Interferon Regulatory Factor 5 (IRF5) Gene Variant rs2004640 is Associated with Carotid Intima Media Thickness in Rheumatoid Arthritis Patients

S Vosslerber, A van Sijl, C.L. Bos, M Peters, A.E. Voskuyl, M.T. Nurmohamed*, C.L. Verweij*

Submitted

*Dual senior authorship
ABSTRACT

INTRODUCTION
Rheumatoid arthritis (RA) is a chronic inflammatory joint disease and is associated with increased cardiovascular (CV) risk. Interferons (IFNs), especially IFNβ, might play a role in atherosclerosis as they are known inhibitors of vascular smooth muscle cell proliferation and intimal hyperplasia. We studied whether functional relevant SNPs in the interferon regulatory factor 5 (IRF5) gene are associated with carotic intima media thickness (cIMT), a surrogate maker for CV disease.

METHODS
In 353 RA patients of the CARRÉ study, IRF5 SNPs rs2004640 and rs4728142 were determined using Taqman Genotyping assay. cIMT was determined in 101 patients by B-mode ultrasonography. Linear regression analyses were used to investigate the association between cIMT and IRF5 genotypes, adjusted for demographic and cardiovascular risk factors.

RESULTS
Patients homozygous for rs2004640 G-allele had higher cIMT compared to those homozygote for the T-allele (p=0.019) and a trend towards higher cIMT was observed (p=0.103) for patients homozygous for the rs4728142 G-allele versus patients with the AA-genotype. Age was an effect-modifier for this association. Linear regression analysis in patients older than 60 years showed that the rs2004640 GG-genotype was associated with higher cIMT (regression coefficient 0.107 (C.I. 0.008; 0.205), p=0.035) compared to the TT-genotype. This remained significant after adjustment for traditional risk factors (regression coefficient 0.111 (C.I. 0.02; 0.202), p=0.020).

CONCLUSIONS
We demonstrate that IRF5 gene variant rs2004640 is associated with preclinical atherosclerosis in RA patients, independent of traditional cardiovascular risk factors. These results might implicate a role for type I IFN in modulating CV disease features in RA.
INTRODUCTION

Rheumatoid arthritis (RA), an inflammatory joint disease, is associated with increased cardiovascular (CV) morbidity and mortality\(^1\)\(^-\)\(^3\), which is not fully explained by traditional CV risk factors\(^4\).

The increased systemic inflammatory state seen in RA patients predisposes them to atherosclerotic disease, but the exact mechanism remains unknown\(^5\)\(^,\)\(^6\). Hence, the search for additional mechanisms linking RA to CV disease is relevant.

An increased inflammatory state (presented by increased immune cell activation and overproduction of inflammatory cytokines) and the reduced presence of endothelial progenitor cells (which can repair endothelial damage) lead to endothelial dysfunction and vascular injury\(^7\). In response to vascular injury, vascular smooth muscle cell proliferation and intimal hyperplasia are induced which sets the atherosclerotic process in motion.

Interferons (IFNs) are known inhibitors of proliferation, especially IFN\(\beta\) has a vascular protective role\(^8\)\(^-\)\(^12\).

Recently, genetic association studies on components of the IFN signaling cascade revealed an association of the \textit{IRF5} gene in complex autoimmune diseases such as SLE\(^{13\text{-}16}\), RA\(^{17,18}\) and MS.\(^{19}\) IRF5 is a member of a family of transcription factors that controls inflammatory and immune responses\(^20\). SNP rs2004640 alters a consensus splice donor site and allows expression of isoforms bearing an alternative exon 1 (exon 1B).\(^{15}\) SNP rs4728142 is positioned in the promotor region of IRF5. Both the IRF5 rs2004640 and rs4728142 SNPs affect IRF5 gene expression and may lead to differences in IFN production and therefore might play a role in the atherogenic process in RA patients. In the current study, we investigate the effect of \textit{IRF5} gene variants on atherosclerosis related clinical parameters and carotid intima media thickness (cIMT) in RA patients.

PATIENTS AND METHODS

PATIENTS

The CARRÉ study is a prospective cohort study investigating CV disease and its risk factors in RA patients\(^21\). In 2000, a random sample of 353 RA patients registered at the Jan van Breemen Research Institute | Reade in Amsterdam, the Netherlands, was drawn. Patients fulfilled the 1987 American College of Rheumatology classification criteria for RA, and were aged between 50 and 75 years\(^22\). An ultrasound study of the carotid artery was performed in 2001 in a randomly selected sub-group of 101 patients. The local ethics committees and institutional review board of the VU University Medical Center in Amsterdam, the Netherlands, approved the study protocol and all participants gave their written informed
consent for the study in accordance to the declaration of Helsinki.

