Identification of a 3′-Untranslated Genetic Variant of \( \text{RARB} \) Associated With Carotid Intima-Media Thickness in Rheumatoid Arthritis: A Genome-Wide Association Study

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Objective. To investigate the genetic background influencing the development of cardiovascular (CV) disease in patients with rheumatoid arthritis (RA).

Methods. We performed a genome-wide association study (GWAS) in which, after quality control and imputation, a total of 6,308,944 polymorphisms across the whole genome were analyzed in 2,989 RA patients of European origin. Data on subclinical atherosclerosis, obtained through assessment of carotid intima-media thickness (CIMT) and presence/absence of carotid plaques by carotid ultrasonography, were available for 1,355 individuals.

Results. A genetic variant of the \( \text{RARB} \) gene (rs116199914) was associated with CIMT values at the genome-wide level of significance (minor allele [G] \( P \) coefficient 0.142, \( P = 1.86 \times 10^{-4} \)). Interestingly, rs116199914 overlapped with regulatory elements in tissues related to CV pathophysiology and immune cells. In addition, biologic pathway enrichment and predictive protein–protein relationship analyses, including suggestive GWAS signals of potential relevance, revealed a functional enrichment of the collagen biosynthesis network related to the presence/absence of carotid plaques (Gene Ontology no. 0032964; false discovery rate–adjusted \( P = 4.01 \times 10^{-5} \)). Furthermore, our data suggest potential influences of the previously described candidate CV risk loci \( \text{NFkB1} \), \( \text{MSRA} \), and \( \text{ZC3HC1} \) (\( P = 8.12 \times 10^{-4} \), \( P = 5.94 \times 10^{-4} \), and \( P = 2.46 \times 10^{-4} \), respectively).

Conclusion. The present findings strongly suggest that genetic variation within \( \text{RARB} \) contributes to the development of subclinical atherosclerosis in patients with RA.

INTRODUCTION

Cardiovascular (CV) disease is the most common cause of morbidity and mortality in patients with rheumatoid arthritis (RA) (1–3). In RA patients, CV disease may develop as a result of an accelerated atherosclerotic process (4). Surrogate markers for subclinical atherosclerosis, i.e., increased carotid intima-media thickness (CIMT) and presence of carotid plaques (5,6), are excellent...
predictors of future CV events. Traditional CV risk factors and chronic inflammation do not fully explain the increased CV predisposition observed in patients with RA, accounting for only ~70% of the population-attributeable risk for CV disease outcomes (7). Cumulative knowledge clearly suggests that genetic factors may play a relevant role in this phenomenon (8), but the specific genetic component of CV disease in RA remains elusive.

Genome-wide association studies (GWAS) constitute a hypothesis-free approach in which millions of common genetic variations across the whole genome are interrogated (9). This strategy has been of great help in elucidating relevant ingredients into the genetics of several complex human diseases (10). The use of this technology has substantially increased the number of established RA susceptibility loci from 3 to >100 during the last decade (11). Nevertheless, there are currently no available GWAS data for haplotype reconstruction and the updated Haplotype Reference Server (MIS) (14), using ShapeIT16 software (version v2.r790).

Genotyping and quality control. Genomic DNA was extracted from peripheral blood using standard procedures. Genotyping was conducted at the Human Genotyping Unit of the National Genotyping Center in Spain, using the GWAS platform Infinium HumanCore BeadChip in an iScan system, according to the protocol recommended by the manufacturer (Illumina). Single-nucleotide polymorphisms (SNPs) with a cluster separation of <0.4 were removed after the calling.

Raw data were subjected to stringent quality control filters using the software Plink (version 1.07) (13). Polymorphisms with call rates of <0.98 and minor allele frequencies of <0.01, as well as those that deviated from Hardy-Weinberg equilibrium ($P < 0.001$), were filtered out. Similarly, samples with <95% successfully called polymorphisms, and 1 subject per pair of first-degree relatives (identity by descent >0.4), were removed. Sex chromosomes were also excluded from the analysis.

To ensure reliability of the results, the associated SNP described below was re-genotyped using a predefined TaqMan 5’ SNP genotyping assay (C_154503570_10) in a 7900HT Fast Real-Time PCR System (Applied Biosoftware), and the TaqMan types were compared with the corresponding imputed data.

