Systemic lupus erythematosus in a multiethnic US Cohort (LUMINA). XXX: association between C-reactive protein (CRP) gene polymorphisms and vascular events


Objectives. To determine if a polymorphic GT\textsuperscript{n} repeat in the intron of the C-reactive protein (CRP) gene associates with occurrence of vascular arterial events in systemic lupus erythematosus (SLE).

Methods. We performed a nested case–control study on the LUMINA cohort of 546 Hispanic, African-American and Caucasian SLE patients. Twenty-five patients who developed vascular arterial events (i.e. myocardial infarction, angina, coronary artery bypass graft surgery, stroke, claudication, gangrene or significant tissue loss and/or arterial peripheral thrombosis) after enrolment were selected as cases and 32 ethnically matched patients with no previous vascular arterial events served as controls. Their CRP gene GT\textsuperscript{n} polymorphism and plasma CRP was determined.

Results. Patients with vascular events had more severe SLE and were more likely to have plasma CRP in the highest quintile of measured values. The overall distribution of GT\textsuperscript{n} alleles for patients with vascular events had a greater number of the GT\textsuperscript{20} variant compared with controls [26.0% of alleles (13/50) vs 15.6% (10/64)]. This greater number of GT\textsuperscript{20} in patients with vascular events was observed for African-Americans [29.2% (7/24) vs 21.0% (8/38)] and Hispanics [33.0% (4/12) vs 0% (0/16)] but not for Caucasians [14.3% (2/14) vs 20.0% (2/10)]. For African-Americans and Hispanics combined (45 patients), the frequency of GT\textsuperscript{20} in those with vascular events (30.6% (11/36) was significantly higher than in those without them (14.8%, 8/54) (P <0.05, one-tailed test for difference in proportions). When patients were categorized according to the number of GT\textsuperscript{20} alleles they carried (thus GT\textsuperscript{20}/GT\textsuperscript{20}, GT\textsuperscript{20}/GT\textsuperscript{x} or GT\textsuperscript{x}/GT\textsuperscript{x}, where x is any allele other than GT\textsuperscript{20}), for both African-Americans and Hispanics the likelihood of vascular arterial events increased in proportion with the GT\textsuperscript{20} dose, and all GT\textsuperscript{20}-homozygous patients developed vascular arterial events.

Conclusions. The CRP GT\textsuperscript{20} variant is more likely to occur in African-American and Hispanic SLE patients than in Caucasian ones, and SLE patients carrying the GT\textsuperscript{20} allele are more likely to develop vascular arterial events.

KEY WORDS: Systemic lupus erythematosus, C-reactive protein.
healthy controls, although the exact mechanism underlying this association is not yet clear [10]. We tested the hypothesis that the CRP intron polymorphism might be associated with the increased occurrence of vascular arterial events observed in SLE patients; such a finding could have important implications in the management of these patients.

Patients and methods

This investigation was a nested case-control study performed in the context of the LUMINA cohort. As has been previously reported, LUMINA (Lupus in Minorities: Nature vs Nurture) is a longitudinal study of outcome of SLE in patients from three ethnic groups (Hispanics, African-Americans and Caucasians) living in these geographical areas of the US: Texas, Alabama and the island of Puerto Rico. The Institutional Review Board of each participating centre approved the LUMINA study, and written informed consent was obtained from each subject according to the Declaration of Helsinki. Each LUMINA patient met SLE classification criteria according to the American College of Rheumatology (ACR) [11, 12], had a disease duration of 5 yr or less at the time of enrolment (T0), had defined ethnicity (all four grandparents of the same ethnicity as the patient), and lived in the geographic catchment areas of the participating institutions. Time of diagnosis (TD) was defined as the date when the patient met the fourth ACR criteria for the classification of SLE. Every patient has a baseline visit (T0); follow-up visits are conducted every 6 months for the first year (T0.5 and T1, respectively) and yearly thereafter (T2, T3 etc. to TL, the last available visit for each patient). During each visit all available medical records are reviewed, a complete history and physical examination are performed, and laboratory tests are obtained. Cases are those SLE patients who developed vascular arterial events subsequent to enrolment [6]; ethnically matched controls are randomly selected LUMINA patients who did not develop a vascular arterial event up to the time of this study and for whom DNA was available.

