Genotype at a promoter polymorphism of the interleukin-6 gene is associated with baseline levels of plasma C-reactive protein

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Abstract

Objective: Baseline concentrations of plasma C-reactive protein (CRP) are associated with coronary heart disease. Interleukin-6 (IL-6) regulates CRP gene expression; a promoter polymorphism (−174G/C) of the IL-6 gene has been shown to influence IL-6 transcription but the relationship between genotype at this polymorphism and circulating levels of inflammatory markers remains unclear. We hypothesised that plasma CRP would be a heritable phenotype that would be influenced by genotype at this polymorphism. Methods: We measured baseline plasma CRP and determined genotypes at the −174G/C polymorphism of the IL-6 gene in 588 members of 98 nuclear families. The heritability of plasma CRP and the association of plasma CRP with genotype were determined using variance components methods. Results: Baseline CRP levels were highly heritable ($h^2 = 0.39, P < 0.0000001$). Presence of the −174C allele was associated with higher baseline CRP levels, both in the whole population ($P = 0.01$), and in the founders only ($n = 128, P = 0.001$). Family-based analyses confirmed the association ($P = 0.02$) suggesting that it arises from chromosomal proximity or identity of the typed polymorphism with a genetic variant influencing baseline CRP levels. Conclusions: Baseline plasma CRP is a significantly heritable cardiovascular risk factor. Levels are associated with genotype at the −174G/C polymorphism of the IL-6 gene. © 2002 Elsevier Science B.V. All rights reserved.

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1. Introduction

To date, 11 prospective studies involving a total of nearly 2000 cases of disease have demonstrated association between the baseline level of plasma C-reactive protein (CRP) and coronary heart disease (CHD) [1]. It is uncertain whether this association is causal; several mechanisms whereby CRP might directly influence atherogenesis have been proposed, but none has been proven [2]. Recent data from intervention trials also suggests that baseline CRP may be a useful marker to identify subjects without overt hyperlipidaemia or previous coronary events who may nevertheless derive benefit from statin therapy, which is thought to be due to a direct anti-inflammatory effect of statins on atheromatous plaques [3]. There is therefore considerable interest in establishing the degree to which baseline CRP is genetically determined, and in identifying genetic polymorphisms that are associated with CRP levels. Baseline plasma CRP levels (that is, plasma CRP levels measured in the absence of any clinically recognised pathology liable to stimulate the acute phase response) have very recently been reported to exhibit high heritabili-

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ty in one study [4], but no genetic polymorphisms associated with baseline levels of plasma CRP have yet been reported. If genetic variability between individuals could be shown to contribute substantially to the overall population variability of CRP, then CRP could potentially be used as an intermediate phenotype for genetic analysis that might be more tractable than the more complex phenotype of clinical CHD, which would be expected to result from a wider variety of genetic and environmental factors. Genetic variants associated with baseline levels of plasma CRP would be strong candidates as CHD susceptibility alleles.

Interleukin-6 (IL-6) is a major determinant of the acute phase response. Expression of the CRP gene during the acute-phase reaction is regulated by IL-6, although the importance of IL-6 in determining baseline plasma levels of CRP is unknown. Genotypes at a recently described polymorphism of the IL-6 promoter (−174G/C), which influences IL-6 gene transcription in vitro, have been examined for association with plasma IL-6 levels in a number of studies, but the results of these studies have been widely discrepant [5–9]. This may be chiefly as a result of random error in relatively modest sample sizes, but may also be partly due to the marked (sixfold) diurnal variability of plasma IL-6, which may limit the intr.individual replicability of plasma IL-6 measurements [10–12]. Baseline CRP concentrations are not subject to such circadian variation and show high reproducibility within individuals over a period of up to 5 years [13–15]. This may explain the apparent superiority of CRP to IL-6 as a predictor of vascular risk that has been observed in some prospective studies, and suggests that CRP may be a more suitable phenotype than IL-6 for investigation regarding any possible genetically determined level of baseline inflammatory activity that may contribute to the risk of atherothrombotic events [16,17]. The relationship between the IL-6 −174G/C polymorphism and baseline plasma CRP levels has been examined in some of those studies that investigated the association between genotype and plasma IL-6 levels. Although no evidence of an association has been found thus far, in some studies the sensitivity of the CRP assay used was limited [9], whilst in others all individuals enrolled were patients with established atherosclerotic disease which may itself have influenced the measured levels of plasma CRP [6,8].

