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Bases Genéticas de la Enfermedad Cardiovascular en Artritis Reumatoide

Genetic Basis of Cardiovascular Disease in Rheumatoid Arthritis

Tesis Doctoral

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ABBREVIATIONS

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[95% CIs]: 95% confidence intervals.

anti-CCP: Anticyclic citrullinated peptide antibodies.

BMI: Body mass index.

CRP: C reactive protein.

CV: Cardiovascular.

CVIE: Cerebrovascular ischemic event.

DC: Dendritic cell.

ESR: Erythrocyte sedimentation rate.

FMD%: Endothelium dependent vasodilatation.

GHS-R: Growth hormone secretagogue receptor.

HDL: High-density lipoprotein.

HIF: Hypoxia inducible factor.

HLA: Human lymphocyte antigen.

IHD: Ischemic heart disease.

IL: Interleukin.

IMT: Intima-media thickness.

INF: Interferon.

IR: Insulin resistance.

LAMs: Leukocyte adhesion molecules.

LD: Linkage disequilibrium.

LDL: Low-density lipoprotein.

LPS: Lipopolysaccharide.

MHC: Major histocompatibility complex.

MI: Myocardial infarction.

MIF: Migration inhibitory factor.

MMP: Matrix metalloproteinase.

NK: Natural Killer.

NO: Nitric oxide.

NOS: Nitric oxide synthase.

NTG%: Endothelium independent vasodilatation

OR: Odds ratio.

oxLDL: oxidized low density lipoprotein.

PAI-1: Plasminogen activator inhibitor type-1.

PBMCs: Peripheral blood mononuclear cells.

RF: Rheumatoid factor.

SE: Shared Epitope.

SR: Scavenger receptor.

TC: Total cholesterol.

TGF: Transforming growth factor.

TLR: Toll-like receptor.

TNF: Tumor necrosis factor.

TNFR: Tumor necrosis factor receptor.

UTR: Untranslated region.

VEGF: Vascular endothelial growth factor.

VSMC: Vascular smooth muscle cell.

ABSTRACT

1. ABSTRACT

Introducción

La artritis reumatoide (AR) es una enfermedad inflamatoria crónica caracterizada por sinovitis persistente, con destrucción del cartílago y hueso adyacente. Es una enfermedad compleja en la que factores genéticos, ambientales y estocásticos se combinan para dar lugar a una patología con una gran variedad de manifestaciones clínicas, gravedad, evolución y respuesta a tratamiento.

A pesar de los avances producidos en los últimos años en el manejo de esta enfermedad, la AR sigue asociada a un incremento de mortalidad, de alrededor de un 50% con respecto a la población general, con una reducción de la esperanza de vida de entre 3 y 10 años. La principal causa de muerte entre pacientes con AR es la patología cardiovascular (CV).

Múltiples estudios epidemiológicos en la AR han mostrado de forma repetida un aumento de la prevalencia de patología CV, tanto clínica como subclínica, así como un aumento de mortalidad por esta causa. Los factores de riesgo CV clásicos como la hipertensión arterial, la diabetes mellitus (DM), el tabaquismo o la dislipemia han sido asociados de forma independiente a un mayor riesgo de enfermedad CV en la AR. Sin embargo, ninguno de estos factores, con la excepción del tabaquismo, parece ser más prevalente en la AR que en la población general. Esto ha llevado a considerar que el exceso de riesgo y de mortalidad CV que sufren los pacientes con AR es debido a procesos específicos o relacionados con la propia enfermedad. En este sentido, la elevación de marcadores inespecíficos de inflamación [como la proteína C reactiva (PCR)], la aparición de auto-anticuerpos [como el factor reumatoide (FR) o los anticuerpos anti péptidos cíclicos citrulinados (anti-CCP)] o el desarrollo de marcadores

de AR severa (como son cambios destructivos en articulaciones o aparición de enfermedad extraarticular), se han asociado a un mayor riesgo y mortalidad CV.

Es importante tener en cuenta que la aterosclerosis es una enfermedad inflamatoria crónica al igual que la AR, existiendo entre ambas numerosas similitudes, como es la presencia a nivel de placa aterosclerótica y sinovial reumatoide de un infiltrado inflamatorio formado principalmente por macrófagos, linfocitos (especialmente CD4+), células musculares lisas de la pared vascular / sinoviocitos, células dendríticas…

Muchas de estas células muestran signos de activación y producen citoquinas pro-inflamatorias y metaloproteinasas de matriz, que contribuyen al crecimiento de la lesión y posterior desestabilización de la placa (en aterosclerosis) / invasión de cartílago y hueso (en la AR). La situación de inflamación crónica y sistémica que produce la AR, en parte debido al aumento de citoquinas circulantes en sangre periférica, actuaría tanto directamente a nivel de la pared vascular como a nivel de otros órganos, para favorecer la aparición y el progreso de las lesiones ateroscleróticas. Uno de los primeros fenómenos que se producen en la aterosclerosis es la aparición de activación / disfunción endotelial, con aumento de la expresión de moléculas de adhesión y quimiocinas, dando lugar al reclutamiento de células inflamatorias desde el lumen vascular. A este fenómeno contribuyen tanto factores de riesgo clásicos de enfermedad CV, como es el aumento de lipoproteínas de baja densidad (LDL), que se acumulan y se oxidan en la región subendotelial, como la mayor concentración de citoquinas circulantes. Monocitos y linfocitos T CD4+ procedentes del lumen vascular migrarán a la pared arterial, donde sufrirán un proceso de activación, con producción y liberación de más citoquinas, quimiocinas y metaloproteinasas de matriz, lo que contribuirá a un mayor reclutamiento de células inflamatorias, su proliferación y activación.

Tanto en la AR como en la aterosclerosis, se han descrito múltiples marcadores genéticos asociados a un mayor riesgo de padecer estas patologías. Algunos marcadores son comunes a ambas, como son ciertos alelos del gen *HLA-DRB1*, mientras que otros, a pesar de su fuerte asociación con la AR, no se han observado asociados a enfermedad CV [como son polimorfismos de los genes proteina fosfatasa de tirosinas, no receptor tipo 22 (*PTPN22*), transductor de señales y activador de la transcripción 4 (*STAT4*), factor asociado al receptor de necrosis tumoral 1 / factor del complemento 5 (*TRAF1/C5*)]. Entre los genes asociados a patología CV en el contexto de la AR se encuentran algunos que codifican para citoquinas y quimiocinas [interleukina 6 (*IL-6*), linfotoxina alfa (*LTA*), ligando de la quimiocina con motivo C-C 21 (*CCL21*)], para proteínas que intervienen en la coagulación [inhibidor de la peptidasa de serpina, subtipo E, miembro 1 (*SERPINE1*)] y para otras proteínas relacionadas con otros factores de riesgo CV [metilén-tetra-hidro-folato reductasa (*MTHFR*), sintasa de óxido nítrico 2, inducible (*NOS2A*), sintasa de óxido nítrico 3 (*NOS3*)].

El objetivo de nuestro trabajo consistió en continuar con el análisis de factores genéticos asociados a enfermedad CV en la AR. Se estudiaron genes que codifican para proteínas implicadas en diferentes niveles de la patogenia de ambas enfermedades, como son citoquinas [factor de necrosis tumoral (*TNFA*)], quimiocinas [receptor de la quimiocina con motivo C-C 5 (*CCR5*)], adipocitoquinas [resistina (*RETN*), adiponectina (*ADIPOQ*)] agentes que promueven la neovascularización [factor de crecimiento vascular endotelial A (*VEGFA*)] o factores que regulan el apetito y el gasto energético [receptor del secretagogo de la hormona de crecimiento (*GHSR*)]:

- TNF-α (codificada por el gen *TNFA*) es una citoquina con una gran variedad de efectos en diferentes tipos celulares. Es producida en grandes cantidades tanto en la articulación inflamada como en la placa aterosclerótica, con un efecto pro-inflamatorio.

Además actúa sobre los adipocitos, por lo que también influencia el metabolismo de lípidos e hidratos de carbono. Esta citoquina juega un papel fundamental en ambas patologías, como demuestra el uso de medicación anti-TNF, que bloquea la interacción de esta citoquina con su receptor. Estos fármacos han mostrado efectividad tanto en el tratamiento de la AR, como en la prevención de patología CV en sujetos afectos de AR. En nuestro trabajo analizaremos un polimorfismo localizado en la región promotora (posición -308, G>A, rs1800629) que se ha asociado a una mayor producción espontánea y en respuesta a estímulos de TNF-α. Esta variante ha sido asociada a enfermedad CV subclínica y a DM, aunque no a la AR ni a enfermedad CV clínica.

- CCR5 (codificada por el gen *CCR5*) es un receptor de quimiocinas que contribuye a la activación y acumulación de macrófagos y linfocitos, además de activación de células endoteliales. La ausencia de esta molécula se ha asociado a una importante disminución del desarrollo de aterosclerosis en modelos murinos. La variación que se estudiará (deleción de 32 pares de bases) se asocia a una disminución de la expresión de este receptor en superficie celular y a una disminución del riesgo de patología CV y probablemente de la AR.

- El tejido adiposo, además de órgano de acumulación de energía, es un órgano endocrino que secreta múltiples proteínas con efectos a nivel metabólico e inmunológico. La resistina (codificada por el gen *RETN*) actúa como un antagonista de la insulina y como un agente proinflamatorio, estimulando la producción de citoquinas y moléculas de adhesión en células endoteliales, linfocitos, monocitos y sinoviocitos. En la AR los niveles de resistina parecen encontrarse levemente elevados en sangre periférica y de forma importante en líquido sinovial, existiendo una correlación positiva entre sus niveles y los de marcadores inespecíficos de inflamación como son la PCR o la velocidad de sedimentación globular. El polimorfismo que se analizará se localiza en

la región promotora del gen (posición -420, C>G, rs1862513) y ha sido previamente asociado a patología cerebrovascular en pacientes diabéticos.

La adiponectina (codificada por el gen *ADIPOQ*) tiene un efecto sensibilizador a la insulina, anti-aterogénico y anti-inflamatorio. En pacientes con síndrome metabólico, como por ejemplo pacientes diabéticos, se han observado niveles más bajos de esta adipocina que en sujetos normales. Estudiaremos dos variantes del gen, una localizada en la región 5' no traducida (5'UTR; posición -11377, rs266729) y otra en el segundo intrón (+276, rs1501299). El primer polimorfismo ha sido asociado a DM tipo 2 y a enfermedad CV subclínica. El segundo polimorfismo no se ha asociado a DM, pero si a un menor riesgo de patología CV clínica.

- La neovascularización es un proceso crucial para el desarrollo tanto del pannus en la AR como de la placa aterosclerótica. Uno de los factores más importantes que estimulan el crecimiento y desarrollo de nuevos vasos es el factor de crecimiento endotelial vascular (codificado por el gen *VEGFA*). Los neovasos generados a partir de los *vasa vasorum* contribuyen al crecimiento de la placa debido a su mayor permeabilidad y fragilidad, lo que da lugar a una mayor extravasación de células inflamatorias y a hemorragias. Los polimorfismos que serán analizados (-1154, G>A, rs1570360, localizado en la región promotora y -634, G>C, rs2010963, localizado en la región 5´UTR) se han asociado a una menor producción de VEGF.

- La ghrelina, a través de su receptor (codificado por el gen *GHSR*) es capaz de estimular de secreción de GH, tiene un efecto orexígeno y regula la homeostasis energética. Este receptor se ha observado en células T, B, monocitos y neutrófilos, sobre los que ejerce un efecto anti-inflamatorio, disminuyendo la síntesis de citoquinas pro-inflamatorias y aumentando la síntesis de citoquinas anti-inflamatorias. También se han observado receptores en cardiomiocitos y células endoteliales, donde ejerce un efecto

antiapoptótico y vasodilatador. En la AR se han observado niveles bajos de ghrelina circulante . Los polimorfismos que serán estudiados (rs509035, localizado en el intrón, rs2922126, localizado en la región promotora y rs512692, localizado en la región 5'UTR) han sido asociados con resistencia a la insulina, síndrome metabólico y niveles bajos de lipoproteínas de alta densidad (HDL).

Resultados

Análisis del gen *TNFA* (rs1800629)

En nuestro estudio observamos una mayor frecuencia de portadores del alelo menor (*TNFA*-A, individuos con genotipos GA y AA) en pacientes con patología CV comparado con pacientes sin eventos CV (37.6% *versus* 27.9%, p = 0.06, OR 1.56 [95% CI 0.95-2.54]). La frecuencia total del alelo menor (*TNFA-A*) también se encontró aumentada en pacientes de AR con patología CV, siendo estas diferencias cercanas a la significación estadística (21.0% *versus* 15.3%, p = 0.053, OR 1.47 [95% CI 0.97-2.22]). En el modelo de regresión de Cox, observamos que los portadores del alelo menor también se asociaron a un mayor HR, especialmente tras ajuste por género, edad de diagnóstico de la AR y presencia del epítopo compartido y de los factores de riesgo CV clásicos (p=0.023, HR 1.72 [95% CI 1.076-2.74]).

Decidimos estratificar nuestra muestra en función de la presencia o ausencia de al menos una copia del epítopo compartido, para lo que introdujimos una interacción en el modelo de regresión de Cox, entre portar al menos una copia del epítopo compartido y al menos una copia del alelo menor del polimorfismo rs1800629 de *TNFA*. Esta interacción resultó significativa, tanto antes como tras el ajuste por las variables ya mencionadas (con la excepción de la presencia o ausencia del epítopo compartido; p= 0.014, p=0.024, respectivamente). Observamos que la asociación entre ser portador del

alelo menor de rs1800629 y patología CV solo resultó significativa en los portadores del epítopo compartido (antes de ajuste: p=0.003, HR 2.23 [95% CI 1.32-3.78]. Tras ajuste: p=0.001, HR 2.43 [95% CI 1.41-4.19]).

Por otro lado, no observamos una influencia significativa de este polimorfismo sobre los marcadores de patología CV subclínica (espesor intima-media carotídeo y vasodilatación dependiente o independiente de endotelio).

Análisis del gen *CCR5* (deleción Δ32, rs333)

La frecuencia de portadores de la delección *CCR5*Δ32 fue significativamente menor en pacientes de AR con patología CV comparado con pacientes sin problemas cardiovasculares (3.4% *versus* 11.3%, p=0.025, OR 0.28 [95% CI 0.06-0.89]). Asimismo, la frecuencia del alelo *CCR5*Δ32 también fue significativamente menor en pacientes con patología CV (1.7% *versus* 5.8%, p=0.024, OR 0.28 [95% CI 0.06-0.88]).

Posteriormente, construimos una regresión de Cox para analizar la influencia de esta variante en la tasa de aparición del primer evento CV, no encontrando una influencia significativa de la variante genética.

Esta deleción en *CCR5* no supuso ninguna influencia significativa sobre los marcadores de patología CV subclínica. Sin embargo, tras ajuste por las previamente mencionadas variables, observamos que los portadores de la deleción se asociaron significativamente a una mayor vasodilatación dependiente de endotelio (FMD%; p=0.024), encontrándose el valor medio de este parámetro en dicho grupo por encima del límite designado para la normalidad.

Análisis del gen *RETN* (rs1862513)

No se observaron diferencias significativas en las frecuencias de alelos o genotipos entre pacientes con o sin patología CV en el análisis del polimorfismo rs1862513 del gen *RETN*. Tampoco se observó una influencia significativa de este polimorfismo en el riesgo de patología CV utilizando modelos de regresión logística ni de regresión de Cox, antes o después de ajuste por las variables ya mencionadas.

Con respecto a la patología CV subclínica, los portadores del alelo menor se asociaron a un menor valor de FMD% (p=0.03), aunque esta diferencia dejó de ser significativa tras ajuste (p=0.20).

Análisis del gen *ADIPOQ* (rs266729 y rs1501299)

No se observaron diferencias significativas en las frecuencias de alelos o genotipos de ambos polimorfismos entre pacientes con o sin patología CV. En el análisis de regresión logística múltiple ajustado por género, edad de diagnóstico de la AR, tiempo de seguimiento y presencia o ausencia de epítopo compartido y factores de riesgo CV clásicos, de nuevo, no se observó ninguna asociación significativa entre alelos o genotipos de ambas variantes y presencia de patología CV. Tras analizar el grado de desequilibrio de ligamiento entre ambos polimorfismos (r^2=0.05), estimamos la frecuencia de las combinaciones alélicas formadas por ambas variantes, en pacientes con y sin patología CV, no encontrando diferencias significativas.

Con respecto a la influencia de ambos polimorfismos en patología CV subclínica, no encontramos diferencias significativas en espesor intima-media carotídeo, vasodilatación dependiente e independiente de endotelio entre genotipos, alelos ni combinaciones alélicas, antes o después de ajustar por género, edad y duración de la AR

en el momento de la realización de la ecografía y presencia o ausencia de epítopo compartido y factores de riesgo CV clásicos.

Análisis del gen *VEGFA* (rs2010963 y rs1570360)

No se observaron diferencias significativas en las frecuencias de alelos o genotipos de ambos polimorfismos entre pacientes con o sin patología CV. En el análisis de regresión logística múltiple ajustado por las variables previamente mencionadas, no se observó ninguna asociación significativa entre alelos o genotipos de ambas variantes y presencia de patología CV. Tampoco cuando se analizaros las combinaciones alélicas de ambos polimorfismos.

Con respecto a la patología CV subclínica, ninguno de los polimorfismos mostró una influencia significativa sobre los valores de de espesor intima-media carotídeo, vasodilatación dependiente o independiente de endotelio.

Análisis del gen *GHSR* (rs509035, rs512692 y rs2922126)

No se observaron diferencias significativas en las frecuencias de alelos o genotipos de los tres polimorfismos entre pacientes con o sin patología CV. En el modelo de regresión logística, antes o después del ajuste por las variables previamente mencionadas, no se observó influencia significativa de ninguno de los tres polimorfismos en el riesgo de patología CV. En el análisis condicional, la homozigosidad del alelo menor de las variantes rs509035 y rs2922126 fue asociado a un efecto protector significativo (OR 0.17 [95% CI 0.038-0.75], p=0.019 y OR 0.52 [95% CI 0.27-0.98], p=0.04, respectivamente). Sin embargo, tras ajuste por las variables ya mencionadas, el efecto dejó de ser estadísticamente significativo (p=0.08, p=0.30, respectivamente).

Con respecto a los haplotipos formados por los tres polimorfismos, tras ajuste, no se observó una asociación significativa con patología CV.

Conclusiones

Este trabajo contribuye a profundizar en la comprensión de las bases genéticas de la patología CV en la AR. Hemos estudiado el papel, como factores de riesgo de enfermedad CV clínica y subclínica, de varios polimorfismos localizados en genes implicados en la patogénesis de ambas afecciones. Todos los polimorfismos analizados habían sido previamente asociados con enfermedades CV o metabólicas, en estudios realizados en población general o en subgrupos (como son diabéticos de tipo 2), de origen caucásico. Es importante señalar que en este estudio es la primera vez que estos polimorfismos han sido analizados como factores de riesgo CV en la AR. Entre las variantes estudiadas de los genes *TNFA*, *CCR5*, *RETN*, *ADIPOQ*, *VEGFA* y *GHSR*, solo las pertenecientes a los dos primeros, *TNFA* y *CCR5*, mostraron una asociación estadísticamente significativa con patología CV.

INTRODUCTION

2. INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized, at the articular level, by persistent synovitis with synovial hyperplasia, cell activation and invasion of the synovium into the adjacent bone and cartilage [1]; and at the systemic level, by systemic inflammation, autoantibodies production and attendant comorbidities [2,3]. RA is a complex genetic disease in the sense that, not only several genes, environmental and stochastic factors interact to cause pathological events [4], but furthermore, this condition is considered a clinical syndrome spanning several disease subsets [5]. These different subsets involve several inflammatory cascades [6], all leading towards a final common pathway in which are present persistent synovial inflammation and associated damage to articular cartilage and underlying bone.

2.1. Pathogenesis of RA

The heritability of this condition [7], together with the presence, in a subset of patients, of autoantibodies that precede the clinical onset [8-10], supports a pathogenetic model in which autoimmune propensity is long-established by the time joint inflammation is 'triggered'. This concept is supported by the observation of an altered cellular composition of the synovial membrane before clinically overt joint inflammation (prearthritis) [11,12], sharing many of the pathological features of established disease [13]. Also, elevated acute phase reactants and even serum cytokines might be reflectors of these early inflammatory changes of the synovial membrane [14]. A triad of genetic factors, environmental factors and nascent breakdown of tolerance directly would lead to changes of the synovial membrane and would pave the way for the initiation of arthritis.

Both family and twin studies support a strong genetic component of susceptibility to RA, with an heritability estimated in 60% [7]: its prevalence can be up to 12% in first-degree relatives of patients with the disease, siblings of patients have an increased risk of disease, compared with that of the general population, between 3 and 15 fold [15] and concordance rates are much higher in monozygotic (15%) than in dizygotic twins (3.6%) [16]. Since RA is a complex disease, it is expected that multiple genetic loci contribute to disease risk, and that the effect of each individual allele is modest.

The strongest and best-known genetic association in RA was found in the Human Lymphocyte Antigen (HLA) region, which lies in the Major Histocompatibility Complex (MHC) in chromosome 6p21, explaining approximately 30% of the total genetic component of susceptibility. The most important association is located in the *HLA-DRB1* gene, with a variety of specific alleles conferring different risks [17,18]. These alleles were collectively termed the shared epitope (SE), since they encode a conserved sequence of amino acids in the third hypervariable region of the *DRB1* chain (Q/R-K/R-R-A-[A]) [19]. The association between *HLA-DRB1* and disease has been consistently replicated in different ethnic population, despite the fact that SE allele frequencies differ significantly between ethnic groups [20]. Also, HLA-SE alleles have been shown to be associated with outcome prediction [21-23] and to interact with environmental factors, such as smoking [24-26]. Several studies have shown evidence of additional RA loci, besides the HLA genes, in the MHC region [27,28], although the interpretation of these associations is difficult by the presence of strong linkage disequilibrium (LD) across this region [29].

The second largest genetic risk to the development of RA is conferred by a variant within the protein tyrosine phosphatase nonreceptor 22 (*PTPN22*) gene (rs2476601, 1858C >T, R620W), located on chromosome 1p13 [30-32]. This observation is the most robust and reproducible genetic association with RA outside the HLA region in

populations of European descent. *PTPN22* encodes a lymphoid-specific phosphatase that inhibits T-cell receptor signaling. This polymorphism results in a gain-of-function of this phosphatase [33,34], provoking the suppression of T cell receptor signaling. This phenomenon could lead to the survival of autoreactive T cells during thymic development and/or a weaker activity of T-regulatory cells [33,35,36].

Another variant associated to disease was found in the *STAT4* gene [37-39]. This protein is a key transcription factor involved in the signaling pathways triggered by Interleukin (IL) -12, IL-23 and type I interferons (IFN) [40], promoting the differentiation of CD4+ T cells to Th1 and Th17 phenotypes, both cell types involved in RA pathogenesis [41,42].

TRAF1/C5 has been identified of as a new risk factor locus [43,44], but also as a predictor of mortality in RA patients [45]. Both Tumor Necrosis Factor (TNF) receptor-associated factor 1 (*TRAF1*) and component 5 (*C5*) are involved in immune regulation and are attractive candidate genes for RA. *TRAF1* is involved in TNF receptors (TNFR) I and II, and CD40 signaling pathways. Mice deficient in *Traf1* show increased T-cell proliferation and activation after the stimulation of TNF or T-cell receptors [46]. On the other hand, the complement system is known to be involved in the pathogenesis of RA [47].

The intergenic region of chromosome 6q23, between the genes *OLIG3* and *TNFAIP3* has also been associated to RA [48-52], with at least three SNPs independently associated, two of them conferring susceptibility (rs6920220 and rs5029937) and one protection (rs13207033). Furthermore, this region has also been reported to be associated with the rate of joint destruction in RA patients [53]. TNFAIP3 is a potent anti-inflammatory protein mainly involved in the negative-feedback regulation of NFκB but it has been shown to have other functions, such as antiapoptotic [54] and tumor suppressor [55] activity.

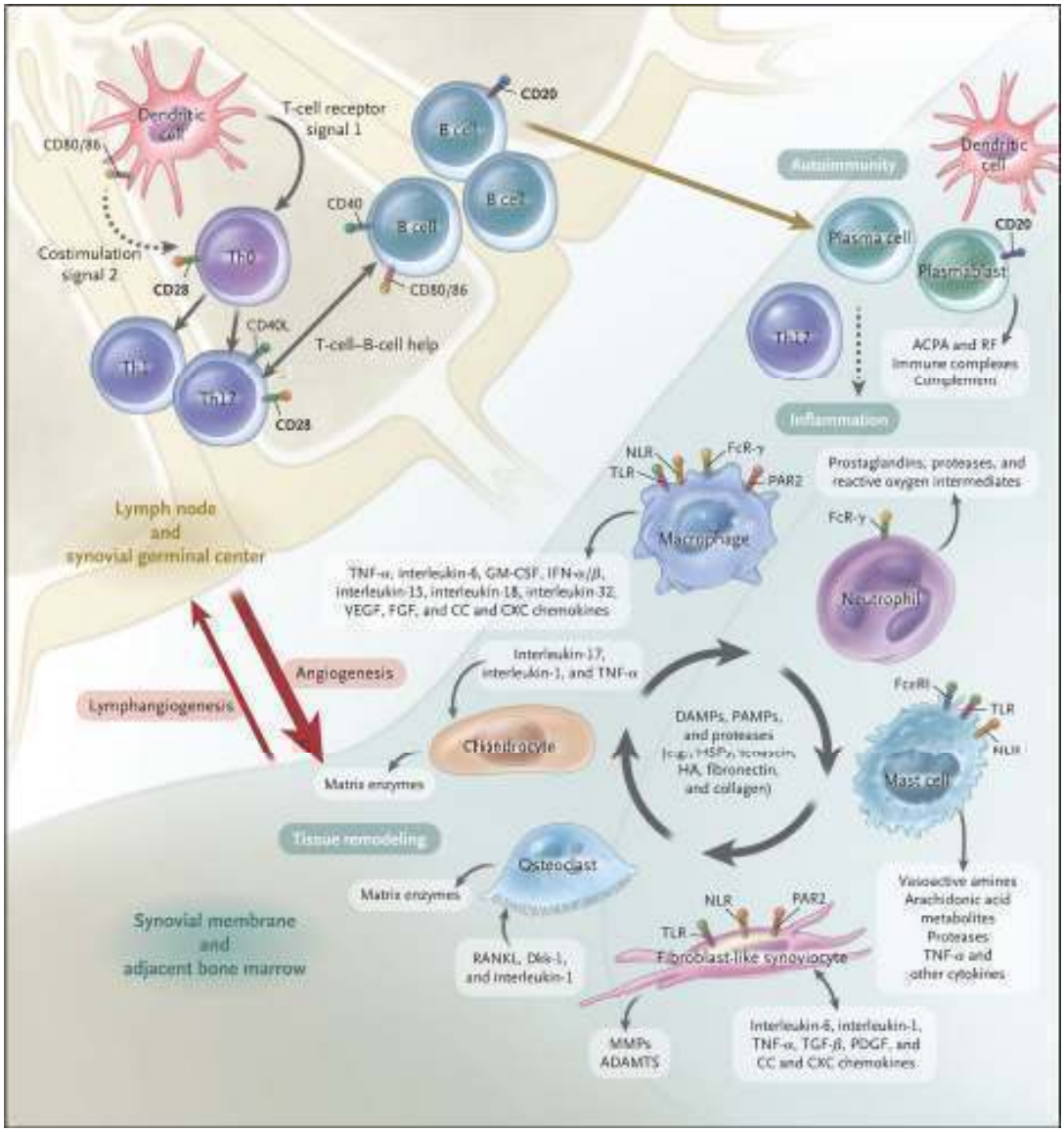
Several studies investigating the possible role of *CTLA4* markers in RA susceptibility have been carried out [56-58]. This molecule is a costimulatory receptor expressed on activated T cells, which downregulates the T-cell response.

Other genes also have been associated to RA, such as *IL2RB* [59,60], the chromosomic regions 12q13 [60,61], 10p15 [60,61] (including the gene *IL2RA* [60]), 4q27 [61,62], *CD40* [61,63] (which has been associated with a higher rate of joint destruction in anticyclic citrullinated peptide antibodies (anti-CCP) positive RA patients [64]), *MMEL1*/*TNFRSF14* [60,61], *AFF3* [59,65].

Nowadays, a integrated model of chronic inflammation explain its perpetuation on the basis of numerous positive feedback loops and failed regulations, involving many cell types, both from the adaptive and innate immune response, plus from host tissue cells, such as synovial fibroblasts, chondrocytes and osteoclasts (**Figure 1**).

The synovial lesion in RA contains a macrophage/fibroblast-rich lining layer overlying interstitial tissues containing a plethora of activated leucocytes including macrophages, dendritic cells (DCs), B cells, CD4/CD8 T cells, mast cells, natural killer (NK) and NKT cells. Angiogenesis within the hypertrophic synovial sublining is an important early event in RA, supporting this infiltrate, driven by the increased metabolic demands and hypoxia of the expanding inflammatory tissue [66,67]. New vessels in the RA pannus are of a characteristic branching morphology [68]. This process reflects the relative abundance of proangiogenetic factors, which exceeds the machinery normally necessary for cellular egress via lymphatics [67]. Vascular endothelial growth factor (VEGF), one of the most important promoters of neovascularization, stimulates the invasive proliferation of these endothelial cells.

Figure 1: Adaptive and Innate Immune Processes within the Joint in Rheumatoid Arthritis



ACPA: anti-citrullinated protein antibodies, ADAMTs: a disintegrin and metalloproteinase with thrombospondin motifs, DAMPs: damage-associated molecular patterns, FGF: fibroblast growth factor, GM-CSF: granulate-macrophage stimulating factor, HSPs: heat shock proteins, INF: interferon, MMPs: matrix metalloproteinases, NLRs: nucleotide-binding domain leucine-rich repeat proteins, PAMPs: pathogen-associated molecular patterns, PAR2: proteinase-activated receptor 2, PDGF: platelet-derived growth factor, RF: rheumatoid factor, TGF: transforming growth factor, TLRs: toll-like receptor, TNF: tumor necrosis factor, VEGF: vascular endothelial growth factor. Adapted from McInnes IB, Schett G. N Engl J Med 2011;365:2205-2219.

Enriched populations of mature antigen-presenting DCs are seen in established RA lesions [69]. The validity of a model in which DC antigen presentation is central to the initiation of this condition has not been yet confirmed due to the lack of a dominant arthritogenic autoantigen. DCs might alternatively contribute to the circumvention of central tolerance by priming the immune system to respond to self antigen that has been post-translationally modified, as might occur following citrullination [70].

Fibroblast-like synoviocytes (FLSs) represent an important player in synovitis initiation, propagation or both, and synovial stromal cells are key in creating a microenvironment that favours inflammatory-cell retention and the perpetuation of immune pathology in RA [71]. These and other cells like B cells and macrophages, express numerous innate immune sensing pathways such as Toll-like receptors (TLRs) [72], NOD-like receptors and molecular components of the inflammasome are expressed [73,74]. TLRs are able to recognize specific structures of invading pathogens [75], as well as endogenous ligands, such as hyaluronan fragments [76]. Ex vivo studies using human RA synovial tissue cultures implicate TLR-dependent pathways in aberrant (auto)antigen presentation, cytokine and matrix metalloproteinase (MMP) production [77,78]. FLSs are also capable of secreting chemokines, largely responsible for the recruitment and retention of leukocytes at these sites [79]. Finally, FLSs have been shown capable of autoantigen presentation to T cells [80].

T cells are one of the most abundant cell types in RA synovium, comprising 30–50% of synovial tissue cells [81]. The majority are CD4+, although CD8+ T cells are also present. RA was historically considered a Th1 disease. However, in very early stages of the disease, Th2 type responses may predominate to promote autoantibody production [82]. Also Th17 cells represent a significant player in human autoimmune inflammation [83].

These cells secrete IL-17 stimulated by IL-6, Transforming Growth Factor (TGF) β and IL-23 [84]. Through direct or indirect effects on various cell types, this highly pleiotropic cytokine can induce inflammation, angiogenesis, osteoclastogenesis, and breakdown of bone and cartilage. Several studies have demonstrated elevated IL-17 levels in blood and synovium of RA patients, with correlations between synovial levels and joint damage [85].

T-regs appear to be overrepresented in the synovium of RA patients, and evidence suggests that this may reflect their functional impairment within the autoimmune microenvironment they are supposed to suppress [86]. Moreover, evidence suggests that the T-cell population as a whole is systemically abnormal in RA, having an excessive proliferative history; the result is a contracted T-cell receptor repertoire and an immunosenescent phenotype [87].