Vascular arterial events were ascertained during clinical visits upon examination by a study physician and/or after a review of patient medical records. Vascular events included in these analyses were those obtained by physician assessment during LUMINA study visits and/or documented in the medical records reviewed for the study visits. These were categorized into three broad groups. Cardiovascular events included the presence of coronary artery disease, either myocardial infarction (ischaemic symptoms and electrocardiography changes and/or biochemical markers of myocardial necrosis) and/or definite or classic angina (substantial chest discomfort provoked by exertion or emotion and relieved by rest or nitroglycerine) and/or a vascular procedure for myocardial infarction (coronary artery bypass graft). Cerebrovascular events included the presence of irreversible or partially reversible motor and/or sensory deficits of sudden or recent onset on the basis of vascular occlusion or insufficiency, complete, incomplete or in evolution, persisting longer than 24 h or lasting less than 24 h with an anatomical correlate. Peripheral vascular events included the presence of arterial claudication (pain in the muscles of the upper or lower extremities, induced by exercise and relieved by rest with absent pulses and/or confirmed by Doppler flow studies and/or angiography) lasting longer than 6 months, and/or evidence of gangrene or significant tissue loss (loss of a digit or a limb), and arterial thrombosis in peripheral arteries (documented by angiogram).

CRP was measured with high sensitivity by immunometric assay (Immulite 2000; Diagnostic Products Corporation, Los Angeles, CA, USA) using patient sera obtained at T0. For CRP genotype determination, white blood cells were isolated and genomic DNA was extracted by proteinase K digestion and salting out. Molecular typing at the CRP intron G10 polymorphism was done by the polymerase chain reaction (PCR) followed by the use of sequencer and Genescan software. Amplification was done using primers that flanked the GT repeats: 5'-6FAM-GTATGACAGGATACTGTCACCT-3' and 5'-CCAGAAAAACAGCAATGTGGAG-3' (Perkin-Elmer, Norwalk, CT, USA). An aliquot of the amplified product was run on a 1.5% agarose gel to verify its integrity, and the sample was electrophoresed on a long-range polyacrylamide gel on a 377 DNA sequencer (ABI-Prism; PE Biosystems, CA, USA). Raw data were collected and the molecular weight of each amplification product was interpolated using Genescan 2.0 software and Genescan-500 Rox size standards (ABI-Prism). The length of the PCR products ranged from 176 to 192 bp, corresponding to introns encoding 16–24 repeats (i.e. GT16 to GT24).

Statistical analyses were performed on parametric data to compare patients with and without vascular events. The χ² test (with Fisher’s exact correction, if appropriate) for proportions and Student’s t test for means were used. All analyses were done using either Statview 5 version 5.0.1 or SAS, version 8.1 (SAS Institute, Cary, NC, USA); all P values are shown but only P ≤ 0.05 was considered significant.

Results

Five hundred and forty-six SLE patients (107 Texan Hispanics, 84 Puerto Rican Hispanics, 200 African-Americans and 155 Caucasians) constituted the LUMINA cohort. The average disease duration for these 546 patients, from diagnosis time (TD) to time of enrolment (T0), and the average follow-up time from T0 to last available visit were 17.3 (16.0) [mean (s.d.)] and 37.6 (33.4) months, respectively. Vascular arterial events occurred in 34 patients since T0 (6.2% of the total); DNA was available for 25 of these; ipso facto they constitute the cases [six Hispanics (five from Texas and one from Puerto Rico), 12 African-Americans, seven Caucasians]. Thirty-four potential controls were chosen randomly for genotyping; DNA was available for 32 of these (eight Hispanics, 19 African-Americans, five Caucasians).

As shown in Table 1, patients having experienced a vascular arterial event were somewhat older than patients who did not, and they were somewhat more likely to be smokers. Patients with vascular arterial events also had a slightly longer follow-up time and a marginally higher damage score as measured by the Systemic Lupus International Collaborating Clinics/ACR (SLICC) damage index (SDI) [13]. There was a somewhat higher proportion of patients with low-density lipoprotein (LDL) cholesterol levels in the cases than in the controls. Patients with vascular events had only marginally higher serum levels of CRP [14.09 (2.49) μg/ml] than patients without them [10.17 (2.72) μg/ml], but patients with vascular events were significantly more likely to have CRP values in the highest quintile of the overall distribution of CRP values (CRP ≥ 17.0 μg/ml; P = 0.007).

The distribution of GT alleles among the 25 cases and 32 controls we examined mirrored the pattern we previously observed in a larger cohort of SLE patients and healthy controls [8]. Thus, GT16 was the common allele in all ethnic groups, accounting for 27.4% (17/62) of all GT alleles in African-Americans to 50.0% (12/24) in Caucasians. GT21 was the second most common allele in Caucasians (25.0%, 6/24) whereas African-Americans showed an over-representation of both GT18 and GT20 (24.2%, 17/70; 24.2%, 15/62 for each allele). In Hispanics the allele distribution was intermediate. When allele distributions of patients with and without vascular events were compared (Fig. 1A), GT20 was observed to be more frequent in the patients with vascular arterial events (26.0%, 13/50) than in those without them (15.6%, 10/64), although the difference was not statistically significant. Over-representation of the GT20 allele was observed for both African-American (29.0%, 7/24 vs 21.0%, 8/38) and Hispanic patients (33.0%, 4/12 vs 0%, 0/16) with vascular arterial events, but not for Caucasians (14.3%, 2/14 vs 20%, 2/10). When...
### Table 1. Baseline socio-economic, demographic, and clinical features of SLE patients with and without vascular arterial events