Imputation methods. After application of the quality control filters, whole-genome SNP genotype imputation in autosomal chromosomes was carried out in the Michigan Imputation Server (MIS) (14), using ShapeIT16 software (version v2.r790) for haplotype reconstruction and the updated Haplotype Reference...
ence Consortium data (version r1.1) as a reference panel, which combine sequencing data from a total of 32,470 individuals from multiple studies (including the 1000 Genomes Project) (15). The quality control filters mentioned above were also applied to the imputed data using Plink. In addition, singletons (r² ≤ 0.2) were excluded. Finally, possible population stratification was controlled by principal components (PC) analysis using Plink and gcta64 and R-base software under GNU Public license v2. The first 10 PCs for each individual were calculated and plotted to identify outliers, and those deviating from the cluster centroid by >4 SD were excluded.

After quality control, 6,308,944 SNPs and 2,989 RA patients remained for analysis in the final data set. Data on demographic, RA clinical, and CV disease–related characteristics are shown in Table 1. Information related to CV events was obtained from the medical records of each patient, with traditional CV risk factors and CV events defined as previously described (3,6). Briefly, individuals were considered to have ischemic heart disease (IHD) if any of the following criteria were satisfied: a recorded diagnosis of ischemic cardiopathy due to an acute coronary syndrome (acute myocardial infarction or unstable angina), abnormal Q waves seen on electrocardiography, and/or >50% stenosis of at least 1 coronary vessel seen on coronary images. A patient was considered to have heart failure based on the Framingham criteria. Cerebrovascular accident was recorded if patients had a stroke and/or transient ischemic attacks (TIAs). Strokes were classified according to their clinical features and were confirmed by computed tomography and/or magnetic resonance imaging. TIAs were diagnosed if the symptoms were self-limited in <24 hours, without residual neurologic damage. Finally, peripheral arterial disease was considered to be present if confirmed by Doppler imaging and arteriography (3,6).

**Subclinical atherosclerosis examination.** Information on subclinical atherosclerosis was available for 1,355 RA patients from the filtered data sets. Subclinical atherosclerosis examination was assessed with a carotid ultrasound technique (evaluation of CIMT and presence/absence of carotid plaques). At the hospitals in Santander, Bilbao, Granada, Córdoba, Tenerife, Valencia, Ciudad Real, and Madrid, the ultrasound examination was performed using a commercial scanner (16,17). Patients from Lugo were assessed by high-resolution B-mode ultrasound (18). CIMT was measured at the far wall of the right and left common carotid arteries over the proximal 15-mm–long segment. CIMT was determined as the average of 3 measurements in each common carotid artery. Consistency of results between these 2 ultrasound methods was previously reported (19), supporting the fact that the use of 2 different instruments to collect CIMT data did not influence the results derived from this analysis. In addition, these studies were performed by experts with high intra- and interobserver reliability, who have collaborated closely in the assessment of subclinical atherosclerosis in RA. Criteria for determining the presence of plaque in the accessible extracranial carotid tree were defined as described by Touboul et al (20).

**Statistical analysis.** Estimations for statistical power were obtained with CaTS Power Calculator for Genetic Studies software, which implements the methods described by Skol et al (21) (Supplementary Tables 1–4, on the Arthritis & Rheumatology web site at http://onlinelibrary.wiley.com/doi/10.1002/art.40734/abstract). All statistical analyses were conducted with Plink. First, we compared the genotype frequencies of all SNPs according to a continuous CV disease outcome variable (CIMT values) by linear regression assuming an additive model. The first 10 PCs, age at the time of the carotid ultrasound examination, and sex were included in the model as covariates. Subsequently, we compared the genotype frequencies of all SNPs according to binary CV disease outcome variables (presence/absence of CV events, IHD, and carotid plaques) by logistic regression on the best-guess genotypes assuming an additive model. The first
10 PCs, age at the time of RA diagnosis, and sex were included as covariates for the presence/absence of CV events and IHD analyses, and the first 10 PCs, age at the time of carotid ultrasound examination, and sex were included as covariates for the presence/absence of carotid plaque analysis. Finally, \( P \) values, beta coefficients, standard errors, odds ratios, and 95% confidence intervals were calculated. The statistical threshold was set at the genome-wide level of significance \( (P < 5 \times 10^{-8}) \).

