Variants of Toll-Like Receptor 4 Modify the Efficacy of Statin Therapy and the Risk of Cardiovascular Events

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Background—Atherosclerosis is increasingly considered to be a chronic inflammatory process. We examined whether genetic variants of the toll-like receptor 4 (TLR4), which are correlated with impaired innate immunity and with progression of carotid atherosclerosis, are also associated with coronary atherosclerosis and predict the risk of cardiovascular events.

Methods and Results—Two polymorphisms of the TLR4 gene (Asp299Gly and Thr399Ile) were determined in 655 men with angiographically documented coronary atherosclerosis. All patients participated in a prospective cholesterol-lowering trial evaluating the effect on coronary artery disease and were randomly assigned to either pravastatin or placebo for 2 years. There were no significant differences between genetically defined subgroups with respect to baseline risk factors, treatment, or in-trial changes of lipid, lipoprotein, or angiographic measurements. Genotype was not associated with progression of atherosclerosis. In the pravastatin group, 299Gly carriers had a lower risk of cardiovascular events during follow-up than noncarriers (2.0% versus 11.5%, \( P=0.045 \)). Among noncarriers, pravastatin reduced the risk of cardiovascular events from 18.1% to 11.5% (\( P=0.03 \)), whereas among 299Gly carriers this risk was strikingly reduced from 29.6% to 2.0% (\( P=0.0002, P=0.025 \) for interaction).

Conclusions—Among symptomatic men with documented coronary artery disease, the TLR4 Asp299Gly polymorphism was associated with the risk of cardiovascular events. This variant also modified the efficacy of pravastatin in preventing cardiovascular events, such that carriers of the variant allele had significantly more benefit from pravastatin treatment.

Key Words: atherosclerosis ■ statins ■ cardiovascular diseases ■ inflammation ■ receptors
associated with reduced extent and progression of carotid atherosclerosis as quantified by B-mode ultrasound.

Inflammation may not only play a role in the progression of early atherosclerosis but may be important in advanced atherosclerosis by determining the stability of atherosclerotic plaques and their proneness to rupture. Compared with lesions causing stable angina, the plaques of patients with acute coronary syndromes contain considerably more inflammatory cells. In particular, the immediate vicinity of the site of plaque rupture is invariably infiltrated by an inflammatory process. Furthermore, C-reactive protein (CRP), a marker of inflammation, has been identified as an independent predictor of death and cardiovascular events in patients with stable and unstable angina, high-risk individuals, and apparently healthy individuals. Statin therapy is most effective in patients with elevated CRP levels, which underscores the hypothesis that statins, beside lipid-lowering effects, have plaque-stabilizing and anti-inflammatory effects.

We hypothesized that TLR4 Asp299Gly and the Thr399Ile polymorphisms would be associated, first, with progression of coronary atherosclerosis, as documented by quantitative coronary angiography, and, second, with the risk of cardiovascular events by affecting plaque stability. Finally, we hypothesized that carriers of the variant allele would respond differently to statin therapy than would noncarriers. We tested these hypotheses in a group of patients with symptomatic CAD who were included in the REGRESS study.

Methods

Study Design

The REGRESS study and design have been described previously. Briefly, REGression GRowth Evaluation Statin Study (REGRESS) was a randomized, placebo-controlled, multicenter study designed to assess the effect of 2 years of treatment with 40 mg pravastatin on progression and regression of angiographically documented coronary atherosclerosis in 885 male patients with a normal to moderately raised serum cholesterol, for example, between 4 and 8 mmol/L (155 to 310 mg/dL), and triglyceride levels <4.0 mmol/L (354 mg/dL). Patients were randomly assigned to receive 40 mg pravastatin once daily or matching placebo. Patients and physicians were blinded to the random assignment throughout the study. A number of substudies were performed in addition to the main angiographic study, including specialized lipid and lipoprotein and genetic studies.

Clinical Outcome Measures

Coronary angiograms were analyzed quantitatively by the Cardiovascular Measurement System (CMS, MEDIS Medical Imaging Systems). The quantitative coronary arteriographic procedures have been described in detail previously. Primary end points were the change in average minimal obstruction diameter (MOD) per patient and the change in average mean segment diameter (MSD) per patient. Change in MOD mainly reflects focal progression-regression of atherosclerosis, and change in MSD mainly reflects diffuse progression-regression of atherosclerosis. If a segment or lesion was adequately visualized in two (preferably orthogonal) projections and free of significant foreshortening in both views, the average values of the parameters in both projections were calculated. To calculate average MOD and MSD per patient, the MOD and MSD of all qualifying segments or obstructions were added and divided by the number of contributing segments or obstructions.

