Genetic and Acquired Inflammatory Conditions Are Synergistically Associated With Early Carotid Atherosclerosis

Hugh S. Markus, DM, FRCP; Robyn Labrum, PhD; Steve Bevan, PhD; Markus Reindl, MD; Georg Egger, MD; Christian J. Wiedermann, MD; Qingbo Xu, PhD; Stefan Kiechl, MD; Johann Willeit, MD

Background and Purpose—If chronic inflammation plays a causal role in atherogenesis, individuals with proinflammatory gene variants would be expected to develop more atherosclerosis. We recently found a synergistic association between 3 functional proinflammatory gene polymorphisms/haplotypes and smoking on carotid intima-media thickness (IMT). We replicated this finding in a second large population and extended the analysis by inclusion of other inflammatory conditions (chronic infection and obesity/abnormal glucose tolerance).

Methods—Common carotid and femoral artery IMT was determined in the Bruneck Study population (n=810). Proinflammatory variants were determined in 3 genes (IL-6 [−174C, −572G, −597A haplotype], IL-1–receptor antagonist [VNTR *2], and endotoxin receptor CD-14 [−159C]).

Results—There was a significant relationship between gene-variant score and carotid IMT: age- and sex-adjusted mean IMT in subjects with 0, 1, and ≥2 gene variants was 936, 987 and 1047 μm, respectively (P=0.001), and synergistic effects of gene-variant score and smoking on IMT measurements (P=0.040). Analogous findings were obtained for obesity/abnormal glucose tolerance and chronic infection. Interactive effects of gene-variant score and a risk factor score composed of the acquired inflammatory conditions were highly significant (P<0.001 each). Results were similar for femoral artery IMT.

Conclusions—These results provide support for a causal role of inflammation in carotid atherosclerosis, and emphasize the importance of gene-gene and gene-environment interactions in this pathogenic pathway. This may help to explain the substantial variability of disease expression in subjects with proinflammatory risk factors such as smoking, diabetes and chronic infection. (Stroke. 2006;37:2253-2259.)

Key Words: atherosclerosis ■ carotid artery ■ genetics ■ inflammation ■ risk factors

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From the Centre for Clinical Neuroscience (H.S.M., R.L., S.B.) and John Parker Chair of Vascular Biology (Q.X.), Department of Cardiac and Vascular Sciences, St George’s University of London, UK; the Department of Neurology (M.R., S.K., J.W.), Innsbruck Medical University, Innsbruck, Austria; the Department of Internal Medicine (C.J.W.), Innsbruck Medical University, Innsbruck, Austria; and the Department of Internal Medicine (G.E.), Bruneck Hospital, Bruneck, Italy.

Correspondence to Professor Hugh Markus, Centre for Clinical Neuroscience, St George’s University of London, London, SW17 ORE. E-mail hmarkus@sgul.ac.uk.

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ularly in individuals with the inflammatory environmental stimulus smoking. A weak overall relationship was found to exist between the number of proinflammatory gene variants (gene-variant score) and common carotid IMT. More excitingly there was a strong synergistic effect of gene-variant score and smoking on IMT.

In genetic association studies replication in an independent population is essential to reduce the possibility of spurious associations and is sometimes considered even more implausible than the original work. Therefore, we have performed replication in a second large population-based survey of carotid atherosclerosis, the Bruneck Study. In a second step we extended the analysis to examine associations with additional inflammatory states (chronic infection and obesity/abnormal glucose tolerance), and with IMT in another vascular territory, the femoral artery.

Methods

Study Subjects

The Bruneck Study is a prospective population-based survey, in a white population from Northern Italy, of the epidemiology and pathogenesis of atherosclerosis.9–10 At the 1990 baseline evaluation, the study population was recruited as a sex- and age-stratified random sample of all inhabitants of Bruneck (Bolzano Province, Italy) 40 to 79 years (125 women and 125 men in the fifth to eighth decades each; n=1000). A total of 93.6% participated, with data assessment completed in 919 subjects. During 1990 and the re-evaluation in 1995, a subgroup of 63 individuals died or moved away. In the remaining population, follow-up was 96.5% complete (n=826). The current evaluation focused on the 1995 examination. The study protocol was approved by the appropriate ethics committees, and all study subjects gave their written informed consent before entering the study.