**DNA EXTRACTION AND GENOTYPING**

Total DNA was extracted from EDTA blood from 353 RA patients using Qiagen’s DNeasy blood & tissue kit (Qiagen) according to the manufacturers’ instructions.

The IRF5 gene variants rs2004640 and rs4728142 were genotyped using the TaqMan SNP Genotyping Assay (Applied Biosystems, CA) according to the manufacturer’s protocol. Allelic discrimination was performed using an ABI Prism 7900HT Sequence Detection System.

**CV RISK FACTORS AND RA RELATED FACTORS**

CV disease history was based on the participant’s medical records, obtained from the participant’s general practitioner or hospital. CV disease consisted of coronary, cerebral or peripheral arterial disease. Prior CV disease was determined according to the methods described by Peters et al. Assessment of CV risk factors was done according to similar standard operation procedures. Body mass index was calculated as the ratio of weight and squared height. Hypertension was defined as a systolic blood pressure (SBP) over 140 mmHg and/or a diastolic blood pressure (DBP) over 90 mmHg and/or the current use of antihypertensive medication. Triglycerides, total cholesterol and high-density lipoprotein (HDLc) cholesterol were determined from fasting blood samples by enzymatic techniques as previously described.

Physical examination was performed to determine the Disease Activity Score in 28 joints (DAS28). Blood samples were drawn for inflammatory variables (i.e., erythrocyte sedimentation rate [ESR] and C-reactive protein [CRP] levels) and IgM rheumatoid factor (IgM-RF) antibodies as described previously. Functional (disability) status was assessed with the Health Assessment Questionnaire, and the presence or absence of erosions on radiographs of hands and feet were recorded (yes/no). Additional data regarding health status, medical history and medication use were assessed by questionnaires.

**CAROTID INTIMA MEDIA THICKNESS (CIMT) MEASUREMENT.**

Measurements were performed with a 7.5-MHz linear probe, connected to a computer equipped with vessel wall movement detection software and an acquisition system (Wall track system, Pie Medical) that enables measurement of the CIMT. After localization of the common carotid artery, cross-sectional measurements were performed 10 mm proximal of the carotid bulb. Sites with mural atherosclerotic plaques were avoided due to difficulty in identifying carotid arterial variables in these regions. The distance between the lumen-intima interface and the leading edge of the media-adventitia interface of the far wall corresponds with CIMT. Measurements of IMT were ECG triggered to the R-peak of the cardiac cycle.
STATISTICAL ANALYSIS

Patients were grouped according to their SNP rs2004640 or rs4728142 allele distribution. Differences in demographics, CV- and RA-related factors between allele groups and genotypes were analyzed using Students t test, Chi square tests and Mann-Whitney-U tests, where appropriate. Linear regression analyses were used to investigate the association between IRF5 alleles / genotypes and cIMT. Associations were adjusted for demographic factors, CV- and RA-related factors on the basis of the literature and their effect on the original estimate of the exposure effect. Statistical analyses were performed using IBM-SPSS version 20.0. P values < 0.05 were considered significant.

RESULTS

PATIENT CHARACTERISTICS

The 353 RA patients from the CARRE cohort were genotyped for IRF5 SNPs rs2004640 and rs4728142. From 101 of these RA patients cIMT was determined. This cohort is referred to as the ‘cIMT cohort’. Genotyping failed in eight patients of the CARRÉ cohort, of which one patients was in the cIMT cohort. The atherosclerosis and RA related clinical characteristics are shown in Table 1. No relevant differences were noted between both cohorts. Allele frequencies of IRF5 rs2004640 and rs4728142 are also shown in table 1.

IRF5 GENE VARIANTS AND ATHEROSCLEROSIS RELATED CLINICAL PARAMETERS IN RA PATIENTS

Firstly, we tested whether IRF5 SNPs rs2004640 or rs4728142 were associated with clinical parameters relevant for atherosclerosis in RA. No significant associations between IRF5 alleles or genotypes and HDL, LDL, systolic blood pressure, diastolic blood pressure, cholesterol, BMI, smoking or prevalent CV disease were observed.

Secondly, we tested whether IRF5 gene variants were associated with RA related clinical parameters (RA duration, DAS28, CRP, presence of erosions and presence of RF). A higher percentage of patients homozygous for the rs2004640 G-allele were positive for RF compared to patients carrying the T-allele (82% versus 68%, p=0.016). No significant associations were observed between IRF5 alleles or genotypes and other RA related characteristics.