The following clinical events (according to prespecified criteria) were analyzed during the study and identified before unblinding: myocardial infarction (fatal or nonfatal); coronary heart disease death (other than known fatal myocardial infarction); nonscheduled PTCA or CABG; stroke and transient ischemic attack; and death (all other causes).

Biochemical and DNA Analyses

Total cholesterol, HDL, and triglycerides were measured on fasting blood samples at the Lipid Reference Laboratory, as published previously. LDL was calculated according to the Friedewald formula.

Genomic DNA was extracted according to a standard protocol. PCR amplification was performed on 1 μL DNA in 10 μL ReddyMix (ABgene) according to methods previously described. Researchers and laboratory personnel had no access to identifiable information and could identify samples by number only.

Statistical Analysis

Within each of the treatment groups, the assumption of Hardy-Weinberg equilibrium was tested by means of gene counting and χ² analysis.

Patients were classified according to genotype combination (normal TLR4; 299Gly-carriership in the presence of 399Ile-carriership; or 299Gly-carriership in the absence of 399Ile-carriership). These groups were compared with respect to relevant baseline characteristics, lipid and lipoprotein concentrations, in-trial changes of lipid and lipoprotein concentrations, and angiographic parameters. Differences in baseline parameters were analyzed with the independent samples t test, 1-way ANOVA, or the Pearson’s χ² test where appropriate. Since triglyceride concentrations had a skewed distribution, the statistical analyses were based on log-transformed data. Changes in lipid concentrations, lipoprotein concentrations, and angiographic measurements during the trial were compared by 1-way ANCOVA, with baseline values as covariates. The interaction between genotype and treatment (placebo or pravastatin) was tested with the interaction test of 2-way ANCOVA, with genotype and treatment as fixed factors and baseline values as covariates. Differences in the rate of events were illustrated with Kaplan-Meier curves and compared by means of a log-rank test. We further used the Cox regression model to test for an interaction between the TLR4 genotype and treatment (pravastatin or placebo).

Results

Frequency of TLR4 Asp299Gly and Thr399Ile Polymorphisms

Of the 885 patients enrolled in REGRESS, DNA was available from 670 individuals. Of these, 655 could be genotyped for both the Asp299Gly and the Thr399Ile polymorphisms. These 655 individuals did not differ significantly in any baseline parameter from the original 885 patients. In the entire cohort, 78 individuals carried the 299Gly variant allele and 69 carried the 399Ile variant allele. The three common genotype combinations (normal TLR4; 299Gly-carriership in the presence of 399Ile-carriership; and 299Gly-carriership in the absence of 399Ile-carriership) were found at frequencies of 0.88, 0.11, and 0.014, respectively. These frequencies did not differ significantly between the two treatment groups (data not shown). For the placebo group, the pravastatin group, and the total cohort, the observed allele frequencies were in Hardy-Weinberg equilibrium.

Baseline Characteristics

When patients were classified according to TLR4 genotype combination, there were no statistically significant differences with respect to CAD risk factors or treatment (Table 1). The individuals with isolated 299Gly-carriership had more severe CAD than individuals with other genotypes, as evidenced by a higher proportion of individuals with 2-vessel...
disease. However, there were only 9 individuals with this genotype, so the data must be interpreted with caution. In both groups, approximately half of the patients were randomly assigned to pravastatin treatment.

**In-Trial Changes of Lipid and Lipoprotein Concentrations and Angiographic Parameters**

The TLR4 genotype did not affect in-trial changes of total cholesterol, LDL cholesterol, triglycerides, or HDL cholesterol (Table 2). Pravastatin affected total cholesterol, LDL, HDL, and triglycerides to a similar extent in all genetic subgroups. Changes in angiographic measurements were also not significantly affected by the Asp299Gly polymorphism, with similar effects of pravastatin treatment in the genetic subgroups.