All studies to date that have examined association between the IL-6 −174 G/C polymorphism and plasma levels of inflammatory markers have been conducted in unrelated individuals. Such studies are potentially susceptible to problems arising from genetic inhomogeneity of the study subjects; although the practical magnitude of this concern is uncertain, it can be avoided if families are studied [18]. Additionally, family studies offer the advantages that they permit the heritability of a phenotype to be estimated, and the requirement for Mendelian transmission provides an in-built check on genotyping accuracy.

We hypothesised that plasma CRP would be a heritable phenotype influenced by the IL-6 −174G/C polymorphism, and have tested this in a large family study.

2. Methods

2.1. Family collection

Ninety-eight British Caucasian families were ascertained on a proband with essential hypertension found to be in the top 5% of the population blood pressure distribution before the age of 65 years. Probands were identified from a hospital hypertension service or via their family physicians. Families of the probands were collected when a sibship of three members available for phenotyping and blood sampling was available, if a parent of the sibship was also available for blood sampling. When no parent was available, a sibship of four was required. The sibship could be in the generation of the proband or of his/her offspring, and when suitable sibships existed in both generations, they were both collected. There was no requirement for any member of the family other than the proband to be classified as hypertensive. However, when a member of a sibship so collected could be determined to be hypertensive (by the same criteria as the probands), then, if a suitable sibship existed in the generation of his/her offspring, that sibship was also collected.

Research personnel administered a detailed questionnaire regarding current and previous health, including the presence of any acute or chronic inflammatory conditions, to all participants. Height, weight and blood pressure (using a Takeda TM2421 automated sphygmomanometer) were measured in all subjects. Blood was drawn for baseline biochemical analysis and urine dipstick testing for haematuria and albuminuria was carried out. All proband and family data were clinically evaluated by one of the investigators (B.K.) to detect the presence of secondary hypertension, including Mendelian forms of genetic hypertension; where this could not be confidently excluded in a family, the family was excluded from the study.

The study conforms with the principles outlined in the Declaration of Helsinki.

2.2. Genotyping

The G/C polymorphism at position −174 of the interleukin-6 gene was typed by polymerase chain reaction (PCR) amplification using primer pairs 5’CACCTGCACCTGGAGACGCCT3’ and 5’TCCCTCACACAGGGCTCGAC3’ under standard conditions using 2 mM MgCl₂, followed by restriction digestion with NlaIII and agarose gel electrophoresis. Control individuals of known genotype were included in each plate, and genotyping was carried out blinded to plasma CRP levels. Mendelian inheritance within families was confirmed using the PedCheck programme [19] and inconsistencies resolved by
re-examination of the raw data, and re-genotyping where necessary.

2.3. CRP measurement

Baseline CRP levels were determined by enzyme-linked immunosorbent assay (ELISA) in plasma anticoagulated with 1:10 0.32% trisodium citrate. Both capture and detection antibodies were rabbit polyclonal anti-human CRP antibodies (Dako), the latter conjugated with horse-radish peroxidase. Binding was visualised with urea hydrogen peroxide and 3,3′,5,5′-tetramethylbenzidine. The reaction was calibrated against international standard 85/506 CRP (NIBSC), threefold serially diluted over a range of 0.1 to 0.00123 mg/l although the range from which samples were read was usually 0.033–0.0037 mg/l. Samples were initially tested at three threefold serially diluted concentrations and each dilution tested in duplicate. For the reading to be valid two of the dilutions had to lie within the appropriate portion of the standard curve and had to be within 10% of one another. If these conditions were not met, the assay was repeated at more appropriate dilutions. The intra-plate relative standard deviation (RSD) was measured by measuring CRP in an aliquoted normal plasma pool on single plates using all available positions. Each sample plate measured at least three dilutions of normal pool and these values were used to correct for differences between plates. The inter-plate RSD was calculated by comparing valid values obtained for samples between plates; each plate had an average of 14.3 comparisons made with other plates. The intra- and inter-assay RSDs were 10.4 and 15.9%, respectively. The lower detection limit of the assay was 0.02 mg/l. Samples were assayed without regard to family relationships to avoid systematic bias.