Ectopic germinal-centre-like structures in the synovia of some patients create a spatially organized microenvironment ideally suited to humoral immune responses. Evidence shows that such structures can support B cell maturation and class switching and thereby promote autoantibody production [88]: B cells can process and present antigenic peptides to pre-primed CD4+ T cells, resulting in classical adaptive humoral responses, and similar cognate interactions with B cells may prime naive T cells in some cases [89]. In fact, co-ligation of the B-cell receptor (BCR) and TLR appears sufficient to induce B-cell activation and antibody production independent of T-cell 'help', and this pathway is a proven mechanism of rheumatoid factor (RF) autoantibody generation in experimental arthritis [90]. RF recognizes the Fc portion of IgG molecules and is a characteristic, although not specific, hallmark of human RA, with approximately 70% of patients being 'seropositive' [91]. Deposition of RF immune complexes occurs in the rheumatoid synovium, where these complexes fix complement, thereby reinforcing B-

cell activation and perpetuating an Fc-receptor-mediated positive-feedback loop [89]. As with ACPA, the precise role of this autoantibody has therefore yet to be fully elucidated, but the RA synovium appears to provide a context in which B-cell tolerance may be broken, autoantibody production enhanced, and an aberrant immune response upheld.

Cells of the monocyte–macrophage lineage and FLSs are important effectors of cartilage and bone destruction in RA. A number of immune pathways such as TLR ligation [92,93] , contact with activated T cells [94], cytokines including IL-17 [95], Fcγ-receptor ligation by immune complexes, and the action of serine proteinases secreted by mast cells and neutrophils [96], converge on these cell types, resulting in the deregulated production of pro-inflammatory cytokines such as TNF-α and IL-1β. Pro-inflammatory cytokines induce chondrocytes, macrophages and FLSs to secrete cartilage-degrading enzymes, including MMPs 1 and 13 and ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) 4 and 5. This is most evident at the interface between cartilage and inflamed synovium – the so-called cartilage–pannus junction, accounting for the striking invasiveness of the inflammatory lesion in RA [97].

2.2. RA and Comorbidity

RA is a condition associated to a disproportionately increased mortality: patients have about a 50% increased risk of premature mortality, and their life expectancy is decreased by 3 to 10 years compared with the general population [98,99]. Despite the substantial improvements in RA management in recent years, there is little epidemiologic evidence for the improvement of mortality: most recent studies demonstrated no significant reduction in mortality in different RA populations worldwide [100,101], and even some authors reported an increased RA-related mortality [102].

Moreover, the dramatic secular declines in the overall mortality rates for the general population [103] has leaded to a widening mortality gap between subjects with RA [104], particularly those positive for RF, and the general population [105]. This is surprising, considering data suggesting that there may be a temporal trend toward milder disease in RA [106,107] and the recent emphasis on tight control of disease activity for all RA patients.

Besides socio demographical prognosis factors such as older age and male sex [101], other independent significant determinants of increased mortality risk are inflammatory markers, such as C reactive protein (CRP) and erythrocyte sedimentation rate (ESR), complex measures of RA activity, such as the Disease Activity Score [101,108], surrogated measures of cumulative disease activity, such as radiographic joint damage and low bone mineral density [109], and clinical indicators of disease severity, including the occurrence of extra-articular manifestations and features of other systemic autoimmune disorders [110,111]. All of these markers, to any extent, are a reflection of the magnitude of the inflammatory burden of this disease. However, other factors independents of RA activity, such as presence of auto antibodies, are also useful for the prediction of poor outcomes and increased mortality in RA patients [101,112], even in the absence of clinical manifestations of rheumatic disease [112,113]. Genetic risk factors for RA also have been linked to an increased mortality risk in the patients: *HLA-DRB1* gene [114] and *TRAF1/C5* [115,116]. Conversely, the *PTPN22* locus was not associated with mortality [114].

The pattern of cause-specific mortality in RA seems to be relatively constant over time, with the major attributable causes of death in RA similar to those of the general population [101,117], therefore being the main cause cardiovascular (CV) diseases [118].

2.3. RA and Cardiovascular disease

Many epidemiological studies [119,120] have reported in a consistent way the increased risk of nearly all forms of CV disease [121-127] and the increased CV mortality [118,123,126-132] among RA patients. The increase in risk and mortality and the reduce life-span affect equally men and women [118,128,133].

Relative to the general population, patients with RA are at an approximately 1.5–2-fold higher risk of myocardial infarction (MI) [119,123,127,129,134], a 1.4–2.7-fold higher risk of stroke [121,123,125,127,135] and a 1.3–1.7-fold higher risk of heart failure [121,136,137]. Regarding mortality rate for any CV disease, RA patients have an overall standardized mortality ratio ranging from 1.5 to 5.0 [132,138], with a 59% increase in death from ischemic heart disease (IHD) and a 52% increase in death from cerebrovascular disease [118]. This morbidity leads to a reduction in lifespan [128,139,140], an important impact on quality of life [141] and an increase in health care costs [142]. Finally, RA patients have a higher prevalence of preclinical atherosclerosis [143-145], an early measure of arterial disease and a validated biomarker of CV risk [146]. Actually the absolute CV risk in RA subjects was equivalent to that in non-RA subjects who were 5–10 years older [147].

In view of these observations, RA can be reasonably considered an independent CV risk factor [148,149], such as diabetes mellitus (DM) [149]: the incidence and prevalence of CV disease in RA are increased to a degree comparable with that in type 2 diabetes mellitus (T2DM), with associated preclinical arterial wall atherosclerotic damage [132,148-150].

CV disease in RA exhibits a peculiar pattern characterized by its premature development, its silent nature and unfavorable CV outcomes [102,151].

Traditional CV risk factors such as smoking, diabetes mellitus, hypertension or hyperlipidemia are independently associated with CV events [152,153], subclinical

atherosclerosis [154,155] and the increased risk of CV mortality in RA [156]. However, these risk factors are not substantially different in RA compared to control populations [122,157,158]. Smoking is the only clearly more frequent classical CV risk factor among RA patients comparing with healthy controls [158]. DM seems also to be more prevalent among RA [158]. Besides, RA itself is an important modifier of these traditional CV risk factors, appearing to have a lower relative impact on CV risk than expected [152,159]. This implies the presence of RA-specific risk factors competing with traditional CV risk factors for their impact on CV disease. This finding has led to the notion that traditional CV risk factors may not account for the difference in CV disease risk between RA patients and controls and therefore RA-associated factors, such as systemic inflammation, must be the sole contributors to the observed increase in CV risk [159-161]. It is likely that this inflammatory amplification of vascular risk is mediated both by direct and indirect effects. For example, high levels of systemic cytokines can contribute directly to endothelial dysfunction and a state of hypercoagulation [161], but can also indirectly contribute to vascular disease by influencing the qualitative nature of lipid particles [162].

Also, due to this distortion of risk, the CV risk profile in RA may not be directly comparable with that in the general population and being underestimate as in other chronic diseases such as chronic kidney disease and diabetes [163,164].

2.4. Atherosclerosis and Inflammation

Atherosclerosis has a chronic inflammatory aetiopathogenesis [165-168]. Atherosclerotic plaques are characterized by an accumulation of blood-borne inflammatory and immune cells (mainly macrophages and T cells, but also mast cells [169], a few B cells [170] and NKT cells [171]), as well as vascular endothelial cells, vascular smooth muscle cells (VSMCs),

DCs [172], extracellular matrix, lipids and acellular lipid-rich debris [170]. Early lesions called 'fatty streaks' consist of subendothelial depositions of lipids, macrophage foam cells loaded with cholesterol and T cells [173]. Over time, a more complex lesion develops, with apoptotic as well as necrotic cells, cell debris and cholesterol crystals forming a necrotic core in the lesion, surrounded by a cap of VSMCs and a collagen-rich matrix. The shoulder region of the plaque, which is where it grows, and the interface between the cap and the core, have particularly abundant accumulations of T cells and macrophages [170,174]. Many of these immune cells show signs of activation and produce pro-inflammatory cytokines such as IFN-γ and TNF–α [175].

Atherosclerosis shares many similarities with other inflammatory/autoimmune diseases [176] (**Table 1**). Indeed, atherosclerosis and RA share a number of similarities [177], including activation of monocytes, T, B, vascular endothelial and mast cells, production of proinflammatory cytokines such as TNF–α, IL-6, IL-12, IL-1 and IL-18, and heightened expression of leukocyte adhesion molecules (LAMs) and MMP [176,178]. These parallels suggest possible mechanisms whereby patients with RA develop an increased risk of atherosclerosis and early death. The increased background level of chronic inflammation, in part due to the release to the systemic circulation of proinflammatory cytokines, might boost a number of proatherogenic functions in the liver, adipose tissue, skeletal muscle, and vascular endothelium, including insulin resistance (IR), dyslipidemia, endothelial activation, and prothrombotic and antifibrinolytic effects [179,180]. All these processes would confer predisposition to CV disease and/or augment its pathogenesis and put an individual at greater risk of developing an acute coronary syndrome or suffering secondary complications thereafter [167,168,181]. Patients with RA have elevated levels of the acute-phase reactant CRP [182], a marker of inflammation associated with increased CV risk [183]. This condition is also associated to an increased

carotid artery intima-media thickness (IMT) [144,145], an early measure of arterial disease and a validated biomarker of CV risk [146].

Table 1. Inflammatory / immunologic similarities between atherosclerosis and rheumatoid arthritis.

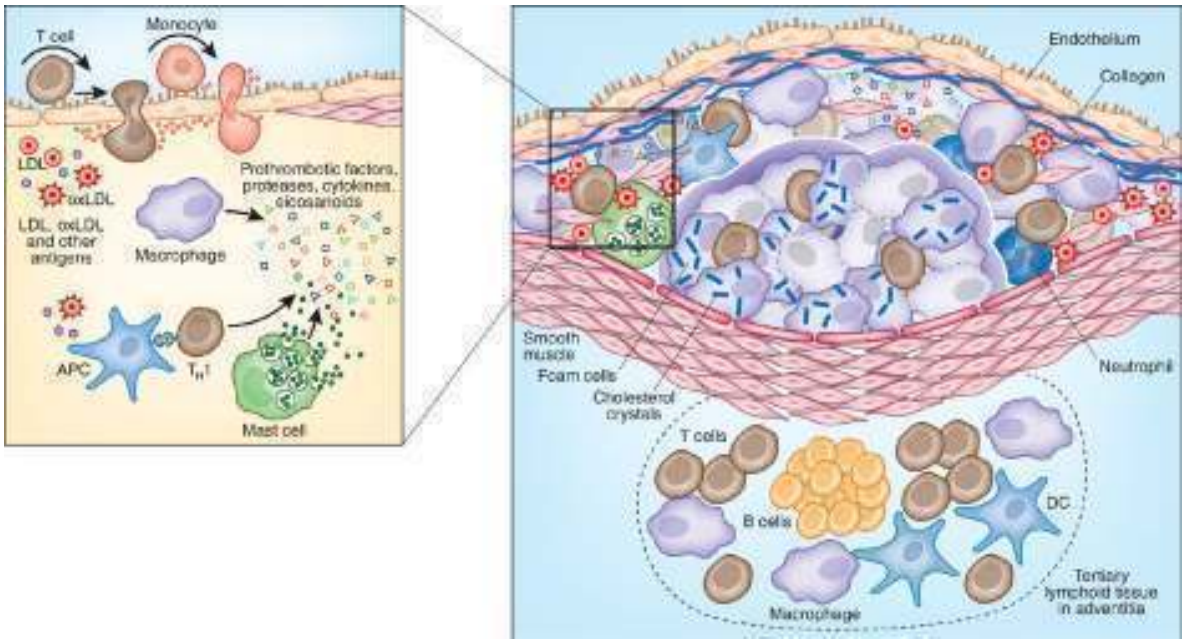
	Atherosclerosis	**Rheumatoid Arthritis**
Macrophage activation		
TNF-α	↑	↑
Metalloproteinase expression	↑	↑
IL-6	↑ (UA)	↑
Mast-cell activation	↑	↑
T-cell activation		
Soluble IL-2 receptor	↑ (UA)	↑
$CD3^+DR^+$	↑ (UA)	↑
$CD4^+CD28^-$	↑ (UA)	↑
$CD4^+$IFN-γ	↑ (UA)	↑
TH1/TH2 balance	↑ TH1	↑ TH1
B-cell activation		
Autoantibodies (ox-LDL, HSP)	0 or ↑	0 or ↑
Rheumatoid factor	0	↑
C-reactive protein	↑ (UA)	↑↑
Adhesion molecules (VCAM-1, ICAM-1, E-selectin, P-selectin)	↑	↑
Endothelin	↑	↑
Neoangiogenesis	↑	↑
Possible antigens	HSP, ox-LDL, Infectious agents	Collagen II, cartilage antigens, HSP, infectious agents

HSP = heat-shock protein; ICAM-1 = intercellular adhesion molecule–1; IFN-γ = interferon-γ; IL = interleukin; ox-LDL = oxidized low-density lipoprotein; TH1 = helper T cell type 1; TH2 = helper T cell type 2; TNF-α = tumor necrosis factor–α; UA = unstable angina; VCAM-1 = vascular cell adhesion molecule-1; ↑ = increase; ↓ = decrease; 0 = no change. Adapted from [176].

2.5. Pathogenesis of Atherosclerosis

Endothelial cell activation contributes early on to atherosclerosis development (**Figure 2**). These cells form the innermost surface of the artery wall and under normal circumstances do not bind leukocytes. Before plaque formation occur early changes that include elevated expression of LAMs and chemokines that recruit inflammatory cells into the arterial wall.

Figure 2: Anatomy of the atherosclerotic plaque.



Adapted from [173].

Triggers of endothelial cell dysfunction, such as high levels of low-density lipoprotein (LDL) cholesterol [184] and others (smoking, hypertension, hyperglycemia…) are associated with increased expression of LAMs, such as vascular cell adhesion molecule 1, which binds monocytes and T lymphocytes, the very cells found in early atherosclerotic plaques. In the case of LDL molecules, once in the arterial wall they become targets for oxidative (by reactive oxygen species) and enzymatic attack (by myeloperoxidase and lipoxygenases). As a consequence, phospholipids are released,

with the capability of initiate innate inflammatory responses and activate endothelial cells and macrophages to express several types of LAMs and chemokines [185,186]. In fact, the lack of these molecules is associated to a reduction of atherosclerotic lesions in mice models [187,188]. The expression of these LAMs is also stimulated by different cytokines such as IFN-γ, TNF-α, IL-1, and IL-4 [189].

Once adherent to the endothelium, leukocytes migrate into the underlying intima in response to chemokines including monocyte chemotactic protein 1, RANTES (CCL5), and fractalkine [190]. Macrophage-colony stimulating factor (produced by endothelial cells and VSMCs [191]) induces monocytes to differentiate into macrophages [192]. This process is necessary for the development of atherosclerosis [193] and it is associated with upregulation of pattern recognition receptors including scavenger receptors (SR) and TLR [192]. SR mediate uptake of oxidized LDL (oxLDL) particles leading to the formation of foam cells, while TLR initiate signalling cascades that lead to inflammatory activation. Uptake by SR does not lead directly to inflammation but can lead to MHC-class-II-restricted antigen presentation of internalized material, thereby linking innate and adaptive immunity [194].

DCs that patrol arteries may take up LDL components for subsequent antigen presentation in regional lymph nodes. In the normal artery wall, resident DCs are thought to promote tolerization to antigen by silencing T cells; however, danger signals generated during atherogenesis may activate DCs, leading to a switch from tolerance to the activation of adaptive immunity [195].

In the T cell population of human plaques, CD4+ cells dominate over CD8+ cells [196]. They are not as abundant as macrophages. Most T cells are of the TCRαβ type and often found in clusters in shoulder regions of the lesion. MHC class II-expressing macrophages and DCs can be detected adjacent to these T cells, suggesting an ongoing

immune activation [175,196]. Clonal expansion of CD4+ T cells has been demonstrated in lesions from humans and mice models [197,198], suggesting that antigen-specific reactions take place in the plaque. Most T cells in atheroma display an activated/memory phenotype [196] and the proportion of activated T cells is particularly high in culprit lesions causing acute coronary syndrome [166,199].

Regarding the "athero-antigens", a few have so far been described. oxLDL is present in lesions, internalize by antigen-presenting cells through the SR pathway, and after proteolytic processing, fragments of the protein component are displayed in cell surface bind to MHC class II molecules. Oxidative modification of LDL breaks tolerance and oxLDL-reactive T cells are localized in plaques, lymph nodes, and in the blood of patients with atherosclerosis [200,201]. Most oxLDL-reactive CD4+ T cells have a Th1 phenotype [200], and are able to promote atherogenesis, a conclusion supported by adoptive-transfer studies in severe combined immunodeficient mice lacking *Apoe* [202]. oxLDL is also able to trigger antibody formation [203]. Antibodies were found to promote clearance of oxLDL, and in some studies, predict coronary events [204]. Other "athero-antigen" identified was heat shock protein-60 [205-207] and antigens derived from certain pathogenic microorganisms, such as Chlamydia pneumoniae [208].

Atherosclerosis is driven by the Th1 response [167,173]. This notion is supported by the finding that the prototypic Th1 cytokine, IFN-γ is produced locally [200,209,210] mainly by Th1 cells, as well as by CD8+ T cells, NKT cells, NK cells, and IL-18-stimulated VSMCs [211,212]. Other Th1 inducing cytokines, such as IL-12 and IL-18 [196,213] can also be found. Consequently, mice models lacking IFN-γ or its receptor [214], IL-12 [215], IL18 [216], TNF-α [217] or T-bet [218] (a transcription factor leading to Th1 differentiation), were

associated to a significant reduction of atherosclerosis. Analogously, administration of a Th1 inhibitory drug, pentoxifyllin [219] also reduces disease.

IFN-γ is a major proatherogenic cytokine, fostering inflammation and extracellular-matrix destabilization. It promotes macrophage and endothelial activation with upregulation of LAMs and MHC class II molecules, enhanced cytokines, chemokines, radicals, proteases, and coagulation factors production and secretion, and inhibits cell proliferation, both endothelial [220] and VSMCs [221], collagen production by the latter [222], and cholesterol efflux [167]. Decreasing the cell and collagen content of the fibrous cap might reduce the stability of the plaque and facilitate its rupture.

Contradictory data have also been presented for Th17 cells. Although IL-17 mRNA seems to be present at low abundance in atherosclerotic plaques, IL-17 protein has been detected in several cell types of human atherosclerotic tissue, including T cells, mast cells, B cells, neutrophils and VSMCs [223].

Another cytokine with a key role in atherosclerosis is TNF-α, which is produced by Th1 cells, macrophages, and NK cells. It is pro-inflammatory and cytotoxic, inducing the production of reactive oxygen and nitrogen species, proteolytic enzymes and pro-thrombotic tissue factor by endothelial cells, and modulating fibrinolysis [224,225]. Also inhibits metabolic enzymes including lipoprotein lipase, which leads to the accumulation of triglyceride-rich lipoproteins in the blood. Such lipoproteins, and the TNF-α levels, have been associated with heart disease in clinical studies [226]. Gene targeting of *TNFA* leads to reduced atherosclerosis [216,217]. In relation with all these functions, TNF-α may modulate the progression of atherosclerotic plaque. Besides, RA patient levels of endothelial progenitor cells (a possible contributor to intimal repair) are low and inversely correlated with TNF-α concentration [227,228].

Inflammation promotes not only the initiation and progression of the atherosclerotic lesion, but also the development of the complicated or disrupted lesion. The most common form of physical disruption that occurs in the advanced or complex plaque involves rupture of the plaque's fibrous cap [229], releasing some of the sequestered contents of the atheroma core and allowing prothrombotic tissue factor to contact coagulation factors in the bloodstream [168]. As mentioned before, inflammatory mechanisms can facilitate disruption or fracture of the fibrous cap. T lymphocytes participate in the inflammatory processes that inhibit the synthesis and promote the degradation of the interstitial collagen matrix that confers tensile strength upon the fibrous cap [230]. IFN-γ limits collagen I and III synthesis by VSMCs (both basal production and TGF-β stimulated production) [222]. Proinflammatory cytokines such as CD40 ligand, IL-1, or TNF-α may also promote plaque disruption by stimulating the expression in endothelial cells, macrophages, and VSMCs of collagen-degrading MMPs, enzymes that catabolize collagen and other macromolecules of the arterial extracellular matrix [231,232]. Postmortem evaluation of coronary arteries from patients with MI found large proportions of activated mast cells at the site of plaque rupture, as well as release of serine proteinases (chymase and tryptase), which may promote collagenolysis via activation of MMP proenzymes [233].

In addition, activated T cells may promote thrombogenesis via expression of the CD40L, which stimulates macrophages to produce the potent procoagulant tissue factor [234]. Proinflammatory cytokines such as IL-1 and TNF-α may also promote thrombosis by increasing tissue factor expression in endothelial cells [235].

2.6. CV Risk Factors

2.6.1. Traditional Risk Factors

In patients with established disease, RA duration has been a fairly consistent RA-specific factor independently associated with CV events [129] and subclinical atherosclerosis [154,236-238], although this association has not been uniform across studies [119,135,136,144,239,240]. There is evidence even supporting an increase risk of CV disease before RA diagnosis [134,241]. This phenomenon could be due to the fact that autoimmunity and systemic inflammation have been shown to precede clinical RA [242]. Also, there is a delay of months to years between articular symptoms onset and RA diagnosis [243] in which CV events could manifest.

Regarding mortality [244], a difference in observed and expected mortality emerged only after 10 years of followup. Acordingly, other studies have not find an increase in mortality before the onset of RA symptoms [245] or in the first 10 years after diagnosis [246].

2.6.1.1. Hypertension

Hypertension is common in patients with RA but it remains unclear whether it is more common than in a comparable general population of subjects without RA [247], although in a recent metaanlysis, the prevalence of this condition was similar to that of subjects without RA [158]. There is evidence for underdiagnosis and undertreatment of hypertension in patients with RA [248] despite the fact that it is the major determinant of target organ damage in these patients [249]. A link between low-grade inflammation and hypertension has been suggested from general population studies [247], although in RA patients this association has not been consistently observed [248,250].

Besides systemic inflammation, also physical inactivity, obesity and medications such as non-steroidal anti-inflammatory drugs, cyclo-oxygenase II inhibitors, corticosteroids, leflunomide and cyclosporine, may lead to high rate and poor control of hypertension in patients with RA [247].

2.6.1.2. Hyper/Dyslipidaemia:

Alterations of the lipid profile have been extensively reported in RA. Although contradictory results were observed regarding the lipid profile before RA onset [251,252], it is considered that before RA symptoms onset and in concordance with raised levels of immunoglobulin M RF, anti-CCP [253] and CRP levels [254] [255], there is already a proatherogenic lipid profile. Also, in naïve treated patients with early RA [256-258], this lipid profile may be detected.

Hyperlipidaemia (high total cholesterol (TC) or LDL) appears to be less common in RA than in non-RA subjects [152,259]. In fact, there are studies showing a lower TC and LDL levels in subjects with an active RA [260,261], as a result from their increased catabolism or increased retention (subendothelial deposition) rather than decreased lipid production [262,263]. This subendothelial lipid deposition might explain the paradox of lower cholesterol levels and increased CV risk in RA [259,264-266].

On the other hand, dyslipidaemia (alterations of individual lipid components and their ratios as defined by specific criteria) may affect up to half of all patients with RA in hospital care [264].

It is important to take into account that antirheumatic treatments, including glucocorticoids, hydroxychloroquine, gold, ciclosporin, as well as the biological agents anti-TNFα, rituximab and tocilizumab, affect lipid levels [267,268].

In vitro animal model and human in vivo studies in subjects without RA demonstrate that the interplay between inflammation and lipid components is far more complex than just alterations of their serum levels [269]: for example, acute phase proteins and inflammation can alter composition and function of high-density lipoprotein (HDL) cholesterol, the enzymes fundamental to its metabolism or the enzymatic content of HDL itself , increasing the susceptibility to oxidation and convert HDL in a more pro-oxidant, pro-atherogenic complex. Such inflammation-induced alterations of structure and function are not confined to HDL but involve also triglycerides and LDL.

2.6.1.3. Diabetes Mellitus

IR has been found to affect both untreated RA patients with recent disease onset [257] and patients with established RA [270]. In the first group, IR has been correlated with inflammatory parameters, as well as disease activity [258], while in the latter IR has been associated with accelerated coronary atherosclerosis.

Inflammatory markers, such as TNF-α, impair the uptake of glucose in skeletal muscle and stimulate lipolysis in the adipose tissue, suggesting one of the mechanisms of IR in RA patients [271]. Subsequently increased lipolysis and fatty acids levels provoke an inflammatory response of macrophages that further stimulates TNF-α and IL-6, with a positive feedback cycle [272].

2.6.1.4. Smoking

Among traditional CV risk factors, smoking is considered one of the strongest environmental elements that are thought to play a relevant role in RA induction and progression, particularly in genetically predisposed individuals [26]. Smoking is associated with severe RA, for instance with more erosive disease or extra-articular

involvement [273]. Smokers are more likely to have positive RF and anti-CCP which are predictors of aggressive RA [273], but it is not clear if smoking confer the same relative risk for CV disease compared to the general population [274].

It is well established that smoking is associated with subclinical atherosclerosis in RA patients [275]. Recently, the additive and multiplicative interaction between heavy smoking (≥ 10 pack-year) and genetic background in seropositive RA in favoring CV risk has been demonstrated in a prospective cohort of female nurses with incident disease [276].

2.6.2. Inflammatory risk factors

Elevated levels of nonspecific systemic inflammatory markers (ESR and CRP), RF, anti-CCP, other inflammatory mediators, RA activity and severity (that is, small and large joint swelling, destructive changes on joint radiographs, rheumatoid nodules, vasculitis, rheumatoid lung disease and corticosteroid use) are statistically significantly associated with increased risk of CV events and/or death [130,147,277-285]. Even the presence of an elevation of nonspecific systemic inflammatory markers (ESR and CRP) or RF in absence of rheumatic disease is a risk factor of CV disease [278,286,287]. This suggests that immune dysregulation may promote CV risk in people with rheumatic disease and also in the general population and further supporting the hypothesis that inflammation and immune dysregulation are responsible, at least in part, for the excess CV risk in RA.

However, the theory that 'high-grade' systemic inflammation in RA may be related to an accelerated atherosclerosis with generalized vascular injury, enhanced CV morbidity and mortality [288], has still not been proved. Although studies in this regard collectively demonstrate worse vascular function and morphology in RA than in controls, have not jet provided definitive answers as to whether this relates to mechanisms operating

before or since the onset of RA, or the relative contribution of current versus cumulative inflammation versus traditional CV risk factors and their interplay. Only one study has suggested that this may be the case in RA [289].

Regarding animal models, they have shown that to knockout or neutralice the *Tnfa* gene was associated to a lower endothelial activation [290] and to a reduction in the extent of atherosclerosis [217], which would suggest that chronically elevated levels of circulating inflammatory cytokines accelerate the process of atherogenesis. However, these results have not been consistently reproduced in humans: only some studies have shown a significant association between subclinical atherosclerosis and systemic inflammatory markers [145,154,237,283,291-293], while others did not showed any association [143,144,236,240,294-297]. This inconsistency could be related to the possibility that chronic exposure to circulating cytokines may have a less potent effect on atherogenesis in RA than the one observed in animal studies or in the general population [298], particularly since inflammatory markers in RA patients are increased many-fold relative to non-RA patients, yet CV disease risk is increased by only approximately 50%. It is important to take into account that most of those studies were cross-sectional, powered only to observe large effects, and enrolled patients with established disease. Since inflammatory markers tend to fluctuate with time and treatment, a single cross-sectional assessment later in the RA disease course may not accurately reflect cumulative exposure.

The strong associations of indirect measures of cumulative inflammation, such as RA duration [144,236] or disease severity [239], with measures of atherosclerosis provide circumstantial evidence of causality (such as the association between active disease and echographic measures of plaque instability [299]), but these measures are indirect and could reflect pathogenic processes other than systemic inflammation.

2.6.3. Genetic risk factors

A specific genetic background, in addition to confer increasing susceptibility to RA, may contribute to the development of subclinical atherosclerotic damage both directly and through the interaction with some traditional and disease-related risk factors [300]. Multiple variants in multiple genes have been investigated in association with CV risk, classic CV risk factors and CV mortality.

2.6.3.1. Influence of polymorphisms of the HLA genes

As previously pointed out, alleles from the *HLA-DRB1* gene are not only risk factors for RA but also for CV disease (especially the *HLA-DRB1**0404 allele) [279]. Carriers of two copies of the SE alleles also were associated with about a two-fold increase of mortality from CV disease [279,280], particularly from IHD [301]. When specific SE genotypes were analyzed, the *HLA-DRB1**01/*04 combination conferred the highest risk of mortality from CV disease, regardless the duration of the disease [279,280,301]. Also, this combination resulted predictive of mortality due to IHD independently of the inflammatory burden [301] (measured as ESR, RF positivity, disability, joint involvement and presence of extra-articular disease).The *HLA-DRB1* gene is also associated with subclinical atherosclerosis: the *0404 allele, together with carrying two copies of the SE, were associated to an impaired endothelium-dependent vasodilation [302] and atherosclerotic plaque [303]. Also, an interaction between alleles of the *HLA-DRB1* gene and other CV risk factors, such as the presence of anti-CCP (which seem to be associated with subclinical atherosclerosis [304]) has been observed. The combination of carrying two copies of the SE alleles, in the presence of anti-CCP and with a smoking background, appears to confer a very high risk of premature death from CV disease [280].

2.6.3.2. Influence of polymorphisms of the TNF superfamily genes

Polymorphisms located in genes belonging to the TNF superfamily were analyzed regarding their association with CV disease or classic CV risk factors. TNF-α is an important pro-inflammatory cytokine, with a central role both in RA and atherosclerosis. Also, as pointed out before, TNF-α disturbs lipoprotein metabolism. A variant located in the promoter region (*TNFA* -1031 (T>C)) has been associated with smaller LDL particles, that have a greater affinity for extracellular matrix and higher susceptibility to oxidation [305]. No other variants of this gene have been studied in association with CV disease in RA.

TNF-β has been implicated in the early stages of the vascular inflammatory process [306], inducing expression of LAMs in endothelial cells. Also, in mice models, its concentration has been correlated with plaque size [307]. The variant located at position 252 (A>G) of the *LTA* gene has been associated with higher transcriptional activity and with a higher risk of MI in RA [308].

Galectin-2 is a soluble beta-galactoside binding lectin, able to interact with TNF-β [309]. There is a polymorphism located in intron 1, at position 3279 (C>T) that is associated to a decreased transcriptional activity of the *LGALS2* gene. Due to its interaction with TNF-β, it has been observed that a decrease in the production of Galectin-2 results in a inhibition of TNF-β secretion into the medium [309]. Although this variant showed association to MI in general population, no association was observed in RA patients [308]. However, it was associated to hypertension [310].

The TNFRII is a type I transmembrane glycoprotein that can be proteolytically cleaved to generate soluble receptor fragments that may function as natural TNF-α antagonists [311]. Unlike TNFRI, TNFRII is generally inducible and mainly expressed by endothelial and immune cells. The interaction with TNF-α leads to the activation of pro-

inflammatory and survival pathways via NF-κB [312]. The polymorphism located at position 196 of the gene *TNFRSF1B* (substitution of Methionine for Arginine) was studied regarding its association with CV disease, observing no association neither with IHD nor stroke. However, this variant was associated to a higher risk of hypertension [313].

2.6.3.3. Influence of polymorphisms of cytokine and chemokine genes

IL-6 is a cytokine characterized by pleiotropic and redundant actions, involved in inflammation, bone metabolism, immunity, endocrine functions. It is also a major regulator of the synthesis of acute phase reactants by the liver [314]. IL-6 is produce by a wide variety of cells, including lymphocytes, monocytes, fibroblasts, endothelial cells and adipocytes. This cytokine is involved in CV disease and in RA: high circulating levels of IL-6 are associated to a higher risk of coronary heart disease and CV mortality [315,316]. Conversely, RA patients have higher IL-6 levels that healthy controls [317]. A variant located in the 5' flanking region (-174 (G>C)) has been associated with higher transcriptional activity and increased levels of IL-6 [318] in general population. Taking into account the previously exposed, this polymorphism has been analyzed regarding its association with CV disease in RA patients, observing a significant association independently from traditional CV risk factors [319]. Also, a significant impairment in endothelium-dependent and independent vasodilation.[320] have been observed. However, this variant was not associated to hypertension [321].

The macrophage migration inhibitory factor (MIF) is a cytokine also produced by a wide variety of cells, such as monocytes/macrophages, lymphocytes, endothelial cells and smooth muscle cells [322]. In RA, MIF stimulates macrophages to release cytokines critical in this condition, such as TNF, IL-1, IL-6, and MMP. In atherosclerosis, plaque

size and macrophages accumulation were found to be directly proportional to increases in MIF levels [322]. Deletion of the *MIF* gene was associated with a marked reduction in lesion frequency and disease severity in atherosclerotic prone mice models [323]. Two variants of *MIF* were studied in their association with CV disease. Both of them had been previously associated with higher plasma MIF levels [324]. However, neither the $CATT_{5–8}$ deletion [325] nor the -173G>C [325,326] polymorphism were associated to CV disease, nor endothelial dysfunction nor carotid IMT [326].

