Human CRP Gene Polymorphism Influences CRP Levels
Implications for the Prediction and Pathogenesis of Coronary Heart Disease


Objective—C-reactive protein (CRP) concentrations are predictive of cardiovascular disease, and levels are heritable, in part. We identified novel polymorphisms in the CRP gene and assessed their influence on CRP level.

Methods and Results—CRP was measured in 250 male army recruits before and after strenuous exercise and perioperatively in 193 coronary artery bypass graft (CABG) patients. Two novel polymorphisms were identified in the CRP gene, −717G>A in the promoter and +1444C>T in the 3′UTR. Among army recruits, CRP was higher in +1444TT homozygotes than +1444 C-allele carriers at baseline (1.04±0.38 versus 0.55±0.06, P=0.014) and at all time points after exercise (2.35±0.68 versus 1.07±0.12, 2.11±0.53 versus 0.88±0.09, and 1.77±0.44 versus 0.71±0.09, P=0.034, P=0.007, and P=0.013, at 2, 48, and 96 hours after exercise, respectively). In the CABG patients, mean CRP (mg/L) rose from 1.97±0.36 at baseline to 167.2±5.07 hours postoperatively. Genotype did not influence CRP at baseline; however, peak post-CABG CRP levels were higher in +1444TT homozygotes compared with +1444C-allele carriers (198±17 versus 164±5, P=0.03).

Conclusions—The CRP gene +1444C>T variant influences basal and stimulated CRP level. These findings have implications both for the prediction and pathogenesis of coronary heart disease. (Arterioscler Thromb Vasc Biol. 2003;23:2063-2069.)

Key Words: genetics ■ inflammation ■ C-reactive protein ■ coronary heart disease ■ risk factors

Inflammation is important in the initiation, progression, and clinical outcome of atherosclerosis.1 Prospective studies indicate association between the levels of C-reactive protein (CRP) and the long-term risk of cardiovascular disease.2–10 In healthy subjects in the steady state, CRP concentrations are 100- to 1000-fold lower than those found during acute infection and inflammation and lie in the range of 0.1 to 10 mg/L. Steady-state CRP concentrations are approximately 2-fold higher in patients with stable coronary heart disease (CHD).11,12 but when an acute coronary syndrome supervenes, CRP concentrations rise acutely, with the level attained predicting the probability of an additional CHD event in the short to intermediate term.13–17 CRP is present in atherosclerotic plaques,18 where it might exert several potential proinflammatory and atherogenic actions that include the binding of oxidized LDL cholesterol,19 induction of adhesion molecule expression,20 activation of complement,21 and stimulation of tissue factor production by monocytes.21 These observations suggest that CRP may be more than just a marker of inflammation and that it may also play an integral role in the pathogenesis of atherosclerosis.

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Although CRP might have an important role in the pathogenesis and prediction of CHD, the factors influencing its basal level and the concentrations achieved during inflammation are incompletely understood. Interleukin-6 (IL-6) is the main inflammatory cytokine stimulus for CRP.22 In the absence of acute inflammation, the concentration of CRP is stable,23 with the absolute level being influenced by age,24,25 sex,26 smoking,7 and body mass index (BMI).24,25 Family studies have also suggested that CRP level is a heritable trait,26 but the genes involved in this regulation are unknown. In this study we have tested the hypothesis that the human CRP gene itself contains common functional polymorphisms that influence CRP level basally and during inflammation.

Methods

Study Groups and Protocols

Studies were conducted in British army recruits studied at baseline and after 48 hours of strenuous physical activity (which induces a
mild inflammatory response) and patients with CHD around the time of coronary artery bypass graft (CABG) surgery, which elicits a major inflammatory response.