2.4. Statistical methods

The presence and significance of covariates of log plasma CRP was assessed by stepwise linear regression using SPSS statistical software (SPSS). Log CRP levels were analysed for association with genotype in the entire population and, separately, in the founders of the families only (i.e., those whose parents did not appear in the pedigree) by analysis of variance. Family-based tests of association of log plasma CRP with the typed marker were carried out using a variance components approach implemented in the QTDT program [20]. The heritability of log plasma CRP was determined using SOLAR [21].

3. Results

The 98 families comprised 588 members with reliable measurements of baseline plasma CRP and genotype information. Seventy-five percent of families comprised between four and seven available members. Characteristics of the subjects studied are shown in Table 1. CRP levels corresponded closely with those described in other populations without inflammatory disease (median 1.26 mg/l, interquartile range 0.51–3.07 mg/l). CRP levels were skewed, and were therefore transformed for further analysis by taking the natural logarithm, which resulted in an approximately Normal distribution (Kolmogorov–Smirnov/Lilliefors statistic 0.027, P = 0.2; Fig. 1). No subject reported symptoms of current inflammatory disease that might have invalidated their baseline CRP measurement. Six subjects had previous histories of inflammatory conditions (inflammatory arthropathy in four cases and inflammatory bowel disease in two cases) that were clinically inactive at the time of blood collection. With one exception, all these subjects had CRP levels below the median. Analyses were conducted both including and excluding these individuals; the significance of the results was unaltered. Age and body mass index (BMI) were highly significant covariates of log CRP (P < 0.001) while age-squared, sex, age-by-sex, and smoking were not (P > 0.05 for all). Prior to adjustment for age and BMI, there was a trend towards association between systolic blood pressure and log plasma CRP (P = 0.05); however, after adjustment for age and BMI, there was no significant association either between systolic or diastolic blood pressure as continuous variables and log CRP, or between hyperten-

![Fig. 1. Distribution of log-transformed baseline plasma CRP in 98 families.](image-url)
Among 588 subjects with G/G, G/C and C/C genotypes, the age and BMI adjusted log CRP levels were 0.01 (S.E. 0.10), 0.29 (0.07) and 0.28 (0.16), respectively, suggesting that the C allele was behaving in a dominant fashion to increase log CRP (Fig. 2). As our initial hypothesis had been that any association would follow a codominant model, this model was tested first. There was significant evidence for association between genotypes and log CRP level in the whole population ($F=3.348, P=0.036$; Table 2) and in the founders ($F=5.640, P=0.005$; Table 2). However, under a dominant model (GG versus GC and CC), there was stronger evidence of association between genotype and log CRP level in the whole population ($F=6.523, P=0.011$; Table 2) and in the founders ($F=11.33, P=0.001$; Table 2). We accounted for multiple hypothesis testing by applying a Bonferroni correction for two analyses: following this, associations observed under the dominant model remained significant. Under the dominant model in the founders, genotype explained 14% of the observed variation in log plasma CRP. Family-based analyses using QTDT gave further support for association between genotype and log CRP ($F=5.18, P=0.0232$). There was a tendency towards higher baseline CRP levels in the founders (mean log CRP of 0.72 in founders compared with 0.05 in nonfounders) but after adjustment for age and BMI this difference was non-significant ($P=0.38$), and there was no evidence for heterogeneity between founders and non-founders regarding the influence of genotype on log CRP ($P=0.4$).

### 4. Discussion

Our study shows that baseline plasma CRP level is a highly heritable phenotype which is associated with genotype at the IL-6 –174G/C polymorphism. The high heritability (0.39) of baseline CRP that we have observed in families ascertained on a hypertensive proband closely agrees with a recent report by Pankow et al. [4] who studied randomly ascertained families and found a heritability of 0.35–0.4 for baseline CRP. This confirms the suitability of our families for such analyses, and indicates that CRP is determined to a significant degree by

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**Table 2**

<table>
<thead>
<tr>
<th>IL6 genotype</th>
<th>All subjects</th>
<th>Founders only</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>Log CRP*</td>
<td>Back-transformed CRP mg/l</td>
</tr>
<tr>
<td>G G</td>
<td>179 (30.4%)</td>
<td>0.01 (0.10)</td>
</tr>
<tr>
<td>G C</td>
<td>321 (54.6%)</td>
<td>0.29 (0.07)*</td>
</tr>
<tr>
<td>C C</td>
<td>88 (15%)</td>
<td>0.28 (0.16)*</td>
</tr>
</tbody>
</table>

* $p=0.011$, **$p=0.001$ for CC and GC genotypes vs. GG genotype.

