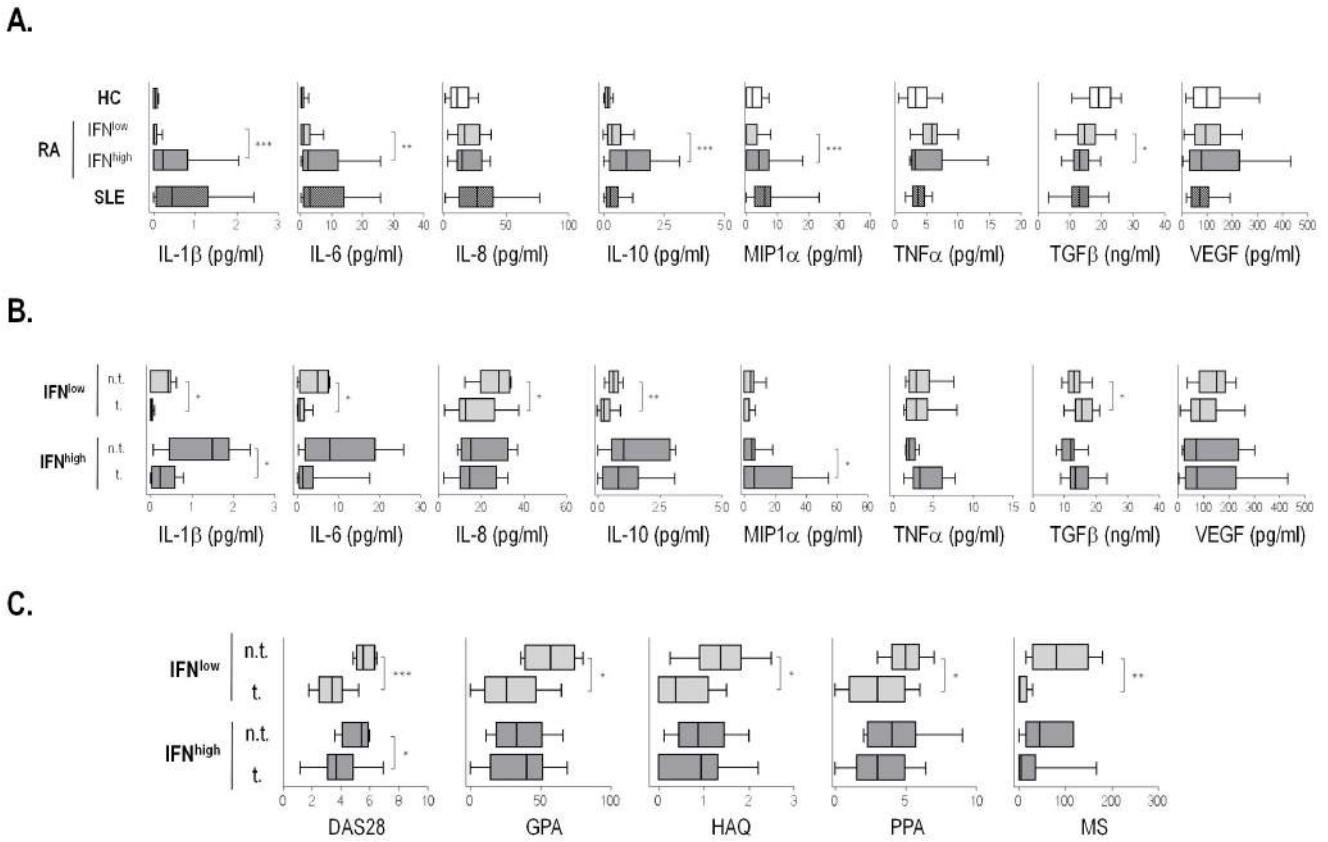

Figure 4. Cytokine profiles and disease features in RA patients are dependent on IFNα serum levels. (A) Proinflammatory cytokines are increased in IFN^{high} patients compared with both HC and IFN^{low} patients. (B) Serum cytokines levels are restored in IFN^{low}-treated patients but not in IFN^{high} ones. (C) Improvement in clinical parameters in IFN^{low}-treated patients compared with IFN^{high} group. Differences between groups were assessed by Mann-Withney U-test. n. t.: non-treated; t.: treated. *$p<0.05$, **$p<0.01$, ***$p<0.001$.
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increased mEPC/EPC ratio found in these patients, suggestive of an impaired EPC function. Actually, recent studies have linked endothelial repair failure in autoimmunity with the IFNα pathway, probably by altering the balance between endothelial cell apoptosis and vascular repair mediated by EPC [15]. This effect has been proposed to be mediated, at least in part, through VEGF repression in EPC [16]. Accordingly, IFN signature in EPC-treated cultures has been associated with impaired functionality and endothelial dysfunction [18]. Moreover, type I IFNs have been linked to atherosclerosis progression and vascular damage in both murine models [17] and human patients [41–43], thus proposing a type I IFN-mediated pathogenic role in CV disease in autoimmune patients. In addition, IFNα pharmacological treatment in non-RA subjects has been associated with CV disease [44,45]. The increased rate of CV events in IFN^{high} patients reported in our study support these findings. Accordingly, Somers *et al.* [42] reported that type I IFNs were independently associated with atherosclerotic development after adjusting for Framingham (traditional) risk factors. Similarly, in SLE, high disease activity is considered a better CV disease predictor than traditional risk factors [46].

In view of our results, we hypothesize that a potential mechanism by which IFNα could increase CV risk may be by promoting a premature EPC differentiation, generating mEPC ($CD133^-$) with little or no vasculogenic and/or repair capability [6], probably similar to the "non-angiogenic phenotype" reported in murine SLE models [16], consequently resulting in a defective EPC-mediated endothelial repair. That is, although counterintuitive, higher EPC counts are not associated with cardioprotection, but endothelial repair failure, because of the high IFNα levels, which are causing a shift towards the mEPC phenotype. In addition, we showed that patients who had experienced CV events exhibited a higher mEPC/EPC ratio, thus linking IFNα, EPC maturation and impaired EPC functionality.

Another interesting finding was the differences in the cytokine patterns of RA patients, which seem to be related to treatment and IFNα levels. In fact, IFN^{high} RA patients showed cytokine disturbances closer to SLE patients, characterized by a proinflammatory profile and higher IL-10 levels, which are associated with disease activity and poor prognosis markers, suggesting that this cytokine could be acting as a proinflammatory mediator in these conditions, as some authors have reported [47,48]. Moreover, the IFN^{high} group exhibited a positive correlation between IFNα and TNFα serum levels, similar to previously reported in SLE patients [49,50]. Although Palucka *et al.* [26] have been proposed a negative cross-regulation between these cytokines, many other associations have been published thereafter, highlighting the relevance of the disease, the experimental model, the sample origin and the characteristics of the patients. It seems that, in some autoimmune disorders, the negative TNF/IFN cross-regulation loop is missed, leading to high serum levels of both cytokines in patients in which they may exert a pathological effect [39,49]. Moreover, different associations of these two mediators have been reported even in a single disease [51], as seen in our

study.Actually, we think that IFN^{high} RA patients might display an impaired endothelial repair partly due to their proinflammatory cytokine network, mainly represented by higher serum levels of IL-1β and IL-6 (a Th17 inducer cytokines) and low TGF-β, compared to their IFN^{low} counterparts. In fact, reported *in vitro* experiments showed that proinflammatory conditions are enough to impair EPC functionality [33,52,53]. Moreover, Mälarstig *et al.* [13] have showed that raised IL-10 levels are associated with poor outcomes and enhanced systemic inflammation in acute coronary syndrome, supporting, at least in part, our findings.

Finally, differences in cytokine levels between treated and untreated patients among IFN^{low} and IFN^{high} groups suggest that IFNα could be a predictive factor for treatment outcomes, being IFN^{high} patients associated with a poor response. This was especially clear for anti-TNF therapy, since clinical response among the previous 6 months was higher in those patients within the IFN^{low} group. Similar conclusions were published by other authors [29,54]. Therefore, this result makes us hypothesize that the IFN^{high} group could benefit from an anti-IFNα therapy [55] rather than traditional DMARDs. However, relatively short follow-up period, differences in treatment duration and the low numbers of patients included do not lead us to achieve consistent conclusions in this issue.

Conclusions

In summary, we show that high IFNα serum levels could identify a group of RA patients with increased disease activity, EPC imbalance, enhanced proinflammatory profile and higher cardiovascular risk, probably due, at least in part, to an impaired endothelial repair. In addition, IFNα could be not only a marker of poor prognosis, but also of poor response to therapy, thus highlighting the relevance of this cytokine as a potential therapeutic target in RA.

Author Contributions

Conceived and designed the experiments: AS. Performed the experiments: JRC BdP PL CP. Analyzed the data: JRC AS. Contributed reagents/materials/analysis tools: MAL FJBG AS. Wrote the paper: JRC AS.

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Review Article

Type I IFNs as biomarkers in rheumatoid arthritis: towards disease profiling and personalized medicine

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Abstract

RA (rheumatoid arthritis) is a chronic rheumatic condition hallmarked by joint inflammation and destruction by self-reactive immune responses. Clinical management of RA patients is often hampered by its heterogeneous nature in both clinical presentation and outcome, thereby highlighting the need for new predictive biomarkers. In this sense, several studies have recently revealed a role for type I IFNs (interferons), mainly IFNα, in the pathogenesis of a subset of RA patients. Genetic variants associated with the type I IFN pathway have been linked with RA development, as well as with clinical features. Moreover, a role for IFNα as a trigger for RA development has also been described. Additionally, a type I IFN signature has been associated with the early diagnosis of RA and clinical outcome prediction in patients undergoing biological drug treatment, two challenging issues for decision-making in the clinical setting. Moreover, these cytokines have been related to endothelial damage and vascular repair failure in different autoimmune disorders. Therefore, together with chronic inflammation and disease features, they could probably account for the increased cardiovascular disease morbidity and mortality of these patients. The main aim of the present review is to provide recent evidence supporting a role for type I IFNs in the immunopathology of RA, as well as to analyse their possible role as biomarkers for disease management.

Key words: autoimmunity, biomarker, endothelial damage, interferon α, rheumatoid arthritis, type I interferon

INTRODUCTION

Type I IFNs (interferons) are a family of cytokines discovered more than 50 years ago by their ability to inhibit (interfere with) viral replication. Since their discovery, they have been the subject of intense research not only from a basic science perspective, but also for their roles in autoimmunity, as well as their therapeutic use in a variety of conditions.

The human type I IFN family comprises 13 functional IFNα genes and single genes for IFNβ, IFNκ, IFNε and IFNω, all derived from one common ancestral gene. All type I IFN cytokines bind to a single heterodimeric receptor composed of two subunits [IFNAR1 and IFNAR2 (IFN-α receptor 1 and 2 respectively)] [1], resulting in the activation of two receptor-associated protein tyrosine kinases JAK1 (Janus kinase 1) and TYK2 (tyrosine kinase 2), which phosphorylate STAT (signal transducer and activator of transcription) 1 and STAT2, leading to their activation and dimerization (Figure 1). Then, the activated STAT1/STAT2 complex assembles with IRF9 (IFN-regulatory factor 9) and this trimeric complex binds to specific DNA target sequences, known as ISREs (IFN-stimulated response elements), thereby activating the transcription of several related genes, coined as IRGs (IFN-response genes) or IFI (IFN-inducible) genes (such as IRF5, IRF7, etc.) (Figure 1) [1]. These IRGs are responsible for the antiviral and immunomodulatory effects triggered by type I IFNs. Actually, type I IFNs have been reported to modulate up to 200 genes, highlighting their pleiotropic character.

During viral infections, although most human cell types are able to secrete type I IFNs, DCs (dendritic cells), and mainly pDCs (plasmacitoid DCs), are the most efficient IFNα producers [2]. Virus and viral RNA/DNA stimulate type I IFN production through cell-associated pattern recognition receptors, such

Abbreviations: CCP, cyclic citrullinated peptide; CV, cardiovascular; DAS, disease activity score; DC, dendritic cell; EPC, endothelial progenitor cell; ET-1, endothelin-1; HCQ, hydroxychloroquine; IFI, IFN-inducible; IFNAR, IFNα receptor; IFN, interferon; IL, interleukin; IL-1RA, IL-1 receptor antagonist; IRAK, interleukin receptor-associated kinase; IRF, IFN-regulatory factor; IRG, IFN-response gene; ISRE, IFN-stimulated response element; JAK, Janus kinase; NSAID, non-steroidal anti-inflammatory drug; pDC, plasmacitoid DC; PRKR, protein kinase R; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; STAT, signal transducer and activator of transcription; Tang cell, angiogenic T-cell; TLR, Toll-like receptor; TNF, tumour necrosis factor; TRAF, TNF receptor-associated factor; TYK2, tyrosine kinase 2; VEGF, vascular endothelial growth factor.

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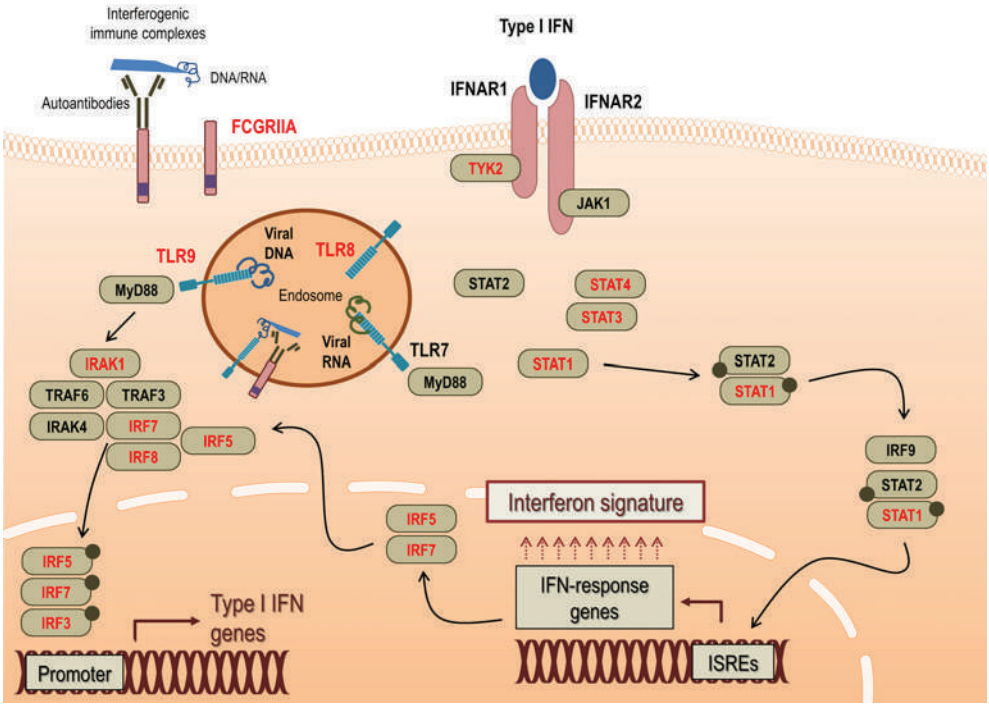

Figure 1 **Type I IFN pathway in pDCs**
Type I IFNs can activate the transcription of IRGs through the JAK/STAT pathway. The type I IFNs can regulate almost 200 genes, whose simultaneous increased expression is referred to as the so-called IFN signature. These genes are responsible for the antiviral effects of type I IFNs and are also involved in the triggering of type I IFNs, as well as in a variety of immunomodulatory effects of type I IFNs. On the other hand, interferogenic immune complexes in autoimmune disorders can stimulate type I IFN gene transcription by engaging FcγRIIa receptors, thereby leading to higher type I IFN expression. Molecules reported to be associated with genetic susceptibility to RA are highlighted in red.

as TLRs (Toll-like receptors), this synthesis being tightly regulated and limited in time. However, interferogenic immune complexes, containing self-nucleic acids, have been reported in some autoimmune disorders [3,4] to induce type I IFNs, predominantly IFNα, after internalization in endosomes via FcγRIIa (Figure 1). At this point, TLR7/TLR9 present in pDC endosomes activate the MyD88 (myeloid differentiation factor 88) adaptor protein, which associates with TRAF3/TRAF6 [TNF (tumour necrosis factor) receptor-associated factor 3 and 6] and IRAK1/IRAK4 [IL (interleukin) receptor-associated kinase 1 and 4]. This complex promotes the phosphorylation and translocation to the nucleus of IRF3, IRF5 and IFR7, which ultimately activate the transcription of type I IFN genes [5,6].

Regarding their functions, type I IFNs efficiently inhibit viral replication, thus being the major innate immune response against viral infections. However, type I IFNs can also carry out a wide range of immunomodulatory functions, including the up-regulation of MHC-I and MHC-II, maturation of DCs, activation natural killer cells, and induction of chemokines, chemokine receptors and co-stimulatory molecules (CD80 and CD86), as well as stimulating B-cell differentiation, antibody production and isotype class switching [5–7]. In addition, these cytokines have been associated with a Th1 shift [5,7]. Therefore type I IFNs can influence both innate and adaptive responses, leading to the activation of cellular and humoral immunity.

TYPE I IFNS AND AUTOIMMUNITY

Owing to their immune-stimulatory effects, type I IFNs and their signalling pathways represent a key factor in the breakdown of tolerance and the subsequent development and/or perpetuation of autoimmune phenomena (Figure 2). Therefore their role in autoimmune diseases has been intensely studied. The most relevant results come from SLE (systemic lupus erythematosus), where IFNα seems to have a prominent role. However, many other autoimmune conditions have been related to the IFN pathway, such as Sjögren syndrome, systemic sclerosis and, more recently, RA (rheumatoid arthritis). Actually, some of these autoimmune conditions (or, specifically, some subsets of patients) may be classified as 'Type I interferonopathies', a recently coined term to cover the broad spectrum of conditions hallmarked by aberrant type I IFN signalling [8,9].

Consistent data indicate a role for type I IFNs (and especially IFNα) in SLE pathogenesis. High plasma levels of IFNα were found in SLE patients [10], and were reported to be associated with serological features, as well as with disease activity [11]. Similar findings were observed when expression arrays were performed in peripheral blood cells and tissue from SLE patients [12]. Additionally, IFNα therapy is associated with the occurrence and increase in the titre of antinuclear antibodies (the prototypical autoantibodies in SLE) in healthy subjects and also with worsening of pre-existing SLE conditions [13,14].

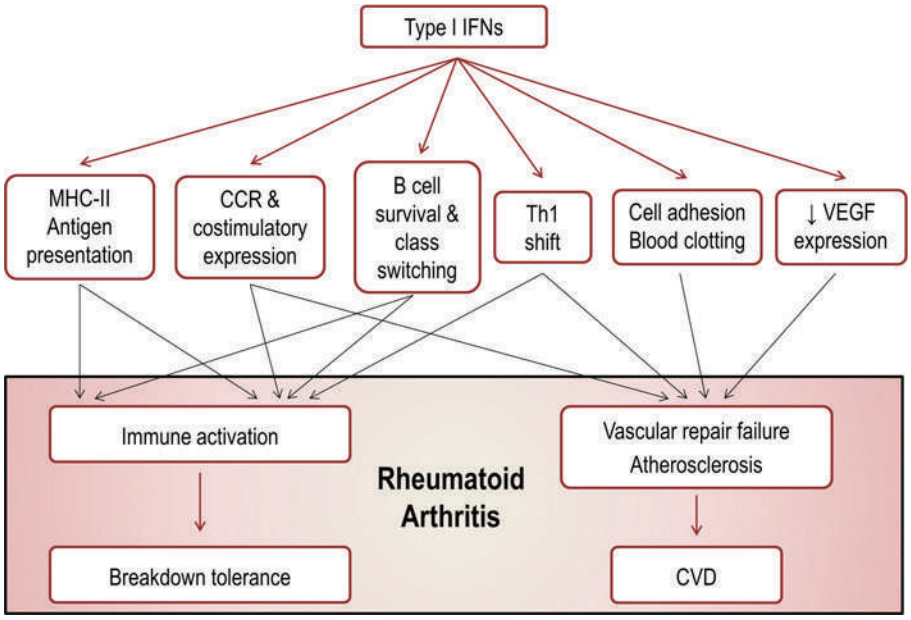

Figure 2 **Proposed roles of type I IFNs on immune activation and CV disease in RA**
Type I IFNs are pleiotropic cytokines that carry out a wide variety of cellular and molecular processes. Immunomodulatory effects of type I IFNs can lead to an enhanced immune activation by affecting both innate and acquired immunity, thus precipitating a breakdown in the tolerance and, hence, onset and perpetuation of the autoimmune phenomena characteristic of RA. Additionally, effects other than immune stimulation may have a role in premature atherosclerosis and impaired vascular repair, therefore promoting CVD (CV disease) progression in RA patients.

Further evidence for a pathogenic function of IFNα in SLE has come from animal models [15], where a role for the genetic background in IFNα signalling [16,17] has been suggested. These findings are in line with the heterogeneity of SLE and thus probably account for the different expression patterns of the type I IFN pathway in different patients [12]. Later studies have delineated the cellular network where IFNα was involved in SLE pathogenesis (reviewed in [18]), with a prominent role for pDCs and immunocomplexes in a self-perpetuating circle of chronic IFN stimulation and B-cell activation. These lines of evidence and the fact that anti-IFNα vaccination resulted in a notable amelioration of clinical features in murine models [19] led to the consideration of IFNα as a therapeutic target in SLE. In this sense, two monoclonal antibodies have been designed and the first clinical trials have been conducted [20,21]. Information concerning clinical benefit it is not yet sufficient to evaluate the efficacy of this therapy. However, despite being a promising therapeutic tool, it should be taken into account that only patients with increased IFNα serum levels or type I IFN signature expression might respond favourably, thus highlighting a clear need for predictive biomarkers in this field.

In spite of the consistent body of evidence that supports a role for IFNα in SLE, it has received little attention in RA (Table 1). RA is a complex disease, characterized by high heterogeneity in genetic predisposition, clinical presentation, disease progression, co-morbidities and response to therapies. Some lines of evidence support a pathogenic role for type I IFNs in RA (Table 2), although some controversy also exists.

In contrast with SLE, up-regulation of type I IFNs have only been found in a subset of RA patients and unexpected differences between the two subtypes of type I IFNs (IFNα and IFNβ) have been reported, thereby highlighting the relevance of these cytokines in the pathogenesis of this disease. Interestingly, IFNβ has predominantly anti-inflammatory properties, inhibits metalloproteinases and plays a role in bone homoeostasis. Proposed explanations for these tissue protection capabilities have been that IFNβ: (i) regulates inflammation and cell migration by decreasing the expression of adhesion molecules, (ii) modulates the balance between (metallo)-proteases and protease inhibitors, thus inhibiting tissue remodelling, and (iii) inhibits cartilage destruction and osteoclastogenesis by inhibiting RANKL (receptor activator of nuclear factor κB ligand)-mediated c-Fos activation [22–27]. Although these regulatory mechanisms of IFNβ may contrast with the immunomodulatory effects proposed for IFNα, IFNβ therapy did not show clinical benefit in RA patients [28], in contrast with the pre-clinical studies. Probably mode of administration and doses may account for the lack of efficacy in this clinical trial. Additionally, the beneficial effect of IFNβ is supposed to be IL-1RA (IL-1 receptor antagonist)-mediated [29]. However, IL-1RA treatment exhibits a modest clinical effect in RA [30,31]. Moreover, the same schedule for IFNβ administration was followed as in multiple sclerosis patients, but these diseases differ in terms of immunopathology and type I IFN activation [32]. As previously stated, differences in type I IFN subtypes (IFNα and IFNβ) arise in RA and may also explain these contradictory findings. It is important to note that, whereas IFNα is mainly

Table 1 Comparison between the state of the art knowledge concerning type I IFN between SLE, the prototypical IFNα-mediated autoimmune condition, and RA

The number of ticks represents the strength of the evidence for each issue.

	SLE	RA
Type I IFN-related genes association with genetic susceptibility	✓✓✓ [45,165–168]	✓✓ [36,45,169,170]
Disease and/or disease-specific autoantibodies occurrence after treatment with type I IFNs	✓✓✓ [13,14,56] (reviewed in [55])	✓ [57–60,63]
Disease triggered in mouse models by IFNα	✓ [15]	✓ [71]
Increased type I IFN signature in peripheral blood from patients (% of patients)	✓✓✓ (up to 90%) [11,12,82] (reviewed in [7])	✓ (25–65%) [68,82,87]
Increased type I IFN plasma levels	✓✓ [10,114]	✓ [75,142]
Association with clinical and serological features	✓✓ [11,12,171] (reviewed in [18])	✓ [68,75,142]
Targeted therapy against type I IFN approved and/or clinical trials performed	✓ [20,21,122,125]	X
Association with CV risk and CV disease occurrence in patients	✓✓ [134,136–138,147,158,172]	✓ [142,143,160,161]

Table 2 Outline of the current evidence for and against a pathogenic role for type I IFNs in RA

Study	For	Against
Pre-clinical studies	Pathogenic role in RA animal models [70,71]	Anti-inflammatory effects (IFNβ) [22,23,26]
	Type I IFN blockade is clinically effective in mouse models [19]	Inhibition of cartilage and bone destruction (IFNβ) [25,27]
	IFNAR-deficient mice do not develop RA [69,71]	Inhibition of vascularization and cell trafficking [173–175]
	IRF-deficient mouse are resistant to RA [43]	Treatment with IFNβ ameliorate clinical symptoms in animal models [27]
	IFNα injection in joints induces RA development in mice [71]	
	IFNα-producing cells detected in synovial tissue [77,79]	
	Increased *ex vivo* IFNα-production by pDCs from RA patients [77]	
Clinical studies	Genetic variants linked to susceptibility and clinical features [35,37,40,42,44,45,47–50,169,176,177]	RA remission after IFNβ treatment [178]
	Viral infections promote disease exacerbations [66,67]	Type I IFN signature is only found in a subset of patients [68,82,87]
	IFNα treatment associated with disease occurrence or aggravation [13,56–64]	
	Induction of RA-specific autoantibodies during IFNα therapy [56,62,65]	
	Increased expression in peripheral blood and target tissue (synovia) [68,82,84,87]	
	Associations with clinical features [68,142]	
	Association with poor clinical response [94–96,98–101,106]	
	Association with erosive lesions [75]	
	Promotion of vascular repair failure [142,143,160,161]	

produced by pDCs, IFNβ is produced widely, with several cell types involved. More recently, a potential role for type III IFNs (IFNλ) in RA has been proposed [33], as common downstream mediators of type I and type III IFNs have been identified and type III IFNs can induce the expression of a number of type I IFN-induced genes [34]. However, the role for IFNλ is yet to be clarified. All of these features make the study of the type I IFN system in RA challenging.

The search for new biomarkers that could assist in the clinical management of RA patients is not only a necessity, but also a constant challenge and, recently, the possibility of considering type I IFNs as biomarkers in RA has emerged.

IRGs AND RA SUSCEPTIBILITY

Extensive research has shed light on the genetic predisposition of RA, thus underlining the relevance of more than 60 risk loci that account for 50% of the total heritability in RA. Most of these

genes are related to antigen presentation, the threshold of immune responses and cytokine regulation. Given the immune effects of type I IFNs and the reported role of their genetic variants in their dysregulation in autoimmunity [5,7], polymorphisms in IRGs may be a plausible explanation of the genetic susceptibility in RA. Actually, among the identified risk genes for RA, a considerable number are involved in the regulation or production of the type I IFN pathway, (Figure 1).

Several studies revealed a role for IRF5 in RA susceptibility, with three polymorphisms being documented [35]. Additional studies confirmed this association, especially in the case of patients harbouring the shared epitope [36,37], thereby (i) restricting the genetic predisposition to a subset of patients with special characteristics, and (ii) associating IFN-related genetic susceptibility to patients with enhanced immune responses to viral antigens [38,39]. Interestingly, the susceptibility to RA associated with IRF5 variants seems to be similar to that of SLE [40]. Furthermore, these variants conferred increased IFN activity in SLE patients, therefore suggesting a functional role of these polymorphisms at the protein level. Whether this effect is also present in RA is still unknown; however, some controversy exists regarding IRF5 and RA susceptibility, probably due to the ethnicity of the subjects and the approach used [41,42]. Likewise, a lack of activation of the IFN pathway in mice lacking IRF1 has been described to result in a decreased frequency and severity of RA [43], and copy number variations in this locus are implicated in RA development in human studies [44]. Additionally, different polymorphisms in STAT4 [45], STAT1 and STAT3 genes [46], implicated in the IFN pathway, have been proposed to be associated with RA development.

Associations with genetic variants of pattern recognition receptors have also been reported. IFI1H is an RNA cytoplasmic receptor that acts as innate immune sensor for viral infection. It can also activate the IFN pathway and has been associated with aberrant IFN signalling in the so-called interferonopathies [47]. Polymorphisms in IFI1H have also been described as risk loci for RA [48,49]. Likewise, several authors have reported increased RA susceptibility due to some alleles in TLR genes being implicated in IFN responses (TLR2, TLR4, TLR8 and TLR9) [50].

Therefore it must be considered that the IFN pathway and IRGs can have a role in RA susceptibility, at least in subgroups of patients with characteristics of severe disease (shared epitope or erosive course).

IFNs AS A TRIGGER OF RA

The first descriptions of type I IFNs in RA patients go back to 1979, when increased levels were described in plasma [51], and later confirmed in synovial fluid [52]. Further studies associated high IFNα serum levels with extra-articular manifestations [53]. However, decreased IFN levels were also reported [54]. Nevertheless, this controversial evidence suggested the existence of dysregulated IFNs in RA patients and proved worthy of further research.

The notion that type I IFNs could play a role in RA came from a clinical setting, since increased autoimmune reactions and occurrence of autoantibodies were detected in patients with viral infections or haematological malignancies upon IFNα therapy [55]. The immune-stimulatory effect of this treatment can precipitate immune-mediated reactions *de novo* or exacerbate an existing autoimmune tendency. This is supported by the observed increase in the titre of antibodies and by the development of clinical disease in patients with pre-existing antibodies [56]. Nevertheless, it must be noted that not all of the patients receiving IFNα developed autoantibodies, and the occurrence of autoantibodies did not necessarily mean autoimmune disease development. This feature highlights IFNα as a trigger of autoimmunity, but also the need for additional mechanisms (genetic background, for instance).

Development of RA in the context of IFNα therapy has been widely documented [57–62], and was associated with rheumatoid factor occurrence in up to 34% of the cases [56], whereas few cases developed anti-CCP (cyclic citrullinated peptide) antibodies and erosive lesions. However, in most of the cases, RA manifestations remitted after IFNα therapy was withdrawn; this did not happen when the patient exhibited anti-CCP antibodies or harboured the shared epitope, and these patients had predominantly a persistent disease. Another remarkable finding of IFN-induced RA is that almost all the patients exhibited a polyarticular onset. This is especially important, since a large number of joints affected at disease onset was associated with an aggressive outcome. Moreover, most patients were unresponsive or showed weak response to NSAIDs (non-steroidal anti-inflammatory drugs), even after cessation of the therapy [61,63–65]. Interestingly though, improvements were reported after hydroxychloroquine treatment [60,65]; however, these cases need to be interpreted with caution, as it is not clear whether these traits can be classified as definite RA by classical criteria.

These findings led us to hypothesize that IFNα may be considered a trigger for RA in predisposed individuals. Furthermore, these patients probably have an aggressive disease phenotype, with a large number of affected joints and a poor response to NSAIDs, but better to antimalarials, in accordance with the inhibitory effect of this therapy on the IFNα pathway.

The idea of IFNs as a trigger of RA is also supported by evidence that infectious agents may not only have a role in the origin of RA, but also in the exacerbation of the disease [66,67]. Accordingly, it has been reported that a subgroup of RA patients exhibited a transcriptional profile that is similar to that of a viral infection [68], with IFN-mediated immunity being strongly up-regulated in these subjects. These patients also exhibit increased anti-CCP positivity, which can be associated with the breakdown of tolerance and increased B-cell activation promoted by IFNα. Moreover, anti-CCP positivity is commonly associated with rapid progression, hence associating IFN-mediated immunity with poor outcome.

Animal models of RA may also provide interesting insights into this field. Defects in DNA clearance were followed by an autoantibody-mediated chronic polyarthritis in mice that resembles human RA [69]. Additionally, nucleic acids by themselves have been described to induce RA development in mouse

models [70] because of their ability to induce IFNα synthesis [71]. Although signalling through IFNAR is needed, PRKR (protein kinase R), a well-known downstream mediator of the IFN pathway, is not required for RA development. Therefore a role for IRF3 and IRF7 in IFN-induced RA would be expected. Other studies have also reported a role of TLR7 and TLR8 in nucleic-acid-induced IFNα production [72]. Actually, all of these mediators are also linked with RA susceptibility in humans. Nevertheless, the frequency of RA development differed among different mouse strains even upon the same stimuli [70], thus reinforcing the involvement of additional mechanisms, such as genetic background, for RA development.

All of these findings are in line with the observation that circulating nucleic acids, and autoantibodies directed against nuclear antigens, can be found in RA patients [73,74]. Moreover, the presence of both nucleic acids and increased IFNα levels has been reported in the synovial fluid, as well as the peripheral blood, of some RA patients with erosive disease [75,76]. Actually, synovial pDCs produce large amounts of IFNα [75,77], which induce further maturation of pDCs, and are able to present arthritogenic antigens to T-cells [78–80], thereby promoting autoimmune phenomena and RA progression.

IFN SIGNATURES IN RA PATIENTS

Signalling through the type I IFN pathway results in an increased expression of several IFN-stimulated genes. This global expression profile is referred to as the 'IFN signature' and it has been profoundly studied in rheumatic diseases. Although the first evidence of rheumatic patients exhibiting an IFN signature came from studies in SLE [81], subsequent studies have demonstrated that several systemic rheumatic conditions, including a subgroup of RA patients, are hallmarked by an IFN signature [82]. This signature in RA was also reported in the synovial membrane, thereby supporting their involvement in the target tissue, similarly to other organ-specific autoimmune disorders [82].

An IFN signature has been reported in 25–65% of RA patients. Some studies reflect that this transcriptomic signature is not different in RA compared with SLE patients [83,84], whereas others have revealed lower IFN activation in RA [82,85]. A previous study of peripheral blood gene expression signatures by Smiljanovic et al. [86] revealed that, despite being shared by SLE and RA patients, the RA IFN signature qualitatively differs from that of SLE patients in terms of target genes and transcription-factor-binding sites and, remarkably, genomic imprints found in RA patients are more heterogeneous, thus suggesting the existence of distinctive transcriptional programmes between SLE and RA. RA patients characterized by this transcriptional profile exhibit increased expression of JAK/STAT mediators, as well as numerous chemokines, chemokine receptors and adhesion proteins, thereby suggesting an underlying enhanced immune activation [68,87]. Additionally, studies by genomic microarrays reported that IFI gene expression patterns are different at the synovial level between SLE patients with arthritic symptoms and RA patients [88]. This finding is in line with the differences observed in joint pathology between these two conditions and supports the idea that type I IFNs are actually more complex than initially considered, being heterogeneous even among different 'related' diseases.

As a consequence, the applicability of the IFN signature is still a matter of debate that needs further investigation. It can be calculated from many different IFN-stimulated genes, that, although most of them show similar expression patterns, differences are notable. In fact, a considerable variability and a wide range of IFN-stimulated gene expression have been reported. Somers et al. [89] stated that PRKR expression in SLE patients was completely independent of that of other IFN-induced genes studied (MX-1, IFI44L, IFIT1 and IFI44). Interestingly, PRKR had been reported to be irrelevant for IFN-induced RA [71], thereby emphasizing the complexity of the type I IFN system. On the other hand, data modelling and analysis also differ among studies [85], and this makes the comparison between different studies difficult. Therefore analysis of a IFN-induced profile based on average gene expression levels ignores co-regulation and interactions of such IFN-induced genes [85]. Moreover, it has been reported that the IFN signature in the rheumatoid synovium could also be induced by TNFα [90,91], thus pointing to a role for TNFα as a modulator of the IFN signature and bringing into question the specificity of the IFN signature as a biomarker of type I signalling in RA. However, whether this mechanism occurs in peripheral blood is unknown.

In conclusion, transcriptomic profiles, such as the IFN signature, could provide a direct insight into the genes and pathways involved in a certain disease in a single patient. Additionally, they are non-invasive techniques, thus being valuable tools for the clinical management of complex diseases. However, several concerns also exist and the results need to be interpreted with caution. In RA, apart from providing new insights into pathogenic mechanisms, the IFN signature also has direct implications in two unmet clinical needs, namely early diagnosis and prediction of therapy response.

IFN AND RA DIAGNOSIS

One of the main challenges in the clinical management of RA is early diagnosis. In this sense, the IFN signature has been reported in the pre-clinical phase of RA and it has been demonstrated to exhibit a consistent predictive value [92,93]. In addition, the IFN signature increased the predictive value of traditional markers (anti-CCP and rheumatoid factor) [92]. Moreover, the fact that the IFN signature is found in patients even before the clinical diagnosis reinforces the hypothesis of IFN as a trigger for RA, thereby ruling out the idea of the IFN signature in RA as an epiphenomenon or consequence of the disease itself.

Genetic variants, infections or dysregulation of the IFN pathway could be potential explanations to account for the presence of the IFN signature before the clinical onset of RA [7]. Regardless of the mechanism, the continuous IFN-mediated activation of the immune system will progressively lead to breakdown of tolerance and the development of autoimmunity. The fact that this signature can be detectable before the clinical onset allows

an opportunity for the establishment of early treatment, which has been reported to result in a better sustained disease control.

IFN AND THE CLINICAL RESPONSE TO TREATMENT

Another major challenge in RA clinical routine is the prediction of therapy outcome, especially to biological therapies, so as to avoid ineffective treatment in potentially unresponsive patients, thereby keeping these patients away from adverse effects and high costs.

Van Baarsen et al. [94] reported data from whole-blood real-time PCR analysis of IRGs in infliximab-treated patients, concluding that a high IFN signature was associated with poor clinical outcome, as measured as a change in DAS (disease activity score) from baseline and EULAR (European League Against Rheumatism) response criteria after 12 weeks. Similarly, Sekiguchi et al. [95] found that sustained low expression of IFN-regulated genes is associated with a good response [ACR (American College of Rheumatology) 50 % criteria] after 22 weeks [95]. Additionally, infliximab treatment decreased anti-CCP titres in patients with a low, but not in those with a high, IFN signature [96], which is in accordance with the differences in anti-CCP antibodies levels depending on the IFN groups [68]. Another plausible explanation could be the existence of an anti-CCP-producing plasma cell clone highly activated by IFN in these patients. However, this latter study did not report clinical differences in response to TNFα blockade. Moreover, contradictory results have been recently reported in a prospective study of RA patients undergoing TNFα-blockade [97] when the IFN signature in neutrophils was analysed. Differences not only in the assayed population, but also in the transcription factors focused on, with more attention paid to the STAT factors in the latter study, may contribute to this controversy.

Similarly, some studies point to a role for the IFN signature as a predictor of a negative response to B-cell therapy in RA. A previous study by Raterman et al. [98] using a whole genome transcript profile approach revealed that, among all of the genes in the human genome, only IRGs were associated with poor clinical response and a change in the DAS28 score after rituximab therapy. Actually, serum IFNα bioactivity at baseline correlated negatively with clinical response after 24 weeks in a similar study [99]. Accordingly, a greater decrease in autoantibody titre was found in patients with a low IFN signature compared with their counterparts with a high IFN signature, thus reinforcing the hypothesis of a resistant B-cell clone probably stimulated by IFNα. Similar results were reported from synovial samples [100], with high IFN-related gene expression being associated with a smaller change in DAS28 score and poor clinical response. Subsequent studies revealed, however, that good responders are characterized by a slight increase in IRG expression early after therapy, whereas this expression was constitutively increased (unchanged) in non-responders [101]. What seems clear is that the lower the IFN signature, the better the clinical response to therapy. Again, a potential explanation of these results could be that high IFN expression is associated with the presence of a B-cell clone insensitive to the effects of rituximab, whose activity and survival may be promoted by B-cell survival factors, such as BLyS [102]. Indeed, BLyS expression has been associated with IFNα in a number of autoimmune diseases [103–105].

