

demia is present years before arthritis develops, which cannot be explained by inflammation itself^{9,10}.

The variation in the production capacity of cytokines is in part genetically determined. TNF and LTA genes are located within the MHC III region of chromosome 6, in close linkage to the class II genes. LTA gene polymorphisms have been associated with variations in the transcriptional activity of LTA¹¹ and in circulating concentrations of C-reactive protein (CRP)¹² and TNF¹³. Additionally, a relationship between the GG genotype in the LTA single-nucleotide polymorphism (SNP) at position 252 and hypertriglyceridemia and decreased high-density lipoprotein (HDL) cholesterol was reported in Korean men¹⁴. Moreover, a recent larger study showed the LTA 252 variant allele to be more frequent in patients with RA than in healthy controls and to be associated with a tripled risk of myocardial infarction¹⁵.

If genetic variants of LTA are important determinants of inflammation and also of dyslipidemia, this could have important implications for understanding the link between these 2 conditions in RA. For this reason we examined the role of the potentially functional LTA 252 A>G polymorphism in RA and whether this polymorphism is related to the severity of the disease and to dyslipidemia, in a nested case-control study. We also attempted to correlate this SNP with serum levels of proinflammatory mediators and fasting lipids.

MATERIALS AND METHODS

Study sample. Consecutive white patients attending the rheumatology outpatient clinics of Hospital Santa Maria, Lisbon, and Hospital Garcia de Orta, Almada, Portugal, on a regular basis and satisfying the 1987 American College of Rheumatology criteria for RA¹⁶ were enrolled in this nested case-control study from a large cohort of patients with RA. Exclusion criteria were ancestry other than white, pregnancy, and breastfeeding. The control group consisted of unrelated healthy white volunteers. Eligibility criteria for controls were the same as for cases, except that controls must not have been diagnosed with RA or other inflammatory disease.

All patients underwent a clinical and laboratory evaluation and the following information was collected: age, sex, body mass index (BMI), age at RA diagnosis, positivity for rheumatoid factor, extraarticular manifestations (rheumatoid nodules, interstitial lung disease, pericardial or pleural effusion, Sjögren's syndrome, vasculitis, and amyloidosis), previous orthopedic surgeries due to RA (including total joint replacement or arthrodesis), current and past medications, comorbidities, and smoking habits. RA disease characteristics and comorbidities were validated by medical records review. Erythrocyte sedimentation rate (ESR) was measured, 28 joints were examined for tenderness and swelling, and the Disease Activity Score (DAS28) ESR¹⁷ was calculated. Functional status was evaluated using the Stanford Health Assessment Questionnaire Disability Index (HAQ)¹⁸. Recent (< 6 months) plain radiographs of hands and feet were reviewed for the presence of erosions. A venous blood sample was collected into EDTA-containing tubes and preserved at -80°C until DNA extraction. An additional blood sample was obtained from 204 participants for measurement of fasting lipid profile, CRP, LTA, TNF, soluble TNF receptor I (sTNFR I), and interleukin 6 (IL-6). Since men and women have different frequency distribution of plasma lipids, this confirmatory group comprised women only; the sample size has a 95% power to detect a difference > 10% in lipid level at a significance level of 0.05.

Three outcomes were defined as surrogate markers of RA severity: (1) the presence of erosions; (2) extraarticular manifestations; and (3) HAQ score > 1. Dyslipidemia was defined as total cholesterol \geq 200 mg/dl or low-density lipoprotein (LDL) cholesterol \geq 130 mg/dl or high-density lipoprotein (HDL) cholesterol < 50 mg/dl for women or < 40 mg/dl for men or triglycerides \geq 150 mg/dl, or the use of lipid-lowering agents.

The protocol was approved by the local Ethics Committee and written informed consent was obtained from all participants.

DNA extraction. DNA was extracted from whole blood using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany), according to manufacturer's instructions.

Genotyping. The LTA 252 A/G polymorphisms were determined by restriction fragment length polymorphism (RFLP) analysis, using the forward primer 5'- CCG TGC TTC GTG CTT TGG ACT A -3' and the reverse primer 5'- AGA GCT GGT GGG GAC ATG TCT G -3' (Invitrogen, San Diego, CA, USA).