**Performance of functional annotations of the associated variants.** In a further step, we evaluated the putative functional implications of the identified CV risk signals by integrating our data with functional annotation data available in public databases, using different bioinformatics approaches. For this purpose, we first identified all of the potential polymorphisms in high linkage disequilibrium (LD; \( r^2 > 0.8 \)) of the associated signals of our GWAS, using the European populations from the 1000 Genomes Project and Plink. All of those potential polymorphism taggers would be considered equally as candidates for prioritizing causality or hypothesizing possible molecular causes of the observed associations in the subsequent bioinformatic approaches. Then, the online tools RegulomeDB (22), HaploReg (version 4.1) (23), and Capture HiC Plotter (CHi-CP) (24) were used to evaluate the possible regulatory effect of the associated signals and their possible implications in the clinical phenotypes analyzed.

**Candidate genomic regions and pathway enrichment analysis.** Finally, we assessed the statistical significance in our GWAS of previously described CV risk–associated genomic regions (±100 kbp 3’ and 5’ of the reported gene) through candidate gene studies (8) and a recently published meta-analysis of ImmunoChip data (25). Regarding the HLA region, a more comprehensive analysis was conducted. We extracted the extended HLA region (29,000,000–34,000,000 bp in chromosome 6) and imputed SNPs, classic HLA alleles at 2- and 4-digits, and polymorphic amino acid positions, as previously described (26–28).

Additionally, a biologic pathway enrichment analysis involving genes that showed suggestive \( P \) values in our study \( (P < 1 \times 10^{-4}) \) was performed by using the tool for that purpose from the Gene Ontology (GO) reference genome project (29,30), powered by the Protein Analysis Through Evolutionary Relationships Classification System (31). Moreover, we conducted a predictive protein–protein interaction analysis among these same markers, using the Search Tool for the Retrieval of Interacting Genes/Proteins database (32). \( P \) values less than 0.05 after correction for multiple testing were considered significant.

**RESULTS**

**Testing for association with CV disease outcomes.** Figure 1 and Supplementary Figure 1 (available on the Arthritis & Rheumatology web site at http://onlinelibrary.wiley.com/doi/10.1002/art.40734/abstract) summarize the overall results obtained for each CV disease outcome analysis performed. Interestingly, a statistically significant signal at the genome-wide level of significance was associated with CIMT values (Figure 1). This signal corresponded with the genetic variant rs116199914, which maps to the 3’-untranslated region (3’-UTR) of the retinoic acid receptor \( \beta \) gene \( (RARB) \) (Table 2). The minor allele (G) of this SNP was significantly related to increased CIMT values \( (\beta = 0.142, \ldots) \).

![Figure 1. Manhattan plot representation of the analysis of carotid intima-media thickness values as the cardiovascular disease outcome. The \(-\log_{10}P\) values are plotted against their physical chromosomal position. The red line represents the genome-wide level of significance \( (P < 5 \times 10^{-8}) \).](https://example.com/figure1.png)
Table 2. Index signals showing the lowest \( P \) values according to the different CV disease outcomes*