More recently, a potential role for the IFN signature in the prediction of clinical response to tocilizumab has been proposed [106], although evidence is limited.

Taken together, in general these results suggest a predictive value for the IFN signature in RA patients undergoing commonly used biological therapies, with a high IFN signature at baseline being associated with poor clinical outcome. This effect, however, may not be specific for RA. Comabella et al. [107] have reported a lack of response to IFNβ therapy in a subgroup of multiple sclerosis patients characterized by an overexpression of type I IFN-induced genes, which is linked to increased secretion of IFNα upon stimulation and elevated activation of DCs [107]. This observation highlights a relevant question concerning type I IFNs: should they be considered ‘as a whole’ or could differences be expected among different types, such as α and β? Mavragani et al. [108] have addressed this question. In a prospective study of RA patients treated with TNFα blockers, they found that both IFNα and IFNβ explained the IFN signature, but the better clinical response was associated with an IFNβ-biased balance, presumably via IL-1Ra production [108], a cytokine antagonist up-regulated by IFNs [109]. These results emphasize the differences between both type I IFNs, suggesting that the IFN signature as a whole should be interpreted with caution. However, IL-1Ra is only modestly effective in RA [30,31]. In addition, these results explain the discrepancies observed in IFNβ-treated RA patients [26,27], as it could be not only the dose of IFNβ, but also its balance with IFNα, that really matters in the clinical context of RA. Finally, these results may confirm a pathogenic role for IFNα in a subgroup of RA patients where this cytokine can counteract the ‘protective’ effects of IFNβ [110].

Since both the IFN signature and its role as a predictive biomarker seem to be present in many autoimmune conditions, it has been proposed that the IFN signature is patient-specific rather than disease-specific. This idea provides a new rationale for therapy indications in autoimmunity based on the relevance of molecular profiling, as it has been adopted in other clinical contexts.

In this scenario, the IFN signature could be a key factor for decision-making in RA treatment, with two possible approaches that need to be considered: (i) the IFN signature as a biomarker to optimize and stratify the clinical management; and (ii) the IFN signature as a therapeutic target. In the first strategy, IFN could be considered a useful tool to stratify patients as suitable candidates (or not) for biological therapies, thus aiming to reduce costs and adverse effects. In the second strategy, the IFN signature could be considered as a target itself [111], with a major role in the pathogenesis of some RA patients, so pharmacological inhibition of this pathway may lead to better disease control. In fact, patients with a high IFN signature might benefit from HCQ (hydroxychloroquine), a treatment that ameliorates IFN-induced RA in some patients and which is able to reduce IFNα production [112–114]. Moreover, antimalarial treatment has been reported to be able to decrease circulating immunocomplexes in

RA patients [115], which are potent IFN stimulators. Moreover, chloroquine was able to block rheumatoid factor production induced by immune complexes in activated B-cells by inhibiting TLR9 signalling [116]. Additionally, HCQ also decreases IL-1, IL-6 and TNFα levels [117], thereby accounting for its beneficial effect in RA patients [118,119]. However, direct evidence for a beneficial effect of HCQ on IFNα production in RA is lacking.

JAK inhibitors are also attractive therapies for these patients not only because of the central involvement of JAK proteins in the IFN pathway, since JAK1 and TYK2 are involved in signal transduction downstream of the IFNAR upon type I IFN ligation [6], but also for their ability to decrease type I IFN synthesis in DCs and synovial cells from RA patients [120,121]. This effect seems to be mediated by suppression of the phosphorylation of STAT1, thereby avoiding broad IRF gene activation.

Finally, IFNα blockade might be advisable in these patients, although there is limited evidence from clinical experience with this therapy. However, despite being initially conceived as a therapy for SLE patients [122], some advances have been made in other type I IFN-mediated conditions [123,124], thus reinforcing again the idea of IFNα as a patient-specific target, rather than a disease-specific one.

Previously, some authors have proposed the use of the type I IFN signature as both a disease target and a pharmacodynamic biomarker [125] in SLE, since differential expression of type I IFN-related genes probably account for different response to anti-IFNα therapy. This personalized approach will possibly benefit a number of patients and it would be also a potential explanation for the lack of success in some clinical trials with biological drugs, since patients are frequently recruited and randomized on the basis of unresponsiveness to traditional therapies, but no attention is paid to the activation pathways, which actually are the underlying mechanisms that need to be interfered with. To the best of our knowledge, no studies have been performed following this scheme, probably because of the lack of standardized and validated procedures for assessment of the type I IFN signature, but pharmacodynamic biomarkers are becoming increasingly important in the context of personalized medicine.

However, despite their clear advantages, expression profiles exhibit a number of limitations that should be considered, such as the differential proportion among leucocyte populations, differential response in expression profiles in these populations, different sources from the isolated RNA, and technical and statistical approaches [126].

IFNα AND VASCULAR DAMAGE

Rheumatic conditions are associated with increased CV (cardiovascular) disease mortality and morbidity [127,128]. Actually, RA patients exhibit a CV risk profile similar to that of a 10-year-older non-RA population or same-aged diabetic patients [129]. This increased risk is unexplained by the effect of traditional CV risk factors [128], but it seems to be influenced by disease parameters [130]. It is clear that chronic inflammation and immune dysregulation underlie, at least in part, this excess risk [130–133]. In this scenario, a potential role for type I IFNs has been shown in autoimmune patients. Because of the relevance of the IFN signature in its pathogenesis, most evidence has been obtained from SLE studies, although some authors have also provided new insights in RA patients as well. However, most of these studies have been confirmed *in vitro* in the presence of IFNα, thereby suggesting that detrimental effects on vascular biology could be attributed to IFNα, whereas the contribution of IFNβ remains unclear.

The IFN signature has been associated with increased and premature direct endothelial damage in murine models of lupus [134] and with endothelial dysfunction in SLE patients [135]. In fact, type I IFNs have been reported to modulate several steps in atherosclerotic plaque progression in murine models of lupus and atherosclerosis [136]. Several studies point to a prominent role of EPC (endothelial progenitor cell) dysfunction mediated by increased type I IFNs. This hypothesis was also confirmed in later studies, thereby revealing detrimental effects of type I IFNs (mainly IFNα) in EPC recruitment, differentiation and functionality. In fact, IFNα skewed the human EPC population to a 'non-angiogenic' phenotype, as characterized by their impaired vasculogenic ability *in vitro* [137]. Interestingly, it has been reported that IFNα treatment down-regulated pro-angiogenic mediators in murine EPC cultures [138], which is in line with decreased VEGF (vascular endothelial growth factor) levels detected in SLE patients [137,139]. Furthermore, IFNα was able to down-regulate angiogenic mediators in several cell types [140,141], thereby promoting a generalized failure of vascular repair. Of note, abrogation of IFN signalling resulted in restoration of normal vascular function. These findings are consistent with an early vascular damage in SLE, suggesting that high IFNα expression could promote CV disease in lupus patients after the onset of the disease, thus explaining the association between the IFN signature and subclinical CV disease reported in SLE patients [89].

Although the IFN signature has not been studied in RA in relation to CV disease, it has been positively correlated with increased immune activation, cell adhesion, blood clotting and fatty acid metabolism pathways [87], which are of relevance in atherosclerotic progression. Our group has recently reported increased IFNα serum levels in a subgroup of RA patients [142]. Of note, these patients presented an EPC imbalance associated with aggressive disease markers and pro-inflammatory cytokines. Probably, this pro-inflammatory environment leads to accelerated EPC maturation and altered vascular repair, which could support the increased rate of CV events observed in these patients [142]. Additionally, we have reported a new detrimental role for IFNα in vascular repair failure in RA patients, since IFNα seems to be associated with lower Tang cell (angiogenic T-cell) counts in patients and in *in vitro* cultures [143]. Tang cells have recently been discovered by their ability to promote vascular repair through co-operation with EPCs, and lower Tang cell counts have been related to vascular disease [144].

Several mechanisms may explain the vascular damage associated with high IFNα expression (Figure 3). Type I IFNs have been linked to destabilization of atherosclerotic plaques [145] and exhibit widespread negative effects on the vasculature by affecting many cell types in atherosclerosis [146]. Besides the

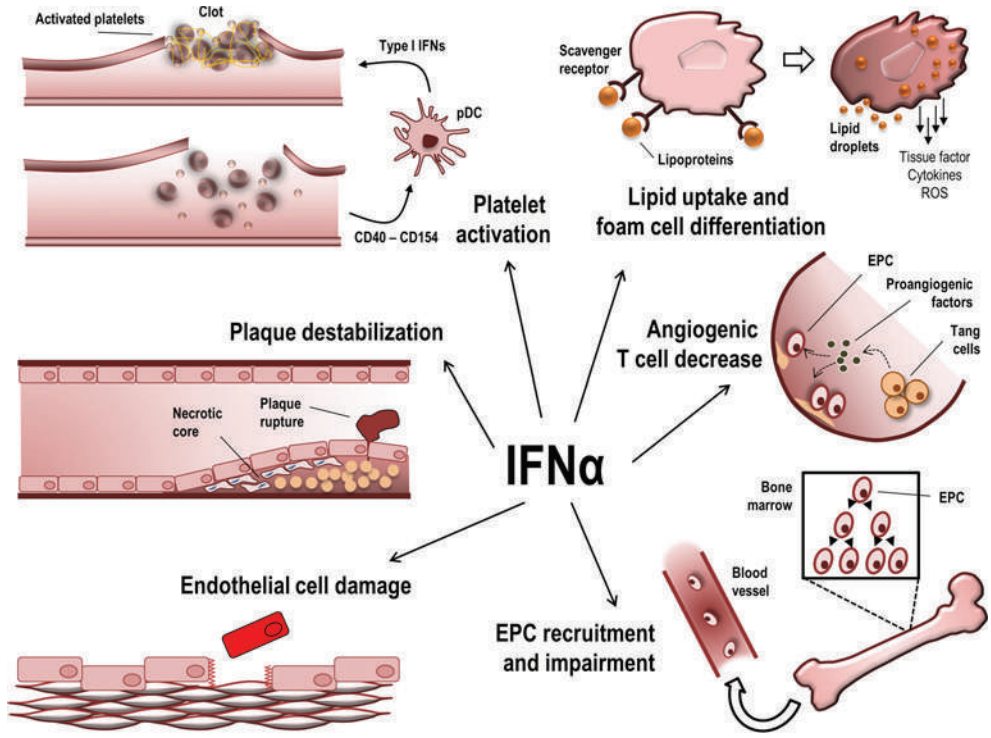

Figure 3 IFNα and vascular damage
IFNα is associated with vascular damage through different ways, including by promoting increased endothelial injury (direct endothelial damage, platelet activation, foam cell differentiation and plaque destabilization) and impaired endothelial repair (EPC dysfunction and Tang cell decrease). ROS, reactive oxygen species.

crucial role of local pDCs, IFNα enhances lipid uptake by macrophages, thereby promoting foam cell differentiation, and they have been associated with vascular disease in SLE patients [147]. In addition, since type I IFNs can increase the expression of co-stimulatory molecules and promote a shift towards Th1 responses, they could induce and amplify a local immune response within the plaque, thus promoting increased plaque instability. Finally, vascular repair is impaired by the effect of IFNα on EPCs and Tang cells.

Apart from VEGF, other molecular mediators behind the negative effects of IFNα on the vasculature have been described. The IFNα pathway has been directly involved in endothelial damage. It has been reported that IFI16, a DNA sensor induced by type I IFNs, promotes endothelial damage and activation and can perpetuate inflammatory responses [148–151]. Interestingly, serum levels of soluble IFI16 are increased in autoimmune patients, especially in RA [150]. Another mediator that deserves to be mentioned is ET-1 (endothelin-1), a potent vasoconstrictor involved in cardiopulmonary pathology, that is inducible by IFNα [152,153] and nucleic acids via TLR3. In fact, ET-1 protein levels increase after IFNα treatment [152] and correlated with IFN-induced proteins [153] in systemic sclerosis patients. Therefore IFN-induced ET-1 levels may account for the adverse cardiopulmonary effects of IFNα therapy and could have a role in IFN-mediated vascular damage. Accordingly, ET-1 levels are increased in serum and synovial fluid from RA patients [154,155], mainly in patients with altered capillaroscopy profiles [155] or extra-articular manifestations [156]. In addition, control of disease activity and inflammation resulted in decreased ET-1 levels and improved cardiac function [157]. Hence ET-1 could have a major role in IFN-induced endothelial damage in RA. Parallel quantification of the IFN signature in these studies would provide insights into the actual role of type I IFNs in vascular damage *in vivo*, but current evidence is limited.

Finally, it is noteworthy that polymorphisms in IRFs have been related to CV disease susceptibility [158,159] and to markers of subclinical atherosclerosis [160,161] in RA patients. Furthermore, pharmacological therapy with IFNα (in non-autoimmune subjects) has also been associated with CV events [162–164], thus reinforcing the pathogenic role for type I IFNs, and especially IFNα, on the vasculature even in the absence of autoimmune milieu.

CONCLUSIONS

Despite the role played by type I IFNs in several autoimmune disorders, evidence supporting their relevance as a trigger for RA is only recently emerging. Increased type I IFN levels may characterize a subgroup of RA patients with distinctive genomic expression profile, as well as specific clinical features. The fact that the IFN signature is found even in the pre-clinical phase of the disease and its potential role as a predictive marker for biologic therapies support the use of IFNα as possible biomarker for different steps in the clinical management of RA. Since the choice

of biological drugs has increased in recent years, and it is becoming increasingly greater as new targets are characterized, type I IFNs are useful biomarkers that should be taken into account for clinical decision-making. The use of type I IFN signatures as pharmacodynamic biomarkers is a promising area not only in RA, but also in a number of autoimmune conditions. Additionally, the involvement of type I IFNs in endothelial damage and vascular repair failure may account for the increased CV risk found in RA patients and could allow, along with other disease features, the identification of patients at risk. Taken together, the inclusion of type I IFN signatures in algorithms for individualized treatments, as well as for CV risk stratification, would notably benefit a number of RA patients.

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Capítulo II: Análisis de las células T angiogénicas en AR

Si bien las EPC tienen un papel crucial en los procesos de reparación endotelial, existen otras poblaciones celulares implicadas en estos mecanismos, como son las células Tang. Sin embargo, presumiblemente por lo reciente de su descubrimiento, esta población celular ha sido poco estudiada y no se había abordado el análisis de esta población celular en pacientes de AR al inicio de esta Tesis Doctoral.

Por tanto, decidimos llevar a cabo el estudio de esta población celular en pacientes de AR con el objetivo de determinar si existía una alteración a nivel de las células Tang en esta patología. Asimismo, investigamos cuáles eran los principales factores que determinaban la frecuencia de esta población en controles sanos y pacientes de AR, analizando conjuntamente parámetros clínicos y factores clásicos de riesgo CV en estos últimos.

Artículo 4: Rodríguez-Carrio J, Alperi-López M, López P, Alonso-Castro S, Ballina-García FJ, Suárez A (2015); *Angiogenic T cells are decreased in Rheumatoid Arthritis patients;* Annals of the Rheumatic Diseases 74(5):921-7.

> Aportación personal al trabajo: participé en todas las fases de este trabajo desde su diseño, el reclutamiento de pacientes, los procedimientos experimentales y el análisis y discusión de los resultados con los coautores. Finalmente, realicé la preparación del manuscrito y las figuras que lo acompañan bajo la supervisión de la Dra. Ana Suárez Díaz.

EXTENDED REPORT

Angiogenic T cells are decreased in rheumatoid arthritis patients

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ABSTRACT

Objective The mechanisms underlying the increased cardiovascular risk (CVR) of rheumatoid arthritis (RA) patients remain unclear. Since the recently discovered angiogenic T cells (Tang) could have a role in endothelial repair through cooperating with endothelial progenitor cells (EPC), the main aim of this study was to analyse the Tang and EPC populations in relation to disease-specific features and traditional CVR factors.

Methods Tang ($CD3^+CD31^+CXCR4^+$) and EPC ($CD34^+VEGFR2^+CD133^+$) populations were quantified by flow cytometry in peripheral blood samples from 103 RA patients and 18 matched healthy controls (HC). Clinical features and traditional CVR factors were obtained from clinical records, and 28-joint Disease Activity Score was used for measuring disease activity. Interferon (IFN) α serum levels were measured by immunoassays.

Results Tang and EPC were strongly decreased in RA patients. In HC, but not in patients, both populations were positively correlated and inversely related to low density lipoprotein- and total-cholesterol levels. Sex, diabetes, dyslipidaemia, hypertension or obesity did not significantly influence Tang in patients, although detected in smokers. However, Tang were closely related to disease activity, autoantibody positivity and IFNα levels. Multiple regression analysis adjusted for traditional CVR factors confirmed that only disease activity, age at diagnosis, antinuclear antibody positivity and smoking habit could predict Tang frequency. Finally, patients who had suffered a CV event since their RA diagnosis presented higher Tang decrease and IFNα levels than those who were CV event-free.

Conclusions Disease-specific parameters, including disease activity, autoantibody profiles and IFNα levels, are associated with Tang decrease in RA, thus probably accounting for CVR.

INTRODUCTION

An increased prevalence of cardiovascular (CV) events, not fully explained by traditional risk factors,[1] has been widely reported in rheumatoid arthritis (RA) patients.[2 3] Therefore, other causes, such as disease activity, chronic inflammation, glucocorticoid treatment and genetic background, have been proposed as disease-related independent risk factors.[3–9] Such factors could increase CV risk by promoting the development of early atherosclerotic lesions and impairing the endothelial repair mechanisms. Moreover, it has been reported that endothelial repair mechanisms were impaired in RA and other autoimmune diseases, partly due to the altered number or function of endothelial progenitor cells (EPC), a haematopoietic-derived population involved in vasculogenesis and vascular repair.[10]

Although EPC levels have been considered as a surrogate marker of CV status in healthy subjects,[11 12] different and even contradictory data about their role in RA patients have been reported. Recently, a novel T cell subset, the so-called angiogenic T cells (Tang),[13] has been described that seems to cooperate with EPCs and enhance endothelial repair function, possibly through the secretion of proangiogenic cytokines. In fact, in vitro experiments showed that Tang depletion could abrogate EPC functionality.[13] Animal models of ischaemia also highlighted the relevance of the Tang population in capillary formation.[13] Tang cells are characterised by the coexpression of CD3, CD31 (platelet endothelial cell adhesion molecule) and CXCR4 (receptor for stromal-cell-derived factor-1) and may express CD4 or CD8. This subset is characterised by the coexpression of naive and memory markers, thus revealing its heterogeneous nature. A recent study in human patients revealed, for the first time, that lower Tang numbers are associated with vascular disease.[14] Thus, Tang may be used as a novel putative biological marker for CV disease.

On the other hand, among other factors that could impair endothelial repair, type I interferons (IFNs) deserve to be noted. IFNα and related cytokines are a family of pleiotropic molecules with potent antiviral effects and an established relevance in systemic autoimmunity.[15] However, increasing evidence points out their roles in endothelial injury and repair failure. In fact, several mechanisms by which IFNα could damage the endothelium have been described.[16–19] In addition, this cytokine has been associated with the occurrence of CV disease in systemic lupus erythematosus independent of traditional CV risk factors.[20] Similar conclusions have been reached by our group when studying RA patients.[21]

Given the very recent description of the Tang population, no such studies in RA patients have yet been reported. Thus, we hypothesised that the increased CV risk of RA patients could be related to altered numbers of Tang cells. Therefore, the main aims of the present study were: (i) to quantify Tang population in RA patients, (ii) to evaluate clinical parameters that could be associated with these cells and (iii) whether IFNα serum levels could influence Tang numbers in these patients.

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MATERIAL AND METHODS

Patients and controls

Our study involved 103 RA patients consecutively recruited from the outpatient clinic from the Rheumatology Department at the Hospital Universitario Central de Asturias, fulfilling the 1987 American College of Rheumatology criteria. Routine clinical examination, including 28-joint Disease Activity Score (DAS28) calculation, was performed during the patients' visit. Then, patients' clinical records were exhaustively revised so as to obtain previous therapies, traditional CV risk factors and histories of previous CV events. Definition and classification of CV events and traditional risk factors (hypertension, diabetes, dyslipidaemia, obesity and smoking) was performed as previously established.[22 23] A CV event was considered if the patient suffered from heart failure, ischaemic heart disease or cerebrovascular accident since their RA diagnosis to the time of sampling. Simultaneously, matched healthy volunteers (n=18; 15 women; age range 23–63 years) without any pathology or treatment were recruited from the Centro Comunitario de Sangre y Tejidos de Asturias. Automatised blood count and serum lipids analysis were carried out for all the participants. Approval for the study was obtained from the Regional Ethics Committee for Clinical Investigation, according to the Declaration of Helsinki and all the participants gave written informed consent.

Flow cytometry analyses

EPC and Tang frequencies were measured by flow cytometry. EPCs were quantified as previously described.[22] Tang were stained with anti-CD3 PerCP-Cy5,5, anti-CD31 fluorescein isothiocyanate (FITC) and CXCR4 PE-Cy7, and those CD31/CXCR4$^+$ were considered Tang (figure 1A) (see online supplementary text).

Cytokine serum level quantification

Serum aliquots were stored at −80°C until cytokine measurements. IFNα and tumour necrosis factor (TNF)α serum levels were analysed by immunoassays (see online supplementary text).

In vitro cultures

Peripheral blood mononuclear cell (PBMC) cultures were carried out to investigate the effect of IFNα, TNFα and patients' serum on Tang frequency in vitro (see online supplementary text).

Statistical analysis

All data are presented as median (IQR) unless otherwise stated. Mann–Whitney, Spearman's ranks, analysis of the variance (ANOVA), χ^2 tests and multivariate regression analysis were used as appropriate. A p value<0.05 was considered statistically significant (see online supplementary text).

RESULTS

Angiogenic–T cells were reduced in RA patients

To evaluate Tang cells in RA patients in relation to EPC-mediated endothelial repair ability and traditional CV risk factors, blood samples from 103 RA patients and 18 healthy controls (HC) were analysed by flow cytometry, quantifying Tang population by means of their CD3, CD31 and CXCR4 expression (figure 1A), whereas EPC populations were determined according to their CD34, CD133 and VEGFR2 expression, as previously described.[24] Demographic and clinical characteristics of patients were summarised in table 1. Results showed a strong decrease of Tang population in RA patients compared with HC, both in absolute numbers (figure 1B) and as a percentage of T cells (2.06 (1.89)% vs 5.52 (4.77)%, p=0.0002). Circulating EPCs, as previously reported, were also decreased in patients (figure 1C).

On the other hand, we observed interesting associations between Tang and CV risk factors in HC that were absent in RA patients (table 2). First, these cells exhibited a strong positive correlation with EPC levels. Of note, this association was found with CD34$^+$CD133$^+$VEGFR2$^+$ cells (the so-called 'true EPC'), but not with total CD34$^+$ or CD34$^+$CD133$^+$ progenitor cells or with the CD34$^+$VEGFR2$^+$ population. In addition, Tang from HC were negatively associated with total cholesterol and low density lipoprotein (LDL)-cholesterol, but not with high density lipoprotein. Furthermore, EPC levels from HC showed similar correlations (total cholesterol: r=−0.573, p=0.013; LDL-cholesterol: r=−0.562, p=0.015), thus supporting the association of both Tang and EPC populations with CV risk factors. Nevertheless, these correlations were completely absent in RA patients. Moreover, male sex and the presence of diabetes, hypertension, dyslipidaemia or obesity did not significantly influence Tang in RA patients, although even lower levels were detected in smokers (p=0.037).

Finally, a stronger Tang–blood decrease was found in the subgroup of RA patients who had suffered a CV event since their RA diagnosis (n=19, time between RA diagnosis and CV event: 70.56±61.11 months) when compared with those without this complication (2.61 (1.88) vs 3.56 (3.75)·10^3 cells/μL, p=0.014). Thus, we analysed the influence of traditional CV risk factors in RA patients with and without previous history of CV events using logistic regression modelling. We found that none of the variables included in the analysis were significantly associated with the occurrence of a CV event (age: p=0.704, male sex: p=0.074, obesity: p=0.958, hypertension: p=0.079, dyslipidaemia: p=0.569, diabetes: p=0.211 and smoking habit: p=0.840). Therefore, traditional CV risk factors did not appear to be the most relevant causes for Tang decrease in RA patients.

Disease activity and autoantibodies influenced Tang in RA patients

Therefore, we aimed to look for disease-specific features which may be involved in Tang reduction in peripheral blood. Among the analysed clinical parameters, the strongest association was detected with DAS28 score (figure 2A), indicating that disease activity plays an important role in Tang decrease. Moreover, Tang and EPC levels remained correlated, although at a lower degree than in HC, in patients with low disease activity (DAS28<2.6, n=27) (figure 2B). This association was completely lost in patients with active disease (DAS28≥2.6, n=76). Other clinical parameters such as tender joint counts (r=−0.260, p=0.009), erythrocyte sedimentation rate (r=−0.330, p=0.001) and age at diagnosis (r=−0.352, p<0.0001), but not disease duration (r=0.009, p=0.929), were negatively correlated with Tang population. In fact, the analysis of RA patients recruited at diagnosis and without treatment (n=7) indicated that Tang were strongly decreased even at early stage of the disease compared with HC (3.98 (4.13)·10^3 vs 8.93 (6.63)·10^3 cells/μL, p=0.006), showing similar levels as they established disease counterparts (3.30 (3.28)·10^3 cells/μL).

Likewise, the analysis of immunological features showed that presence of autoantibodies was also related with Tang–blood population, since patients presenting rheumatoid factor (RF), anticyclic citrullinated peptide antibody (anti-CCP) or antinuclear antibody (ANA) exhibited lower Tang levels than their negative counterparts (figure 2C). In fact, Tang were negatively

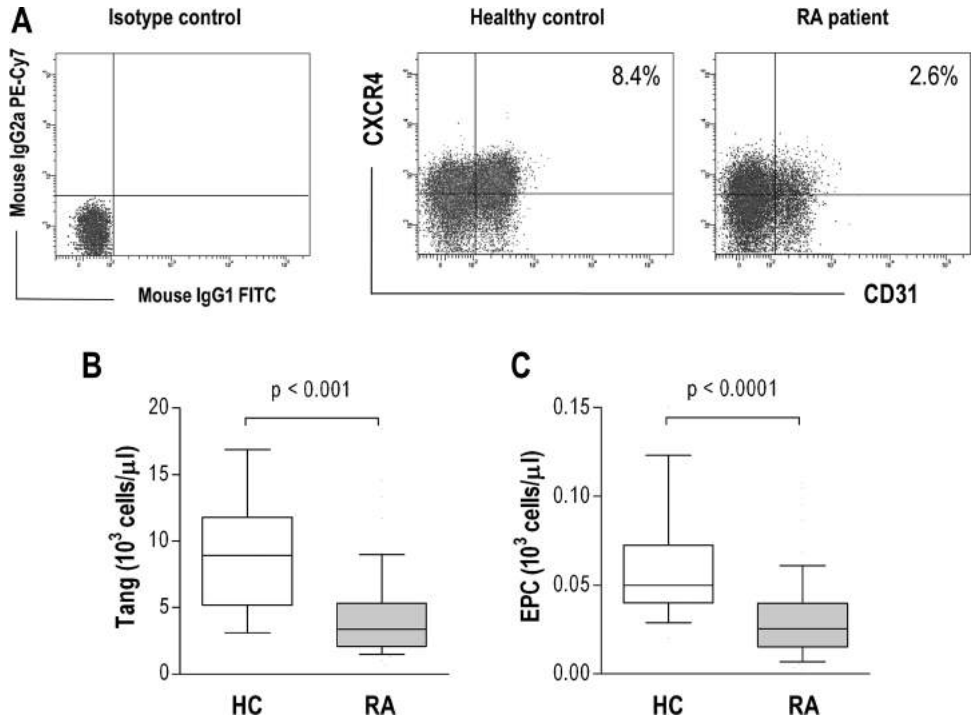

Figure 1 Tang and endothelial progenitor cell (EPC) are decreased in peripheral blood of rheumatoid arthritis (RA) patients. (A) Representative CD31 versus CXCR4 dot-plots of a healthy controls (HC) and a RA patient. Gated CD3 lymphocytes were analysed for CD31 and CXCR4 expression by flow cytometry. Tang population was identified as the triple-positive CD3/CD31/CXCR4 cells in the lymphocyte gate. Quadrants were set according to the fluorescence signal provided by the isotype controls. Box plots represent Tang (B) and EPC (C) peripheral blood reduction in RA patients compared with HC. Differences were evaluated by Mann–Whitney U test.

associated with RF and ANA titres (r=−0.473, p<0.0001 and r=−0.252, p=0.011, respectively). Additionally, patients with previous CV events presented higher frequency of anti-CCP (88.9% vs 62.5%, p=0.031) and a trend for RF positivity (83.3% vs 62.5%, p=0.091).

Therefore, in order to evaluate the relevance of clinical and immunological features in determining Tang frequencies, a multivariate linear regression analysis were performed. After adjusting for traditional CV risk factors (age, sex, dyslipidaemia, diabetes, hypertension, obesity and smoking), only disease activity (B (95% CI) −0.546 (−1.008 to −0.339), p=0.0001), age at diagnosis (−0.041 (−0.088 to −0.018), p=0.003), ANA positivity (−1.046 (−1.959 to −0.184), p=0.019) and smoking (−0.732 (−2.189 to −0.374), p=0.006) showed a significant effect in predicting Tang levels, thus supporting that, except for smoking, disease-specific parameters rather than traditional CV risk factors, are implicated in Tang numbers in RA patients.

IFNα levels were associated with Tang decrease in peripheral blood

In addition to clinical parameters, other factors involved in RA pathogenesis and inflammation burden could have a role in Tang frequency reduction. Thus, to evaluate possible serum markers associated with endothelial damage, we quantified circulating IFNα and TNFα, two cytokines involved in the pathogenesis of several autoimmune diseases. Results showed that serum levels of IFNα were significantly increased in patients (p=0.004) (see online supplementary table S1) and correlated inversely with Tang (figure 3A). Although IFNα was undetectable in the 49.3% of patients, this association remained significant in the IFNα-detectable subgroup (r=−0.233, p=0.048), which displayed lower Tang levels (2.64 (3.03)·10^3 vs 4.51 (4.48)·10^3 cells/µL, p=0.004). This relationship was completely absent in HC (r=−0.028, p=0.918), probably because IFNα was undetectable in most of them (88%). No associations with total CD3 or CD31 cells were found, suggesting that detrimental effects were specific for Tang population rather than a generalised effect on T cells. Moreover, RA patients with previous CV events exhibited higher IFNα serum levels compared with those without them (p=0.019, 78% of IFNα positive). On the other hand, TNFα levels, also increased in patients (p=0.014), failed to exhibit a significant association with Tang numbers (see online supplementary figure S1A), although patients with the highest levels (>80th percentile, 39.49 pg/mL) showed a trend to lower Tang counts (2.49 (1.74)·10^3 vs 3.92 (3.93)·10^3 cells/µL, p=0.081). No significant differences were detected in patients with previous CV events (p=0.573).

Finally, culture assays were performed in order to evaluate the effects of these cytokines on Tang population. Thus, PBMCs were cultured for 4 days in medium alone or in the presence of IFNα (1000 U/mL). Tang frequency was significantly reduced (up to a 26.6%) in IFNα-treated cells (figure 3B), although the total amount of viable T lymphocytes was similar in both cultures (p=0.516). Therefore, to determine the possible effect of the IFNα present in RA serum, PBMCs were cultured in medium supplemented with 10% of pooled sera from either HC or RA patients and with increasing concentrations of anti-IFNα or control rabbit IgG antibodies added to RA serum. At day 4, RA serum-treated cells displayed a strong Tang decrease compared with those HC-treated that was partially restored, dose-dependently, by anti-IFNα blockade. No differences in total CD3 counts were observed after IFNα or RA serum treatment. Moreover, the Annexin V/7-Actinomycin D (AAD) staining showed that both early and late Tang apoptosis was not different

Table 1 Demographic and clinical parameters of RA patients

	RA patients (n=103)
Gender (female : male)	83:20
Age at sampling, years (mean±SD)	54.81±14.37
Disease features	
Disease duration, years (mean (range))	5.58 (0–30.00)
Age at diagnosis, years	47.83 (13.92)
Disease activity (DAS28)	3.49 (1.90)
Tender joint count	2.00 (4.00)
Swollen joint count	1.00 (3.00)
Patient global assessment (0–100)	37 (36.50)
ESR, mm/h	13.00 (22.50)
CRP, mg/L	2.00 (3.90)
HAQ (0–3)	0.87 (1.21)
RF (+), n (%)	65 (63.1)
αCCP (+), n (%)	66 (64.0)
ANA (+), n (%)	51 (49.1)
Shared epitope, n (%)	41 (39.8)
Erosive disease, n (%)	48 (46.6)
Traditional CV risk factors, n (%)	
Dyslipidaemia	36 (34.9)
Hypertension	35 (33.9)
Diabetes	9 (8.7)
Obesity (BMI>30)	20 (19.4)
Smoking habit	34 (33.0)
CV events, n (%)	
Previous CV events	18 (17.4)
Ischaemic heart disease	8 (7.7)
Heart failure	8 (7.7)
Cerebrovascular accidents	2 (1.9)
Treatments, n (%)	
None or NSAIDs	7 (6.7)
Glucocorticoids	56 (54.3)
Methotrexate	77 (74.7)
TNFα blockers	44 (42.7)
Tocilizumab	12 (11.6)
Statins	20 (19.4)

Categorical variables are summarised as n (%), and continuous one as median (IQR) unless otherwise was stated.
αCCP, cyclic citrullinated peptide antibody; ANA, antinuclear antibody; BMI, Body Mass Index; CRP, C reactive protein; CV, cardiovascular; DAS28, 28-joing Disease Activity Score; ESR, Erythrocyte Sedimentation Rate; HAQ, Health Assessment Questionnaire; NSAIDs, non-steroidal anti-inflammatory drugs; RA, rheumatoid arthritis; RF, rheumatoid factor; TNFα, tumour necrosis factor α.

Table 2 Associations of angiogenic T cells with EPC populations and CV risk factors

	HC	RA
EPC populations		
$CD34^+VEGFR2^+CD133^+$ (EPC)	r=0.886 p<0.0001	r=−0.052 p=0.679
CD34	r=0.252 p=0.313	r=0.126 p=0.263
$CD34^+CD133^+$	r=0.318 p=0.198	r=0.142 p=0.205
$CD34^+VEGFR2^+$	r=0.362 p=0.139	r=−0.010 p=0.933
Traditional CV risk factors		
Total cholesterol (mg/dL)	r=−0.688 p=0.002	r=0.040 p=0.709
HDL-cholesterol (mg/dL)	r=−0.099 p=0.695	r=0.112 p=0.300
LDL-cholesterol (mg/mL)	r=−0.670 p=0.002	r=0.107 p=0.325
Male sex	p=0.360	p=0.282
Diabetes		p=0.712
Hypertension		p=0.570
Obesity (BMI >30)		p=0.119
Smoking habit		p=0.037

Associations between Tang and continuous variables were assessed by Spearman rank correlations (r coefficient and p value are shown), and Mann–Whitney U test was used with categorical variables (p value is shown).
BMI, Body Mass Index; CV, cardiovascular; EPC, endothelial progenitor cell; HC, healthy controls; HDL, high density lipoprotein; LDL, low density lipoprotein; RA, rheumatoid arthritis.

in IFN-treated cultures than in the negative control (early: 0.53 ±0.21 vs 0.82±0.28, p=0.114; late: 0.16±0.20 vs 0.08±0.07, p=0.771). However, IFNα seemed to be able to downregulate CXCR4 expression (mean fluorescence intensity (MFI) in Tang: 7630.50±1575.82 vs 5318.50±3253.80; in CD3 cells: 4359 ±799.23 vs 3606.25±454.77), thus being one potential mechanism to explain lower Tang numbers.

These results point out the detrimental role of IFNα on Tang subset and the involvement of other serum factors in this effect. In fact, TNFα was also able to slightly decrease Tang frequencies (7.4%) in vitro (see online supplementary figure S2B), although no associations were observed in patients.

DISCUSSION

In recent years, several studies have been performed to explore the mechanisms underlying the increased CV risk and endothelial damage observed in RA patients. The results presented herein are the first reporting a new factor that seems to be implicated in this condition: the recently described subpopulation of immune cells, the so-called Tang.

It has been suggested that Tang cells may be used as a biomarker for CV risk and endothelial function.[13] Accordingly, the evaluation of Tang cells in healthy individuals performed in this work showed that this population was negatively correlated with total and LDL-cholesterol levels. In addition, Tang were positively associated with $CD34^+VEGFR2^+CD133^+$ cells, the true EPC population, but not with other phenotypes ($CD34^+VEGFR2^+$ or $CD34^+CD133^+$). This result highlights the special relevance of CD133 labelling for EPC measurements by flow cytometry.[25] Although a positive correlation between Tang and EPC colonies in vitro has been previously reported,[13] this is the first study showing a correlation between EPC and Tang in human peripheral blood. These findings suggest a connection between decreased Tang numbers and increased CV risk.

The most important finding of our work, however, was the striking circulating Tang decrease detected in RA patients, even at diagnosis, which, additionally, was unrelated to EPC levels and traditional CV risk factors, except for smoking. Instead, disease activity and presence of autoantibodies seemed to have detrimental effects on the Tang population. These cells were decreased in a disease activity-dependent manner in RA patients, thus suggesting that specific disease features were implicated in Tang decrease. In fact, the association between EPC and Tang was partially recovered in patients with low disease activity. These findings are in line with the idea that an accurate control of the disease will have a positive impact on CV risk management.[26] However, our data could support an alternative role of Tang in chronic

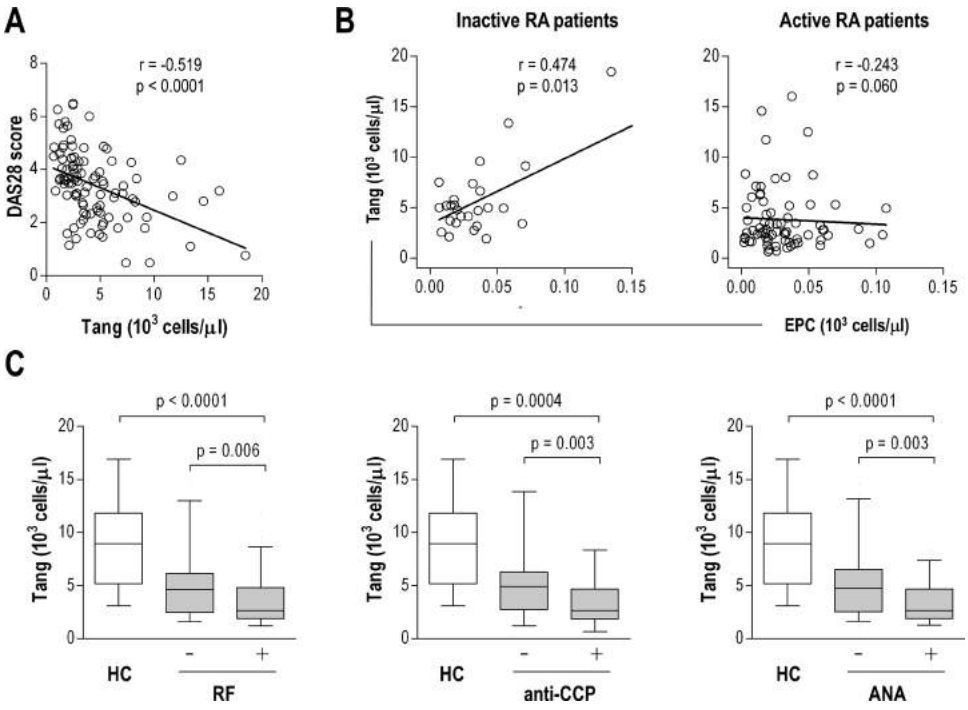

Figure 2 Disease activity and autoantibody positivity were associated with Tang decrease. (A) Tang cells were decreased in rheumatoid arthritis (RA) patients in a disease activity-dependent manner and (B) were positively correlated with endothelial progenitor cells (EPC) populations in inactive patients (Disease Activity Score (DAS)<2.6, n=27) but not in active ones (DAS≥2.6, n=66). (C) Autoantibodies positivity (rheumatoid factor (RF): n=65, α cyclic citrullinated peptide antibody (CCP): n=66 and antinuclear antibody (ANA): n=51) was associated with Tang reduction. Correlations were assessed by Spearman ranks test and differences were evaluated by Mann–Whitney U test.

inflammation, since Tang behaviour under this situation is yet unknown. In fact, it might be expected that Tang–blood cells would migrate to the inflamed tissues, thus explaining the low circulating counts and the inverse relationship with inflammatory markers. Therefore, we cannot exclude that Tang cells in patients with an active inflammatory disease could be involved in the chronic inflammation rather than in angiogenic repair.