The polymerase chain reaction was performed in a 20- μ l reaction mixture containing 100 ng genomic DNA, 40 μ M dNTP (Invitrogen), 2 mM MgCl₂ (Finnzymes, Espoo, Finland), 0.4 μ M of each primer, and 0.02 U/ μ l of the enzyme Phusion™ DNA Polymerase (Finnzymes). The cycle conditions were denaturation of the template DNA in a cycle of 98°C during 30 s; amplification of target DNA for 35 cycles of 98°C for 10 s, 71°C for 10 s, and 72°C for 12 s; and final extension in a cycle of 72°C for 7 min.

The amplified products [741 base pairs (bp)] were digested at 37°C with NcoI enzyme (New England Biolabs, Hitchin, UK) and evaluated in 2% agarose gels. Fragments with 545 and 196 bp were defined as G/G genotype; 741, 545, and 196 bp as A/G genotype; and undigested fragments with 741 bp as A/A genotype. Genotyping was repeated in 10% of the samples for purposes of quality control.

Cytokine measurement. The cytokines LTA, TNF, sTNFR I, and IL-6 were analyzed using the Bender MedSystems (Vienna, Austria) bead-based assay for quantitative detection of soluble human analytes by flow cytometry. The protocol was performed according to the manufacturer's instructions.

Statistical analysis. Continuous variables are presented as means \pm SD or medians and 25th to 75th percentiles, depending on whether the data were normally distributed. Categorical variables are reported as absolute values and proportions.

Consistency of genotype frequencies with the Hardy-Weinberg equilibrium was examined using a chi-squared goodness-of-fit test on a contingency table of observed compared with expected genotype frequencies. Power was calculated under the log-additive model at 2-sided α = 0.05, using Quanto version 1.2.4¹⁹.

Association of LTA 252 A>G alleles and genotypes with the diagnosis of RA was analyzed by chi-squared tests. Within RA we assessed the independent relationship between genotypes and disease severity and dyslipidemia using logistic regression. If a significant association was identified, logistic regression analysis was also used to adjust for confounders. Student's t test or the Mann-Whitney U test was used to compare levels of lipids and inflammatory mediators between cases and controls and 1-way ANOVA and Kruskal-Wallis tests were used to assess differences among genotypes, as appropriate.

Statistical analysis was carried out using SPSS version 17.0 for Windows software (SPSS Inc., Chicago, IL, USA), and a 2-tailed p value < 0.05 was considered significant.

RESULTS

Association of LTA 252 polymorphism with RA diagnosis. The study group consisted of 657 white participants, 388 patients with RA and 269 unrelated healthy controls. Women were 88.7% of cases and 90.3% of controls. The genotype frequency of the LTA 252 A>G polymorphism was in agreement with that predicted by the Hardy-Weinberg

equation in both the RA ($p = 0.30$) and the control population ($p = 0.12$). The distribution of genotypes and alleles in patients and controls is shown in Table 1.

In RA we identified 50.8% A/A homozygotes, 39.4% A/G heterozygotes, and 9.8% G/G homozygotes, resulting in allele frequencies of 70.5% and 29.5% for A and G alleles, respectively. The common allele frequency was significantly higher in RA ($p = 0.018$) and the A/A genotype was associated with an increased likelihood of having RA (OR 1.772, 95% CI 1.076–2.921, $p = 0.02$). After correction for age and sex, this association remained statistically significant (OR 2.293, 95% CI 1.226–4.089, $p = 0.009$). The sample size had an 80% power to detect a size-effect ≥ 1.4 under a log-additive genetic model at a 0.05 significance level for a risk allele frequency of 64%.

LTA 252 genotypes and RA characteristics. Characteristics of RA subjects are presented in Table 2. Patients carrying the

LTA G/G genotype were younger ($p = 0.001$) and also younger at onset of RA ($p < 0.0001$) than those carrying A/A or A/G genotypes, regardless of similar disease duration. Fewer patients with the A/A genotype were smokers ($p = 0.02$), otherwise the 3 groups presented similar median disease activity, proportion of rheumatoid factor positivity, and joint surgeries, as well as therapeutic options (use of corticosteroids, methotrexate, or biological agents). The prevalence of comorbid conditions did not differ across LTA genotypes.

