Toll Receptor Polymorphisms and Carotid Artery Intima-Media Thickness

Robyn Labrum, PhD; Steve Bevan, PhD; Matthias Sitzer, MD; Matthias Lorenz, MD; Hugh S. Markus, FRCP

Background and Purpose—Inflammation is a key mechanism in atherosclerosis. Variation in genes encoding inflammatory responses may therefore influence atherosclerosis risk possibly through interaction with chronic infections and proinflammatory environmental risk factors such as smoking, diabetes, and obesity. The Toll-like receptor family (TLRs) genes TLR2 and TLR4, both involved in the inflammatory process, are potential candidates and TLR-4 has been previously associated with cardiovascular disease, although other studies have failed to confirm this.

Methods—A total of 3000 individuals from the prospective community-based Carotid Atherosclerosis Progression Study (CAPS) were genotyped for single nucleotide polymorphisms: TLR2 (Arg753Gln, –16934 A/T) and TLR4 (D299G, T399I). Associations were determined with common carotid artery intima-media thickness (IMT) at baseline and also progression of IMT over the 3-year follow-up period. Gene–environment interactions with high sensitive C-reactive protein, smoking, body mass index, and diabetes were determined.

Results—There was no association between single nucleotide polymorphisms or haplotypes in either TLR4 or TLR2 and either baseline IMT or progression of IMT over the 3-year follow up. There were no interactions among the three proinflammatory risk factors. No genotype or haplotype was associated with high sensitive C-reactive protein.

Conclusions—In this large community population, we found no evidence for genetic variation in these two TLRs being risk factors for increased IMT either directly or through interaction with proinflammatory risk factors. We were unable to confirm associations with the TLR4 polymorphisms reported in previous smaller studies. (Stroke. 2007;38:1179-1184.)

Key Words: atherosclerosis — carotid artery — genetics — inflammation — ultrasound
have been associated with reduced risk of cardiovascular disease, including carotid artery intima-media thickness (IMT)\textsuperscript{11} and acute coronary event risk.\textsuperscript{12} However, other studies failed to replicate these findings in coronary artery disease,\textsuperscript{13} IMT,\textsuperscript{14} and stroke.\textsuperscript{15} The conflicting findings with TLR4 may reflect the small sample sizes in most studies as well as the differing populations studied. Another important factor is that few have examined gene–environment interactions with potential proinflammatory conventional risk factors. If inflammation is a mechanism by which some of these risk factors mediate atherosclerosis, the need to assess gene–environment interactions becomes important.\textsuperscript{16}

Risk factors mediate atherosclerosis, the need to assess gene–environment interactions becomes important.\textsuperscript{17}

Vascular risk factors were assessed using a standardized computer-assisted interview performed by a physician experienced in vascular medicine. Risk factors determined included pack-years of cigarette smoking and smoking category (never/ex/current smoker), history of arterial hypertension, history of diabetes mellitus, and body mass index (BMI).\textsuperscript{21} Socioeconomic status was measured using a four-point scale previously applied to German populations for coronary risk factor studies.\textsuperscript{22} The mean value of three supine blood pressure measurements was taken as the actual arterial blood pressure.\textsuperscript{23} Fasting blood samples were taken for estimation of serum cholesterol and glycosylated hemoglobin A1. Total serum cholesterol was determined enzymatically using a commercial kit (Boehringer). Baseline high sensitive C-reactive protein (hs-CRP) circulating levels were measured using an IMMAGE automatic immunoassay system (Beckmann-Coulter). Informed written consent was obtained from all participants, and the study protocol was approved by the ethical review committee of the Hospital of J.W. Goethe-University Frankfurt am Main.