### Army Volunteer Exercise Study

After obtaining ethical approval from the Defense Medical Services Clinical Research Committee, recruits at the Army Training Regiment, Bassingbourn, UK, were enrolled at the start of an 11-week period of basic training. At the end of this period, recruits embarked on an intensive 48-hour final military endurance exercise (FME) that has been shown to induce an acute inflammatory response. A total of 250 consecutive white subjects were recruited. Venous blood samples were drawn from the antecubital vein at induction to basic training and then on 3 occasions (2, 48, and 96 hours) after return from the FME. Samples were centrifuged (3500g, 10 minutes), and plasma and cells were separated and frozen at −20°C until analysis.

### Coronary Artery Bypass Study

The study had hospital ethics committee approval, and written informed consent was obtained from all participants. All patients undergoing elective first-time CABG surgery at the Middlesex Hospital (London, UK) were invited to take part in the study. Subjects with evidence of a preexisting inflammatory state or unstable coronary artery disease were excluded. Subjects were also excluded who suffered potentially confounding infective postoperative complications or circulatory failure requiring inotropic support. Aspirin was omitted routinely 10 days before surgery. Blood samples were drawn preoperatively and then again daily for the first 5 postoperative days. These were centrifuged immediately (3500g, 10 minutes), and plasma and cells separated and frozen at −20°C until analysis.

### Assays of CRP, Interleukin-6, and Fibrinogen

C-reactive protein was measured on a BN Prospec (Dade Behring). Interassay and intraassay coefficients of variation were <4% and <2%, respectively, with a detection limit of 0.20 mg/L. IL-6 concentration was measured using a commercial assay (R&D Systems) by staff blind to all subject data. Interassay and intraassay coefficients of variation were 3% and 4%, respectively, with a detection limit of 0.70 pg/mL. Fibrinogen concentration was determined by a semiautomated Clauss assay in an MDA-180 coagulometer (Organon Teknika) using the manufacturer’s reagents and calibrated with the 7th British Standard (NIBSC).

### Polymorphism Screening of the Human CRP Gene

Polymorphisms were identified by single-strand conformation polymorphism analysis of heat-denatured DNA fragments generated by polymerase chain reaction (PCR) amplification of overlapping fragments from the gene from 32 unrelated individuals (primers in online data supplement). Mobility shifts were confirmed by sequencing on an ABI 377 sequencer.

### Genotyping of Polymorphisms

Two newly identified polymorphisms (−717G>A and +1444C>T) were genotyped by PCR and restriction fragment-length polymorphism analysis using primer pairs 5'-ACTGGACTTTTACTGAGGGC-3'/5'-ATTCCCATCTAATGTTAGGAAACT-3' and SacII for −717G>A and 5'-AGCTCGTAAACTATGCTGGGCGA-3'/5'-CTTCTCAGCTCTTCCCTATGATG-3' and Bsp 126I for +1444C>T (see online data supplement). In both cases, 1 of the primer sequences was mismatched to force an allele-specific restriction enzyme site into the PCR product. Additionally, a previously identified silent polymorphism in exon 2 (+1059G>C) was genotyped using the published method.

### Statistical Analysis

Nonnormal data were log-transformed before analysis, but data are presented as geometric means and approximate SDs for the sake of clarity. Differences in CRP between genotypes were assessed by ANOVA and by Student’s t tests for unpaired data. One-way ANCOVA was also performed with age, sex, BMI (kg/m²), smoking, diabetic status, therapy with statins or angiotensin-converting enzyme inhibitors, length of cardiopulmonary bypass, operation duration, and aortic cross-clamp time as covariates in the CABG data set and age, BMI, and smoking status as covariates in the army group. A χ² test was used to test for Hardy-Weinberg equilibrium. Linkage disequilibrium between sites was estimated using the method of Chakravarti et al. P<0.05 was considered to be statistically significant.