On the other hand, Tang were also associated with late age at diagnosis and the presence of ANA, RF and anti-CCP antibodies, all of them previously associated with poor prognosis and CV risk in RA.[27 28] Moreover, autoantibody positivity has been previously related to CV events in RA[29] and other clinical conditions.[30 31] Therefore, our data suggest that the negative effect of autoantibodies on Tang could be one of the underlying mechanisms by which they influenced CV risk, at least in RA.

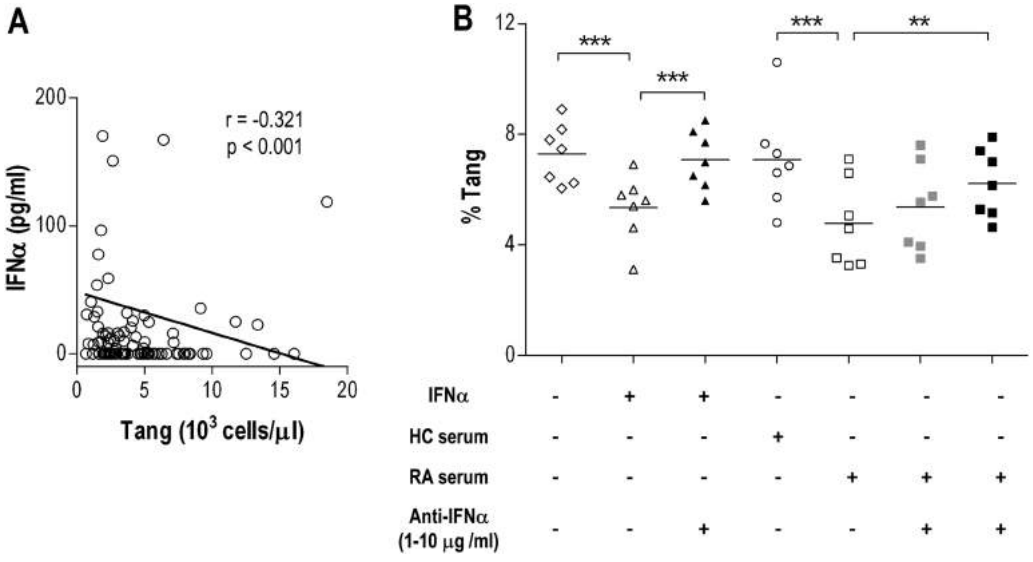

Figure 3 Interferon (IFN)α exhibited negative effects on Tang population. (A) IFNα serum levels were negatively correlated with Tang frequency in rheumatoid arthritis (RA) patients. (B) Peripheral blood mononuclear cell (PBMC) cultured in the presence or absence (medium) of 1000 U/mL of recombinant human IFNα prompted an in vitro Tang reduction, which was totally abrogated by IFNα blockade. A similar decrease was observed when culturing in the presence of RA pool serum (IFNα: 159.65 pg/mL; TNFα: 88.63 pg/mL) compared with those healthy controls sera-treated (IFNα: undetectable; TNFα: 5.09 pg/mL). Serum RA-treated reduction was partially recovered by IFNα blockade in a dose-dependent fashion: 1 μg/mL (▣) and 10 μg/mL (■). Independent cultures were performed with freshly isolated PBMC from different blood donors (n=7). Correlations were analysed by Spearman ranks test and differences between among treatments were evaluated by a repeated measures analysis of the variance (ANOVA) and Bonferroni post hoc test. Horizontal bars represent the mean value ***p<0.001, **p<0.01.

According to our results, disease-specific features rather than traditional CV risk factors, apart from smoking, appear to be associated with Tang reduction in RA, thus supporting the idea that new factors should be taken into account in CV risk assessment in RA. In line with this, an interesting result of this work was the suggested harmful role played by IFNα on Tang cells. Type I IFN signature has been widely associated with the pathogenesis of autoimmunity, first in systemic lupus erythematosus (SLE),[32] but currently also in a subset of RA and other disorders.[15 33 34] Moreover, type I IFNs have been associated with disease activity[35] and clinical features[36 37] as well as with atherosclerosis markers[19 38 39] and CV disease.[20] In fact, different ways by which IFNα could damage the endothelium have been described.[40] Additionally, IFNα treatment has been associated with increased CV events in non-RA subjects.[41–43] Thus, the role of IFNα in Tang decrease reported here may suggest a new way by which this cytokine could have a negative impact on endothelial repair and CV risk, in the subgroup of RA patients with the IFNα 'signature'.[15 34] Recently, systemic disease has been associated with the occurrence of CV events in RA patients.[44] Our results are in line with all these findings, since patients with higher IFNα levels are characterised by a higher rate of CV events and lower Tang frequencies. Thus, in addition to inflammatory and disease-specific markers, high IFNα levels might be helpful in the identification of RA patients with high CV risk.

Finally, the analysis of patients with a history of CV events may support the use of Tang as a putative marker of endothelial damage and CV disease in RA, as was suggested in other pathologies.[14] These patients exhibited lower Tang counts than those CV-free, highlighting the role of Tang cells in vascular repair. These patients also displayed increased levels of IFNα, previously associated with the development of premature atherosclerosis[20 39] and CV disease[19] in lupus. Accordingly, type I IFN signature has been found to be upregulated even several years after CV event occurrence.[17] Therefore, Tang cells could be an interesting target in RA and CV disease.

In conclusion, our data indicate that peripheral Tang decrease, in addition to an altered EPC function, is associated with the increased CV risk in RA patients, probably by impairing endothelial repair. These low Tang levels are closely related to disease-specific parameters. Specifically, high disease activity and autoantibody positivity are strong indicators of Tang reduction, whereas presence of high IFNα levels could be considered an additional factor in a subgroup of patients. We cannot exclude, however, that severe disease, chronic inflammation and IFNα can directly promote endothelial dysfunction, thus increasing CV risk independently of Tang population. In any case, these disease features could be interesting tools to account for CV risk in RA patients. Although further studies are needed to investigate the functionality of these cells in inflammatory conditions, increasing Tang number and/or function might be a promising intervention in RA patients, mainly in those with high risk or history of CV disease. In this sense, IFNα blockade[45] could be a valuable therapy for patients with high levels of this cytokine.

Contributors JR-C performed most of the flow cytometry analyses and data collection as well as wrote the manuscript. PL participated in immunoassays measurements and experimental procedures. MA-L, SA-C and JB-G were in charge of patients' recruitment and clinical data collection. AS conceived and coordinated the study, collected the data, performed the statistical analyses and corrected the manuscript. All the authors read and approved the final version of the manuscript.

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Patient consent Obtained.

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Capítulo III: Estudio de las micropartículas circulantes en AR

Las MP circulantes son unos mediadores muy interesantes en el estudio del daño endotelial y la enfermedad CV, tanto por su capacidad para desencadenar diferentes tipos de respuestas biológicas, como por las ventajas que ofrecen como biomarcadores. Aunque la investigación de las MP en pacientes con enfermedad CV y disfunción endotelial ha aportado evidencias de gran interés para entender los procesos patogénicos asociados a la progresión de la disfunción endotelial, el estudio de las MP en AR no había recibido la misma atención que otras patologías y se había centrado en su papel patogénico en las respuestas inflamatorias implicadas en la patogénesis de la enfermedad. Además, la utilización de diferentes metodologías para la identificación y cuantificación de MP dificulta enormemente la comparación de los resultados obtenidos en los diferentes trabajos publicados.

Hasta el inicio la presente Tesis Doctoral, no se había estudiado la presencia y distribución de diferentes tipos de MP circulantes en pacientes de AR, en relación a los parámetros clínicos y los factores clásicos de riesgo de forma simultánea, lo cual es de gran relevancia al coexistir un estado de activación inmunitaria con la activación endotelial. Por tanto, decidimos abordar este estudio optimizando además un nuevo protocolo para la identificación de MP circulantes procedentes de muestras de plasma mediante citometría de flujo.

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> Aportación personal al trabajo: en este estudio corrió a mi cargo la mayor parte del trabajo experimental, tanto en la parte realizada con muestras de pacientes como los estudios in vitro. Asimismo, llevé a cabo el análisis y discusión de los resultados con el resto de los coautores. Por último, realicé la redacción del manuscrito y las figuras que lo acompañan en colaboración con la Dra. Ana Suárez Díaz.

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Original Paper

Altered profile of circulating microparticles in rheumatoid arthritis patients

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Abstract

Microparticles (MPs) could be considered biomarkers of cell damage and activation as well as novel signalling structures. Since rheumatoid arthritis (RA) is characterized by immune and endothelial activation, the main aim of the present study was to analyse MP counts in RA patients. Citrated-blood samples were obtained from 114 RA patients, 33 healthy controls (HC) and 72 individuals with marked cardiovascular (CV) risk without autoimmune manifestations (CVR). MPs were analysed in platelet-poor plasma (PPP) and different subsets were identified by their surface markers: platelet- ($CD41^+$), endothelial- ($CD146^+$), granulocyte- ($CD66^+$), monocyte- ($CD14^+$) and Tang- ($CD3^+CD31^+$) derived. Disease activity score (DAS28), clinical and immunological parameters as well as traditional CV risk factors (diabetes, hypertension, dyslipidaemia and obesity) were registered from clinical records and all data were integrated using Principal Component Analysis (PCA). Absolute MP number was increased in RA patients compared with HC and positively correlated with traditional CV risk factors, similar to that of CVR subjects. In addition, frequency of the different MP subsets was different in RA patients and significantly associated with disease features. Moreover, *in vitro* assays revealed that MPs isolated from RA patients were able to promote endothelial activation and exhibited detrimental effects on human microvascular endothelial cells (HMEC-I) endothelial cell functionality. Circulating MPs from RA patients displayed quantitative and qualitative alterations that are the result of both disease-specific and traditional CV risk factors. Accordingly, this MP pool exhibited *in vitro* detrimental effects on endothelial cells, thus supporting their role as biomarkers of vascular damage.

Key words: angiogenic T-cell, cardiovascular risk factor, endothelial damage, microparticle, rheumatoid arthritis

INTRODUCTION

Microparticles (MPs) are small membrane vesicles (0.1–1.0 μm) constitutively released by many cell types under physiologically conditions, but enhanced in many pathological situations, mainly associated with cell damage. Largely considered as inert cell debris, previous studies have demonstrated they could have a role in intercellular communication [1]. They have been demonstrated to harbour nucleic acids, signalling molecules, cytokines and even organelles, thereby supporting their active role in cell biology [2,3]. These facts have led to more attention being focused on MPs, since they could exert different effects depending on the conditions under which they originated as well as on the cell type from which they have been released. Accordingly, MPs exhibit an array of surface markers derived from their parental cell that can be used to assess their origin [4].

Increased levels of MPs have been reported in patients with malignancies, infections, systemic inflammation, autoimmune and vascular diseases, among other pathological states [5]. Thus, circulating MPs have been commonly considered as biomarkers of injury, since they originate after cell activation and apoptosis, or are actively released upon specific activating receptors signals [2,6]. This is especially relevant in the context of cardiovascular (CV) disease, since MPs derived from different cell types implicated in the etiopathology of the disease (endothelial cells, lymphocytes, monocytes, smooth muscle cells and platelets) have been found to be increased in patients [7,8]. Actually, MPs from platelets and endothelial cells are proposed to play a

Abbreviations: APC, allophycocyanin; BMI, body mass index; CV, cardiovascular; CVR, cardiovascular risk; HMEC-I, human microvascular endothelial cells; EMP, endothelial-derived MP; ESR, erythrocyte sedimentation rate; GMP, granulocyte-derived MP; HC, healthy controls; DAS28, disease activity score (28 joints); MoMP, monocyte-derived MP; MP, microparticle; PCA, Principal Component Analysis; PE, phycoerythrin; PMP, platelet-derived MP; PPP, platelet-poor plasma; PRP, platelet-rich plasma; RA, rheumatoid arthritis; Tang, angiogenic T-cell; Tang-MP, Tang-derived MP; TNFα, tumour necrosis factor α; VEGFR2, vascular endothelial growth factor receptor 2_1; VPD, Violet Proliferation Dye 450.

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role in thrombogenesis as well as in endothelial activation [9,10]. Moreover, platelet-derived MPs are known to be able to activate neutrophils [11,12], thereby promoting an innate immune activation that leads to neutrophil extracellular traps (NET) formation [13]. These mechanisms are also involved in vascular damage, thus supporting the link between MPs, inflammation and CV disease. In fact, the chronic inflammation associated with many autoimmune disorders could underlie the increased prevalence of CV events reported in these patients [14,15]. However, the role played by MP subsets in these situations remains unknown. Therefore, knowledge of these events could help to identify patients at risk and improve specific therapies. Additionally, they represent accessible and valuable biomarkers of different tissues (especially the vasculature) that are difficult to reach and study.

Taking into account these considerations, rheumatoid arthritis (RA), an autoimmune condition in which both immune and endothelial activation can be found, provides an interesting scenario to analyse MPs subsets. Previous evidence is limited and results concerning disease associations and MP subsets are heterogeneous (reviewed in [1]). A plausible explanation to account for these discrepancies, also found in other pathologies, is the lack of standardized protocols to analyse MPs. Because of their small size and the large heterogeneity of plasma MPs, most studies are performed by flow cytometry, although other methods have also been used. Traditionally, MPs had been identified by annexin V-binding in their surface [16], but previous evidence has brought into question this methodology, since annexin V-negative MPs express specific surface markers [17,18] and have been reported to have clinical relevance [19]. Consequently, many authors have chosen different approaches to avoid annexin staining, such as MP total labelling [20] or no labelling [4].

With the aim of estimating the contribution of MPs to RA pathogenesis, this work simultaneously analysed total and platelet-, endothelial-, granulocyte- and monocyte-derived MPs in relation to disease-specific parameters as well as traditional CV risk factors. In addition, since we have recently proposed a role for angiogenic T-cells (Tang) in RA [21], we aimed to evaluate whether Tang-derived MPs can be found and if associations with clinical parameters could provide new insights into this T-cell subset. Finally, *in vitro* studies were performed to estimate the potential deleterious effect of RA-MPs on vascular endothelium.

MATERIALS AND METHODS

Patients

We conducted a case–control study involving 114 RA patients fulfilling the 2010 American College of Rheumatology RA criteria, consecutively recruited from the Department of Rheumatology (Hospital Universitario Central de Asturias, Oviedo). Routine clinical examination, including disease activity score (28 joints) (DAS28) calculation, was performed at the time of sampling. Medical records were revised in order to register clinical and immunological parameters, medications, traditional CV risk factors and previous CV events. Definition and classification of CV events and traditional risk factors (hypertension, diabetes, dyslipidaemia, obesity and smoking) were performed as previously established [22,23].

Simultaneously, 33 healthy volunteers within a similar age range and gender as patients were recruited from the same population, and a group of 72 individuals with different traditional CV risk factors were recruited from their primary care referral centre (Table 1).

Automatized complete blood count and serum lipids analysis were carried out for all the participants. Approval for the study was obtained from the Regional Ethics Committee for Clinical Investigation, in compliance with the Declaration of Helsinki. All the participants gave written informed consent prior to study inclusion.

Blood sampling and isolation of platelet-poor plasma

A fasting blood sample was obtained by venipuncture in 4.5 ml citrate-containing tubes (BD Vacutainer), which were transferred to the laboratory and centrifuged at 3000 ***g*** for 15 min at room temperature to obtain platelet-poor plasma (PPP) within a maximum of 2 h after blood collection. The resulting plasma was divided in three aliquots and stored at $-80\,^{\circ}$C until analysis.

Analysis of MPs by flow cytometry

PPP aliquots were thawed at room temperature and 200 μl were transferred into new tubes and centrifuged at 13000 rpm for 30 min at $15\,^{\circ}$C. Then, the upper 180 μl were carefully discarded and the initial volume was restored with 0.22 μm double- filtered PBS. To identify cell-derived MPs, a Violet Proliferation Dye 450 (VPD, BD Biosciences) staining was performed, thus avoiding annexin V limitations [24]. Hence, 150 μl were transferred into a new tube, brought to a final volume of 1 ml with double-filtered PBS and 1 μl of 1 mM VPD was added. After incubation at $37\,^{\circ}$C for 15 min, staining was stopped by placing the samples immediately on ice for 20 min. Finally, VPD-stained MP suspensions were divided into different tubes and pairs of antibodies were added to identify specific MP subsets: anti-CD41–FITC (Immunostep) and anti-CD146– allophycocyanin (APC) (Miltenyi Biotech); anti-CD14– phycoerythrin (PE) (Miltenyi) and anti-CD66b–APC (BD); anti-CD3–APC (Immunostep) and anti-CD31–PE (Immunostep). Antibodies were previously centrifuged (16 060 ***g*** rpm, 10 min, $4\,^{\circ}$C) in order to avoid aggregates [25]. Incubation was performed at room temperature for 15 min and then MP suspensions were transferred into Stepcount tubes (Immunostep), which allow absolute quantification. Tubes were immediately processed by flow cytometry.

Samples were analysed in a FACS Canto II flow cytometer. Forward scatter (FSC) and side scatter (SSC) were adjusted to logarithmic gain. The MP gate was designed using latex microbeads (Sigma–Aldrich) and confirmed with platelet-rich plasma (PRP) [26], setting 1.1 and 0.3 μm as the upper and lower detection limits. Below 0.3 μm, only debris seemed to be detected after analysing double-filtered PBS. Unstained MPs were used to set the threshold for VPD-positive signal and unstained negative control of VPD-stained MPs to establish specific antibody fluorescence. No spillover between VPD and the fluorochromes assayed was registered. Acquisition was performed until

Table 1 Demographic and clinical parameters of RA patients

Categorical variables are summarized as numbers (percentage), and continuous variables as medians (interquartile range) unless otherwise stated *[median (range)]. [a]*P* value <0.01 (HC compared with RA: $P = 0.696$). [b]*P* value <0.01 (CVR compared with RA: $P = 0.460$). DAS28, disease activity score (28 joints); HAQ, Health Assessment Questionnaire; RF, rheumatoid factor; αCCP, cyclic citrullinated peptide antibody; ANA, antinuclear antibody; NSAIDS, non-steroidal anti-inflammatory drugs. Differences were assessed by Kruskal–Wallis and Dunn–Bonferroni multiple comparisons tests and chi-square test, as appropriate.

Parameter	HC ($n = 33$)	CVR ($n = 72$)	RA patients ($n = 114$)
Gender (female:male) (*n*)	25:8	38:34[a]	90:24
Age at sampling (years)*	44.29 (23–72)[b]	57.41 (33–69)	55.04 (22–87)
Disease features			
Disease duration (years)			4.91 (7.19)
Age at diagnosis (years)			48.41 (14.62)
Disease activity (DAS28)			3.61 (1.97)
Tender joint count			2.00 (6.00)
Swollen joint count			1.00 (4.00)
Patient global assessment (0–100)			40.00 (41.25)
ESR (mm/h)			15.50 (23.00)
Patient pain assessment (0–10)			4.00 (4.00)
HAQ (0–3)			0.87 (1.22)
RF (+) (*n*)			70 (61.4%)
αCCP (+) (*n*)			70 (61.4%)
ANA (+) (*n*)			58 (50.8%)
Shared epitope (*n*)			47 (41.2%)
Erosive disease (*n*)			49 (42.9%)
Traditional CV risk factors (*n*)			
Dyslipidaemia			40 (35.3%)
Hypertension			38 (33.3%)
Diabetes (Type 2)			9 (7.8%)
Obesity (BMI >30)			22 (19.3%)
Smoking habit			38 (33.3%)
Number of traditional CV risk factors*			1.00 (0–4)
Previous CV events			18 (15.7%)
Treatments (*n*)			
None or NSAIDs			12 (10.5%)
Glucocorticoids			61 (53.5%)
Methotrexate			80 (70.1%)
TNFα blockers			45 (39.4%)
Tocilizumab			12 (10.5%)
Statins			20 (17.5%)

10 000 microbeads from Stepcount tubes were acquired (<4 min/tube) at medium rate. All samples were processed and analysed batchwise to minimize technical variations.

Total and subset specific cell-derived MPs (absolute number/ml plasma) were calculated according to the MP counts acquired, the total number of microbeads from Stepcount tubes and the dilution performed during sample preparation.

In vitro assays with HMEC-I cells

The *in vitro* effects of plasma-isolated MPs on endothelium were evaluated using human microvascular endothelial cells (HMEC-I) cells. To this end, pooled MP suspensions from healthy controls (HC), cardiovascular risk (CVR) individuals and RA patients were prepared from ten representative subjects of each group [HC: 1.85×10^6 total MPs/ml, 9.3×10^4 platelet-derived MPs (PMP)/ml, 968.87 endothelial-derived MPs (EMP)/ml, 244.01 granulocyte-derived MPs (GMP)/ml, 190.57 Tang-derived MPs (Tang-MP)/ml and 3.8×10^3 monocyte-derived MPs (MoMP)/ml; CVR: 2.2×10^6 total MPs/ml, 9.9×10^4 PMP/ml, 500.23 EMP/ml, 172.64 GMP/ml, 227.61 Tang-MP/ml and 2.8×10^3 MoMP/ml; and RA: 4.1×10^6 total MPs/ml, 12.4×10^4 PMP/ml, 2200.6 EMP/ml, 1024.86 GMP/ml, 829.65 Tang-MP/ml and 5.1×10^3 MoMP/ml]. Similarly, age and gender distribution did not differ among groups ($P = 0.70$ and $P = 0.327$, respectively). PPP was centrifuged as previously described and supernatants were discarded and replaced with HMEC-I complete medium so as to eliminate residual plasma containing soluble mediators. Then, the volume

was adjusted to obtain uniform total MP concentrations in the different pools and serial dilutions were performed according to the experimental design. Two different pools were prepared per group and assayed simultaneously. HMEC-I cells were cultured in MCDB131 medium (Sigma) supplemented with 10% foetal calf serum (PAA, Belgium), 100 μg/ml streptomycin and ampicillin (PAA), 2 mM glutamine (Sigma), 1 μg/ml hydrocortisone (Sigma) and 10 ng/ml epidermal growth factor (Immunostep) in a humidified incubator with 5% CO_2 at 37°C. Assays were performed within the second and sixth passages.

For angiogenesis assays, 96-well plates were coated with 50 μl of Matrigel (BD) and left for 30 min at 37°C for polymerization. Then, 50 000 HMEC-I cells resuspended in 100 μl of complete medium were added to each well, followed by the addition of MP suspensions (50 μl) at different concentrations in duplicate. After 16 h of culture, both tube formation and branching points were quantified on one focal plane in three non-overlapping fields per well (27) at 40× magnification, using a Motic AE2000 (Motic) inverted microscope equipped with a compatible digital camera (Moticam 2000, Motic).

To assay endothelial activation, HMEC-I cells were cultured in 24-well plates with MP suspensions at different concentrations for 16 h. Then, cells were washed and stained with Fixable Viability dye e450 nm (eBioscience) for 30 min in the dark. Next, cells were washed and stained with different antibodies: anti- vascular endothelial growth factor receptor 2 (VEGFR2)–PE (R&D), anti-CD144–APC (Milteny) and anti-CD62E–APC (Immunostep) for 30 min at 4°C. Finally, cells were washed again to eliminate non-binding antibodies and immediately analysed by flow cytometry.

TNFα quantification

Serum aliquots were stored at −80°C until cytokine measurements. Tumour necrosis factor α (TNFα) serum levels were quantified using a BD OptEIA kit (BD) following the manufacturer's instructions. The detection limit was 1.95 pg/ml.

Statistical analysis

Data are expressed as median (interquartile range) unless otherwise stated. Differences between MP concentrations were assessed using the Kruskal–Wallis test with Dunn–Bonferroni correction for multiple comparisons test, whereas correlations were studied using the Spearman ranks test. TNFα effect on MP counts was studied by multivariate linear regression analysis adjusted for traditional CV risk factors. MPs counts were log-transformed for normalization prior to regression analyses. Because of the high number of parameters studied, a Principal Component Analysis (PCA) was performed, including traditional CV risk factors and demographic, clinical and inflammatory parameters. The number of components retained was based on 18 values (>1) and loadings >0.5 were used to identify the variables comprising a component. Principal component scores were calculated for each patient and used for multivariate regression analysis. Results from *in vitro* assays were analysed by one-way ANOVA with Dunnett post-hoc test. SPSS 19.0, R 3.0.3 and GraphPad Prism 5.0 for Windows were used.

RESULTS

MP counts were increased in RA patients

Circulating MPs were quantified by flow cytometry in plasma samples from 114 RA patients (Table 1), 33 HC and 72 individuals with different traditional CV risk factors (Supplementary Table S1). The strategy used to identify MPs is presented in Figure 1. The MP gate was set within 0.3 and 1.1 μm using latex microbeads and PRP, with most of the detected MPs being smaller than 0.6 μm (Figure 1A). Total cell-derived MPs were defined as those events within this gate and positive for VPD staining (Figure 1B). Specific MP subsets were identified by their surface markers: $CD41^+$ (PMP), $CD146^+$ (EMP), $CD14^+$ (MoMP), $CD66b^+$ (GMP) and $CD3^+CD31^+$ (Tang-MP) (Figure 1C). Gates were adjusted by the signal provided by the negative control of VPD-stained MPs.

Absolute counts of total MPs in patients and controls and that of different subsets are summarized in Figure 2. Total number of MPs was significantly increased in CVR individuals compared with HC [3.14 (2.6) $\times 10^6$ compared with 2.10 (1.23) $\times 10^6$ MPs/ml] and further increased in RA patients [4.21 (3.02) $\times 10^6$ MPs/ml]. Although platelets were the main source of MPs among the analysed subsets, they did not show a significant increase in any group. However, the absolute number of MPs derived from endothelial cells, granulocytes and Tang lymphocytes was significantly increased as was the number of total MPs in RA patients. Accordingly, the frequency of these MP subsets out of the total MPs was also increased in RA patients compared with HC [EMP: 0.05 (0.10) compared with 0.02 (0.067)%, $P = 0.029$; GMP: 0.02 (0.03) compared with 0.01 (0.00)%, $P = 0.001$; Tang-MP: 8.16 (11.70) compared with 1.20 (6.76)%, $P < 0.0001$]. Conversely, no differences were registered in the different MP subsets between the CVR group and HC. Therefore, RA patients exhibit not only a quantitative increase but also an altered MP profile.

MP profile was associated with traditional CV risk factors and disease-specific parameters

Next, we wondered whether traditional CV risk factors and/or disease-specific parameters could account for the MP alterations detected in RA patients. Notably, total MPs number was positively associated with some traditional CV risk factors in both RA and CVR patients. Specifically, similar significant correlations were detected with triacylglycerols (triglycerides) (RA: $r = 0.390$, $P < 0.0002$; CVR: $r = 0.358$, $P = 0.012$), total/high-density lipoprotein (HDL)-cholesterol ratio (RA: $r = 0.319$, $P = 0.004$; CVR: $r = 0.298$, $P = 0.040$) and body mass index (BMI) (RA: $r = 0.232$, $P = 0.021$; CVR: $r = 0.304$, $P = 0.022$). Also, the number of traditional CV risk factors correlated with total MP counts ($r = 0.221$, $P = 0.030$). However, none of these associations were observed with any specific MP subset.

Nevertheless, the striking increase in the absolute number and the differences in MP composition detected in RA patients could not be explained by the presence of traditional CVR factors. Therefore, cellular damage and/or activation related to disease specific parameters may play a role. In this sense, interesting associations were found: EMP counts correlated positively with disease duration ($r = 0.285$, $P = 0.005$); GMP with

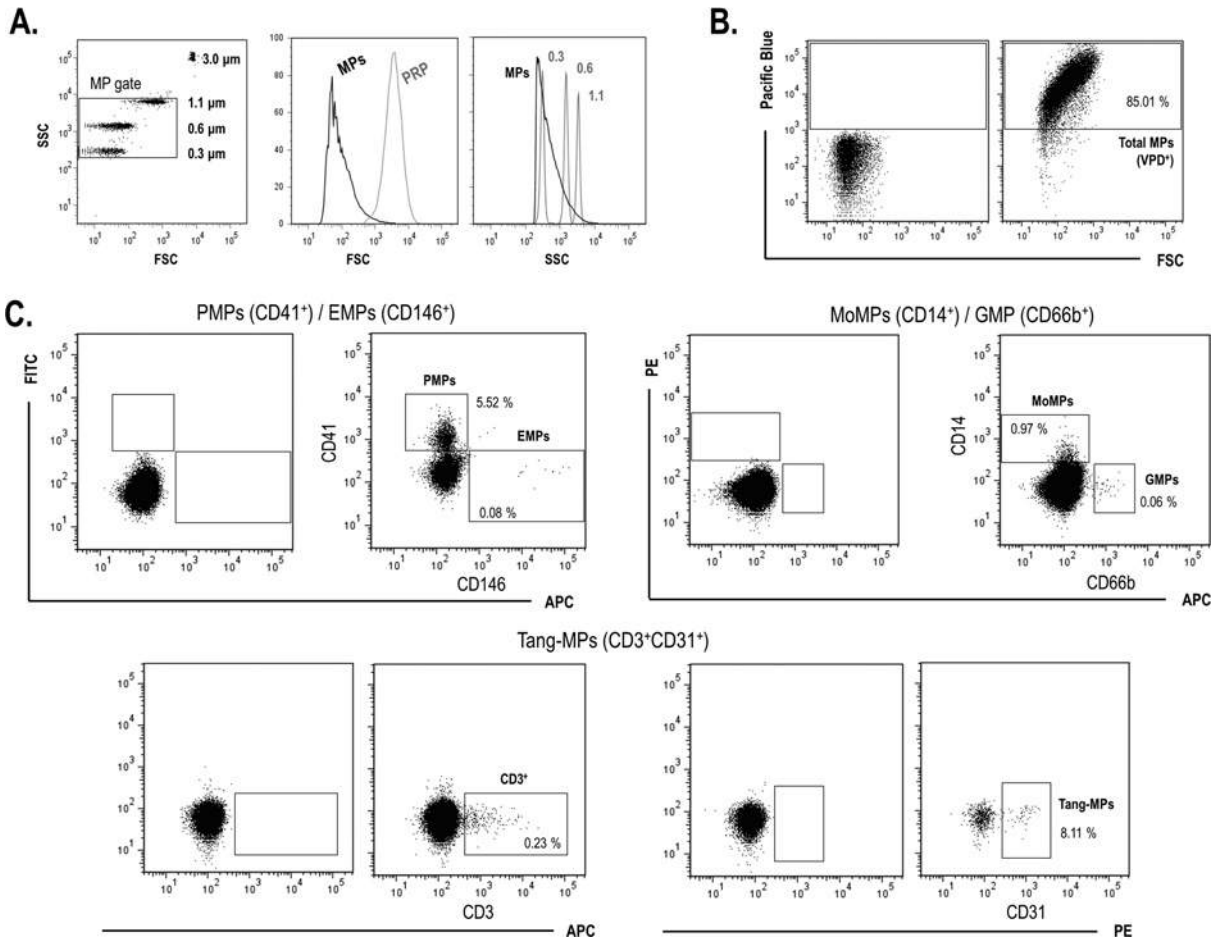

Figure 1 **Gating strategy for MP analysis**

(**A**) Latex beads were used to calibrate FSC and SSC logarithmic gain and to design the MP gate. Analysis of MP suspension revealed that most MPs had this size. PRP sample was prepared to confirm MP gate. (**B**) Total MPs were defined as those events VPD^+. Threshold was adjusted with an unstained MP suspension. (**C**) Gating strategy for PMPs ($CD41^+$ MPs), EMPs ($CD146^+$ MPs), GMPs ($CD66b^+$ MPs), MoMPs ($CD14^+$ MP) and Tang-MPs ($CD3^+$ MPs were first gated and evaluated by their $CD31^+$ expression and those $CD3^+CD31^+$ double-positive were defined as Tang-MPs).

DAS28 ($r = 0.271$, $P = 0.032$), erythrocyte sedimentation rate (ESR) ($r = 0.233$, $P = 0.022$) and age at diagnosis ($r = 0.233$, $P = 0.021$); Tang-MP with DAS28 ($r = 0.275$, $P = 0.007$), tender ($r = 0.229$, $P = 0.026$) and swollen ($r = 0.306$, $P = 0.003$) joint counts and MoMP with RF (rheumatoid factor) titre ($r = 0.240$, $P = 0.041$). Interestingly, patients on tocilizumab treatment exhibited lower Tang-MPs ($P = 0.050$) and GMPs ($P = 0.011$), whereas methotrexate usage was related to decreased Tang-MP counts ($P = 0.033$), probably associated with the lower DAS28 ($P < 0.001$ and $P = 0.008$, respectively) found in these patients. Therapies used in the CVR did not result in different MPs counts in any of the subsets analysed.

All these results indicated that a large number of parameters, including traditional CV risk and disease specific factors, accounted for total and specific MP subsets in RA patients. Therefore, we employed a PCA to reduce this number into a small set of components that could explain most of the variance of the MP counts. This method also provides an integrative approach for the different factors included, avoiding potential collinearity bias and multiple testing concerns. PCA was conducted with the parameters summarized in Table 2 and all of them exhibited communalities higher than 0.5. The Kaiser–Meyer–Olkin test provided a good adequacy of the data (0.687) as did the Bartlett test of sphericity ($P = 10^{-49}$). Results of the PCA provided four components with eigen values >1, which were interpreted based on the loadings relating the variable to the component. Thus, loadings higher than 0.5 were used to identify the variables that define each component. As seen in Table 2, disease-specific parameters loaded on the first component ('rheumatic-related'), whereas traditional CV risk factors loaded on the second component ('traditional CV-related'), only disease duration loaded on the third component ('duration-related') as ESR did on the fourth ('inflammation-related'). This model explained 69.0 % of the total variance.

Finally, we analysed whether principal components were associated with the different MP subsets by multiple regression

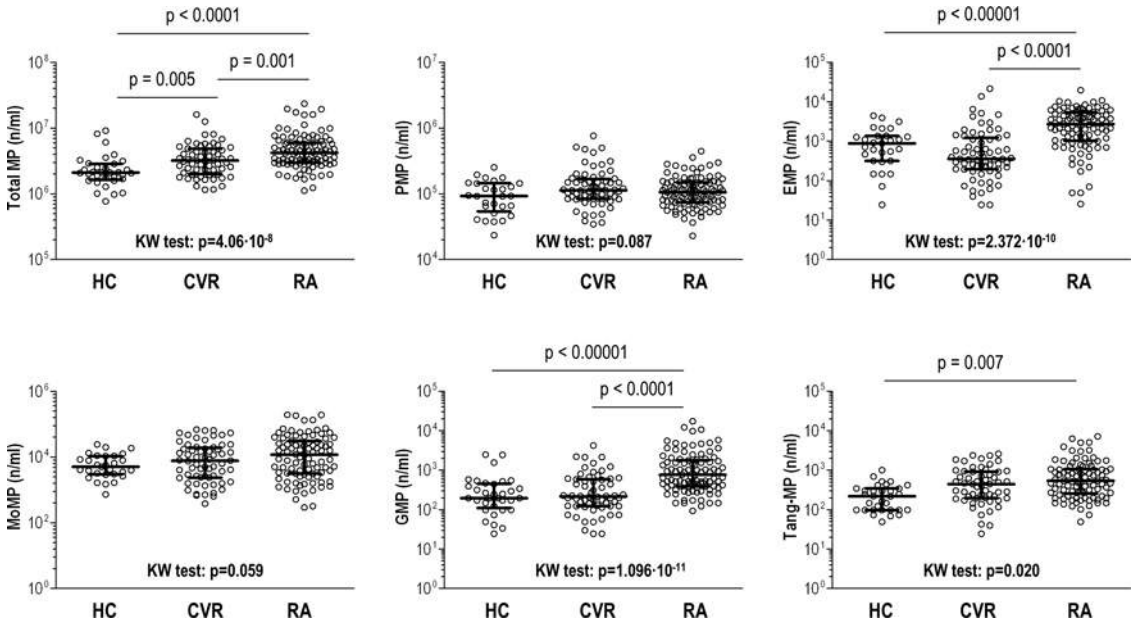

Figure 2 Total and specific MP subsets in patients and controls
Absolute number of total MPs and PMPs, EMPs, MoMPs, GMPs and Tang-MPs was analysed in 114 RA patients, 72 individuals with traditional CVR factors and 33 HC. Horizontal lines represent median and interquartile range. Differences were assessed by Kruskal–Wallis with Dunn–Bonferroni multiple comparison tests. Kruskal–Wallis *P* value for each subset is indicated at the bottom. Only significant *P* values from multiple comparisons tests are indicated.

Table 2 Component loadings from PCA
Variables included in the analysis and their corresponding loading on each component are shown. Variables were assigned to each component based on loadings >0.5. Loadings in bold indicate the component on a variable loaded the highest.

	Component loadings			
Variable	**C1**	**C2**	**C3**	**C4**
Disease duration (years)	0.123	0.330	**0.580**	−0.470
Tender joints count	**0.774**	−0.356	−0.280	0.014
Swollen joints count	**0.701**	−0.330	−0.255	−0.019
Health Assessment Questionaire (0–3)	**0.706**	0.140	0.031	−0.203
Patient pain assessment (1–10)	**0.665**	0.054	0.066	−0.315
Global patient assessment (1–100)	**0.861**	−0.002	0.037	−0.107
ESR (mm/h)	0.262	0.172	0.574	**0.704**
Disease activity (DAS28)	**0.844**	−0.104	0.135	0.360
BMI (kg/m^2)	0.293	**0.786**	−0.126	0.055
Total/HDL colesterol ratio	0.031	**0.622**	−0.422	0.180
Number of traditional CV risk factors	0.068	**0.715**	−0.411	0.034
Age at sampling (years)	0.143	**0.581**	0.322	−0.050

analysis (Table 3), each MP subset being adjusted for the four components. Interestingly, we found that traditional CVR factors (component 2) can predict total MP numbers, whereas the counts of specific MP subsets are only explained by disease-specific parameters (components 1 and 3). These observations support our previous findings.

TNFα levels correlated with Tang-MPs unless traditional CV risk factors were present

Elevated production of TNFα, a cytokine involved in RA pathogenesis, has been related to cell activation, apoptosis and endothelial damage. Therefore, to evaluate whether it could play a role in MP release, serum levels of this cytokine were quantified in RA patients and HC.

In spite of the increased levels of TNFα present in RA patients [8.42 (9.12) compared with 5.35 (4.25) pg/ml, $P = 0.001$], they were unrelated to the total MP number ($r = 0.037$, $P = 0.730$). Further analysis of MP subsets showed Tang-MP counts were slightly associated with TNFα ($r = 0.171$, $P = 0.097$), but this correlation becomes relevant in RA patients without any traditional CV risk factor ($n = 24$, $r = 0.669$, $P < 0.0001$). Moreover, this association was also apparent, although at a lower level, in patients with less than two traditional CV risk factors ($n = 51$, $r = 0.459$, $P = 0.001$), and in those with less than three factors

Table 3 Multivariate regression analyses of MP subsets in RA patients

Multivariate regression analyses of MP subsets (as dependent variables) and the four components obtained by PCA (as predictors) associated with specific disease variables: rheumatic-related (Rhe-rel), traditional CV-related (tCV-rel), disease duration-related (Dur-rel) and inflammation-related (Infl-rel). Results are expressed as β coefficient and (*P* value) for each PCA component. Significant coefficients are highlighted in bold.