**Risk of Cardiovascular Events**

Carriership of the 299Gly allele did not significantly affect the risk of cardiovascular events in the entire cohort, when compared with noncarriers (11.5% versus 14.9%, \( P = 0.58 \)) (Table 3). However, in the pravastatin group, carriers of the variant 299Gly-allele had a significantly lower risk of cardiovascular events than noncarriers (2.0% versus 11.5%, \( P = 0.045 \)). Pravastatin reduced the risk of cardiovascular events in the entire cohort by 50% (19.0% versus 10.0%, \( P = 0.0007 \)). Strikingly, among noncarriers, the risk of cardiovascular events was reduced from 18.1% to 11.5% (\( P = 0.03 \), whereas among 299Gly carriers, the risk was reduced significantly more from 29.6% to 2.0% (\( P = 0.0002 \)) (Figure 1).

Testing for interaction between the Asp299Gly genotype and treatment group revealed that the efficacy of pravastatin in reducing the time to first cardiovascular event was significantly different between the genetic groups (\( P = 0.025 \)).

Subdivision of the group of 299Gly-carriers according to carriership of the 399Ile variant allele resulted in genotype groups that were too small to detect significant differences and interactions. The least common genotype combination (299Gly-carriership in the absence of 399Ile-carriership)

### TABLE 1. Baseline Characteristics, Severity of Coronary Atherosclerosis, and Treatment According to Toll-Like Receptor 4 Genotype

<table>
<thead>
<tr>
<th></th>
<th>299AspAsp (n=577)</th>
<th>299Gly-Positive 399Ile-Positive (n=69)</th>
<th>299Gly-Positive 399Ile-Negative (n=9)</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Demographic</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age, y</td>
<td>56±8</td>
<td>56±8</td>
<td>57±9</td>
<td>0.76</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>26±3</td>
<td>26±3</td>
<td>25±2</td>
<td>0.84</td>
</tr>
<tr>
<td>Systolic blood pressure, mm Hg</td>
<td>135±18</td>
<td>136±20</td>
<td>130±12</td>
<td>0.69</td>
</tr>
<tr>
<td>Diastolic blood pressure, mm Hg</td>
<td>82±10</td>
<td>80±10</td>
<td>78±8</td>
<td>0.22</td>
</tr>
<tr>
<td>Current or former smoker, n (%)</td>
<td>509 (88)</td>
<td>58 (84)</td>
<td>7 (78)</td>
<td>0.43</td>
</tr>
<tr>
<td>Current smoker, n (%)</td>
<td>166 (29)</td>
<td>14 (20)</td>
<td>2 (22)</td>
<td>0.31</td>
</tr>
<tr>
<td>Total cholesterol, mmol/L</td>
<td>6.0±0.9</td>
<td>6.0±0.7</td>
<td>5.9±0.9</td>
<td>0.85</td>
</tr>
<tr>
<td>LDL cholesterol, mmol/L</td>
<td>4.3±0.8</td>
<td>4.3±0.6</td>
<td>4.3±0.9</td>
<td>0.99</td>
</tr>
<tr>
<td>HDL cholesterol, mmol/L</td>
<td>0.9±0.2</td>
<td>0.9±0.2</td>
<td>0.9±0.2</td>
<td>0.78</td>
</tr>
<tr>
<td>Triglycerides, mmol/L</td>
<td>1.67 (0.49–4.03)</td>
<td>1.70 (0.46–3.92)</td>
<td>1.10 (0.63–2.32)</td>
<td>0.22</td>
</tr>
<tr>
<td>Coronary artery disease</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>History of myocardial infarction, n (%)</td>
<td>279 (48)</td>
<td>28 (41)</td>
<td>5 (56)</td>
<td>0.43</td>
</tr>
<tr>
<td>Left ventricular ejection fraction, %</td>
<td>70±12</td>
<td>71±14</td>
<td>63±15</td>
<td>0.46</td>
</tr>
<tr>
<td>Mean segment diameter, mm</td>
<td>2.80±0.48</td>
<td>2.87±0.41</td>
<td>2.66±0.63</td>
<td>0.24</td>
</tr>
<tr>
<td>Minimal obstruction diameter, mm</td>
<td>1.89±0.55</td>
<td>1.91±0.39</td>
<td>1.91±0.51</td>
<td>0.96</td>
</tr>
<tr>
<td>Stenosis, %</td>
<td>35±13</td>
<td>33±8</td>
<td>33±8</td>
<td>0.38</td>
</tr>
<tr>
<td>Coronary artery disease, n (%)</td>
<td>249 (43)</td>
<td>32 (46)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>1 Vessel</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 Vessels</td>
<td>195 (34)</td>
<td>22 (32)</td>
<td>7 (78)</td>
<td>0.014</td>
</tr>
<tr>
<td>3 Vessels</td>
<td>131 (23)</td>
<td>15 (22)</td>
<td>2 (22)</td>
<td></td>
</tr>
<tr>
<td>Medication</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nitrates, n (%)</td>
<td>311 (54)</td>
<td>38 (55)</td>
<td>4 (44)</td>
<td>0.83</td>
</tr>
<tr>
<td>( \beta )-Blockers, n (%)</td>
<td>432 (75)</td>
<td>50 (72)</td>
<td>8 (89)</td>
<td>0.57</td>
</tr>
<tr>
<td>Calcium channel blockers, n (%)</td>
<td>343 (59)</td>
<td>43 (62)</td>
<td>5 (56)</td>
<td>0.87</td>
</tr>
<tr>
<td>ACE inhibitors, n (%)</td>
<td>61 (11)</td>
<td>8 (12)</td>
<td>1 (11)</td>
<td>0.97</td>
</tr>
<tr>
<td>Diuretics, n (%)</td>
<td>58 (10)</td>
<td>10 (14)</td>
<td>0 (0)</td>
<td>0.31</td>
</tr>
<tr>
<td>Platelet aggregation inhibitors, n (%)</td>
<td>327 (57)</td>
<td>33 (48)</td>
<td>7 (78)</td>
<td>0.16</td>
</tr>
</tbody>
</table>