The chemokine CCL21 has been implicated in the pathogenesis of both RA and atherosclerosis: regarding the first, it has been implicated in the organization of lymphoid tissue affected by RA [327]. In the latter, by recruiting T cells and macrophages to the atherosclerotic lesions and by promoting inflammatory responses in these cells [328]. A polymorphism from this gene (rs2812378) was analyzed regarding its influence in CV mortality, not finding any significant association [329].

2.6.3.4. Influence of polymorphisms of the nitric oxide synthase genes

Endothelial dysfunction constitutes one of the primary causes of initiation of atherosclerosis. Since the endothelium is a major source of nitric oxide (NO) in the vasculature, loss of normal cellular function would result in altered nitric oxide synthase (NOS) function and NO synthesis. The endothelium has a constitutive supply of NO from the endothelial NOS (eNOS, NOS3) and under certain conditions such as inflammation, can produce excessive NO from the inducible isoform of NOS (iNOS, NOS2) [330]. In normal conditions, NO exerts vasodilatadory and anti-inflammatory effects on the vascular wall (inhibiting the expression of LAMs and chemokines). However, produced in great excess by the NOS3 of endothelial cells and macrophages, can induce injury to the endothelium, though the production of NO derived radicals [330].

Both genes have been studied in connection to CV disease. The polymorphism of the *NOS3* gene located at exon 7, leading to a glutamate to aspartate substitution at position 298, was not associated to a higher risk of CV events [331]. The functional effects of this variant in the enzymatic activity are uncertain [332]. On the other hand, two variants of the iNOS gene (*NOS2A*) located in the promoter region were analyzed: the microsatellite CCTTT (with a influence in the gene transcriptional activity [333]) and the (C>T) substitution at position -786. Neither both variants showed a significant association with the risk of CV disease [331]. However, in carriers of the *HLA-DRB1**0404 allele, these three variants showed association with CV disease [331], suggesting an interaction among different genes.

2.6.3.5. Other polymorphisms studied

Regarding other variants strongly associated to RA, besides HLA, such as *PTPN22*, *STAT4* and *TRAF1/C5*, none of them showed a significant association with neither clinical nor subclinical CV disease [334]. Besides, *TRAF1/C5* polymorphism was not associated with a higher mortality from CV disease [335].

The 5,10-methylene tetrahydrofolate reductase is an enzyme that catalyze the transformation of methionine to cysteine. The lack of this enzyme leads to the accumulation of homocysteine, an independent nontraditional risk factor for CV disease. Homocysteine is directly toxic to endothelial cells, increases low-density lipoprotein oxidation, and has prothrombotic effects [336]. The 677 (C>T) and the 1298 (A>C) *MTHFR* gene polymorphisms, that are associated to a lower enzyme activity [337] [338] and higher homocysteine plasma levels, were studied regarding their association

with CV disease in RA. Only the *MTHFR* 1298 (A>C) gene polymorphism showed association to CV disease and endothelial dysfunction [339].

Leptin is a non-glycosylated peptide hormone, encoded by the gene *LEP* in humans [340]. In animal models, its synthesis is regulated by food intake, sex hormones and inflammatory mediators, and its levels are negatively correlated with glucocorticoids and positively with insulin [341-345]. Leptin levels have also been found increased in humans in several inflammatory diseases, including obesity, metabolic syndrome, RA and atherosclerosis [346-348]. Direct pro-inflammatory activities of leptin on immune response have been shown in human and murine macrophages [349,350], human neutrophils [351], NK cells [352], dendritic cells [353], T lymphocytes [354] and synovial fibroblasts [355]. On the basis of these studies, leptin has been investigated as a marker of disease activity in RA patients. On this regard, controversial results have been published [356,357]. Furthermore, anti-TNF-α antibody treatment did not have any effect on serum levels of leptin in RA patients [358,359]. Recently, the influence of a polymorphism located in the 5'UTR (rs2167270 (19 G>A)) in the risk of clinical or subclinical CV disease in RA patients was analyzed. Unfortunately, no significant association was found [360]. This variant had been previously associated with leptin levels [361] and obesity [362].

The plasminogen activator inhibitor type-1 (PAI-1) is the primary physiologic inhibitor of plasminogen activation in blood. During intravascular fibrinolysis, endothelial-derived plasminogen activator converts plasminogen to the active protease plasmin, which is able to degrade fibrin. Endogenous plasminogen activator is rapidly neutralized by PAI-1 [363]. Because of its major role in the regulation of the fibrinolytic process, PAI-1 overexpression may compromise normal fibrin clearance mechanisms

and promote pathological fibrin deposition and thrombotic events. A polymorphism of this gene, located in the promoter region (where one allele has a sequence of four guanosines (4G) and the other has five guanosines (5G) at position − 675, upstream from the mRNA initiation point) has been previously associated to CV disease and venous thrombotic episodes [364] in general population. In RA patients, this variant also has been associated with IHD [313].

JUSTIFICATION AND OBJECTIVES

3. JUSTIFICATION AND OBJECTIVES

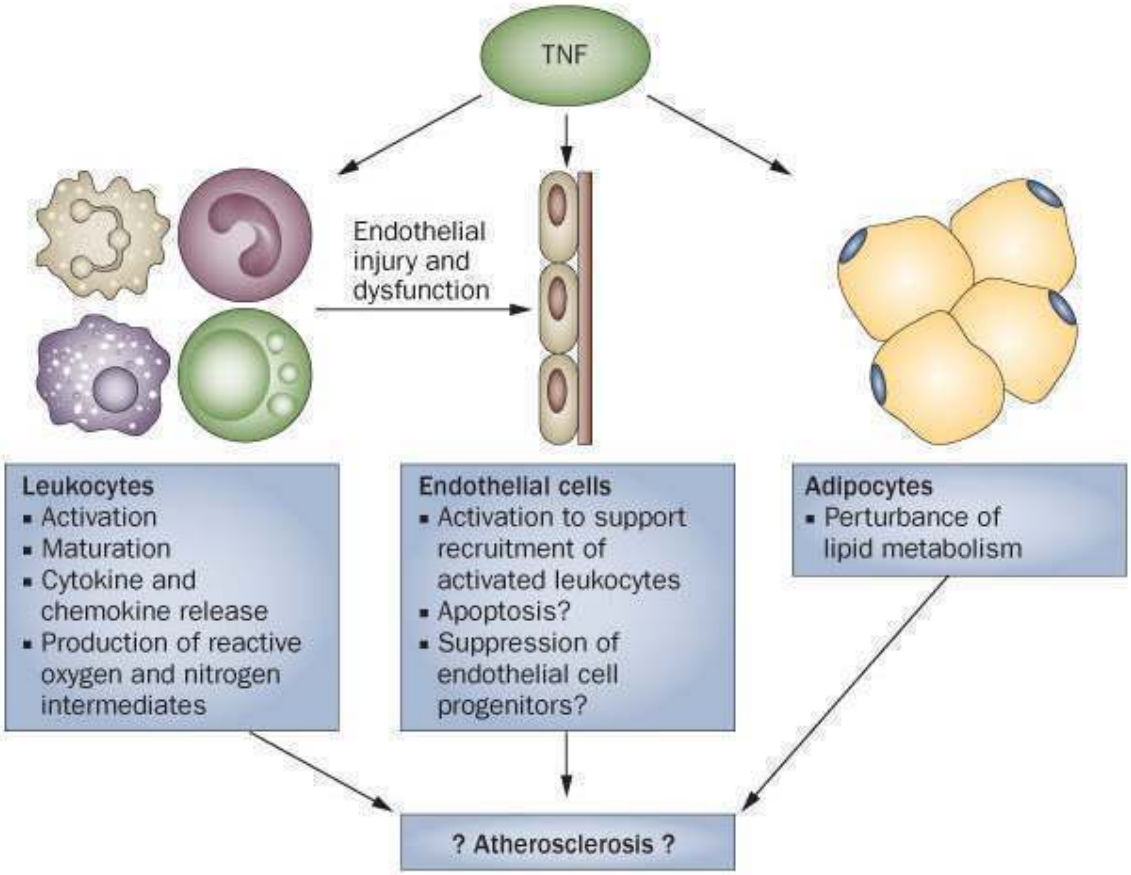
RA and atherosclerosis are both complex diseases in which genetic and environmental factors interact, leading to a chronic inflammatory condition. Besides, both diseases are related, sharing pathophysiological mechanisms. In both disorders, inflammatory mediators play a central role in the initiation and development of the rheumatoid pannus / atheromatous plaque.

Cytokines are small glycoproteins that function primarily as messengers in the immune system via autocrine, paracrine or endocrine manners. Cytokines bind specific receptor complexes, which, in turn, signal via transduction pathways to modulate gene expression within target cells. More than 100 cytokines within large, structurally related super-families have been described, and these mediate a large variety of regulatory and effectors functions within the immune system and beyond. Cytokines tend to be regulated in a coordinated manner; however, some seem to occupy pivotal positions within this hierarchy [96]. TNF-α is considered a pleiotropic inflammatory cytokine with a central role in many pathophysiologic states and in associated comorbidities that affect more than just the primary target tissue. It has a homotrimeric structure, able to bind to two receptors, TNFRI and TNFRII, and thereby, be able to influence a variety of molecular and cellular events that contribute to several disease states [365]. After synthesis in the endoplasmic reticulum, TNF-α is trafficked to the cell membrane where it remains as a functional membrane protein, or is solubilized via the action of a membrane-bound cleaving enzyme, TNF-α converting enzyme (TACE). TNF-α regulates leukocyte activation, maturation, cytokine and chemokine release, and production of reactive oxygen and nitrogen intermediates [366]. As such, it is a central

regulator of inflammatory cascades during both initiation and amplification of inflammatory reactions.

TNF-α probably promotes the inflammatory cascade within the arterial wall during development of atherosclerosis (**Figure 3**). It is produced by Th1 cells, macrophages, and NK cells.

Figure 3: Contribution of Tumor Necrosis Factor to atherosclerosis.



TNF: Tumor Necrosis Factor. Adapted from [366].

This cytokine activates endothelial cells to express adhesion molecules as well as proinflammatory cytokine and chemokine receptors. It also promotes endothelial cell injury [367], enhancing apoptosis and suppressing the activities of endothelial cell

progenitors that could sustain endothelial repair [368]. In RA patients, higher TNF-α concentration correlates with lower levels of circulating endothelial progenitor cells [227].

TNF-α has also been implicated in promoting endothelial injury through recruitment of immune cells, such as neutrophils, which can mediate tissue destruction [369]. In addition, TNF-α promotes oxidative stress, and can directly impair NO bioavailability with consequent promotion of endothelial dysfunction [224,225]. TNF-α also promotes the expression of tissue factor.

TNF-α has been implicated in the functional modulation of a variety of other tissue-specific cell types, including chondrocytes, osteoclasts, hepatocytes, neurons and adipocytes. Through the latter cell type, TNF-α might contribute to regulation of lipid and glucose metabolism [370,371], which has direct clinical implications in the acute setting, for necessary advantageous metabolic responses to injury or severe infection, and in the chronic setting, for increased vascular risk.

Another evidence of the involvement of the TNF-α in atherosclerosis pathogenesis is the reduced risk of CV events observed in those RA patients treated with TNF-α antagonist drugs [372].

TNF-α production may have a genetic component up to 60% [373]. Its synthesis is tightly regulated at the level of gene transcription. It is important to take into account that determining whether a particular polymorphism is directly responsible for a phenotype and disease association is difficult when the polymorphism is within the MHC region, due to strong LD between alleles across the MHC. The minor allele of the -308 (G>A) polymorphism, located in the promoter region of the *TNFA* gene, is strongly associated with the MHC haplotype A1, B8, DR3 [374], which is also associated with high TNF production [375,376] and autoimmune diseases, including insulin-dependent diabetes mellitus [377] and systemic lupus erythematosus [378]. The minor allele of the *TNFA* variant

is also associated with enhanced spontaneous or stimulated TNF-α production in both in vitro and in vivo [379,380]. However, so far evidence suggests that circulating TNF-α levels do not seem to correspond with the − 308 polymorphisms: circulating TNF-α level might be under a multifactoral regulatory process, meanwhile local TNF-α concentration might be of greater importance and under more control by specific polymorphisms [380].

Due to the influence of this polymorphism in TNF-α production, an association with RA risk and response to therapy, especially anti-TNF therapy was predicted. However, in one hand the association with RA has been replicated with inconsistent results and in a recent metaanalysis, it seems that this polymorphism may represent a significant risk factor for RA in Latin Americans, but not in Europeans [381]. On the other hand, this variant is neither a predictor of clinical response to anti-TNF treatment [382,383].

Regarding CV disease, this variant has not been associated to subclinical atherosclerosis in Caucasian [303] no Indian [384]. With respect to clinical CV disease, the results have been contradictory, although in a recent metaanlysis, it seems that this variant is not associated to CV disease [385]. However, this variant was associated to CV disease in patients with end stage renal disease [386] or DM [387].

Chemotactic cytokines, or chemokines, are small signalling proteins which main function is to attract and stimulate specific subsets of leukocytes, therefore playing a major role in acute and chronic inflammatory processes [388]. Both in RA and atherosclerosis, several chemokines have been detected in synovial tissue and atheromatous plaques mediating not only migration, but also maturation and activation of leukocytes and other inflammatory cells present in the lesions [389,390]. CCR5 is a heptahelical serpentine G protein-coupled receptor that binds multiple ligands,

including CCL3 (MIP-1α), CCL4 (MIP-1β), CCL5 (RANTES) and CCL8 (MCP-2). Of these ligands, MIP-1α and MIP-1β are considered to be selective to CCR5 [391]. CCR5 is expressed on macrophages, monocytes, Th1 cells, immature DCs and also in non-immune cells such as endothelial cells and VSMCs [392-396]. This molecule contributes to the survival [397] and accumulation [398] of macrophages during inflammation as well as to the recruitment and activation of T cells [399]. It participates in osteoclasts formation [393] and VSMCs activation and secretion of tissue factor [394]. Consistent with its role in these cell types, CCR5 is considered to play a part both in RA and atherosclerosis.

Regarding atherosclerosis prone mice models, CCR5 is implicated in the development and progression of the atheromatous plaque [396,400,401]. Knockout mice studies have showed a reduction in plaque formation associated to a lower macrophage, Th1 and smooth muscle cell accumulation and increased expression of anti-inflammatory cytokines such as IL-10. Furthermore, studies using an antagonist of the CCR5 and CCR3 [402] or a recombinant RANTES receptor antagonist [403] have demonstrated an attenuation of atherogenesis in *Ldlr* knock-out mice.

A naturally occurring variant of the *CCR5* gene, known as *CCR5*Δ32 (dbSNP rs333), exists at allele frequencies of typically 10% in European populations [404] and it is defined by a 32-bp deletion that leads to a truncated nonfunctional receptor [405]. Consequently, in individuals homozygous for *CCR5*Δ32, the CCR5 receptor is eliminated from the cell surface [406] and in heterozygous individuals, surface expression is reduced by 20% to 30% relative to the wild-type [407].

It is not clear the association between this variant and CV disease. Several publications have demonstrated a protective effect of the *CCR5*Δ32 allele with CV disease [408-412] including reduced CRP, increased plasma HDL, decreased plasma triglycerides, decreased carotid IMT, reduced incidence of CV disease for a 10-year period, and even

a greater incidence of longevity. However, other studies have demonstrated no relationship with CV events [413-417] and in a single study, an increased CV risk associated with *CCR5*Δ32 allele and an earlier age of onset of MI in women was reported [418]. These inconsistencies may reflect differences in study design, power, or population, and additional data will be helpful to understand the role of this *CCR5* polymorphism.

Regarding RA, the association between the *CCR5*Δ32 and this condition is also controversial [419]. Some studies could not confirm a link [420,421] although a meta-analysis (5 studies/1790 RA patients) suggested a protective effect [422]. Regarding *CCR5*Δ32 variant and disease severity, also conflicting results have been reported [420,423,424]. Several studies observed a milder disease (such as less swollen joints, less morning stiffness and lower frequency of RF positivity) in RA patients carrying this deletion [423,424], although another found a non significant more severe disease in RA patients carrying *CCR5*Δ32 [420].

Adipose tissue was previously regarded merely as an energy-storing organ, but now it is recognised as an active endocrine organ, secreting a variety of proteins that influence metabolism [425], known as 'adipokines' or 'adipocytokines'. These molecules orchestrate via endocrine, paracrine, autocrine and juxtacrine mechanisms different processes, including food intake, insulin sensitivity, immunity and inflammation [426,427]. Adipocytokines induce their activities through the binding to selective transmembrane receptors on different cell types. At present, the most studied adipocytokines are adiponectin, resistin, leptin and TNF-α.

Resistin is secreted from adipocytes in mice and its main action is to antagonize the action of insulin [428]: mice over expressing the *Retn* gene are insulin-resistant through

increased levels of serum resistin [428,429], while mice without *Retn* show decreased fasting plasma glucose levels [430]. However, in humans resistin is believed to play a role in inflammatory responses [431]: *RETN* appears to be mainly expressed in monocytes, macrophages [432,433], as well in synoviocytes [434], while isolated human primary adipocytes and preadipocytes do not express *RETN* [434]. Its expression and secretion may be regulated by innate inflammatory signals, such as endotoxin and proinflammatory cytokines, like TNF-α [435-440]. In turn, recombinant resistin up-regulates the expression of pro-inflammatory cytokines and LAMs on human endothelial cells [441,442], peripheral blood mononuclear cells (PBMC) [435], synoviocites from RA patients [435] and adipocytes [434]. Consistent with this observations, resistin has been shown to induce pro-inflammatory activities in chronic inflammatory diseases, including RA and atherosclerosis [435,442-445].

Plasma resistin levels are highly correlated with levels of diverse inflammatory markers in healty subjects [431] , although in fully adjusted models, only soluble TNFRII (an index of TNF-α systemic activation [446]), phospholipase A2 and IL-6 but not CRP, remained positively associated with resistin levels. Several small studies have reported that circulating resistin levels are increased with obesity [447,448], and T2DM [449], although not all reports have been consistent in this regard [450]. In the latter, resistin levels are strongly associated with plasma soluble TNFRII [431], but not with measures of adiposity or IR. Regarding CV disease, no clear association with subclinical atherosclerosis, such as coronary arterial calcification, has been observed [431,451].

In RA, the reports regarding resistin serum levels are not consistent, but it seems that, at least in Caucasians, or serum levels are not significantly different comparing with healthy subjects [347,436], or the increased levels observed in some reports are not due to the disase itself, as the difference does not remain significant after adjust for body mass

index (BMI), inflammation, or both [452]. However, in Japanese populations, RA is an independent factor associated to higher resistin levels [453].

Regarding synovial fluid, there is a significant higher level of extracellular resistin compared with matched blood samples [436,454] and compared with synovial fluid from patients with a degenerative/traumatic joint disease [436,445,454].

In synovial tissue, macrophages (CD68), B lymphocytes (CD20) and plasma cells (CD138) but not T lymphocytes (CD3) showed colocalisation with resistin [445].

In RA patients, resistin levels are correlated with markers of inflammation such as circulating levels of IL-1Ra [453,455] and TNF- α [453]. In fact, treatment with anti-TNF-alpha therapy results in a rapid reduction of serum resistin levels [456]. Also, some studies have shown a correlation between resistin and levels of CRP, ESR and leucocytes count [445,453,456,457], although these findings have not been always replicated [435]. Also, association between resistin levels and DAS28 in RA patients has been described [445], as well as a weak association with a higher Larsen score [452].

In RA resistin levels did not correlate neither with anthropometric parameters, IR [457,458] nor coronary arterial calcification [458].

The variant located in the promoter region at position −420 (C>G) of the *RETN* gene is associated to an enhanced promoter activity and therefore to higher resistin plasma levels [459-461], especially in Asian populations. The haplotype defined by the minor allele of this variant and the minor allele of another polymorphism located at -358 was associated to the higher resistin plasma levels [462]. However, the latter is likely monophormic in Caucasians (http://www.hapmap.org/index.html.en). Conversely, in healthy Caucasian populations, the -420 polymorphism seemed not to be associated with plasma levels [463-465] or only account for a minor variation in circulating resistin [451,466]. The molecular mechanism underlying this apparent ethnic difference is uncertain

but also might relate to reported variation in regulation of mRNA transcription across ethnicity [467]. This variant, in healthy subjects, has been associated with soluble TNFRII levels [451].

Reports of the -420 *RETN* variant associations with BMI or other measures of adiposity in populations of European descent have been inconsistent in the literature: some have reported no association [451,461,464,468], while others did so only in subgroup analyses [469-471]. Regarding T2DM, contradictory results have been observed: the −420 variant was reported a primary determinant of susceptibility [472] in Japanese population, meanwhile other studies did not find an association neither in Caucasian [461,473-475] nor in Chinese [476] populations. However, both in Caucasian [474] and in Japanese T2DM patients [460], the -420 polymorphism has been associated to cerebrovascular disease. This variant has not been associated to coronay arterial disease in Caucasian [465] and in a Chinese population the results are conflictive [476,477]. Also in Caucasians, there was neither association with carotid atherosclerosis nor with MI [478] nor mortality form all causes nor with CV mortality [465].

Adiponectin (encoded by the *ADIPOQ* gene) is the most abundant adipokine in human plasma [479,480]. It was previously thought to be secreted by adipocytes only [479], but has been shown to be expressed by other cell types, including osteoblasts and synovial cells such as RA synovial fibroblasts [481]. Peripheral levels show a pronounce differences between men and women with about 1.5 times higher concentrations in women [482,483]. Adiponectin has an insulin-sensitising, anti-inflammatory and anti-atherogenic properties, playing a critical role in the development of IR [484]. This adipocytokine exerts its insulin-sensitising effects in the liver by suppressing gluconeogenesis and in the skeletal muscle by enhancing fatty acid oxidation [485]. Furthermore, adiponectin exhibits anti-inflammatory and atheroprotective actions in various tissues by suppressing the

expression of LAMs [486] and SR [487], reducing the expression of TNF-α [488], raising NO production [489], suppressing the proliferation and migration of VSMCs [490] and reducing neointimal formation in response to vascular injury (in mice) [491]. Also it suppresses the proliferation of myelomonocytic progenitors [488]. Opposed to the antiinflammatory and antiatherogenic properties of adiponectin in CV and metabolic diseases [492-494], in RA this adipokine seems to exert proinflammatory effects: in vitro and in animal studies demonstrated the ability of adiponectin to activate proinflammatory pathways in synoviocytes [481,495] (especially those derived from RA subjects in comparison with those derived from osteoarthritis), leading to production of chemokines, cytokines and matrix-degrading enzymes (especially CCL20, CXCL11, MMP10, MMP3) [496,497], chondrocytes, leading to production of IL-6, IL-8 [497] and MMP3 [498], and osteoclasts [499] that may mediate damage to cartilage and bone. In this regard, adiponectine levels have been linked with erosive damage in RA [500-502] and erosive osteoarthritis [503].

Plasma adiponectin levels have been reported as decreased in states of obesity [482,504,505], T2DM [504,506-509] and coronary arterial disease [510-517]. Adiponectin plays an important role in the pathogenesis of T2DM and low levels of adiponectin serve as predictor for develop of IR and T2DM [506,518].

However, in RA, plasma levels were found higher than in healthy control [347] and synovial levels were higher compared with osteoarthritis patients [454].

ADIPOQ is placed on chromosome 3q27. This genomic region has been shown to segregate with IR traits and T2DM in several family studies [519,520]. A recent, systematic analysis of this locus in Europeans suggests that this gene is organized in two blocks of high LD separated by a region of low LD placed in the middle of the first intron [483].

Adiponectin levels are highly heritable (30–70%) [521-523] and various polymorphisms located in the *ADIPOQ* gene showed a major influence in adiponectine plasma levels

[524-526]. However, the variants that will be analyzed in our work have not been consistenly associated to this adipocytokine peripheral levels, nor in Caucasian [523,524,526,527] nor in Asian [525] populations. It is unclear whether these differences in association across studies reflect differences in genetic background or modifying environmental factors between populations are the result of discrepancies in ascertainment criteria or phenotyping methods or simply represent statistical fluctuations [523]. However, both have been associated to CV and metabolic diseases.

The rs266729 variant, located in the 5' flanking region, position −11377, has been associated with T2DM in Caucasians in two recent metaanalysis [528,529], although in a previous one no association was described [523]. Regarding CV disease, there was association with ischemic stroke [530] in non-diabetic Chinese subjects. However, Caucasians did not show association with clinical CV disease [531,532] but was related to a significant (>50%) coronary stenoses [531] and to a greater carotid IMT [533].

Regarding the rs1501299 polymorphism, located in the second intron at position +276, it was not found associated with T2DM in various metanalysis [523,528], although, in T2DM subjects, the minor allele was associated to a reduced CV risk [523]. In non diabetic population, no association was observed in two different studies with Caucasian subjects of north European ancestry [531,534]. However, in a cohort of Caucasians of south European ancestry, an association was observed between this variant and a lower risk of CV disease [535]. Regarding subclinical atherosclerosis, association with carotid IMT or ischemic stroke was described neither in Asians [536], nor in Caucasians [537]. No association with coronary stenoses was observed in north European Caucasian population [531].

Neovascularization is an important process in the development and instabilization of atherosclerotic plaques [538]. The immature vessels induced by pro-angiogenic factors can contribute directly (through intraplaque hemorrhage) or indirectly (by supplying inflammatory cells) to the instability of the plaque [539-541]. It has been observed that treatment of mice models prone to atherosclerosis with recombinant human VEGF causes an increase in plaque growth [542]. As the atheromatous plaque grows and the intima thickens, oxygen diffusion from the lumen becomes more difficult, resulting in low oxygen tension and activation of proangiogenic substances. Most neovessels in the plaque originate from branches of vasa vasorum [543] and typically are dysmorph and immature [544]. One of the most important pro-angiogenic factors is VEGF. Hypoxia, through hypoxia inducible factor (HIF)-1 [545], is able to increase the expression of both VEGF and VEGF receptors, facilitating angiogenic processes and enabling endothelial cells to stimulate the receptors through autophosphorylation [546]. HIF-1 expression can be induced also by inflammatory [547], oxidative [548], vasoactive [549] and thrombotic factors [550]. On the other hand, inflammation may also regulate angiogenesis independent of HIF-1 [551]: pro-inflammatory cytokines such as IL-6, IL-1β and TNF-α are able to increase *VEGFA* expression [552,553].

VEGF can act as an endothelial-cell mitogen and as a modulator of changes in vascular permeability [554]: it is able to stimulate endothelial cell proliferation and growth and to prevent apoptosis in ischemic conditions [555]. Besides, it is able to modulate the recruitment of leucocytes, increasing the vascular permeability [556], by increasing the expression of various LAMs in endothelial cells of newly formed vessels [557,558]. It is also able to stimulate the production and release of chemokines, such as monocyte chemoattractant protein-1 [559,560], and to directly stimulate the migration and activation of monocytes through its receptor Flt-1 [561]. In turn, activated monocytes produce TNF-α

and IL-1β and various MMP. Although these enzymes, from a physiological perspective, clear the road for growing neovessels, they also contribute to the weakening of the fibrous cap and thus to the instability of the plaque [562].

Different evidences have supported a role of VEGF in RA angiogenic processes [563,564]. As in the atheromatous plaque, VEGF expression is enhanced in response to the hypoxic conditions in the RA joint [66,565] and by a variety of pro-inflammatory cytokines, such as TNF-α, IL-1, IL-6, IL-17, IL-18, NO, hepatocyte growth factor, macrophage MIF, endothelin-1 and prostaglandins [66,564,566-569]. Accordingly, RA patients show elevated levels of *VEGFA* expression in synovial fluids [570] and serum, with a correlation between serum VEGF with RA severity [571].

Several polymorphisms have been described within the *VEGFA* promoter and 5´UTR regions, which regulate *VEGFA* expression at the post-transcriptional level [572,573]. We selected two variants with functional relevance: the -1154 G>A (rs1570360) located within the *VEGFA* promoter and -634 G>C (rs2010963) located within 5´UTR [574]. Minor allele of the -1154 variant has been associated to a lower *VEGFA* transcription [574]. The -634 polymorphism seems to affect the activity of the internal ribosomal entry site B [574], reducing *VEGFA* translation of the normally secreted VEGF isoforms [574]. Accordingly, the major allele is associated to higher serum VEGF levels [573,575,576]. In turn, the -1154 variant has been associated to greater VEGF production by PBMC [574,577], but not to higher peripheral levels [578].

Despite the functional relevance of these polymorphisms, no association with the risk of RA have been observed, nor in Caucasian [579], nor in Asian populations [580]. Regarding other conditions, the -634 variant has been associated to severe diabetic complications [573], neurodegenerative disorders [574], Behçet disease [581] and giant cell arteritis [582] and the presence of severe ischemic complications [583] in this disease. Regarding CV disease,

this variant showed association only in subjects with T2DM [576]. In turn, the -1154 polymorphism has not been associated with CV disease [584,585].

Immune responses and cytokine expression are influence by alterations in circulating hormones and nutrients, and, in turn, proinflammatory cytokines and immune-derived growth factors produced during inflammation influence hormone secretion, neuroendocrine function and metabolism [586]. Growth hormone is one of the most studied endocrine factors in this regard owed to the many effects that exert on cells and organs of the hematopoietic and immune systems, including effects on cell survival, T-cell development, cytokine regulation and the proliferation of lymphoid cells [587]. In turn, it has been described a 28-amino-acid peptide, known as ghrelin, that binds to the growth hormone secretagogue receptor (GHS-R). Through this receptor it exerts its main endocrine functions, such as stimulation of GH secretion from the pituitary (independent from that of hypothalamic GH-releasing hormone), induction of food intake [588] and regulation of energy homeostasis [589,590]. This peptide is mainly synthesized and secreted by the X/A-like cells in the oxyntic glands of the stomach [591,592]. However, lower ghrelin expression and secretion have been also demonstrated in the gastrointestinal tract, pancreas, brain, pituitary gland, kidney, lung, placenta and the heart [590,591]. Acylation on the third serine residue, promoted by ghrelin O-acyltransferase [593] is essential for binding to the GHS-R1a [594], a highly functional conserved G-protein-coupled receptor [595]. Besides the endocrine, other ghrelin functions have been reported: release of various hormones like ACTH, cortisol and prolactin [596]; modulation of cell proliferation and survival; glucose metabolism, pancreatic function, and gastric acid secretion [590,597]; and immune modulation. Both ghrelin and GHS-R have been detected on the surface of immune cells: T cells, B cells, neutrophils, and

monocytes [598,599]. This peptide is able to inhibit the expression of proinflammatory cytokines such as IL-1β, IL-6 and TNF-α in T lymphocytes and monocytes [598], and TNF-α and H_2O_2-induced cytokine release in endothelial cells [600]. It also augments the anti-inflammatory cytokine production and chemokine levels in experimental arthritis [601,602]. Accordingly with these anti-inflammatory functions, lower ghrelin plasma levels have been observed in RA patients compared to controls [603] and treatment with anti-TNF-α drugs increases ghrelin concentrations [604].

GHS-R has also been found in heart, blood vessels and endothelial cells [605], suggesting that ghrelin could have direct effects on CV regulation, cardiac structure and function as well as atherosclerotic lesions [600]. Ghrelin has showed effects as an inotropic regulator, enhancing the cardiac output [605-607] through a non-GHS-R 1a receptor, leading to the release of cyclooxygenase metabolites from endothelial cells [608]. Also ghrelin has a direct vasodilatation effect [609,610], by stimulating NO production and release from the endothelium [611,612], and by antagonizing the effect of endothelin1 [613], in an endothelium-independent mechanism. Also, this molecule has a cardioprotective effect, protecting the cardiomyocytes against apoptosis [614], both in ischemia-reperfusion animal models [615,616] and in congestive heart failure [617]. In this condition, the expression in atrium and ventricles of ghrelin is decreased, while the GHS-R1a expression is increased [618]. It has been observed that ghrelin receptors are also up-regulated in atherosclerotic coronary arteries [619].

Different studies have associated different *GHSR* variants with obesity [620], coronary artery disease [621] and left ventricular hypertrophy [622]. The rs509035 variant, located in the intron has been associated to insulin-resistance traits [623] and lower HDL levels in women [624]. On the other hand, the rs2922126 variant, located in the promoter region, has been linked to metabolic syndrome, an increased waist circumference and increased

fast blood glucose in women [624]. None of these polymorphisms, besides the rs512692, located in the 5'UTR region, have been associated to a higher risk of developing RA [625].