Variable	Total MPs	PMPs	EMPs	GMPs	Tang-MPs	MoMPs
C1 (Rhe-rel)	−0.085 (0.387)	−0.007 (0.948)	−0.131 (0.290)	**0.296** (**0.014**)	**0.319** (**0.010**)	0.209 (0.835)
C2 (tCV-rel)	**0.290** (**0.004**)	0.100 (0.331)	0.039 (0.743)	−0.107 (0.350)	−0.008 (0.944)	−0.105 (0.389)
C3 (Dur-rel)	−0.014 (0.889)	−0.017 (0.869)	**0.329** (**0.008**)	−0.053 (0.650)	0.073 (0.540)	−0.173 (0.167)
C4 (Infl-rel)	0.058 (0.551)	0.061 (0.548)	−0.119 (0.312)	0.091 (0.427)	0.118 (0.316)	0.164 (0.186)

($n = 73$, $r = 0.244$, $P = 0.038$), indicating that the higher the number of traditional CV risk factors, the lower the TNFα contribution to MP release. Actually, analysing all RA patients in a multivariate regression model including traditional CV risk factors, TNFα was associated with Tang-MP counts ($r = 0.259$, $P = 0.012$). No association with other MP subsets was found.

MPs from RA patients promoted endothelial disturbance *in vitro*

Finally, since MPs have been linked to CV risk and endothelial activation, we performed *in vitro* experiments to evaluate whether circulating MPs isolated from HC, RA patients or individuals with traditional CVR factors could affect angiogenic tube formation and endothelial activation in HMEC-I cells.

Angiogenesis assays on Matrigel were conducted after adding HC-, CVR- or RA-MP pools at different concentrations (0.5–8×10^6 MP/ml), selected according to the range of total MP counts in controls (Figure 3A). Results showed that the number of both branching points and tubes were dose-dependently inhibited by RA-MPs, whereas no effect was seen with HC- or CVR-MPs (Figure 3B). Interestingly, MPs from RA patients exhibited an anti-angiogenic effect at 1×10^6 MP/ml, a lower concentration than is usually found in plasma.

Additionally, MP-mediated activation of endothelial cells was estimated by analysing by flow cytometry different endothelial-specific markers, as well as cell viability, in HMEC-I cells cultured in the presence of the different MP pools (Figure 3C). No cytotoxic effect was seen under any of the conditions tested, thus excluding that viability could affect endothelial functionality. However, we observed that RA-, but not HC- or CVR-MPs, increased the expression of CD62E, CD144 and VEGFR2 (all $P < 0.050$), thus suggesting the promotion of an activated endothelial status.

Finally, to assess whether these findings could be attributed to a specific cell-derived MP subset, the amount of each MP subset present in the cultures was compared with the effects found in angiogenic assays. Figure 4 shows that the detrimental effect observed with total RA MPs was also detected when the different subsets were analysed, but it seems to be different depending on their cellular origin. Analysing the effects at physiological levels (median value in HC), striking differences between RA and HC were observed with MoMPs and PMPs but not with GMPs and Tang-MPs, thus suggesting that the deleterious effect of Tang- and GMP-RA MPs could be due to the increased proportion within the total MP count, whereas MoMPs and PMPs from RA patients could have a detrimental effect by themselves.

Therefore, MPs from RA patients are able to disturb *in vitro* endothelial functionality dose-dependently, maybe due to endothelial activation, whereas HC- and CVR-MPs did not promote this effect, even at greater concentrations. These results are a proof of concept that supports functional qualitative and quantitative effects associated with a skewed composition of RA MPs.

DISCUSSION

The study reported here shows relevant differences in the number and composition of circulating MPs in RA patients compared with HC and individuals with traditional CV risk factors. Additionally, the main finding is that this altered MP pool is the result of disease features as well as traditional CV risk factors, as was supported using a PCA approach. This distinct profile could underlie the detrimental effects exhibited by RA-MPs in endothelial cell assays, presumably by promoting an endothelial activation status. The results presented support the use of MPs as biomarkers of endothelial damage in RA patients, with potential use for clinicians in decision making and CV risk stratification.

In line with our results, other studies performed with RA patients showed increased MP counts, total or specific from different cell subsets and associated with some clinical features [27–29]. However, evidence is limited and results are heterogeneous and even contradictory, probably because of the different methodologies used. We have developed an MP total labelling strategy so as to (i) identify virtually all MPs and not only those derived from apoptosis and (ii) avoid the technical drawbacks associated with annexin V staining. Recent evidence suggests the relevance of annexin V-negative MPs in many conditions. Actually, Nielsen et al. reported that in systemic lupus erythematosus patients only annexin V-negative MPs were increased and associated with clinical parameters [19], whereas other authors have reported that most MPs did not express annexin V [17,18,30], so limiting the study to this subset could bias the conclusions. On the other hand, freezing steps and several other factors are thought to affect the annexin V binding-fraction [26,31–33], thus making the comparison between different studies difficult and emphasizing the need for alternative protocols. To this end, studies using different reagents have been published [18,34–37] to overcome annexin V disadvantages.

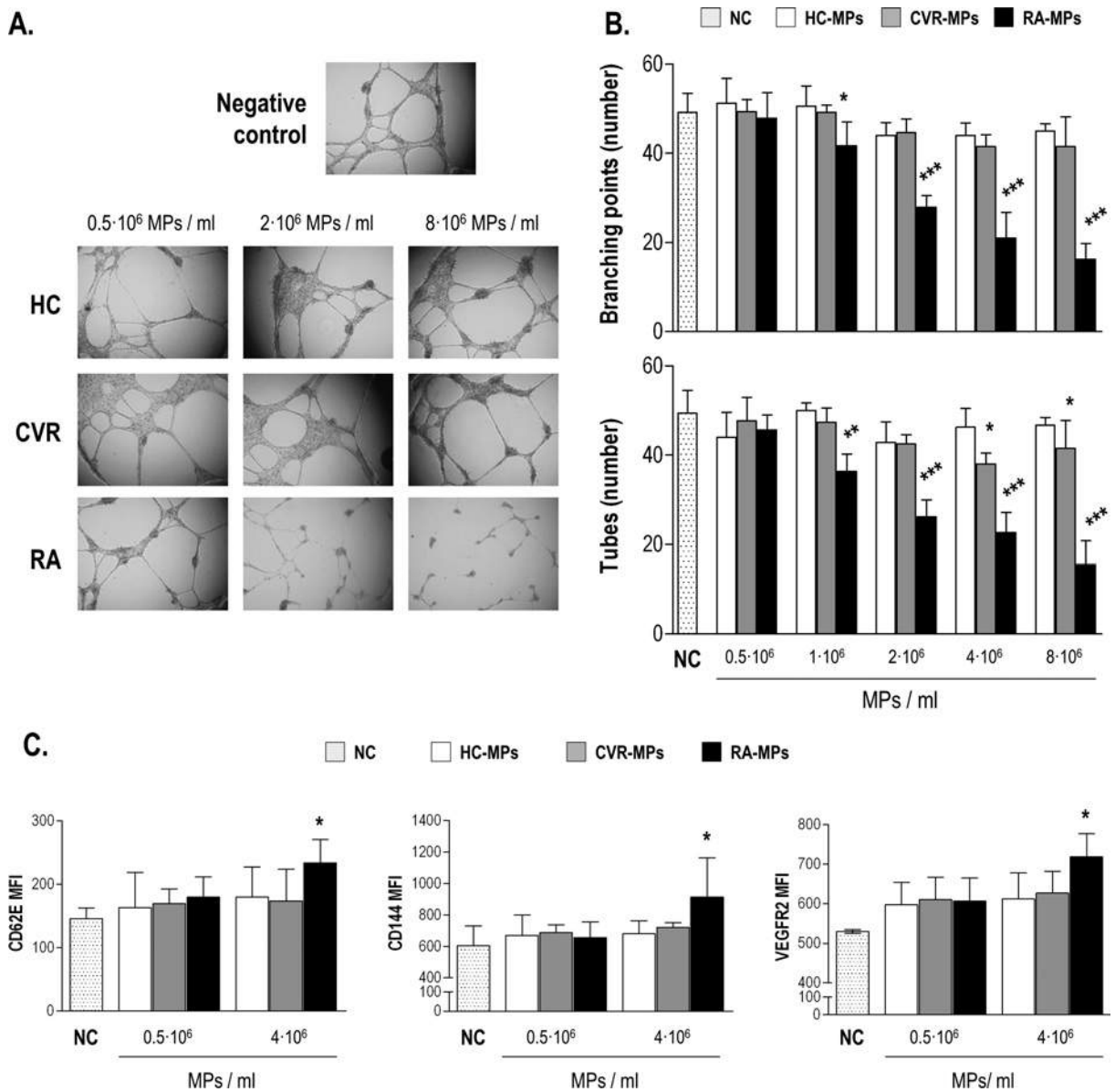

Figure 3 ***In vitro*** **effects of MPs isolated from patients and controls**

HMEC-I cells were cultured alone (negative control, NC) or in the presence of MP pools at different concentrations isolated from RA patients, individuals with traditional CVR factors or HC. (**A**) Representative microphotographs (×40) of HMEC-I cells cultured on Matrigel coated plates to perform angiogenic assays. (**B**) Branching points and tube numbers identified in different cultures ($n = 8$). (**C**) Flow cytometry analysis of CD62E, CD144 and VEGFR2 expression on HMEC-I cells cultured in the presence of the different MP pools ($n = 4$). Bars represent means ± S.D. and differences between each treatment and the negative control were assessed with one-way ANOVA and Dunnett post-hoc test.

Because of their role in inflammation, angiogenesis and vascular reactivity, MPs have been extensively studied in RA and rheumatic diseases [1]. However, this is the first study in which PCA was used as an integrative tool to analyse MP counts, thus avoiding multiple-testing concerns and supporting previous results. This work clearly indicates that total MP counts can be explained by traditional CV risk factors in individuals at risk (RA and CVR subjects), but RA patients exhibited a profile of increased MP subsets that is only explained by disease-specific factors. Results from CVR individuals as positive CV-risk control allows us to confirm that MP disturbances in RA are specific to the disease itself and independent of comorbidities. Additionally, although PCA component scores are independent in the whole RA group, rheumatic- and traditional CV-related components were positively correlated in the patients who had a history of CV events ($r = 0.602$, $P = 0.008$; $n = 18$) but not in the CV-free group ($r = 0.032$, $P = 0.759$), revealing the relationship between these two features. These results are in line with previous evidence about the interplay of traditional CV risk factors and disease parameters in RA [38,39]. Moreover, disease-specific factors

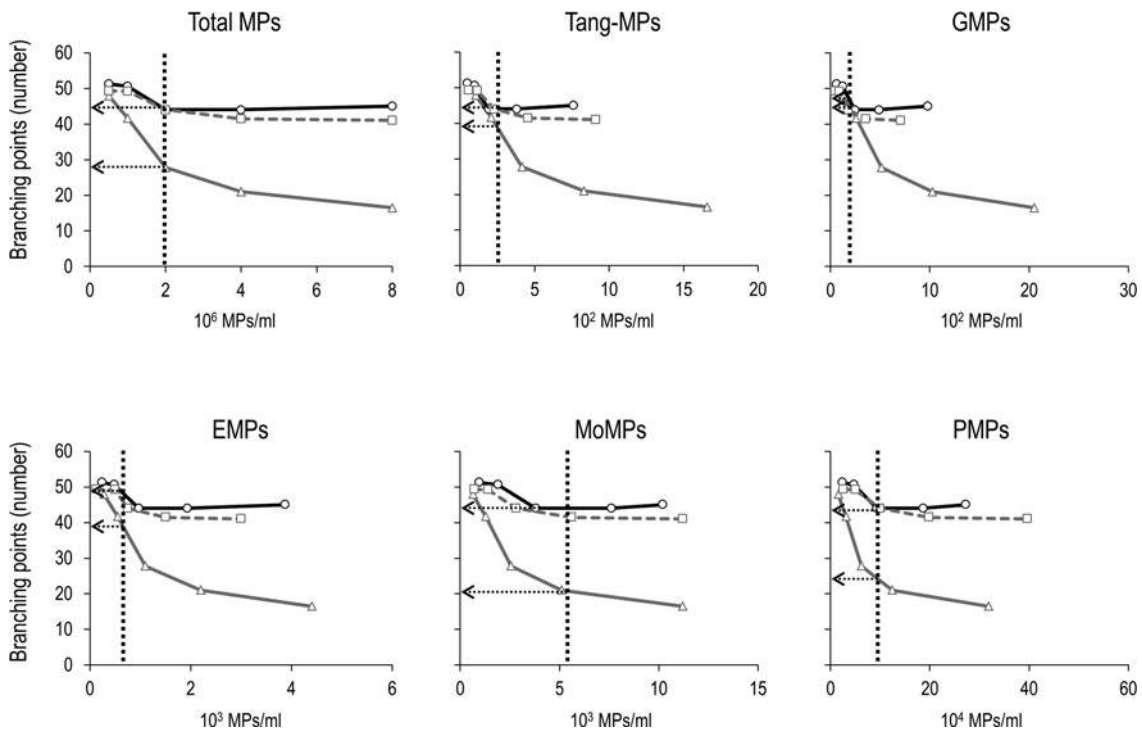

Figure 4 ***In vitro* effects analysed according to different MP subsets**

The *in vitro* effect of MPs (measured as number of branching points) was analysed separately according to the different subsets studied. RA MPs (triangles, grey full line) exhibited greater detrimental effect on branching point numbers than those from HC (circles, black line) and CVR (squares, broken grey line) even at the same concentration, although differences were observed when PMPs and MoMPs were compared with GMPs and Tang-MPs. Vertical dotted lines represents the median values found in HC.

associated with MP subsets in the present study (disease duration, DAS28 and age at diagnosis) have been associated with CV disease in RA [38,40,41], thereby supporting the relevance of MPs as biomarkers of endothelial activation and damage in RA patients.

Another interesting finding of this work is the role played by MPs derived from Tang cells, a previously-discovered T-cell subset that enhances endothelial repair through cooperating with endothelial progenitor cells [42]. Although no differences in the frequency of MPs derived from T-lymphocytes were detected, both frequency and absolute number of Tang-MPs were increased in RA patients. Interestingly, this increased Tang-MP formation could account for the decreased Tang cell counts previously reported in RA [21]. Moreover, our research revealed a DAS28-dependent Tang-MP shedding, thus linking DAS28 with impaired endothelial repair. Actually, the disease parameters positively associated with Tang-MP in this work were the same as have been found to be negatively associated with Tang frequency [21]. Consequently, Tang-MPs could be considered as a surrogate biomarker of endothelial damage and vascular repair failure. Furthermore, the association between TNFα, a proapoptotic cytokine increased in RA, and Tang-MPs reinforces this hypothesis and the link between disease-specific parameters and MP release. Again, the finding that the presence of traditional CV risk factors disturbs this association confirms the interplay between traditional CV risk factors and disease features.

In spite of the striking increase in total MPs in patients, there were no differences compared with controls in the PMP counts, suggesting that the RA-specific MP profile may not simply be due to a general MP increase, but rather specific mechanisms targeting different cell populations may be implicated. Accordingly, no associations were detected between PMPs and PCA components, whereas EMPs, GMPs and Tang-MPs correlated positively with disease-related parameters. Another plausible explanation is that therapies would interfere with platelet function; however, no effect of the concomitant medications was observed either when untreated patients (allowed to use non-steroidal anti-inflammatory drugs) were analysed, thus excluding a confounding effect of drug usage on platelet activation. Hence, we could attribute these results to the analysis performed in our study. In fact, increased PMP counts in RA were observed when annexin-V binding was used [28,29]; however, when alternative procedures were performed, opposite results were achieved [19,29]. This leads us to hypothesize that the total labelling protocol performed in this work could mask the differences in Annexin V-positive PMPs due to an elevated number of negative-events. Therefore, although possible platelet activation during PPP isolation cannot be ruled out, our functional assays indicated that the *in vitro* detrimental effects of MPs from RA patients depend on qualitative alterations in PMPs

rather than differences in absolute counts, contrary to what was observed with Tang-MPs or GMPs (Figure 4).

The fact that the RA MP profile can only be explained by disease-specific features leads us to think that these MPs may have a role in RA pathogenesis. Recent studies analysing the MP proteome support this idea [43]. Accordingly, differential 'MP signature' was found in synovial fluid from RA patients compared with other arthritic diseases [44]. Thus, in this pathological situation, MPs could be acting in a vicious circle: disease-related cell injury could generate a RA-specific MP pool which in turn might worsen specific clinical features, such as damaging the vascular endothelium, with subsequent increase in CV risk. Accordingly, MPs from RA patients have been reported to be able to modulate chemokines and cytokines from synoviocytes [45], thus probably amplifying inflammatory responses.

Finally, the existence of a RA-specific MP profile was supported by our *in vitro* assays, since they revealed that effects on endothelial cells depend on the MP pool rather than the concentration (even within physiological concentrations). Specifically, RA-MPs were able to inhibit HMEC-I Matrigel tube formation in a dose-dependent manner, whereas MPs derived from HC and CVR individuals failed to exhibit similar results. This detrimental effect may be due to the promotion of endothelial activation, as was indicated by flow cytometry analysis of endothelial markers. In fact, endothelial activation has been associated with impaired endothelial function in a variety of conditions [46], including RA [47]. A role for MPs in CV disease and endothelial function has been previously reported [48,49], but this is the first study where MPs isolated from RA patients have been assayed. Despite providing limited evidence, these results could support the role of MPs as active players in RA pathogenesis, proving worthy of further research.

However, it should be noted that not all MPs are proatherogenic. Actually, some groups have revealed anticoagulant and protective effects of some MP subsets [50–52], in contrast with the procoagulant and deleterious results reported by others [10,27,45,48,53]. Interestingly, these diverse effects could be attributed to the exposure to different mediators, such as activated protein C, tissue factor or von Willebrand factor, among MP subsets. Although these effects cannot be excluded using the actual data, our results from *in vitro* assays point to a pathogenic role of MPs in RA patients.

Some remarks about the current study should be made. First, despite covering the same age range, HC were younger that RA and CVR patients. However, no associations between age and MP counts were detected in any group. Additionally, age was included in the PCA in order to correct for potential differences. On the other hand, the lack of a standardized protocol to determine cell-derived MPs is the main limitation in the field of MPs. The fact that we have developed a new protocol enabling the determination of the total MPs could make the comparison of our results with other studies difficult, since usually only apoptosis-derived MPs were analysed. However, this is a common problem in the field, and a balance between innovative methods and potential results should be considered. Nevertheless, our findings are relatively similar to others obtained by different methods. Finally, although our data did not allow direct determination of the detrimental effects of each specific MP subset, the *in vitro* results suggest differences between them. Further studies are needed to confirm this hypothesis, however, MP separation procedures from plasma have not been implemented yet. Furthermore, circulating MPs are present *in vivo* as a (heterogeneous) group, so 'individual' *in vitro* effects of a single population need to be considered with caution. In conclusion, the findings of the present study reveal that RA patients exhibited not only increased MP counts but also a qualitatively altered MP profile that is associated with disease-specific and CV risk factors. Moreover, this MP profile could be capable of disturbing the vascular endothelium. In addition, increased Tang-MPs, probably associated with the DAS28-dependent Tang decrease, could have a role in endothelial repair failure in these patients, thereby supporting the use of both Tang and Tang-MPs as biomarkers of endothelial repair failure.

CLINICAL PERSPECTIVES

- It is known that the number of cell-derived microparticles (MPs) is associated with endothelial dysfunction and impaired vascular repair.
- RA patients exhibit a specific MP profile that is associated with both disease-specific features and traditional CVR factors. This specific profile could underlie the detrimental effects on endothelial cells *in vitro*, presumably by promoting endothelial activation.
- MPs could be considered as biomarkers of endothelial damage in RA patients, with potential use for clinicians in decision making and CV risk stratification.

AUTHOR CONTRIBUTION

Javier Rodríguez-Carrio performed most of the experimental procedures, carried out statistical analyses and drafted the manuscript. Patricia López and Santiago Carro-Esteban performed some experimental procedures. Mercedes Alperi-López, Sara Alonso-Castro and Francisco Ballina-García were in charge of patients recruitment and clinical data collection. Ana Suárez conceived the study, designed the protocols and drafted and edited the manuscript. All authors read and approved the final version of the manuscript.

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Capítulo IV: Papel de la amplitud de distribución eritrocitaria como biomarcador de riesgo cardiovascular

Dado el enfoque traslacional del segundo objetivo de este proyecto de investigación, resultaría interesante investigar el papel de parámetros clínicos como biomarcadores de riesgo CV en pacientes de AR, con el objetivo de aportar evidencias para su implementación en el manejo clínico del riesgo CV, así como para analizar qué mecanismos subyacen a su capacidad indicadora y aportar nuevas evidencias al campo de estudio.

En este punto, observamos que el parámetro amplitud de distribución eritrocitaria (ADE, RDW: *Red cell Distribution Width*) estaba cobrando importancia como biomarcador emergente de riesgo CV en población general, así como en diferentes cuadros clínicos de enfermedad CV como factor pronóstico.

Dado que la posible capacidad como predictor del RDW parecía estar asociado a la inflamación, parecía un candidato prometedor como biomarcador de riesgo CV en AR, por lo que decidimos profundizar en su estudio.

Inicialmente, llevamos a cabo un estudio retrospectivo a partir de una muestra de pacientes de AR reclutados previamente por nuestro grupo, en el que se estudió el poder predictor de eventos CV del RDW. Basándonos en los resultados obtenidos, realizamos un estudio posterior con diseño transversal e incluyendo un mayor número de pacientes, en el que se analizaron las asociaciones del RDW con biomarcadores de daño y reparación endotelial.

Artículo 6: Rodríguez-Carrio J, Alperi-López M, López P, Alonso-Castro S, Ballina-García FJ, Suárez A (2015); *Red Cell Distribution Width is associated with cardiovascular risk and disease parameters in Rheumatoid Arthritis;* Rheumatology (Oxford) 54(4):641-6.

> Aportación personal al trabajo: mi contribución en este trabajo se centró en el diseño del estudio, procesamiento de las variables analizadas y su análisis estadístico, así como la discusión de los resultados con los coautores. Además, llevé a cabo la redacción del manuscrito y la preparación de las figuras bajo la supervisión de la Dra. Ana Suárez Díaz.

Artículo 7: Rodríguez-Carrio J, Alperi-López M, López P, Alonso-Castro S, Carro-Esteban SR, Ballina-García FJ, Suárez A (2015); *Red Cell Distribution Width is associated with*

cardiovascular risk and disease parameters in Rheumatoid Arthritis; Atherosclerosis 240(1):131-6.

Aportación personal al trabajo: en este trabajo corrió a mi cargo la mayor parte de la labor experimental, así como el análisis e interpretación de los resultados. Finalmente, llevé a cabo la preparación del manuscrito y las figuras bajo la supervisión de la Dra. Ana Suárez Díaz.

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Red cell distribution width is associated with cardiovascular risk and disease parameters in rheumatoid arthritis

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Abstract

Objective. Since red cell distribution width (RDW) has been associated with cardiovascular (CV) disease and inflammation in several conditions, the main aim of this study was to evaluate its prognostic value in RA patients and its potential associations with clinical features.

Methods. The history of CV events was retrospectively reviewed in 160 RA patients and RDW was recorded at disease onset and 6 and 12 months after diagnosis to calculate the accumulated value [area under the curve (AUC) RDW] and change during the first year (ΔRDW). In addition, RDW was analysed in 110 patients with established disease in relation to clinical features.

Results. Increased RDW at diagnosis and AUC RDW were able to predict the occurrence of CV events in RA patients [hazard ratio (HR) 1.247 (95% CI 1.079, 1.441), $P = 0.003$ and HR 1.038 (95% CI 1.018, 1.059), $P = 0.0001$, respectively] after adjusting by potential confounding factors. Receiver operating characteristic curve analyses revealed a better power of discrimination for the AUC RDW ($P = 3.394 \times 10^{-5}$). In addition, an increase in RDW during the first year was associated with poor CV outcome ($P = 0.010$). On the other hand, RDW in patients with established RA was significantly associated with disease activity, acute phase reactants and severity.

Conclusion. RDW at disease onset may be used as an early marker of CV risk in RA, whereas in patients with established disease it was related to the activity of the disease. These findings suggest that RDW can be considered as a surrogate marker of inflammation and, consequently, CV risk in RA patients.

Key words: red cell distribution width, rheumatoid arthritis, cardiovascular disease, biomarker.

Introduction

RA is an autoimmune disease characterized by joint inflammation and destruction. RA patients exhibit increased prevalence and severity of cardiovascular (CV) disease compared with healthy controls, but traditional CV risk factors fail to account for this increase [1]. Immune dysregulation, chronic underlying inflammation and genetic factors are therefore thought to explain this excess risk [2–4].

Early control of the disease could lead to sustained clinical remission by controlling inflammation and thereby preventing erosive progression, extra-articular manifestations and CV disease [5], since disease progression is a key factor for RA prognosis and CV risk [6]. An accelerated atherosclerotic process is thought to be an early event in RA [7]. The identification of predictive early markers for CV disease is therefore a relevant challenge in RA investigation.

Red cell distribution width (RDW) is a measurement of the heterogeneity in size of the circulating erythrocytes (anisocytosis). Commonly regarded as a useful index for differential diagnosis in anaemia [8], it has frequently been ignored in other disorders. Nevertheless, it has recently been reported that elevated RDW values are associated with poor CV prognosis in several CV conditions [9]. In addition, it has been proposed as a marker of mortality

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not only in these patients but also in the general population [10]. Although the mechanisms underlying this effect remain unclear, it has been hypothesized that increased RDW may reflect an inflammatory burden in these conditions [11], thus suggesting a possible alteration in chronic inflammatory diseases such as RA. However, the potential associations of inflammation and disease parameters with RDW in RA patients and whether it might have a prognostic value in the development of CV events in this disease are still unknown. Consequently, the main aim of the present work was to evaluate the prognostic value of RDW determination at early stages of RA and to study whether RDW could be associated with disease-specific parameters in RA patients with established disease.

Materials and methods

RA patients

All the patients ($n = 160$) fulfilled the 2010 ACR criteria, attended the RA Early Diagnosis Outpatient Clinic at the Hospital Universitario Central de Asturias (HUCA) and were registered in the clinical database (see the flowchart in supplementary Fig. S1, available at *Rheumatology* Online). For the retrospective analysis, clinical records were examined until October 2013 in order to obtain a history of CV events, RDW (at diagnosis as well as 6 and 12 months thereafter) and clinical parameters. The definition and classification of CV events and traditional risk factors were as previously established [12]. A CV event was considered if the patient suffered from heart failure, ischaemic heart disease or cerebrovascular accident since their RA diagnosis. CV events were clustered together in order to globally analyse CV disease in RA patients, as previously reported [4, 12]. Also, a blood sample was obtained from 110 of these patients, representatives of the initial cohort, in which complete clinical and laboratory parameters were registered at the time of sampling. Approval for the study was obtained from the Regional Ethics Committee for Clinical Investigation at HUCA, Oviedo, and all the participants gave written informed consent according to the Declaration of Helsinki.

RDW

A complete blood count (including RDW) was performed in the haematology laboratory (HUCA) using an ADVIA 2120 automated haematology analyser (Siemens, Munich, Germany). The cut-off of 14% was identified as the upper limit by trained haematologists. Patients were classified as having anaemia if they had haemoglobin <13 g/dl and/or haematocrit <37 for men and <12 g/dl and/or <35 for women.

Statistical analysis

Results are presented as the median [interquartile range (IQR)] for continuous variables or *n* (%) for categorical ones. The integral of RDW in the first year after diagnosis [area under the curve (AUC) RDW] was calculated in order to evaluate the total RDW accumulated over time (0, 6 and 12 months). ΔRDW was the difference between RDW at 12 months and at diagnosis. The association between changes in RDW during the first year of the disease and CV event occurrence was analysed by using ΔRDW as a categorical variable (>0, increased). Mann–Witney *U*, Wilcoxon and chi-squared tests were used for comparisons as appropriate. Spearman's rank correlation test was used to analyse correlations.

The discrimination of RDW for CV event occurrence was assessed using the AUC of the receiver operating characteristic (ROC) curve. A Cox regression model adjusted for age, disease duration and sex was used to estimate the influence of RDW (as time-dependent variable) on CV event occurrence. Hazard ratios (HRs) and 95% CIs were provided to indicate the strength of the associations. Survival analyses were performed using the Kaplan–Meier method and comparisons were made using the log-rank test. We used the time since 12 months after diagnosis and the occurrence of a CV event, or the end of the retrospective study for those who had not suffered from a CV event, as the survival time.

A *P*-value <0.050 was considered to be statistically significant and all statistical analyses were performed with SPSS 18.0 (IBM, Armonk, NY, USA), R software version 2.15 (R Project for Statistical Computing, Vienna, Austria) and GraphPad Prism 5.00 for Windows (GraphPad Software, La Jolla, CA, USA).

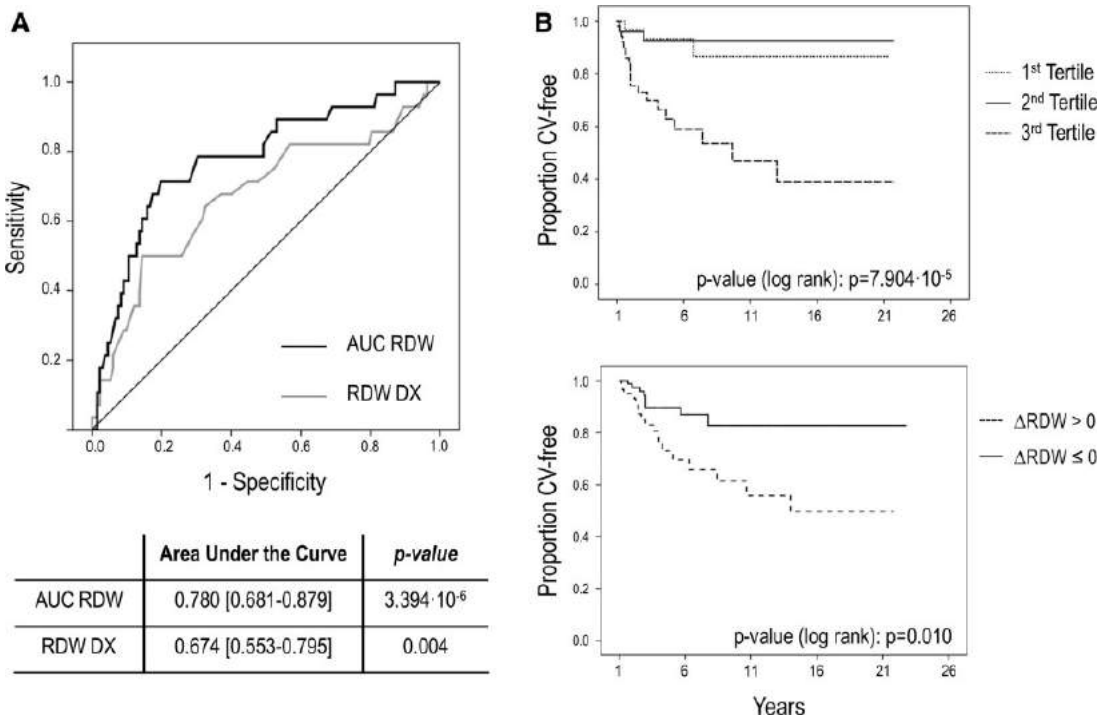
Results

RDW and CV events in RA patients

Patients included in the retrospective study were 131/160 (81.8%) women, had a median disease duration of 5.50 years (range 1–30) and 28/160 (17.5%) had experienced a CV event (see supplementary Table S1, available at *Rheumatology* Online).

Analysing the whole RA group, no differences were detected between RDW at diagnosis (RDW DX) and 1 year later [13.90 (S.D. 1.70) *vs* 13.96 (S.D. 1.65), $P = 0.898$]. However, the change along the first year (ΔRDW) was greater in patients who had suffered from a CV event than in those who were CV event-free [0.64 (S.D. 0.48) *vs* −0.06 (S.D. 1.59), $P = 0.025$]. In addition, the CV event group exhibited a higher RDW DX [14.81 (S.D. 2.48) *vs* 13.60 (S.D. 1.44), $P = 0.004$], and even greater differences were observed when analysing the total RDW accumulated (AUC RDW) in the first year after diagnosis [180.06 (S.D. 17.22) *vs* 164.83 (S.D. 15.76), $P = 3.386 \times 10^{-6}$). Cox regression analyses adjusted for sex, age at diagnosis and disease duration revealed that both RDW DX [HR 1.247 (95% CI 1.079, 1.441), $P = 0.003$] and AUC RDW [HR 1.038 (95% CI 1.018, 1.059), $P = 0.0001$] were predictors of CV events. Interestingly, neither disease duration [median 5.50 years (IQR 4.18–8.89 4.71) *vs* 5.41 (IQR 4.04–8.46)] nor time of follow-up [median 2.01 years (IQR 1.25–3.00) *vs* 3.08 (IQR 1.00–5.00)] differed between groups ($P = 0.743$ and $P = 0.204$, respectively). ROC analyses showed that RDW DX has a moderate discriminative power for the occurrence of CV events, being good in the case of AUC RDW (Fig. 1A). Actually, choosing the

Fig. 1 RDW as a predictive factor of CV events in RA patients



	Area Under the Curve	*p-value*
AUC RDW	0.780 [0.681-0.879]	$3.394 \cdot 10^{-6}$
RDW DX	0.674 [0.553-0.795]	0.004

(**A**) ROC analysis of AUC RDW and RDW DX. Choosing the 75th percentile of AUC RDW (173.3) as the cut-off point, we found a positive predictive value of 45.00% (95% CI 29.27, 61.51) and a negative predictive value of 91.67% (95% CI 85.20, 95.92). (**B**) Kaplan–Meier analyses of CV-free survival. Patients were classified according to AUC RDW tertiles and according to changes in RDW after the first year after diagnosis (ΔRDW). Time to CV event (years) started at 1 year after diagnosis. Comparisons between groups were assessed by log-rank test. RDW: red cell distribution width; CV: cardiovascular; ROC: receiver operating characteristic; AUC: area under the curve; RDW DX: RDW at diagnosis; AUC RDW: accumulated RDW during the first year.

75th percentile of the AUC RDW (173.3) as the cut-off point, we found a specificity of 83.33% (95% CI 75.86, 89.25) and a sensitivity of 64.29% (95% CI 44.07, 81.33) for CV events.

Furthermore, Kaplan–Meier models were used to confirm the value of repeated RDW measurements during early RA to predict the occurrence of CV events. Fig. 1B shows that the highest AUC RDW tertile was associated with a higher rate of CV events. Similar results were obtained when the tertiles of RDW DX were analysed ($P = 0.015$). Moreover, an RDW increase in the first year ($\Delta RDW > 0$) was associated with poor CV outcome.

Finally, these analyses were performed excluding patients with signs of anaemia ($n = 29$). All the associations remained significant, thus excluding the possible effect of erythrocyte disorders.

RDW and disease parameters

Next, to evaluate whether RDW could be associated with clinical features or inflammatory markers, we analysed RDW and disease parameters at the time of sampling in 110 RA patients with established disease. Analysis of clinical and demographic features according to RDW tertiles (Table 1) showed that patients in the highest tertile were older than, but had a similar age at diagnosis to the other groups, thus having a significantly longer disease duration. They were also more likely to be overweight, while other traditional CV risk factors did not show significant differences. Furthermore, they were less likely to be treated with tocilizumab, but no difference was detected with other therapies.

Regarding disease features, it is remarkable that patients in the highest tertile exhibited higher 28-joint DAS (DAS28) scores and other severity markers, thus suggesting an association of RDW with progression of the disease. Moreover, Spearman's rank correlation test showed that RDW was significantly associated with CRP ($r = 0.288$, $P = 0.005$), ESR ($r = 0.279$, $P = 0.006$), DAS28 ($r = 0.298$, $P = 0.001$) and HAQ ($r = 0.305$, $P = 0.002$), thus suggesting an association of RDW with disease activity and severity.

Discussion

Although several studies have suggested that inflammation and disease-specific features underlie the increased

Table 1 Characteristics of patients according to red cell distribution width tertiles

	First tertile (<13.60) (*n* = 36)	Second tertile (13.60–14.42) (*n* = 37)	Third tertile (>14.42) (*n* = 37)
Age at sampling, median (range), years	52.25 (22–87)	54.95 (32–79)	60.25 (28–87)**
Female, *n* (%)	31 (86.1)	30 (81.0)	29 (78.3)
BMI, mean (S.D.)	23.33 (6.98)	26.33 (9.14)*	28.22 (7.07)**
HTA, *n* (%)	7 (19.4)	13 (35.1)	13 (35.1)
Diabetes, *n* (%)	1 (2.7)	4 (8.1)	4 (8.1)
Dyslipidaemia, *n* (%)	10 (19.6)	14 (28.0)	13 (25.4)
Smokers, *n* (%)	8 (22.2)	14 (37.8)	11 (29.7)
Age at diagnosis, median (range), years	45.50 (18–85)	47.83 (21–77)	49.58 (23–80)
Swollen joint count, mean (S.D.)	2.00 (3.00)	1.50 (4.25)	2.00 (4.00)*
Tender joint count, mean (S.D.)	0.00 (1.00)	1.50 (4.00)*	1.00 (3.00)
Global patient assessment (0–100), mean (S.D.)	15.00 (37.00)	32.50 (32.50)*	45.00 (24.00)*
CRP, mean (S.D.), mg/dl	1.05 (3.03)	2.00 (3.85)	3.00 (7.30)*
ESR, mean (S.D.), mm	9.00 (16.50)	16.50 (21.00)*	13.00 (33.00)*
DAS28, mean (S.D.)	2.76 (2.03)	3.59 (1.13)*	3.73 (1.94)**
HAQ, mean (S.D.)	0.31 (0.91)	0.87 (1.16)*	1.12 (0.86)**
Patient pain assessment (0–10), mean (S.D.)	2.50 (4.50)	3.50 (4.25)	5.00 (3.15)**
Disease duration, median (range), years	3.41 (1–16)	5.75 (1–22)	7.08 (2–30)*
RF, *n* (%)	20 (55.5)	20 (54.0)	27 (72.9)
αCCP, *n* (%)	20 (55.5)	22 (59.4)	22 (59.4)
ANA, *n* (%)	16 (44.4)	15 (40.5)	19 (51.3)
Erosive disease, *n* (%)	12 (33.3)	13 (35.1)	18 (48.6)
SE, *n* (%)	9 (25.0)	9 (24.3)	15 (40.5) *
Glucocorticoids, *n* (%)	17 (47.2)	21 (56.7)	24 (64.8)
MTX, *n* (%)	30 (83.3)	28 (75.6)	28 (75.6)
TNFα-blockers, *n* (%)	11 (30.5)	17 (45.9)	17 (45.9)
Tocilizumab, *n* (%)	8 (22.2)	2 (5.5)	2 (5.5)*
Statins, *n* (%)	4 (11.1)	4 (10.8)	7 (18.9)

Differences compared with the first tertile were assessed by the χ^2 test or Mann–Witney *U* test, as appropriate. *$P < 0.050$, **$P < 0.010$. αCCP: cyclic citrullinated peptide antibody; DAS28: 28-joint DAS; HTA: hypertension; RDW: red cell distribution width; SE: shared epitope.

CV risk in RA patients, the actual origin of this phenomenon is still unknown [1, 3]. Therefore new biomarkers allowing early identification of patients at risk are needed for the clinical management of these patients. In this regard, the present work supports the use of cumulative RDW measurements in early disease as a potential CV predictive marker.