| CV disease outcome, Chr. | Position in Chr. (GRCh37) | SNP ID         | GENCODE gene | Change       | Minor allele | MAF     | \( P \)            | \( \beta \) [SE] or OR (95% CI) |
|-------------------------|---------------------------|----------------|--------------|--------------|--------------|---------|-------------------|---------------------------------|
| **CIMT values**         |                           |                |              |              |              |         |                   |                                 |
| 3                       | 25.638.355                | rs116199914    | RARB (3′-UTR) | G<\(\alpha\)  | G            | 0.012   | \(1.86 \times 10^{-8}\)† | 0.142 [0.025]                   |
| 3                       | 25.622.694                | rs77388418     | RARB (intronic) | C<\(\epsilon\)T | C            | 0.014   | \(2.07 \times 10^{-7}\) | 0.124 [0.024]                   |
| 12                      | 63.337.536                | rs1695024      | PRDM10 (intronic) | A<\(\epsilon\)G | A            | 0.230   | \(2.64 \times 10^{-7}\) | 0.031 [0.006]                   |
| 11                      | 129.852.180               | rs111703287    | LMNA (intronic) | T<\(\epsilon\)C | T            | 0.014   | \(3.90 \times 10^{-7}\) | 0.119 [0.023]                   |
| **CV events**           |                           |                |              |              |              |         |                   |                                 |
| 1                       | 166.485.891               | rs6684311      | 27 kb 3′ of RP11-276E17.2 | G<\(\epsilon\)C | G            | 0.189   | \(2.85 \times 10^{-7}\) | 1.68 (1.38–2.05)               |
| **IHD**                 |                           |                |              |              |              |         |                   |                                 |
| 1                       | 245.338.976               | rs112844193    | KIF26B (intronic) | T<\(\epsilon\)C | T            | 0.054   | \(1.35 \times 10^{-7}\) | 2.67 (1.85–3.85)               |
| 7                       | 120.966.790               | rs3779381      | WNT16 (intronic) | G<\(\epsilon\)A | G            | 0.283   | \(2.09 \times 10^{-7}\) | 1.77 (1.23–2.19)               |
| 1                       | 156.057.417               | rs112941217    | LMNA (intronic) | C<\(\epsilon\)T | C            | 0.030   | \(4.67 \times 10^{-7}\) | 4.81 (2.61–8.87)               |
| **Carotid plaques**     |                           |                |              |              |              |         |                   |                                 |
| 17                      | 15.008.430                | rs8066891      | 123 bp 3′ of RP11-924A14.1 | G<\(\epsilon\)A | G            | 0.171   | \(4.47 \times 10^{-6}\) | 0.58 (0.46–0.73)               |
| 9                       | 29.148.449                | rs12683261     | 259 bp 5′ of MIR873 | A<\(\epsilon\)G | A            | 0.031   | \(4.57 \times 10^{-6}\) | 0.25 (0.14–0.45)               |
| 1                       | 240.599.906               | rs9727451      | FN2 (intronic) | A<\(\epsilon\)G | A            | 0.087   | \(4.69 \times 10^{-6}\) | 2.13 (1.54–2.95)               |
| 4                       | 166.579.647               | rs2611206      | 26 kb 5′ of RP11-340B18.1 | A<\(\epsilon\)G | A            | 0.126   | \(4.84 \times 10^{-6}\) | 0.53 (0.41–0.69)               |

* CV = cardiovascular; Chr. = chromosome; SNP = single-nucleotide polymorphism; MAF = minor allele frequency; OR = odds ratio; 95% CI = 95% confidence interval; CIMT = carotid intima-media thickness; 3′-UTR = 3′-untranslated region; IHD = ischemic heart disease.
† Statistically significant at the genome-wide level of significance.
of North and Western European ancestry, British in England and Scotland, Toscani in Italy, and Finnish in Finland). Since no proxies were identified, we functionally annotated just the rs116199914 polymorphism. As this SNP is located in the 3′-UTR of the RARB gene, we used bioinformatic tools aimed at exploring annotations of the noncoding genome with putative regulatory effects on gene expression (including effect on regulatory motifs, chromatin state, and protein binding, as well as expression from expression quantitative trait locus studies) in GEO, ENCODE, Roadmap Epigenomics, and promoter CHi-C data sets, and published literature.

Interestingly, RegulomeDB results suggested that rs116199914 may represent a DNA element with relevant regulatory effects (score 6). Additional functional implications were suggested with both HaploReg version 4.1. and CHi-C. In particular, overlapping with histone marks in tissues related to CV pathophysiology and cells of the immune system was observed (Figure 2). Specifically, rs116199914 was described to overlap with the enhancer histone mark H3K4me1 and the promoter histone mark H3K9ac in fetal heart, and with histone marks enriched at promoters and enhancers in immune cells (23). Furthermore, as derived from the CHi-C data sets, rs116199914 was reported to interact with, among others, NF-κB inhibitor–interacting Ras-like 1 gene (NKIRAS1) in total CD4 Mycosis fungoides cells and total CD8 cells (35) (Supplementary Figure 2, on the Arthritis & Rheumatology web site at http://onlinelibrary.wiley.com/doi/10.1002/art.40734/abstract). In addition, rs116199914 was described to affect the sequence-specific binding for NFAT (23).