Clinical History and Examination

Current and past smoking status was assessed in each subject. Regular alcohol consumption was quantified in terms of grams per day. Hypertension was defined as blood pressure (mean of 3 measurements) ≥140/90 mm Hg or the use of antihypertensive drugs. Diabetes mellitus (DM) was coded as present for fasting glucose levels ≥140 mg/dL (7.8 mmol/L) or a 2-hour value (oral glucose tolerance test) ≥200 mg/dL (11.1 mmol/L; WHO definition). Impaired glucose tolerance (IGT) was defined by fasting glucose levels <140 mg/dL and a 2-hour value ≥140 mg/dL and <200 mg/dL. High, moderate, and low socioeconomic status were assumed if the subject had ≥12, 11 to 9, or ≤8 years of education or the average monthly income of the person with the highest income in the household was ≥$2000, $1000 to 2000, or ≤$1000, respectively.

To identify subjects with chronic infective and inflammatory conditions we began an extensive screening consisting of 2 phases.11 The first step involved a detailed self-reported medical andmedication history, thorough clinical examination, spirometry, extensive laboratory evaluations including urinary analysis, and a review of the Bruneck Hospital databases and other medical records. If the data were inconclusive, in a second step individuals were referred for further examinations. The diagnosis of common chronic infections, as previously described,11 was established according to standard diagnostic criteria by an expert committee including specialists from various medical fields. The individual diseases defined as chronic infection were: chronic obstructive disease 141, chronic bronchitis 86, chronic upper respiratory infection 3, recurrent/chronic urinary infection 34, periodontitis 19, recurrent/chronic skin infection 14 and chronic pancreatitis/diverticulitis 7. Some individuals had more than one condition and, therefore, the total is greater than the number of subjects (261) with any total with chronic infection.

Laboratory Methods

Blood samples were drawn after an overnight fast. In subjects with acute infection, blood drawing was delayed for ≥6 weeks, ie, until ≥4 to 5 weeks after recovery from infectious illness. High-density lipoprotein cholesterol was determined enzymatically (CHOD-PAP method, Merck; coefficient of variation (CV) 2.2% to 2.4%). Low-density lipoprotein cholesterol was calculated with the Friedewald formula except in subjects with triglycerides >4.52 mmol/L in whom it was directly measured. IL-6 was measured by an enzyme amplified sensitivity immunoassay (Medgenics) with standards performed in duplicates and samples as singles; assay sensitivity was 3 pg mL−1, interassay CV below 8% (2.2% to 7.5%) and intra-assay CV below 6% (4.4% to 5.6%). Other parameters were assayed by standard methods.9–10

The following polymorphisms previously studied in the CAPS population were determined, namely IL-6 to 174, −572 and −592 on chromosome 7p21, IL-1 receptor antagonist variable number tandem repeat (IL-1RN-VNTR) on chromosome 2q14, and the endotoxin receptor CD14−159 on chromosome 5q31. The IL-6 polymorphisms IL-1RN VNTR and CD14−159 polymorphisms were genotyped as in the previous study.8 Genotypes were assigned by investigators blinded to subject identity.

Scanning Protocol and Definition of Ultrasound End Points

The ultrasound protocol involves the scanning of the right and left internal (bulbous and distal segments) and common carotid arteries (CCA; proximal and distal segments), and of the femoral arteries 40 mm proximal and 10 mm distal to the bifurcation into the superficial and deep branches. Scanning was performed with a 5-MHz imaging probe and a 5-MHz Doppler probe.11 IMT was measured at plaque-free sections of the far wall of the femoral and CCA (intra-observer coefficient of variation, 7.9%, n=100) as the distance between the lumen-intima and media-adventitia interfaces.11 In the analysis the mean maximum IMT of the left and right arteries was used.

Statistical Analysis

Data were analyzed using SPSS. Analysis was performed in 2 parts. First, a replication of the previous associations found in the CAPS study using an identical statistical approach to the initial study was per-
formed. The hypothesis tested was whether the number of proinflammatory gene variants, for this study the IL-6 haplotype, the IL1RN VNTR and the CD14 SNP, is associated with increasing IMT, and whether there was an interaction with smoking. Second, we extended the model as outlined above. Generalized linear models were used to determine relationships between genotypes and levels of IMT, IL-6 and other markers of inflammation. Haplotype frequencies were estimated using Phase 2.0 using the default parameters with the additional step of 10 iterations for the final estimation. Phase 2 gives probabilities for the correctness of haplotype allocation; 96.3% had a probability >90%.