RDW is a reproducible, automated and widely used parameter that is easily available in all health facilities. Moreover, it has been previously optimized and validated and it is included in the total blood cell count, so it does not add any further costs nor does it need any special technical requirements. In addition, since it is measured frequently, monitoring is relatively simple, which was shown in our study to increase its prognostic value.

The results herein clearly indicate a predictive value of RDW in RA patients as a CV risk biomarker. The prognostic value is improved if a cumulative measurement is taken into account. Importantly, these associations were independent of the presence of anaemia, and RDW remained an independent predictor of CV disease after being adjusted for potential confounding factors. These findings support the use of RDW as a novel risk indicator for CV disease [8]. Although the mechanistic links between RDW and CV disease remain unclear, it has been hypothesized that increased values are linked to the inflammatory burden. Accordingly, inflammation has been reported to be associated with high RDW levels [11, 13], probably because of impaired erythrocyte maturation [14].

In line with this, we have shown that RDW is associated with markers of activity, inflammation and severity in patients with established disease. Accordingly, current studies have revealed an increased CV risk in patients with aggressive disease [6]. In fact, our results support the idea that inflammatory burden is behind CV risk excess in RA patients, since our patients exhibited a positive correlation between RDW and acute phase reactants. Moreover, as opposed to what happens with acute phase reactants, RDW is not influenced by recent infections. Consequently, it provides a consistent measure of inflammation—and disease activity—in patients with infections or other conditions that can alter acute phase reactants. RDW could therefore be a potential index to provide additional information about the disease activity of RA patients. Furthermore, RDW is associated not only

with inflammation and disease-specific parameters, but also with traditional CV risk factors, such as age or BMI, thus being an integrative measure of different underlying factors implicated in CV disease susceptibility.

In conclusion, we found that RDW changes in the first year after diagnosis may predict CV disease occurrence. In line with the concept of window of opportunity [15, 16], our results may be particularly relevant to clinical practice, since monitoring RDW during the first stage of the disease could allow for control of inflammation, thus possibly leading to a lower rate of CV events. Accordingly, since RDW is also associated with disease activity and inflammation in patients with established disease, aggressive treatment to reduce disease activity in early RA patients has been related to lower CV risk and mortality rates [7, 17–20].

Rheumatology key messages

- Red cell distribution width, especially the cumulative measurement, may represent a simple and useful cardiovascular risk biomarker in early RA.
- Red cell distribution width correlated with inflammation, disease activity and severity in RA patients.

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Supplementary data

Supplementary data are available at *Rheumatology* Online.

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Red cell distribution width is associated with endothelial progenitor cell depletion and vascular-related mediators in rheumatoid arthritis

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ABSTRACT

Objectives: The role of Red Cell Distribution Width (RDW) as a predictor of cardiovascular (CV) events has been proposed in a variety of conditions, including Rheumatoid Arthritis (RA). However, the mechanisms underlying this effect are still unknown. Since inflammation and Endothelial Progenitor Cells (EPCs) imbalance have been reported in RA patients to be related to CV disease, we wondered whether RDW could be linked to endothelial repair failure in RA.

Methods: EPCs ($CD34^+VEGFR2^+CD133^+$) were quantified by flow cytometry in peripheral blood samples from 194 RA patients. IFNα, TNFα, IFNγ, IL-8, VEGF, GM-CSF, MCP-1, ICAM-1, EGF, Leptin and Resistin serum levels were quantified by immunoassays. Clinical and immunological parameters as well as history of traditional CV risk factors and CV events were registered from medical records. RDW was measured in complete blood cell count analyses.

Results: RDW was negatively related to EPC counts in patients with established disease (>1 year, $n = 125$) ($r = -0.306$, $p < 0.001$). Moreover, RDW was independently associated to an EPC depletion in the whole group (β [95% CI]: −3.537 [−6.162, −0.911], $p = 0.009$) after adjusting for clinical parameters, disease duration, treatments and traditional CV risk factors. Additionally, RDW was positively correlated with IFNα serum levels, a cytokine related to endothelial damage, and with IL-8, VEGF and neutrophil to lymphocyte ratio, thus supporting the association with inflammation and vascular remodelling.

Conclusions: RDW was associated to EPC depletion and increased levels of different mediators linked to endothelial damage and vascular repair failure, thereby shedding new light on the nature of RDW as CV-predictor.

1. Introduction

Red Cell Distribution Width (RDW) is a traditional marker of anisocytosis, widely used for anaemia diagnosis. However, recent evidence points to a role for RDW as a cardiovascular risk biomarker [1]. Additionally, other authors have linked high RDW measurements with increased markers of inflammation [2]. These findings make RDW an attractive candidate to be regarded as CV risk biomarker in autoimmune conditions and, especially, in Rheumatoid Arthritis (RA) [3]. We have recently looked into this possibility, concluding that RDW could be a CV risk biomarker in RA patients [4].

However, the actual mechanisms that underlie the predictive role of RDW as a CV risk biomarker in RA remain unclear. Disease activity [3] and inflammation [5] have been linked to CV disease in RA probably because of the effects they could cause on vascular damage and repair. In this scenario, circulating Endothelial Progenitor Cells (EPC) could play a pivotal role in endothelial damage repair. Decreased or functionally impaired EPCs have been reported in RA, associated in most cases with disease activity and inflammatory mediators [6–8], and linked to CV disease development [9–11]. In this context, we wondered whether RDW would be associated to an endothelial repair failure. Due to the growing relevance of EPCs in the field of rheumatic diseases, this would be of special interest in the clinical setting, since it would provide information regarding EPC imbalance through an easy, inexpensive

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and validated parameter.

On the other hand, although RDW has been associated with inflammatory burden, the exact mechanisms that underlie this effect remain unknown. Only associations with general markers of inflammation (that is, CRP and ESR) have been reported [2,12]. Due to the relevance of the cytokine network in RA pathogenesis [13] and CV disease susceptibility [14], dissecting the actual circuits within the inflammatory burden that account for the RDW values in RA, would provide valuable insights about the nature of RDW as a biomarker and, additionally, new strategies for CV risk management in RA.

Consequently, the main aim of this study was to explore the mechanisms that underlie the role of RDW as a CV risk biomarker in RA patients and evaluate its potential associations with proven biomarkers of endothelial damage and impaired vascular repair.

2. Material and methods

2.1. Patients

Our study involved 194 RA patients consecutively recruited from the Rheumatology Department at Hospital Universitario Central de Asturias (HUCA). All patients fulfilled American College of Rheumatology 2010 criteria. Routine clinical examination included DAS28 score calculation and assessment of ongoing therapies and concomitant traditional CV risk factors. Furthermore, clinical medical records were revised to register past CV events. Traditional CV risk factors and CV events were classified as previously described [10,15]. A fasting blood sample was obtained by venipuncture. A complete blood count (including RDW) was performed in an ADVIA 2120 automated haematology analyser (Siemens) in the haematology laboratory at the HUCA.

Approval for the study was obtained from the Regional Ethics Committee for Clinical Investigation, in compliance with the Declaration of Helsinki, and all the participants gave written informed consent.

2.2. Flow cytometry analysis

Circulating EPCs were quantified in blood samples by flow cytometry as previously described [10]. Briefly, 100 μl of whole blood were preincubated with FcR Blocking Reagent (Miltenyi Biotec) for 20 min at 4 °C. Then, anti-CD34 FITC (BD Biosciences), anti-VEGFR2 PE (R&D) and anti-CD133 APC (Miltenyi) or isotype-matched controls were added and incubated for 30 min at 4 °C. Finally, red cells were lysed and cells were washed twice with PBS. Stained cells were acquired in a FACS Canto II (BD Biosciences) at a low rate until more than 100,000 events in the lymphocyte gate and 100 events in the $CD34^+$ gate were registered. EPCs were identified as $CD34^+CD133^+VEGFR2^+$ cells within the lymphocyte gate.

2.3. Quantification of serum cytokine levels

Serum aliquots were obtained from RA patients and were stored at −80 °C until cytokine measurements were conducted. IFNα serum levels were measured in 194 patients (69 early and 125 long-standing RA, whereas other cytokines were analysed in 129 individuals (29 and 110, respectively). IFNα, IL-8, VEGF and GM-CSF serum levels were quantified using a Cytometric Bead Array Flex Set (BD) in a BD FACS Canto II flow cytometer using FCAP Array v.1.0.1, following the manufacturer's instructions. The theoretical detection limits were 1.25 pg/ml, 1.2 pg/ml, 4.5 pg/ml and 0.2 pg/ml, respectively.

IFNγ serum levels were assessed using an OptEIA kit (BD) following the manufacturer's instructions (detection limit: 0.58 pg/ml). Levels of ICAM-1, TNFα, MCP-1, EGF, leptin and resistin were quantified using Mini ELISA Development Kits (PeproTech), according to the manufacturer's instructions (detection limits were: 23 pg/ml, 3.9 pg/ml, 8 pg/ml, 8 pg/ml, 63 pg/ml and 24 pg/ml, respectively).

2.4. Statistical analysis

Data are expressed as median (interquartile range) or n (%), as appropriate. Since data were not normally distributed, spearman rank's test was used to analyse correlations and Mann–Withney U test was used to assess differences between groups. Variables were log-transformed to achieve normal distribution prior to multiple regression analyses. A p-value <0.050 was considered as statistically significant. Statistical analyses were performed under SPSS v. 19.0 and R software v. 2.15.1.

3. Results

3.1. EPC depletion correlates with RDW levels

Given the association between RDW and CV disease in RA patients, we aimed to evaluate whether this parameter could be related to EPC counts, a surrogate marker of endothelial repair. To this end we quantified circulating EPCs by flow cytometry in 194 RA patients (Table 1) with different disease duration (from 0 to 258 months). Our data revealed that EPC counts correlated negatively with disease duration (Fig. 1A). In fact, long-standing (>1 year) RA patients (n = 125) exhibited an EPC depletion compared to their early counterparts (0.025(0.027) vs 0.060(0.060)·10^3/μl, p = 0.012), whereas RDW did not differ between groups (p = 0.103).

Although the analysis of the whole RA group showed a negative association between RDW and EPC counts (r = −0.186, p = 0.014), this correlation was stronger in long-standing patients but was not detected in the early group (Fig. 1B). Multivariate regression analyses confirm that this effect was detected in long-standing patients even after adjusting for traditional CV risk factors (hypertension, dyslipidaemia, diabetes, BMI and smoking habit) and treatments (glucocorticoids, methotrexate, TNFα-blockers, tocilizumab and statins) as possible confounding variables (β[95% CI], p-value: −4.182 [−6.394, −1.970], p = 0.0003), but not in their early counterparts (−1.779 [−9.583, 6.025], p = 0.634). Interestingly, RDW did not correlate with total CD34+ or CD133+ progenitors (r = 0.044, p = 0.559; and r = 0.049, p = 0.441; respectively), thus confirming that this effect was specific for the EPC population and not due to a generalized mobilization of bone-marrow progenitors.

On the other hand, the analysis of clinical parameters revealed that RDW was associated with inflammation, disease activity and severity in the long-standing group, but not in the early stage disease group (Table 2). Interestingly, RDW was also associated with traditional CV risk factors (age and BMI) in these patients. Moreover, these results were consistent after controlling for traditional CV risk factors (β, p-value: age at sampling: 0.257, p = 0.037; disease duration: 0.367, p < 0.001; BMI: 0.435, p = 0.012; CRP: 0.249, p = 0.032; ESR: 0.259, p = 0.024; PGA: 0.415, p < 0.0001; DAS28: 0.272, p = 0.018 and HAQ: 0.267, p = 0.019), hence excluding any potential effect of comorbidities-derived inflammation. Therefore, a multivariate regression analysis using EPC counts as dependent variable was performed in the whole group, including the demographic and clinical parameters associated with RDW (as shown in Table 2) as well as traditional CV risk factors and immunomodulatory treatments as potential confounding variables. Our results confirm that RDW was independently associated with an EPC depletion (β [95% CI]: −3.537

Table 1
Clinical characteristics of RA patients.

	RA patients (n = 194)	Disease duration		
		≤1 year (n = 69)	>1 year (n = 125)	p-value
Gender (female:male)	159:35	27:12	102:23	0.861
Age at sampling, years (median (range))	53 (24–87)	51 (24–79)	57 (22–84)	0.117
Disease features				
Disease duration, years	2.62 (5.60)	0.32 (0.92)	5.25 (6.33)	0.0001
Age at diagnosis, years (median (range))	49 (18–85)	50 (18–78)	49 (18–85)	0.212
Disease activity (DAS28)	3.79 (2.10)	5.03 (2.27)	3.49 (1.92)	0.0001
Tender Joint Count	3.00 (7.00)	8.00 (9.00)	2.00 (5.00)	0.0001
Swollen Joint Count	2.00 (5.00)	5.00 (5.00)	1.00 (3.00)	0.0001
Patient Global Assessment (0–100)	40.50 (40.00)	51.50 (41.00)	37.00 (35.50)	0.002
ESR, mm/h	16.00 (23.00)	26.50 (29.50)	14.00 (21.00)	0.136
CRP, mg/l	2.17 (5.53)	3.25 (8.13)	2.00 (4.35)	0.480
HAQ (0–3)	0.93 (1.13)	1.27 (1.11)	0.87 (1.18)	0.001
RF (+), n (%)	109 (56.1)	32 (46.3)	77 (61.6)	0.038
αCCP (+), n (%)	110 (56.7)	35 (50.7)	75 (60.0)	0.353
ANA (+), n (%)	79 (40.7)	24 (34.7)	55 (44.0)	0.640
Traditional CV risk factors, n(%)				
Dyslipidemia	65 (33.5)	24 (34.7)	41 (32.8)	0.390
Hypertension	50 (25.7)	14 (20.2)	36 (28.8)	0.742
Diabetes	17 (8.7)	5 (7.2)	12 (9.6)	0.976
Obesity (BMI > 30)	26 (13.4)	7 (10.1)	19 (15.2)	0.358
Smoking habit	52 (26.8)	17 (24.6)	35 (28.0)	0.589
Previous CV events, n(%)				
Previous CV events	33 (17.0)	9 (13.0)	24 (19.2)	0.293
Ischaemic heart disease	14 (7.2)	5 (7.2)	9 (7.2)	
Heart failure	15 (7.7)	4 (5.7)	11 (8.8)	
Cerebrovascular accidents	4 (2.0)	0 (0.0)	4 (3.2)	
Treatments, n(%)				
None or NSAIDs	35 (18.0)	35 (50.7)	0 (0.0)	
Glucocorticoids	98 (50.5)	24 (34.7)	74 (59.2)	0.001
Methotrexate	126 (64.9)	27 (39.1)	99 (79.2)	0.0001
TNFα blockers	49 (25.2)	2 (2.8)	47 (37.2)	0.0001
Tocilizumab	12 (6.1)	0 (0.0)	12 (9.6)	
Statins	24 (12.3)	3 (4.3)	21 (16.8)	0.203

Data are expressed as median (interquartile range) for continuous variables and n(%) for categorical ones, unless otherwise stated. Statistical differences between patients and onset and with long-standing disease were assessed by Mann–Withney U or x^2 tests, as appropriate.

[−6.162, −0.911], p = 0.009), in addition to the effect of disease activity (−0.142 [−0.280, −0.004], p = 0.044). These results indicate that RDW could be used as a clinical biomarker of EPC depletion in RA patients, even after adjusting for traditional CV risk factors and therapies, hence suggesting that it could reflect the impaired EPC-mediated endothelial repair capability associated with the disease course.

3.2. RDW and vascular mediators

The altered balance between endothelial damage and repair that underlie vascular injury in RA patients is influenced by cytokines and other vascular and inflammatory mediators [16–23]. Thus, we aimed to explore the associations between RDW and several mediators involved in these processes: cytokines related to Th1 pathogenesis (TNFα and IFNγ), endothelial damage (IFNα) or vascular remodelling (IL-8, VEGF, GM-CSF and MCP-1); adipokines (leptin and resistin); endothelial factors (ICAM-1 and EGF); and haematological markers of inflammation (neutrophil to lymphocyte (N/L) ratio). It is important to note that no differences in RDW were observed due to glucocorticoids (p = 0.348), methotrexate (p = 0.271) or TNFα blockers (p = 0.158) usage, although a lower

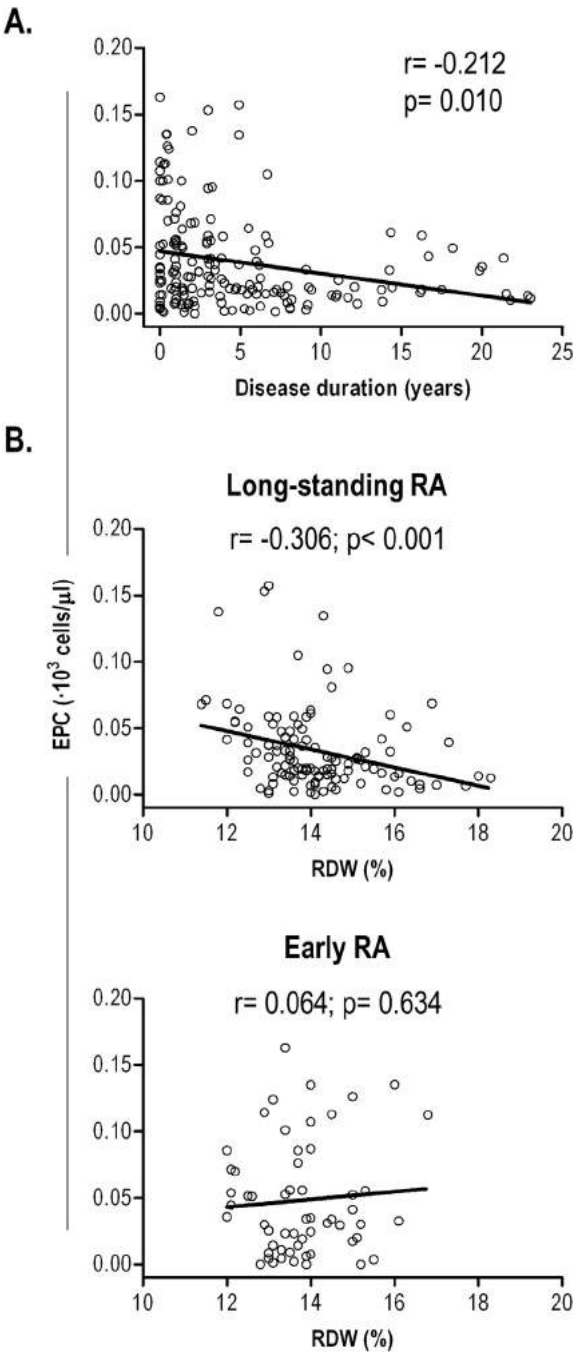

Fig. 1. Association between EPC counts and disease features in RA patients. (A) EPC counts are negatively associated with disease duration. (B) RDW paralleled EPC depletion in long-standing patients but not in their early counterparts. Correlations were assessed using the Spearman ranks correlation test.

RDW was detected in patients on tocilizumab therapy (p = 0.036).

Again, RDW failed to exhibit any significant association with the analyzed mediators in early RA patients, whereas IFNα, IL-8, VEGF and N/L ratio showed a positive correlation in the long-standing group (Table 3). However, significant differences in these mediators between long-standing and early RA patients were only found in IFNγ, MCP-1 and resistin levels (Table 4). Exclusion of patients with previous CV events did not change these findings.

It is noteworthy that RDW was associated with IFNα in the entire RA group. This association remained significant after controlling for

Table 2
Associations between RDW and clinical features in RA patients.

RA patients (n = 194)		Disease duration	
		≤1 year (n = 69)	>1 year (n = 125)
Age at sampling	r = 0.119	r = −0.077	**r = 0.214**
	p = 0.101	p = 0.728	**p = 0.018**
Disease duration	**r = 0.236**	r = −0.081	**r = 0.239**
	p = 0.001	p = 0.508	**p = 0.009**
BMI	**r = 0.223**	r = 0.046	**r = 0.297**
	p = 0.001	p = 0.818	**p = 0.004**
CRP	r = 0.120	r = −0.097	**r = 0.281**
	p = 0.146	p = 0.522	**p = 0.004**
ESR	r = 0.169	r = −0.043	**r = 0.297**
	p = 0.060	p = 0.758	**p = 0.002**
Patient Global Assessment	r = 0.165	r = −0.075	**r = 0.394**
	p = 0.047	p = 0.603	**p < 0.0001**
DAS28	r = 0.180	r = 0.210	**r = 0.341**
	p = 0.078	p = 0.080	**p < 0.0001**
HAQ	r = 0.141	r = −0.125	**r = 0.320**
	p = 0.077	p = 0.397	**p < 0.001**

Correlations were assessed by Spearman rank's test.
Associations found to be statistically significant are highlighted in bold.

Table 3
Associations between RDW and cytokines and other mediators.

RA patients (n = 194)		Disease duration	
		≤1 year (n = 69)	>1 year (n = 125)
TNFα (pg/ml)	r = 0.091	r = 0.042	r = 0.079
	p = 0.358	p = 0.893	p = 0.459
IFNγ (pg/ml)	r = 0.109	r = 0.388	r = 0.089
	p = 0.285	p = 0.239	p = 0.470
IFNα (pg/ml)	**R = 0.211**	r = 0.202	**r = 0.222**
	p = 0.005	p = 0.109	**p = 0.020**
IL-8 (pg/ml)	**r = 0.193**	r = −0.197	**r = 0.260**
	p = 0.049	p = 0.519	**p = 0.012**
VEGF (pg/ml)	**r = 0.212**	r = −0.039	**r = 0.244**
	p = 0.029	p = 0.900	**p = 0.019**
GM-CSF (pg/ml)	r = 0.161	r = 0.157	r = 0.140
	p = 0.099	p = 0.609	p = 0.181
MCP-1 (pg/ml)	r = 0.129	r = 0.102	r = 0.091
	p = 0.189	p = 0.739	p = 0.387
ICAM-1 (ng/ml)	r = −0.010	r = −0.083	r = 0.019
	p = 0.923	p = 0.787	p = 0.854
EGF (pg/ml)	r = 0.020	r = −0.420	r = 0.030
	p = 0.840	p = 0.153	p = 0.779
Leptin (ng/ml)	r = 0.168	r = 0.116	r = 0.141
	p = 0.089	p = 0.705	p = 0.183
Resistin (ng/ml)	r = −0.142	r = −0.327	r = −0.102
	p = 0.181	p = 0.276	p = 0.377
N/L ratio	**r = 0.265**	r = −0.327	**r = 0.269**
	p = 0.006	p = 0.227	**p = 0.009**

Correlations were assessed by Spearman rank's test.
Associations found to be statistically significant are highlighted in bold.

disease duration, activity, ESR and exposure to glucocorticoids, methotrexate, TNFα blockers and tocilizumab (0.011[0.001, 0.021], p = 0.032). Actually, recently-diagnosed patients showed a similar correlation trend to their long-standing counterparts, whereas the significant associations detected for IL-8, VEGF and N/L ratio were exclusively due to long-standing patients and were completely absent in the early group. In addition, IFNα levels were strongly correlated with RDW in patients with previous CV events (r = 0.524, p = 0.002; n = 33), this group having higher levels of this cytokine than their CV-free counterparts (41.16 ± 79.31 vs 23.30 ± 66.10, p = 0.030). Of all the studied molecules, this relationship was unique for IFNα, a cytokine previously associated with endothelial damage and CV disease in RA patients. These results support the idea of RDW as a marker of endothelial damage and repair failure in RA, hallmarked by an EPC imbalance and increased IFNα serum levels.

Table 4
Cytokine levels in RA patients according to disease duration.

	RA patients (n = 194)	Disease duration	
		≤1 year (n = 69)	>1 year (n = 125)
TNFα (pg/ml)	350.69 ± 441.43	319.63 ± 194.83	355.81 ± 470.34
IFNγ (pg/ml)	8.93 ± 15.99	3.75 ± 1.39	9.78 ± 17.11**
IFNα (pg/ml)	37.86 ± 70.95	18.12 ± 20.24	34.02 ± 76.00
IL-8 (pg/ml)	51.02 ± 28.83	60.58 ± 46.24	49.04 ± 23.64
VEGF (pg/ml)	123.57 ± 42.09	133.63 ± 49.31	121.49 ± 40.43
GM-CSF (pg/ml)	40.71 ± 45.93	66.19 ± 82.33	35.45 ± 32.34
MCP-1 (pg/ml)	389.91 ± 381.50	234.40 ± 113.84	422.02 ± 409.00**
ICAM-1 (ng/ml)	280.28 ± 148.69	313.41 ± 175.47	273.44 ± 142.68
EGF (pg/ml)	113.59 ± 100.65	109.30 ± 43.89	167.33 ± 169.68
Leptin (ng/ml)	15.79 ± 22.03	10.30 ± 6.60	16.93 ± 23.89
Resistin (ng/ml)	10.23 ± 4.06	13.11 ± 5.03	9.64 ± 3.58**
N/L ratio	3.51 ± 2.94	3.20 ± 1.90	3.56 ± 3.09

Data are expressed as mean ± standard deviation. Differences between early and long-standing RA patients were assessed by Mann–Withney U test. *p < 0.050, **p < 0.010, ***p < 0.001.

On the other hand, the association of RDW with IL-8 and VEGF in long-standing RA patients suggests its potential role as biomarker of vascular remodelling. Moreover, IL-8 was found to be independently related to RDW after adjusting for disease duration, activity, ESR and treatments (0.062[0.004, 0.120], p = 0.037). Finally, the correlation between RDW and the N/L ratio in these patients supports the use of both haematological parameters as inflammatory markers.

These results seem to link RDW with vascular remodelling processes, thereby supporting its association with vascular damage and repair failure.

4. Discussion

This study provides novel insights about the nature of RDW as a CV risk biomarker in RA, thereby shedding new light into the mechanisms underlying the predictive value of RDW previously reported [4] in these patients. Our main results revealed that RDW was associated with EPC depletion as well as with markers of vascular damage and remodelling processes, all of them features which usually occur during the disease course and are linked to CV disease development. These findings were restricted to the long-standing RA group, whereas RDW in patients at the early stage of the disease remain unrelated to these parameters. In addition, our results remain significant after adjusting for traditional CV risk factors and exposure to treatments, thereby supporting the independent role for RDW as biomarker of CV risk.

CV disease occurrence in RA patients is the result of traditional CV risk factors and disease parameters [3,5,24,25]. Although the actual mechanisms are still unknown, disease parameters seem to influence CV risk by their effects on endothelial repair and damage. In this scenario, the role played by EPCs deserves a mention. EPCs have been reported to be depleted or functionally impaired in RA patients [6–11], associated with disease activity and duration. This situation was also found in other rheumatic and inflammatory diseases and supports the role for EPC depletion in the increased CV risk found in these patients [26,27]. Therefore, identification of EPC depletion may be of special interest for these conditions, but in this sense, biomarkers are lacking. This is the first study in reporting that RDW could be independently related to EPC depletion, thereby supporting a new dimension for this well-known parameter in the clinical setting.

Our results highlight important differences between recent onset and long-standing RA patients, which probably reflects a progressive vascular impairment during the course of the disease.

Whereas RDW was related to markers of vascular damage, inflammation and disease activity in patients with established disease, these associations were missing in the early group. Even the associations with age or BMI, which are independent of disease parameters, are not present in the early group. It is plausible that changes in the cytokine and inflammation networks are taking place in the early patients, so the associations of RDW with disease parameters could be different to those seen in patients with established disease [28]. Nevertheless, including all clinical parameters in a regression model, our results confirm that RDW was an independent predictor of EPC depletion in the whole RA group. Moreover, the fact that early changes in RDW are predictors of CV prognosis in RA patients [4] suggests that this parameter is prematurely altered, even earlier than usual molecular or cellular signs of vascular damage. These results highlight the differences between these two stages of the disease and are in line with the concept of 'window of opportunity', whose implication in CV disease prediction in RA has been recently proposed [29–32].

We have previously reported that RDW measurements during the early phase of the disease are predictors of CV occurrence in RA patients [4]. Accordingly, RDW was associated to markers of impaired endothelial repair and vascular remodelling, thereby supporting, to some extent, our previous findings. Additionally, differences between early and established RA patients give us relevant clues on the mechanisms underlying the role for RDW as predictive biomarker. The associations with EPC and vascular mediators observed in long-standing patients could be a consequence of a cumulative exposure to inflammation, immune dysregulation and traditional CV risk factors. However, as these changes are linked to disease course, they cannot me monitored in the early stage, thereby explaining the lack of associations in this group. All these lines of evidence herein presented here lead us to think that RDW could be a very early marker of vascular damage, since small changes in RDW can predict CV outcome even after other phenomena become evident. This is especially relevant since RDW has several advantages as a biomarker [33]. The different nature of RDW as a biomarker (since it is not a classic cell or molecular marker) probably may account for its early sensitivity and proves RDW may have a differential consideration than the classic well-known biomarkers. Actually, RDW has been related to atherosclerosis plaque formation [34] and also to endothelial dysfunction [35,36], which is the subclinical step prior to atherosclerosis plaque formation. Endothelial dysfunction has been found in recent onset RA patients, even in the absence of traditional CV risk factors [37].

On the other hand, RDW was associated with IL-8 and VEGF, factors linked to active vascular remodelling processes, in long-standing but not in early patients. Both endothelial injury and EPC depletion are associated with disease duration, so vascular remodelling mechanisms are expected not to be so active in the early stage. This could explain why there was no association between these molecules and RDW in the early group. However, in patients with longer disease duration, because of the chronic exposure of EPCs to multiple risk factors, an EPC exhaustion can be detected, thereby resulting in a decreased endothelial reparative capability. During this stage, reparative mechanisms are not able to counteract endothelial damage, which becomes clinically relevant. The association between RDW and IL-8 in a multivariate model provide potential mechanistic insights about RDW as a biomarker. IL-8 is an inflammatory chemokine released by macrophages, which is involved in neutrophils chemotaxis and induction of adhesion molecules in endothelial cells. Neutrophils have a relevant role in atherosclerosis [21,38], especially in the early steps [39]. Moreover, neutrophils have been connected with enhanced vascular damage and impaired vascular repair in autoimmunity [40], with a central role for type I IFNs, thereby linking IL-8, neutrophils, type I IFNs and impaired vascular repair. Additionally, IL-8 has been proposed as a biomarker for risk stratification of CV disease patients [41] and also for the identification of patients at risk of developing a CV event [42]. Therefore, our findings point to a role for IL-8-neutrophils axis in CV disease in RA patients.

In line with this, RDW was associated with N/L ratio, which has been reported to be a marker of inflammation and CV risk in different conditions [43]. Although the potential role of this parameter in RA is unknown, it is supported by the involvement of neutrophils in RA pathogenesis [44] as well as in atherosclerosis progression [39,40]. Thus, the correlation with RDW made the N/L ratio a new candidate as a risk biomarker that should be taken into account in future studies with RA patients.

Similarly, another interesting result is the association between RDW and IFNα serum levels. Of the studied mediators, this is the only molecule that presented the same trend in all patients, although statistical significance was not reached in early patients. In recent years, a growing body of evidence has proposed a role for IFNα (and type I IFNs) in endothelial damage and repair failure [45]. IFNα has been reported to promote abnormal vasculogenesis by altering EPC phenotype and functionality [46–48] in SLE patients and murine models of lupus and atherosclerosis. We have reported an EPC imbalance and increased CV events occurrence associated with IFNα serum levels in RA patients [10]. Additionally, genetic variants of interferon regulatory factors, linked to increased signalling through type I IFN intracellular pathway, have been associated with markers of early atherosclerosis and CV disease occurrence in RA patients [49,50].

In conclusion, our results support that RDW changes during the early stage of RA could be a predictor of CV occurrence because it may be considered as a very early biomarker of subclinical endothelial damage, opposed to other molecular or cellular markers which are undetectable at this stage. After this period, RDW was associated with disease activity and severity as well as with vascular remodelling mechanisms, which may be probably triggered by the progression of the subclinical damage, becoming evident at a cellular and molecular level. However, the increased IFNα levels and the EPC depletion presented by these patients could account for the vascular repair failure, thereby leading to atherosclerosis and CV disease progression in RA. All these findings reinforce the potential use of RDW to stratify CV risk in RA in the clinical setting.

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Capítulo V: Determinación de autoanticuerpos anti-HDL en pacientes de AR

La literatura científica más reciente ha probado la participación de diferentes tipos de anticuerpos en el desarrollo de enfermedad CV, lo cual va en línea con la hipótesis del origen inflamatorio de la aterosclerosis. Por tanto resulta lógico pensar que en enfermedades autoinmunes sistémicas, debido a la pérdida de tolerancia frente a antígenos propios, este mecanismo pueda tener incluso mayor relevancia. Este hecho ha sido ampliamente estudiado en LES, donde se han caracterizado un buen número de autoanticuerpos con capacidad proaterogénica, entre los que se encuentran los autoanticuerpos anti-HDL y anti-Apo A-1. Sin embargo, la presencia de mecanismos patogénicos compartidos entre ambas patologías, así como evidencias previas de otros grupos, hace pensar que estos autoanticuerpos podrían estar presentes también en AR. La presencia de estos autoanticuerpos es relevante dado que podrían tener un papel pronóstico y se trata además de biomarcadores fácilmente implementables en los laboratorios de rutina clínica.

A la vista de estos antecedentes, decidimos investigar la presencia y niveles de anticuerpos anti-HDL de isotipo IgG en pacientes de AR y sus posibles relaciones con los niveles séricos de lipoproteínas, parámetros clínicos, factores clásicos de riesgo CV y su relación con mediadores de inflamación y daño endotelial.

Artículo 8: Rodríguez-Carrio J, Alperi-López M, López P, Ballina-García FJ, Abal F, Suárez A (2015); *Antibodies to High Density Lipoprotein are associated with inflammation and cardiovascular disease in Rheumatoid Arthritis patients;* Clinical Science (enviado).

Aportación personal al trabajo: mi contribución en este trabajo se centró en el diseño e implementación del protocolo de determinación de estos autoanticuerpos, así como la realización de la mayor parte de los inmunoensayos. Igualmente, realicé el análisis de los resultados y contribuí a la interpretación de los mismos. Finalmente, llevé a cabo la redacción del manuscrito y la preparación de las figuras, bajo la supervisión de la Dra. Ana Suárez Díaz.

Antibodies to High Density Lipoprotein are associated with inflammation and cardiovascular disease in Rheumatoid Arthritis patients

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ABSTRACT

Since factors other than chronic inflammation may underlie the altered lipid profiles in Rheumatoid Arthritis (RA) patients, and the presence of antibodies against HDL lipoproteins has been reported in other autoimmune conditions, we undertook this study to evaluate whether they are present in RA and associated with clinical and cardiovascular (CV) parameters. To this end, IgG anti-HDL antibodies and total IgG serum levels were quantified in 212 RA patients, 131 sex- and age-matched healthy controls (HC) and 52 subjects with traditional CV risk factors. A subgroup of 13 RA patients was prospectively followed upon TNFα-blockade. TNFα, IFNα, MIP1α, IFNγ, IL-8, VEGF, GM-CSF, IL-17, MCP1, SDF-1α, resistin and leptin serum levels were quantified by immunoassays.

IgG anti-HDL levels were higher in RA patients compared to HC ($p<0.0001$) and CVR subjects ($p=0.015$). Differences with HC remained after correction for total IgG levels ($p<0.003$). Anti-HDL/IgG were negatively associated with HDL levels in RA (-1.182[-1.823, -0.541], $p=0.0003$) after adjusting for demographical, clinical, inflammatory parameters and treatments. RA patients with high levels of anti-HDL/IgG (n=40, 18.8%) were more likely to have experienced a CV event ($p<0.0001$), and exhibited increased levels of several proinflammatory mediators (CRP, IFNα, MIP1α, IFNγ, IL-8, GM-CSF, IL-17 and MCP1). Finally, change in anti-HDL antibodies upon TNFα-blockade was independently associated with increasing HDL levels. Overall, IgG anti-HDL antibodies are increased in RA independently of traditional CV risk factors and associated with a proinflammatory milieu and impaired lipid blood profile, which may contribute to the increased rate of CV events in these patients.

Key words: anti-HDL antibodies, HDL, cardiovascular risk, chronic inflammation, Rheumatoid Arthritis, anti-TNFα therapy

INTRODUCTION

Rheumatoid Arthritis (RA) is associated with greater rates of cardiovascular disease (CVD) morbidity than the general population. This greater risk cannot be explained only by traditional CV risk factors [1], and different non-traditional factors, such as genetic background, chronic inflammation or exposure to treatments seem to be involved [2].

Serum lipids and lipoproteins are pivotal players in the field of CVD. Raised low-density lipoproteins (LDL)-cholesterol and reduced high-density lipoprotein (HDL)-cholesterol levels are well established risk factors in the general population. In RA, however, the association between serum lipids and CVD seems to be more complex. Active RA patients tend to exhibit lower levels of lipids than the general population but an excess of CV risk. Control of the disease using different disease-modifying antirheumatic drugs (DMARDs) is usually accompanied by increasing lipid levels, mainly in HDL-cholesterol, to variable degrees [3;4]. The phenomenon that a condition associated with increased CVD morbidity was associated with reduced lipid levels was termed as "lipid paradox" [4;5], and several lines of evidence point towards a role of chronic inflammation as the underlying factor. However, the mechanisms that potentially drive the interactions between inflammation and lipid metabolism are not entirely understood.

HDL lipoproteins are important in preventing the development of atherosclerotic lesions and endothelial homeostasis [6], and chronic inflammation has been described not only to be able to decrease HDL levels [4] but also to impair their protective functions [7], by modification of its composition [8]. Moreover, effective disease activity control leads to normalization of HDL levels in some studies [9;10], but not in others [11], whereas other authors found that a good clinical outcome may induce restoration of HDL functions rather than changes in levels [12]. Consequently, some gaps remain in the understanding of changes in lipids in RA and this warrants further studies on the potential contribution of emergent players.

Recently, the hypothesis that certain autoantibodies may have a role in CVD development in chronic autoimmune diseases has emerged [13]. The presence of anti-HDL IgG antibodies has been reported in Systemic Lupus Erythematosus (SLE) patients [14;15], associated with severe disease and inflammatory burden. Interestingly, anti-HDL antibodies can interfere with the anti-inflammatory and anti-oxidant intrinsic functions of HDL [16]. However, the presence and possible clinical role of anti-HDL IgG antibodies in RA patients remains unknown.

Therefore, the main aims of this study were (i) to investigate whether anti-HDL IgG are present in RA patients, (ii) whether they could be related to the altered lipid profile, clinical characteristics and traditional CV risk factors and (iii) to analyze if this antibodies might be associated with lipid changes upon anti-TNFα treatment.

MATERIAL AND METHODS

Patients and controls

This was a cross-sectional case-control study with three different groups of individuals enrolled (Table 1). Our study involved 212 RA patients recruited from the Department of Rheumatology at Hospital Universitario Central de Asturias. All of them fulfilled the 2010 American College of Rheumatology classification criteria for RA. A complete clinical examination, including Disease Activity Score 28-joints (DAS28) calculation, was performed on each patient on the day of their clinic appointment and a blood sample was drawn by venipuncture. Also, clinical records were retrospectively revised so as to register traditional CV risk factors and the history of CV events. Definition and classification of CV events and traditional risk factors (hypertension, diabetes, dyslipidemia, obesity and smoking) were performed as previously established [17]. In addition, a subgroup of 13 RA patients (12 women, median age 43 (range: 30 – 65), DAS28 5.08(1.93), 38.5% RF+, 46.1% anti-CCP+), candidates for TNFα–blockers, was prospectively followed and a blood sample was drawn immediately before and 3 months after anti-TNFα therapy. Clinical response after TNFα-blockade was evaluated by EULAR criteria [18].

Simultaneously, 131 gender- and age-matched healthy volunteers (HC) were recruited from the same population and a group of 52 individuals with marked traditional CV risk factors [tCVR: 22 (42.3%) diabetes, 32 (61.5%) hypertension, 32 (61.5%) dyslipidemia, 19 (36.5) smoking and 17 (32.6%) obesity] was recruited from their primary care referral center.