The objective of our work is to deepen in the understanding of the genetic risk factors for CV disease in RA patients. To accomplish this goal, we proposed to study different polymorphisms belonging to genes that have been implicated both in RA and CV disease. We selected genes that code for proteins involved at different levels of the pathology of both conditions, such as cytokines (*TNFA*), chemokines (*CCR5*), adipokines (*RETN*, *ADIPOQ*), molecules that promote neovascularization (*VEGFA*) and factors that regulated energy balance and intake (*GHSR*).

The specific objectives for this study are:

a) To analyze the role of the *TNFA* −308 (rs1800629) polymorphism in the risk of clinical and subclinical CV disease in RA patients.
b) To study if in RA patients, the deletion Δ32 of the *CCR5* gene (dbSNP rs333) is associated to a lower risk of clinical or subclinical CV disease.
c) To asses the influence of the *RETN* polymorphism located at -420 (rs1862513) in RA patients regarding the risk of clinical or subclinical CV disease.
d) To evaluate the association between the *ADIPOQ* rs266729 (−11377) and rs1501299 (+276) variants and CV disease, both clinical and subclinical, in RA patients.
e) To analyze if the *VEGFA* -634 (rs2010963) and -1154 (rs1570360) polymorphisms are a risk factor in RA patients of clinical or subclinical CV disease.
f) To asses the influence in clinical and subclinical CV disease of the *GHSR* rs509035, rs512692 and rs2922126 polymorphisms in RA patients.

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TNFA −308 (rs1800629) polymorphism is associated with a higher risk of cardiovascular disease in patients with rheumatoid arthritis

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ABSTRACT

Objective: To assess the influence of the *TNFA* rs1800629 (G > A) polymorphism in the risk of cardiovascular (CV) disease and subclinical atherosclerosis in patients with rheumatoid arthritis (RA).
Methods: 587 patients fulfilling the 1987 American College of Rheumatology classification criteria for RA were studied. Patients were genotyped for the *TNFA* rs1800629 polymorphism using predesigned TaqMan single nucleotide polymorphism genotyping assay. Also, *HLA-DRB1* genotyping was performed using molecular based methods. Carotid artery intima-media thickness, flow-mediated endothelium-dependent and endothelium independent vasodilatation, used as surrogate markers of subclinical atherosclerosis, were measured in a subgroup of patients.
Results: We observed a higher frequency of carriers of the minor allele A among the patients with CV disease (with 37.6% vs. without 27.9%, $p = 0.06$, OR 1.56 [95% confidence interval-CI 0.95–2.54]). Carriers of the minor allele A exhibited a higher risk of CV events after adjustment for demographic and traditional CV risk factors ($p = 0.023$, HR 1.72 [95% CI 1.076–2.74]). Also, a significant interaction between this polymorphism and the presence of the rheumatoid shared epitope (SE) was observed ($p = 0.024$). Due to this, the association between carriers of the minor allele A and CV disease was only present in carriers of the SE, even after adjustment ($p = 0.001$, HR 2.43 [95% CI 1.41–4.19]). No significant association between the TNFA variant and the surrogate markers of subclinical atherosclerosis was observed.
Conclusion: Our results show that *TNFA* rs1800629 gene polymorphism is associated with predisposition to CV complications in patients with RA. This predisposition is restricted to individuals carrying the rheumatoid SE.

1. Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease associated with high risk of cardiovascular (CV) events due to accelerated atherosclerosis [1,2]. In this regard, CV disease is the most common cause of mortality in patients with RA [3]. Both traditional (classic) [4] and non-traditional CV risk factors such as chronic inflammation [5] have been implicated in the elevated CV morbidity observed in these patients.

Since the outcome of patients with RA is strongly linked to the development of accelerated atherosclerosis and CV complications [1–3] and RA is a complex polygenic disease, an issue of major importance is to determine the genetic implication in the risk of

Abbreviations: HLA, human leukocyte antigen; TNF, tumor necrosis factor; RA, rheumatoid arthritis; CV, cardiovascular; OR, odds ratio; CI, confidence interval; FMD, endothelium-dependent-flow-mediated vasodilatation-(post-ischemia); NTG, endothelium independent- (post-nitroglycerin) vasodilatation; RF, rheumatoid factor; Anti-CCP, anti-cyclic citrullinated peptide antibodies; IMT, intima-media thickness; ANOVA, analysis of variance; ANCOVA, analysis of covariance.

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CV morbidity of these patients. With respect to this, we previously observed a contribution of the human leukocyte antigen (*HLA*)-*DRB1* gene to the risk of endothelial dysfunction, CV events and CV mortality in Spanish individuals with RA [5,6]. Also, we reported a contribution of the inducible and endothelial nitric oxide synthase (*NOS2A* and *NOS3*) gene polymorphisms to CV event risk in patients with RA [7]. Moreover, we have recently reported an implication of the methylene tetrahydrofolate reductase (*MTHFR*) A1298C gene polymorphism in the increased risk of atherosclerosis of patients with RA [8].

Tumor necrosis factor-alpha (TNF-α) is a potent immunomediator and proinflammatory cytokine that has been implicated in the pathogenesis of a large number of human diseases, including RA [9] and atherosclerosis [10]. TNF-α mediates a wide variety of effector functions that are of major importance in the pathogenesis of RA, including endothelial cell activation and chemokine amplification leading to leukocyte accumulation, osteoclast and chondrocyte activation promoting joint destruction, nociceptor sensitization and development of metabolic syndrome [9]. In atherosclerosis, TNF-α activates inflammatory and endothelial cells, induce a prothrombotic state as well as insulin resistance and dyslipidemia [10].

TNF-α synthesis is tightly regulated at the level of gene transcription, and its production may have a genetic component of approximately 60% [11]. A single nucleotide polymorphism located at position −308 in the *TNFA* promoter (G > A, rs1800629) has been associated with enhanced spontaneous or stimulated TNF-α production both *in vitro* and *in vivo* [12,13]. However, circulating TNF-α level does not seem to be influenced by this polymorphism. While *TNFA* rs1800629 polymorphism has been considered to be a risk factor in some autoimmune diseases such as insulin-dependent diabetes mellitus and systemic lupus erythematosus [12], the potential association of this polymorphism with RA has shown contradictory results. In this regard, a recent meta-analysis disclosed that this polymorphism may be a risk factor for RA in Latin Americans but not in Europeans [14]. Moreover, *TNFA* polymorphism has not been confirmed to be a predictor of the clinical response to anti-TNF treatment in RA [15,16].

Since the incidence of coronary artery disease is a function of the development and progression of atherosclerosis, the use of noninvasive surrogate markers of atherosclerosis may be useful for the diagnosis of CV disease through the identification of subclinical disease. Noninvasive imaging techniques provide an approach for identifying high-risk individuals who may benefit from active intervention to prevent clinical disease. They offer a unique opportunity to study the relation of surrogate markers to the development of atherosclerosis. Among them, ultrasound techniques based on flow velocity and intima thickness are considered an efficient way to measure the subclinical atherosclerosis. There are some other noninvasive imaging modalities such as coronary, and aorta imaging, left ventricular echocardiography imaging, electron-beam computed tomography, magnetic resonance imaging, and ankle–brachial index.

Taking into account all these considerations and the central role played by TNF-α in RA and atherosclerosis, in the present study we aimed to determine the influence of *TNFA* rs1800629 polymorphism in the increased incidence of CV events observed in patients with RA. Considering the previously reported high experience of our group in the assessment of subclinical atherosclerosis in different rheumatic diseases using ultrasound techniques, in the present study we also assessed whether the *TNFA* rs1800629 (G > A) gene polymorphism might be associated with an increased risk of subclinical atherosclerosis manifested by the presence of endothelial dysfunction or increased carotid artery intima-media wall thickness (IMT).

2. Patients and methods

2.1. Patients and study protocol

Between March 1996 and March 2008, 608 consecutive patients that fulfilled the 1987 American College of Rheumatology classification criteria for RA [17] were recruited from the rheumatology outpatient clinics of Hospital Xeral-Calde, Lugo and Hospital Clínico San Carlos, Madrid, Spain. A DNA sample (see below) was extracted from these patients at the time of recruitment. Between December 2009 and January 2010 patient's clinical records were examined until patient's death, loss of follow-up or December 1st, 2009. Sociodemographical and clinical data regarding clinical manifestations, classic CV risk factors and history of CV events were registered. Clinical definitions for CV events and classic CV risk factors were established as previously described [5,18]. In this regard, patients were considered to have diabetes mellitus if before disease diagnosis they had been diagnosed as having diabetes mellitus by their family physicians or if 2 fasting plasma glucose levels on different days at the time of disease diagnosis or over the extended follow-up were >125 mg/dl [5]. Smoking habit was considered to be present in those patients who smoked at the time of disease diagnosis, during the follow-up or who had smoked within the 10 years before the onset of RA symptoms or the disease diagnosis.

A CV event was considered to be present if the patient had ischemic heart disease, heart failure, cerebrovascular accident or peripheral arteriopathy.

The definition of ischemic heart disease (IHD) included acute coronary syndromes with or without persistent ST-segment elevation and chronic coronary heart disease. IHD was diagnosed if any of the following criteria were satisfied: a recorded diagnosis of ischemic cardiopathy, on account of some acute coronary syndrome (acute myocardial infarction or unstable angina), the presence of pathological Q waves in the electrocardiogram, and coronary images showing >50% stenosis of at least one coronary vessel [5]. Data regarding the clinical presentation of heart failure were also collected from all patients, based on the Framingham criteria [18]. A patient was considered to have a cerebrovascular accident when he/she had a stroke and/or transient ischemic attacks (TIAs). Strokes were classified according to their clinical features and they were confirmed by computed tomography and/or magnetic resonance imaging. TIAs were diagnosed if the symptoms were self-limited in less than 24 h, without residual neurological damage [5]. Peripheral arterial disease was considered to be present if it was confirmed by Doppler and arteriography [19]. Information on the main demographic characteristics, CV risk factors and CV events of patients in whom genotyping success was achieved (n = 587 [97%]) is shown in Table 1. Genotype and allele distribution in our RA sample was similar to that previously described in non-RA healthy Spanish population [20].

Since Hospital Xeral-Calde and Hospital Clínico San Carlos are the referral centers for the population of each respective area, the first CV event was defined as an event (case) of CV complication diagnosed at the hospital in a patient without a previous history of CV disease. Based on previously established protocols of management, all patients on methotrexate therapy were treated with folate supplementation.

To determine the potential association between the *TNFA* rs1800629 polymorphism and the presence of subclinical atherosclerosis, between March 2007 and September 2009 a random subgroup of patients from the Lugo cohort with no previous history of CV events was selected. Presence of endothelial dysfunction was assessed by a brachial artery reactivity study in 130 patients. Flow-mediated endothelium-dependent vasodilatation-FMD (post-ischemia) and endothelium-independent-NTG (post-nitroglycerin) vasodilatation were measured by brachial ultra-

Table 1
Demographic characteristics and genotype distribution of the patients with rheumatoid arthritis included in the study.

Variables	$n = 587$
Females	435 (74.1)
Age of patients at the time of disease diagnosis, years, median [IQR]	56 [45–65]
Time follow-up, years, median [IQR]	13.5 [7–18]
Anti-CCP positive ($n = 401$)	243 (60.6)
Rheumatoid factor positive ($n = 581$)	435 (74.9)
Shared epitope ($n = 526$)	344 (65.4)
HLA-DR3 ($n = 479$)	57 (11.9)
Cardiovascular events	93 (15.8)
Ischemic heart disease	51 (8.7)
Cerebrovascular accidents	27 (4.6)
Heart failure	23 (3.9)
Peripheral arteriopathy	7 (1.2)
Hypertension ($n = 583$)	219 (37.6)
Diabetes mellitus ($n = 583$)	67 (11.5)
Dyslipidemia ($n = 583$)	244 (41.9)
Obesity ($n = 583$)	59 (10.1)
Smoking habit ($n = 581$)	103 (17.7)
TNFA rs1800629	
GG	414 (70.5)
GA	156 (26.6)
AA	17 (2.9)
G	984 (83.8)
A	190 (16.2)

Except where indicated otherwise, values are *n* (%). IQR, interquartile range; anti-CCP, anti-cyclic citrullinated peptide antibodies.

sonography as previously reported [6,21]. A value of FMD less than 7% was considered pathologic, indicating the presence of endothelial dysfunction [21]. Intra-observer variability for FMD and NTG was 1.3% and 1.9%, respectively, based on the repeat of the brachial ultrasonography in 32 healthy controls. Assessment of endothelial function of patients undergoing anti-TNF therapy was performed 24–48 h before drug administration. Also, carotid ultrasonography studies were performed in 108 patients to determine the carotid artery IMT. It was assessed in the right common carotid artery as previously reported [22]. Based on a second carotid ultrasonography performed to 20 RA patients and 20 healthy controls within a week after the first assessment, the correlation coefficient for carotid IMT was 0.98.

The subject's written consent was obtained according to the declaration of Helsinki, and the design of the work was approved by the Ethics Committee of Galicia (Spain). The Ethics Committee of the Hospital Clinico San Carlos (Madrid) also approved the study.

2.2. Genotyping

2.2.1. TNFA genotyping

DNA from patients was obtained from whole peripheral blood, using standard methods.

Subjects were genotyped to determine *TNFA* rs1800629 status using TaqMan Assays-on-Demand from Applied Biosystems following the manufacturer's protocol and analyzed using the ABI 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The typing was successful in 587 patients (96.5%). Also, 10% of the samples were re-genotyped at random. However, no differences were observed with the results obtained before.

2.2.2. Shared epitope and DR3 determination

Several *HLA-DRB1* alleles (*HLA-DRB1**0401, *0404, *0405, *0408, *0101, *0102, *1001, and *1402) are associated with susceptibility to RA. These alleles encode a conserved amino acid sequence (QKRAA, QRRAA, or RRRAA), called the shared epitope (SE), at position 70–74 in the third hypervariable region of the HLA-DRβ1 molecule [23].

HLA-DRB1 typing was carried out using a reverse dot-blot kit with sequence-specific oligonucleotide (SSO) probes (Dynal RELITM SSO HLA-DRB1 typing kit; Dynal Biotech, Bromborough, UK). When necessary, high-resolution typing of *HLA-DRB1**03 samples was performed using Dynal AllSetTM SSP DRB1*03.

In our RA sample, 65.4% of the patients had at least one copy of the rheumatoid SE, which was a frequency higher than that described in non-RA Spanish individuals [24].

2.3. Statistical analysis

Comparison of means was performed using *T*-test. Comparison of proportion between 2 or more group was carried out using χ^2 test or Fisher test, when required. Hardy–Weinberg (HWE) equilibrium was tested in the RA patients with and without CV disease. Both groups were in HWE (RA with CV disease $p = 0.99$, RA without CV disease $p = 0.60$). Strength of association between CV events and genotypes of the *TNFA* rs1800629 polymorphism was estimated using odds ratios (ORs) and 95% confidence intervals (CIs), via multiple logistic regression; estimates were further adjusted for gender, age at RA diagnosis, time of follow-up, presence or absence of SE and classic (traditional) CV risk factors (hypertension, diabetes mellitus, dyslipidemia, obesity and smoking habit) as potential confounders. The study had an 80% power for detecting an OR ≥ 2. Later, a statistical interaction between *TNFA* rs1800629 polymorphism and presence or absence of SE was introduced in the logistic regression model. A Cox regression model was used to estimate the influence of the *TNFA* rs1800629 polymorphism on CV disease. We used the occurrence of at least one CV event as the outcome and the survival time when the first CV event occurred. The survival time of individuals without CV events was the age at patient's death, loss of follow-up or December 1st, 2009. Patients who died of any other causes different from CV events were considered as not having CV events. Proportional hazard assumption was tested using Schoenfeld residuals. Results were expressed as hazard ratios (HRs) with 95% confidence intervals [95% CIs] and were computed as both crude and adjusted for age at RA diagnosis, gender and classic CV risk factors. The selected variables used for adjustment were selected due to their association with the outcome (CV event) and the exposure (rs1800629 genotype) and because they produced a change greater than 10% in the HR.

The association between genotypes of the *TNFA* rs1800629 gene polymorphism and carotid IMT, FMD%-endothelium dependent vasodilatation and NTG%-endothelium independent vasodilatation was also tested using analysis of covariance (ANCOVA) adjusting for gender, age and duration of the disease at the time of the ultrasonographic study, and presence or absence of the SE and traditional CV risk factors. This study had 80% power to detect a difference in carotid intima-media thickness of 0.1 mm or higher, a difference of 2.5% or higher in FMD-endothelium-dependent vasodilatation and a difference of 3.5% or higher in NTG-endothelium-independent vasodilatation. Statistical significance was defined as $p \leq 0.05$. Calculations were performed with STATA 10 (STATA Corporation, College Station, Texas).

3. Experimental results

3.1. Influence of TNFA rs1800629 polymorphism in the risk of CV events

We compared the genotypic and allelic frequencies of the *TNFA* rs1800629 polymorphism between the subgroup of RA patients that suffered CV events and those who did not experience such CV complications (Table 2). We found an increased frequency of homozygous and heterozygous for the minor allele A (GA + AA

Table 2
Differences between RA patients with CV events or without CV events according to *TNFA* rs1800629 polymorphism.

TNFA rs1800629 genotype *n* (%)	With CV events	Without CV events	*p*	OR [95% CI]
GG	58 (62.4)	356 (72.1)		1
GA	31 (33.3)	125 (25.3)	0.087	1.52 [0.94–2.46]
AA	4 (4.3)	13 (2.6)	0.280	1.89 [0.60–5.99]
GA + AA	35 (37.6)	138 (27.9)	0.060	1.56 [0.95–2.54]
Allele 2*n* (%)				
G	147 (79.0)	837 (84.7)		1
A	39 (21.0)	151 (15.3)	0.053	1.47 [0.97–2.22]

CV, cardiovascular; OR [95% CI], odds ratio with 95% confidence interval.

genotypes) among patients who experienced CV events (37.6% vs. 27.9% in those without CV events, $p = 0.06$, OR 1.56 [95% CI 0.95–2.54]). Also, the minor allele A frequency was increased among the RA patients with CV events (21.0% vs. 15.3%, $p = 0.053$, OR 1.47 [95% CI 0.97–2.22]).

Taking into account these results, we performed a Cox regression model to account for the variation of risk of the first CV event through time according to the *TNFA* rs1800629 polymorphism, assuming a dominant model of effect (carriers of the minor allele A vs. non carriers: GA + AA vs. GG). In the crude analysis we observed a non significant increased high risk for the carriers of the minor allele A over time ($p = 0.071$, HR 1.49 [95% CI 0.97–2.31]). However, after adjustment for classic CV risk factors, a significantly increased risk of CV events among carriers of the minor allele A was found ($p = 0.023$, HR 1.72 [95% CI 1.076–2.74]). This latter model was adjusted for the presence or absence of at least one copy of the alleles belonging to the rheumatoid SE, therefore ascertaining the independency of both variables SE and the *TNFA* rs1800629 polymorphism.

3.2. Influence of TNFA rs1800629 polymorphism in the risk of CV events, according to the presence or absence of the SE

In a further step we stratified our sample according to the presence of absence of the SE (Table 3). To do so, in Cox regression model described above we established a statistical interaction between the presence or the absence of the SE and the fact of carrying or not at least one copy of the minor allele A of the *TNFA* rs1800629 polymorphism. We found a significant interaction ($p = 0.014$), meaning that the effect of the *TNFA* rs18006229 polymorphism in the risk of CV events was different according to the presence or absence of SE. We observed that being a carrier of the minor allele A was associated with a higher risk of CV events but only in those RA patients with at least one copy of the SE ($p = 0.003$, HR 2.23 [95% CI 1.32–3.78]). This association between both variables was still maintained after adjustment for gender, age and classic CV risk factors ($p = 0.024$), and the significant association between carriers of the minor allele A and CV disease was only confirmed in those RA patients carrying the rheumatoid SE ($p = 0.001$, HR 2.43 [95% CI 1.41–4.19]). In this regard, SE negative RA patients showed no association between this *TNFA* polymorphism and CV events (unadjusted $p = 0.26$, adjusted $p = 0.44$) (Table 3).

Table 3
Association between *TNFA* rs1800629 polymorphism and the presence of cardiovascular events, according to a dominant pattern of effect, taking into account an interaction between this polymorphism and the presence or absence of shared epitope.

	p	HR [95% CI]	*p*[a]	HR [95% CI][a]
GA + AA vs. GG				
SE+	0.003	2.23 [1.32–3.78]	0.001	2.43 [1.41–4.19]
SE−	0.26		0.44	
Interaction	0.014		0.024	

SE, shared epitope; HR [95% CI], hazard ratio with 95% confidence interval.

[a] Analyses adjusted for gender, age at rheumatoid arthritis diagnosis, presence or absence of rheumatoid factor, hypertension, diabetes, dyslipidemia, obesity and smoking habit.

In a recent study we described a significant association between the *MHTFR* rs1801131 polymorphism and the *HLA-DRB1* 0401*/0404*alleles with CV disease [5,8]. Taking into account these results, in the present study we assessed the combined effect of these two polymorphisms along with the *TNFA* rs1800629 polymorphism. Therefore, we analyzed the combined effect of these three genetic risk factors creating a variable ranging from 0 to 3 according to the number of genetic risk factors that were present. For this purpose we defined the following genetic risk factor: (1) being carrier of the minor allele C of the *MHTFR* rs1801131 polymorphism, (2) being carrier of the minor allele A of the *TNFA* rs1800629 variant and (3) the presence of at least one copy of the *HLA-DRB1* 0401*or 0404* alleles. We considered the number of genetic risk factors that were present regardless of the type of genetic risk factor that was present. Using this new variable we performed a Cox regression model that was adjusted for gender, age at RA disease diagnosis, presence or absence of rheumatoid factor, hypertension, diabetes, dyslipidemia, obesity and smoking habit. Following this procedure we observed that only when these three genetic risk factors together were present the association with CV disease was significant ($p = 0.019$, HR 3.07 95% CI [1.21–7.79]).

3.3. Influence of TNFA rs1800629 polymorphism in the presence of subclinical atherosclerosis

Regarding the surrogate markers of subclinical atherosclerosis, we observed that patients who were homozygous for the minor allele A had higher values of carotid IMT and lower values of FMD% and NTG%. However, the low number of patients carrying this genotype (3 from the group in whom carotid ultrasonography was performed and 4 from those in whom a brachial ultrasonography was carried out) did not allow us to draw any strong conclusion (Table 4A). On the other hand, when we grouped the patients who carried of the minor allele A (GA + AA) and compared them with the remaining patients (those carrying the GG genotype), we did not observe significant differences regarding any surrogate marker of subclinical atherosclerosis. Also, in the ANCOVA model (Table 4B) and after adjustment for demographical and classic CV risk factors, we did not find significant differences regarding carotid IMT values, FMD% and NTG%.

3.4. HLA-DR3 and CV disease

Due to the linkage disequilibrium between the *TNFA* rs1800629 polymorphism and HLA-DR3, we assessed the presence of HLA-DR3 in a subsample of both cohorts ($n = 481$). Fifty-seven patients (11.9%) carried at least a copy of HLA-DR3. We observed a significant association between the presence of HLA-DR3 and the presence of the minor allele A of *TNFA* rs1800629 polymorphism ($p < 0.0001$). Due to this linkage between HLA-DR3 and *TNFA* rs1800629 polymorphism, we assessed the effect of HLA-DR3 in

Table 4
(A) Comparison of carotid artery intima-media thickness, flow-mediated endothelium-dependent (post-ischemia) vasodilatation (FMD) and endothelium-independent (post-nitroglycerin) vasodilatation (NTG), according to *TNFA* rs1800629 polymorphism. (B) Comparison of carotid artery intima-media thickness, flow-mediated-endothelium dependent vasodilatation and endothelium-independent vasodilatation, according a recessive pattern of effect of *TNFA* rs1800629 polymorphism in an ANCOVA model.

	IMT mm, median (IQR)	*p*
(A)		
GG (*n* = 70)	0.74 (0.19)	
AG (*n* = 35)	0.71 (0.16)	
AA (*n* = 3)	0.86 (0.18)	
GA + AA vs. GG	0.72 (0.16)	0.67

	FMD%, median (IQR)	*p*	NTG% median (IQR)	*p*
GG (*n* = 84)	6.12 (5.49)		17.29 (7.71)	
AG (*n* = 42)	5.25 (4.00)		17.70 (7.73)	
AA (*n* = 4)	3.03 (2.21)		10.88 (6.16)	
GA + AA vs. GG	5.05 (3.91)	0.25	17.10 (7.79)	0.89

	IMT	FMD%	NTG%
(B)			
p GA + AA vs. GG[a]	0.92	0.11	0.40

FMD, flow-mediated endothelium-dependent vasodilatation; NTG, endothelium-independent (post nitroglycerin) vasodilatation; IMT, carotid artery intima-media thickness; SD, standard deviation.

[a] Analyses adjusted for gender, age at the time of ultrasonography performance, follow-up time and presence or absence of SE, hypertension, diabetes, dyslipidemia, obesity and smoking habit.

the risk of suffering CV events. However, no significant associations were observed ($p = 0.74$).

4. Discussion

The present study showed that RA patients carrying the minor allele A of the *TNFA* rs1800629 (G > A) polymorphism are associated with a higher risk of CV events, after adjustment for demographic and classic CV risk factors.

Taking into account that the presence the rheumatoid SE has been repeatedly associated with endothelial dysfunction, CV events and a poor outcome of RA [5,6,25,26], we aimed to analyze the effect of the *TNFA* rs1800629 (G > A) polymorphism stratifying our series of patients according to the presence or absence of the rheumatoid SE. For this purpose, we introduced an interaction between the *TNFA* polymorphism and the SE in the Cox regression model. Following this procedure we observed that the association between the *TNFA* variant and CV events was only restricted to those patients carrying the SE.

The higher CV risk and accelerated atherogenesis observed in patients with RA cannot only be explained by the presence of traditional CV risk factors [1,4]. This fact led to the notion that RA or a high-grade systemic inflammatory status act as independent risk factors, conferring predisposition to the pathogenesis of the atherosclerotic disease or accelerating the disease process in affected individuals [27]. Therefore, the interaction between SE and *TNFA* may implicate that the alterations produced by the *TNFA* rs1800629 itself are not strong enough to yield an increase in the risk of CV events.

Regardless of potential controversies, most data suggest a potential role of the *TNFA* rs1800629 polymorphism in the regulation of *TNFA* gene expression [12]. However, no association between this *TNFA* variant and CV disease was observed in subjects without RA [28–33]. Therefore, it is plausible to think that some additional factors, such as those induced by the presence of the rheumatoid SE in patients with RA, may be required to experience a clinically evident CV effect mediated by the *TNFA* variant. Although our data may also suggest that patients with RA carrying the A allele of the *TNFA* may have more severe subclinical atherosclerosis manifested by increased carotid IMT and worse values of FMD% and NTG%, the low number of patients analyzed who were homozygous for the *TNFA* rs1800629 AA genotype did not allow us to raise any strong conclusion about this finding.

Our results are in accordance with the previously described deleterious effects of TNF-α over endothelial cells such as promotion of endothelial dysfunction through impairing nitric oxide bioavailability, cell injury [34], and apoptosis and suppression of the activities of endothelial progenitor cells that may sustain endothelial repair [35]. TNF-α also promotes endothelial injury through recruitment of immune cells, such as neutrophils, which can mediate tissue destruction. On the other hand, anti-TNF therapy has been associated with an improvement of endothelial function in RA patients with severe disease refractory to conventional disease modifying anti-rheumatic drugs [36]. Also, registry databases have shown that anti-TNF therapy may reduce the CV risk in patients with RA, potentially through a reduction in the inflammatory load [37].

5. Conclusions

Our findings suggest that the *TNFA* rs1800629 (G > A) gene polymorphism is associated with predisposition to CV complications in patients with RA. This predisposition seems to be restricted to individuals carrying the rheumatoid SE. Taking together; these results reinforce the potential implication of a genetic component in the development of CV disease in patients with RA.

Competing interest

The authors declare that they have no competing interest.

Author's contributions

LRR carried out genotyping, participated in the design of the study, data analysis and helped to draft the manuscript. CGJ performed the ultrasonographic studies and participated in the design of the study, data analysis and helped to draft the manuscript RPM participated in genotyping and data analysis. TRV participated in the acquisition and interpretation of data. LR participated in the acquisition and interpretation of data. JAM-F participated in the acquisition and interpretation of data. BF has been involved in the acquisition and interpretation of data and in revising it critically for important intellectual content. JM has made substantial contributions to conception and design of the study, acquisition of data, coordination and helped to draft the manuscript and has given final approval of the version to be published. MAG-G has made substantial contributions to conception and design of the study, acquisition of data, coordination and helped to draft the manuscript and has given final approval of the version to be published.

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RESEARCH ARTICLE Open Access

CCR5Δ32 variant and cardiovascular disease in patients with rheumatoid arthritis: a cohort study

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Abstract

Introduction: The aim of our study was to analyze the influence of the *CCR5Δ32* polymorphism in the risk of cardiovascular (CV) events and subclinical atherosclerosis among patients with rheumatoid arthritis (RA).

Methods: A total of 645 patients fulfilling the American Rheumatism Association 1987 revised classification criteria for RA were studied. Patients were genotyped for the *CCR5* rs333 polymorphism using predesigned TaqMan assays. Also, *HLA DRB1* genotyping was performed using molecular-based methods. Carotid intima-media thickness, flow-mediated endothelium-dependent dilatation (FMD) and endothelium-independent vasodilatation, which were used as surrogate markers of subclinical atherosclerosis, were measured in a subgroup of patients with no clinical CV disease.

Results: A lower frequency of carriers of the *CCR5Δ32* allele among patients with CV events (3.4% versus 11.3%, $P = 0.025$, odds ratio 0.28, 95% confidence interval (95% CI) 0.06 to 0.89) was observed. However, after adjusting for gender, age at time of RA diagnosis, and the presence of shared epitope, rheumatoid factor and classic CV risk factors in the Cox regression analysis, this reduction of CV events in *CCR5Δ32* allele carriers was slightly outside the range of significance ($P = 0.097$; hazard ratio 0.37 (95% CI 0.12 to 1.19)). Carriers of the *CCR5Δ32* deletion also showed higher FMD values than the remaining patients (*CCR5/CCR5Δ32* patients: 7.03% ± 6.61% versus *CCR5/CCR5* patients: 5.51% ± 4.66%). This difference was statistically significant when analysis of covariance was performed ($P = 0.024$).

Conclusions: Our results show a potential influence of the *CCR5Δ32* deletion on the risk of CV disease among patients with RA. This may be due to a protective effect of this allelic variant against the development of vascular endothelial dysfunction.

Keywords: rheumatoid arthritis, atherosclerosis, cardiovascular disease, genetics, *CCR5Δ32*, rs333

Introduction

CCR5 is a G protein-coupled receptor that is expressed on macrophages, monocytes, Th1 cells, immature dendritic cells, endothelial cells and vascular smooth muscle cells (VSMCs) [1-4]. The activation of this molecule through one of its ligands contributes to the survival and accumulation of macrophages [5] during inflammation, to the recruitment and activation of T cells [6] and to the activation and secretion of tissue factor [2] of VSMCs. It also participates in osteoclast formation [1]. Consistent with its roles, CCR5 is considered to play a role in both rheumatoid arthritis (RA) and atherosclerosis [7].

The *CCR5Δ32* (dbSNP rs333) polymorphism is defined by a 32-bp deletion that leads to a truncated nonfunctional receptor [8], which is eliminated from the cell surface in homozygous individuals or its expression is reduced by 20% to 30% in heterozygous individuals [9]. Several studies have demonstrated a protective effect of the *CCR5Δ32* allele in patients with CV disease [10,11], although others have demonstrated no association [12-14].

CCR5Δ32 deletion also was suggested to have a protective effect on RA susceptibility in a Spanish cohort [15]. Although other studies could not confirm a protective effect of this variant [16], a meta-analysis has suggested a protective effect [17]. When the influence of the

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CCR5Δ32 polymorphism and disease severity were analyzed, conflicting results were reported [16,18]. These contradictions may be the result of differences in study design, study power or the populations assessed; therefore, additional data will be helpful to understand the role of *CCR5* gene polymorphisms. Taking all of these considerations together, the aim of the present study was to analyze the influence of the *CCR5Δ32* polymorphism on the risk of CV events and subclinical atherosclerosis in patients with RA.

Materials and methods

Patients and study protocol

Between March 1996 and March 2008, 660 consecutive patients who fulfilled the American Rheumatism Association 1987 revised classification criteria for RA [19] were recruited from the rheumatology outpatient clinics of Hospital Xeral-Calde (Lugo, Spain) and Hospital Clínico San Carlos (Madrid, Spain). DNA samples were extracted from these patients at the time of recruitment. Between December 2009 and January 2010, patients' clinical records were examined until death, loss of follow-up or 1 December 2009. Sociodemographic and clinical data regarding clinical manifestations, classic CV risk factors and history of CV events were registered. Clinical definitions for CV events and classic CV risk factors were established as previously described [20,21]. Information on the main demographic characteristics, CV risk factors and CV events of patients in whom genotyping success was achieved (n = 645 (97.7%)) is shown in Table 1. Hospital Xeral-Calde and Hospital Clinico San Carlos are the referral centers for the population of each respective area. The first CV event was defined as an event (case) of CV complication diagnosed at the hospital in a patient without a history of CV disease.