The severity of RA was analyzed for the presence of erosions, extraarticular features, and increased disability (HAQ score > 1). Hands and feet radiographs were available for review in 72% of patients. In univariate logistic regression the presence of erosions was more likely in older patients ($p < 0.0001$), with older age at RA onset ($p = 0.03$), longer disease duration ($p = 0.0001$), being a woman ($p = 0.03$), and

Table 1. Lymphotoxin- α (LTA) 252 genotype and allele frequencies. Values calculated with the G/G genotype as reference.

| | Patients with RA, n = 388 | Control Subjects, n = 269 | p | OR (95% CI) |
|-------------------|---------------------------|---------------------------|-------|---------------------|
| LTA 252 genotypes | | | | |
| A/A | 197 (50.8%) | 117 (43.5%) | 0.025 | 1.772 (1.076–2.921) |
| A/G | 153 (39.4%) | 112 (41.6%) | 0.160 | 1.438 (0.867–2.386) |
| G/G | 38 (9.8%) | 40 (14.9%) | | |
| Allele frequency | | | | |
| A allele | 547 (70.5%) | 346 (64.3%) | 0.018 | 1.325 (1.049–1.675) |
| G allele | 229 (29.5%) | 192 (35.7%) | 0.018 | 0.754 (0.597–0.954) |

Table 2. Demographic and clinical characteristics of patients with rheumatoid arthritis (RA) according to the lymphotoxin- α A>G genotype. Data are expressed as median (25th–75th percentile) or proportions (%).

| Characteristics | RA Total, n = 388 | A/A, n = 197 | A/G, n = 153 | G/G, n = 38 | p |
|------------------------------------|-------------------|------------------|------------------|------------------|----------|
| Women | 344 (88.7) | 173 (87.8) | 137 (89.5) | 34 (89.5) | 0.87 |
| Age, yrs | 58 (48–67) | 59 (49.5–67) | 59 (50.5–67) | 45 (35–59) | 0.001 |
| Body mass index, kg/m ² | 26.7 (23.1–29.7) | 27.2 (23.5–29.2) | 26.6 (23.5–30.6) | 24.7 (21.4–28.1) | 0.19 |
| Waist, cm | 87.2 (11.3) | 87.5 (10) | 87.6 (12) | 85.9 (15) | 0.57 |
| Current smoker | 52 (13.4) | 12 (6.1) | 34 (22.2) | 6 (15.8) | 0.02 |
| Age at RA onset | 44 (32–55) | 45 (34.5–55.5) | 46 (33–56) | 34 (22–43) | < 0.0001 |
| Disease duration, yrs | 10 (4.4–18) | 10 (4.7–19.3) | 10 (4.2–18.9) | 10 (4.1–18.2) | 0.88 |
| Rheumatoid factor-positive | 284 (73.2) | 139 (70.6) | 116 (75.8) | 29 (76.3) | 0.58 |
| DAS28 | 4.1 (3.0–5.3) | 3.9 (2.9–5.2) | 3.9 (3.0–5.5) | 4.5 (3.5–5.1) | 0.65 |
| HAQ | 1.125 (0.5–1.87) | 1 (0.5–1.75) | 1.25 (0.62–1.87) | 1.25 (0.25–1.84) | 0.58 |
| Erosions, n = 284 | 235 (82.7) | 133 (87.5) | 85 (80.2) | 17 (65.4) | 0.015 |
| Extraarticular features | 96 (24.7) | 52 (26.4) | 36 (23.5) | 8 (21.1) | 0.71 |
| Joint surgery | 52 (13.4) | 22 (11.2) | 28 (18.3) | 2 (5.3) | 0.34 |
| Corticosteroids | 262 (67.5) | 136 (69) | 105 (68.6) | 21 (55.3) | 0.46 |
| Methotrexate | 292 (75.3) | 146 (74.1) | 121 (79) | 25 (65.8) | 0.43 |
| Biologics | 74 (19) | 37 (18.8) | 32 (20.9) | 5 (12.8) | 0.51 |

DAS: Disease Activity Score; HAQ: Health Assessment Questionnaire.