### Results

#### Identification of Polymorphisms

Approximately 4.5 kb of the CRP gene from 2075 bp upstream of the transcription start site to 996 bp downstream of exon 2 was screened. Two novel polymorphisms were identified, a −717G>A substitution in the gene promoter and a +1444C>T variant in the 3'UTR (the latter with reference to GenBank sequence M11880). These variants have been designated rs2794521 and rs1130864, respectively, on the NCBI SNP database (http://www.ncbi.nlm.nih.gov/SNP; accessed April 21, 2003). No coding region polymorphisms were identified on this screen, although a silent +1059G>C polymorphism has been reported previously.

#### Demographic Characteristics of Study Groups

### Army Recruits

Two hundred fifty white male army recruits were successfully enrolled. Baseline demographic characteristics of the study participants are shown in Table 1. Apart from a slightly higher basal IL-6 level among +1444TT homozygotes, there were no systematic differences between individuals of differing CRP genotype.

### CABG Patients

There were 349 elective first-time CABG cases, from which 275 were invited to participate and all but 5 agreed. Data from 27 subjects were removed before analysis (13 lacked baseline samples, 9 had additional surgical procedures at the time of CABG, 2 operations were done without cardiopulmonary bypass, and 3 patients did not undergo surgery). Two hundred forty-three subjects were successfully recruited into the study. One hundred sixty-nine (69%) were taking a statin and 69

### TABLE 1. Baseline Demographic Factors in Army Cohort by CRP +1444C>T Genotype

<table>
<thead>
<tr>
<th></th>
<th>Overall</th>
<th>CC+CT</th>
<th>TT</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.†</td>
<td>219</td>
<td>206</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>Age, y</td>
<td>19.4±2.1</td>
<td>19.7±2.1</td>
<td>19.6±2.6</td>
<td>0.47</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>22.4±2.5</td>
<td>22.3±2.5</td>
<td>23.2±2.8</td>
<td>0.27</td>
</tr>
<tr>
<td>Systolic BP, mm Hg</td>
<td>122±11</td>
<td>123±11</td>
<td>120±12</td>
<td>0.32</td>
</tr>
<tr>
<td>Diastolic BP, mm Hg</td>
<td>71±9</td>
<td>71±9</td>
<td>69±9</td>
<td>0.42</td>
</tr>
<tr>
<td>Smokers, %</td>
<td>48</td>
<td>49</td>
<td>54</td>
<td>0.50</td>
</tr>
<tr>
<td>Basal IL-6, pg/mL</td>
<td>0.64±0.78</td>
<td>0.62±0.67</td>
<td>0.96±1.55</td>
<td>0.06</td>
</tr>
<tr>
<td>Peak IL-6, pg/mL</td>
<td>1.29±1.0</td>
<td>1.29±1.1</td>
<td>1.23±0.56</td>
<td>0.94</td>
</tr>
</tbody>
</table>

*For comparison of groups (CC+CT) vs TT.
†No. of subjects for whom genotypes and cytokine measures were available at baseline.
Allele and Genotype Frequencies

The allele and genotype frequencies were in Hardy-Weinberg equilibrium in both the army recruits and CABG patients (Tables 3 and 4). There was evidence of significant allelic association between the −717G>A and +1444C>T polymorphisms (Δ = −0.37, P < 0.005 in the army group and Δ = −0.39, P < 0.0005 in the CABG patients). Homozygotes for the +1444T allele were more common among CABG patients (11.8%) than the army recruits (5.7%), χ² = 4.9, P = 0.02. However, there was no significant difference in allele frequency or genotype distribution between the CABG and army groups for the −717G>A and +1059G>C polymorphisms.

Acute-Phase Response

Acute-Phase Response After Final Military Exercise

Summary data for the CRP, IL-6, and fibrinogen and responses to exercise are presented in Figure 1A. At baseline, mean levels of IL-6, fibrinogen, and CRP were 0.66±0.04 pg/mL, 2.65±0.04g/L, and 0.59±0.04 mg/L, respectively. After the 48-hour intensive FME, there was evidence of an acute-phase response reflected by significant increases in all 3 markers. Changes in IL-6 and CRP were the most marked, with peak increases of approximately 2-fold after return from exercise. There were significant correlations between the peak levels of IL-6 and CRP (r = 0.27, P < 0.0005) and IL-6 and fibrinogen (r = 0.34, P < 0.0005).