### Ultrasound Imaging

For ultrasonic examinations, a 7.5- to 10.0-MHz linear array transducer was used (P700SE; Phillips Medical System). Preprocessing configurations (log gain compensation [60 dB] and image persistence) were held constant during all examinations. The gain was adjusted so that the least dense arterial wall interface was just visible. Using antero-oblique insonation, far-wall carotid IMT was visualized within the common carotid artery 20 to 60 mm proximally from the flow divider on both sides. The images were digitally captured during the systole of a single heartbeat on a personal computer using S-VHS PC-EYE 2-frame grabber (ELTEC Elektronik GmbH) in 16-bit R-G-B packing mode (748×576 pixel) for offline measurements. Vertical and horizontal calibration measurements were performed every 100th measurement using an ultrasound assurance phantom. The method used for IMT measurements, which used a semiautomatic image analysis approach, and inter/intraobserver reproducibility, has been described in detail previously.\textsuperscript{24} The presence of carotid plaque was also determined defined as a focal region of IMT thickening of >1.7 mm as previously described.\textsuperscript{25} This 1.7-mm cutoff was predefined for the CAPS study\textsuperscript{26} based on a previous described definition.\textsuperscript{27} Plaques were identified in cross-sectional imaging allowing near-wall plaques to be detected and were imaged also using the color mode so nonechogenic plaques were detected.

### Methods

Blood was taken in EDTA tubes and stored at −80°C. DNA was extracted from leukocytes in 2981 using Nucleon Kits (Tepnel Life Sciences). All genotyping assays were performed blind to patient details. Genotyping methods are summarized in Table 1.

### Materials and Methods

#### Subjects

We studied subjects from the prospective “Carotid Atherosclerosis Progression Study” (CAPS).\textsuperscript{28} All members of a German primary health care service population (n=32 708) who lived within a radius of 50 km from five study sites in Western Germany were invited to participate. Within a predefined time limit, 6962 (21.3%) agreed to participate. Of these, 5056 (from four of the five study sites) were invited to follow-up examination after 3 years and 3583 (67%) participated. Demographic and risk factor profiles of those invited and not invited were very similar. Forty-eight subjects died during the follow-up period. Mean (SD) duration of follow up between the two carotid duplex examinations was 38.53 (4.32) months. The first 3000 individuals in whom repeat carotid IMT measurements were performed were included in the current study.
Polymerase chain reaction was performed in a total reaction volume of 15 µL with the following conditions: denaturation 95°C for 5 minutes followed by 45 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds with a final cycle of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 10 minutes apart from TLR2-16934 A/T, the polymerase chain reaction for which was performed with denaturation 95°C for 5 minutes followed by 45 cycles of 95°C for 30 seconds, 68 to 50°C for 30 seconds, 72°C for 30 seconds with a final cycle of 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 10 minutes. Restriction enzyme digestion was performed according to the manufacturer’s instructions.

Statistical Analysis
Analyses were performed with both baseline and progression IMT values. Data were analyzed using SPSS (version 10.0). Mean IMT values between the right and left common carotid artery were used in all analyses. Baseline IMT values were skewed and the reciprocal was used to normalize the distributions before parametric analysis. The progression IMT values represent the absolute change in IMT over the 3-year period. These values were normally distributed and therefore the original values were used for analysis. For analysis of hs-CRP logarithmically transformed values were used. For analysis of association between genotype and hs-CRP when there were less than five subjects in a homozygous genotype, the subjects were considered with the heterozygote.

Associations with both individual single nucleotide polymorphisms (SNPs) and haplotypes was performed. Haplotypes were constructed and frequencies were calculated using PHASE 2.0 software (www.stat.washington.edu/stephens/software.html). Additive, dominant, and recessive allele models were tested to determine the effects of heterozygosity. Age- and sex-adjusted and subsequently multivariate analysis adjusting for age, sex, and vascular risk factors (smoking, BMI, history of diabetes mellitus, total cholesterol, and history of arterial hypertension) was performed. Multiple linear regression was used to determine any relationships between genotypes/haplotypes and mean IMT levels with specific gene–hs-CRP, gene–smoking, gene–diabetes, and gene–BMI interaction terms included, respectively.

Gene–environment interactions were examined using binary logistic regression with the interaction term as a covariate in the analysis. Both recessive and dominant models were examined in this manner as is usual practice for such studies. Correction for multiple testing was applied for the number of individual risk factors per genotype to achieve a probability value for significance to be assumed before analysis was undertaken.