ALLELIC DISTRIBUTION OF IRF5 GENE VARIANTS AND CIMT LEVELS IN RA PATIENTS

When comparing cIMT between RA patients with different IRF5 genotypes, it appeared that there was an allele-dose effect for the rs2004640 genotypes. cIMT was lower in patients with a TT-genotype and a gradual increase in cIMT was observed in patients with the GT- and GG-genotype respectively. A significant difference in cIMT between RA patients
homozygous for the rs2004640 T-allele compared to those homozygote for the G-allele was observed (p=0.0191). (Figure 1). Age was an effect-modifier for these associations and the strongest association between cIMT and rs2004640 was seen in patients older than 60 (n=60). Linear regression analysis in these patients showed that the rs2004640 GG-genotype was associated with higher cIMT (regression coefficient 0.107 (C.I. 0.008; 0.205), p=0.035) which remained significant after adjustment for demographic and cardiovascular risk factors (sex, cholesterol, blood pressure and smoking) (regression coefficient 0.111 (C.I. 0.02; 0.202), p=0.020). Adjustment for positivity for RF, which was shown to be related to rs2004640 genotype, did not alter this association (regression coefficient 0.106 (C.I. 0.007; 0.206), p=0.037). Although significance was not reached for rs4728142, a comparable trend towards an association between the rs4728142 GG-genotype and enhanced cIMT levels in RA patients was observed (regression coefficient 0.071 (C.I. 0.031; 0.173), p=0.164). Since rs2004640 and rs4728142 are in strong linkage disequilibrium, linear regression was also

Table 1. Baseline characteristics

<table>
<thead>
<tr>
<th></th>
<th>CARRE cohort (n=353)</th>
<th>cIMT cohort (n = 101)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Demographics</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age, yrs</td>
<td>63 ± 8</td>
<td>62 ± 7</td>
</tr>
<tr>
<td>female, %</td>
<td>66</td>
<td>60</td>
</tr>
<tr>
<td><strong>RA characteristics</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RA duration, yrs</td>
<td>7 (4-10)</td>
<td>7 (4-10)</td>
</tr>
<tr>
<td>DAS28-score</td>
<td>3.9 ±1.4</td>
<td>3.5 ±1.2</td>
</tr>
<tr>
<td>ESR, mm/hr</td>
<td>18 (9-31)</td>
<td>14 (8-30)</td>
</tr>
<tr>
<td>CRP, mg/l</td>
<td>7 (3-18)</td>
<td>6 (3-15)</td>
</tr>
<tr>
<td>IgM RF positive, %</td>
<td>72</td>
<td>72</td>
</tr>
<tr>
<td>Erosive diseases, %</td>
<td>82</td>
<td>82</td>
</tr>
<tr>
<td><strong>Medication</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypertension med use, %</td>
<td>26</td>
<td>25</td>
</tr>
<tr>
<td>Statin use, %</td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td><strong>Atherosclerosis characteristics</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cholesterol, mmol/L</td>
<td>5.6 (5.0-6.5)</td>
<td>5.6 (5.0-6.5)</td>
</tr>
<tr>
<td>LDL, mmol/L</td>
<td>3.6 (3.1-4.4)</td>
<td>3.5 (3.0-4.4)</td>
</tr>
<tr>
<td>HDL, mmol/L</td>
<td>1.4 (1.1-1.7)</td>
<td>1.5 (1.1-1.7)</td>
</tr>
<tr>
<td>Triglycerids, mmol/L</td>
<td>1.3 (1.0-1.8)</td>
<td>1.3 (1.0-1.8)</td>
</tr>
<tr>
<td>Body Mass Index</td>
<td>26 (24-29)</td>
<td>26 (23-28)</td>
</tr>
<tr>
<td>Systolic blood pressure, mmHg</td>
<td>140 (130-155)</td>
<td>140 (130-151)</td>
</tr>
<tr>
<td>Diastolic blood pressure, mmHg</td>
<td>80 (75-85)</td>
<td>85 (75-85)</td>
</tr>
<tr>
<td><strong>IRFs minor allele frequencies</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs2004640 (G)</td>
<td>0.481</td>
<td>0.445</td>
</tr>
<tr>
<td>rs4728142 (A)</td>
<td>0.418</td>
<td>0.419</td>
</tr>
</tbody>
</table>

DAS: Disease activity Score; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; RF: rheumatoid factor; LDL: Low-density lipoprotein; HDL: High-density lipoprotein. Continuous variables are presented as mean ± standard deviation, median (interquartile range) or percentages where appropriate.
Genetic Variation in IRF5 is Associated with cIMT in Rheumatoid Arthritis

3.1 performed in patients with the rs2004640 GG and rs4728142 GG genotype versus patients carrying both the rs2004640 TT and rs4728142 AA genotype, respectively. A trend towards a significant association between the double genotypes and cIMT was observed (regression coefficient 0.105 (C.I. 0.012; 0.222), p=0.076).

Discussion

We demonstrated for the first time that the IRF5 rs2004640 G-allele is related to higher cIMT in RA, particularly in patients older than 60 years.