Acute-Phase Response After CABG Surgery

Mean IL-6 (pg/mL) reached a peak 6 hours after surgery, increasing more than 50-fold from 4.5±0.2 to 232±14 (P < 0.0005) (Figure 1B). IL-6 levels remained significantly elevated throughout the sampling period (mean IL-6 96 hours after CABG, 28.0±6.4, P < 0.0005 versus baseline). Mean CRP (mg/L) rose 83-fold from 1.97±0.80 to a peak of 167.2±5.0 72 hours after surgery (P < 0.0005). There were significant correlations between peak CRP and peak IL-6 levels (r = 0.10, P = 0.0015). CRP levels declined after this time point, although they remained significantly higher than baseline by the final sampling point (mean CRP day 5 post-CABG, 78.5±4.0, P < 0.0005 versus baseline). Mean fibrinogen levels reached a peak of 7.4±0.2 g/L on the fourth postoperative day. Fibrinogen remained significantly elevated up to the time of hospital discharge compared with baseline (6.9±0.5 versus 3.7±0.1, P < 0.0005).

Effect of CRP Genotype on Basal and Stimulated CRP Level

Army Volunteer Exercise Study

Before training, subjects homozygous for the +1444T allele had significantly higher CRP values than +1444C-
allele carriers, \( P = 0.014 \) (Figure 2A). CRP values were 0.54\pm0.05, 0.56\pm0.07, and 1.04\pm0.38 mg/L for CC, CT, and TT genotypes, respectively. Similarly, at all time points after FME, +1444TT homozygotes also had higher CRP concentrations than the C-allele carriers (\( P = 0.034, P = 0.007, \) and \( P = 0.013 \) at 2, 48, and 96 hours after FME, respectively). The association between +1444C>T genotype and CRP levels persisted after adjustment for age, sex, BMI, smoking, and IL-6 as well as genotypes for the -717G>A and +1059G>C CRP polymorphisms (ANCOVA, \( P = 0.023, P = 0.005, P = 0.007, \) and \( P = 0.013 \) at baseline and 2, 48, and 96 hours after FME). In a multiple regression analysis age, BMI, smoking status, and the +1444C>T polymorphism influenced CRP level. The +1444C>T polymorphism accounted for 3.7\%, 2.0\%, 4.0\%, and 4.5\% of the total CRP variance at baseline and 2, 48, and 96 hours after FME. In contrast, baseline CRP levels were not influenced by -717G>A or +1059G>C genotype, nor was there any significant association between these 2 polymorphisms and postexercise CRP.

Although initial analysis indicated that +1059GG homozygotes had a higher CRP 2 hours after FME than +1059C-allele carriers, this effect was lost after multivariate analysis and was accounted for by the observation that all +1059 GG homozygotes were also homozygous for the +1444T allele.

**CABG Study**

Preoperative CRP levels were higher for +1444TT homozygotes than +1444C-allele carriers, although this difference was not statistically significant. CRP values were 1.9\pm0.4, 2.0\pm0.8, and 2.9\pm0.9 mg/L for CC, CT, and TT genotypes, respectively. However, at all times beyond the first 24 hours after surgery and despite the

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**TABLE 4. CRP Genotype Distributions and Rare Allele Frequency in CABG Patients**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Rare Allele Frequency (95% CI)</th>
<th>No. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRP promoter</td>
<td>-717G&gt;A</td>
<td>0.27 (0.23 to 0.32)</td>
</tr>
<tr>
<td>CRP Exon 2</td>
<td>+1059G&gt;C</td>
<td>0.07 (0.05 to 0.10)</td>
</tr>
<tr>
<td>CRP 3’UTR</td>
<td>+1444C&gt;T</td>
<td>0.33 (0.28 to 0.38)</td>
</tr>
</tbody>
</table>

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**Figure 1.** A, Summary data for the CRP, IL-6, and fibrinogen response to exercise. B, Summary data for the response to CABG. Fn indicates fibrinogen.