Results
Demographic characteristics of the study population are given in Table 2. Genotyping was successful in 2955 of the 3000 cases. All genotypes for the polymorphisms studied were in Hardy-Weinberg equilibrium. Allele and haplotype frequencies are shown in Table 3.

Associations With Baseline Intima-Media Thickness
There were no associations between TLR 2 and 4 individual SNPs or haplotypes on univariate analysis or multivariate analysis controlling for age and sex and cardiovascular risk factors. Mean IMT values, 95% confidence intervals, and probability values for change in IMT are given for SNPs (additive model) in Table 4 and haplotypes (dominant model) in Table 5. Similar results were obtained when analyzing data using dominant and recessive models (data not shown). There were no significant interactions between any of the SNPs or haplotypes and smoking, BMI, or diabetes mellitus when analyzed individually using additive, dominant, and recessive models (data not shown). There was no association between hs-CRP and any genotype or haplotype (data not shown).

We also examined whether each gene may be making a small contribution to IMT variability acting through gene–environment interactions as has recently been described for cytokine genes2 by calculating a combined gene score. Based on evidence from the literature, the TLR2 “AC” haplotype and the TLR4 “GC” haplotype were designated proinflammatory (because variants have been shown to have an “antiinflammatory” action, it follows that the wild types would be “proinflammatory”). A gene-variant score in which 2 represented individuals homozygous for 2 inflammatory haplotypes and 0 was homozygous for none was also calculated. No
significantly increase in IMT was noted with increasing gene variant score ($P=0.775$) (Table 6). A proinflammatory environmental risk factor score was also calculated as 0 for no proinflammatory stimuli, 1 for a single proinflammatory stimulus, and 2 for two or more proinflammatory stimuli with the proinflammatory stimuli being current/ex-smoker, BMI $>30$ kg/m², and a positive history of diabetes. A significant increase in IMT was noted with increasing proinflammatory risk score (Table 6), but no interaction was found between the gene variant and proinflammatory risk factor scores ($P=0.385$).

### Association With Intima-Media Thickness Progression

There was no association between TLR2 or 4 SNPs (Table 4) or haplotypes (Table 5) and common carotid artery IMT progression over the 3-year follow-up period. Results were similar, with no significant associations, when baseline IMT was controlled for. There were no significant interactions between any of the SNPs or haplotypes and smoking, BMI, or diabetes mellitus when analyzed individually using additive, dominant, and recessive models (data not shown).

### TABLE 4. Results of Multivariate Analysis for Individuals Single Nucleotide Polymorphisms

<table>
<thead>
<tr>
<th>Single Nucleotide Polymorphism</th>
<th>Baseline IMT</th>
<th>IMT Progression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean IMT (mm)</td>
<td>95% CI</td>
</tr>
<tr>
<td>TLR2 Arg753Gln CT</td>
<td>0.729</td>
<td>0.719–0.741</td>
</tr>
<tr>
<td></td>
<td>0.733</td>
<td>0.723–0.739</td>
</tr>
<tr>
<td></td>
<td>0.742</td>
<td>0.713–0.734</td>
</tr>
<tr>
<td>TLR2 −16934 A/T AA</td>
<td>0.730</td>
<td>0.724–0.735</td>
</tr>
<tr>
<td></td>
<td>0.731</td>
<td>0.712–0.754</td>
</tr>
<tr>
<td></td>
<td>0.724</td>
<td>0.599–0.886</td>
</tr>
<tr>
<td>TLR4 D299G GG</td>
<td>0.728</td>
<td>0.722–0.733</td>
</tr>
<tr>
<td></td>
<td>0.714</td>
<td>0.696–0.731</td>
</tr>
<tr>
<td></td>
<td>0.757</td>
<td>0.585–0.929</td>
</tr>
<tr>
<td>TLR4 T399I CC</td>
<td>0.728</td>
<td>0.723–0.734</td>
</tr>
<tr>
<td></td>
<td>0.717</td>
<td>0.697–0.737</td>
</tr>
<tr>
<td></td>
<td>0.717</td>
<td>0.576–0.858</td>
</tr>
</tbody>
</table>

**IMT** indicates intima-media thickness.
Additive gene scores were also calculated for IMT progression values (as described for the cross-sectional analysis of baseline IMT). Again, no significant difference in change in IMT was noted for increasing gene variant score ($P=0.922$). In addition, no interaction was noted between the gene variant score and proinflammatory risk factor score ($P=0.896$).