<table>
<thead>
<tr>
<th>Variable</th>
<th>Yes (25 patients)</th>
<th>No (32 patients)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethnicity (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hispanic</td>
<td>24</td>
<td>25</td>
<td>0.143</td>
</tr>
<tr>
<td>African-American</td>
<td>48</td>
<td>59</td>
<td>0.063</td>
</tr>
<tr>
<td>Caucasian</td>
<td>28</td>
<td>16</td>
<td>0.069</td>
</tr>
<tr>
<td>Age (yr): mean (S.D.)</td>
<td>43.1 (13.9)</td>
<td>36.4 (12.9)</td>
<td>0.016</td>
</tr>
<tr>
<td>Gender: % women</td>
<td>88</td>
<td>97</td>
<td>0.098</td>
</tr>
<tr>
<td>Years of education: mean (S.D.)</td>
<td>12.2 (3.7)</td>
<td>12.1 (3.8)</td>
<td>0.098</td>
</tr>
<tr>
<td>Health insurance: % having</td>
<td>64</td>
<td>66</td>
<td>0.116</td>
</tr>
<tr>
<td>Poverty line: % below</td>
<td>52</td>
<td>31</td>
<td>0.069</td>
</tr>
<tr>
<td>Ever smoked: %</td>
<td>28</td>
<td>9</td>
<td>0.116</td>
</tr>
<tr>
<td>Disease duration (TD – T0) (months): mean (S.D.)</td>
<td>20.1 (16.1)</td>
<td>16.8 (14.4)</td>
<td>0.420</td>
</tr>
<tr>
<td>Follow-up time (T0 – TL) (months): mean (S.D.)</td>
<td>71.7 (29.8)</td>
<td>53.4 (43.4)</td>
<td>0.081</td>
</tr>
<tr>
<td>SLAM* score: mean (S.D.)</td>
<td>8.8 (4.1)</td>
<td>9.9 (6.9)</td>
<td>0.098</td>
</tr>
<tr>
<td>SLICC/SDI* score: mean (S.D.)</td>
<td>1.1 (1.3)</td>
<td>0.5 (1.2)</td>
<td>0.098</td>
</tr>
<tr>
<td>Serum cholesterol &gt;200 mg/dl: %</td>
<td>20</td>
<td>20</td>
<td>1.000</td>
</tr>
<tr>
<td>LDL* cholesterol &gt;130 mg/dl: %</td>
<td>24</td>
<td>7</td>
<td>0.081</td>
</tr>
<tr>
<td>HDL* cholesterol &lt;35 mg/dl: %</td>
<td>56</td>
<td>47</td>
<td>0.495</td>
</tr>
<tr>
<td>CRP &gt;17 μg/ml (%)*</td>
<td>36</td>
<td>6</td>
<td>0.007</td>
</tr>
<tr>
<td>Antiphospholipid antibody-positive: %</td>
<td>32</td>
<td>22</td>
<td>0.393</td>
</tr>
<tr>
<td>Maximum prednisone dose (mg per day)</td>
<td>10.4 (19.5)</td>
<td>18.9 (25.0)</td>
<td>0.171</td>
</tr>
<tr>
<td>Ever on immunosuppressive drugsg: %</td>
<td>28</td>
<td>19</td>
<td>0.786</td>
</tr>
<tr>
<td>Ever taking hydroxychloroquine: %</td>
<td>80</td>
<td>69</td>
<td>0.400</td>
</tr>
</tbody>
</table>

*Only P values ≤ 0.10 are shown.

TD, time of diagnosis.

TL, time of last visit.

\*Systemic Lupus Activity Measure.

\*Systemic Lupus International Collaborating Clinics/American College of Rheumatology Damage Index.

\*Low-density lipoprotein.

\*High-density lipoprotein.

\*Top quintile of CRP distribution.

\*IgG and/or IgM antiphospholipid antibody and/or the lupus anticoagulant.

\*Either oral or intravenous cyclophosphamide or oral azathioprine.

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**Fig. 1.** Influence of ethnicity and vascular arterial events on CRP allele polymorphism. (A) The allele frequencies for the CRP intron GT\* polymorphism in SLE patients with vascular arterial events (VE) or without VE (controls) was determined for all patients and for African-Americans, Hispanics and Caucasians separately. (B) Allele frequencies for African-Americans and Hispanics combined. Note breaks in the ordinate axes and the changing scales. Numbers in brackets indicate the number of patients analysed. The asterisk indicates a significantly increased frequency of the GT\* variant in VE cases versus controls (P < 0.05).
African-Americans and Hispanics were combined (Fig. 1B) the difference in GT<sup>20</sup> frequency between patients with and without vascular arterial events achieved statistical significance (30.6%, 11/36 vs 14.8%, 8/54; P < 0.05, one-tailed test for difference in proportions).