**Figure 2.** A, Postexercise CRP levels by CRP +1444C>T genotype. B, Post-CABG CRP levels by CRP +1444C>T genotype.
large rise in CRP that resulted, mean CRP levels were higher in +1444TT homozygotes than +1444C-allele carriers (Figure 2B). This was most marked at the 48- and 72-hour time points, although the difference was only significant at the 72-hour time point \((P = 0.19\) and \(P = 0.032\) at 48 and 72 hours, respectively). The effect of +1444C>T genotype on peak CRP levels remained significant after multivariate analysis, \(P = 0.01\). Apart from CRP, +1444C>T genotype, significant predictors of CRP level were BMI and operation duration. There was no significant interaction between statin therapy and genotype on CRP level either at baseline \((P = 0.29)\) or at peak \((P = 0.69)\). However, after adjustment for statin therapy, there was still evidence of a difference in maximum CRP by genotype \((P = 0.05)\). There was no evidence of any significant association of CRP with +1059G>C genotype on CRP levels at any time point either at baseline or after CABG.

**Discussion**

We have identified 2 novel common CRP polymorphisms, 1 in the gene promoter \((-717G>A)\) and the other in the 3’ untranslated region \((+1444C>T)\). In 2 independent studies, a strong, consistent, and independent association was detected between the +1444C>T polymorphism and CRP level. These findings clearly implicate genetic factors in the regulation of CRP level and are the first to identify a candidate functional polymorphism in the gene itself.

**CRP Genotype Is Associated With CRP Level in 2 Models of the Acute-Phase Response**

In both our studies, homozygosity for the +1444T allele was significantly and independently associated with a greater peak CRP level after an inflammatory stimulus. Importantly, these associations were preserved after accounting for factors known to influence CRP level, including age, sex, BMI, smoking, diabetes, and IL-6. This association was detected despite differences in the absolute basal level of CRP in the 2 study groups (one a healthy cohort, the other a group with severe CHD) and in the nature and magnitude of the inflammatory stimulus. In the first study of healthy volunteers, the +1444C>T polymorphism was also associated with an approximately 2-fold difference in basal CRP \((0.55 \text{ versus } 1.04 \text{ mg/L for +1444C-allele carriers and +1444TT homozygotes, respectively})\), and this difference was preserved after adjustment for age, BMI, smoking, and baseline IL-6. This magnitude of the difference in CRP level by genotype has been associated, in prospective cohort studies, with a 25% to 50% increase in the long-term risk of cardiovascular events.\(^{31}\) Although no statistically significant association was detected between CRP genotype and basal CRP in patients with CHD, it should be noted first that the absolute level of CRP was higher in this group than in the healthy army recruits (presumably reflecting a preexisting low-grade inflammatory stimulus in these atherosclerotic patients) and second that many were taking statins that are recognized as modulating CRP level. These factors might have served to dilute any genetic association. Despite this, and consistent with the findings in the army recruits, a clear association was still detected between the CRP +1444C>T polymorphism and peak post-CPR level after the inflammatory stimulus of CABG surgery. Overall, baseline and peak IL-6 correlated with baseline and peak CRP, respectively, but our study was not powered to detect an interaction between the IL-6 stimulus and CRP genotype in the determination of CRP level. It would be important to explore the possibility of such an interaction in future studies.