The IRF5 rs2004640 gene variants may be involved in the atherosclerotic process in rheumatoid arthritis in different ways. IRF5 is a master regulator of type I IFN-activity and functions as a transcription factor when phosphorylated, leading to expression of downstream interferon response genes as well as production of type I IFN itself. The rs2004640 IRF5 SNP is located 2bp near the intron-exon boundary for exon 1b and creates an exon donor splice site which enables transcription of exon 1b. Splicing of IRF5 is highly complex and multiple IRF5 isoforms are initiated at each promoter. Different isoforms can contain either exon 1a, 1b or 1c, depending on the promoter where transcription is initiated. The IRF5 rs2004640 T allele, the allele that enables transcription of exon 1b, is associated with higher mRNA levels of IRF5. Furthermore, it is known that the IRF5 isoforms differ in their ability to transactivate type I IFN genes, e.g. IFNα or IFNβ. Thus the IRF5 rs2004640 T-allele is likely to enhance the expression of IRF5 and successively type I IFN, including IFNβ. IFNβ is well known for its protective role in vascular diseases. Zhang et al showed that IFNβ can play a prominent anti-atherosclerosis,
anti-inflammation, and anti-proliferation role by reducing angiotensin II-accelerated increase in vascular smooth muscle cell proliferation and intimal hyperplasia. However, it also has been described that IFNβ accelerates lesion formation in atherosclerotic mouse models.\textsuperscript{29} In our study, we focused on the relation of \textit{IRF5} and carotid intima media thickness (cIMT), which is an early marker for atherosclerosis that does not necessarily reflect plaque formation.\textsuperscript{30} In the present study, the association between rs2004640 G-allele and cIMT implicates that this \textit{IRF5} gene variant is associated with protection against atherosclerosis, most likely via decreased production of IFNβ. Influence of IFNβ on IMT may thus especially be effective in situations with high angiotensin activity, because high angiotensin-converting enzyme activity is associated with IMT. It seems relevant to further explore the role of \textit{IRF5} genetics and IFNβ production in RA patients in relation to a larger panel of cardiovascular disease-related parameters, including cIMT and plaque formation.

Notably, for the \textit{IRF5} rs4728142 SNP, only a trend towards an association with cIMT was found. Rs4728142, which is located in the promoter region of \textit{IRF5}, was also associated with autoimmune diseases such as SLE and MS.\textsuperscript{14,19} Although the relation to \textit{IRF5} mRNA levels was not studied yet, an increased binding of SP1 to the A allele of rs4728142 was observed. Our results suggest that the effect of rs4728142 on activation of the type I IFN pathway is less prominent than the effect of rs2004640 but further functional studies are needed to fully support this observation.

Our data showed that age is an effect modifier for the association between \textit{IRF5} gene variants and cIMT. We therefore stratified the results in patients above and below the age of 60. As a consequence, the absence of an inhibitory effect of the \textit{IRF5} rs2004640 G allele was only observed in this group of patients. After adjustment for confounders the association remained statistically significant. Furthermore, we observed that RF positivity was associated with the rs2004640 TT-genotype. CV disease is more prominent in seropositive RA, and the percentage of patients that are RF positive is higher in the patient group with the rs2004640-GG genotype. This indicates that there might be a link (either direct or indirect) between seropositivity and type I IFN activity, possibly via \textit{IRF5} genetics. This is in line with previous studies that showed that the association between \textit{IRF5} genetics is stronger in seronegative RA. Unfortunately, this study was not powered to study the relationship between \textit{IRF5} genetics and cIMT in seronegative versus seropositive RA, but it would be highly relevant to further investigate the relationship between seropositivity, cardiovascular disease and type I IFNs in RA.

Altogether, these finding implicate an important role of the \textit{IRF5} transcription pathway in atherosclerosis in patients with RA. Further studies are needed to demonstrate whether this is mediated via modulation of IFNβ production or through a different process. Furthermore, it might be interesting to investigate whether \textit{IRF5} gene variants influence atherosclerosis in non-RA patients.
CONCLUSIONS

Our data show an association between IRF5 and cIMT, an established marker for future atherosclerotic disease. Further appreciation and delineation of the precise mechanism on how IRF5 and possibly IFNβ attenuate CV risk in RA is important for clinical practice and future therapies.

ACKNOWLEDGEMENTS

This work was partly supported by European Community Sixth Framework Programmes Autorome (From Immune Responses in Rare Autoimmune Diseases to novel Therapeutic Intervention Strategies—a personalized Medicine approach) and Autocure (Curing autoimmune diseases) and the Dutch Centre for Medical Systems Biology (CMSB). These sponsors had no involvement in the study design, analysis or interpretation of the data and publications.
REFERENCES