Those genotypes/haplotypes that were associated in the original study with higher IL-6 levels were designated “inflammatory haplotypes.” Alleles associated with increased levels were: IL-6, −174C, −572G and −597A, IL-1RN *2 and CD-14 −159C. To model the effects of increasing gene-variant score of inflammatory genotypes on IMT, a gene-variant score was calculated indicating the number of proinflammatory variants for which the individual was homozygous with a range of 0 to 2, where 2 represented individuals homozygous for ≥2 inflammatory genotypes/haplotypes and 0 homozygous for none. Age- and sex-adjusted and multivariate analyses adjusting for age, sex and vascular risk factors were performed as in the CAPS Study. Interactions between genetic and acquired inflammatory conditions were determined by entering the interactions terms in the regression model. All probability values were 2-sided.

### Results

Baseline characteristics of the 810 individuals are shown in Table 1: 368 (45.4%) were current/ex-smokers, 198 (24.4%), had obesity/IGT/DM, and 261 (32.2%) chronic infection. Allele and haplotype frequencies (Table 2) were similar to those obtained in the CAPS population. All genotypes tested were in Hardy-Weinberg equilibrium.

### Replication Study

The number of proinflammatory gene variants (gene-variant score) was 0 in 592 (73.1%), 1 in 197 (24.3%) and ≥2 in 21

### Table 3. Replication Study: CCA IMT According to the Gene-Variant Score and Smoking Status

<table>
<thead>
<tr>
<th>Gene-Variant Score</th>
<th>Nonsmokers</th>
<th>Current/Exsmokers</th>
<th>Nonsmokers</th>
<th>Current/Exsmokers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Mean (95% CI)*</td>
<td>n</td>
<td>Mean (95% CI)*</td>
</tr>
<tr>
<td>0</td>
<td>318</td>
<td>918 (894–941)</td>
<td>274</td>
<td>957 (931–982)</td>
</tr>
<tr>
<td>1</td>
<td>117</td>
<td>941 (903–979)</td>
<td>80</td>
<td>1054 (1008–1100)</td>
</tr>
<tr>
<td>≥2</td>
<td>7</td>
<td>947 (782–1112)</td>
<td>14</td>
<td>1089 (985–1193)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Gene variant score</th>
<th>P=0.001</th>
<th>Smoking</th>
<th>P=0.007</th>
<th>Interaction</th>
<th>P=0.071</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smoking</td>
<td>P=0.002</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Interaction</td>
<td>P=0.040</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Means (μm) and 95% CI adjusted for age and sex; ‡means (μm) adjusted for age, sex, social status, alcohol consumption, body mass index, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol and hypertension.
There was a highly significant relationship between gene-variant score and CCA IMT: age- and sex-adjusted mean CCA IMT in subjects with 0, 1 and ≥2 gene variants was 936, 987 and 1047 μm, respectively (P < 0.001).

There was a significant interaction between gene-variant score and smoking on carotid IMT (Table 3).

Extension Study
In the extension study there were highly significant interactions with the additional proinflammatory risk factor obesity/IGT/DM and chronic infection (Table 4). An inflammatory risk factor score was constructed from the number of inflammatory risk factors: smoking, obesity/IGT/DM and chronic infection. This score was 0 in 256 (31.6%), 1 in 326 (40.2%) and ≥2 in 228 (28.1%) subjects. As to potential effects on CCA IMT, there was a highly significant interaction (P < 0.001) between the gene-variant score and the risk-factor score (Figure, A; P < 0.001): age-, sex- and risk factor–adjusted mean CCA IMT (95% CI) values increased with the number of inflammatory risk conditions (0, 1 and ≥2). In subjects with a gene-variant score of 0, values were 933 μm (903 to 962), 925 μm (900 to 950), and 960 μm (929 to 991), respectively. However, the increase in IMT with increasing gene-variant score was steeper in subjects with a gene-variant score of 1, being 906 μm (859 to 953), 995 μm (950 to 1040), and 1061 μm (1008 to 1114), and even more accentuated in those with a gene-variant score of ≥2, being 866 μm (644 to 1087), 890 μm (779 to 1001) and 1443 μm (1285 to 1600), respectively.