Since the GT<sup>20</sup> allele was over-represented in African-American and Hispanic patients with vascular arterial events, we investigated whether GT<sup>20</sup> and the risk of vascular arterial events was associated in these ethnic groups. To compensate for the small sample size, we categorized patients into three possible genotypes: GT<sup>30</sup> homozygotes (GT<sup>30</sup>GT<sup>20</sup>) (3.5% of all patients), GT<sup>20</sup> heterozygotes (GT<sup>30</sup>GT<sup>20</sup>) (33.3%) and any other genotype (GT<sup>30</sup>GT<sup>x</sup>) (63.2%). Although formal χ<sup>2</sup> trend analysis could not be performed due to the absence of GT<sup>20</sup> homozygotes without vascular events, a positive relationship between GT<sup>20</sup> dose and vascular event risk was apparent (Fig. 2), i.e. homozygous patients exhibited the highest rate of occurrence of vascular events. Remarkably, the two African-American GT<sup>30</sup>GT<sup>20</sup> patients (representing 6.5% of all African-Americans examined) both developed vascular events, as did all four of the Hispanic GT<sup>30</sup>GT<sup>x</sup> patients (representing 28.6% of all Hispanics examined). The pattern was not seen in Caucasians.

**Discussion**

Several recent studies have indicated that concentrations of CRP might be influenced by different kinds of CRP gene polymorphisms [14–16], including the GT dinucleotide repeat within the intron of the CRP gene [10]. Two of the GT alleles most frequently present in the general population, GT<sup>16</sup> and GT<sup>21</sup>, are associated with low basal levels of CRP. Indeed, this observation was supported by Russell et al., who showed that CRP haplotypes containing GT<sup>16</sup> or GT<sup>21</sup> alleles were associated with low or intermediate plasma levels of CRP in lupus patients [16]. Conversely, the GT<sup>20</sup> allele, which is over-represented in some ethnic minorities, such as African-Americans, had previously been associated with high basal levels of CRP [10]. The present study is the first to indicate that an association exists between CRP gene polymorphism and cardiovascular events in SLE patients. Importantly, this disease association is revealed by a GT variant already known to predispose to higher basal concentrations of CRP [10].

In a previous study that examined another CRP gene polymorphism also shown to be associated with low levels of CRP (1059G/C polymorphism), Zee and Ridker failed to find an association with arterial thrombotic events [14]. It is possible that the association we report here for GT<sup>20</sup> only reveals itself within the context of SLE. This is a likely scenario as, during inflammation, cytokines and other proinflammatory molecules might up-regulate the CRP gene and reveal effects not seen in the absence of inflammation.

We are of course aware that a larger study is required to establish unequivocally an association between the CRP GT<sup>20</sup> allele and vascular arterial events. Indeed, given the relative paucity of patients with vascular arterial events in the LUMINA cohort, most of our comparisons suffer from a lack of statistical power. Furthermore, since vascular arterial events may occur at any time after enrolment of patients, in many cases several years from disease onset, some of the patients we included as controls may develop arterial events in the future; it is also possible that we may have missed subclinical events in some cases and controls. Nevertheless, supporting the validity of our patient classification system are the results of univariate comparisons of cases and controls (Table 1). Thus as expected, cases were more likely to be older, to have ever smoked and to have accrued more damage, and were more likely to have abnormal LDL cholesterol and CRP values. Therefore, despite the recognized weaknesses of our study, we believe that the observed association between CRP polymorphism and vascular events is real.

The results of this study confirm that the extent of CRP GT polymorphism is variable among different ethnic groups, the GT<sup>20</sup> variant being more frequent in African-Americans and Hispanics than it is in Caucasians. SLE patients carrying the GT<sup>20</sup> allele are more likely to have vascular arterial events and the risk seems to be dose-dependent. One interpretation of these data is that certain CRP gene polymorphisms might predispose SLE patients to the development of cardiovascular complications. On the other hand since this association is more apparent for...
African-American and Hispanic patients than for Caucasian ones, the GT\textsuperscript{20} allele could be in linkage disequilibrium with an unknown susceptibility gene or genes. Risk factors for vascular arterial events known to elevate CRP, such as smoking, are predicted to strengthen the association, and this might explain the ethnic difference. It should also be noted that, as these patients continue to be monitored, some controls may ultimately become cases. Therefore, if our initial observations are confirmed by future analyses of additional patients in the LUMINA cohort, risk assessment for the occurrence of vascular events in SLE might ultimately incorporate GT\textsuperscript{20} as a variable.

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References