having the A/A genotype (OR 3.706, 95% CI 1.447–9.448, $p = 0.006$). Factors identified to be significantly associated with both A/A genotype and erosions, thus acting as potential confounders, included age ($p = 0.001$ and $p < 0.0001$, respectively) and age at disease onset ($p < 0.0001$, $p = 0.03$). Following adjustment for confounders, the effect of genotype on erosions was no longer statistically significant (OR 1.799, 95% CI 0.600–5.397, $p = 0.29$). We did not find any statistically significant association between the A/A genotype and the presence of extraarticular manifestations (OR 1.345, 95% CI 0.580–3.121, $p = 0.49$) or disability (OR 1.031, 95% CI 0.510–2.084, $p = 0.932$) either in the crude model or following adjustment for potential confounders (extraarticular manifestations, OR 1.292, 95% CI 0.537–3.110, $p = 0.56$; disability, OR 1.290, 95% CI 0.620–2.682, $p = 0.49$).

LTA genotypes and dyslipidemia. Fifty patients (25.4%) with LTA 252 A/A, 27 (17.6%) with A/G, and 4 (7.7%) with G/G genotype had been previously diagnosed with dyslipidemia ($p = 0.23$) and were receiving treatment with lipid-lowering agents. As dyslipidemia could be underdiagnosed and/or undertreated, we measured fasting cholesterol, HDL cholesterol, LDL cholesterol, and triglycerides in 204 participants (94 patients with RA and 110 controls, all women). Disease characteristics of this confirmatory group were similar to the whole population. Dyslipidemia was highly prevalent in both patients (75.5%) and controls (67.3%). In our confirmatory group, the presence of dyslipidemia was more likely in older participants (OR 1.047, 95% CI 1.022–1.072, $p < 0.001$), with higher BMI (OR 1.010, 95% CI 1.001–1.101, $p = 0.04$), and in those carrying the A/A genotype (OR 2.773, 95% CI 1.209–6.361, $p = 0.01$). Following adjustment for confounders (age and BMI), and in patients also for DAS28 and corticosteroid dose because active RA and steroids may alter lipid profile, the association between dyslipidemia and genotype remained significant only in patients with RA (Table 3). The A/A genotype was independently associated with dyslipidemia (OR 18.3, 95% CI 2.91–94.80, $p = 0.002$), as well as with higher levels of triglycerides ($p = 0.01$) in patients not treated with lipid-lowering agents. There was also a trend toward higher total cholesterol levels among patients carrying the A/A genotype ($p = 0.08$). HDL and LDL cholesterol were not significantly different among genotypes (Table 4).

Inflammatory markers. Plasma LTA levels were below the limit of detection in the majority of samples and could not be analyzed further. Plasma levels of CRP, TNF, sTNFR I, and IL-6 were compared in patients with RA and controls (Table 5). As expected, we found significantly higher CRP (10 ± 2 mg/l vs 3 ± 2.8 ; $p < 0.0001$), TNF (13.3 ± 30.3 pg/ml vs 5.1 ± 4.9 pg/ml; $p < 0.0001$), sTNFR I (1.8 ± 1.2 pg/ml vs 1.5 ± 0.9 ; $p = 0.07$), and IL-6 (4.8 ± 2.4 pg/ml vs 2.4 pg/ml; $p = 0.04$) levels in patients compared to healthy controls.

Table 3. Association of dyslipidemia with lymphotoxin- α 252 genotypes in patients with rheumatoid arthritis. OR adjusted for age, body mass index, current prednisone dose, and 28-joint count Disease Activity Index (DAS28).

| | Crude OR (95% CI) | p | Adjusted OR (95% CI) | p |
|-----------------|----------------------|---------|-------------------------|-------|
| Genotype | | | | |
| A/A | 18.00 (4.01–80.71) | < 0.001 | 18.30 (2.91–94.80) | 0.002 |
| A/G | 5.85 (1.46–23.37) | 0.012 | 5.10 (0.89–29.15) | 0.08 |
| G/G (reference) | 1 | | 1 | |
| Age | 1.039 (1.004–1.075) | 0.030 | — | |
| Body mass index | 1.148 (1.023–1.289) | 0.019 | — | |
| Prednisone dose | 1.055 (0.926–1.218) | 0.095 | — | |
| DAS28 | 0.722 (0.507–1.029) | 0.072 | — | |

Patients with the G/G genotype presented CRP levels almost 3 times higher (13.1 ± 8.9 mg/l) than those with the A/A genotype (5.3 ± 4.9 mg/l; $p = 0.007$) and this difference remained significant after adjustment for disease activity ($p = 0.001$). No significant differences in TNF or IL-6 levels were identified among genotypes in patients or in controls.