Candidate genes and pathway analysis. We also determined the statistical significance, in our GWAS, of previously described CV risk genes by candidate studies (8) and a recently published meta-analysis of ImmunoChip data (25). P values of <0.05 were observed across most of the evaluated loci (Supplementary Table 5, http://onlinelibrary.wiley.com/doi/10.1002/art.40734/abstract). Among them, the lowest P values were detected for associations of the NFkB1 and methionine sulfoxide reductase A gene (MSRA) regions with the presence of CV events \( P = 8.12 \times 10^{-4} \) and \( P = 5.94 \times 10^{-4} \), respectively, as well as the zinc-finger C3HC-type containing 1 gene (ZC3HC1) region with CIMT values \( P = 2.46 \times 10^{-4} \). The association between NFKB1 and CV events remained statistically significant after correction for multiple testing (rs227361, false discovery rate–adjusted \( P = 4.50 \times 10^{-2} \)). Regarding the HLA system, no statistically significant results were observed across this genomic region (Supplementary Figure 3, http://onlinelibrary.wiley.com/doi/10.1002/art.40734/abstract).

In addition, analysis of possible biologic pathway enrichments and predictive protein–protein relationships was performed for the gene products of loci that showed \( P \) values of potential relevance in our study \( (P < 1 \times 10^{-3}) \). In this regard, the molecular network of the selected proteins related to the presence/absence of carotid plaques had significantly more interactions than expected (number of nodes 51, number of edges 8, average node degree 0.314, clustering coefficient 0.235; expected number of edges 3, protein–protein interaction enrichment \( P = 1.68 \times 10^{-2} \)) (Figure 3). In accordance with the functional enrichments of the network, the most significantly associated GO term corresponded to "collagen biosynthetic process" (GO number 0032964) (false discovery rate–adjusted \( P = 4.01 \times 10^{-3} \)). No statistically significant results were obtained when these analyses were performed according to CIMT values, presence/absence of CV events, or IHD.

### DISCUSSION

During the last decade, the genetic basis of the increased predisposition to CV disease observed in RA patients has been comprehensively investigated using a candidate gene strategy (8). However, not until the present study have GWAS data been generated and analyzed. Therefore, the results presented here may represent a turning point for better understanding of the pathogenic mechanisms underlying this severe complication of RA.

A genetic marker of the RARB gene (rs116199914) was associated, at the genome-wide level of significance, with...
subclinical atherosclerosis, assessed by CIMT. Interestingly, this signal overlaps with promoter and enhancer histone marks in fetal heart and immune cells. In addition, rs116199914 has been described to interact with the gene NKIRAS1. These data are striking, as NKIRAS1 encodes a crucial protein for the inhibition of NF-κB (36,37), which is one of the most relevant molecules involved in inflammation processes (38) and is considered to be a key regulator of several atherosclerosis genes (39). A previous candidate gene study demonstrated the influence of a promoter genetic variant in the NF-κB coding gene (NFKB1) on the risk of developing CV events among patients with RA (39). Additionally, the use of drugs that block cytokines of the NF-κB signaling pathway has been described as a promising therapeutic strategy to attenuate the heightened CV risk in patients with RA (40,41) and to provide a beneficial effect on surrogate CV disease markers in those patients (42,43).

In addition, the associated variant identified in our study was shown to affect sequence-specific binding of NFAT, which regulates inducible gene transcription during the immune response (44–46). Originally, NFAT was described as being mainly expressed in activated T cells (46) and other immune cells (45). Currently, its regulatory roles in blood vessels and heart tissue are well established (47–49). Furthermore, a role of this molecule in angiogenic processes has been confirmed (48). Consistent with this, cumulative knowledge clearly demonstrates that the chronic inflammation observed in patients with RA, critical for the development of atherosclerosis, is often accompanied by imbalanced angiogenesis (50). In accordance with that, increased serum levels of the angiogenic molecule angiopoietin 2 have been found to correlate with the development of CV events in patients with RA (50).