Similar results were found with femoral artery IMT. Age- and sex-adjusted femoral artery IMT in subjects with a gene-variant score of 0, 1 or ≥2 was 928, 946 and 1016 μm respectively (P = 0.082). There was a significant interactive effect of gene-variant score and smoking on IMT, and significant interactions were also seen for obesity/IGT/DM and chronic infection (Table 5). Again, there was a strong interaction (P < 0.001) between gene-variant score and inflammatory risk score (Figure, B). Age-, sex- and risk factor–adjusted mean femoral artery IMT (95% CI) values increased with the number of inflammatory risk conditions, and again the slope of the relationship increased as gene-variant score increased. Values for those with risk-factor

<table>
<thead>
<tr>
<th>Gene-Variant Score</th>
<th>No Chronic Infection</th>
<th>Chronic Infection</th>
<th>Multivariate Analysis</th>
</tr>
</thead>
<tbody>
<tr>
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<td>n Mean (95% CI)*</td>
<td>n Mean (95% CI)‡</td>
</tr>
<tr>
<td>0</td>
<td>408 928 (908–949)</td>
<td>184 949 (918–981)</td>
<td>408 933 (913–953)</td>
</tr>
<tr>
<td>1</td>
<td>128 942 (906–978)</td>
<td>69 1058 (1009–1108)</td>
<td>128 939 (904–974)</td>
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<tr>
<td>≥2</td>
<td>13 877 (766–988)</td>
<td>8 1322 (1180–1464)</td>
<td>13 871 (764–979)</td>
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</table>

<table>
<thead>
<tr>
<th>Gene-Variant Score</th>
<th>No Obesity/IGT/DM</th>
<th>Obesity/IGT/DM</th>
<th>Multivariate Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n Mean (95% CI)*</td>
<td>n Mean (95% CI)‡</td>
<td>n Mean (95% CI)‡</td>
</tr>
<tr>
<td>0</td>
<td>442 930 (911–949)</td>
<td>150 952 (919–985)</td>
<td>442 938 (920–957)</td>
</tr>
<tr>
<td>1</td>
<td>152 977 (944–1009)</td>
<td>45 1025 (965–1085)</td>
<td>152 977 (946–1008)</td>
</tr>
<tr>
<td>≥2</td>
<td>18 922 (829–1015)</td>
<td>3 1797 (1568–2026)</td>
<td>18 911 (820–1001)</td>
</tr>
</tbody>
</table>

*Means (μm) adjusted for age and sex; ‡means (μm) adjusted for age, sex, social status, alcohol consumption, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol and hypertension.
scores of 0, 1 and ≥2 were: 888 μm (858 to 918), 932 μm (907 to 957), and 953 μm (922 to 985), respectively, in subjects with a gene-variant score of 0; 858 μm (810 to 905), 925 μm (879 to 971) and 1065 μm (1011 to 1118) in those with a gene variant score of 1; 803 μm (580 to 1026), 899 μm (787 to 1011), and 1369 μm (1210 to 1528) for a score of ≥2.

All associations were consistently found in men and women. Further adjustment for statins and other common types of medication, and for other potential vascular risk factors (lipoprotein-a ferritin, fibrinogen and microalbuminuria) had virtually no effect on the results obtained (data not shown).

The contribution of individual haplotypes/genotypes to the relationship was explored (Table 6). All individual components were associated with IMT and showed a synergism with inflammatory factors except for IL-1RN with carotid IMT (although associations were present with femoral IMT) and CD14 with femoral artery (although associations were present with carotid IMT).

Levels of IL-6 and other markers of inflammation increased with the number of proinflammatory gene variants, but only in the presence of inflammatory risk conditions (P<0.05 for interaction with all variables listed below). In detail, age- and sex-adjusted geometric means of IL-6 in subjects with inflammatory risk conditions and 0, 1 and ≥2 gene variants were 4.5, 6.9 and 7.2 pg/mL (P=0.006 for trend). Corresponding values for high-sensitivity C-reactive protein were 2.5, 3.6 and 6.2 mg/L (P=0.004 for trend), for osteoprotegerin 4.1, 4.3 and 6.2 pmol/L (P=0.007 for trend) and for fibrinogen 300, 329 and 335 mg/dL (P=0.069 for trend).