Endothelial dysfunction was assessed between March 2007 and September 2009 in a random subgroup of patients in the Lugo cohort with no history of CV disease. Flow-mediated endothelium-dependent vasodilatation FMD (postischemia) and endothelium-independent vasodilatation NTG (postnitroglycerin) were assessed on the basis of a brachial artery reactivity study in 127 patients as previously reported [22,23]. Intraobserver variability was 1.3% and 1.9%, respectively, based on repeat ultrasonography in 32 healthy controls. Assessment of the endothelial function of patients undergoing anti-TNF therapy was performed 24 to 48 hours before drug administration. Carotid artery intima-media thickness (IMT) was determined in 105 patients as previously reported [23,24]. The correlation coefficient was 0.98 based on repeat ultrasonography in 20 RA patients and 20 healthy controls. Participants' written consent was obtained according to the Declaration of Helsinki, and the design of the study was approved by the Ethics Committee of Galicia (Spain) and the Hospital Clinico San Carlos (Madrid).

Table 1 Demographic characteristics and genotype distribution of the patients with rheumatoid arthritis included in the study[a]

Variables	Patients (N = 645)
Females	484 (75.0)
Median patient age at time of disease diagnosis, years (IQR)	56 (45 to 65)
Median follow-up, years (IQR)	13 (7 to 19)
Anti-CCP-positive (N = 470)	283 (60.2)
Rheumatoid factor-positive (N = 635)	474 (74.7)
Shared epitope (N = 579)	366 (63.2)
Cardiovascular events	87 (13.5)
Ischemic heart disease	47 (7.3)
Cerebrovascular accidents	19 (2.9)
Heart failure	23 (3.6)
Peripheral arteriopathy	10 (1.6)
Hypertension (N = 640)	248 (38.8)
Diabetes mellitus (N = 637)	74 (11.6)
Dyslipidemia (N = 621)	282 (45.4)
Obesity (N = 610)	66 (10.8)
Smoking habit (N = 621)	112 (18.0)
CCR5Δ32 rs333	
CCR5/CCR5	579 (89.8)
CCR5 /CCR5Δ32	64 (9.9)
CCR5Δ32 /CCR5Δ32	2 (0.3)
CCR5	1,222 (94.7)
CCR5Δ32	68 (5.3)

[a]Anti-CCP: anticyclic citrullinated peptide antibodies; IQR: interquartile range. Values are n (%) except where indicated otherwise.

Genotyping

CCR5 genotyping

DNA from patients was obtained from whole peripheral blood using standard methods. Participants were genotyped to determine *CCR5* status using TaqMan Assays-on-Demand from Applied Biosystems following the manufacturer's protocol and analyzed using the Applied Biosystems 7900 HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The typing was successful in 645 patients (97.7%). Ten percent of the samples were regenotyped at random. We observed no differences from the results obtained before.

Shared epitope determination

Several *HLA-DRB1* alleles are associated with susceptibility to RA, encoding a conserved amino acid sequence at positions 70 to 74 in the third hypervariable region, called the "shared epitope" [25]. *HLA DRB1* typing was carried out using a reverse dot-blot kit with sequence-specific oligonucleotide probes (Dynal RELI SSO HLA-DRB1 Typing Kit; Dynal Biotech, Bromborough, UK). In

our sample, 63.2% of the patients had at least one copy of the rheumatoid shared epitope, a frequency higher than that found previously in Spanish individuals without RA [26].

Statistical analysis

Comparison of means was performed using a *t*-test. Comparison of proportions between two or more groups was carried out using a χ^2 test or Fisher's exact test when required. Hardy-Weinberg equilibrium (HWE) was tested in the RA patients with and without CV disease. Both groups were in HWE (P = 0.87 and P = 0.93, respectively). The study had 80% power for detecting an odds ratio (OR) ≥ 2. A Cox regression model was used to estimate the influence of the *CCR5* polymorphism on CV disease. We used the occurrence of at least one CV event as the outcome. Survival time was defined as "age of the subjects at" or "elapsed time between RA diagnosis and" the first CV event, patient's death, loss of follow-up or 1 December 2009. Patients who died as a result of any cause other than CV events were considered not to have had CV events. Proportional hazards assumptions were tested using Schoenfeld residuals. The results are expressed as hazard ratios (HRs) with 95% confidence intervals (95% CIs) and were computed as both crude analysis and adjusted for age at RA diagnosis, gender and classic CV risk factors. The selected variables used for adjustment were selected on the basis of their association with the outcome (CV event) and the exposure (*CCR5* genotype) and because they produced a change > 10% in the HR.

The association between *CCR5Δ32* and carotid IMT, FMD and NTG was also tested using analysis of covariance (ANCOVA) adjusting for gender, age and duration of the disease at the time of ultrasonography as well as for the presence or absence of the shared epitope and traditional CV risk factors. This study had 80% power to detect a difference in carotid IMT of 0.1 mm or higher, a difference of 2.5% or higher in FMD and a difference of 3.5% or higher in NTG. Statistical significance was defined as $P \leq 0.05$. Calculations were performed with Stata version 10 software (StataCorp LP, College Station, TX, USA).

Results

Influence of the *CCR5Δ32* polymorphism on the risk of cardiovascular events

We compared the genotypic and allelic frequencies of the *CCR5Δ32* polymorphism between the subgroups of RA patients with and without CV events (Table 2). We found a decreased frequency of carriers of the deletion (*CCR5*/*CCR5Δ32* + *CCR5Δ32*/*CCR5Δ32*) among the patients with CV events (3.4% versus 11.3%, P = 0.025, OR 0.28 (95% CI 0.06 to 0.89)). Likewise, the *CCR5Δ32* allele frequency was also decreased among the RA patients with CV events (1.7% versus 5.8%, P = 0.024, OR 0.28 (95% CI 0.06 to 0.88)).

We performed Cox regression analysis to account for the variation of risk of the first CV event over time according to the *CCR5*Δ32 variant, assuming a dominant model of effect (carriers versus noncarriers of the deletion) (Table 3). When age was used as a measure of survival time, to carry at least a copy of the *CCR5Δ32* allele was not associated with a lower risk of CV disease over time, in both the crude and adjusted analyses (P = 0.14 and P = 0.14, respectively). When elapsed time from RA diagnosis was used, the reduction of CV events in carriers was slightly outside the range of significance in the crude analysis (P = 0.078, HR 0.35 (95% CI 0.11 to 1.12)) and in the adjusted analysis (P = 0.097, HR 0.37 (95% CI 0.12 to 1.19)).

Influence of the *CCR5Δ32* polymorphism in subclinical atherosclerosis

Owing to the small number of homozygotes for the *CCR5Δ32* deletion, none of those patients underwent ultrasonography for the assessment of subclinical atherosclerosis. Therefore, the comparisons were performed between heterozygote and homozygote subjects with two copies of the allele without the 32-bp deletion. In the

Table 2 Differences between rheumatoid arthritis patients with or without cardiovascular events according to *CCR5Δ32* polymorphism[a]

	RA patients, *n* (%)			
***CCR5* genotype**	**With CV events**	**Without CV events**	***P* value**	**OR (95% CI)**
CCR5/CCR5	84 (96.6)	495 (88.7)		1
CCR5/CCR5Δ32	3 (3.4)	61 (10.9)	0.029	0.29 (0.06 to 0.92)
CCR5Δ32/CCR5Δ32	0 (0.0)	2 (0.4)	0.99	0.0 (0.0 to 31.63)
CCR5Δ32 carriers	3 (3.4)	63 (11.3)	0.025	0.28 (0.06 to 0.89)
Allele 2				
CCR5	171 (98.3)	1,051 (94.2)		1
CCR5Δ32	3 (1.7)	65 (5.8)	0.024	0.28 (0.06 to 0.88)

[a]CV: cardiovascular; OR (95% CI): odds ratio with 95% confidence interval; RA: rheumatoid arthritis.

Table 3 Cox regression model to estimate the influence of the *CCR5Δ32* polymorphism in the risk of cardiovascular disease in patients with rheumatoid arthritis[a]

Patient group characteristics	*P* value	HR (95% CI)	*P* value[b]	HR (95% CI)[b]
Carriers vs. noncarriers[c]	0.14	0.42 (0.13 to 1.33)	0.14	0.42 (0.13 to 1.33)
Carriers vs. noncarriers[d]	0.078	0.35 (0.11 to 1.12)	0.097	0.37 (0.12 to 1.19)

[a]HR (95% CI): hazard ratio with 95% confidence interval. [b]Analyses adjusted for gender, age at rheumatoid arthritis (RA) diagnosis, presence or absence of shared epitope, rheumatoid factor, hypertension, diabetes, dyslipidemia, obesity and smoking habit. [c]Using as survival time the patient's age at the time of the first cardiovascular event, patient's death, loss of follow-up or 1 December 2009. [d]Using as survival time the elapsed time between RA diagnosis and the time of the first cardiovascular event, the patient's death, loss of follow-up or 1 December 2009.

unadjusted analysis, we did not observe a significant difference regarding carotid IMT, FMD or NTG ($P = 0.32$, $P = 0.28$ and $P = 0.64$, respectively) (Table 4). However, in the adjusted ANCOVA, we observed a significant association between being heterozygous for the *CCR5Δ32* deletion and a higher FMD ($P = 0.024$) (Table 5). In this regard, the mean FMD percentage among heterozygotes was higher than in those without the allelic variation (7.03% ± 6.61% versus 5.51% ± 4.66%, respectively). Interestingly, the mean FMD percentage among heterozygous patients was considered normal [23].

Discussion

This study is the first to address the role of the *CCR5Δ32* deletion in the risk of CV disease in RA patients. We observed a lower frequency of this variant among the patients with CV complications. However, in the Cox regression model, the potential protective role of the *CCR5Δ32* deletion was slightly outside the range of significance. Regarding the surrogate markers of subclinical atherosclerosis, we observed that RA patients with a copy of the *CCR5* allele containing the 32-bp deletion had a higher FMD value. In fact, the mean FMD value in those patients was over the cutoff point for normal endothelial function observed in our echocardiography laboratory. These observations suggest a protective effect of the *CCR5Δ32* deletion against the development of endothelial dysfunction, an early step in the atherogenic process, in patients with RA. Although no association of *CCR5Δ32* deletion with carotid IMT was observed in our series, a significantly lower carotid IMT in the common carotid artery was found in individuals carrying the *CCR5Δ32* deletion in the Bruneck study [27], which is a prospective population-based survey of the epidemiological pathogenesis of atherosclerosis. Since FMD constitutes a physiologic assessment of endothelial dysfunction and carotid IMT is an anatomic structural measure of subclinical atherosclerosis, it is logical that FMD might be a more useful diagnostic marker than carotid IMT in the early stages of the disease. In this regard, no relationship between carotid IMT and brachial artery FMD was found in middle-aged men without a history of CV disease who were considered to be at low or intermediate risk for future CV events based on current risk stratification algorithms [28]. Brachial FMD and carotid IMT values may indicate distinct and independent stages in the complex pathways leading to accelerated atherosclerosis in patients with RA. It was recently reported that, in patients with RA without CV disease, the association between FMD and carotid IMT values was observed only in patients with long disease duration [29].

As we pointed out in the Introduction, CCR5 seems to play an important role in the development of atherosclerosis. In rodent knockout models, the lack of CCR5 was associated with a reduction in plaque formation and macrophages, Th1 and smooth muscle cell accumulation, and increased expression of anti-inflammatory cytokines such as IL-10 [4,30,31]. Furthermore, studies using an antagonist of the CCR5 and CXCR3 chemokine receptors [32] or a recombinant RANTES (regulated upon activation, normal T cell expressed and secreted) receptor antagonist [33] have demonstrated an attenuation of atherogenesis in low-density lipoprotein receptor-null mice. In humans, the presence of the *CCR5Δ32*

Table 4 Comparison of carotid artery intima-media thickness, flow-mediated endothelium-dependent (postischemia) vasodilatation and endothelium-independent vasodilatation according to the *CCR5Δ32* polymorphism distribution[a]

CCR5Δ32 polymorphism	Mean IMT, mm (SD)	*P* value	Mean FMD % (SD)	*P* value	Mean NTG % (SD)	*P* value
CCR5/CCR5 ($n = 95$)	0.73 (0.16)					
CCR5/CCR5Δ32 ($n = 10$)	0.79 (0.32)					
CCR5Δ32/CCR5Δ32 ($n = 0$)	-	0.32				
CCR5/CCR5 ($n = 113$)			5.51 (4.66)		17.2 (7.64)	
CCR5/CCR5Δ32 ($n = 14$)			7.03 (6.61)		18.21 (8.45)	
CCR5Δ32/CCR5Δ32 ($n = 0$)			-	0.28	-	0.64

[a]IMT: intima-media thickness; FMD: flow-mediated endothelium-dependent (postischemia) vasodilatation; NTG: endothelium-independent (postnitroglycerin) vasodilatation.

Table 5 Comparison of carotid artery intima-media thickness, flow-mediated endothelium-dependent (postischemia) vasodilatation and endothelium-independent vasodilatation according to a recessive pattern of effect of *CCR5Δ32* polymorphism in an analysis of covariance model[a]

	P value		
***CCR5Δ32* group**	**IMT**	**FMD**	**NTG**
Carriers vs. noncarriers	0.77	0.024	0.11

[a]FMD: flow-mediated endothelium-dependent vasodilatation; IMT: carotid artery intima-media thickness; NTG: endothelium-independent (postnitroglycerin) vasodilatation. Analyses were adjusted for gender, age at the time of ultrasonography, follow-up time, and presence or absence of rheumatoid shared epitope, hypertension, diabetes, dyslipidemia, obesity and smoking habit.

deletion, when associated with lower or even absent expression of the CCR5 molecule on the cell surface [9], has also been associated with a lower risk of CV disease in some studies [10,11]. In the present study, we observed better endothelial function in response to ischemia among those RA patients carrying the *CCR5Δ32* deletion. However, this fact was not associated with a strong reduction in the risk of CV disease. Since endothelial dysfunction is an early step in the atherogenic process, these observations might appear to be contradictory. However, RA is a chronic inflammatory disease, and it is well known that the persistence of chronic inflammatory burden is of major importance in the development of CV events in these patients [20]. Because of that, it is possible that a chronic inflammatory status might overcome the potential protective effect that the *CCR5Δ32* deletion may have against the progression of the atherogenic process.

Conclusions

In summary, our results show a potential influence of the *CCR5Δ32* deletion on the risk of CV disease in patients with RA. This may be due to a protective effect of this allelic variant against the development of vascular endothelial dysfunction. However, further studies need to be carried out to replicate our findings and confirm the role of this molecule in the atherosclerosis disease observed in patients with RA.

Abbreviations

ANCOVA: analysis of covariance; anti-CCP: anti-cyclic citrullinated peptide antibodies; bp: base pair; CI: confidence interval; CV: cardiovascular; FMD: endothelium-dependent flow-mediated vasodilatation (postischemia); HLA: human leukocyte antigen; HR: hazard ratio; IMT: intima-media thickness; NTG: endothelium-independent (postnitroglycerin) vasodilatation; RA: rheumatoid arthritis; RF: rheumatoid factor; TNF: tumor necrosis factor.

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Authors' contributions

LRR and MGB carried out genotyping, participated in the design of the study and the data analysis, and helped to draft the manuscript. CGJ performed the ultrasonographic studies, participated in the design of the study and the data analysis, and helped to draft the manuscript RPM participated in genotyping and data analysis. TRV, JAMF and LR participated in the acquisition and interpretation of data. BF was involved in the acquisition and interpretation of data and in revising it critically for important intellectual content. JM and MAGG made substantial contributions to the conception and design of the study, the acquisition of data, study coordination, helped to draft the manuscript, and gave final approval of the version to be published. All authors read and approved the final version of the manuscript for publication. MAGG and JM share senior authorship of this manuscript.

Competing interests

The authors declare that they have no competing interests.

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Lack of association between *RETN* rs1862513 polymorphism and cardiovascular disease in patients with rheumatoid arthritis

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Abstract

Objective

To assess the influence of the RETN rs1862513 polymorphism in the risk of cardiovascular (CV) disease and subclinical atherosclerosis in patients with rheumatoid arthritis (RA).

Methods

Six hundred and sixty-eight patients fulfilling the 1987 American College of Rheumatology classification criteria for RA, seen at the rheumatology outpatient clinics of Hospital Xeral-Calde, Lugo, and Hospital San Carlos, Madrid, Spain, were studied. Patients were genotyped for the RETN rs1862513 polymorphism using predesigned TaqMan single nucleotide polymorphism genotyping assay. Also, HLA-DRB1 genotyping was performed using molecular based methods. Carotid intima-media thickness (IMT), flow-mediated endothelium-dependent and endothelium independent vasodilatation, used as surrogate markers of subclinical atherosclerosis, were measured in a subgroup of patients.

Results

No significant differences in the genotypic or in the allelic distribution between RA patients with or without CV disease were found. In this regard, we only observed a slight increased frequency of homozygous and heterozygous for the minor allele G (CG+GG genotypes) among patients who experienced CV events compared to those without CV events (53.04% vs. 52.62%, p=0.94). A higher frequency of classic CV risk factors was observed among the carriers of the minor allele G. However, in the adjusted logistic regression model no association between the RETN variant and CV disease was found (p=0.50). Also, when surrogate markers of subclinical atherosclerosis were assessed, in the adjusted ANCOVA model only a trend towards a higher carotid IMT was found among allele G carriers (p=0.06).

Conclusion

RETN rs1862513 polymorphism does not seem to be a genetic risk factor for both clinically evident CV disease and subclinical atherosclerosis in patients with RA.

Key words

rheumatoid arthritis, atherosclerosis, cardiovascular disease, genetics, *RETN*, rs1862513

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Introduction

Rheumatoid arthritis (RA) is a chronic disease associated with an accelerated atherosclerosis (1-3), causing an increased cardiovascular (CV) morbidity and mortality (4). This accelerated atherosclerosis is a consequence of both traditional CV risk factors (5, 6) and the presence of a chronic systemic inflammatory status (7-9). With respect to this, a strong correlation between systemic inflammation and CV disease has been observed among RA subjects (8, 10).

Resistin is an adipokine that in humans is mainly expressed in monocytes and macrophages (11, 12). Unlike mice (13), human isolated primary adipocytes and preadipocytes do not express this adipokine (14). It is believed that resistin plays a role in inflammatory responses (15). Resistin expression and secretion is regulated by innate inflammatory signals such as endotoxins or LPS (16) and proinflammatory cytokines (17-21), such as TNF-α. Interestingly, high levels of resistin have been found in synovial fluid from patients with RA (22). Also, resistin serum levels are higher among RA patients compared to healthy controls (22). A positive correlation between C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) with serum resistin has also been observed in RA patients (23-26). Also, higher DAS28 (23) and Larsen score (24) seem to be associated to higher resistin levels.

A *RETN* polymorphism located at -420 (rs1862513 C>G) has been associated to an enhanced *RETN* promoter activity, resulting in higher resistin plasma levels (25) in Asian populations. However, in Caucasians, this influence seems to be much weaker (26-29).

Taking into account the role of this adipokine in the inflammatory response and the emerging role of chronic inflammation in atherosclerosis (30), association between polymorphism of *RETN* rs1862513 and CV disease has been investigated. In nondiabetic Caucasian samples, no association between this polymorphism and coronary arterial calcification (29), angiographic coronary arterial disease (28), occurrence of myocardial infarction (31), CV mortality (28) or carotid atherosclerosis (31) was described. However, in Caucasian (32) and Japanese (33) diabetic patients, this polymorphism has been associated with cerebrovascular disease.

Taking into account the potential role played by this adipokine in RA and that *RETN* rs1862513 polymorphism may play a role in CV disease in those subject with an underlying chronic inflammatory diseases (34), we decided to analyse the potential role of this polymorphism to develop CV disease in RA patients.

Material and methods

Patients and study protocol

Between March 1996 and March 2008, 696 consecutive patients, fulfilling the 1987 American College of Rheumatology classification criteria for RA (35), were recruited from the rheumatology outpatient clinics of Hospital Xeral-Calde, Lugo and Hospital Clínico San Carlos, Madrid, Spain. A DNA sample (see below) was extracted from these patients at the time of recruitment. Between December 2009 and January 2010 patient's clinical records were examined until patient's death, loss of follow-up or December 1st, 2009. Socio-demographical and clinical data regarding clinical manifestations, traditional CV risk factors and history of CV events were registered. Clinical definitions for CV events and risk factors were established as previously described (7, 36). In this regard, patients were considered to have diabetes mellitus if before disease diagnosis they had been diagnosed as having diabetes mellitus by their family physicians or if 2 fasting plasma glucose levels on different days at the time of disease diagnosis or over the extended follow-up were >125 mg/dl (7). Smoking habit was considered to be present in those patients who smoked at the time of disease diagnosis, during the follow-up or who had smoked within the 10 years before the onset of RA symptoms or the disease diagnosis. A CV event was considered to be present if the patient had ischaemic heart disease, heart failure, cerebrovascular accident or peripheral arteriopathy.

The definition of ischaemic heart disease (IHD) included acute coronary

syndromes with or without persistent ST-segment elevation and chronic coronary heart disease. IHD was diagnosed if any of the following criteria were satisfied: a recorded diagnosis of ischaemic cardiopathy, on account of some acute coronary syndrome (acute myocardial infarction or unstable angina), the presence of pathological Q waves in the electrocardiogram, and coronary images showing >50% stenosis of at least one coronary vessel (7). Data regarding the clinical presentation of heart failure were also collected for all patients, based on the Framingham criteria (36). A patient was considered to have a cerebrovascular accident when he/she had a stroke and/or transient ischaemic attacks (TIAs). Strokes were classified according to their clinical features and they were confirmed by computed tomography and/or magnetic resonance imaging. TIAs were diagnosed if the symptoms were self-limited in less than 24 hours, without residual neurological damage (37). Peripheral arterial disease was considered to be present if it was confirmed by Doppler and arteriography (38). Information on their main demographic characteristics, CV risk factors and CV events are shown in Table I.

Since Hospital Xeral-Calde and Hospital Clínico San Carlos are the referral centres for the population of each respective area, the first CV event was defined as an event (case) of CV complication diagnosed at the hospital in a patient without a previous history of CV disease. Based on previously established protocols of management, all patients on methotrexate therapy were treated with folate supplementation.

To determine the potential association between *RETN* rs1862513 gene polymorphisms and the presence of subclinical atherosclerosis, between March 2007 and September 2009 a random subgroup of patients among the Lugo cohort with no previous history of CV events was selected. Presence of endothelial dysfunction was assessed by a brachial artery reactivity study in 124 patients. Flow-mediated endothelium-dependent dilatation FMD (post-ischaemia) and endothelium independent- NTG (post-nitroglycerin) vasodilatation were measured by brachial ultrasonography as previously reported (39, 40). A value of FMD less than 7% was considered pathologic, indicating the presence of endothelial dysfunction (40). Intra-observer variability for FMD and NTG was 1.3% and 1.9%, respectively, based on the repeat of the brachial ultrasonography in 32 healthy controls. Assessment of endothelial function of those patients undergoing anti-TNF therapy was performed 24-48 hours before its administration. Also, carotid ultrasonography studies were performed in 104 patients to determine the carotid artery intima-media thickness (IMT). It was assessed in the right common carotid artery as previously reported (40, 41).

Informed consent was obtained from all patients. The local institutional committees approved the study.

Table I. Demographic characteristics and genotype distribution of the patients with rheumatoid arthritis included in the study.

Variables	n=668	
Females	497	(74.40)
Age of patients at the time of disease diagnosis, years, median [IQR]	56.0	(45.0–65.3)
Time follow-up, years, median [IQR]	13.3	(6.8–22.9)
anti-CCP positive (n=487)	285	(58.52)
Rheumatoid Factor positive (n=652)	480	(73.62)
Shared epitope (n=598)	375	(62.71)
Cardiovascular events	115	(17.22)
Ischaemic heart disease	62	(9.28)
Cerebrovascular accidents	32	(4.79)
Heart failure	30	(4.49)
Peripheral arteriopathy	13	(1.95)
Hypertension (n=663)	265	(39.97)
Diabetes mellitus (n=661)	84	(12.71)
Dyslipidemia (n=645)	299	(46.36)
Obesity (n=627)	67	(10.69)
Smoking habit (n=637)	112	(17.58)
RETN rs1862513		
CC	316	(47.31)
CG	276	(41.32)
GG	76	(11.38)
C	908	(67.96)
G	428	(32.04)

Except where indicated otherwise, values are n (%). IQR: Interquartile range. Anti-CCP: anti-cyclic citrullinated peptide antibodies.

Genotyping

– RETN *genotyping*

DNA from patients was obtained from peripheral blood, using standard methods.

Six hundred and ninety-six subjects were genotyped to determine *RETN* rs1862513 status using TaqMan Assays-on-Demand from Applied Biosystems following the manufacturer's protocol and analysed using the ABI 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The typing was successful in 668 patients (95.98%).

– *Shared epitope determination*

Several *HLA-DRB1* alleles (*HLA-DRB1**0401, *0404, *0405, *0408, *0101, *0102, *1001, *1402) are associated with susceptibility to rheumatoid arthritis. These alleles encode a conserved amino acid sequence (QKRAA, QRRAA, or RRRAA), called the shared epitope, at position 70-74 in the third hypervariable region of the HLA-DRβ1 molecule (42).

HLA-DRB1 typing was carried out using a reverse dot-blot kit with sequence-specific oligonucleotide (SSO) probes (Dynal RELITM SSO HLA-DRB1 typing kit; Dynal Biotech, Bromborough, UK). When necessary, high-resolution typing of HLA-DRB1*03 samples was performed using Dynal AllSetTM SSP DRB1*03.

Statistical analysis

Comparison of means was performed using *t*-test. Comparison of proportion between 2 or more groups was carried out using χ^2 test or Fisher's exact test, when required.

Strength of association between CV events and genotypes of *RETN* rs1862513 polymorphism was estimated using odds ratios (OR) and 95% confidence intervals (CI), via multiple logistic regression; estimates were further adjusted by gender, age at RA diagnosis, time of follow-up, presence or absence of shared epitope and traditional CV risk factors (hypertension, diabetes mellitus, dyslipidemia, obesity and smoking habit) as potential confounders. A dominant pattern of effect was considered for the RETN variant (CG+GG *vs*. CC).

A Cox regression model was used to estimate the influence of the *RETN* rs1862513 polymorphism on CV disease. We used the occurrence of at least one CV event as the outcome and the survival time when the first CV event occurred. The survival time of individuals without CV events was the age at patient's death, loss to follow-up or December 1st, 2009. Patients who died of any other causes different from CV events were considered as not having CV events. Proportional hazard assumption was tested using Schoenfeld residuals. Results were expressed as hazard ratios (HRs) with 95% confidence intervals [95% CIs] and were computed as both crude and adjusted for age at RA diagnosis, gender and classic CV risk factors.

The association between genotypes and alleles of the *RETN* rs1862513 gene polymorphism and carotid IMT, FMD%-endothelium dependent vasodilatation and NTG%-endothelium independent vasodilatation was tested using unpaired *t*-test, to compare between 2 groups, and one-way analysis of variance (ANOVA), to compare among more than two groups. Moreover, we also tested association between these parameters and alleles using analysis of covariance (ANCOVA) adjusting by gender, age and duration of the disease at the time of the ultrasonographic study, and presence or absence of shared epitope and traditional CV risk factors.

Statistical significance was defined as p≤0.05. Calculations were performed with STATA 10 (STATA Corporation, College Station, Texas).

Table II. Differences between RA patients with CV events or without CV events according to the *RETN* rs1862513 polymorphism.

RETN rs1862513	with CV events	without CV events	*p*-value	OR [95% CI]
Genotype n (%)				
CC	54 (46.96)	262 (47.38)		1
CG	50 (43.48)	226 (40.87)	0.74	1.07 [0.69–1.68]
GG	11 (9.57)	65 (11.75)	0.58	0.82 [0.38–1.73]
CG+GG	61 (53.04)	291 (52.62)	0.94	1.02 [0.68–1.52]
Allele 2n (%)				
C	158 (68.70)	750 (67.81)		1
G	72 (31.30)	356 (32.19)	0.79	0.96 [0.70–1.32]

CV: Cardiovascular. OR [95% CI]: Odds Ratio with 95% Confidence Interval.

Table III. Demographic characteristics and CV risk factor distribution in carriers and non carriers of the minor allele G of the *RETN* rs1862513 polymorphism.

	Variables		
RETN rs1862513	CC	CG+GG	*p*-value
Females	238 (75.32)	259 (73.58)	0.61
Age of patients at the time of disease diagnosis, years, median [IQR]	56 (46-66)	56.4 (45-65)	0.66
Time follow up, years, median [IQR]	13 (7.7-19.0)	14.4 (6.4-19.3)	0.27
anti-CCP positive (n=487)	132 (58.15)	153 (58.85)	0.88
Rheumatoid Factor positive (n=652)	226 (72.67)	254 (74.49)	0.60
Shared epitope (n=598)	173 (61.79)	202 (63.52)	0.66
Hypertension (n=663)	112 (35.90)	153 (43.59)	0.04
Diabetes mellitus (n=661)	32 (10.26)	52 (14.90)	0.07
Dyslipidemia (n=645)	130 (42.90)	169 (49.42)	0.10
Obesity (n=627)	33 (11.07)	34 (10.33)	0.77
Smoking habit (n=637)	45 (14.90)	67 (20.00)	0.09

Except where indicated otherwise, values are n (%). IQR: Interquartile Range. Anti-CCP: anti-cyclic citrullinated peptide antibodies.

Results

Influence of RETN rs1862513 gene polymorphism in the risk of CV disease in patients with RA

After the examination of all patients' clinical records, we observed that 115 (17.22%) patients had experienced CV events after de diagnosis of RA.

We compared the genotypic and allelic frequencies of *RETN* rs1862513 polymorphism between the subgroup of patients who experienced CV disease and the remaining patients with RA. No statistically significant difference between both groups was observed (Table II). In this regard, we only observed a slight increased frequency of homozygous and heterozygous for the minor allele G (CG+GG genotypes) among patients who experienced CV events compared to those without CV events (53.04% *vs*. 52.62%, *p*=0.94).

In a further step we analysed the distribution of the clinical characteristics and CV risk factors between carriers and non carriers of the minor allele G (Table III). We observed a higher frequency of classic CV risk factors among the carriers of the minor allele G (hypertension in non G carriers 35.90% *vs*. G carriers 43.59%, *p*=0.04, diabetes mellitus 10.26% *vs*. 14.90%, *p*=0.07, dyslipidemia 42.90% *vs*. 49.42%, *p*=0.10, smoking habit 14.90% *vs*. 20.00%, *p*=0.09), except for obesity (*p*=0.77).

We wanted to take into account this different distribution of classic CV risk

Table IV. Logistic regression model to explain the presence of cardiovascular disease in rheumatoid arthritis patients according to *RETN* rs1862513 genotype, adjusted for classic CV risk factors.

	p-value*	OR [95% CI]*
G carriers *vs*. non carriers	0.50	0.83 [0.50-1.40]

*Analyses adjusted for gender, age at rheumatoid arthritis diagnosis, follow-up time, presence or absence of shared epitope, hypertension, diabetes mellitus, dyslipidemia, obesity and smoking habit.
OR [95% CI]: Odds Ratio with 95% Confidence Interval.

factors between carriers and non carriers of the minor allele in the influence of the *RETN* rs1862513 polymorphism on the risk of CV disease in RA patients. Therefore we constructed a logistic regression model to explain the presence of CV disease according to *RETN* rs1862513 polymorphism. However, the adjusted model showed no association between the *RETN* variant and CV disease (p=0.50) (Table IV).

In a further step, we specifically assessed the influence of this polymorphism in the occurrence of cardiac ischaemic events or cerebral ischaemic events. Again, no significant associations were found in both the adjusted (p=0.96, p=0.73 respectively) and in the unadjusted (p=0.25, p=0.25 respectively) analyses.

We analysed the occurrence of CV events over a median of 13.4 years (interquartile range 6.8-18.8). We calculated the HR for the *RETN* variant, assuming a dominant pattern of effect, in a crude and adjusted model. However, we did not observe any significant association (unadjusted p=0.75, adjusted p=0.44).

RETN *rs1862513 polymorphism and the presence of subclinical atherosclerosis*

When we studied the carotid IMT according the *RETN* rs1862513 genotypes, we observed, a trend for a higher carotid IMT (p=0.09) and a significantly lower EDV% values among allele G carriers (p=0.03). No significant differences were found regarding EIV (p=0.20) (Table V A). However, in the adjusted ANCOVA model, only a trend towards a higher carotid IMT was found among allele G carriers (p=0.06) (Table V B).