Automated serum lipids analysis was carried out on all the participants from fresh blood samples. Serum samples were stored at -80ºC until laboratory measurements were performed. Approval for the study was obtained from the Regional Ethics Committee for Clinical Investigation, in compliance with the

Declaration of Helsinki. All the participants gave written informed consent prior to their inclusion in the study.

Determination of anti-HDL antibodies

IgG antibodies against HDL were measured in all serum samples by ELISA as previously described [14] with slight modification. ELISA Maxisorp plates (Nunc) were half-coated overnight at 4º C with 20 μg/ml human HDL-cholesterol (Sigma) in 70% ethanol (test half) or ethanol alone (control half). Plates were blocked with PBS 1% BSA (Sigma) for 1 hour at room temperature and then washed with PBS. Serum samples, previously diluted 1:50 in PBS containing 0.1% BSA, and standard curves from pooled sera (diluted 1:16 to 1:512) were incubated for 2 hours at room temperature. After that, three washes with TBS were performed and alkaline phosphatase-conjugated anti-human IgG (1:1000) (Immunostep) was added. Finally, the plate was washed twice with TBS followed by addition of p-nitrophenylphosphate (Sigma) in dietanolamine buffer. Absorbance at 405 nm was recorded and signal from the control half of the plate was subtracted to that of the test half. Anti-HDL Arbitrary Units (AU) were calculated for each sample according to the standard curves.

Similarly, total IgG was quantified by conventional ELISA techniques and AU values obtained from the anti-HDL ELISA were corrected using total IgG levels (anti-HDL/IgG).

Quantification of cytokine serum levels

Analyses of serum cytokines were performed in a subsample of RA patients (n=129). IFNα, MIP1α, IL-8, IL-17A, VEGF and GM-CSF serum levels were quantified using a Cytometric Bead Array Flex Set (BD) in a BD FACS Canto II flow cytometer using FCAP Array v.1.0.1, following the manufacturer's instructions. The theoretical detection limits were 1.25 pg/ml, 0.6 pg/ml, 1.2 pg/ml, 1.25 pg/ml, 4.5 pg/ml and 0.2 pg/ml, respectively.

IFNγ serum levels were assessed using an OptEIA kit (BD) following the manufacturer's instructions (detection limit: 0.58 pg/ml). Levels of TNFα, MCP-1, leptin and resistin were quantified using Mini ELISA Development Kits (PeproTech), according to the manufacturer's instructions (detection limits were: 3.9 pg/ml, 8 pg/ml, 63 pg/ml and 24 pg/ml, respectively).

Statistical analysis

Data are expressed as mean ± standard deviation or median (interquartile range), as appropriate. Categorical variables were expressed as n(%) and analyzed using chi-square tests. Spearman rank's test was used to analyze correlations, whereas one-way ANOVA (Bonferroni multiple comparisons test), Kruskal-Wallis (Dunn-Bonferroni correction for multiple comparisons test) or Mann-Withney U tests were used to assess differences between groups. Variables were log-transformed to achieve normal distribution prior to multiple regression analyses. A p-value <0.050 was considered as statistically significant. Statistical analyses were performed under SPSS v. 19.0 and R software v. 2.15.1.

RESULTS

Anti-HDL antibodies as predictors of altered HDL-cholesterol levels in RA patients

Anti-HDL IgG levels were quantified in serum samples from the groups summarized in Table 1, RA patients exhibiting increased amounts of these antibodies both as arbitrary units (AU) and after correction for total IgG levels (anti-HDL/IgG). The tCVR group did not exhibit significant differences with HC, suggesting that anti-HDL antibodies were present in RA independently of comorbidities.

No differences were observed in anti-HDL antibodies (p=0.859) between RA patients recruited at onset (n=47) and their long-standing counterparts. However, serum levels of HDL-cholesterol were negatively associated with anti-HDL/IgG in recent onset RA patients (Figure 1), but not in the long-standing group (r= -0.051, p=0.553), suggesting that disease features or treatments could influence the lipid profile in RA. In fact, although no differences in HDL-cholesterol were detected between HC and the whole RA group, patients recruited at onset exhibited decreased levels compared to their long-standing counterparts (51.60±15.62 vs 62.98±17.23, Bonferroni p<0.001) and the HC group (p=0.021).

Therefore, to evaluate whether anti-HDL/IgG antibodies could influence the lipid profile in RA patients, a multiple linear regression analysis adjusted for age, markers of inflammation (CRP and ESR), disease activity and duration as well as treatments received was performed. Results showed that anti-HDL/IgG antibodies significantly predicted low HDL-cholesterol levels (Table 2), and similar effect was obtained analyzing anti-HDL AU (-0.587 [-0.957, -0.216], p=0.002). Moreover, when the total:HDL-cholesterol ratio was entered as the dependent variable, anti-HDL/IgG was found to be the only independent predictor (0.188 [0.123, 0.253],

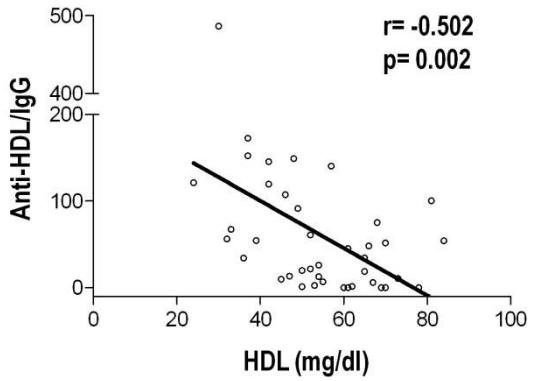
p=8.95·10^{-8}).Of note, despite not reaching significance, was that a slight effect for the TNFα-blockers usage in this analysis (-0.512 [-1.067, 0.043], p=0.070) may be hypothesized. All these results support a role for anti-HDL/IgG antibodies in the altered lipid profile of RA patients.

Table 1: Demographic parameters, serum lipids and anti-HDL antibodies on the populations studied

	HC	RA	tCVR[1]	*p-value*
n	131	212	52	
Gender (f/m)	97/34	175/37	37/15	0.080
Age at sampling, years (mean (range))	52 (26 – 80)	53 (18 – 87)	55 (33 – 68)	0.452
Total-cholesterol, mg/dl	205.91±33.56	207.42±35.71	216.60±42.97	0.476
HDL-cholesterol, mg/dl	58.79±14.00	60.56±17.60	57.91±14.30	0.515
LDL-cholesterol, mg/dl	126.46±29.35	122.60±32.38	136.00±50	0.175
Total/HDL-cholesterol ratio	3.67±1.00	3.72±1.35	3.93±1.13	0.211
Anti-HDL, AU (median (range))	37.75 (0 – 1126.35)	187.02 * (0 – 8681.37)	61.15 (0 – 2972.26)	< 0.0001
Anti-HDL/IgG, (median (range))	18.92 (0 – 417.00)	35.78 ** (0 – 2500.10)	18.81 (0 – 1379.23)	0.005

Continuous variable are summarized as mean ± standard deviation, unless otherwise stated. Differences were assessed by Kruskal-Wallis test with Dunn-Bonferroni correction for multiple comparisons, or chi-square test, as appropriated. [1]tCVR population (diabetes n=22, hypertension n=32, dyslipidemia n=32, smoking n=19 and obesity n=17) *HC vs. RA: p<0.0001, CVR vs RA: p=0.015. **HC vs. RA p=0.003.

Figure 1: Anti-HDL antibodies and HDL-cholesterol levels in early RA patients.



Anti-HDL/IgG antibodies were negatively correlated with HDL-cholesterol levels in RA patients recruited at onset (n=47). Correlation was assessed by Mann-Withney U test.

Anti-HDL antibodies and clinical features

Since our results suggest that development of anti-HDL antibodies could be an early event in some RA patients, we hypothesize that the presence of high levels of these autoantibodies, in addition to their effect on blood lipids, might identify a subgroup of RA patients with special clinical or immunological characteristics. Thus, patients were classified into two groups using the HC 90th percentile of anti-HDL/IgG antibodies (169.80) as cut off. Disease parameters, treatments, CV factors and serum levels of several cytokines were evaluated in accordance (Table 3).

No significant differences in the frequency of anti-HDL^{high} patients (40/212, 18.8%) were observed between recent onset and long-standing RA (p=0.292). However, anti-HDL^{high} patients tended to be increased among males (12/40, 30.0% vs 28/172, 16.3% in females), exhibited larger disease duration, high CRP levels and increased frequency of erosive disease. Although no differences in disease activity were found,

patients in remission (DAS28≤2.6) were less likely to exhibit high anti-HDL/IgG levels than those with moderate or active disease (DAS>2.6) (9.75% vs 23.7%, p=0.030). Interestingly, anti-HDL^{high} patients were not associated to either RF- or anti-CCP positivity, thereby excluding widespread autoantibody production or a potential effect of cross-reactivity with other autoantibodies. Similarly, no differences in received therapies were registered. However, it is noteworthy that anti-HDL^{high} patients were much more likely to have suffered from a CV event than their anti-HDL^{low} counterparts, whereas the distribution of traditional CV risk factors, except for dyslipidemia, was similar between groups. Actually, in a multivariate regression analysis where age, gender, traditional CV risk factors,

Table 2: Effect of anti-HDL/IgG antibodies on HDL-cholesterol levels in RA patients

	B [95% CI]	*p-value*
Anti-HDL/IgG	-1.182 [-1.823, -0.541]	0.0003
Age	-0.010 [-0.168, 0.148]	0.904
CRP	-0.118 [-0.475, 0.238]	0.513
ESR	-0.019 [-0.157, 0.120]	0.791
Disease duration	1.949 [-0.722, 4.620]	0.628
DAS28 score	0.484 [-1.487, 2.455]	0.628
Glucocorticoids	4.584 [-0.379, 9.547]	0.070
Methotrexate	2.148 [-3.511, 7.808]	0.454
TNFα blockers	2.050 [-3.385, 7.485]	0.457

Linear multivariate regression analysis was performed using HDL-cholesterol levels as dependent variable.

disease activity and duration, autoantibodies, inflammatory markers and treatments entered as dependent variables, only anti-HDL and hypertension remained significant predictors of CV disease (p<0.0001 and p=0.021, respectively).

On the other hand, anti-HDL^{high} patients displayed higher IFNα levels, a cytokine associated with vascular damage, and were related to a higher systemic inflammation, as several proinflammatory mediators were upregulated (MIP1α, IFNγ, IL-8, GM-CSF, IL-17A and MCP-1), which is in line with the increased levels of CRP also found in this group.

Hence, anti-HDL^{high} RA patients were hallmarked by a proinflammatory milieu, IFNα production and increased prevalence of CV events, suggesting the use of anti-HDL antibodies as biomarkers for CV risk.

Effect of TNFα-blockade on anti-HDL antibodies

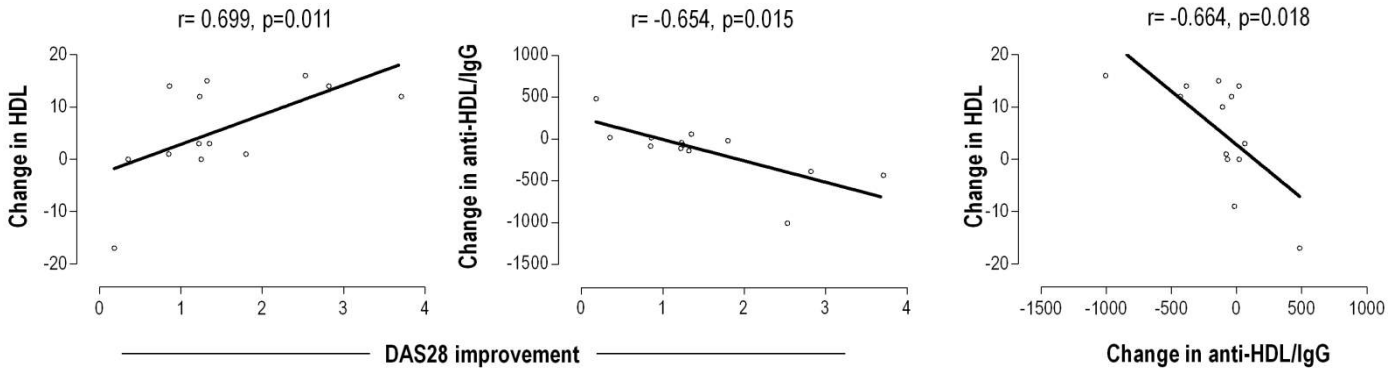
Since TNFα-blockade has been related to change in lipid profiles in RA patients [4;19], we aimed to test the possible effect of TNFα antagonists on anti-HDL antibody production. To this end, a subgroup of RA patients (n=13) candidates for TNFα blockade were prospectively followed for 3 months and changes on lipid profile, anti-HDL and total IgG levels as well as disease outcome were analyzed.

First, we observed that DAS28 improvement was accompanied by both an increase in HDL-cholesterol levels and a decrease in anti-HDL/IgG values (Figure 2). Interestingly, changes in anti-HDL/IgG paralleled those of HDL-cholesterol, thus supporting the relationship between decrease of anti-HDL production and recovery of HDL-cholesterol levels. Equivalent results were obtained when the change on total:HDL-cholesterol ratio was analyzed, confirming the role of these autoantibodies in the partial restoration of lipid profile.

Finally, we performed a multiple regression analyses to exclude the possible effect of disease activity as a confounding factor. Results revealed that changes in anti-HDL/IgG antibodies upon TNFα-blockade independently predicted change on HDL-cholesterol

levels after adjusting for CRP decrease and EULAR clinical response (-3.164 [-5.510, -0.818], p=0.015), thereby supporting their role in the lipid profile maintenance in patients on biologic therapy. However, TNFα blockade did not result in a significant change on HDL-cholesterol (58.00±15.89 vs 62.00±14.44, p=0.170) or anti-HDL/IgG levels (143.99(319.93) vs 62.62(178.13), p=0.101).

Figure 2: Anti-HDL antibodies, HDL-cholesterol levels and clinical improvement upon TNFα-blockade.



Clinical improvement (measured as difference in DAS28) after 3 months in patients on anti-TNFα therapy (n=13) was associated with the change in HDL-cholesterol and anti-HDL antibodies, being the former negatively correlated. Subsequent multivariate analysis revealed an independent effect of anti-HDL antibodies. Correlations were assessed by Mann-Withney U test.

DISCUSSION

Although a growing number of studies support the link between RA pathogenesis and CVD development, and several lines of evidence point to an altered lipid profile in RA patients, the connection between CVD and lipid profiles in RA is poorly understood. The results presented in this paper suggest the involvement of a new player, anti-HDL antibodies, in this scenario.

Altered lipid profiles in RA patients have been widely reported (reviewed in [4]), gradually associated with the number of fulfilled ACR criteria [20]. Most authors have explained these results on the basis of an inflammatory burden [20-22] and poor disease activity control [3;20]. However, results are quite heterogeneous and even contradictory. On the one hand, in spite of the main role of inflammation in the so-called “lipid paradox” in RA [5], the magnitude of the differences in lipid levels explained by inflammation is limited [23]. On the other hand, patients with similar degrees of disease activity control, and thus similar levels of immunosuppression, may exhibit important differences in lipid levels [4;24], thus highlighting a role for the involvement for mechanisms other than inflammation. The results presented here indicate anti-HDL antibodies as the missing link that could explain these discrepancies. Actually, our results confirm that anti-HDL antibodies can explain low HDL-cholesterol levels even after adjustment for several potential confounders, including inflammation, demographic parameters, treatments and disease activity and duration, hence supporting its independent effect. Moreover, our findings are strengthened by the fact that anti-HDL antibodies could also explain total:HDL-cholesterol ratio, which is the preferred parameter for CV stratification in RA patients [25]. However, since we studied anti-HDL antibodies and some papers reported very modest changes on total:HDL-cholesterol ratio [3], we decided to primarily focus our results on HDL-cholesterol levels.

Our data show that anti-HDL antibodies were not associated with traditional CV risk factors in RA, and individuals with traditional CV risk did not exhibit increased anti-HDL levels compared to the control population. Similarly, increased anti-HDL levels in RA patients were not associated with increased total IgG levels. This leads us to hypothesize that anti-HDL antibodies may identify a subgroup of RA patients with specific clinical and immunological characteristics that

Table 3: RA patients classified according to levels of anti-HDL antibodies

	anti-HDL^{low} (n=172)	anti-HDL^{high} (n=40)	*p-value*
Age (years)	53.87 (16.22)	56.58 (22.69)	0.184
Gender (female:male)	144:28	28:12	0.053
Disease features			
Disease duration (years)	2.12 (4.81)	4.33 (8.00)	0.010
TJC	3.00 (8.00)	2.00 (7.00)	0.993
SJC	1.00 (5.00)	1.50 (4.00)	0.830
CRP	1.00 (2.70)	2.85 (6.43)	<0.001
ESR	18.00 (23.00)	21.00 (28.00)	0.193
DAS28 score	3.67 (2.45)	4.04 (1.84)	0.443
RF	88 (51.1)	28 (70.0)	0.095
Anti-CCP	92 (53.4)	23 (65.0)	0.325
Age at diagnosis	50.00 (17.00)	49.67 (20.00)	0.880
Shared epitope (n=141)	64 (57.1)	18 (62.0)	0.632
Erosions (n=129)	32 (34.4)	19 (55.8)	0.029
Blood lipids, *mean±SD*			
Total cholesterol (mg/dl)	206.00±36.20	211.05±34.44	0.419
HDL-cholesterol (mg/dl)	61.80±17.93	55.17±16.13	0.050
LDL-cholesterol (mg/dl)	121.75±33.61	124.70±27.92	0.513
Total:HDL-cholesterol ratio	3.63±1.18	4.23±1.92	0.060
Treatments, n(%)			
None or NSAIDs	41 (23.8)	6 (15.0)	0.207
GC	80 (46.5)	23 (57.5)	0.248
MTX	111 (64.5)	28 (70.0)	0.669
TNFα-blockers	36 (20.9)	12 (30.0)	0.248
Tocilizumab	7 (4.0)	5 (12.5)	0.173
Traditional CV risk factors, n(%)			
Hypertension	51 (29.6)	14 (35.0)	0.517
Dyslipidemia	36 (20.9)	16 (40.0)	0.013
Diabetes	16 (9.3)	6 (15.0)	0.285
Obesity (n=129)	22 (23.9)	7 (22.5)	0.880
Smoking	61 (37.6)	13 (32.5)	0.708
History of CV events, n(%)	22 (12.7)	16 (40.0)	<0.0001
Cytokines (n=129)	n=95	n=34	
TNFα (pg/ml)	216.14 (291.32)	312.04 (266.50)	0.451
IFNα (pg/ml)	0.00 (15.83)	16.06 (95.92)	0.006
MIP1α (pg/ml)	7.12 (19.74)	21.75 (76.47)	<0.001
IFNγ (pg/ml)	4.20 (3.44)	7.15 (8.78)	0.004
IL-8 (pg/ml)	41.78 (10.79)	46.85 (18.68)	0.006
VEGF (pg/ml)	112.43 (42.04)	117.29 (50.55)	0.313
GM-CSF (pg/ml)	26.76 (6.88)	29.75 (19.55)	0.033
IL-17A (pg/ml)	3.71 (25.74)	26.33 (101.43)	0.002
MCP-1 (pg/ml)	282.04 (325.38)	382.54 (794.84)	0.024
SDF-1α (ng/ml)	2.74 (6.69)	3.97 (7.35)	0.402
Resistin (ng/ml)	9.3 (5.27)	10.33 (4.90)	0.158
Leptin (ng/ml)	10.34 (11.31)	14.24 (15.57)	0.402

Continuous variables were summarized as median (interquartile range), unless otherwise stated. Differences were assessed by Mann Withney U or chi-square tests, as appropriated. Anti-CCP: anti-Cyclic Citrullinated Peptide antibody, CRP: C-reactive protein, CV: cardiovascular, GC: glucocorticoids, DAS28: Disease Activity Score (28 joints), ESR: Erythrocyte Sedimentation Rate, HAQ: Health Assessment Questionnaire, MTX: methotrexate, TSJ: tender joint count, SJC: swollen joint cont.

are more likely to suffer from a CV event. This is supported by the fact that these autoantibodies were present at disease onset. Recently, Wick and colleagues reported the presence of anti-Apo A1 antibodies in patients with periodontitis associated with atherosclerosis burden [26]. Interestingly, periodontal inflammation has been hypothesized to have an early role in RA development [27;28], and the lipid profile of RA patients has been reported to be altered even before RA diagnosis [23], hence suggesting that both anti-HDL and altered lipid profiles are early events in RA pathogenesis, at least in a subset of patients, presumably driven by an inflammatory environment.

The findings herein presented show that patients with high anti-HDL levels exhibited an enhanced systemic immune response, characterized by increased levels of a number of proinflammatory cytokines. Although we cannot know whether this inflammatory milieu is the cause or the consequence of anti-HDL production, we thought that previous inflammatory events could underlie anti-HDL production which, in turn, can amplify inflammatory responses, by several mechanisms. Actually, anti-inflammatory and antioxidant properties of HDL-cholesterol particles are impaired by anti-HDL antibodies [16]. This "HDL dysfunction" leads to higher levels of oxidized lipids (mainly oxLDL) [16;29] and increased levels of proinflammatory mediators.

HDL particles can inhibit the production of IL-1β and TNFα by interfering with the crosstalk between T cells and monocytes [30]. Similarly, HDL can decrease MCP-1 production by human macrophages [7] and some apolipoproteins are able to decrease the IL-8 production in neutrophils [31]. Moreover, auto-antibodies can directly promote monocyte and endothelial cells activation [32], and in particular, anti-Apo A1 antibodies have been reported to induce the production of IL-8 by human macrophages [33]. These findings regarding IL-8 are especially important, since this chemokine is the main chemoattractant for neutrophils, and neutrophil recruitment and activation are important steps in atherosclerotic plaque formation [34]. Additionally, neutrophils can enhance the oxidative burst, thus promoting oxLDL formation and also impeding the HDL protective effects by chlorination [35], thereby stimulating a positive feedback loop between inflammation, lipid oxidation and atherosclerosis. Finally, the fact that IgG auto-antibodies can activate MyD88 [36] as well as TLR intracellular pathways [37] could explain the increased IFNα levels in anti-HDL^{high} RA patients. Previous data from our group also highlight the relevance of the IL-8-neutrophil axis as emergent biomarkers of CV risk in RA patients [38]. Neutrophils have been associated with enhanced vascular injury in autoimmunity, type I IFNs having a crucial role in this scenario [39;40]. Additionally, type I IFNs have been reported to impair endothelial repair by several mechanisms (reviewed in [41]). Therefore, anti-HDL antibodies may identify a group of patients with an enhanced systemic proinflammatory response that could be linked to an increased rate of CV disease.

This study is the first to analyze anti-HDL antibodies in RA, but the results are in line with previous studies on anti-Apo A1 antibodies in RA [33;42]. The fact that we obtain a slightly higher amount of "positive" patients for these antibodies compared with those working with anti-Apo A1 is easily explained as Apo A1 is only one component of the HDL lipoproteins. Moreover, as HDL particles contains many other proteins [43] and Apo A1 is displaced by other components in situations of inflammation [8], we thought that focusing on total HDL particles would be a better approach in the clinical setting than relying on a unique target. In fact, correspondence between both anti-HDL and anti-Apo A1 positivity was only found in around 60% of SLE patients, thus underlining the relevance of other antigens on HDL particles [15]. However, which antibody approach provides the best predictive power remains unknown.

Another interesting finding from this work is the role of anti-HDL antibodies in the lipid outcome after TNFα-blockade in RA patients. This treatment has been associated with HDL-cholesterol increases in half of the studies evaluated in a recent review [3], thereby again suggesting a role for additional factors involved in the lipid profile maintenance in RA. To date, anti-TNFα agents have been hypothesized to change lipid levels by an indirect counteracting inflammation and the derepression of genes involved in lipid metabolism [44;45]. Our results point towards an independent effect on anti-HDL reduction, even after adjusting for changes in CRP as well as EULAR response, and may explain the modification on HDL function in RA patients undergoing TNFα-blockade [12]. However, our results did not exhibit statistical differences in lipid levels upon treatment, which is in line with the short period of follow up [3]. Longer-term studies on the effect of TNFα-blockade on anti-HDL antibodies and HDL function are warranted. Nevertheless, these results are a proof of concept that anti-HDL antibodies could have a role in the change in lipid profiles upon anti-TNFα therapy.

Our results are of special interest for the clinical setting, as anti-HDL determination may be a useful tool in the CV risk stratification in RA. Traditional CV risk factors fail to fully explain the CVD morbidity in RA [1], and some modifications have been proposed [25], but they also rely on lipid levels, so additional biomarkers are needed. Anti-HDL stratification could be an additional clinical target, as lipid lowering agents displayed beneficial effects on CV endpoints in RA patients [46] and on HDL function [47]. Actually, Anti-Apo A1 levels provided incremental prognostic information over the Framingham risk score for CVD development in RA [42].

Finally, this study has some limitations that should be stated. Prospective analyses on the TNFα-blockade was conducted on a small group and followed for a short period, which probably accounts for the lack of significant changes on lipid levels. In addition, the tCVD group was smaller when compared to both HC and RA, thus resulting in a considerable deviation of the data in this subset. Moreover, this group was composed of patients with different traditional CV risk factors but all were CVD-naïve. It is plausible to think that some of these patients, mainly those with higher anti-HDL levels, are about to develop a CV event in the short term, hence also explaining the great heterogeneity found in this group.

In summary, we report for the first time the potential role for anti-HDL antibodies in the altered lipid profile in RA patients, independently of the inflammatory burden. These antibodies are linked with an enhanced proinflammatory milieu, which can underlie the increased rate of CVD in these patients. Moreover, our results point to the role of anti-HDL antibodies in the lipid outcome upon TNFα-blockade after adjusting for the EULAR response. Collectively, our data point towards a clear role for these antibodies, and thus the humoral immune response, in the connection between lipid profile and CV disease in RA. These antibodies could be a promising tool for CV risk stratification and management in this condition.

CLINICAL PERSPECTIVES

- Despite exhibiting an increased cardiovascular risk, the associations between lipid profiles and cardiovascular disease in RA are poorly understood.
- RA patients exhibit increased levels of anti-HDL IgG antibodies, which are associated with altered lipid profile, enhanced proinflammatory mediators and increased cardiovascular rate, but not with traditional cardiovascular risk factors. Decreasing anti-HDL levels could mediate, at least in part, the beneficial effect of TNFα-blockade on serum lipids levels.
- Anti-HDL antibodies could have a pivotal role in the connection between lipid abnormalities and inflammation in RA. Therefore, anti-HDL antibodies may be considered as promising biomarkers of cardiovascular risk in RA patients, with potential use in the clinical setting for CV risk stratification and early treatment consideration.

AUTHOR CONTRIBUTION

Javier Rodríguez-Carrio performed most of the experimental procedures, carried out the statistical analyses and drafted the manuscript. Patricia López performed some experimental procedures. Mercedes Alperi-López, Francisco J. Ballina-García and Francisco Abal were in charge of patients' recruitment and clinical data collection. Ana Suárez conceived the study, designed the protocols and drafted and edited the manuscript. All authors read and approved the final version of the manuscript.

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Capítulo VI: Efecto de la terapia con agentes bloqueantes del TNFα

Como objetivo final de este proyecto, se valoró la realización de un estudio prospectivo en pacientes de AR antes y después de la instauración de un tratamiento, con el objetivo de estudiar los cambios que se producían en los biomarcadores anteriormente descritos.

Se eligió realizar este estudio en el contexto de la terapia con bloqueantes del TNFα debido a que ofrecía importantes ventajas para este campo de estudio: se trata de una terapia muy específica, su mecanismo de acción, y por tanto las vías de control de la inflamación por las que actúa, están notablemente caracterizadas, y se ha asociado en estudios epidemiológicos con menores tasas de enfermedad CV, así como con cierto grado de restauración de la función endotelial a corto plazo. A la vista de estas observaciones, y con los resultados obtenidos en los anteriores capítulos de esta Tesis Doctoral, decidimos estudiar cuál era el efecto del tratamiento con agentes bloqueantes del TNFα sobre los biomarcadores anteriormente descritos y evaluar si el cambio en éstos se asociaba a la respuesta clínica.

Este objetivo fue abordado en un grupo de pacientes de AR candidatos a tratamiento con TNFα que fue seguido prospectivamente durante 3 meses. En ellos se evaluaron las EPC, Tang, MP derivadas de células Tang, anticuerpos anti-HDL IgG (resultados recogidos en el artículo 8), así como algunas citocinas y factores de crecimiento solubles. Además, debido a que algunos autores sugerían la existencia de un fenómeno de inmunosenescencia en AR, que podía ser contrarrestado mediante el tratamiento con agentes anti-TNFα y cuyos mediadores a nivel celular parecen jugar un papel en la enfermedad CV, decidimos ampliar nuestro proyecto al estudio de la inmunosenescencia, y su posible contribución al riesgo CV, en pacientes de AR.

Artículo 9: Rodríguez-Carrio J, Alperi-López M, López P, Alonso-Castro S, Ballina-García FJ, Suárez A (2015); *TNFα polymorphism as marker of immunosenescence for Rheumatoid Arthritis;* Experimental Gerontology 61:123-129.

> Aportación personal al trabajo: en lo referente a este trabajo, realicé la mayor parte de la labor experimental, el análisis de los resultados, así como la discusión de los resultados con los coautores. Además, realicé la preparación del manuscrito y las figuras bajo la supervisión de la Dra. Ana Suárez Díaz.

Artículo 10: Rodríguez-Carrio J, Alperi-López M, López P, Ballina-García FJ, Suárez A (2015); *Good response to Tumour Necrosis Factor alpha blockade results in an Angiogenic T Cell recovery in Rheumatoid Arthritis patients;* Rheumatology (Oxford) (in press).

Aportación personal al trabajo: en este trabajo corrió a mi cargo la mayor parte de la labor experimental, así como el análisis e interpretación de los resultados. Finalmente, llevé a cabo la preparación del manuscrito y las figuras bajo la supervisión de la Dra. Ana Suárez Díaz.

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TNFα polymorphism as marker of immunosenescence for rheumatoid arthritis patients

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ABSTRACT

Background: Expansion of $CD4^+CD28^{null}$, a common feature of immunosenescence, which has been reported in rheumatoid arthritis (RA) patients, may also be associated with a $CD4^+$ imbalance. Although the increase of $CD4^+CD28^{null}$ cells has been related to TNFα exposure, nothing is known about the possible role of genetic variants of this cytokine.
Methods: Participants were genotyped for TNFA rs1800629 (−308 G > A) and frequency of the $CD4^+CD28^{null}$, regulatory T cells and Th1 cells subsets were quantified in peripheral blood samples by flow cytometry in 129 RA patients and 33 healthy controls.
Results: The expansion of $CD4^+CD28^{null}$ cells in RA patients was associated with TNFA genotype, even at diagnosis, and linked to markers of aggressive disease in patient carriers of the minor allele. Analysis of regulatory T cells and IFNγ-$CD4^+$ expression suggested that defective suppression and/or Th1-shift could underlie the expansion of this population in these patients. Finally, although treatment with TNFα-blockers reduced $CD4^+CD28^{null}$ cells in most patients, only those carriers of the common GG genotype reached values within the range of HC and showed a disease activity improvement correlated to this decrease.
Conclusions: Our results provide evidence for a genetic basis of the premature immunosenescence of RA patients and highlight its potential role in clinical outcome after TNFα blockade.

1. Introduction

Since life expectancy has increased in recent decades, research into the immunobiology of aging is of outstanding relevance. The progressive deterioration of the immune system, termed immunosenescence, leads the elderly to become more susceptible to some types of diseases such as cancer, infections, chronic inflammation or autoimmunity. Actually, some features of immunosenescence are also linked to the development of rheumatoid arthritis (RA) even in young or middle aged individuals (Lindstrom and Robinson, 2010).

Immunosenescence is associated with an impaired $CD4^+$ phenotype, with a decline in numbers of naïve $CD4^+$ cells and increased proportion of highly differentiated effector and memory $CD4^+$ cells, such as the $CD28^{null}$ population (Weyand et al., 2003). In fact, the expansion of $CD4^+CD28^{null}$ cells has been associated with immunosenescence in a variety of conditions, not exclusively elderly, and could be considered as a marker of immunosenescence itself. $CD4^+CD28^{null}$ cells exhibit a special immunophenotype characterized by the loss of CD28 costimulatory molecule and that of CD7, accompanied by the gain of expression of CD57 and killer immunoglobulin-like receptors (KIRs). Functionally, this population is composed by terminal differentiated cells that exhibit a cytotoxic and proinflammatory profile (Weyand et al., 2003; Namekawa et al., 1998) and are resistant to apoptosis (Schirmer et al., 1998).

RA is associated with a premature immunosenescence, as shown by lower numbers of T cell excision circles, perturbation of the T-cell repertoire and premature telomeres shortening (Thewissen et al., 2005; Wagner et al., 1998; Fujii et al., 2009), findings linked to a $CD4^+CD28^{null}$ expansion (Thewissen et al., 2007a). This premature aging of the immune system seems to be independent of disease duration and treatments (Koetz et al., 2000; Schmidt et al., 1996a; Schonland et al., 2003) and its origin remains unknown. Actually, $CD4^+$ repertoire imbalance is present in the early phases of the disease (Koetz et al., 2000), thus pointing to a potential role for genetic factors predisposing to immunosenescence in RA.

Taking this into consideration, it is interesting to note that $CD4^+$-$CD28^{null}$ cells have been related to the exposure to TNFα (Bryl et al., 2001), a strong proinflammatory cytokine playing a relevant role in RA. It is noteworthy that TNFα expression is highly determined by

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several genetic variants (Wilson et al., 1997), of which rs1800629 (−308 G > A) has been reported to be associated with high TNFα production in the case of the minor (A) allele. Moreover, the rs1800629 genotype has been associated with clinical features and treatment response in RA patients (Toonen et al., 2012). Interestingly, this polymorphism has been linked to longevity in healthy individuals (Cardelli et al., 2008). However, whether this genetic variant is associated with immunosenescence in RA is still unknown.

The main aim of this study is to determine the possible role of the TNFα rs1800629 genotype as a marker for premature immunosenescence in RA patients analyzing $CD28^{null}$ cells and $CD4^{+}$ imbalance. Additionally, we evaluate the effect of TNFα-blocking treatment on this population.

2. Material and methods

2.1. Patients and controls

Our study involved 129 RA patients, consecutively recruited from the outpatient clinic from the Rheumatology Department at the Hospital Universitario Central de Asturias (HUCA), fulfilling the 2010 American College of Rheumatology criteria. Routine clinical examination, including 28-joint disease activity score (DAS28) calculation as well as blood sample collection were performed during the patients' visit. Then, patients' clinical records were revised in order to register previous therapies and traditional CV risk factors. Global patient health was also assessed in a subset of patients (n = 54) using a SF-36 Questionnaire, and both physical and mental component scores were calculated (Ware et al., 1995). An additional subgroup of 13 RA patients (12 women, median age 43 (range: 30–65), DAS28 5.08(1.93), 38.5% RF+, 46.1% anti-CCP+), naïve to TNFα antagonists, was recruited and a blood sample was drawn immediately before and 3 months after anti-TNFα therapy.

Matched healthy volunteers (n = 33) without any pathology or treatment (25 women, median age 49 (range: 35–60)) were recruited from the Centro Comunitario de Sangre y Tejidos de Asturias at the same time as patients. Approval for the study was obtained from the Regional Ethics Committee for Clinical Investigation, according to the Declaration of Helsinki and all the participants gave written informed consent.

2.2. Flow cytometry analyses

The frequency of $CD4^{+}CD28^{null}$ and $CD4^{+}CD25^{high}FOXP3^{+}$ (regulatory T cells, Treg) cells in peripheral blood was analyzed by flow cytometry. For $CD4^{+}CD28^{null}$ population, peripheral blood was immunostained with anti-CD3 PerCP-Cy 5.5 (BD Biosciences, Germany), anti-CD4 CF-Blue (Immunostep, Salamanca) and anti-CD28 APC-Cy7 (BD) or isotype controls (all from BD) for 30 min at 4 °C. Then, 2 ml of FACS Lysing Solution (BD) was

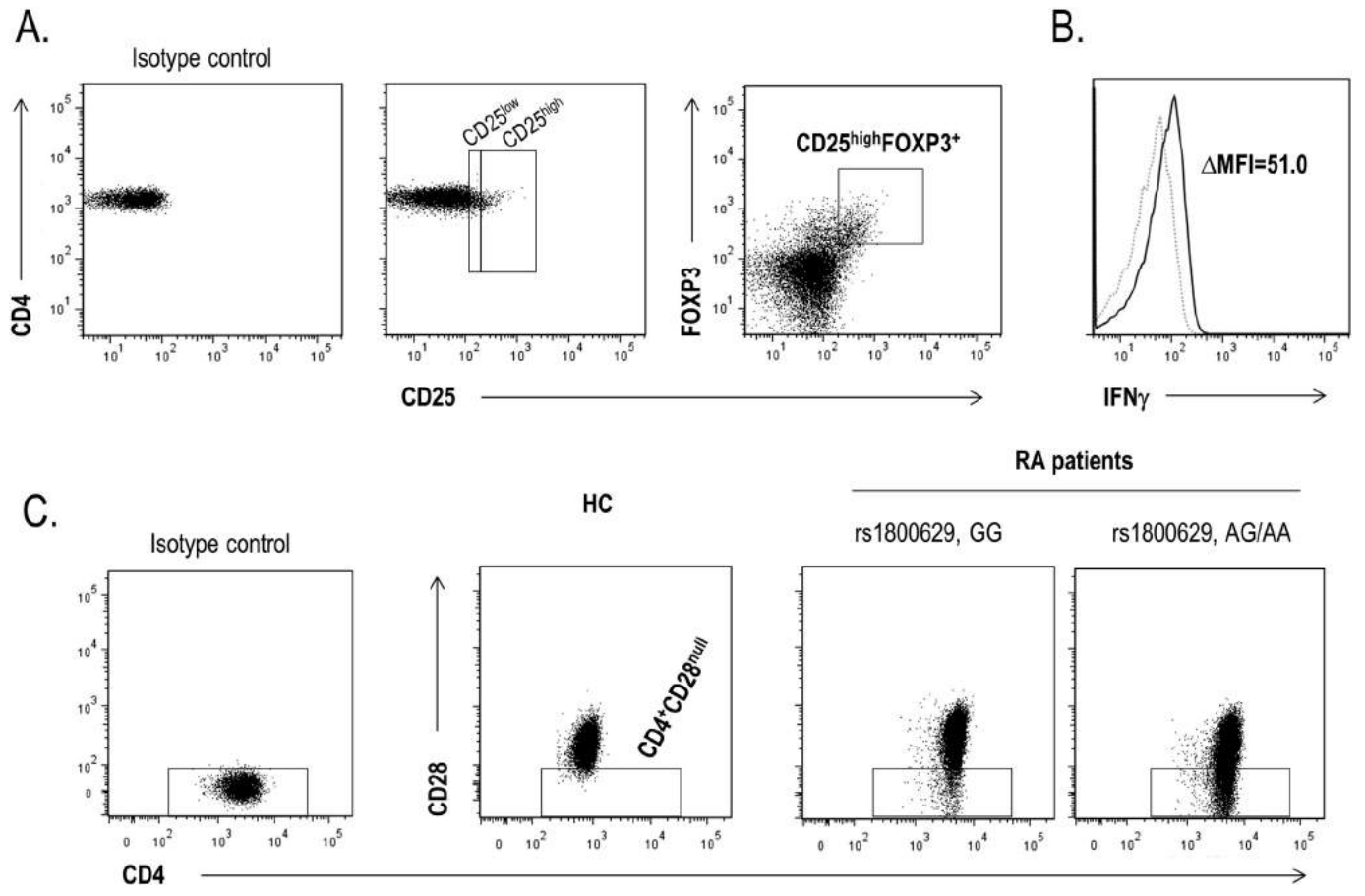

Fig. 1. FACS analyses of the studied populations. Representative dot-plots of the analyses performed to analyze the different T cell subsets. (A) Treg cells: $CD4^{+}$ gated lymphocytes were evaluated for their CD25 expression and those $CD25^{high}$ (brightest CD25 expression (de et al., 2012)) and $FOXP3^{+}$ were considered as Treg. (B) $CD4^{+}$ IFNγ expression: intracellular IFNγ levels in $CD4^{+}$ T cells (black solid line) was determined and that of isotype control was subtracted (gray dotted line) to calculate MFI. (C) $CD4^{+}CD28^{null}$ population: gated $CD4^{+}$ lymphocytes were evaluated for their expression of CD28, being those below the signal provided by the isotype control considered as $CD4^{+}CD28^{null}$ cells. A representative dot-plot from a healthy control (HC) and two RA patients with different rs1800629 genotype are shown.

added to each tube and left for 5 min at 4 °C. Finally, cells were centrifuged (1600 rpm, 5 min, room temperature) and washed twice with sterile PBS. Tubes were analyzed in a FACS Canto II flow cytometer and 30,000 events within the $CD3^+$ gate were acquired. The frequency of $CD4^+CD28^{null}$ cells was calculated as the percentage of CD28-negative cells in the gated $CD3^+CD4^+$ population and expressed as the percentage within the total lymphocyte gate.