**Discussion**

This large population-based study confirms that proinflammatory variants in genes encoding components of the systemic inflammatory response are associated with early carotid atherosclerosis as assessed by IMT. We replicated a synergistic association of proinflammatory gene-variant score and smoking on carotid IMT as previously found in CAPS.8 The current study extends these findings to demonstrate similar interactions with obesity/IGT/DM, and with chronic infection. Furthermore, we replicated the previous findings in an independent vascular territory, the femoral artery. In analogy to CAPS, the proinflammatory gene-variant score was associated with higher IL-6 levels in individuals with inflammatory risk factors but not in those without. This population had a similar age and gender distribution to the CAPS population (supplemental Table I, available online at http://stroke.ahajournals.org) and was also white.8 Hypertension, current

**TABLE 5. Extension Study: Femoral Artery IMT According to the Gene-Variant Score and Various Inflammatory Risk Conditions**

<table>
<thead>
<tr>
<th>Gene-Variant Score</th>
<th>Age/Sex-Adjusted Analysis</th>
<th>Multivariate Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nonsmokers</td>
<td>Current/Exsmokers</td>
</tr>
<tr>
<td>0</td>
<td>n</td>
<td>Mean (95% CI)*</td>
</tr>
<tr>
<td>1</td>
<td>n</td>
<td>Mean (95% CI)*</td>
</tr>
<tr>
<td>≥2</td>
<td>n</td>
<td>Mean (95% CI)*</td>
</tr>
</tbody>
</table>

*Means (μm) adjusted for age and sex; †means (μm) adjusted for age, sex, social status, alcohol consumption, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol and hypertension.
smoking and diabetes were more common although mean BMI was lower.

Replication of our previous findings in a second large dataset, with a high level of significance, makes it likely that the associations between proinflammatory gene variants and IMT, and in particular the interaction with inflammatory risk factors, represent true findings rather than spurious associations. Adequate modeling of gene-environment interactions resulted in highly significant associations and prominent and important to recognize that drawing valid causal inferences from its application depends on more extensive assumptions than are required in randomized controlled trials. It has been implemented to determine causality in a number of disease states including cardiovascular disease and cancer, although it is important to recognize that drawing valid causal inferences from its application depends on more extensive assumptions than are required in randomized controlled trials. In our study, emergence of a strong association between IMT, a valid surrogate of vascular risk, and proinflammatory gene variants, and especially the synergistic effect of gene-variant score and inflammatory risk factors, provides support for a causal role of inflammation in atherosclerotic vascular disease.

Our findings lend further support to the hypothesis that inflammation and chronic infection play a causal role in cardiovascular disease. Many studies have shown that raised levels of circulating inflammatory markers, and particularly C-reactive protein, are prospectively associated with increased cardiovascular disease risk. This does not necessarily imply causality but may be an expression of subclinical disease (atherosclerosis) usually present years before the symptomatic event (reverse causality). Genetic association studies and the principle of Mendelian randomization are approaches suitable to resolve whether associations are causal, or represent confounding attributable to reverse causality. Such an approach was first used to determine whether the relationship between low-serum cholesterol and cancer could be attributable to reverse causation, with presymptomatic tumors reducing cholesterol levels. It has been implemented to determine causality in a number of disease states including cardiovascular disease and cancer, although it is important to recognize that drawing valid causal inferences from its application depends on more extensive assumptions than are required in randomized controlled trials. In our study, emergence of a strong association between IMT, a valid surrogate of vascular risk, and proinflammatory gene variants, and especially the synergistic effect of gene-variant score and inflammatory risk factors, provides support for a causal role of inflammation in atherosclerotic vascular disease.

Our results also support the view that inflammation acts as a mediator of the increased cardiovascular disease risk seen in smokers, chronic infection and obesity/IGT/DM and assists in identifying susceptibility genes for atherosclerosis in subjects with these risk factors.

The choice of polymorphism in this study was the same as in CAPS except for the IL-1β-31 polymorphism which in CAPS was combined in a haplotype with the IL1-RN polymorphism, but was not considered in the Bruneck Study.
because of a very low frequency in this population. All the proinflammatory gene variants had been associated with increased IL-6 levels in the presence of acquired inflammatory conditions. Initial studies suggested the G, rather than C, allele of the IL-6 to 174 polymorphism was associated with increased IL-6 levels, but further studies from the same group found increased levels associated with the C allele as we found in both the CAPS and Bruneck populations.

In conclusion, these results provide further support for a causal role of chronic inflammation in atherosclerosis, and emphasize the importance of gene-environment interactions in this pathogenic pathway. The associations we report are with IMT and although IMT is a marker of atherosclerosis, it may also relate to other pathologies. Therefore, it is important these finding are replicated in patients with clinical end points or more advanced atherosclerosis with plaque or stenosis on imaging.

**Disclosures**

None.

**References**

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