Data are shown as mean±SD or as n (%). The statistical analysis for triglyceride levels was performed on log-transformed values, but untransformed median (minimum-maximum) values are given in the table.
occurred only 9 times (3 on placebo, 6 on pravastatin). Although the prevalence of cardiovascular events appeared similar in the 399-positive and 399-negative group, no conclusions can be made.

**Discussion**

We examined whether the Asp299Gly and Thr399Ile polymorphisms of the TLR4 gene influenced the progression of coronary atherosclerosis and the risk of cardiovascular events in a large cohort of men with symptomatic CAD. Our results revealed an important interaction between these genetic variants and pravastatin treatment on the risk of cardiovascular events. In particular, the efficacy of pravastatin treatment in preventing cardiovascular events was significantly higher in 299Gly carriers than in noncarriers. This observation builds on previous reports in which we attempted to identify genetic factors that affect the clinical presentation of the patients and their responses to pravastatin therapy.26–28

**TABLE 3. Incidence of Cardiovascular Events According to Toll-Like Receptor 4 Genotype and Treatment**

<table>
<thead>
<tr>
<th>Genotype and Treatment</th>
<th>Placebo</th>
<th>Pravastatin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo</td>
<td>18.1% (54/299)</td>
<td>14.9% (14/29)</td>
</tr>
<tr>
<td>Pravastatin</td>
<td>11.5% (32/278)</td>
<td>11.5% (32/278)</td>
</tr>
</tbody>
</table>

**Frequency of TLR4 Asp299Gly and Thr399Ile Polymorphisms**

Genotyping of study participants revealed that the allele frequency of the 299Gly variant was 5.9% in the REGRESS cohort. In the Bruneck study, the allele frequency was 3.5%, and in three other populations they were reported to be 6.6%, 7.9%, and 3.3%. These allele frequencies are all within the expected range. In addition, the frequencies of the three common genotype combinations (normal TLR4; 299Gly carriership in the presence of 399Ile-carriership; and 299Gly carriership in the absence of 399Ile-carriership) were similar to those described in previous reports. Thus, the genotype combinations that were associated with decreased progression of carotid atherosclerosis (299Gly-carriership with and without 399Ile-carriership) do not occur at a substantially lower frequency in the REGRESS cohort of men with symptomatic CAD than in random population samples.
parameters of arterial lumen size. In addition, the time span of 2 years may have been too short to detect a small difference.

Blunted inflammatory response leads to ineffective removal of infectious agents, which may lead to persistence or even progression of the inflammatory trigger. Persistent triggering of the innate immune system may be especially harmful in an atherosclerotic plaque in which abundant TLR4 is present to initiate an inflammatory response. It has been hypothesized that such an inflammatory response activates resident cells and macrophages in the atherosclerotic plaque. Systemic LPS administration yielded more proinflammatory cytokine gene expression in the aorta of rabbits with diet-induced atheroma than in rabbits without atheroma. Thus, at some point in the natural course of atherosclerosis, the beneficial effect of a blunted immunologic response in 299Gly carriers may be outweighed by persistent inflammatory triggering as the result of ineffective removal of the proinflammatory agent. This balance depends on the amount of TLR4 present and thus on the severity of atherosclerosis. In the REGRESS cohort of men with advanced atherosclerosis, this enhanced inflammatory response may have led to plaque inflammation and instability.