For the Treg subset, peripheral blood was extracellularly stained with anti-CD4 APC-Cy7 (BD), CD25 FITC (BD) for 30 min at 4 °C. Then, cells were lysed, fixed and permeabilized (FOXP3 transcription factor staining kit, eBioscience, California) and intracellularly stained with anti-FOXP3 PE and anti-IFNγ PerCP-Cy 5.5 (both from eBiosciences) or isotype controls (eBioscience). Samples were analyzed and 50,000 $CD4^+$ lymphocytes were acquired. Isotype controls were used to set up the CD25-negative population and $CD25^{high}$ population selected according to the CD25 brighter expression (de et al., 2012). This population was analyzed for the expression of $FOXP3^+$ according to the signal provided by the isotype control. $CD4^+CD25^{high}FOXP3^+$ cells were considered as regulatory T cells (Treg) and were quantified as the percentage within the total lymphocyte gate.

Additionally, $CD4^+$ cells were analyzed for the intensity of IFNγ intracellular staining (measured as mean fluorescence intensity, MFI) (Thewissen et al., 2007a).

2.3. TNFA rs1800629 polymorphism genotyping

DNA from 121 patients (93.7%) and all the controls was obtained from whole peripheral blood using standard methods.

Subjects were genotyped to determine TNFA rs1800629 status by analyzing the Tm of the probe/target duplex after PCR amplification and hybridization with fluorescent-labeled probes matched with one sequence variant (LighCycler; Roche Diagnostics, Germany), as previously stated (Lopez et al., 2006). The primers used were 5′-CCT GCA TCC TGT CTG GAA GTT A and 5′-CTG CAC CTT CTG TCT CGG TTT. The hybridization probes (TIB MOLBIOL, Germany) were AAC CCC GTC CCC ATG CCC C-F and LC Red 640-CCA AAC CTA TTG CCT CCA TTT CTT TTG GGG AC. Samples were analyzed in batches with negative and positive controls.

2.4. Cytokine serum levels

Serum aliquots were stored at −80 °C until cytokine measurement. TNFα serum levels were quantified using a Mini EDK kit (Peprotech) following the manufacturer's instructions (detection limit: 3.9 pg/ml), whereas IFNγ serum levels were assessed by an OptEIA kit (BD) (detection limit: 0.58 pg/ml).

2.5. Statistical analysis

Data are expressed as median (interquartile range) for continuous variables, whereas n(%) was used in categorical ones. Clinical and laboratory variables were compared using Mann–Whitney U test, two way ANOVA or χ^2 square test, as appropriate. Correlations were assessed by a Spearman's rank or Pearson tests as appropriate. For the prospective analyses, paired T test was used. Additionally, multivariate regression analyses were performed to ascertain the effect of $CD4^+CD28^{null}$ population on DAS28 score improvement. Since comparisons were

Table 1
Clinical characteristics of the whole group of RA patients and grouped according to rs1800629 polymorphism.

	RA patients (n = 129)	rs1800629 genotype		
		GG (n = 91)	AG/AA (n = 30)	p-Value
Gender (female:male)	103:26	74:17	23:7	0.579
Age at sampling, years (median (range))	54 (22–82)	55 (22–79)	53 (18–82)	0.904
Disease features				
Disease duration, years	4.50 (7.15)	5.16 (7.25)	3.83 (8.92)	0.457
Age at diagnosis, years (median (range))	47 (18–80)	49 (18–80)	47 (22–75)	0.831
Disease activity (DAS28)	3.65 (2.08)	3.68 (2.08)	3.52 (2.61)	0.820
Tender joint count	2.00 (6.75)	2.00 (6.00)	1.00 (7.00)	0.639
Swollen joint count	1.00 (4.00)	1.00 (4.00)	1.00 (6.00)	0.098
Patient global assessment (0–100)	40.00 (45.00)	41.00 (47.00)	32.50 (52.50)	0.776
ESR, mm/h	16.00 (22.75)	18.00 (23.00)	10.00 (19.50)	0.038
Patient pain assessment (0–10)	4.00 (4.00)	5.00 (4.00)	4.00 (3.00)	0.718
HAQ (0–3)	0.87 (1.13)	1.00 (1.20)	0.93 (1.14)	0.189
SF-36 physical component (0–100)	51.00 (33.5)	51.00 (30.00)	52.00 (41.50)	0.600
SF-36 mental component (0–100)	72.00 (36.50)	73.00 (29.00)	72.00 (52.50)	0.970
RF (+), n(%)	78 (60.4)	56 (61.5)	17 (56.6)	0.864
αCCP (+), n(%)	78 (60.4)	56 (61.5)	17 (56.6)	0.864
ANA (+), n(%)	61 (47.2)	43 (47.2)	14 (46.6)	0.782
Shared epitope, n(%)	57 (44.1)	45 (49.4)	11 (36.6)	0.201
Erosive disease, n(%)	50 (38.7)	40 (43.9)	7 (23.3)	0.129
CV risk factors, n(%)				
Dyslipidemia	45 (34.8)	33 (36.2)	9 (30.0)	0.483
Hypertension	42 (32.5)	28 (30.7)	11 (36.6)	0.574
Diabetes (type II)	12 (9.3)	6 (6.8)	6 (16.6)	0.100
Obesity (BMI > 30)	29 (22.4)	20 (21.9)	7 (23.3)	0.899
Smoking habit	43 (33.3)	31 (34.0)	9 (30.0)	0.655
Previous CV events	19 (14.7)	12 (13.1)	5 (16.6)	0.496
Treatments, n(%)				
None or NSAIDs	19 (14.7)	11 (12.0)	6 (20.0)	0.279
Glucocorticoids	72 (55.8)	49 (53.8)	20 (66.6)	0.265
Methotrexate	90 (69.7)	64 (70.3)	20 (66.6)	0.586
TNFα blockers	45 (34.8)	38 (41.5)	5 (16.6)	0.010
Tocilizumab	12 (9.3)	8 (8.7)	3 (10.0)	0.869
Statins	24 (18.6)	18 (19.7)	4 (13.3)	0.387

Categorical variables are expressed as n(%) whereas median (interquartile range) was used for continuous ones, unless otherwise stated. RA patients were compared by rs1800629 polymorphism using the Mann–Whitney U or χ^2 square test, as appropriate. p-Values are indicated in the right column.

performed between groups with different sample sizes (n), Hedge's g statistic was calculated for each reported significant outcome in our study (Nakagawa and Cuthill, 2007), with values of g > 0.8 considered of a large effect. Power analysis was performed according as previously described (Dupont and Plummer, 1998).

SPSS 18.0 and GraphPad Prism 5.00 packages were used for statistical analysis. A p-value < 0.050 was considered statistically significant.

3. Results

3.1. Association between TNFα rs1800629 genotype and CD4$^+$ subpopulations in RA patients

RA patients and HC were classified into two groups (GG and AG/AA) according to the TNFα rs1800629 genotype. To evaluate immunosenescence and CD4$^+$ imbalance, the frequency of CD28^{null} and Treg (CD25^{high}FOXP3$^+$) cells as well as intracellular IFNγ expression in CD4$^+$ lymphocytes were analyzed (Fig. 1). No differences in disease features were observed between groups, although treatment with anti-TNFα blockers was less frequent in AG/AA patients (Table 1).

The entire RA group exhibited a noticeable increase in CD4$^+$CD28^{null} cells compared to HC ($p < 0.0001$, Hedge's $g = 1.08$). No differences between genotypes were observed in HC. However, patient carriers of the minor allele (AG/AA genotypes) exhibited higher levels of this population than GG-homozygous ($p < 0.0001$, Hedge's $g = 2.35$) (Fig. 2A). In order to exclude a potential confounding effect due to differential distribution of TNFα blockers between genotypes, a two way ANOVA was performed, thereby confirming that both rs1800629 genotype and TNFα blockers usage had an effect on CD4$^+$CD28^{null} subset (both $p < 0.050$), whereas the interaction between factors did not achieve statistical significance ($p = 0.590$). This result ruled out the possibility of any interaction between these variables, thus allowing us to study both factors independently. Moreover, AG/AA patients at diagnosis presented a higher frequency of CD4$^+$CD28^{null} cells compared to their established-disease counterparts, whereas no differences were found in patients with the GG genotype (Fig. 2B). Also, CD4$^+$CD28^{null} subset correlated with several parameters of disease severity and disability in the AG/AA group (Table 2). No associations were found related to age or sex. These results support premature immunosenescence in RA patients that was associated with TNFα genotype and linked with clinical markers of aggressive disease.

Additionally, both TNFα and IFNγ serum levels were increased in RA patients compared to HC (249.36(278.91) vs 42.54(113.20) pg/ml, $p < 0.001$; and 4.48(5.05) vs 3.00(3.12) pg/ml, $p = 0.002$, respectively). TNFα serum levels were associated with CD4$^+$CD28^{null} frequency in the whole group ($r = 0.246$, $p = 0.002$), although no associations were found when patients and HC were analyzed independently. No associations were found with IFNγ serum levels.

Table 2
Associations between CD4$^+$CD28^{null} cells and clinical features in RA patients according to rs1800629 genotype.

	GG (n = 91)	AG/AA (n = 30)
Tender joints count	r = 0.045 p = 0.683	r = 0.399 p = 0.029
Swollen joints count	r = −0.120 p = 0.276	r = 0.442 p = 0.014
Patient pain assessment	r = −0.027 p = 0.805	r = 0.490 p = 0.006
Health assessment questionnaire	r = −0.158 p = 0.150	r = 0.469 p = 0.009
Global patient assessment	r = 0.110 p = 0.312	r = 0.456 p = 0.011
DAS28 score	r = −0.043 p = 0.694	r = 0.510 p = 0.004
Physical component (SF-36)	r = 0.192 p = 0.229	r = −0.771 p = 0.002
Mental component (SF-36)	r = 0.259 p = 0.102	r = −0.732 p = 0.004

Correlations were analyzed using the Spearman's rank test.

On the other hand, Treg frequency in patients did not differ between genotypes (GG: 0.360(0.383)% vs AG/AA: 0.297(0.252)%, $p = 0.103$), and was similar to HC (0.338(0.319)%, $p = 0.906$). However, this population was negatively correlated with CD4$^+$CD28^{null} cells in HC and also in patients with the GG genotype, but not in carriers of the A allele (Table 3). Additionally, IFNγ expression in CD4$^+$ cells was positively correlated with CD4$^+$CD28^{null} cells in AG/AA patients, but not in HC or GG patients. Again, no differences in IFNγ expression were detected between genotypes (GG: 50.00(28.00) vs AG/GG: 52.50(55.00), $p = 0.370$), but it was increased in patients compared with HC (49.50(37.00) vs 29.00(17.00), $p < 0.0001$). Therefore, our results suggest that Th1 shift and compromised Treg subset could underlie CD4^{+-}CD28^{null} expansion in RA patient carriers of the high TNFα-producing genotype, thus linking immunosenescence with CD4$^+$ imbalance in RA patients.

3.2. Effect of TNFα blockade on CD4$^+$CD28^{null} population

In view of these results, we hypothesized that the CD4$^+$CD28^{null} population could be affected by anti-TNFα therapy in these patients. The analysis of the whole RA group revealed a significantly lower frequency of CD4$^+$CD28^{null} cells in patients undergoing this therapy (Fig. 3A). However, when genotypes were taken into account, this effect was clear in GG-homozygous ($p = 0.003$) but not in carriers of the AG/AA genotype ($p = 0.414$), thereby suggesting a role for TNF-related

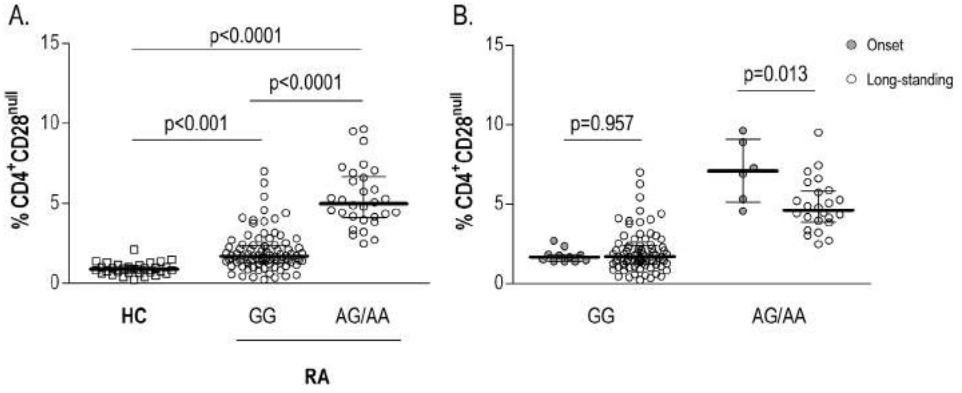

Fig. 2. Analysis of CD4$^+$CD28^{null} cell frequency among HC and RA patients. (A) Frequency of CD4$^+$CD28^{null} cells in HC (n = 33) and RA patients (n = 129). RA patients were grouped according to their rs1800629 genotype (GG: n = 91; AG/AA: n = 30). (C) CD4$^+$CD28^{null} population in rs1800629 AG/AA RA patients is increased at diagnosis (n = 6) compared to established disease (n = 24), but not in those carrying GG genotype (n = 11 and n = 80, respectively) (clear circles, long-standing RA patients; gray circles, RA patients at diagnosis). Differences were assessed using the Mann–Whitney U test.

Table 3
Associations between $CD4^+CD28^{null}$ cells and other $CD4^+$ subsets.

	HC (n = 33)	RA (n = 129)	rs1800629 genotype	
			GG (n = 91)	AG/AA (n = 30)
$CD4^+CD25^{high}FOXP3^+$ (%)	r = −0.446	r = −0.241	r = −0.244	r = −0.204
	p = 0.009	p = 0.007	p = 0.024	p = 0.280
IFNγ (MFI, $CD4^+$)	r = −0.259	r = 0.241	r = 0.177	r = 0.561
	p = 0.146	p = 0.008	p = 0.104	p = 0.001

Correlations were analyzed using the Spearman's rank test.

immunosenescence in the immunological and clinical response to TNFα blockade in RA patients.

Given the low number of patient carriers of the AA/AG genotype included in the group, to confirm these results and to assess whether these differences in clinical response could underlie these findings, we recruited a new group of 13 RA patients, naïve to TNFα blockers, to perform a prospective study analyzing the $CD4^+CD28^{null}$ population before and after 3-months of anti-TNFα therapy.

As expected, after anti-TNFα therapy, $CD4^+CD28^{null}$ frequency showed a notable decrease in most individuals (Fig. 3B). In fact, analysis by genotypes showed significant differences in both groups (GG: p = 0.017; AA/AG: p = 0.043), although of the GG-homozygous patients most (7/8) reached values within the range of HC (mean ± 2SD), whereas this effect was not seen in the AG/AA group (0/5). Moreover, we detected an interesting association with the clinical response to treatment. DAS28 improvement after therapy was inversely correlated with the change in $CD4^+CD28^{null}$ cells in patient carriers of the GG genotype but not in those AG/AA (Fig. 3C). This finding was studied by linear regression analysis adjusted by $CD4^+CD28^{null}$ frequency pre-treatment, as notable differences exist between genotypes. This analysis was performed choosing the change in DAS28 score as the dependent variable, and it revealed that $CD4^+CD28^{null}$ decrease predicts DAS28 improvement in GG-homozygous (β[95% CI] −2.324[−3.449, −1.200], p = 0.003) but not in the AG/AA group. Statistical analysis revealed a power of 0.977 for the multivariate regression of GG group, whereas a value of 0.320 was obtained for the AG/AA one.

These results indicate that anti-TNFα therapy may ameliorate the immunosenescence associated with RA disease in patients harboring the GG genotype and suggest that the reduction of $CD4^+CD28^{null}$ cells may be a new mechanism that could partly explain the better response of TNF rs1800629 GG patients to TNFα blockers, thus suggesting an association between immunosenescence and clinical outcome.

4. Discussion

The findings presented here support a genetic basis for immunosenescence in RA and a connection between clinical parameters and the immunosenescent profile of patients. Additionally, we

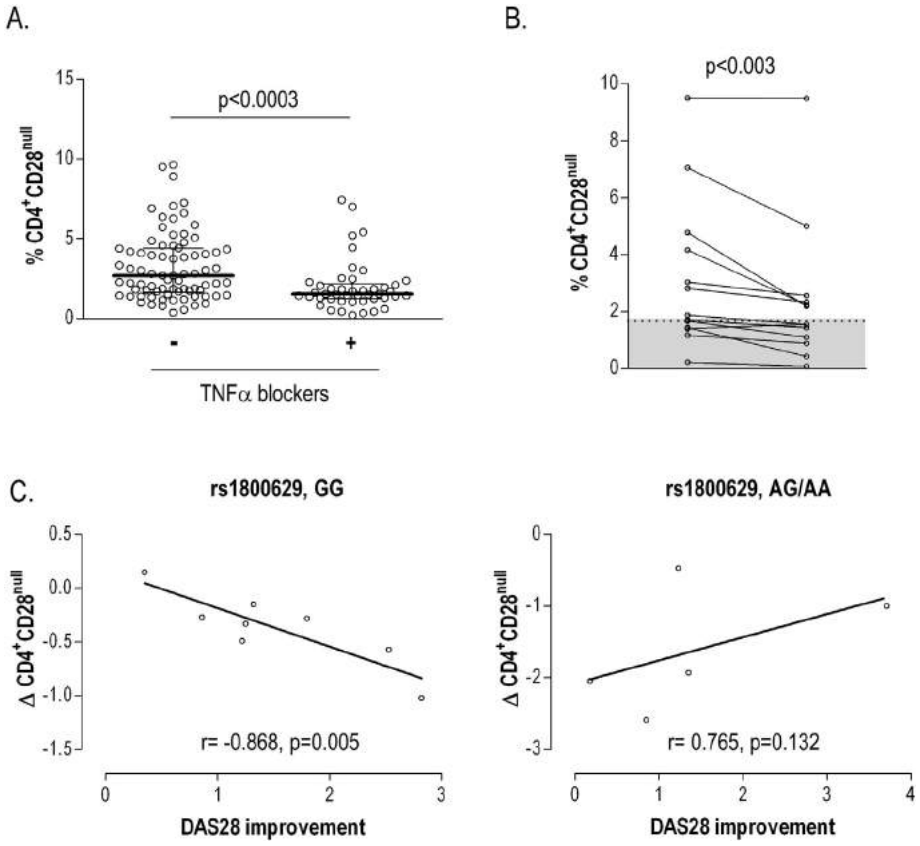

Fig. 3. Effect of anti-TNFα therapy on $CD4^+CD28^{null}$ population. (A) Frequency of $CD4^+CD28^{null}$ population depending on TNFα blockers usage (no TNFα blockers: n = 84; TNFα blockers: n = 45). (B) Changes in $CD4^+CD28^{null}$ population before and after three months of anti-TNFα therapy (n = 13). gray-shaded area represents the area covered by mean ± 2SD of $CD4^{+-}CD28^{null}$ frequency in HC. (C) Association between the change in $CD4^+CD28^{null}$ population after anti-TNFα treatment and the improvement in DAS28 score during the same period depending on rs1800629 genotype (GG: n = 8; AG/AA: n = 5). Mann–Whitney U test, paired T test and Pearson correlation test were used as appropriate.

conclude that these factors may have a role in the clinical outcome to anti-TNFα.

During the process of immunosenescence, $CD4^+CD28^{null}$ cells develop survival strategies and effector functions that are relevant for the disease manifestations of RA. Actually, most $CD4^+CD28^{null}$ cells are autoreactive cells in RA patients (Schmidt et al., 1996b), their cytotoxic features are relevant to the pathogenesis (Namekawa et al., 1998; Griffiths et al., 1992) and the expansion of this population is associated with aggressive disease (Pawlik et al., 2003). However, little is known about the association between clinical markers and $CD4^+CD28^{null}$.

Our results provide some evidence that immunosenescence is associated with clinical features depending on genetic variants. Therefore, the association between the $CD4^+CD28^{null}$ subset and TNFα polymorphism demonstrated in this work could be of interest in the daily clinical routine in order to identify and stratify RA patients by risk, thus improving clinical management in this heterogeneous condition. Currently, few predictive markers are available (anti-CCP positivity, shared epitope) and immunosenescence as a predictive marker is not taken into account. However, Goronzy and colleagues reported that $CD4^+CD28^{null}$ frequency, and other markers of immunosenescence, can be considered as a predictive factor for erosive course in RA (Goronzy et al., 2004). Furthermore, markers of immunosenescence may also be of interest in the management of individuals at risk of developing RA, in order to establish earlier therapies. In fact, diagnosis of early/pre-clinical RA is currently a matter of outstanding interest, because of its relevance to achieving disease remission and avoiding long-term disability (van Nies et al., 2010).

On the other hand, since chronic exposure to TNFα could also underlie increased $CD4^+CD28^{null}$ frequency (Bryl et al., 2001), and therefore, contribute to immunosenescence in RA, we wondered whether TNFα blockade could reverse or ameliorate this process. In fact, the reversion of T-cell senescence (Goldberg et al., 2007) and "rejuvenation" of the immune system (Warrington et al., 2003; Weyand et al., 2009) have been proposed as therapeutic approaches. Our findings revealed that the effect of anti-TNFα therapy on $CD4^+CD28^{null}$ depends again on the rs1800629 polymorphism. It seems that immunosenescent status in carriers of the GG genotype is lower than that of their AG/AA-counterparts. This could explain why the former were almost able to reduce this population to within the range of healthy individuals, opposite to the situation in the latter, even after adjusting for the pre-treatment $CD4^+CD28^{null}$ frequency. Additionally, $CD4^+CD28^{null}$ decrease in patients carrying GG genotype was associated with DAS28 improvement, although no effect was observed in patients carrying AG/AA variant. These findings highlight the relevance of immunosenescence in the clinical outcome of patients undergoing this therapy. Moreover, these results could explain, at least in part, the better clinical response reported in patients with this genotype (Maxwell et al., 2008; O'Rielly et al., 2009). Interestingly, because the $CD4^+CD28^{null}$ reduction paralleled the DAS28 score decrease; it could be thought that this effect was due to clinical improvement. However, we think this possibility can be ruled out, since DAS28 was only related to the size of $CD28^{null}$ population in AA/AG patients. Therefore, we think this result was a specific effect of the TNFα blockade on this subset, rather than an epiphenomenon of clinical outcome.

On the other hand, since a previous study suggested that $CD4^+CD28^{null}$ cells were resistant to in vitro immunoregulation by Treg subset (Thewissen et al., 2007b), we analyzed for the first time, the association between $CD4^+CD28^{null}$ and Treg subsets in RA patients. Our findings point to a compromise of Treg subset that could explain the lower degree of correlation between Treg subset and $CD4^+CD28^{null}$ cells in patients compared to HC. Interestingly, this is especially clear in patients carrying the AG/AA genotype. However, whether Treg impairment in patients could lead to $CD4^+CD28^{null}$ expansion or if immunosenescence may have a role in Treg compromise in patients cannot be determined from our study.

In spite of the relevance of immunosenescence in RA, it is not commonly used in prognostic or predictive studies. Instead, "calendar" age of the patients is considered as a marker of aging. However, although some studies propose a role for age as a predictive factor in clinical response (Atzeni et al., 2014), others did not find such an association (Hyrich et al., 2006). A plausible explanation for this controversy is that age in years of RA patients is not a good marker, since premature aging of RA patients has been reported and seems to be different among patients. As a result, "real" or "biological" age of RA patients, that is, immunosenescence, should be taken into account. The concept that a gap exists between "calendar" and "biological" age is supported by the fact that whereas $CD4^+CD28^{null}$ frequency associated with age in healthy individuals, that is not the case for autoimmune patients, as we also observed in our patients. In addition, $CD4^+CD28^{null}$ cells in RA patients seem to be unrelated to disease duration (Martens et al., 1997), reinforcing the idea that immunosenescence is not simply associated to lengths of time, but underlying specific mechanisms are involved.

Finally, $CD4^+CD28^{null}$ could have a role in the main comorbidity in RA, that is, cardiovascular disease (CVD) (del Rincon et al., 2001). The $CD4^+CD28^{null}$ population has been reported to be increased in patients with cardiovascular conditions (Liuzzo et al., 1999), thus suggesting a role for premature immunosenescence in these patients. Although no associations were found in our study with CVD or CV risk in RA patients, it is interesting to note that clinical features associated with increased $CD4^+CD28^{null}$ frequency have been previously related to CVD susceptibility in RA (Rodriguez-Rodriguez et al., 2011; Turesson et al., 2007). Moreover, CVD in RA is the result of the interaction between genetic factors and immune dysregulation (Gonzalez-Gay et al., 2007). Interestingly, the same situation seems to explain $CD4^+CD28^{null}$ numbers, according to our findings.

The main limitation of our study was the low number of patients with the genotype AG/AA in the prospective study, which did not lead us to properly evaluate the effect of the change in $CD4^+CD28^{null}$ numbers on disease activity improvement.

In conclusion, we have identified a genetic basis to account for $CD4^+CD28^{null}$ expansion and premature immunosenescence in RA patients, the associations with clinical parameters and the role in clinical outcome after TNFα blockade in these patients. Our results suggest a role for immunosenescence as a marker for RA, thereby highlighting its potential use in the clinical management of RA.

Conflict of interest

The authors declared no conflict of interest.

Acknowledgments

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Good response to tumour necrosis factor alpha blockade results in an angiogenic T cell recovery in rheumatoid arthritis patients

SIR, The increased cardiovascular (CV) morbidity of RA is associated with disease activity and the underlying inflammatory burden [1], although the actual mechanisms by which they play a role in CV susceptibility remain unclear. It has been proposed that angiogenic T cells (Tang), in cooperation with endothelial progenitor cells (EPCs), play a role in vascular repair. However, both populations are decreased in the circulation of RA patients, Tang being strongly related to disease activity [2]. Actually, disease activity may have a role in microparticle shedding from Tang, thereby suggesting a detrimental role for disease activity in this subset [3] that could potentially account for the decreased Tang levels in RA. Therefore, we aimed to analyse the effect of clinical response to TNFα-blockade on circulating Tang, EPCs and Tang-derived microparticles (Tang-MPs).

A total of 13 RA patients naive to TNFα blockers were longitudinally recruited [age: 48 (range 32–65) years, 1 man and 12 women, disease duration 1.50 (range 1.00–7.17) years, 5 RF^+, 6 anti-CCP^+, 3 active smokers], and a blood sample was taken immediately before as well as 3 months after anti-TNFα therapy (11 golimumab and 2 etanercept). All patients were on concomitant MTX, and 10 (76.9%) were also on low-dose glucocorticoid therapy. Clinical and immunological parameters and fasting lipid analysis were assessed at each patient visit. Clinical response was determined following the EULAR criteria [4]. EPCs ($CD34^+VEGFR2^+CD133^+$), Tang ($CD3^+CD31^+CXCR4^+$) and Tang-MPs were analysed by flow cytometry, as previously described [2, 3]. Serum TNFα, VEGF, IL-8, leptin and stromal cell-derived factor 1 (SDF-1α) levels were measured by immunoassays. A total of 33 healthy individuals [age: 49 (range 35–59) years, 11 men and 22 women] were used as healthy controls (HCs) (supplementary Table S1, available at *Rheumatology* Online). Differences were assessed by paired tests, and Hedges' *g* statistic was calculated to measure size effect ($g > 0.8$ was considered as a large effect) [5]. Data were expressed as mean (interquartile range) or median (S.D.) as appropriate. Our study had a calculated power of 0.95 with $\alpha = 0.05$. Approval from the local ethics committee (Comité de Ética de Investigación Clínica del Principado de Asturias) was obtained. Written informed consent was obtained from all participants.

Both Tang [4.03 (5.22) *vs* 7.57 (8.85) $\times 10^3$ cells/μl, $P = 0.002$; $g = 0.82$] and EPC [0.007 (0.021) *vs* 0.034 (0.024) $\times 10^3$ cells/μl, $P = 0.005$; $g = 1.72$] populations increased after anti-TNFα therapy. However, whereas Tang reached levels similar to those found in HCs [10.37 (7.14), $P = 0.522$], EPCs remained below normal levels [0.052 (0.039), $P = 0.030$]. The DAS28 score was significantly decreased after 3 months [4.05 (1.98) *vs* 5.15 (1.74), $P < 0.001$]. Moreover, the DAS28 score improvement paralleled Tang increase after treatment ($r = 0.676$, $P = 0.011$), but not that of EPC ($r = 0.225$, $P = 0.449$). Multivariate analysis adjusted by MTX and glucocorticoid dosages and smoking habit revealed that DAS28 improvement was the only predictor of Tang increase [$\beta = 0.906$ (95% CI 1.811, 0.001); $P = 0.050$], thereby highlighting the dependence of Tang on disease activity, and excluding the potential effects of concomitant DMARDs.

Additionally, Tang frequency after treatment was related to traditional CV risk factors (total-cholesterol/HDL ratio: $r = -0.629$, $P = 0.028$), as has been reported in HCs [3], but not at baseline ($P = 0.236$). Similar findings were observed with EPCs (baseline: $r = 0.484$, $P = 0.131$; after therapy: $r = -0.741$, $P = 0.006$), suggesting that disease activity control is a crucial step that should be targeted as aggressively as possible as part of the RA clinical routine.

Further analysis of patients according to clinical response, showed a greater Tang increase after treatment in good responders ($n = 5$) compared with moderate/non-responders ($n = 8$) [5.83 (5.32) *vs* 2.50 (2.42) $\times 10^3$ cells/μl, $P = 0.030$; $g = 0.96$]. In fact, whereas good responders reached Tang values similar to those of HCs [11.47 (11.98) *vs* 10.37 (7.14) $\times 10^3$ cells/μl, $P = 0.598$], a trend to a lower frequency was detected in moderate/non-responders [6.24 (7.55), $P = 0.115$] (Fig. 1). Moreover, only good responders displayed a parallel increase in Tang and EPCs (correlation between the change in both populations: $r = 0.900$, $P = 0.037$), suggesting a more favourable pattern of vascular repair in these patients through cooperation between the two involved populations. Analysis of serum biomarkers revealed that TNFα blockade was associated with decreasing VEGF [122.84 (41.87) *vs* 105.76 (32.60) pg/ml, $P = 0.002$] leptin [16.07 (10.94) *vs* 13.58 (9.45) ng/ml, $P = 0.014$] and SDF-1α levels [5.09 (5.33) *vs* 3.49 (4.67) ng/ml, $P = 0.002$] in the whole group, whereas IL-8 and TNFα were only reduced in good responders [TNFα: 451.05 (265.98) *vs* 150.14 (153.47) pg/ml, $P = 0.045$; IL-8: 43.41 (6.92) *vs* 33.84 (3.78) pg/ml, $P = 0.040$]. Interestingly, the decrease in TNFα levels paralleled EPC recovery ($r = 0.900$, $P = 0.036$), whereas that of leptin paralleled a decrease in EPCs ($r = 0.850$, $P = 0.050$) and Tang ($r = 0.850$, $P = 0.050$), thus suggesting that tighter control of inflammatory and vascular-related mediators is associated with better Tang and EPC recovery.

Finally, Tang-MP shedding was decreased after TNFα blockade in all patients [3929.91 (9374.93) *vs* 7859.83 (9798.62) MP/ml, $P = 0.021$; $g = 0.60$], but to a greater

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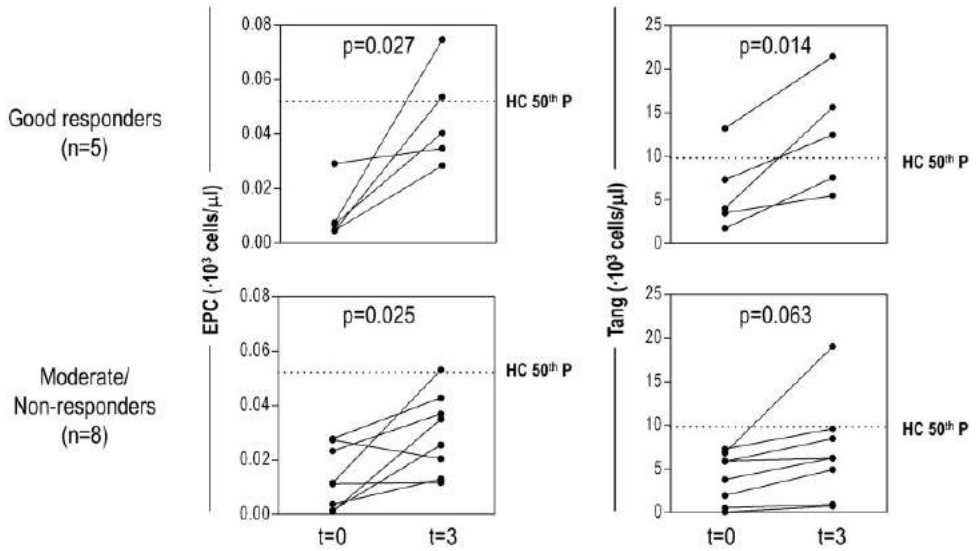
FIG. 1 Tang and EPC recovery after anti-TNFα therapy



Patients were grouped by their clinical response according to EULAR criteria, and graphs indicate frequency before ($t = 0$) and 3 months after ($t = 3$) anti-TNFα therapy. Each dot represents one patient. Dotted line represents the HC median value (50th percentile). Differences were assessed by paired *t*-test. Tang: angiogenic T cells; EPCs: endothelial progenitor cells; HCs: healthy controls.

extent in good responders [280.70 (1038.62) MP/ml, $P = 0.006$; $g = 2.36$), in line with the greater Tang-recovery in this group.

In summary, our results confirm that disease activity control is responsible for the Tang recovery in RA patients undergoing TNFα blockade therapy. Additionally, lower Tang-MP shedding supports this hypothesis. This mechanism could underlie the clinical benefit of TNFα blockers on CV endpoints, which is dependent on clinical response [6, 7] and therefore on disease activity, a parameter closely related to the maintenance of this population [2]. Although total EPC recovery after anti-TNFα therapy has been reported [8], technical and clinical differences between the two studies should be noted. However, larger studies are needed to confirm these findings.

Rheumatology key message

- Good response to TNFα-blockers leads to an angiogenic T-cell recovery in RA.

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Supplementary data

Supplementary data are available at *Rheumatology* Online.

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Discusión

4. Discusión

Consideraciones iniciales

Durante el desarrollo del proyecto de investigación que compone la presente Tesis Doctoral, se ha abordado el estudio de diferentes mediadores de diversa naturaleza y origen que pueden tener un papel determinante en el daño endotelial y el riesgo CV en el contexto de la AR. Además, se ha profundizado en los mecanismos que subyacen al papel de estos mediadores, tratando de arrojar luz sobre el origen de las alteraciones que pueden estar implicadas, desde un punto de vista mecanístico, en este proceso.

En los artículos que integran los resultados experimentales de esta Tesis Doctoral, se incluye la discusión detallada de los resultados obtenidos en cada uno de ellos, por lo que corresponde a este apartado proporcionar una visión global de los hallazgos más importantes de este trabajo, poniendo en relevancia las relaciones que existen entre ellos y situándolos en el contexto actual de la investigación acerca del daño endotelial y el riesgo CV en AR.

Origen del daño endotelial y el riesgo CV en AR

Tras la constatación del incremento de morbi-mortalidad por enfermedad CV en pacientes de AR, la dimensión temporal del riesgo CV en pacientes de AR ha resultado una constante en la investigación en el campo durante los últimos años.

Un buen número de estudios han observado que la duración de la enfermedad constituye un parámetro predictor del desarrollo de enfermedad CV clínica y subclínica (Chung *et al*, 2005;Gabriel *et al*, 2003;Gonzalez-Juanatey *et al*, 2003;Naz *et al*, 2008). De hecho, la EULAR señala en su documento de recomendaciones para el manejo clínico del riesgo CV en pacientes con artritis inflamatorias (Peters *et al*, 2010), la duración de la enfermedad superior a 10 años como uno de los criterios para estratificar pacientes con especial riesgo. Como resulta lógico, estas observaciones son atribuidas a una exposición más prolongada del paciente a un estado proinflamatorio.

Sin embargo, este hallazgo no ha sido observado consistentemente en todos los estudios. Recientemente, dos estudios de diseño prospectivo y con un número considerable de pacientes, reflejan que, si bien el índice de actividad de la enfermedad es un factor predictor del desarrollo de enfermedad CV, la duración de la enfermedad no se asocia con la aparición de eventos CV (Arts *et al*, 2015a;Arts *et al*, 2015b). De hecho, los autores no

observan un incremento de riesgo CV tras 10 años de duración de la enfermedad (Arts *et al*, 2015a).

Estas discrepancias pueden ser explicadas por el hecho de que trabajos anteriores muestran conclusiones que son resultado de otras estrategias de diagnóstico y tratamiento de la AR, mientras que estudios más recientes, en los que el diagnóstico precoz y las nuevas estrategias terapéuticas suponen el estándar para el manejo clínico de estos pacientes, muestran una tendencia hacia un curso de la enfermedad menos agresivo y más controlado. Por tanto, en estudios más recientes, si bien los pacientes se ven expuestos a un estado proinflamatorio como consecuencia de la enfermedad, la magnitud de la carga inflamatoria en éstos es de menor grado. Presumiblemente, este efecto puede explicar que el tiempo de exposición no sea un factor tan relevante, en tanto que la carga inflamatoria a la que los pacientes se ven expuestos es menor. Estas consideraciones, que pueden ser el origen de la heterogeneidad de estos resultados, resaltan la necesidad del estudio conjunto de duración y actividad de la enfermedad. Ha de ser tenido en cuenta que ambos parámetros pueden verse asociados, de forma artificial, como consecuencia de la instauración de tratamientos y la respuesta a los mismos. Es por ello que resulta imprescindible un correcto análisis que permita tener en cuenta esta interacción.

Los resultados obtenidos en esta Tesis Doctoral aportan nuevas ideas en este punto. A la vista de los resultados obtenidos en los artículos 1 y 2, la duración de la enfermedad se acompaña de una depleción progresiva de EPC circulantes en pacientes de AR, si bien esta asociación es más evidente en pacientes con bajos niveles de IFNα y queda enmascarada en pacientes IFN^{high}. Ya sea por un agotamiento del reservorio medular que impida una movilización adecuada hacia la periferia, a una excesiva migración hacia la membrana sinovial inflamada como consecuencia de fenómenos de quimiotaxis, o bien debido a una maduración acelerada de las EPC circulantes hacia el fenotipo CD133^{-} (mEPC) en la periferia, la duración de la enfermedad parece acompañarse de una alteración de las EPC que conllevaría una reparación endotelial defectiva de forma generalizada. En línea con estas observaciones, los resultados del artículo 5 mostraron que el nivel de MP derivadas de células endoteliales, que constituye un marcador de daño endotelial, aumenta con la duración de la enfermedad. Estos resultados parecen reflejar una situación de daño endotelial asociado con la evolución de la enfermedad, que no podría ser reparado debido a una afectación progresiva de los mecanismos de reparación vascular, en este caso, la población de EPC circulantes. De hecho, el RDW, propuesto como biomarcador de riesgo CV en el artículo 6, se encontró asociado con la depleción de EPC circulantes y con marcadores de procesos de remodelado vascular en pacientes con una duración de la enfermedad

superior a 1 año, pero no en pacientes con una evolución menor, donde no cabe que éstos sean tan aparentes (artículo 7), lo que apoyaría este razonamiento.