DISCUSSION

RA is both genetically and clinically a heterogeneous disease. Although its causes remain largely unknown, the importance of proinflammatory cytokines in the pathophysiology of RA has been emphasized recently. Polymorphisms in cytokine genes are associated with different cytokine transcriptional levels, and these variations might affect not only the frequency of the disease, but also its phenotypic expression. All these clues point toward functional cytokine gene polymorphisms being potential additional risk factors for RA.

We report a significant association of the LTA A allele with RA in whites. In particular, the A/A genotype was associated with almost doubled odds of diagnosis of RA. However, the disease risk allele was not associated with surrogate markers of RA severity.

Our study population comprised a relatively large and homogeneous group of whites mostly from south Portugal. Patients with RA were followed regularly in a cohort study at the participating institutions for several years, the disease was well characterized, and detailed clinical information was confirmed from patient files. The prevalence of the variant G allele in healthy controls was identical to that reported in large European control populations¹², but lower when compared to Asian populations^{20,21}. Nevertheless, this study has several limitations, including the fact that we did not test for other potentially relevant polymorphisms of the TNF cluster that might be in linkage disequilibrium with the studied one.

There are few studies addressing the LTA 252 A>G SNP

Table 4. Lymphotoxin- α 252 A>G genotypes and fasting lipid levels in participants not receiving lipid-lowering therapy. Results are means \pm SD.

| Lipid Levels (mg/dl) | Patients with RA | | | Healthy Control Population | | |
|----------------------|------------------|--------------------------|--------------|----------------------------|--------------|--------------|
| | A/A, n = 34 | A/G, n = 27 | G/G, n = 13 | A/A, n = 25 | A/G, n = 38 | G/G, n = 21 |
| Total cholesterol | 212 \pm 32 | 200 \pm 37 | 197 \pm 38 | 200 \pm 32 | 205 \pm 32 | 197 \pm 26 |
| HDL | 65 \pm 24 | 64 \pm 14 | 64 \pm 13 | 59 \pm 13 | 62 \pm 11 | 65 \pm 15 |
| LDL | 129 \pm 24 | 120 \pm 33 | 113 \pm 32 | 124 \pm 33 | 127 \pm 26 | 119 \pm 22 |
| Triglycerides | 108 \pm 41* | 98 \pm 36 [†] | 81 \pm 24 | 94 \pm 31 | 94 \pm 39 | 92 \pm 47 |

* Adjusted $p_{A/A \text{ vs } G/G} = 0.01$ and $^{\dagger} p_{A/G \text{ vs } G/G} = 0.05$. HDL: high-density lipoprotein; LDL: low-density lipoprotein.

Table 5. Serum levels of inflammatory mediators in patients and healthy controls according to genotype.

| Serum Levels | Patients with RA | | | Controls | | |
|----------------|------------------|----------------|-----------------|----------------|----------------|-----------------------------|
| | A/A, n = 45 | A/G, n = 36 | G/G, n = 13 | A/A, n = 33 | A/G, n = 51 | G/G, n = 26 |
| ESR, mm/h | 35 \pm 18 | 39 \pm 30 | 41 \pm 19 | 19 \pm 10 | 21 \pm 12 | 22 \pm 16 |
| CRP, mg/l | 5.3 \pm 4.9 | 15.1 \pm 39 | 13.1 \pm 8.9* | 3.3 \pm 2.8 | 2.7 \pm 2.3 | 3.6 \pm 3.6 |
| TNF, pg/ml | 10.5 \pm 17.4 | 18.6 \pm 44 | 7.9 \pm 12.5 | 6.2 \pm 5.9 | 4.8 \pm 5 | 4.3 \pm 3.6 |
| sTNFR I, pg/ml | 1.73 \pm 0.9 | 1.90 \pm 1.4 | 1.79 \pm 0.8 | 1.51 \pm 1.0 | 1.33 \pm 0.7 | 1.91 \pm 0.8 [†] |
| IL-6, pg/ml | 3.6 \pm 9.1 | 7.6 \pm 17.5 | LOD | LOD | LOD | LOD |