Table V. A. Comparison of carotid artery intima-media thickness, Flow-mediated endothelium dependent (post-ischaemia) vasodilatation (FMD) and endothelium independent (post-nitroglycerin) vasodilatation (NTG), according to *RETN* rs1862513 polymorphism. **B**. Comparison of carotid artery intima-media thicknesss, Flow-mediated endothelium dependent (post-ischaemia) vasodilatation (FMD) and endothelium independent (post-nitroglycerin) vasodilatation (NTG), according to *RETN* rs1862513 polymorphism in an adjusted ANCOVA model.

A

	IMT mm, mean (SD)	*p*-value		
CC (n=51)	0.71 (0.14)			
CG+GG (n=53)	0.76 (0.20)			
Model		0.09		
C	0.74 (0.17)			
G	0.74 (0.19)			
		0.84		

	FMD%, mean (SD)	*p*-value	NTG% mean (SD)	*p*-value
CC (n=63)	6.75 (5.39)		18.00 (8.19)	
CG+GG (n=61)	4.79 (4.58)		16.27 (6.71)	
Model		0.03		0.20
C	6.20 (5.29)		17.60 (7.77)	
G	4.87 (4.46)		16.14 (6.82)	
		0.06		0.16

B

	IMT	FMD	NTG
p carrier G *vs*. non carrier G*	0.06	0.20	0.59

* Analyses adjusted for gender, age at brachial ultrasonography performance, follow-up time, presence or absence of shared epitope, hypertension, diabetes, dyslipidemia, obesity and smoking habit. FMD: Flow-mediated endothelium-dependent Vasodilatation. NTG: Endothelium independent (post nitroglycerin) vasodilatation. IMT: Carotid artery intima-media thickness. SD: Standard Deviation.

Discussion

Data from the present study show that the *RETN* rs1862513 polymorphism does not seem to be a genetic risk factor for CV disease in patients with RA. Previous studies on gene polymorphisms associated with susceptibility to RA have shown contradictory results in terms of genetic association with the increased risk of CV disease observed in patients with this condition. In this regard, an association of *HLA-DRB1**04 shared epitope alleles with increased incidence of CV events (7), CV mortality (7) and endothelial dysfunction (39) has been reported in Spanish individuals with RA. This association of *HLA-DRB1* alleles with CV disease in patients with RA was also confirmed in British individuals (43, 44). However, we could not establish an association with clinically evident CV disease or subclinical atherosclerosis in Spaniards when the influence of other gene variants located outside the MHC region (*PTPN22*, *STAT4* and *TRAF1/C5*) which are also associated with increased disease susceptibility to RA was studied (45). Although an association of endothelial dysfunction with genes implicated in the inflammatory response such as *IL6* was observed in patients with RA (46), no association between subclinical atherosclerosis or CV events with other gene polymorphisms such as *MIF-173* was found (47). In contrast, we recently observed that the *methylene tetrahydrofolate reductase* 1298 A>C gene polymorphism confers an increased risk for subclinical atherosclerosis and CV events in patients with RA (48). Therefore, the search for potential gene candidates that may influence the development of CV disease in patients with RA needs further investigation. Interestingly, an association of the *RETN* rs1862513

polymorphism with CV disease (specifically cerebrovascular ischaemic disease) in Caucasian (32) and Japanese (33) diabetic patients has been reported. However, it is important to highlight that this *RETN* rs1862513 polymorphism does not seem to be associated with susceptibility to diabetes mellitus in Caucasian subjects (25, 32, 49, 50).

Taking together all these observations we feel that the lack of association of this *RETN* rs1862513 polymorphism with CV disease (28, 29, 31), except when CV disease was specifically assessed in diabetic patients (32, 33), suggests that the *RETN* variant may increase the risk of CV disease only if another underlying predisposing disease is present. RA can also be considered a predisposing condition for CV disease (4, 7). A chronic inflammatory status seems to be responsible for the higher risk of CV disease observed in patients with this RA (7, 8). Nevertheless, in the present study we could not find a significant association between the *RETN* rs1862513 polymorphism and the presence of clinically evident CV disease in patients with RA. Regarding subclinical atherosclerosis, carriers of the minor allele G seemed to be associated with greater carotid IMT, even after adjustment for classic CV risk factors, although this potential association did not reach statistical significance (p=0.06). Therefore, this result would require confirmation in a larger patients' sample. On the other hand, no strong association between the *RETN* rs1862513 polymorphism and endothelial function was observed.

A potential limitation of this work was the lack of determination of serum resistin concentration in all the RA patients assessed in the present study. However, this *RETN* variant seems to exert only a small influence in the serum levels of resistin (26-29). In this regard, as previously described (51), serum resisting levels were assessed in a representative subsample of 39 patients with RA included in the present study. However, no statistically significant differences in the serum resistin concentrations were found when these 39 patients were stratified according to the *RETN* rs1862513 genotypes (data not shown).

Resistin seems to play a role in the pathophysiology of RA. Its production is induced by and in turn induces cytokine synthesis such as TNF-α and IL-6 (16-21), both playing a pivotal role in RA (52-53) and atherosclerosis pathogenesis (54-55). In this regard, a strong correlation between serum resistin levels and inflammatory markers such as C-reactive protein has been observed in patients with RA undergoing TNF-alpha antagonist therapy due to severe disease refractory to conventional disease modifying anti-rheumatic drugs (51). Moreover, resistin seems to exert a deleterious effect on the human cartilage by altering the proteoglycan synthesis (56).

In conclusion, the *RETN* rs1862513 polymorphism does not seem to be a genetic risk factor for CV disease in RA.

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BRIEF COMMUNICATION

Lack of association between *ADIPOQ* rs266729 and *ADIPOQ* rs1501299 polymorphisms and cardiovascular disease in rheumatoid arthritis patients

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Abstract

To assess the potential association between *ADIPOQ* rs266729 and rs1501299 gene polymorphisms, either isolated or in combination, and cardiovascular disease in patients with rheumatoid arthritis (RA), 674 patients seen at the rheumatology outpatient clinics of Hospital Xeral-Calde, Lugo, and Hospital San Carlos, Madrid, Spain, were analyzed. Genotyping was performed using predesigned TaqMan assays (Applied Biosystems, Foster City, CA). Carotid intima-media thickness, flow-mediated endothelium-dependent and endothelium-independent post-nitroglycerin vasodilatation, which are used as surrogate markers of subclinical atherosclerosis, were measured in a subsample. No significant differences in the genotype, allele or allele combination frequencies of both polymorphisms were found between RA patients with or without cardiovascular events or subclinical atherosclerosis. Therefore, *ADIPOQ* rs266729 and rs1501299 polymorphisms do not seem to be associated with cardiovascular disease in RA.

The aim of the present study was to analyze the association between two polymorphisms of the adipokine gene (*ADIPOQ*), rs266729 (−11377, C > G) and rs1501299 (+276, G > T), and the presence of clinically evident cardiovascular (CV) disease or subclinical atherosclerosis in a series of rheumatoid arthritis (RA) patients. RA is a chronic autoimmune condition associated to a higher risk of CV disease (1) because of an accelerated atherosclerosis (2). Adiponectin is a protein secreted by adipocytes and released in the circulation of human healthy subjects at relatively high levels (3). Adiponectin exerts insulin-sensitizing effects by suppressing gluconeogenesis in the liver and by enhancing fatty acid oxidation in the skeletal muscle (4). Also, adiponectin exhibits anti-inflammatory (5) and atheroprotective actions by reducing the expression of vascular adhesion molecules (6), scavenger receptors (7), inflammatory cytokines such as TNF-α (8) and raising nitric oxide production (9). Moreover, adiponectin suppresses the proliferation and migration of smooth muscle cells into the atheromatous plaque (10). Plasma adiponectin levels have been found decreased in obesity (11–13), type 2 diabetes (14) and coronary artery disease (15, 16).

Polymorphisms located in the *ADIPOQ* gene exert a high influence on adiponectin serum levels (17, 18). However, it seems that these genetic variants do not significantly influence

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the risk of CV disease (19). The *ADIPOQ* rs266729 and rs1501299 polymorphisms have not shown a clear association with serum adiponectin levels (18). Conversely, the minor allele G of *ADIPOQ* rs266729 polymorphism (20, 21) and the major allele G of the rs1501299 polymorphism (18, 22) seem to be associated with CV disease.

The RA patients studied in the present work were recruited between March 1996 and March 2008 from the rheumatology outpatient clinics of Hospital Xeral-Calde, Lugo, and Hospital Clínico San Carlos, Madrid, Spain. All patients fulfilled the 1987 American College of Rheumatology classification criteria for RA (23), and at the time of recruitment DNA samples were extracted. Between December 2009 and January 2010, patient's clinical records were examined until patient's death, loss of follow-up or 1 December 2009. Sociodemographical and clinical data regarding clinical manifestations, traditional CV risk factors and history of CV events were registered (24, 25). A CV event was considered to be present if the patient had ischemic heart disease, heart failure, cerebrovascular accident or peripheral arteriopathy.

Between March 2007 and September 2009, a subgroup of patients from the Lugo cohort with no previous history of CV events was selected at random to establish the existence of subclinical atherosclerosis using as surrogate markers the presence of endothelial dysfunction [determined by brachial ultrasonography assessing brachial artery reactivity: flow-mediated endothelium-dependent (FMD, post-ischemia) vasodilatation and endothelium-independent (NTG, post-nitroglycerin) vasodilatation] (24, 26) and the carotid artery intima-media thickness (IMT) determined by carotid ultrasonography (27, 28). Informed consent was obtained from all patients. The local institutional committees approved the study. Typing of both *ADIPOQ* polymorphisms was performed using TaqMan Assays-on-Demand from Applied Biosystems following the manufacturer's protocol and analyzed using the ABI 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). Typing was successful in 96.8% of the sample ($n = 674$). The main features of the patients assessed in this study were the following: 495 females (73.44%), the median age at RA diagnosis in years was 56 [interquatile rank (IQR) 45–65] and the median time of follow-up in years was 13 (6.6–18.1). Of those patients on whom data were available, 58.47% (290/496) were anti-cyclic citrulinated peptide antibody positive, 73.10% (481/658) were rheumatoid factor positive and 62.85% (379/603) had at least one copy of the rheumatoid shared epitope (SE). Also, 119 (17.66%) had experienced clinically evident CV disease [ischemic heart disease in 65 patients (9.64%), cerebrovascular accidents in 34 subjects (5.04%), congestive heart failure in 30 patients (4.45%) and peripheral arteriopathy in 15 patients (2.23%)]. Regarding classic CV risk factors, 40.15% (269/670) had hypertension, 12.76% (85/666) had diabetes mellitus, 46.54% (303/651) had dyslipidemia, 10.78% (68/631) were obese and 18.04% (116/643) were current or former smokers.

Genotype data was checked for deviation from Hardy–Weinberg equilibrium (HWE) using http://ihg.gsf.de/cgi-bin/hw/hwa1.pl. Neither RA patients with CV disease nor those RA patients without CV disease had a deviation from HWE for the *ADIPOQ* rs1501299 and the rs266729 polymorphisms.

We compared the genotype and allele frequencies of the *ADIPOQ* rs266729 and rs1501299 variants between the RA patients with and without clinically evident CV disease using the chi-squared test. However, we did not observe any significant differences (Table 1). We performed a multiple logistic regression adjusted for gender, age at RA diagnosis, time of follow-up, presence or absence of the rheumatoid SE and traditional CV risk factors (hypertension, diabetes mellitus, dyslipidemia, obesity and smoking habit) as potential confounders to account for the influence of these variables in the association between *ADIPOQ* polymorphisms

Table 1 Differences between RA patients with or without cardiovascular disease according to *ADIPOQ* rs266729 and *ADIPOQ* rs1501299 polymorphisms

	With CV disease	Without CV disease	*P*	OR (95% CI)
ADIPOQ rs266729				
CC	67 (56.30)	327 (58.92)	—	1
CG	46 (38.66)	188 (33.87)	0.40	1.19 (0.77–1.85)
GG	6 (5.04)	40 (7.21)	0.49	0.73 (0.24–1.84)
Allele 2n (%)				
C	180 (75.63)	842 (75.86)	—	1
G	58 (24.37)	268 (24.14)	0.94	1.01 (0.72–1.42)
ADIPOQ rs1501299				
GG	69 (57.98)	287 (51.71)	—	1
GT	44 (36.97)	224 (40.36)	0.34	0.82 (0.53–1.26)
TT	6 (5.04)	44 (7.93)	0.21	0.57 (0.19–1.41)
Allele 2n (%)				
G	182 (76.47)	798 (71.89)	—	1
T	56 (23.53)	312 (28.11)	0.15	0.79 (0.56–1.10)

CV, cardiovascular; OR (95% CI), odds ratio with 95% confidence interval.

and CV disease. Again, we did not find any significant association between any of these polymorphisms and CV disease (rs266729 adjusted $P = 0.84$, rs1501299 adjusted $P = 0.11$). In a further step, to assess the independency of both polymorphisms in their association with clinically evident CV disease, we performed a conditional analysis. However, no significant association was observed (rs266729 adjusted $P = 0.50$, rs1501299 adjusted $P = 0.082$).

We also analyzed the combined association of both polymorphisms with CV disease. We estimated the linkage disequilibrium (LD) between both variants and their allelic combinations using the UNPHASED software (29). We observed a low LD ($r^2 = 0.05$) and three allelic combination with a frequency on RA patients without CV disease higher than 5% (Table 2). No significant differences were observed among the combinations.

Regarding subclinical atherosclerosis, we compared the mean values of carotid IMT, endothelium dependent (FMD) or independent (NTG) vasodilatation according to the rs266729 or the rs1501299 genotypes (using the ANOVA test) and alleles (using an unpaired *t*-test). No significant differences were observed in both cases [*ADIPOQ* rs266729: carotid IMT CC ($n = 53$) mean (SD) 0.73 mm (0.15), CG ($n = 44$) 0.76 mm (0.21), GG ($n = 9$) 0.65 mm (0.14), $P = 0.21$; FMD CC ($n = 65$) 6.43% (5.34), CG ($n = 51$) 4.88% (4.67), GG ($n = 11$) 6.55% (4.32), $P = 0.22$; NTG CC ($n = 65$) 17.57% (7.94), CG ($n = 51$) 16.82% (7.10), GG ($n = 11$) 16.84% (8.16), $P = 0.86$. *ADIPOQ* rs1501299: carotid IMT GG ($n = 53$) 0.74 mm (0.21), GT ($n = 49$) 00.72 mm (0.15), TT ($n = 4$) 0.75 mm (0.10), $P = 0.83$; FMD GG ($n = 67$) 5.35% (5.61), GT ($n = 56$) 6.37% (4.25), TT ($n = 4$) 5.88% (5.07), $P = 0.54$; NTG GG ($n = 67$) 17.02% (8.05), GT ($n = 56$) 17.54% (7.23), TT ($n = 4$) 15.65% (4.57), $P = 0.85$). We also performed an analysis of covariance (ANCOVA) to test the association between the surrogate markers of subclinical atherosclerosis and the *ADIPOQ* variants, adjusting for gender, age and duration of the disease at the time of the ultrasonographic study, and presence or absence of SE and traditional CV risk factors. No association was observed (*ADIPOQ* rs266729 carotid IMT $P = 0.59$, FMD $P = 0.52$, NTG $P = 0.86$; *ADIPOQ* rs1501299 carotid IMT $P = 0.27$, FMD $P = 0.87$, NTG $P = 0.61$). Also, no association between the polymorphisms, analyzed simultaneously in a conditional analysis, and any of the surrogate markers of subclinical atherosclerosis was found (data not shown).

As previously described (30), serum adiponectin levels were assessed in a representative subsample of 39 patients with RA included in the present study. However, no statistically significant differences in the serum adiponectin concentrations were found when we stratified the patients according to the *ADIPOQ* rs266729 or rs1501299 genotypes (data not shown).

Both *ADIPOQ* polymorphisms analyzed in this study have been associated to CV disease in non-RA individuals. The minor allele G of *ADIPOQ* rs266729 polymorphism was associated with CV disease (16, 20, 21), and the minor allele T of the rs1501299 variant was associated with a reduced frequency of CV disease (18, 31). However, in the present study neither of these two *ADIPOQ* variants showed a significant association with clinically evident CV disease or with subclinical atherosclerosis in patients with RA.

The accelerated atherosclerosis observed in RA is a consequence of both traditional CV risk factors (32, 33) and the presence of chronic systemic inflammation (24, 34). In this regard, a strong correlation between systemic inflammation and both clinically evident CV disease and subclinical atherosclerosis has been observed (24, 35). In patients with severe RA, high-grade inflammation was independently negatively correlated with serum adiponectin (30), a finding that was similar to what has been reported in non-RA subjects. Also, as in non-RA subjects, the metabolic syndrome features of glucose and atherogenic dyslipidemia were inversely related to serum adiponectin concentrations in patients with severe RA refractory to conventional therapies (30). However, no association between adiponectin levels and disease activity, joint damage (36, 37), insulin resistance (30, 38) and coronary artery calcification score determined by electron beam computed tomography (38) has been observed in RA.

Our study exhibited two potential limitations: a power of 70%–75% for detecting odds ratio (OR) = 0.5 or lower and the lack of a replication cohort. However, in line

Table 2 Distribution of allelic combinations of *ADIPOQ* rs266729 and *ADIPOQ* rs1501299 polymorphisms in rheumatoid arthritis patients with or without cardiovascular disease

ADIPOQ rs266729–*ADIPOQ* rs1501299	With CV disease	Without CV disease	*P*	OR (95% CI)	*P*[a]	OR (95% CI)[a]
C-G	126 (52.94)	545 (49.10)	—	1	—	1
G-G	56 (23.53)	253 (22.79)	0.81	0.96 (0.98–1.36)	0.74	0.93 (0.60–1.44)
C-T	54 (22.69)	297 (26.76)	0.18	0.79 (0.55–1.11)	0.17	0.73 (0.47–1.14)
G-T[b]	2 (0.84)	15 (1.35)	—	—	—	—

CV, cardiovascular; OR (95% CI), odds ratio with 95% confidence interval.

[a]Analyses adjusted for gender, age at rheumatoid arthritis diagnosis, follow-up time and presence or absence of shared epitope, hypertension, diabetes mellitus, dyslipidemia, obesity and smoking habit.

[b]The allelic combination G-T was not included in the analysis because of its low frequency.

with the observations described above, our data show no significant association of *ADIPOQ* rs266729 and rs1501299 polymorphisms, either as isolated or in combination, with the risk of clinically evident CV disease or with any surrogate marker of subclinical atherosclerosis in RA patients.

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Vascular endothelial growth factor A and cardiovascular disease in rheumatoid arthritis patients

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Key words
atherosclerosis; cardiovascular disease; genetics; *VEGFA*; rheumatoid arthritis; rs2010963; rs1570360

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Abstract

To determine the contribution of the vascular endothelial growth factor A (*VEGFA*) rs2010963 (-634 G>C) and rs1570360 (-1154 G>A) polymorphisms to the risk of cardiovascular (CV) disease in a series of patients with rheumatoid arthritis (RA). Six hundred sixty-one patients fulfilling the 1987 American College of Rheumatology classification criteria for RA, seen at the rheumatology outpatient clinics of the Hospital Xeral-Calde, Lugo, and the Hospital San Carlos, Madrid, Spain, were studied. Patients were genotyped for the *VEGFA* rs2010963 (-634 G>C) and rs1570360 (-1154 G>A) polymorphisms using predesigned TaqMan single nucleotide polymorphism (SNP) genotyping assay (Applied Biosystems, Foster City, CA). Also, human leukocyte antigen (*HLA*) *DRB1* genotyping was performed using molecular-based methods. Clinical histories of the patients were reviewed for the presence of CV events that were considered to be present if the patient had ischemic heart disease, heart failure, cerebrovascular accident, or peripheral arteriopathy. Also, a subgroup of patients without the history of CV events was assessed for the presence of subclinical atherosclerosis manifested by the presence of endothelial dysfunction by brachial artery reactivity ($n = 126$) and increased carotid artery intima–media thickness ($n = 105$) using high resolution Doppler ultrasonography. No significant association between the *VEGFA* rs2010963 and the rs1570360 polymorphisms (neither isolated nor joined as allelic combinations) with clinically evident CV disease was found in this series of patients with RA. It was also the case when we examined the contribution of these polymorphisms to the development of subclinical atherosclerosis. *VEGFA* polymorphisms do not seem to exert a significant influence on the risk of CV disease in patients with RA.

Introduction

Atherosclerosis is a chronic inflammatory disease characterized by lipid-containing inflammatory lesions of large- and medium-sized arteries (1). Neovascularization plays an important role both in the development and instabilization of atherosclerotic plaques (2, 3). Most neovessels in the plaque originate from branches of vasa vasorum (4).

It has been shown that these neovessels growing into the plaque through angiogenesis are dysmorphic and immature (5), contributing to the instability of the plaque through intraplaque hemorrhage or by supplying inflammatory cells (3, 6, 7). One of the most important pro-angiogenic factors is the vascular endothelial growth factor (VEGF). This factor may play an important role in atherosclerosis as treatment with recombinant human VEGF causes an increase in the plaque growth (8, 9). It exerts different actions in the endothelial cells: stimulates proliferation and growth, prevents apoptosis (10), and increases vascular permeability (11) by augmenting the expression of adhesion molecules (12–14)

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†Drs MAG-G and JM shared senior authorship in this study.

and modulating the recruitment of leucocytes. VEGF can also stimulate monocytes (15), increasing the expression of pro-inflammatory cytokines and metalloproteinases.

Expression of the 5′ UTR and promoter region of the *VEGFA* gene is performed at the post-transcriptional level (16, 17). The minor allele of the rs1570360 (-1154 G>A) polymorphism (18, 19), located within the promoter and the major allele of the rs2010963 (-634 G>C) polymorphism, located within 5′ UTR (20, 21) have been related to a lower *VEGFA* gene expression and lower VEGF circulating levels. In this regard, functional studies have shown that the *VEGFA* -634 G allele is associated with lower circulating VEGF levels *in vivo,* reduced VEGF transcription, and less internal ribosomal entry site B-mediated VEGF expression (21). With respect to this, we described a potential role of *VEGFA* -634 G→C polymorphism in the clinical spectrum of manifestations of patients with giant cell arteritis (GCA), a systemic vasculitis involving middle- and large-sized blood vessels (22). In this regard, the G allele was significantly overrepresented in biopsy-proven GCA patients with ischemic complications and, additionally, a higher risk of developing severe ischemic complications was observed for -634 GG homozygous individuals. Moreover, an implication of *VEGF* -634 G→C polymorphism was observed in the development of severe diabetic complications (20) and several autoimmune and inflammatory disorders, such as Behçet's disease (23), a condition that encompasses a group of multisystemic complications secondary to occlusive vasculitis.

Rheumatoid arthritis (RA) is a chronic inflammatory disease associated with an accelerated atherosclerosis (24, 25), which is responsible for an increased cardiovascular (CV) morbidity and mortality (26). The chronic systemic inflammatory status, independent of the traditional CV risk factors, plays a pivotal role in the development of the accelerated atherogenesis observed in the patients with RA (24, 27–30). Interestingly, elevated levels of VEGF have been observed in the serum of patients with RA (31) and these levels seem to be correlated with the severity of the disease (32).

Taking into account all these considerations, the aim of this study was to determine the contribution of the *VEGFA* rs2010963 (-634 G>C) and rs1570360 (-1154 G>A) polymorphisms to the risk of CV disease in a series of patients with RA.

Patients and methods

Patients

Six hundred sixty-one consecutive patients, fulfilling the 1987 American College of Rheumatology classification criteria for RA (33), seen at the rheumatology outpatient clinics of the Hospital Xeral-Calde, Lugo, and the Hospital Clínico San Carlos, Madrid, Spain, between March 1996 and March 2008 were assessed for the *VEGFA* rs2010963 (-634 G>C) and rs1570360 (-1154 G>A) polymorphisms.

Study protocol

At the time of recruitment, socio-demographical and clinical data regarding clinical manifestations, traditional CV risk factors and previous history of CV events were registered. Clinical definitions for CV events and traditional (classic) CV risk factors were established as previously described (27, 34). Smoking habit encompassed to those patients who smoked at the time of disease diagnosis, during the follow-up, or who had smoked within the 10 years before the onset of RA symptoms or the disease diagnosis. A CV event was considered to be present if the patient had ischemic heart disease, heart failure, cerebrovascular accident, or peripheral arteriopathy.

Patients were prospectively followed and assessed every 3–6 months. Clinical records were examined until patient's death, loss of follow-up, or December 2009. Information on the main demographic characteristics, traditional CV risk factors, and CV events of this cohort of patients with RA is shown in Table 1.

As Hospital Xeral-Calde and Hospital Clínico San Carlos are the referral centers for the population of each respective area, the first CV event was defined as an event (case) of CV complication diagnosed at the hospital in a patient without a previous history of CV disease. Specific information on CV events was collected based on the patients' medical records. On the basis of previously established protocols of

Table 1 Distribution of demographic and RA-related characteristics, cardiovascular disease events and cardiovascular risk factors, and genotype distribution of the patients with RA included in the study[a]

Variables	$n = 661$
Females	484 (73.22)
Age of patients at the time of disease diagnosis, years, median [IQR]	56.00 [45.33–65.37]
Time follow-up, years, median [IQR]	13.42 [6.95–19.00]
Anti-CCP positive ($n = 484$)	288 (59.50)
Rheumatoid factor positive ($n = 646$)	476 (73.68)
Shared epitope, presence ($n = 600$)	376 (62.67)
One extra-articular manifestations	181 (27.38)
Two or more extra-articular manifestations	47 (7.11)
Subcutaneous nodules	94 (14.22)
Sjogren's syndrome	147 (22.24)
Vasculitis	12 (1.82)
Pulmonary involvement	18 (2.72)
RA subjects with cardiovascular disease	113 (17.10)
Ischemic heart disease	62 (9.38)
Cerebrovascular accidents	32 (4.84)
Heart failure	26 (3.93)
Peripheral arteriopathy	14 (2.12)
Hypertension ($n = 657$)	267 (40.64)
Diabetes mellitus ($n = 653$)	83 (12.71)
Dyslipidemia ($n = 640$)	296 (46.25)
Obesity ($n = 619$)	68 (10.99)
Smoking habit ($n = 631$)	113 (17.91)

Anti-CCP, anti-cyclic citrullinated peptide antibodies; IQR, interquartile range; RA, rheumatoid arthritis.

[a]Except where indicated otherwise, values are *n* (%).

management, all patients on methotrexate therapy were treated with folate supplementation.

To determine the potential association between *VEGFA* rs2010963 (-634 G>C) and rs1570360 (-1154 G>A) gene polymorphisms and the presence of subclinical atherosclerosis between March 2007 and September 2009, a random subgroup of patients from the Lugo cohort with no previous history of CV events was selected. The presence of endothelial dysfunction was assessed by a brachial artery reactivity study in 126 patients. In these patients, flow-mediated endothelium-dependent (post-ischemia) dilatation (FMD) and endothelium-independent (post-nitroglycerin) vasodilatation (NTG) were measured by brachial ultrasonography as previously reported (35, 36). A value of FMD <7% was considered pathologic, indicating the presence of endothelial dysfunction (36). Intraobserver variability for FMD and NTG was 1.3% and 1.9%, respectively, based on the repeat of the brachial ultrasonography in 32 healthy controls. To minimize the effect of TNF-antagonist therapy, assessment of endothelial function in patients undergoing anti-TNF therapy was performed 24–48 h before administration of these drugs (36). Also, carotid ultrasonography studies were performed in 105 patients to determine the carotid artery intima–media thickness (IMT). It was assessed in the right common carotid artery as previously reported (36, 37). Informed consent was obtained from all patients. The local institutional committees approved the study.

Genotyping

VEGFA rs2010963 (-634 G>C) and rs1570360 (-1154 G>A) genotyping

DNA from patients was obtained from peripheral blood, using standard methods. Subjects were genotyped to determine the *VEGFA* rs2010963 (-634 G>C) and rs1570360 (-1154 G>A) polymorphisms status using TaqMan Assays-on-Demand from Applied Biosystems following the manufacturer's protocol and analyzed using the ABI 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA).

Shared epitope determination

Several human leukocyte antigen (*HLA*)-*DRB1* alleles (*HLA-DRB1* *0101, *0102, *0401, *0404, *0405, *0408, *1001, and *1402) are associated with susceptibility to RA. These alleles encode a conserved amino acid sequence (QKRAA, QRRAA, or RRRAA), called the shared epitope, at position 70–74 in the third hypervariable region of the HLA-DRβ1 molecule (38). *HLA-DRB1* typing was carried out using a reverse dot-blot kit with sequence-specific oligonucleotide (SSO) probes (Dynal RELITM SSO *HLA-DRB1* typing kit; Dynal Biotech, Bromborough, UK). The presence of the shared epitope was defined as to carry at least one or any combination of the following *HLA-DRB1* alleles: *0101, *0102, *0401, *0404, *0405, *0408, *1001, and *1402.

Statistical analysis

All genotype data were checked for deviation from Hardy–Weinberg equilibrium (HWE) using http://ihg.gsf.de/cgi-bin/hw/hwa1.pl. None of the patients from this series had a deviation from HWE for one of both *VEGFA* polymorphisms. Linkage disequilibrium values (r2) and allelic combinations were generated using UNPHASED software (39).

Comparison of proportions was carried out using chi-squared test or Fisher test, when required. Strength of association between CV events and genotypes, alleles or allelic combinations of the *VEGFA* polymorphisms was estimated using odds ratios (OR) and 95% confidence intervals (CI) via multiple logistic regression; estimates were further adjusted for gender, age at RA diagnosis, time of follow-up, absence or presence of the rheumatoid shared epitope, and traditional CV risk factors (hypertension, diabetes mellitus, dyslipidemia, obesity, and smoking habit) as potential confounders. The association between genotypes, alleles, or allele combinations of the *VEGFA* polymorphisms and carotid IMT, FMD, or NTG was tested using unpaired *t*-test to compare between two groups and by one-way analysis of variance (ANOVA) to establish comparisons among more than two groups. Moreover, we also tested association between these parameters and alleles using analysis of covariance (ANCOVA) adjusting for gender, age, and duration of the disease at the time of the ultrasonographic study, and absence or presence of shared epitope and traditional CV risk factors. The Bonferroni correction for multiple testing was applied and significant *P* value changed from <0.05 to <0.005. Calculations were performed with STATA 10 (STATA Corporation, College Station, TX).

Results

Influence of the *VEGFA* rs2010963 (-634 G>C) and rs1570360 (-1154 G>A) polymorphisms in the risk of clinically evident CV disease in RA patients

Genotype and allele distribution of the patients with RA assessed in the study is shown in Table 2. In our RA sample set, 37.33% of the patients were shared epitope negative, 48.00% had one copy of the shared epitope and 14.67% had two copies. The most prevalent *HLA-DRB1* genotypes were one copy of the *0401 allele + one copy of a shared epitope negative allele (16.21%), one copy of the *0101 allele + one copy of a shared epitope negative allele (11.13%), and one copy of the *0405 allele + one copy of a shared epitope negative allele (7.03%).

The power of this study to detect a difference between absence or presence of CV disease in RA patients with an estimated OR between 1.5 and 2.0, a type I error rate of 0.05, a dominant inheritance mode, and 0.15% of population risk, was between 48% and 89% for the rs2010963 polymorphism and between 49% and 91% for the rs1570360 polymorphism.

Table 2 Genotype and allele frequencies of the *VEGFA* polymorphisms in the whole RA cohort and according to the presence or absence of clinically evident CV disease[a]

	All RA sample	With CV disease	Without CV disease	*P*	OR [95% CI]
VEGFA -634					
GG	271 (41.00)	48 (42.48)	223 (40.69)	—	1
GC	315 (47.66)	52 (46.02)	263 (47.99)	0.70	0.92 [0.58–1.45]
CC	75 (11.35)	13 (11.50)	62 (11.31)	0.94	0.97 [0.47–2.00]
Allele 2*n* (%)					
G	857 (64.83)	148 (64.49)	709 (64.69)	—	1
C	465 (35.17)	78 (34.51)	387 (35.31)	0.82	0.97 [0.71–1.32]
VEGFA -1154					
GG	327 (49.47)	49 (43.36)	278 (50.73)	—	1
AG	270 (40.85)	50 (44.25)	220 (40.15)	0.25	1.29 [0.82–2.03]
AA	64 (9.68)	14 (12.39)	50 (9.12)	0.17	1.59 [0.77–3.23]
Allele 2*n* (%)					
G	924 (69.89)	148 (65.49)	776 (70.80)	—	1
A	398 (30.11)	78 (34.51)	320 (29.20)	0.11	1.28 [0.93–1.75]

CV, cardiovascular; OR [95% CI], odds ratio with 95% confidence interval; RA, rheumatoid arthritis; *VEGFA*, vascular endothelial growth factor.
[a] r^2 *VEGFA* -634/-1154:0.27.