**Association With Carotid Plaque**

There were 162 with carotid plaque at baseline. There was no association between any polymorphism and carotid plaque (TLR 2 16934 $P=0.414$, TLR 2 753 $P=0.315$, TLR4 299 $P=0.145$, TLR 399 $P=0.789$; all analyses based on an additive model but similar form other models).

**Discussion**

In this large community population, we found no association between polymorphisms or haplotypes in either the TLR2 or TLR4 genes. There was no association with either IMT values at baseline or progression of IMT over a 3-year period. Furthermore, we found no evidence of any interaction with potential proinflammatory cardiovascular risk factors.

The genes for TLR2 and TLR4 present attractive candidates for atherosclerosis because they are important in recognizing evolutionary highly conserved molecular motifs in pathogens. Expression of TLRs is upregulated in atherosclerotic lesions, and in addition, variation in both the coding and regulatory regions of both genes has been associated with altered susceptibility to inflammatory conditions. Associations between the TLR genes and chronic infections on the one hand and conventional proinflammatory risk factors on the other hand would be important. However, we found no evidence of any interactions between the polymorphisms studied and hs-CRP, smoking, diabetes, or obesity with either baseline or progression IMT.

Inflammatory processes may play a role at a number of stages during the atherosclerotic process. They have been implicated not only in early atherosclerosis, but also in plaque instability leading to conversion of asymptomatic to symptomatic disease. Measurement of IMT only allows investigation of risk factors for the former. Therefore, further studies are required to determine whether these genetic variants are important in later stages of the disease process and in conversion to plaque instability. We also looked at the associations with carotid plaque and found no associations, but the number of subjects with plaque was small and therefore the power to detect an association was low. A variety of definitions have been used for carotid plaque; we used $>1.7$ mm, which was predefined at the start of the CAPS study, but cutoffs ranging from 1.1 to 1.7 mm have been used. The higher cutoff we used may partly explain the low plaque prevalence.

This study has a number of strengths. Associations were determined in a well-characterized large population. The use of a continuous variable such as IMT provides considerable statistical power. IMT values were available not only at baseline, but also at 3 years allowing associations with progression to be determined. Importantly, IMT values in the CAPS population have been associated with emergent cardiovascular end points during follow up. A potential limitation of our study is the possibility that variants in TLR2 or TLR4 other than those we studied may play a role in atherosclerosis. However, we chose variants that have been implicated functionally in previous studies and associated with altered inflammatory responses and in the case of TLR4, which have been previously associated with cardiovascular disease. A further limitation of the progression part of our study is that there was only a small increase in IMT over the 3-year follow-up period, and measurement error may further reduce the ability to detect associations. However, we have previously found associations between IMT progression over the same period and conventional risk factors, and the results were consistent with those with baseline IMT, which represents lifelong exposure to risk factors.

In conclusion, in this large community population, we found no evidence that genetic variants in either TLR2 or TLR4 have emphasized the importance of gene–environment interactions. It has been suggested that a number of conventional cardiovascular risk factors such as smoking, diabetes, and obesity may act, at least in part, by inducing a chronic inflammatory response. Consistent with this, interactions between these risk factors and a number of cytokine and other proinflammatory genes have been reported. In these studies, associations between the genetic variants and IMT itself were weak or not significant but became highly significant once interactions with proinflammatory environmental risk factors were accounted for. In view of the role of TLRs in the inflammatory process, one might hypothesize that similar interactions between the TLR genes and chronic infections on the one hand and conventional proinflammatory risk factors on the other hand would be important. However, we found no evidence of any interactions between the polymorphisms studied and hs-CRP, smoking, diabetes, or obesity with either baseline or progression IMT.
TLR4 are risk factors for increased IMT either directly or through interaction with proinflammatory cardiovascular risk factors.

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Disclosures
None.

References
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