Sin embargo, este deterioro progresivo no es extrapolable a otros mecanismos de reparación. A partir de los resultados presentados en el artículo 4, se observa que la frecuencia alterada de células Tang en pacientes de AR se correlaciona con la actividad de la enfermedad, no habiendo asociación con el tiempo de evolución. De hecho, se observó que la depleción de células Tang ya era manifiesta en pacientes al diagnóstico y sin tratamiento. El estudio de MP derivadas de células Tang confirmó que el daño en éstas parece obedecer únicamente al índice de actividad. Además, el control de la actividad de la enfermedad mediante el tratamiento con agentes bloqueantes del TNFα se tradujo en una normalización de los niveles de células Tang circulantes, de forma paralela a la mejora clínica y la reducción de mediadores proinflamatorios. Sin embargo, pese a que el grado de control de la actividad obtenido fue suficiente para normalizar la frecuencia de las células Tang, éste no produjo el mismo efecto en la población de EPC, sugiriendo nuevamente la participación de otros parámetros en el control de esta población. Estos resultados ponen de manifiesto, por tanto, la existencia de una afectación paulatina de los mecanismos de reparación endotelial debido al deterioro de las EPC, que además se puede amplificar como resultado de periodos de alta actividad de la enfermedad, por el compromiso de las Tang. Como consecuencia de lo anterior, se podría pensar que los pacientes de AR pueden tener alterados distintos mecanismos de reparación endotelial de forma diferente según sus características clínicas, por lo que el origen del daño y, por tanto, la forma de abordarlo, puede ser diferente en distintos tipos de pacientes.

Por otro lado, son varios los autores que han observado que el desarrollo de enfermedad CV en pacientes de AR es un evento relativamente temprano en el curso de la enfermedad, apareciendo dentro de los primeros 10 años de duración de la misma (Kremers *et al*, 2008;Franklin *et al*, 2010;Holmqvist *et al*, 2012;Kerola *et al*, 2012;Lindhardsen *et al*, 2011). Estos resultados son consistentes con evidencias proporcionadas por otros autores en relación a cambios subclínicos relacionados con la enfermedad CV. Así, se ha observado que el grosor íntima-media carotídeo en pacientes de AR con menos de un año de duración se encuentra incrementado respecto a individuos control (Ahmed *et al*, 2010;Hannawi *et al*, 2007;Turiel *et al*, 2009). Del mismo modo, se ha descrito que la función endotelial en pacientes de AR está alterada ya en los primeros meses tras el diagnóstico (Bergholm *et al*, 2002;Foster *et al*, 2012;Hannawi *et al*, 2009), incluso en pacientes jóvenes en ausencia de factores tradicionales de riesgo CV (Vaudo *et al*, 2004).

El hecho de que haya evidencias de enfermedad cardiovascular subclínica en pacientes de AR al diagnóstico, hace pensar que si bien el tiempo de evolución puede ser un factor importante para la progresión del daño endotelial, otros factores han de explicar el desarrollo de éstas en pacientes de forma previa al progreso de la afectación articular. En este escenario, la alteración de las células Tang sugiere un papel de la alteración temprana de las respuestas del sistema inmunitario.

Aunque a priori podría resultar controvertida la participación de mecanismos patogénicos antes del diagnóstico de la enfermedad, se ha de tener en cuenta que existe una ventana temporal desde la aparición de la disregulación inmunitaria hasta la aparición de los primeros síntomas a nivel sinovial que dan lugar al diagnóstico clínico de la AR (Tak, 2001). Como se ha mencionado en apartados anteriores, la autoinmunidad a nivel sistémico precede el desarrollo de la afectación articular (van de Sande *et al*, 2011), como atestigua la presencia de niveles séricos elevados de citocinas y autoanticuerpos con anterioridad al diagnóstico clínico (Deane *et al*, 2010;Jorgensen *et al*, 2008;Kokkonen *et al*, 2010;Rantapaa-Dahlqvist *et al*, 2003).

Este fenómeno podría tener un papel en el desarrollo de aterosclerosis precoz y disfunción endotelial tempranos en AR o, más correctamente, en pacientes con AR preclínica (Gerlag *et al*, 2012). Se ha descrito además que el perfil lipídico está alterado en estos individuos antes del diagnóstico definitivo de AR (Steiner & Urowitz, 2009), lo cual puede contribuir al desarrollo de disfunción endotelial temprana.

En este punto, es interesante considerar la participación de factores genéticos. Diferentes estudios han puesto de manifiesto que las variantes génicas pueden tener un papel en la susceptibilidad a enfermedad CV en pacientes de AR (Rodriguez-Rodriguez *et al*, 2012). Teniendo en cuenta que se ha visto una asociación entre diferentes alelos del locus *HLA-DRB1* con el desarrollo tanto de eventos CV así como aterosclerosis subclínica (Gonzalez-Gay *et al*, 2007), se podría suponer que estas variantes génicas se relacionan con una mayor susceptibilidad y severidad de la AR que podría explicar de forma indirecta una mayor prevalencia de enfermedad CV en estos pacientes. Sin embargo, otros loci clásicamente asociados con susceptibilidad a AR (*PTPN22*, *STAT4*, *TRAF1/C5*) no se han asociado con enfermedad CV o aterosclerosis subclínica. Resultados más recientes en otros loci (*LTA*, *CCR5*, *IL6*, *SERPINE1*, *TNFA*, *MTHFR*, *IRF5*) pueden sugerir la participación de mecanismos patogénicos diferentes a los relacionados con la patogénesis de la AR, que podrían actuar de forma adicional, contribuyendo así al exceso de riesgo CV y severidad de la enfermedad CV en AR en comparación con la población general.

A nivel clínico se ha descrito recientemente que la instauración temprana de tratamiento en pacientes con AR de reciente comienzo se acompaña de una mejoría en el perfil de factores clásicos de riesgo CV, así como en los marcadores de inflamación sistémica (Ajeganova *et al*, 2013;Georgiadis *et al*, 2008). Además, la supresión de la inflamación en las fases iniciales de la enfermedad se ha asociado a una reducción del riesgo CV (Dessein *et al*, 2002;Dessein *et al*, 2005a), apoyando igualmente la presencia de un riesgo CV incrementado en AR ya al inicio de la enfermedad.

Parte de los resultados obtenidos en esta Tesis aportan evidencias que se alinean con estas ideas. Por un lado, se encuentran niveles séricos elevados de IFNα en una fracción de pacientes de AR, ya al diagnóstico y con independencia de la duración de enfermedad. Este marcador está asociado en estos pacientes con un disbalance temprano mEPC/EPC, así como con un descenso más acusado de células Tang. Además, estos pacientes se caracterizan por exhibir niveles más elevados de citocinas proinflamatorias en suero en comparación con aquellos pacientes IFN^{low}. Cabe destacar que la activación de la vía de los IFN de tipo I ha sido descrita en pacientes al diagnóstico e incluso en la fase preclínica de la enfermedad (Lubbers *et al*, 2013;van der Pouw Kraan TC *et al*, 2007). Por otro lado, se observa que un porcentaje importante de pacientes de AR al diagnóstico presenta anticuerpos anti-HDL IgG, asociados de forma independiente a un perfil lipídico alterado. En este caso, si bien no se ha analizado la presencia de estos anticuerpos en la fase previa al diagnóstico de AR, es importante señalar que se ha descrito su presencia en pacientes con enfermedad periodontal en asociación con marcadores de aterosclerosis precoz (Wick *et al*, 2013), lo cual resulta de interés si se tiene en cuenta que se ha sugerido que la inflamación periodontal puede jugar un papel como mecanismo desencadenante de la AR (Chen *et al*, 2013;de *et al*, 2009). En resumen, estos resultados apoyan el papel patogénico de una disregulación de la respuesta inmunitaria en el contexto del riesgo CV en la fase más precoz de la AR.

Esta situación pone el foco sobre el estudio de los mediadores de inflamación, ya sea de tipo clínico o subclínico, durante la fase más temprana de la enfermedad. Sin embargo, resulta complejo el estudio de la asociación entre inflamación y enfermedad CV en AR, debido a que puede haber variaciones tanto en el grado de inflamación a lo largo del curso de la enfermedad como en el grado de respuesta que ésta puede desencadenar en un contexto de inflamación crónica. Asimismo, el diseño transversal con un análisis de una medición puntual no resulta una buena aproximación para el estudio de este fenómeno, en tanto que, por un lado, sólo se observarían efectos de cierta magnitud y, por otro lado, no permiten contemplar posibles fluctuaciones de los niveles de inflamación sistémica.

Además, si bien se hipotetiza cierto grado de inflamación en las primeras fases de la enfermedad, algunos pacientes pueden exhibir una inflamación subclínica de reducida magnitud que puede no ser cuantificable mediante los marcadores rutinarios, como los reactantes de fase aguda. Por tanto, las características de este campo sugieren la necesidad de contar con marcadores tempranos, con relativa sensibilidad y la posibilidad de recoger información de carácter acumulativo.

En este escenario se enmarcan los resultados obtenidos en el estudio del RDW como un biomarcador pronóstico de enfermedad CV en AR. Por un lado, el RDW es un biomarcador que no se ve influido por infecciones agudas (Hu *et al*, 2013), que tienen como resultado alteraciones en los niveles de reactantes de fase aguda y que podrían dificultar el uso de estos marcadores en pacientes de AR, siendo un riesgo especialmente delicado en pacientes con altas dosis de inmunosupresores. Por otro lado, aunque el RDW se informa como un parámetro puntual, se podría considerar que recoge, en cierto modo, información de carácter acumulativo. Esto es debido a que se calcula en base a la heterogeneidad en tamaño de los eritrocitos, y habida cuenta de que éstos cuentan con una vida media de 3 meses, el valor de RDW en un momento puntual recoge la respuesta de los eritrocitos ante diferentes estímulos nocivos que hayan podido alterar su morfología como consecuencia de la exposición a éstos. Asimismo, debido a su propia naturaleza, el RDW podría ser capaz de detectar fenómenos de inflamación subclínica, que los reactantes de fase aguda no son capaces de detectar, debido a que el efecto sobre los eritrocitos podría "amplificar" este estímulo, haciéndolo así apreciable. Estas características hacen del RDW un biomarcador muy interesante y con múltiples ventajas para su posible implementación en el manejo clínico del riesgo CV en pacientes de AR, y posiblemente en otras enfermedades sistémicas.

En resumen, se podría decir que los resultados de esta Tesis Doctoral apoyan el origen multifactorial del daño endotelial que subyace al riesgo CV en AR, asociándose los diferentes biomarcadores estudiados a distintos mecanismos de daño. Así, mientras que EPC y Tang se asocian principalmente con la duración y la actividad de la enfermedad, respectivamente, el daño asociado con IFNα y anti-HDL tiene como origen una alteración inmunológica presente en un subgrupo de pacientes y de forma independiente a la duración de la enfermedad. Igualmente, el RDW reflejaría una situación de disregulación inmunitaria, en este caso en forma de inflamación subclínica temprana, Por último, el perfil de MP indica un daño celular asociado con esta patología, que refleja no sólo un daño endotelial sino un compromiso de la reparación endotelial mediada por células Tang.

Interacción entre factores clásicos y no clásicos de riesgo CV en AR

Actualmente se acepta que los factores clásicos de riesgo CV interaccionan con los factores específicos de la enfermedad (también denominados factores no clásicos) en el desarrollo de enfermedad CV en pacientes de AR. Diferentes estudios retrospectivos (Wallberg-Jonsson *et al*, 1999) y prospectivos (Gonzalez-Gay *et al*, 2007;Innala *et al*, 2011) coinciden en señalar este hecho.

La interacción entre ambos tipos de factores puede ser consecuencia de la existencia de similitudes y mecanismos compartidos entre la patogénesis de la AR y la aterosclerosis, como ha sido descrito por algunos autores (Tabla 8) (Bartoloni *et al*, 2011;Pasceri & Yeh, 1999).

Tabla 8: Similitudes entre la aterosclerosis y la AR

	Aterosclerosis	AR
Activación de macrófagos		
TNFα	↑	↑
Expresión de MMP	↑	↑
IL-6	↑ (angina inestable)	↑
Activación de mastocitos	↑	↑
Activación de neutrófilos	↑	↑
Activación de células T		
sIL-2R	↑ (angina inestable)	↑
$CD3^+DR^+$	↑ (angina inestable)	↑
$CD4^+CD28^-$	↑ (angina inestable)	↑
$CD4^+IFN\gamma^+$	↑ (angina inestable)	↑↑
Balance Th1/Th2	↑ Th1	↑ Th1
Respuesta Th17	↑ / -	↑
Activación de células B		
Autoanticuerpos	↑ / -	↑↑
IL relacionadas	↑ / -	↑
PCR	↑ (angina inestable)	↑↑
Moléculas de adhesión	↑	↑
Endotelina	↑	↑
Neovascularización	↑	↑
Antígenos caracterizados	HSP, oxLDL, agentes infecciosos	Colágeno clase II, antígenos del cartílago, HSP, agentes infecciosos

Parece lógico pensar entonces que la participación de mecanismos patogénicos desencadenados por la AR favorecería el progreso de la disfunción endotelial hacia lesiones ateroscleróticas más severas, como recientemente se ha sugerido (Sodergren *et al*, 2010). Además, esta hipótesis permite explicar no sólo la razón de la rápida progresión de las lesiones ateroscleróticas en AR, sino también su severidad y extensión morfológica, que parece estar incrementada en pacientes de AR en comparación con individuos sin enfermedad autoinmune sistémica concomitante (Hollan *et al*, 2007;Hollan *et al*, 2008), al verse implicados mecanismos patogénicos adicionales en los primeros que no están presentes en estos últimos.

Nuestros resultados pueden avalar en cierto modo estas hipótesis. Por un lado, hemos observado que, aunque los niveles de células Tang en la población control se asocian con factores clásicos, un conjunto de factores clásicos y factores asociados a la enfermedad (actividad de la enfermedad, edad al diagnóstico, presencia de ANA y tabaquismo) se relacionan con estos niveles en pacientes de AR.

De la misma manera, los resultados obtenidos en el estudio de las MP han proporcionado resultados interesantes en este punto. Por un lado, si bien el número total de MP circulantes puede ser explicado por factores clásicos, lo cual está de acuerdo con el diferente número de MP halladas en controles sanos, pacientes con riesgo CV clásico en ausencia de enfermedad autoinmune y pacientes de AR, la frecuencia de los diferentes tipos de MP se relacionó con parámetros clínicos exclusivamente. Del mismo modo, el análisis de la relación entre el número de MP derivadas de células Tang y los niveles séricos de TNFα fue dependiente del número de factores clásicos de riesgo CV, sugiriendo así que a mayor contribución de éstos, menor contribución cabía esperar del TNFα en la producción de estas MP. Por último, el análisis de componentes principales realizado en este trabajo aportó otro resultado relevante: pese a que las variables componentes son, por definición, incorreladas entre sí, cuando se analizó la correlación entre la componente que resumía las variables de los factores clásicos de riesgo CV y la componente relacionada con los parámetros clínicos de actividad de la enfermedad, se observó que ambas estaban fuertemente correlacionadas en el subgrupo de pacientes que presentaban una historia previa de enfermedad CV, siendo su coeficiente de correlación próximo a 0 en los individuos que no mostraban esta complicación.

Estos resultados apoyan por tanto la existencia de una interacción entre ambos tipos de factores y podrían sugerir el análisis de MP como una aproximación relevante para el estudio de estas interacciones, que pueden ser evaluadas de una forma más directa en

comparación con los estudios epidemiológicos (Figura 12). Además, un análisis basado en MP permitiría el estudio de estas interacciones en situaciones de cambio, como en diferentes fases de la enfermedad, ante diferentes tratamientos, etc.

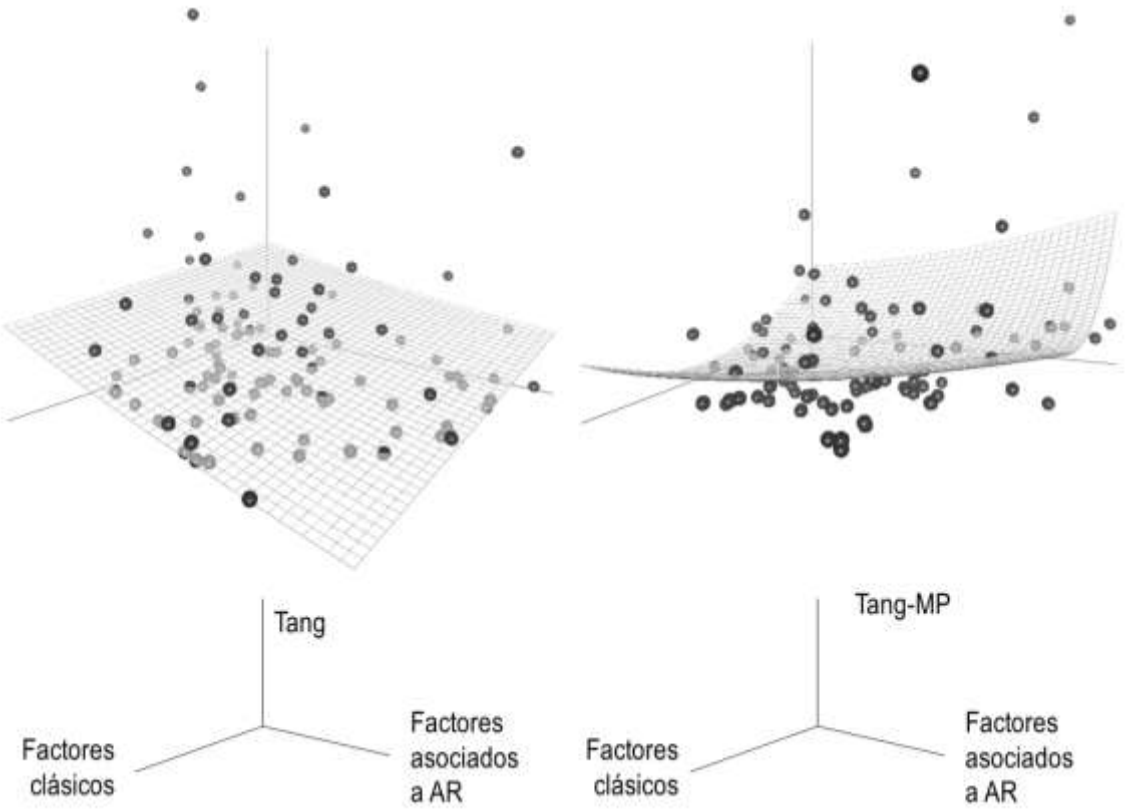

Figura 12 | Asociación de Tang y Tang-MP con factores clásicos y factores asociados a AR. Los gráficos muestran la asociación entre la frecuencia de células Tang o de Tang-MP con factores de riesgo clásicos y asociados a AR. Las variables que recogen los factores de riesgo (clásicos y asociados a AR) se muestran como las componentes principales calculadas a partir de las variables originales.

En respuesta al tratamiento, la interacción entre factores clásicos y no clásicos parece ser diferente. En los resultados obtenidos en el artículo 4, se observa que en pacientes de AR las células Tang no se asocian con los factores clásicos, mientras que la actividad de la enfermedad parece ser el principal factor que explica sus niveles. De forma similar, en el artículo 10, se observa que los factores clásicos no se asocian con esta población celular en las muestras previas al tratamiento con anti-TNFα; mientras que se observa una relación entre ambos parámetros una vez se ha controlado la actividad de la enfermedad.

De forma global, estos resultados proporcionan evidencias experimentales que avalan la hipótesis teórica de la competencia entre los factores clásicos y los específicos de la enfermedad propuestos por Symmons y Gabriel (Symmons & Gabriel, 2011). Asimismo,

proporcionan nuevas evidencias que sustentan de forma empírica la recomendación EULAR de tratamiento agresivo de la enfermedad como intervención para la reducción del riesgo CV en pacientes de AR (Peters *et al*, 2010).

Bloqueo del TNFα, respuesta clínica y riesgo CV

En línea con lo anteriormente expuesto, se ha observado que el control de la actividad de la enfermedad se asocia con una menor prevalencia de enfermedad CV en estudios epidemiológicos (Choi *et al*, 2002;Meek *et al*, 2014;Solomon *et al*, 2015) y con una menor disfunción endotelial (de *et al*, 2015) en pacientes de AR. En este aspecto, se ha estudiado en profundidad el efecto del tratamiento con agentes bloqueantes del TNFα, concluyéndose así que la respuesta satisfactoria a este tratamiento se traduce en menores tasas de enfermedad CV (Dixon *et al*, 2007), lo cual podría ser debido a una reducción de disfunción endotelial (Gonzalez-Juanatey *et al*, 2012). Resulta lógico pensar que las funciones ateroprotectoras consecuencia del tratamiento con agentes anti-TNFα puedan resultar del bloqueo de los mecanismos deletéreos desencadenados por el TNFα sobre el endotelio y otros tejidos (alteración del perfil lipídico, resistencia a la insulina, activación endotelial, activación de la coagulación,…) (McKellar *et al*, 2009), que contribuyen al progreso de la aterosclerosis (Dixon & Symmons, 2007). Sin embargo, existen resultados contradictorios en la literatura actual (Chung *et al*, 2003;Kwon *et al*, 2003;Listing *et al*, 2008;Mann *et al*, 2004;Setoguchi *et al*, 2008;Van *et al*, 2005).

En los últimos años se ha visto que pacientes con angina muestran un aumento en sangre periférica de una subpoblación de células $CD4^+$ que carecen de la expresión de la molécula CD28 ($CD4^+CD28^{null}$) (Liuzzo *et al*, 2007). Esta población se ha relacionado con fenómenos de inestabilización de placas de ateroma, debido a su capacidad de producir elevados niveles de citocinas proinflamatorias y moléculas citotóxicas (Dumitriu *et al*, 2009) que explican su capacidad de inducción de apoptosis en células endoteliales *in vitro* (Nakajima *et al*, 2002), proponiendo así una vía de participación en la patogénesis de la enfermedad CV. Esta población celular se ha visto aumentada también en pacientes de AR, particularmente en aquellos con manifestaciones extraarticulares y en asociación con marcadores de disfunción endotelial (Gerli *et al*, 2004;Pawlik *et al*, 2003). En línea con las observaciones anteriores, el TNFα parece jugar un papel crucial en la diferenciación de esta población (Bryl *et al*, 2001), y de hecho algunas evidencias señalan que el bloqueo de esta citocina se acompaña de una reducción en los niveles de esta población tanto *in vitro* (Rizzello *et al*, 2006) como *in vivo* (Pawlik *et al*, 2004).

Los resultados presentados en esta Tesis Doctoral en el artículo 9, sugieren que la elevación de esta población celular en pacientes de AR se presenta mayoritariamente en individuos portadores del alelo minoritario *TNFA* -308 A*, cuya asociación con la susceptibilidad a enfermedad CV en esta patología ha sido recientemente descrita (Rodriguez-Rodriguez *et al*, 2011). De este modo, una de las causas que subyacen al mayor riesgo de enfermedad CV en los individuos portadores de este alelo, podría ser la expansión de esta población. Sin embargo, nuestros resultados no muestran una asociación directa entre la presencia de enfermedad CV y el aumento de esta población, más allá de su asociación con parámetros de actividad y severidad de la enfermedad. Diferencias en la presentación clínica de la enfermedad CV (los pacientes con enfermedad isquémica y accidentes cerebrovasculares muestran mayores niveles de esta población, si bien se no se observan diferencias estadísticamente significativas, posiblemente por no contar con un tamaño muestral adecuado), así como que el diseño retrospectivo del estudio no permita contemplar la posibilidad de que la frecuencia de esta población se reduzca tras el evento CV, son importantes limitaciones que pueden ser la base de estas discrepancias.

Asimismo, se observa que el tratamiento con bloqueantes del TNFα en este grupo de pacientes se asocia con una reducción menos significativa y no asociada a la mejoría clínica. No obstante, se precisan más estudios con mayor número de pacientes para el análisis de las diferencias en la respuesta clínica según los diferentes alelos de este locus y su relación con la población $CD4^{+}CD28^{null}$.

También en relación con la respuesta al tratamiento con agentes bloqueantes del TNFα, los resultados obtenidos en esta Tesis Doctoral revelan que los individuos respondedores muestran una mayor recuperación de células Tang circulantes, en línea con una menor liberación de MP derivadas de esta población celular, así como en asociación con el cambio en la población de EPC. En cuanto a los mediadores solubles, se encuentra una reducción más pronunciada en los niveles de TNFα e IL-8 en los individuos respondedores. Finalmente, se observa que el tratamiento con agentes anti-TNFα se acompañó de una reducción en los niveles de anti-HDL IgG que se asociaron de forma independiente con la restauración de los niveles de HDL.

En general, nuestros resultados aportan evidencias que avalan el papel ateroprotector del tratamiento con bloqueantes del TNFα a diferentes niveles, dando cuenta de la validez como biomarcadores de los diferentes mediadores analizados.

Perspectivas futuras

Estratificación del riesgo CV

El hecho de que el riesgo CV se haya encontrado aumentado ya en las primeras fases de la enfermedad (Franklin *et al*, 2010;Kerola *et al*, 2012;Kremers *et al*, 2008), unido a la observación de que la instauración de un tratamiento temprano y la respuesta efectiva al mismo se acompañen tanto del control de algunos factores clásicos y no clásicos de riesgo CV (Georgiadis *et al*, 2008), como de la restauración de indicadores de disfunción endotelial y progresión aterosclerótica a corto (Hannawi *et al*, 2009;Georgiadis *et al*, 2008) y largo plazo (Turiel *et al*, 2010), así como una menor incidencia de enfermedad CV a largo plazo (Ajeganova *et al*, 2013), sugiere la existencia de una ventana temporal donde sería conveniente realizar un control estricto de la enfermedad, no sólo desde el punto de vista articular, sino poniendo especial atención también en el riesgo CV.

Con este objetivo, sería imprescindible contar con herramientas que permitiesen una monitorización del riesgo CV de los pacientes, de forma que se pudiese realizar una estratificación del riesgo en base a los mismos, permitiendo así diferentes intervenciones terapéuticas de prevención de una forma proporcional al riesgo.

El avance en el conocimiento del papel de los factores de riesgo y su interrelación ha llevado en los últimos años a un cambio en el paradigma del estudio del riesgo CV, pasando los factores de riesgo de ser estudiados de forma individual a ser considerados de forma conjunta bajo el concepto de "riesgo global", el cual proporciona una fuerza predictiva de mayor valor que cualquier factor de forma independiente (Grundy *et al*, 1998;Pearson *et al*, 2003;Pyorala *et al*, 1994). Este hecho justifica el uso de algoritmos para el cálculo del riesgo CV global, que se basan en la presencia y grado de diferentes factores de riesgo CV, como las escalas de Framingham (*Framingham Risk Score*), SCORE (*Systematic Coronary Risk Evaluation*) o REGICOR (*Registre Geroní del Cor*).

Sin embargo, estos algoritmos presentan algunas limitaciones. Se ha visto en algunos estudios que una proporción relevante de pacientes con enfermedad CV no presenta factores clásicos de riesgo (Oh *et al*, 2010). Además, se ha observado que el control de estos factores no se traduce consistentemente en un menor riesgo cardiovascular real en las poblaciones analizadas (Ford *et al*, 2009).

Estos algoritmos han sido realizados a partir de grandes cohortes de población general e incluyen solamente, en consecuencia, factores clásicos de riesgo CV. El uso de estos algoritmos en situaciones patológicas que puedan alterar la presentación clínica de los

factores clásicos, o bien, que impliquen a otros factores de riesgo explicaría la mala adecuación de estos algoritmos en algunas situaciones. De hecho, son varios los estudios que sugieren la relevancia de otros factores de riesgo cardiovascular que no están recogidos dentro de los llamados factores clásicos (Bianchi *et al*, 2007;Muntner *et al*, 2004).

En el caso de la AR, estos algoritmos han mostrado una escasa efectividad, en consonancia con lo anteriormente expuesto (del Rincon *et al*, 2001a;Gomez-Vaquero *et al*, 2013). A la vista de la participación de los factores específicos de la enfermedad en el riesgo CV de los pacientes de AR, resulta lógico que la consideración únicamente de los factores clásicos no resulte un enfoque adecuado. Es por ello que en los últimos años se ha considerado la introducción de otros factores de riesgo con el objetivo de mejorar la valoración del riesgo CV en estos pacientes.

En este sentido, algunos expertos apoyan la inclusión de pruebas de imagen, como la ecografía carotídea, de forma paralela a la valoración del riesgo CV mediante el algoritmo SCORE en pacientes de AR con sospecha de especial riesgo (Corrales *et al*, 2014;Gonzalez-Gay *et al*, 2012). Por otro lado, la EULAR ha propuesto algunas recomendaciones específicamente para la valoración del riesgo CV en artritis inflamatorias (Peters *et al*, 2010).

Los resultados obtenidos en esta Tesis Doctoral podrían ser de futura aplicación en este campo. Por un lado, se podría sugerir el uso del RDW como un potencial biomarcador temprano de ayuda en la estratificación del riesgo CV en estos pacientes ya al diagnóstico de AR, si bien se requieren más estudios para confirmar los resultados obtenidos en este trabajo, así como un análisis de su posible estandarización inter-centros, que podría ser el principal inconveniente de este parámetro (Lippi *et al*, 2014). No obstante, el hecho de poder contar en el ámbito clínico con un biomarcador que permita reflejar la alteración de los mecanismos de reparación endotelial, debidos tanto al descenso en la frecuencia de EPC como al aumento de los niveles séricos de citocinas proinflamatorias, especialmente de aquellas asociadas a procesos de daño y remodelado vascular, sería de gran utilidad.

Del mismo modo, los anticuerpos anti-HDL podrían ser unos candidatos atractivos en este punto. Al hecho de que el trabajo con autoanticuerpos, tanto como variable clínica para la toma de decisiones como en su parte técnica y de estandarización, sea un procedimiento común en el ámbito clínico, se le une el hecho de que la positividad para autoanticuerpos anti-HDL IgG nos permite identificar pacientes con un perfil sérico de citocinas muy relevantes para el daño endotelial y el riesgo cardiovascular en AR (Tabla 9). Identificar conjuntamente este perfil a través de un único marcador, en este caso la

positividad para anticuerpos anti-HDL, resulta muy ventajoso y factible, puesto que los niveles de citocinas resultan más complejos de medir de forma rutinaria y su significado clínico a nivel individual es difícil de interpretar.

Tabla 9: Ambiente proinflamatorio en individuos con autoanticuerpos anti-HDL IgG

Perfil lipídico alterado	• Perfil proaterogénico • Disfunción de HDL (pérdida de función antiinflamatoria y antioxidante) • Daño endotelial directo • Activación de plaquetas
↑ IFNα	• Diferenciación y activación de células espumosas • Alteración de la reparación endotelial por EPC y Tang • Inestabilización de placas
↑ MIP1α	• Activación y quimiotaxis de monocitos • Inestabilización de placas
↑ IFNγ	• Diferenciación de células espumosas • Diferenciación de células $CD4^{+}CD28^{null}$
↑ IL-8	• Activación y quimiotaxis de neutrófilos • Activación de MMP • Expresión de moléculas de adhesión en células endoteliales
↑ GM-CSF	• Activación y quimiotaxis de monocitos • Producción de IL-23
↑ IL-17A	• Activación de formación de *Neutrophil Extracellular Traps* (NETs), implicadas directa e indirectamente en el daño endotelial y su progresión en pacientes con enfermedades autoinmunes sistémicas
↑ MCP-1	• Quimiotaxis de monocitos

Además, cabe destacar que los resultados obtenidos en el estudio de estos autoanticuerpos fueron independientes de los factores clásicos, por lo que es esperable que la inclusión de estos biomarcadores mejore notablemente el poder predictor de los algoritmos basados exclusivamente en factores clásicos, como se ha observado con los anticuerpos anti-Apo A1 recientemente (Keller *et al*, 2012;Vuilleumier *et al*, 2010a). De hecho, aunque el SCORE incorpora información sobre los niveles de HDL mediante el ratio Colesterol total/HDL, y pese a ser ésta la forma preferida para el análisis de estos niveles en AR (Peters *et al*, 2010), se ha descrito que la inclusión de los niveles de HDL de forma independiente mejora la predicción de este modelo, especialmente en aquellos individuos con niveles extremos de esta lipoproteína (Cooney *et al*, 2009), como podría ser el caso de los pacientes de AR. Este hecho podría ser debido a que la inclusión de los niveles de HDL de forma independiente permite aportar, en cierto modo, información ligada a las funciones del

HDL más allá de su papel en el transporte reverso de colesterol (Calabresi *et al*, 2003), resaltando así el papel crucial de estas lipoproteínas en el riesgo CV.

Riesgo CV en fase preclínica

El avance del conocimiento acerca de los mecanismos que gobiernan la patogénesis de la AR ha dado lugar a numerosas evidencias clínicas y experimentales que coinciden en señalar las diferencias existentes ente la AR precoz o de reciente comienzo y la AR establecida. De forma paralela, se ha ido avanzando en el estudio del daño endotelial y el riesgo CV en relación a ambas fases de la enfermedad, si bien es un campo relativamente reciente.

El hecho de que varios estudios hayan observado disfunción endotelial en pacientes de AR al diagnóstico (Vaudo *et al*, 2005), e incluso lesiones ateroscleróticas en algunos casos (Hannawi *et al*, 2007), hace pensar que los mecanismos de daño endotelial y la alteración de los procesos de reparación han de preceder al diagnóstico clínico de la enfermedad. El hecho de que se ha observado la presencia de citocinas proinflamatorias y autoanticuerpos en el suero en individuos durante la fase preclínica de la enfermedad, sugiere que los fenómenos que conducen al incremento del riesgo CV en AR ya están actuando durante esta fase. Existen algunas evidencias de que algunos mediadores pueden condicionar el riesgo CV en la fase preclínica, como la implicación de diferentes variantes génicas en la disfunción endotelial, así como el hecho de que se haya descrito un perfil lipídico alterado (Myasoedova *et al*, 2010a;Steiner & Urowitz, 2009) y una elevación en marcadores de activación endotelial (Rantapaa-Dahlqvist *et al*, 2007) en individuos con artritis preclínica.

Sería por tanto interesante analizar los biomarcadores propuestos en esta Tesis Doctoral durante la fase preclínica de la enfermedad con el objetivo de profundizar en el estudio de estos mecanismos. Este enfoque nos permitiría delimitar la contribución de los mecanismos patogénicos de la AR al riesgo CV y nos proporcionaría un escenario muy interesante para estudiar si el incremento de riesgo CV en AR es diferente según la etapa de la enfermedad o si, por el contrario, el incremento de daño endotelial que sugieren algunos trabajos actuales es un mero producto de la acumulación pasiva de daño durante el tiempo de exposición analizado. Asimismo, de forma equivalente a lo hallado acerca de la importancia de la ventana de oportunidad en el abordaje terapéutico de la AR, el estudio de estos parámetros nos permitiría proporcionar nuevas evidencias que sean de ayuda para la mejora del manejo clínico del riesgo CV en esta patología.

Conslusions

5. Conclusions

1. IFNα can be detected in serum in a subgroup of RA patients, in higher levels than healthy controls and comparable to those from SLE patients. IFNα serum levels are associated with an EPC imbalance characterized by a shift towards a mature EPC phenotype which hides the disease duration-related EPC decrease in RA. High IFNα serum levels identify RA patients with higher disease activity, presence of autoantibodies, increased levels of proinflammatory cytokines and occurrence of CV events.

2. Tang are negatively correlated with total- and low density lipoproteins-cholesterol levels and they also exhibit a positive correlation with EPC frequency in healthy controls. Decreased Tang counts are found in RA patients in association with disease activity, age at diagnosis, antinuclear autoantibodies and smoking habit. Low disease activity is related to a partial recovery of EPC to Tang correlation. RA patients who have suffered from a cardiovascular event exhibited a greater Tang depletion.

3. IFNα serum levels are negatively associated with Tang frequency in peripheral blood in RA patients. This cytokine also prompts an *in vitro* decrease in Tang frequency which can be abrogated by IFNα blockade.

4. RA patients exhibit an altered MP profile characterized by an increase in total MP counts as well as in the number of endothelial-, granulocyte- and Tang-derived MP compared to both healthy controls and patients with traditional CV risk factors. Whereas the total number of MP is related to traditional CV risk factors, the numbers of specific MP subsets are associated with disease-related parameters. MP from RA patients, but not those from healthy controls or individuals with traditional CV risk factors, promote endothelial activation and exhibited detrimental effects on endothelial functionality *in vitro*.

5. Both RDW at diagnosis and 1-year cumulative RDW predict the occurrence of CV events in RA patients. An increase in RDW during the first year also predicts reduced CV-free survival during the follow up.

6. RDW is negatively correlated with EPC frequency in long-standing RA patients, but not in those with early disease. After controlling for clinical parameters, disease duration, traditional CV risk factors and treatments, RDW is an independent predictor of EPC depletion in peripheral blood.

7. RDW is associated with disease activity, duration and severity in long-standing RA patients, as well as with IFNα, IL-8 and VEGF levels. RDW is independently associated with IL-8 serum levels after adjusting for disease duration and activity, ESR and treatments.

8. IgG anti-HDL antibodies are increased in RA patients compared with healthy controls, even after correcting for total IgG levels, and are associated with an impaired lipid blood profile, especially at the disease onset. RA patients with high levels of IgG anti-HDL antibodies are more likely to have suffered a CV event and exhibit increased levels of proinflammatory mediators.

9. $CD4^{+}CD28^{null}$ cells are expanded in RA patients linked to the TNFA -308 minor allele and related to markers of aggressive disease, but not with cardiovascular disease. Poor regulatory T cell-mediated suppression or Th1 shift could underlie this expansion. After TNFα-blockers usage, lower decrease of this population, unrelated to clinical improvement, is achieved in these patients in comparison to those with the common genotype.

10. Change in IgG anti-HDL antibodies is related to change in HDL levels in RA patients upon TNFα blockade, even after correcting for clinical response and CRP decrease.

11. Good response to TNFα-blockade is related to an angiogenic T cell recovery which parallels change in EPC, and also with decreased Tang-derived MP shedding. Frequency of Tang after TNFα blockade is associated with total to HDL-cholesterol ratio.

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5. Bibliografía

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Anexo

Anexo: Informe de factor de impacto de las publicaciones presentadas

Los resultados obtenidos como consecuencia de la realización de la presente Tesis Doctoral han dado lugar a 10 artículos, cuyos indicadores de calidad según la base de datos Journal of Citation Reports (edición 2013) se indica a continuación:

	Revista	Índice de impacto	Posición	Cuartil	Categoría
Artículo 1	Rheumatology (Oxford)	4,435	6/30	Q1	Rheumatology
Artículo 2	PLOS One	3,534	8/55	Q1	Multidisciplinary sciences
Artículo 3	Clinical Science (London)	5,629	14/124	Q1	Medicine, research & experimental
Artículo 4	Annals of the Rheumatic Diseases	9,270	2/30	Q1	Rheumatology
Artículo 5	Clinical Science (London)	5,629	14/124	Q1	Medicine, research & experimental
Artículo 6	Rheumatology (Oxford)	4,435	6/30	Q1	Rheumatology
Artículo 7	Atherosclerosis	3,971	15/65	Q1	Peripheral vascular disease
Artículo 9	Experimental gerontology	3,529	8/49	Q1	Geriatrics & gerontology
Artículo 10	Rheumatology (Oxford)	4,435	6/30	Q1	Rheumatology