Our results suggest a functional impact of the genetic variant RARB rs116199914. In this regard, it could be speculated that the interaction between this polymorphism and NKIRAS1 modulates the expression of the latter, affecting the inhibition of NF-κB. This may trigger the regulation of genes encoding proinflammatory cytokines, adhesion molecules, chemokines, and inducible nitric oxide synthase, thus contributing to endothelial damage and subsequently to CV disease. Similarly, since RARB rs116199914 has been described to affect the sequence-specific binding of NFAT as noted above, it may be reasonable to consider that this phenomenon modulates the expression of genes related to angiogenic processes in atherosclerosis.

Additional suggestive signals of potential relevance were observed when both the presence/absence of CV events (including IHD) and subclinical atherosclerosis were tested.
However, those signals did not reach the genome-wide level of significance, probably due to insufficient statistical power to detect risk variants with low-to-moderate effects. Biologic pathway enrichment and protein–protein interaction analyses revealed a functional enrichment of the collagen biosynthesis network according to the presence/absence of carotid plaques. This result is consistent with the fact that collagen constitutes the main component of the fibrous cap of the carotid plaque and contributes to its structural integrity and vulnerability (51). Indeed, a recent Metabochip analysis performed in American patients with RA revealed a suggestive association between a genetic variant in the Col1(IV) gene (\(\alpha\)) patients with RA revealed a suggestive association between a genetic variant in the Col1(IV) gene (\(\alpha\)) patients with RA revealed a suggestive association between a genetic variant in the Col1(IV) gene (\(\alpha\)) patients with RA revealed a suggestive association between a genetic variant in the Col1(IV) gene (\(\alpha\)) patients with RA revealed a suggestive association between a genetic variant in the Col1(IV) gene (\(\alpha\)) and contributes to its structural integrity and vulnerability (51).

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Finally, our results support the implication of the previously reported candidate CV risk gene \(NFKB1\) and suggest a potential influence of both \(MSRA\) and \(ZC3HC1\) in the development of CV disease in RA. In contrast, a relevant influence of the HLA region in this process, though suggested previously by others (8), was not supported by our data.

There is evidence that current CV risk screening and management strategies underestimate the actual degree of predisposition to CV in patients with RA. In this context, genetic markers related to the development of CV disease in patients with RA may be used as additional tools to identify those patients at high CV risk, who may definitively benefit from active therapy to prevent CV events. Accordingly, the results of our study may help in the design of efficient tools to identify RA patients who are more likely to develop CV disease based on their genetic background.

A potential major limitation of the present study is the lack of replication of the discovery findings in an independent cohort of patients with RA. In addition, the study could have been underpowered to detect associations with small effect size. Further investigations to confirm our results are needed. Interestingly, Karpouzas et al reported that the frequency of unstable, noncalcified plaques is increased among patients with RA (53). Unstable plaques are very dangerous since they are particularly susceptible to disruption. Vulnerable plaques are generally characterized as those having a thin inflamed fibrous cap over a very large lipid core. Since the conventional carotid ultrasound technique performed in our study did not allow us to identify the presence of unstable plaques, we believe further investigations aimed at identifying a potential role of the genetic variant \(RARB\) rs116199914 in the risk of unstable plaques should be conducted.

In conclusion, through a whole-genome screening of common genetic variation, we have identified \(RARB\) rs116199914 as the main genetic variant associated with CIMT values in patients with RA. This finding could potentially lead to an improved ability to predict and screen for this condition and initiate treatment to prevent life-threatening CV events in RA patients.

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Corrigendum

In the article by Chong et al in the September 2013 issue of *Arthritis & Rheumatism* (now *Arthritis & Rheumatology*) (Fibroblast Growth Factor 2 Drives Changes in Gene Expression Following Injury to Murine Cartilage In Vitro and In Vivo) [page 2346–2355], there was an error in Figure 1B (lower row: Midzone): the same image appears for “Control (24h)” and “Control (0 mins).” This did not affect the study results or conclusions reported in the article. A corrected Figure 1B is shown below.

The authors regret the error.