* Adjusted $p_{G/G \text{ vs } A/A} = 0.001$; $^{\dagger} p_{G/G \text{ vs } A/G} = 0.02$. ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; TNF: tumor necrosis factor; sTNFR I: soluble TNF receptor I; LOD: concentrations below the limit of detection.

in RA, most of them with a small number of patients, which might have contributed to the conflicting results. Takeuchi, *et al* reported an increased frequency of the LTA A allele in Japanese DRB1*0405-positive patients²⁰, apparently independent of MHC class II genes, in a study that included 103 patients with RA. In contrast, Panoulas, *et al* recently found increased carriage of the G allele among 388 British patients¹⁵. Others report no relation of this SNP with RA^{22,23}. In addition to sample size issues, a possible explanation for these different results is that genetic susceptibility related to the LTA gene may vary in different ethnic groups. In addition, different inclusion criteria and lack of homogeneity of the studied populations might contribute to further confounding factors. Another hypothesis is that differences are due to linkage disequilibrium with other genes of the MHC more relevant to RA susceptibility.

Although LTA 252 A>G is an intronic polymorphism and some controversy exists whether this is a functional polymorphic site, there is compelling evidence suggesting that the LTA 252 G/G genotype is linked to enhanced expression of LTA and higher serum concentrations of inflammatory markers^{11,12,13,24}. This is important, as the increased proinflammatory environment could influence the phenotypic expression of RA. We observed a strong association between the LTA 252 G/G genotype and younger age at RA onset that has not been previously reported. Interestingly, the influence of the allelic variation of the LTA 252 on the

mean age at onset of psoriasis was described by Balding, *et al* in patients with psoriatic arthritis²⁵, thus suggesting the influence of the G/G genotype on the beginning of the disease at youngest age. We also measured inflammatory mediators in a confirmatory group and found higher CRP levels in patients possessing the G/G genotype, but no significant differences could be detected in TNF, sTNFR I, or IL-6 levels. CRP levels are influenced by genetic factors and the LTA 252 G allele was associated with high levels in population studies^{11,26}. The relatively small number of individuals carrying the G/G genotype as well as the high dispersion of measured values could have contributed to the lack of association with other inflammatory mediators. Another limitation is the cross-sectional design of the study and the fact that patients were at different stages of the disease and under treatment for years.

We found a strong association between dyslipidemia and the A/A genotype in patients with RA that could not be explained by other factors, but this was not observed in the control population. In particular, triglyceride levels were higher among patients carrying the A allele, independent of age, disease activity, or corticosteroid use. Panoulas, *et al*¹⁵ also found significantly higher total cholesterol, as well as a trend toward higher LDL and triglyceride levels in patients with RA carrying the LTA 252 A/A genotype. Moreover, higher fasting triglycerides were previously reported in healthy white men possessing the LTA A allele²⁷ in contrast

with results from Korean men¹⁴, but sex and ethnic-related variations could account for the different results. Together, these observations suggest that the dyslipidemia that accompanies and even precedes RA is not only a consequence of inflammation but is also genetically determined. However, these results need replication in larger population samples.

Our study shows that the LTA 252 A allele is associated with increased risk of developing RA in Portuguese whites. Moreover, the A/A genotype translates to a phenotype that is more prone to dyslipidemia, raising the possibility that a genetic element contributes to the disordered lipid metabolism encountered in patients with RA as well as in individuals who eventually develop RA. This SNP also influences age of disease onset and levels of CRP, but it does not seem to be a major determinant of RA severity. Although our study involved a relatively large number of subjects, replication is needed in other cohorts for testing the robustness of these results. If confirmed, these findings will contribute to understanding the underlying mechanisms for the cardiovascular burden in RA.

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