We analyzed the genotype and allele distribution of both *VEGFA* polymorphisms regarding the absence or presence of clinically evident CV disease in RA patients (Table 2). However, no significant differences in the genotype and allele ($P = 0.82$) frequencies were found for the *VEGFA* -634 variant. It was also the case for the *VEGFA* -1154 polymorphism ($P = 0.11$).

In a further step, we constructed a logistic regression model to explain the presence of CV disease according to the *VEGFA* rs2010963 (-634 G>C) and rs1570360 (-1154 G>A) allele distribution that was further adjusted for classic CV risk factors (Table 3). Neither of these polymorphisms showed a significant association before or after adjustment for classic CV risk factors with clinically evident CV disease (rs2010963, adjusted $P = 0.54$, rs1570360 adjusted $P = 0.11$).

We also analyzed the combined influence of the *VEGFA* -634 and the *VEGFA*-1154 gene polymorphisms in the risk of CV disease comparing the frequency of their estimated allelic combinations (Table 4). Only three combinations were considered (the fourth combination was excluded from the analysis because of its low frequency: 0.15% among the subjects without CV disease and 0% in those with CV disease). No significant differences were observed among combinations, before or after adjustment.

Table 3 Logistic regression model to explain the presence of CV disease according to the *VEGFA* allele distribution

	P	OR [95% CI]	*P*[a]	OR [95% CI][a]
VEGFA -634				
C *vs* G	0.82	0.97 [0.71–1.30]	0.54	0.89 [0.61–1.30]
VEGFA -1154				
A *vs* G	0.11	1.28 [0.94–1.73]	0.11	1.37 [0.93–2.01]

CV, cardiovascular; OR [95% CI], odds ratio with 95% confidence interval; *VEGFA*, vascular endothelial growth factor A.
[a]Analyses adjusted for gender, age at the time of rheumatoid arthritis diagnosis, follow-up time, and presence or absence of shared epitope, hypertension, diabetes mellitus, dyslipidemia, obesity, and smoking habit.

Influence of the *VEGFA* rs2010963 (-634 G>C) and rs1570360 (-1154 G>A) polymorphisms in the risk of subclinical CV disease in RA patients

As an increased frequency of subclinical atherosclerosis has been observed in RA patients without clinically evident CV disease (35, 40), we also aimed to establish the potential influence of these two *VEGFA* polymorphisms in the development of subclinical atherosclerosis using two well-defined surrogate markers of atherosclerosis. However, we did not observe any significant association between the *VEGFA* rs2010963 (-634 G>C) or the rs1570360 (-1154 G>A) polymorphism and the values of carotid IMT, FMD, or NTG (Table 5). In the ANCOVA model adjusted for gender, age at the time of the ultrasonographic assessment, follow-up time, and absence or presence of shared epitope and traditional CV risk factors, no significant differences were found according to *VEGFA* rs2010963 (-634 G>C) or the rs1570360 (-1154 G>A) alleles (Table 5). Moreover, no association between allelic combinations and any of these two surrogate markers of subclinical atherosclerosis was found (data not shown).

Discussion

This is the first study that specifically assesses the influence of *VEGFA* polymorphisms in the risk of both clinically evident and subclinical CV disease in a large series of patients with RA. Neither the rs2010963 nor the rs1570360 variants (isolated or joined as allelic combinations) had any

Table 4 Distribution of allelic combinations of the *VEGFA* polymorphisms according to the presence of CV disease

VEGFA -634 to -1154	With CV disease	Without CV disease	*P*	OR [95% CI]	*P*[a]	OR [95% CI][a]
G-G	70 (30.97)	391 (35.74)	—	1	—	1
C-G	78 (34.51)	385 (35.19)	0.49	1.13 [0.80–1.61]	0.81	1.06 [0.68–1.65]
G-A	78 (34.51)	318 (29.07)	0.082	1.37 [0.96–1.95]	0.13	1.41 [0.90–2.21]

CV, cardiovascular; OR [95% CI], odds ratio with 95% confidence interval; *VEGFA*, vascular endothelial growth factor A.
[a]Analyses adjusted for gender, age at the time of rheumatoid arthritis diagnosis, follow-up time, and presence or absence of shared epitope, hypertension, diabetes mellitus, dyslipidemia, obesity, and smoking habit.

significant association with clinically evident CV disease or with surrogate markers of subclinical atherosclerosis. Genotype and allele distribution of both the alleles in RA patients with and without CV disease were similar to the ones previously published in healthy controls from Spain (41).

Two previous studies search for associations between these two *VEGFA* variants and RA. However, neither of them found a significant association with disease susceptibility (41, 42), despite the evidence that supports a role of VEGF in the RA angiogenic processes and therefore in the development of the inflammatory pannus (43) and despite the effect that both polymorphisms exert on VEGF production. It is important to note that both the rheumatoid joint and the atherosclerotic plaque are hypoxic environments (44, 45). Hypoxia has been associated with an increase of inflammatory cells and mediators and with a direct increase in the production of VEGF (46, 47). Moreover, pro-inflammatory cytokines, such as TNF-α, increase the production of VEGF (46). Also, angiogenesis itself is implicated in inflammation and the development of both the atherosclerotic plaque and the synovial pannus which, in turn, contributes to hypoxia. However, it is known that other factors may also contribute to the angiogenesis process in the synovium and the atherosclerotic lesions (48, 49). Therefore, it is possible that other pro-angiogenic factors might be able to compensate the lower production of VEGF associated with these mutations. However, it is possible that the stimulus of VEGF production exerted by hypoxia and/or pro-inflammatory cytokines might be strong enough to overcome the effects of these polymorphisms. Alternatively, a plausible explanation for the lack of association of these two functional *VEGFA* polymorphisms with CV disease may be the paradoxical effect that VEGF may exert. For example, in vascular disorders leading to vessel occlusion, such as coronary arteriopathy, hypoxia-induced VEGF may play a compensatory role leading to new vessel formation (50). With respect to this, in animal models

Table 5 *VEGFA* -634 and -1154 polymorphisms and surrogate markers for atherosclerosis[a]

	IMT mm, mean (SD)	*P*	*n*	FMD%, mean (SD)	*P*	NTG% mean (SD)	*P*
VEGFA -634							
GG ($n = 45$)	0.74 (0.20)	—	49	5.72 (4.62)	—	16.90 (6.94)	—
GC ($n = 50$)	0.73 (0.16)	—	66	5.59 (5.58)	—	17.13 (8.62)	—
CC ($n = 10$)	0.75 (0.18)	—	11	6.35 (2.70)	—	17.82 (5.14)	—
ANOVA Model	—	0.95	—	—	0.90	—	0.94
G ($n = 140$)	0.74 (0.19)	—	164	5.67 (5.00)	—	16.99 (7.62)	—
C ($n = 70$)	0.74 (0.16)	—	88	5.78 (5.00)	—	17.30 (7.85)	—
t-test model	—	0.95	—	—	0.66	—	0.76
VEGFA -1154							
GG ($n = 48$)	0.75 (0.20)	—	63	5.74 (5.36)	—	17.31 (7.62)	—
GA ($n = 49$)	0.71 (0.14)	—	54	5.8 (4.86)	—	16.99 (8.07)	—
AA ($n = 8$)	0.84 (0.18)	—	9	4.89 (3.23)	—	16.27 (6.54)	—
ANOVA model	—	0.12	—	—	0.89	—	0.93
G ($n = 145$)	0.74 (0.19)	—	180	5.76 (5.19)	—	17.21 (7.72)	—
A ($n = 65$)	0.74 (0.16)	—	72	5.57 (4.49)	—	16.81 (7.65)	—
t-test model	—	0.97	—	—	0.79	—	0.71
ANCOVA model							
VEGFA -634, p C *vs* G[b]	—	0.65	—	—	0.64	—	0.89
VEGFA -1154, p A *vs* G[b]	—	0.86	—	—	0.64	—	0.52

ANOVA, analysis of variance; ANCOVA, analysis of covariance; FMD, flow-mediated endothelium-dependent vasodilatation; IMT, intima–media thickness; NTG, endothelium-independent (post-nitroglycerin) vasodilatation; SD, standard deviation; *VEGFA*, vascular endothelial growth factor A.
[a]Comparison of the carotid IMT, and brachial FMD and NTG results according to the *VEGFA* -634 and -1154 genotypes and alleles in an ANOVA, *t*-test, and adjusted ANCOVA models.
[b]Analyses adjusted for gender, age at the time of ultrasonography assessment, follow-up time, and presence or absence of shared epitope, hypertension, diabetes, dyslipidemia, obesity, and smoking habit.

of myocardial infarction, gene therapy with *VEGFA* improves collateral circulation (51), increases myocardial blood flow, and vasodilatation in response to adenosine (52) and also improves wall motion (53). Moreover, the minor allele C of the *VEGFA* rs2010963 (-634 G>C) variant (associated with higher VEGF levels) was associated with a lower frequency of severe ischemic complications in patients with biopsy-proven GCA patients (22).

Our findings suggest that the two *VEGFA* polymorphisms analyzed in this work do not appear to be associated with clinically evident CV disease or subclinical atherosclerosis in patients with RA. The search for potential genes that may influence the increased risk of accelerated atherogenesis in patients with RA is warranted.

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Letters to the Editors

Analysis of the influence of the ghrelin receptor rs509035, rs512692 and rs2922126 polymorphisms in the risk of cardiovascular disease in patients with rheumatoid arthritis

Sirs,

Cardiovascular (CV) events due to accelerated atherosclerosis constitute the leading cause of mortality in patients with rheumatoid arthritis (RA) (1). Chronic systemic inflammation predicts the progression of atherosclerosis and contributes to the increased incidence of CV events observed in RA (2). The mechanisms involved in inflammation related CV disease in RA require further study.

Ghrelin is a 28-amino-acid gastric peptide that was discovered in 1999 and was identified as the endogenous ligand for the growth hormone secretagogue receptor (3). Ghrelin receptor (GHSR) is a seven-transmembrane domain G protein-coupled receptor located both in the central nervous system (the pituitary and hypothalamus) and in a wide variety of peripheral tissues including the heart, blood vessels and endothelial cells (4). The ghrelin/GHSR system exerts antiinflammatory effects, both inhibiting proinflammatory cytokine release (IL-1beta, IL-6, and TNF-alpha) in monocytes, T cells and endothelial cells and increasing the production of antiinflammatory cytokines and chemokines (5). This system also appears to exert a cardioprotective effect, protecting myocites against ischaemia and having cardiotropic actions (6).

In RA the ghrelin/GHSR system also seems to exert a protective effect in the vascular system. We reported that anti-TNF-α therapy increased serum levels of ghrelin (7), which, in turn, was associated with a reductions in soluble P-selectin serum level, a biomarker of endothelial activation that predicts CV event rates.

Polymorphisms located in the *GHSR* gene have been associated with CV disease and classic CV risk factors (6) In the present study we assessed for first time the potential the effect of tree polymorphisms the *GHSR* gene (8) on the risk of clinically evident CV disease in patients with RA.

Six hundred and fifty-nine consecutive patients, fulfilling the 1987 American College of Rheumatology classification criteria for RA (9), seen at the rheumatology outpatient clinics of Hospital Xeral-Calde, Lugo and Hospital Clínico San Carlos, Madrid, Spain, between March 1996 and March 2008 were assessed for the *GHSR* rs509035, rs512692 and rs2922126 polymorphisms. Patients were genotyped for the *GHSR* polymorphisms using predesigned TaqMan single nucleotide polymorphism genotyping assay as previously reported (8). Also, *HLA-DRB1* genotyping was performed using molecular based methods. A CV event was considered to be present if the patient had ischaemic heart disease, heart failure, cerebrovascular accident or peripheral arteriopathy.

The local institutional committees approved the study.

No deviation from Hardy-Weinberg equilibrium for any *GHSR* polymorphisms was found in patients with or without CV events. Statistical significance was defined as p≤0.05. Calculations were performed with STATA 10 (STATA Corporation, College Station, Texas).

None of these tree polymorphisms were associated with susceptibility to RA (8).

No significant differences in the allele or genotype frequencies of the *GHSR* variants between RA patients with or without CV disease were found (Table I). In the unadjusted logistic regression model patients homozygotes for the minor allele of the rs509035 and rs2922126 polymorphisms showed a non-significant trend towards a protective effect against clinically evident CV disease (odds ratio - OR 0.37 [95% confidence interval - CI 0.09–1.04], p=0.05 and OR 0.55 [95% CI 0.28–1.08], p=0.06, respectively). However, when an adjustment for gender, age at RA diagnosis, time of follow-up, presence or absence of the rheumatoid shared epitope and classic CV risk factors was made, this protective trend remained out of the range of significance. Likewise, the haplotype analysis showed no statistically significant differences in the CV risk after adjustment for classic CV risk factors.

Table I. Differences in genotype and allele frequencies of *GHSR* rs509035, rs512692 and rs2922126 polymorphisms between RA patients with CV disease or without CV disease.

	With CV disease	Without CV disease	*p*-value	OR [95% CI]
GHSR rs509035				
GG	70 (60.34)	331 (60.96)		1
GA	42 (36.21)	164 (30.20)	0.38	1.21 [0.77–1.89]
AA	4 (3.45)	48 (8.84)	0.07	0.39 [0.10–1.13]
AA *vs*. GG+GA			0.05	0.37 [0.09–1.04]
Alleles 2n (%)				
G	182 (78.45)	826 (76.06)		1
A	50 (21.55)	260 (23.94)	0.44	0.87 [0.61–1.25]
GHSR rs512692				
AA	77 (66.38)	354 (65.19)		1
AT	34 (29.31)	162 (29.83)	0.87	0.96 [0.60–1.54]
TT	5 (4.31)	27 (4.97)	0.75	0.85 [0.25–2.34]
Alleles 2n (%)				
A	188 (81.03)	870 (80.11)		1
T	44 (18.97)	216 (19.89)	0.75	0.94 [0.65–1.37]
GHSR rs2922126				
TT	55 (47.41)	235 (43.28)		1
TA	49 (42.24)	214 (39.41)	0.92	0.98 [0.62–1.53]
AA	12 (10.34)	94 (17.31)	0.07	0.55 [0.26–1.11]
AA *vs*. TT+TA			0.06	0.55 [0.28–1.08]
Alleles 2n (%)				
T	159 (68.53)	684 (62.98)		1
A	73 (31.47)	402 (37.02)	0.11	0.78 [0.57–1.07]

CV: Cardiovascular; OR [95% CI]: Odds Ratio with 95% Confidence Interval.

RA is a polygenic disease. Previous studies on gene polymorphisms associated with susceptibility to the disease have shown contradictory results in terms of gene association with increased risk of CV disease in RA. In this regard, an association of HLA-DRB1*04 shared epitope alleles with increased incidence of CV events has been reported in Spanish individuals with RA (2). Also, an association of endothelial dysfunction with genes implicated in the inflammatory response such as *IL6* was observed in patients with RA (10). In contrast, in the same cohort, no association with clinically evident CV disease was observed when other gene variants located outside the MHC region (*PTPN22*, *STAT4* and *TRAF1*/*C5*) which are also associated with increased disease susceptibility were studied (11). Likewise, no association between CV events and RA with other gene polymorphisms such as *MIF-173* was found (12).

The results found in the present study indicate that *GHSR* rs509035, rs512692 and rs2922126 polymorphisms are not risk factors for clinically evident CV disease in patients with RA. Therefore, the genetic influence in the development of CV disease in patients with RA is still far from being understood. It is possible that complex gene interactions might influence the development of accelerated atherosclerosis observed in these patients. The search for the potential influence of other genes associated with the inflammatory response in the development of CV disease in patients with RA is warranted.

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DISCUSSION

5. DISCUSSION

In our work we studied the influence of several genetic polymorphisms, belonging to genes implicated in the immune response, in the risk of clinical and subclinical CV disease in patients affected with RA. The genes were selected due to its relationship with the pathophysiology of both diseases, and the genetic variants analyzed had not been previously studied as risk factors of CV disease in RA, although some of them had been associated with one or both conditions.

As previously pointed out in the *Introduction* section, the risk of CV disease and CV mortality is greatly increased among RA patients[118,121-132]. Classic CV risk factors, such as hypertension or DM do not account for this higher risk [122,157,158] and RA itself (or the systemic inflammation of this condition) is considered an independent risk factor for CV disease, at the same level of DM [148,149]. It is important to take into account that atherosclerosis, as RA, is a chronic inflammatory disease [165-168], and inflamed synovium and atherosclerotic plaque are strikingly similar in a number of respects. Both lesions present elevated levels of cytokines such as TNF [626], IL-6, IL-12, IL-1 and IL-18, reflecting the local stimulation of macrophages by activated T cells. In addition, contain an exaggerated matrix response and involve local cellular components, including respectively, synovial fibroblasts, chondrocytes and osteoclasts, and vascular smooth muscle, fibroblast and endothelial cells [176]. Moreover, the T cells implicated in the pathogenesis of both conditions are predominantly of Th1 or Th17 phenotypes [627]. Also, high levels of MMPs are expressed in both lesions. Taking into account all these observations, we could hypothesize that the increased background level of chronic inflammation in RA might confer a predisposition to CV disease and/or augment its

pathogenesis and put an individual at greater risk of developing clinical or subclinical CV disease or suffering secondary complications thereafter.

5.1. *TNFA* -308 variant and CV disease in RA

The first inflammatory factor that we analyzed regarding its role in the pathogenesis of clinical and subclinical CV disease in RA patients, due to the central part that plays in both diseases [366,628] was the TNF-α. This is a cytokine with a pleiotropic function in immunity, inflammation, control of cell proliferation, differentiation and apoptosis [629]. In the acute situation, local production of TNF-α is clearly beneficial, increasing the expression of adhesion molecules on the vascular endothelium that allow immune cells, in particular neutrophils and macrophages, to translocate to sites of tissue damage and infection [630]. Furthermore, TNF-α activates phagocytes to engulf and clear infectious agents and cellular debris. However, systemic or prolonged exposure to TNF-α may be harmful.

The TNF family of proteins includes secreted cytokines and membrane proteins that bind to cell surface receptors, including TNF-α, lymphotoxin-α (LT-α or TNF-β), CD40 ligand, Fas ligand and several other proteins. TNF-α is synthesized as a monomeric type 2 transmembrane precursor protein (tmTNF), which cytoplasmic tail is cleaved by the TNF-α converting enzyme (TACE, a matrix metalloprotease), releasing the circulating or soluble cytokine (sTNF) [631]. The membrane bound TNF can operate both as a ligand and as a receptor: binding by its receptor or anti-TNF drugs induce a signal backwards into the ligand-expressing cell [632].

sTNF and tmTNF are produced by a wide range of immune cells, particularly activated macrophages (which are the main source of this cytokine) [633], but also T and B lymphocytes, NK cells, DCs and monocytes. sTNF and tmTNF are biologically active,

and their relative amounts are determined by several factors including the cell type and activation status, the stimuli triggering TNF-α production, and TACE activity and expression of endogenous TACE inhibitors [634].

sTNF and tmTNF interact with two structurally related but functionally distinct TNFR, TNFRI (p55, CD120a) and TNFRII (p75, CD120b), mediating their biological functions [635]. TNFR1 is constitutively expressed in practically all cell types except erythrocytes, whereas TNFR2 is generally inducible and mainly expressed by endothelial and immune cells.

TNF-α has an important role in the initiation, development and complication of the atherosclerotic lesion. Regarding initiation, endothelial dysfunction is considered an early event in the evolution of atherogenesis, as well as a surrogate marker of risk of CV disease. TNF-α plays an important part in this process, activating the endothelial cells to support the recruitment of activated leukocytes to the inflammatory lesion, though the expression of adhesion molecules, proinflammatory cytokine and chemokine receptors, and the synthesis and release of inflammatory cytokines and chemokines. TNF-α also promotes endothelial cell injury [367], directly by enhancing cell apoptosis or suppressing the activities of endothelial cell progenitors that could sustain endothelial repair [368]. Also indirectly, through the recruitment of immune cells, such as neutrophils, which can mediate tissue destruction [369], or though the promotion of oxidative stress or direct impairment of NO bioavailability. Consistent with these findings, patients with inflammatory rheumatic conditions, such as RA, have evidence of endothelial dysfunction [636,637], and treatment with anti-TNF therapy is associated to an amelioration of this phenomenon [638,639].

Regarding atherosclerosis progression, TNF-α levels have been associated with obesity and decreased insulin sensitivity [271,640]. This cytokine has been implicated in the

functional modulation of a variety of tissue-specific cell types, such as adipocytes, where TNF-α contributes to regulate the lipid and glucose metabolism [370,371]. In line with this observations, treatment with infliximab improved insulin sensitivity, both in RA and ankylosing spondylitis patients [641-643].

Finally, regarding plaque rupture and complication, TNF-α has dual effects on extracellular matrix homeostasis, inducing the release of metalloproteinases responsible for matrix degradation [644,645] and suppressing the synthesis of matrix proteins [646].

According to the actions of TNF-α in the atherosclerotic plaque, its serum levels are higher in patients with clinical coronary arterial disease, comparing with controls, and higher in MI compared with angina [647]. Also, higher TNF-α levels are predictive for the first [648,649] and recurrent [650] CV events.

Due to the systemic effects of this cytokine, TNF-α synthesis is tightly regulated, mainly at the level of gene transcription. *TNFA* is located on the short arm of chromosome 6 within the MHC. Genetic alterations in this locus are known to be involved directly in high TNF-α production. There is a biallelic G to A transition polymorphism located at position -308 in the *TNFA* promoter, in a region able to bind nuclear proteins and modulate transcription [651]. The minor allele (A) is strongly associated with the MHC haplotype HLA-A1, B8, DR3 [374], which is, in turn, associated with high TNF-α production [375,376]. Besides, this polymorphism seems to have a significant effect on transcriptional activity [652-654], with the minor variant associated to a greater activity, although these results were not consistently replicated [655-657]. On the other hand, this variation has been associated to a higher TNF-α production in whole blood cultures stimulated with lipopolysaccharide (LPS) [658] or anti-CD3 and anti-CD28 [379]. However, these results were not always replicated [659,660]. So far, evidence suggests that circulating TNF-α levels do not seem to fully correspond with the −308 *TNFA*

promoter polymorphism, probably because serum levels depend of a multifactoral regulatory process. However, local TNF-α concentration might be of greater importance and under more control by specific polymorphisms [661].

Regarding RA, this polymorphism has not showed association with a higher risk to this condition [381], despite the high circulating and synovial fluid levels of TNF-α and the central role placed in RA pathogenesis. With respect to CV disease, the results of the association with the -308 *TNFA* polymorphism are conflicting in Caucasians. Some studies reported an association between this variant and CV disease: in one study was observed a link between minor allele and unstable angina, but not MI, in no obese subjects [662]. Also, in a Spaniard cohort, an association between a higher risk of clinical coronary disease and the minor allele was observed, but only in patients with T2DM [663]. It is important to point out that this variant itself is not a risk factor for T2DM [664]. On the other hand, many other studies did not find an association to MI [665-668], coronary arterial disease [669,670] or congestive heart failure [661]. Similarly, in a recent meta-analysis, no association with CV disease and this variant was found [385]. Regarding subclinical atherosclerosis, this variant has not been associated neither in Caucasian [303] nor Indian [384] populations.

In Asian populations, -308 *TNFA* SNP was associated with a lower risk of CV disease: in a Chinese population, minor allele was associated to a lower risk of MI, although only in non smokers [671]. In an independent Chinese and in two Korean populations, a lower risk for ischemic stroke associated with the minor allele was observed [672-674]. Finally, in the above mentioned meta-analysis, a protective effect to ischemic stroke in Asian population was detected [385].

We observed a significant association between the minor allele of the -308 variant of the *TNFA* gene and clinical CV disease in a longitudinal cohort of Spanish RA patients. In a

next step, we decided to account for the influence of the SE in the association between the -308 *TNFA* variant and CV disease, due to to the previously observed association between SE and CV disease in RA [182] and the described LD between the -308 TNFA variant and certain HLA alleles, specially DR3. Although a LD between TNFA and HLA-DR3 was detected in our population, no evidence of association of DR3 with CV disease was found. In addiiton, no association between the -308 TNFA and the SE was observed in our cohort. However, we observed that the TNFA minor allele was a risk factor for clinical CV diseased only in patients with at least a copy of the SE, even after adjustment for classical CV risk factors.

Regarding subclinical CV disease, although the minor allele showed higher carotid IMT and lower endothelial vasodilatation values with respect to the major allele, these differences were not significant.

These results are in concordance with the data previously reported of association of *TNFA* polymorphism and CV disease in Spaniard population [663], where the association was only significant in subjects with T2DM. As previously noted, both RA and T2DM are independent risk factors for CV disease and this genetic variant is not a risk factor neither for RA nor T2DM. Taking into account that most of the association studies in Caucasian population were performed in general population and showed no association with CV disease, we could hypothesize that this variant has a mild effect in the pathogenesis of atherosclerosis and therefore in the risk of CV disease and only when it is analysed in the context of another condition associated to CV disease the effect of the polymorphism becomes apparent. In the line with this hypothesis is also the observation that the -308 TNFA only showed association in patients carrying the SE, due to the association of these HLA alleles with a higher inflammatory burden in RA and with more endothelial dysfunction, higher risk of CV events and a poorer outcome of RA.

5.2. *CCR5*Δ32 variant and CV disease in RA

CCR5 is a chemokine receptor that could potentially be involved in plaque development [675]. Both CCR5 and its ligands (CCL3, CCL4, CCL5, CCL8) are expressed on cells located in atherosclerotic plaque, such as macrophages, T cells, VSMCs and coronary endothelial cells [394,676-678]. This chemokine system mediates the arrest and transendothelial diapedesis of monoytes and T lymphocytes [679]. In addition, CCL5 can be stored and released from α-granules by platelets and its deposition and immobilization on activated aortic endothelium or neointimal lesions constitutes an important mechanism, by which platelets contribute to exacerbation of lesion formation [680].

Observations taken from animal models also support the role of CCR5 in CV disease. In atherosclerosis prone mice (owed to the knock out for the *Apoe* gene), the lack of *Ccr5* protected against advanced atherosclerosis regardless the diet (normal or high fat diet), although did not alter the development of early atherosclerotic lesions [400,681]. This would indicate that in the early stages, *Ccr5* does not play an important role [682] in plaque formation. These knock out mice were also protects against neointima formation after arterial wire-injury [683], through up-regulation of the anti-inflammatory cytokine IL-10 by neointimal VSMCs. Deletion of this gene is also associated with an increase in the count of circulating endothelial progenitor cells, a putative atheroprotective factor [400]. In a different mice model (knock out for *Ldlr* gene), lack of *Ccr5* was not associated to changes in plaque size but to an improved plaque stability [684]. In agreement with this observation, in humans it has been observed an up-regulation of *CCR5* mRNA expression in unstable carotid atherosclerotic plaques in comparison with stable plaques [685]. In mice, it has been also observed that CCR5 is implicated in the recruitment of monocytes [686,687] as well as T cells [688] into atherosclerotic lesions. Despite all these

evidences, it is important to take into account that, due to differences in atherosclerotic disease [689], in monocyte subsets [690] and species differences in the complement of chemokines [691], to extrapolate results from mouse models to humans must be done carefully.

There is mutation in the *CCR5* gene that reduces its cell surface expression, acting as a naturally occurring gene deletion. It consists in a 32-base pair deletion (*CCR5*Δ32) that causes a shift in the reading frame, which creates an early stop codon and the production of a truncated protein that is retained in the endoplasmic reticulum [692]. In homozygote for this polymorphism, the receptor is completely eliminated from cell surface [406], while in heterozygote, expression is reduced to 20-30% of the wild type expression [407].

This polymorphism is present in various Caucasian populations at a frequency of about 9%, while it is almost absent in some African, Japanese and Chinese ethnic groups [405,693]. Among European white populations, this variant exhibits a north to south gradient of prevalence, with allelic frequencies highest in Denmark and Northern France and lowest in Corsica [694].

This polymorphism has been associated with RA and with CV disease. Regarding the first, the association with RA risk and severity is conflictive. Some studies have showed an association between this deletion and lower disease activity but not with lower risk of disease [423,695]. Other have found an association with both lower disease activity and lower risk [696] while others have found only association with lower risk, but not with severity. Besides, various studies did not find any association nor with risk nor with severity [697,698]. In 2006 was published a meta-analysis to summarize the influence of the *CCR5*Δ32 variant in the risk of RA, observing an significant association between the polymorphism and lower risk of RA [422]. However, more studies have been published later, and all but one [696] failed to observe any association [420,421,699].

The *CCR5*Δ32 variant has also been studied regarding its influence in CV disease, both clinical and subclinical, comprising populations of different origins, such as Caucasian (both from northern and southern Europe), populations from Turkey and India. However, this is the first time the influence of this polymorphism is analysed in the risk of CV disease in RA patients. In our work, we observed a significant association between the *CCR5*Δ32 variant and a lower risk of clinical CV disease, when we considered our study as a case control analysis. In previous transversal studies, it was observed an association between the *CCR5*Δ32 variant and a lower risk of MI in a Spaniard male population[409], in a northern European Caucasian female population [418] (but only in subjects younger of 55 years) and in a southern European Caucasian population (regardless the age) [408]. However these results have not been consistently replicated: in another case control study of younger of 55 years with MI of northern [413] or eastern [414] European Caucasian ancestry, no differences were observed regarding the genotypic or allelic distribution of the *CCR5*Δ32 deletion. Furthermore, in a Turkish population, the deleted variant was associated with a higher risk of MI [700].

When we performed a longitudinal analysis, studying the rate of occurrence of the first CV event, *CCR5*Δ32 no longer remained associated. In the only longitudinal analysis published [411], it was observed a lower incidence of CV events (cerebral, cardiac and peripheral) associated to the minor allele. Regarding subclinical atherosclerosis, in our study we observed an association of the *CCR5*Δ32 variant with a higher (normal) value of subclavial artery vasodilatation in response to ischemia and no association with carotid IMT. This was also the first study analysing the influence of the *CCR5*Δ32 variant in endothelial dysfunction. Previously, the association between this variant and subclinical atherosclerosis has been studied using carotid IMT or coronary arterial disease as surrogate markers. Regarding carotid IMT, conflictive results were observed:

in one study an association with lower carotid IMT was found [411], meanwhile in another, no association was observed [417]. Various studies have analysed the influence of this polymorphism in the risk of coronary arterial disease associated or not to coronary chest pain, and in general, no association was observed, nor in Caucasian [410,413,415], nor in Indian populations [416].

The systemic inflammation in RA promotes endothelial cell activation, characterized by loss of vascular integrity, increased expression of adhesion molecules, production of several cytokines and chemokines and upregulation of HLA molecules, leading to endothelial dysfunction. Chemokines are necessary in this pathophysiological process, attracting and guiding immune cells from the lumen into the vascular wall. The decrease in cell surface expression of CCR5 would result in a diminished flow of immune cells to the arterial wall, diminishing the inflammatory reaction within the wall and therefore the level of endothelial dysfunction. These phenomena are in concordance with the association observed in our work between the deletion in *CCR5* and normal average endothelial function, measured as subclavial artery vasodilatation in response to ischemia. However, oppose to what could be expected, we found no association between this variant and a lower carotid IMT. The persistence in time of endothelial dysfunction would lead to an increased accumulation of monocytes and other inflammatory cell in the vessel wall and the progressive development of the atherosclerotic plaques [701,702]. Although not published in our paper, we observed in our sample an increased of carotid IMT with the elapsed time between RA diagnosis and the moment the subclinical CV disease was assessed (Spearman´s rho = 0.32, p = 0.0009). Accordingly, we observed a worsening of the endothelial function with time (Spearman´s rho = - 0.19, p = 0.033). However, when we analyzed our population according to the *CCR5* genotype, we observed an increase in carotid IMT values

regardless the genotype (non carriers: Spearman´s rho = 0.29, p = 0.005; carriers Spearman´s rho = 0.87, p = 0.003), and a worsening of endothelial function over time but only in those RA patients non carrying the deletion (non carriers: Spearman´s rho = - 0.23, p = 0.014; carriers Spearman´s rho = 0.23, p = 0.44). We hypothesize that the reason why in *CCR5*Δ32 carriers the carotid IMT continue to increase in time despite a not worsening in endothelial dependent vasodilatation is, as pointed out previously [703], because these two surrogate markers of subclinical atherosclerosis may be measuring two different but related aspects of the pathophysiology of this condition. Despite the lack of correlation between these two parameters in healthy subjects [703], our group observed a correlation in RA patient with long established disease [704]. CCR5 could be an important factor contributing to endothelial dysfunction but far less important for the development of structural vascular alterations.