Cross-sectional studies have indicated that environmental and demographic factors such as age, sex, smoking, obesity, and diabetes account for only 22% to 30% of the interindividual variability in steady-state CRP,\(^{32}\) and family studies have suggested that CRP level is a heritable trait.\(^{26}\) However, our study is the first to implicate a single-nucleotide polymorphism in a potential regulatory region of the CRP gene itself in the determination of CRP level. However, it is likely that other important genetic influences exist. Recently, a polymorphism in the gene that encodes IL-6, the major cytokine stimulus for CRP production, was also found to be associated with CRP level.\(^{33}\)

**Is the +1444C>T Variant Functional?**

Our study cannot provide direct evidence for a functional role for the +1444C>T CRP polymorphism. One explanation for our observations is that this polymorphism is a marker for an as-yet unidentified variant in the vicinity of the CRP locus. If the +1444C>T variant was shown to be functional in in vitro studies, its effects might be mediated through modulation of mRNA stability, as has been reported for a common functional 3’UTR variant in the prothrombin gene.\(^{34}\) Regulation of mRNA stability is a potentially important step in CRP production, because CRP mRNA is known to have a short half-life of approximately 2.5 hours.\(^{35}\)

**Limitations of the Present Study**

First, the number of subjects with the TT genotype was relatively few, and most participants were men. Therefore, additional studies in larger cohorts in which women are adequately represented are needed. Second, because this study is unable to define whether the +1444C>T polymorphism is functional or a marker in linkage disequilibrium with a functional variant elsewhere in the gene, it might be regarded as being hypothesis generating.

**Implications of Present Findings for Pathogenesis of Atherosclerosis and for Risk Prediction**

It is still not clear whether CRP contributes to the pathogenesis of atherosclerosis or is acting as a marker of distinct inflammatory processes or products that have a causal role in atherogenesis. CRP has the capacity to bind oxidized LDL and to induce adhesion molecule and tissue factor expression in endothelial cells and monocytes, respectively. Critically, CRP has also been found within atherosclerotic plaques.\(^{18}\) In patients with CHD, the circulating level of CRP correlates inversely with endothelium-dependent vasodilatation, which is itself predictive of
adverse outcome.36 If these observations reflect a pathogenetic role for CRP and if the lifetime exposure to CRP is important, then the identification of genetic factors like the +1444C>T polymorphism that are associated not only with steady-state CRP but also with the temporal profile of its rise and fall after an inflammatory stimulus may help to refine assessments of susceptibility to CAD. Although not the main aim of the present study, we identified an excess of +1444TT homozygotes among CABG patients when compared with the healthy army recruits (11.8% versus 5.7%; \( \chi^2 = 4.9; \) \( P = 0.02 \)). This finding is in keeping with the hypothesis that this variant increases susceptibility to CHD, although this will require confirmation in an appropriately powered case-control study. Alternatively, if CRP is marker for, rather than a mediator of, atherosclerosis, then the important prognostic value of a particular CRP level37 may differ according to an individual’s genotype, because an elevation in CRP that results from inflammation may have a different implication for future risk than an elevation in CRP that is the result of an individual’s genotype. If this is the case, then there may eventually be a need to establish genotype-specific risk thresholds for CRP in the prediction of CHD risk. An additional potentially important consequence of the identification of a genetic variant associated with differences in CRP concentration is that it will allow clarification of the link between CRP and vascular disease by using Mendelian randomisation to test causality.38

Acknowledgments

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References

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In the November 2003 issue of *Arteriosclerosis, Thrombosis, and Vascular Biology*, in the article entitled “Human CRP Gene Polymorphism Influences CRP Levels: Implications for the Prediction and Pathogenesis of Coronary Heart Disease” by Brull et al (*Arterioscler Thromb Vasc Biol*, 2003;23:2063–2069), the authors have identified two typographical errors. One of the primers for detection of the −717 polymorphism should have read 5′-ATCCCATCTATGAGTGAGAACC-3′ and not 5′-ATTCCCATCTATGAGTGAGAACCT-3′ as stated. Also, the common allele for this polymorphism should have been labeled A (Sac II uncut) and the rare allele G (Sac II cut), rather than the other way round. The authors apologize for these errors.