In summary, our observations would be explained by a model in which the extent of plaque inflammation, and thus the risk of plaque rupture, is determined by 3 factors: (1) the amount of inflammatory trigger that is capable of activating TLR4: This factor may be affected, in turn, by the efficacy of TLR4 in its removal; (2) the amount of TLR4 present in the vessel wall; and (3) the efficacy of TLR4 in mounting a local inflammatory response. This model would explain the striking reduction of cardiovascular events observed in 299Gly carriers through the use of statin therapy, compared with those who did not. Statins are known to reduce LDL cholesterol, and thus oxidized LDL, which is a potent upregulator of TLR4. Thus, in 299Gly carriers using statin therapy, a persistent inflammatory triggering caused by ineffective removal may have been negated by a reduction of TLR4 and a genotype-dependent inefficient initiation of local inflammation.

Limitations
There are several potential limitations to the present study. First, the events defined in REGRESS include a number of different clinical entities, for example, nonscheduled percutaneous or surgical revascularization, nonfatal myocardial infarction, and fatal myocardial infarction. Nevertheless, the large majority were events in which atherosclerotic plaque rupture and subsequent thrombotic occlusion are the underlying pathophysiological processes. The fact that these plaque rupture–related events led to a range of clinical outcomes may have depended on numerous other factors and does not negate our findings. Second, the conclusions obviously apply only to symptomatic men with documented CAD. However, this population is representative for the majority of male patients with CAD in the Netherlands.

Finally, the frequency of isolated 299Gly-carriership (ie, in the absence of 399Ile-carriership) was low in our cohort,
which is consistent with previous reports. These reports have suggested that the genotype at residue 399 influences the effect of the Asp299Gly polymorphism; 299Gly-carryer in the presence of 399Ile-carrier predicts decreased TLR4 function, but 299Gly-carryer in the absence of 399Ile-carrier yields a TLR4 protein that functions even worse. We did perform analyses to evaluate whether the interaction between Asp299Gly genotype and pravastatin treatment was affected by the Thr399Ile polymorphism as well. However, the results do not allow any conclusions because of the low frequency of this genotype combination. An even larger cohort of patients, or one with a higher prevalence of this genotype combination, will be required to address this issue.

Conclusions

We observed that among symptomatic men with documented CAD, the TLR4 Asp299Gly polymorphism was associated with the risk of cardiovascular events. This genetic variant also predicted the efficacy of pravastatin in preventing cardiovascular events such that carriers of the variant allele had substantially more benefit from pravastatin treatment. A substantial amount of experimental evidence exists regarding the role of inflammation in determining the risk of plaque rupture-related events. However, clinical data are limited. The relevance of our observation lies in the fact that it shows genetic variance in the innate immune system to be associated with the occurrence of plaque rupture-related clinical events. In addition, to our knowledge, this is the first observation of an interaction between genotype and statin treatment in reducing the risk of clinical cardiovascular events without an effect on lipid parameters.

Acknowledgments

The REGRESS study was sponsored by Bristol-Myers Squibb Co, Princeton, NJ. W.R.P. Agema was supported by the Netherlands Heart Foundation (grant 1999.210), The Hague, The Netherlands, and a grant from the Interuniversity Cardiology Institute of the Netherlands, Utrecht, The Netherlands. J.J.P. Kastelein is an Established Investigator from the Interuniversity Cardiology Institute of the Netherlands, The Hague, The Netherlands. J.J.P. Kastelein is an Established Investigator from the Interuniversity Cardiology Institute of the Netherlands, The Hague, The Netherlands. J.J.P. Kastelein is an Established Investigator from the Interuniversity Cardiology Institute of the Netherlands, The Hague, The Netherlands. J.J.P. Kastelein is an Established Investigator from the Interuniversity Cardiology Institute of the Netherlands, The Hague, The Netherlands.

References

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_Circulation._ 2003;107:2416-2421; originally published online May 12, 2003;
doi: 10.1161/01.CIR.0000068311.40161.28
_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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