Regarding clinical CV disease, we observed a protective effect of the *CCR5* deletion. Bridging the gap, this result is in concordance with the observation in a mice model lacking the *Ccr5* gene in which atherosclerotic plaque showed a more stable structure but no change in size [684]. If the lack of *CCR5* has a similar effect in humans, it would explain the lower incidence of CV events in RA patients carrying the *CCR5* deletion, despite similar carotid IMT.

5.3. Adipokines and CV disease in RA

There has been growing evidence that the dominant cell type of adipose tissue, the adipocyte, has the ability to synthesize and release proinflammatory molecules, complement factors, signaling molecules, growth factors, and adhesion molecules [425], such as TNF-α, resistin, and adiponectin [705], therefore playing a role in inflammation. Besides TNF-α, two adipocytokines were studied in our work: resistin and adiponectin.

Regarding resistine, in humans, this molecule is mainly expressed by monocytes, macrophages [432,433], and synoviocytes [434], and plays a pro-inflammatory role [431], up-regulating the expression of cytokines and adhesion molecules on human endothelial cells [441,442,706], stimulating the synthesis of TNF-α, IL-6, IL-1β and resistin in PBMC [435], of TNF-α and IL-6 in synoviocites from RA patients [435] and of IL6, IL8, TNF-α, CCL2, MMP3 and visfatin from adipocytes [434]. In agreement with this observations, in healthy subjects, resitin levels are positively correlated with inflammatory markers, such as IL-6 [431,707,708]. Despite the link between inflammation and metabolic conditions such as obesity, IR and T2DM, studies in human cohorts have not consistently observed an increased systemic resistin in these conditions [447-450,707,709-714]. Also, regarding RA, resistin peripheral levels in Caucasian seem to be similar or only slightly elevated compared to healthy subjects [347,436,452]. However, in synovial fluid, resistin levels are higher compared with matched blood samples [436,454] and compared with the synovial fluid from patients with a degenerative/traumatic joint disease [436,445,454], suggesting local production in the joint. As in healthy subjects, there is a positive correlation between systemic inflammation markers, such as TNF- α, CRP or ESR, and resistin plasma [453] [455] and synovial [454] levels. Also, a weak association with specific markers of inflammation such as DAS28 [445] or Larsen score [452] have been described. Finally, as previously pointed out in non RA patients, no higher resistin levels in RA patients with obesity or IR have been observed [457,458].

Regarding CV disease, resistin levels were not associated with carotid IMT/endothelial dysfunction/coronary arterial calcification nor in healthy subjects [431,451,707,708,715,716] nor in T2DM patients [715], although in hypertensive patients, there was a positive correlation between both [717]. However, circulating levels of resistin were associated with an increased risk of incident ischemic stroke [718] and acute coronary syndrome [719-721]. In RA

patients, resistin levels did not correlate neither with anthropometric parameters, IR [457,458] nor coronary arterial calcification [458].

In our study we analyzed for the first time the influence of the -420 polymorphism of the *RETN* gene in the risk of clinical or subclinical CV disease in RA patients. We choose this variant owed to the enhanced promoter activity associated to this mutation [461]. Previously this polymorphism was associated to higher resistin plasma level, especially in Asian populations [459-461]. Apparently this is due to the presence of another variant in LD located at the position -358: the combination of the minor allele of both variants it is associated to the highest resistin plasma levels [462] in Japanese population. The much lower variability of this latter polymorphism in Caucasian populations is probably the responsible for the -420 polymorphism small effect in circulating resistin [451,463-466].

This gene variant has been inconsistently associated to metabolic conditions such as obesity or T2DM. Regarding obesity, some studies have reported no association [451,461,464,468], while others did so only in subgroup analyses [469-471]. When the influence of this polymorphism in T2DM was analyzed, contradictory results were observed [461,472-476], although in a recent meta-analysis no association between this variant and T2DM was observed [722]. Regarding CV disease, in non-RA population, this polymorphism has been associated to cerebrovascular disease, but only in T2DM patients, both in Asian [460] and Caucasian [474] populations. Regarding coronary arterial disease, no association was observed in Caucasian [465,478] and the results in Asians are conflictive [476,477]. No association with subclinical atherosclerosis, such as carotid IMT [478] or coronary arterial calcification [451], has been observed in Caucasians.

In our study we observed, regarding total clinical CV disease, no association with this *RETN* polymorphism. This result is in consonant with the previously published in

Caucasian populations, as we previously pointed out. Although not included in the published paper, and owed to the previous association with cerebrovascular disease, we analyzed the influence of this variant in the risk of cerebrovascular ischemic events (CVIE): no significant association was observed, although the difference in prevalence of carriers of the minor allele between RA patients with and without CVIE was larger than between patient with or without any CV event (62.5% vs. 52.6%, p = 0.27). After adjust by classic CV risk factors, no significant association was observed (p = 0.87).

Regarding subclinical CV disease, the carriers of the minor allele were associated to a significant lower flow mediated endothelial dependent vasodilatation (4.8% vs. 6.8%, p = 0.03), and to a non significant higher carotid IMT (0.76mm vs. 0.71mm, p = 0.09). After adjusted analysis by classic CV risk factors, the variable became and remained, respectably, non significant. In view of these results, as commented for adiponectin, we cannot confirm the role of this variant in the pathogenesis of CV disease in RA patients. It is interesting to take into account that the only association of this polymorphism with CV disease was described in T2DM patients. Therefore, we could hypothesized that this polymorphism in order to become an independent CV risk factor must combine with other CV risk factors, such as T2DM. Taking into account that RA is a CV risk factor similar to DM, we had hypothesized that in the context of this condition, the -420 *RETN* polymorphism would appear as an independent CV risk factor, although these results do not confirm this hypothesis.

The other adipocytokine analyzed in our work was adiponectin. It was originally described as only secreted by adipocytes. However, it has been observed to be expressed by other cell types, including osteoblasts [723] and synovial cells such as RA synovial fibroblasts [481].

Adiponectin can exert either a pro or an anti inflammatory role depending on which cell acts. Regarding metabolic and CV diseases [492-494], this molecule exerts an anti-inflammatory effect, suppressing the expression of vascular adhesion molecules [486] and SR [487], reducing the expression of and the response to TNF-α [488,724], raising NO production [489], suppressing the proliferation and migration of smooth muscle cells [490] and reducing neointimal formation in response to vascular injury (in mice) [491]. According to these findings, adiponectin plasma levels are negatively correlated with inflammatory markers such as IL-6, TNF-α or CRP [725-727], and decreased adiponectin levels have been reported in obesity [482,504,505], T2DM [504,506-509], hypertension [728,729], dyslipemia [730-732] and coronary arterial disease [510-517,733-735]. In fact, low levels of adiponectin are predictive for the development of IR and T2DM [506,518,736,737].

Despite all these anti-inflammatory effects, at the articular level adiponectin induces the production of pro-inflammatory cytokines, chemokines, MMP, VEGF and adhesion molecules, in synoviocytes, lymphocytes, endothelial cells and chondrocytes [481,495-498]. It also is able to increase osteoclast formation indirectly through stimulating RANKL and inhibiting OPG production in osteoblasts [499]. As a reflection of these phenomena, there is a positive correlation between articular damage and adiponective plasma levels [500-502]. Besides, both plasma [347,452,738] and synovial [454,738] adiponectin levels are higher in RA patients compared with healthy controls or osteoarthritis patients, respectively. However, no correlation between synovial adiponectin levels and unspecific inflammatory markers such a as CRP or ESR have been described [454,738], and, regarding plasma levels, results are non consistent: one study has described no association with CRP or ESR [738] while other has observed a negative correlation between both [739] (as in no RA subjects). In RA, plasma levels were higher than synovial levels [738], which

would suggest that plasma adiponectin is produced in peripheral fat stores, which secrete adiponectin into the blood stream.

Despite the pro-inflammatory effect at the articular level in RA patients, as in healty subjects, a similar association regarding metabolic traits and adiponectin levels has been observed. In both groups, higher levels of this adipocytokine are associated to a healthier lipid profile (lower triglycerides/HDL cholesterol ratio and TC/HDL cholesterol ratio) and glucose metabolism (lower fasting plasma glucose levels) [739]. Regarding the association between adiponectin levels and BMI in RA, the results have not being consistent: some studies have not found any correlation between plasma [738,739] or synovial [454,738] adiponectin levels and BMI, meanwhile others have found a significant negative correlation [452,502] with plasma concentration.

As we pointed out in the introduction, adiponectin plasma levels are highly heritable [521-523] and various polymorphisms located in the *ADIPOQ* gene showed a great influence in adiponectine levels [524-526]. In our study, we selected two variant (rs266729 and rs1501299), each of them located in one of the LD blocks of the *ADIPOQ* gene. Neither of the two polymorphisms analyzed in our study have been consistently associated with adiponectin plasma concentration [523,524,526,527]. However, they have been associated to metabolic and CV disease. The minor allele of the rs266729 polymorphism has been associated with T2DM in Caucasians in two recent metaanalysis [528,529], although in a previous one no association was described [523]. It has been also associated with clinical CV disease [530] in non-diabetic Chinese subject and in Caucasians [531,532], and with subclinical CV disease, such as significant (>50%) coronary stenosis [531] and a greater carotid IMT [533].

On the other hand, the rs1501299 polymorphism has not been associated with T2DM [523,528]. Regarding CV disease, the minor allele of this variant has been associated to a

lower risk of CV disease in Caucasian subjects of south European ancestry [535] and in T2DM patients [523]. However, no association with CV disease was observed in two different studies in non diabetic Caucasian subjects of north European ancestry [531,534]. Regarding subclinical atherosclerosis, association with carotid IMT or ischemic stroke was described neither in Asians [536], nor in Caucasians [537]. No association with coronary stenosis was observed in a north European Caucasian population [531].

In our study we assessed for first time the influence of the *ADIPOQ* rs266729 and rs1501299 polymorphisms, either isolated or in combination, in the risk of clinically evident CV disease or in the presence of subclinical atherosclerosis in a large series of patients with RA. We observed no significant association, neither with clinical, nor with subclinical atherosclerosis. We observed a higher frequency of the minor allele of the rs1501299 polymorphism among RA patients without CV disease (28.1% vs. 23.5%), although the difference was not statistical significant ($p = 0.15$). In the logistic regression analysis and in the conditional analysis, either adjusted or unadjusted, we observed again a non significant protective effect of the minor allele regarding clinical CV disease. Regarding subclinical atherosclerosis, no differences were observed according to genotypes or alleles of this variant.

The other *ADIPOQ* variant studied, rs266729, showed no association with clinically evident CV disease or with subclinical atherosclerosis. The combined effect of both polymorphisms did not show a significant association with the risk of CV disease or with the presence of subclinical atherosclerosis assessed by three surrogate markers of subclinical atherosclerosis.

With the data we gathered, we can not confirm the role of neither of both variant in the risk of clinical or subclinical CV disease among RA patients. With regard to the rs1501299 polymorphism, our results are in line with the previously published in no-RA

population, although the differences did not reach statistical significance, maybe due to a not large enough sample size. However, regarding the other polymorphism (rs266729), we observed a much smaller differences between RA patients with and without CV disease (both clinical and subclinical), and likely the results would not improve despite a larger sample size.

5.4. *VEGFA* polymorphisms and CV disease in RA

Among the similarities between the atherosclerotic plaque and the rheumatoid synovial is the presence of hypoxia in both lesions [66,565]. This condition stimulates the growth of new vessels that carry the nutrients and oxygen needed to the hypoxic tissue. Hypoxia is the main environmental stress factor that triggers the production of VEGF, both through mechanism dependent and independent of the HIF regulated elements in the *VEGFA* gene [545]. The HIF-independent pathway is mediated by other transcription factors whose expression is also induced by the lack of oxygen [551,740] and other factors, such as cytokines and growth factors presents in both lesions: IL-6, IL-1β, TNF-α, platelet-derived growth factor, basic fibroblast growth factor , epidermal growth factor and TGFs [552,553,741]. Also, ox-LDL upregulates VEGF expression in macrophages and endothelial cells, though a HIF independent mechanism [742].

The predominant cellular target of VEGF is the endothelial cell, in which promotes proliferation, sprouting, migration, tube formation and expression of several antiapoptotic proteins [555], being a potent survival factor. VEGF also increases vascular permeability [556] and causes vasodilatation through the induction of NOS [743]. This angiogenic factor also binds the VEGF receptors present on hemopoietic stem cells (promoting their survival and mobilization from the bone marrow), monocytes

(inducing monocyte chemoattraction) [561], osteoblasts (promoting osteoblast-mediated bone formation) and VSMCs (increasing their proliferation) [744].

As the atherosclerotic plaque develops, the expression of VEGF increases, leading to a correlation between the extent and progression of the atherosclerotic lesion and the extent of plaque neovascularization: while normal arterial segments show no or low VEGF expression, in early atherosclerotic lesions and atheromatous plaques, activated endothelial cells, macrophages, and partially differentiated VSMCs show a clear VEGF positivity. Finally, in advanced atherosclerotic lesions associated with extensive neovascularization, intense VEGF expresion is observed in endothelial cells of microvessels and infiltrating macrophages [575,745]. Accordingly, neovascularization is more common at the sites of infiltration by chronic inflammatory cells, owed to the proangiogenic stimulus provide by these cells [746].

Plaque inflammation and neovascularization are associated to a higher risk of rupture and to an increased potential to become symptomatic [747]. Higher intimal vessels counts are seen in those patients with symptomatic carotid and coronary disease compared with subjects who were symptoms free [748]. Accordingly with the observations regarding VEGF expression and atherosclerosis, serum VEGF concentration is correlated with risk factors for atherosclerosis [749], such as hypercholesterolemia [750] and smoking [749]. Also, the serum levels of this factor are increased in subjects with clinical and subclinical atherosclerosis, compared with healthy controls [751-753], and in subjects with acute MI compared with chronic coronary ischemic disease [754]. Also, there has been described a correlation between plasma VEGF levels and the size of coronary collateral vessels in cases of chronic ischemia [754], although higher VEGF levels have been associated with worst outcome after MI [755] and higher CV mortality [756].

In our work we analyzed two polymorphisms of the *VEGFA* gene located in the 5' UTR region: the rs2010963 polymorphism, located at -634 and rs1570360m located at -1154. Both variants regulate *VEGFA* expression at the post-transcriptional level [572,573], affecting both gene expression and plasma levels.

The major allele C of the -634 variant has been associated both to an enhanced *VEGFA* expression at both transcriptional and translational levels [574,757] and to higher serum levels [573,575,576]. However, the expression of VEGF in PBMCs in response to LPS was greater in homozygote for the minor allele G [758]. This polymorphism has been associated to other diseases, such as severe diabetic complications [573], neurodegenerative disorders [574], Behçet disease [581] and giant cell arteritis [582]. Regarding the latter disease, the major allele of this -634 polymorphism was significantly associated with a higher risk of severe ischemic complications [583]. Its association with CV disease has been also studied. In Caucasians, no association with coronary atherosclerosis [584], carotid IMT or MI [759] was observed, in non T2DM subjects. However, the major allele C has been associated with MI in Caucasians with T2DM [576]. In Asian populations, the major allele of this variant has been associated with a greater extension of coronary atherosclerosis [760], but not no a higher risk of stroke [761].

On the other hand, the mayor allele G of the -1154 variant has been associated to a greater VEGF production by PBMC [574,577], although not significant differences in serum VEGF has been described [578]. This variant has not been associated with coronary atherosclerosis in Caucasians [584,585], although it has been associated with clinical coronary ischemic disease in an Indian population [762]. No association with a higher risk of ischemic stroke was observed [761] [763] in Asian populations. This variant has been associated the higher risk of hypertensive nephropathy in Hispanics [764]

Despite the functional relevance of these polymorphisms, no association with the risk of RA have been observed, nor in Caucasian [579], nor in Asian populations [580].

In our work we observed no significant association between any of the two *VEGFA* polymorphisms and neither clinical nor subclinical CV disease in RA patients. As we previously pointed out, these polymorphisms influence *VEGFA* transcription, being associated both to a lower transcriptional activity. Besides, due to the effects of VEGF in the atherosclerotic plaque, it was plausible to hypothesize that the -634 and -1154 variants would have a significant effect in atherosclerosis, clinical or subclinical. The apparent lack of influence of these polymorphisms in the risk of CV disease could be owed to a paradoxical effect played by VEGF in ischemic conditions [562]: on one hand, VEGF is implicated in the development and complication of atherosclerotic plaques, leading to tissue ischemia. However, on the other hand this factor is also essential in the development of collateral circulation that would appear in response to tissue ischemia provoked by the atheromatous plaque. Therefore, a higher VEGF production would be associated, not only to a major plaque growth but also to a greater collateral vessel circulation development. The lack of association in Caucasian populations of both polymorphisms with CV disease [584,585,759] maybe would reflect this compensatory phenomenon. However, it seems that in T2DM, the pro-atherogenic effect of VEGF surpass the protective effect that this factor may exert [576]. We had hypothesized that in RA we would observe a similar effect, due to that, as T2DM, RA is an independent CV risk factor. However, no significant effect was observed in RA.

5.5. *GHSR* polymorphisms and CV disease in RA

In our work, we decided to study the influence of the GHS-R in the risk of CV disease in RA. The growth hormone secretagogues induce a significant growth hormone production from pituitary cells via their binding to a specific cell surface receptor, GHS-R [595,765], which endogenous ligand is ghrelin [591]. This secretagogue is mainly expressed by the X/A-like cells in the stomach, but also by B cells, T cells, monocytes and NK cells [765]. Its acylated form has demonstrated its ability to stimulate GH release from pituitary cells [597], to induce food intake and to increase adiposity [597]. The GHS-R is also expressed in many organs within the body including pancreatic, lymphoid, reproductive and gastrointestinal tissues. Within the immune system, it is expressed in human B and T cell, PBMCs, as well as immune organs [765].

The expression of ghrelin and GHS-R are regulated during states of acute and chronic inflammation, stress and aging [603]. In example, the activation of T cells via T-cell receptor ligation up-regulates the expression of grelin and GHS-R [598]. Conversely, ghrelin, through the GHS-R, can directly signal and induce alterations in cytokine and LAMs expression in immune cells, probably through the ability of ghrelin to modulated NFκB signaling [587]. The main effect of ghrelin in T cells, monocytes/macrophages and endothelial cells is anti-inflammatory, inhibiting the release of pro-inflammatory cytokines such as IL-1α, IL-1β, TNF-α and IL-6, in T-cell receptor activated T cells, in LPS activated monocytes and DCs, in mononuclear and T cells stimulated with leptin and in endothelial cells stimulated with TNF-α [598,600,766]. In the latter, also diminishes the expression of the vascular cell adhesion molecule-1 and monocyte chemoattractant protein-1 [767]. Ghrelin also stimulates the production of anti-inflammatory cytokines such as IL-10 in macrophages [768]. It is able to suppresses both Th1 (IL-2 and IFN-γ) and Th2 (IL-4 and IL-10) cytokine mRNA expression in mice [769] and to inhibit splenic T

cell proliferation induced by stimulation with immobilized anti-CD3 antibody. Conversely, the suppression of the endogenous ghrelin expression in human T cells results in increased levels of IFN-γ, IL-17 and other pro-inflammatory cytokines and chemokines upon T-cell receptor activation [766].

Besides its anti-inflammatory effects, ghrelin exerts a vasodilatory effect [605], antagonizing the action of endothelin-1 [613], increasing the effect of acetylcholine [611], decreasing the sympathetic nervous activity [770,771] and raising the production of endothelial NOS [611]. Also, ghrelin stimulates proliferation [772] and exerts an antiapoptotic effects in cardiomyocytes and endothelial cells [614,773,774]. Hexarelin, a syntetic agonist of the GSH-R, decreases apoptosis induced by angiotensin-II in cultured rat cardiomyocytes [775]. Linked to those effect in cardiomyocytes, ghrelin also improves left ventricular function during ischemia-reperfusion injury [776] and, in mice models of heart failure, ghrelin improves left ventricular dysfunction and attenuates the development of cardiac cachexia [606]. In patients with chronic heart failure, ghrelin also improves cardiac function and decreases systemic vascular resistance [609].

As a consequence of the anti-inflammatory, vascular and cardiac effects exerted by ghrelin, this peptide is considered to play a protective role in atherosclerosis and CV disease.

A positive correlation between ghrelin levels and classic biomarkers of inflammation, such as TNF-α, IL-6 and IL-1β has been observed [765]. In line with these observations, elevated ghrelin levels have been reported in inflammatory conditions, such as ankylosing spondylitis, ANCA-associated vasculitis, celiac disease and inflammatory bowel diseases, with normal levels during remission of these inflammatory diseases [777-779]. However, in RA ghrelin levels have been reported similar [780] or decreased [603]

compared with healthy subjects. Also in metabolic conditions, such as obesity and IR [781], low ghrelin levels have been found.

In our work we analyzed the role of 3 polymorphism of the *GHSR* gene: rs512692, located in the 5'UTR region, rs509035, located in the intron and rs2922126, located in the promoter region. Previously, these 3 variants had showed no association with RA [625].

These polymorphisms have been analyzed with regard to metabolic condition and CV disease, with conflicting results. The variant rs572169 (in complete LD with rs509035 in the CEU population) in Caucasians showed no association with T2DM [782], glucose intolerance, obesity [783,784] nor BMI [785]. However, in another studies, the rs509035 variant was associated to different measures of glucose metabolism (fasting glucose, 2h insulin) [623] and obesity in Caucasians [786]. Also, rs572169 was associated to lower HDL values [787] in Caucasian but not in Chinese population [624]. On the other hand, the rs512692 variant has showed association with obesity in Caucasians [786], while the rs2922126 polymorphism showed no association [784]. However, in Chinese population rs2922126 was associated to metabolic syndrome [624].

Regarding CV disease, both rs509035 and rs512692 were associated in Caucasians [621]. Also rs509035 was associated with left ventricle hypertrophy [622], while rs512692 was not.

In our study, we found no association either with clinical or subclinical CV disease for any of the variant. The 3 polymorphisms showed a non significant higher frequency of the minor allele among those RA patients without CV disease. Regarding both rs509035 and rs512692 our results are the opposite to those previously published [621], in which the minor allele showed a higher frequency among the patients with CV disease, although our results were non significant. In conclusion, we can not confirm the implication of

the rs512692, rs509035 nor rs2922126 polymorphisms in the pathogenesis of either clinical or subclinical CV disease in RA.

CONCLUSIONS

6. CONCLUSIONS

Our work contributes to the understanding of the genetic basis of CV pathology in RA. We have studied the role as risk factors for clinical and subclinical CV disease of various polymorphisms located in genes involved in the pathogenesis of both conditions. All the genetic variants had been previously associated with CV or metabolic diseases in Caucasian general population or in subgroup of patients affected with classic CV risk factors, such as T2DM. This was the first time that these polymorphisms were analyzed in a RA population. Among the variants studied of *TNFA*, *CCR5*, *RETN*, *ADIPOQ*, *VEGFA* and *GHSR*, only the first two showed a significant association with CV disease. Nonetheless, these results need to be replicated to assert their accuracy.

PERSPECTIVES

7. PERSPECTIVES

In the last years, it has taken place an important advance in the knowledge of the genetic bases of CV disease. However, in aggregate the discovered variants explain only a small fraction of the heritability of CV disease (estimated to be up to 30–60% in the general population [788]), probably in part, due to the limited power of previous studies to discover effects of modest size. Until now, the research performed in RA patients was hypothesis-driven candidate gene association studies, such as the ones performed in this work. This approach provides a focused view of genomic regions of interest, allowing targeting putative functional variant. Also, this design is specially useful when allele frequencies are low, effect sizes are small, or the study population is limited [789]. However this kind of approach has its own pitfalls, such as the lack of replication of results, presence of false positives and little account for genetic heterogeneity. To overcome these issues, in part would be necessary to increase the number of studied subjects, both cases and controls. To that end, it would be necessary the joint collaboration of different groups in order to replicate the results in different populations. Also, pooling various cohorts would allow increase the detection of modest genetic effects.

Another research paradigm for population-based genetic association studies are the hypothesis-free genome-wide association studies (GWAS), in which, from hundreds of thousands to millions of single nucleotide polymorphisms, distributed along the whole genome, are analyzed at once. This year (2011) has been published a meta-analysis of GWAS for CV disease performed in Caucasian general population [790]. In this work, 10 of the 12 previously identified variants in GWAS achieved genome-wide significance (p-value $< 5\times10^{-8}$) and 13 new loci were detected and replicated. We consider that to

perform this kind of analysis in the context of RA would be a major step-forward in the understanding of the genetic bases of this condition. Also, taking into account that chronic inflammatory diseases share to some degree a genetic background, it would be really interesting to perform a more "directed" genotyping array such as the "Immunochip" that contains a little less than 200.000 polymorphisms, previously associated with major autoimmune and inflammatory diseases. On the other hand, in the last years was release another genotyping array designed to test about 200.000 polymorphisms of interest for metabolic and atherosclerotic / CV disease traits. Regardless the platform to use, it would be necessary again the joint efforts of multiples groups to pool enough subjects to achieve sufficient statistical power.

The better understanding of the genetic basis of CV disease in RA will grant a better comprehension of the signaling pathways and different molecules implicated in the pathogenesis of this condition. This knowledge will be useful in the discovery of new therapeutic targets and in the development of new drugs.

ANNEX

8. ANNEX

8.1. Patients and Study Protocol

Between March 1996 and March 2008, consecutive patients that fulfilled the 1987 American College of Rheumatology classification criteria for RA [791] were recruited from the rheumatology outpatient clinics of Hospital Clínico San Carlos, Madrid and Hospital Xeral-Calde, Lugo, Spain. A DNA sample (see below) was extracted from these patients at the time of recruitment. Between December 2009 and January 2010, patient's clinical records were examined until patient's death, loss of follow-up or December 1st, 2009. Socio-demographical and clinical data regarding clinical manifestations, classic CV risk factors and history of CV events were registered.

Regarding classic CV risk factors, patients were considered to have dyslipidemia if they had hypercholesterolemia and/or hypertriglyceridemia (defined as diagnosis of hypercholesterolemia or hypertriglyceridemia by the patients' family physicians prior to the diagnosis of RA, or total cholesterol and/or triglyceride levels in fasting plasma >240 mg/dl and 160 mg/dl, respectively, at the time of disease diagnosis or over the extended followup). Patients were considered to have hypertension if before the diagnosis of RA they had been diagnosed as having hypertension by their family physicians, or if at the time of disease diagnosis or over the extended followup they had blood pressure >150/90 mm Hg in 2 different examinations performed on different days. Patients were considered to have diabetes mellitus if before disease diagnosis they had been diagnosed as having diabetes mellitus by their family physicians or if 2 fasting plasma glucose levels on different days at the time of disease diagnosis or over the extended followup were >125 mg/dl. Obesity was defined as body mass index (calculated as weight in kg divided by height in m2) >30 kg/m2 at enrollment or over

the extended followup. Smoking history was treated as a dichotomous variable (heavy versus nonheavy smoking) in this analysis. Heavy smokers comprised patients who smoked at the time of disease diagnosis or during the followup or who had smoked within the 10 years before the onset of RA symptoms or the disease diagnosis, and the remaining patients (nonheavy smokers) were those who had never smoked or had stopped smoking at least 10 years before the disease onset.

Regarding CV events, IHD included acute coronary syndromes with or without persistent ST-segment elevation and chronic coronary heart disease. IHD was diagnosed if any of the following criteria were satisfied: a recorded diagnosis of ischemic cardiopathy on account of some acute coronary syndrome (acute MI or unstable angina), the presence of pathologic Q waves in the electrocardiogram, and coronary images showing >50% stenosis of at least 1 coronary vessel. Cerebrovascular accident was defined as the presence of stroke (confirmed by computed tomography and/or magnetic resonance imaging) and/or transient ischemic attack (diagnosed if the symptoms were self limited in <24 hours, without residual neurologic damage). Heart failure was defined based on the Framingham criteria [792]. Peripheral arterial disease was considered to be present if it was confirmed by Doppler and arteriography.

Hospital Clinico San Carlos and Hospital Xeral-Calde are the referral centers for the population of each respective area; the first CV event was defined as an event (case) of CV complication diagnosed at the hospital in a patient without a previous history of CV disease.

Endothelial dysfunction was assessed by measure of the brachial artery reactivity, between March 2007 and September 2009 in a random subgroup of patients from the Lugo cohort with no previous history of CV disease. B-mode scan of the right brachial artery, in a longitudinal section 2–12 cm proximal to the antecubital fossa, was

performed in supine participants using a vascular software for 2-dimensional imaging, color and spectral Doppler, an internal electrocardiogram (EKG) monitor, and a 7.5-MHz phased-array transducer Hewlett-Packard SONOS 5500 system (Hewlett-Packard, Palo Alto, CA). The anterior and posterior intima-media interfaces were used to define the baseline artery diameter, calculated as the average of measurements made during 4 cardiac cycles at end diastole. Timing of each image frame with respect to the cardiac cycle was determined with simultaneous EKG recordings on the ultrasound system digital monitor. During image acquisition, anatomic landmarks were noted to maintain the same image of the artery throughout the study using a specific stereotactic clamp.

Flow-mediated endothelium-dependent vasodilatation (FMD%; post-ischemia) was assessed using a forearm blood pressure cuff inflated on the ipsilateral wrist to at least 50 mm Hg above resting systolic blood pressure for 5 minutes, and then was released. FMD% (an increase in brachial artery diameter) was measured 30–60 seconds after cuff release. A midartery pulsed Doppler signal was obtained within the first 15 seconds after cuff deflation to assess hyperemic velocity. Endothelium-independent (NTG%; post-nitroglycerin) vasodilatation was assessed administering 400 μg of sublingual nitroglycerin, which acts directly on vessel smooth muscle to cause vasodilatation. NTG% was measured 4 minutes after nitroglycerin intake. In all cases a cardiologist (CG-J) analyzed all of the ultrasound data offline and was blind to the clinical information. Intra-observer variability was 1.3% and 1.9%, respectively, based on the repeat of the ultrasonography in 32 healthy controls. Assessment of endothelial function of patients undergoing anti-TNF therapy was performed 24-48 hours before drug administration.

Carotid intima-media thickness (IMT) was measured in the right common carotid artery, using high-resolution B-mode ultrasound (Hewlett Packard SONOS 5500), with

a 10-MHz linear transducer. Patients and controls were examined in supine position with the neck extended and the chin turned contralateral to the side being examined. Measurement of IMT was assessed in the common carotid artery 1 cm distal to the carotid bifurcation in the posterior wall. All measurements were made manually on digitized still images that were obtained during ultrasound scanning. They were calculated as the average of measurement during 3 cardiac cycles at end-diastole. Measurements of IMT were performed by 2 cardiologists and in all cases the cardiologists were blinded to clinical information of the subjects and both agreed on the results. The reproducibility of the IMT measurements was evaluated in 20 patients and 20 controls within 1 week of the first examination. The correlation coefficient for IMT was 0.986.

The subject's written consent was obtained according to the declaration of Helsinki, and the design of the work was approved by the Ethics Committee of Hospital Clinico San Carlos (Madrid) and Galicia (Spain).

8.2. Genotyping

DNA from patients was obtained from whole peripheral blood, using standard methods. All the polymorphisms were genotyped using TaqMan Assays-on-Demand from Applied Biosystems, following the manufacturer's protocol and analyzed using the ABI 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The typing successful rate was over 95%. 10% of the samples were re-genotyped at random. No differences were observed with the results obtained before.

8.3. Statistical Analysis

Comparison of means was performed using T-test. Comparison of proportion between 2 or more group was carried out using χ^2 test or Fisher test, when required. Hardy-Weinberg (HWE) equilibrium for each polymorphism was tested in the RA patients with and without CV disease, using http://ihg.gsf.de/cgi-bin/hw/hwa1.pl. When requered, LD values (r2) and allelic combinations were generated using UNPHASED software. Strength of association between CV events and genotypes, alleles or allelic combinations of the studied polymorphisms was estimated using odds ratios (OR) and 95% confidence intervals [95% CIs] via multiple logistic regression. Also a Cox regression model was used to estimate the influence of some polymorphisms on CV disease. We used the occurrence of at least one CV event as the outcome. Survival time was defined as "age of the subjects at" or "elapsed time between RA diagnosis and" the fist CV event, patient's death, loss of follow-up or December 1st, 2009. Patients who died of any other causes different from CV events were considered as not having CV events. Proportional hazard assumption was tested using Schoenfeld residuals. Results were expressed as hazard ratios (HRs) with [95% CIs] and were computed as both crude and adjusted for age at RA diagnosis, gender and presence of classic CV risk factors. The selected variables used for adjustment were selected due to their association with the outcome (CV event) and the exposure (genotype) and because they produced a change greater than 10% in the HR.

The association between the polymorphisms and carotid IMT, FMD% and NTG% was also tested using analysis of covariance (ANCOVA) adjusting for gender, age and duration of the disease at the time of the ultrasonography study, and presence or absence of the SE and traditional CV risk factors. Calculations were performed with STATA 10 (STATA Corporation, College Station, Texas).

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9. REFERENCES

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