* Log CRP values are mean (standard error).
genetic factors rather than exclusively by random, individual-specific responses to concurrent inflammatory stimuli. In view of the association between CRP and atherothrombotic events, the high heritability of CRP suggests that CRP may have value as an intermediate phenotype in genetic studies of CHD susceptibility. In addition to our findings regarding the heritability of CRP, this study is the first to our knowledge that demonstrates association between any genetic polymorphism and the baseline plasma level of CRP. The study is also the largest thus far to investigate the relationship between the IL-6 −174 G/C polymorphism and any inflammatory cytokine. Its family-based design allows estimation of the heritability of plasma CRP, and acts as a control for population stratification.

The IL6 −174G/C polymorphism is a credible biological candidate for involvement in the regulation of the inflammatory response; it is situated close to a binding site for the glucocorticoid receptor and the nucleotide substitution creates a potential binding site for the transcription factor NF-1 [22]. However, previous population genetic studies are inconclusive regarding which of the alleles is associated with higher plasma levels of inflammatory cytokines. Fishman et al. showed in 92 healthy subjects that the mean plasma IL-6 level was lower in those of CC genotype than in those of GG or GC genotype (P=0.02), suggesting a recessive effect of the C allele in lowering plasma IL-6 [5]. However, Rauramaa et al. found no effect of the polymorphism on baseline plasma IL-6 levels in 92 asymptomatic males [7]. Subsequently Jones et al. presented data on 231 patients with abdominal aortic aneurysms and showed a borderline significant (P=0.047) codominant effect of the polymorphism on plasma IL-6 levels, the presence of the C allele being associated with higher plasma IL-6 levels in a codominant fashion [6]. That study also investigated the effect of genotype at this polymorphism on plasma CRP; no significant association was observed. Margaglione et al. found no association between genotype and plasma levels of either CRP or IL-6 in 598 randomly ascertained individuals, but the reproducibility of the plasma assays used in that study was low at lower levels of CRP and IL-6; this resulted in the use of the plasma phenotypes as dichotomous rather than continuous variables, which may have diminished the power of that study to detect any genetic effect [9]. Most recently, Brull et al. found no association between genotype and plasma IL-6 levels in a study of 127 patients undergoing CABG, although a recessive effect of the C allele to increase plasma IL-6 levels 6 h after CABG appeared to be present (P=0.04) [8]. Those studies that have examined clinical vascular phenotypes with respect to these genotypes have likewise tended to be discrepant: the C allele was associated with a lesser severity of carotid artery atherosclerosis in the study of Rauramaa et al., but it was associated with a higher cardiovascular mortality in aortic aneurysm patients in the study of Jones et al.

The reasons for the discrepancies among these other studies, and between those studies and the present study, are unclear. However, most of the previous studies have involved substantially smaller samples than the present study, and the levels of statistical significance of the observed associations have typically been modest, so the discrepancies may be due to random error. The tendency to random error may have been exacerbated by the marked diurnal variability of plasma IL-6, since timed samples were not taken in most of these studies. Some studies enrolled healthy individuals, whereas other studies enrolled patients with clinically apparent atherosclerotic disease, in whom the presence of the disease itself may have confounded any genetic influence on plasma CRP or IL-6 levels. All studies have all been performed in European Caucasian populations and the allele frequencies in these studies have been in good agreement with each other; however, only the present study has a family-based design, which can eliminate the risk of false-positive results due to undetected subtle population stratification. Our data show highly significant association between the C allele and baseline plasma CRP levels, and provide the strongest evidence so far for an effect of this polymorphism on the regulation of baseline plasma cytokine levels.

One limitation of our study is that plasma IL-6 levels were not measured (as it was not possible to obtain blood samples from all subjects at standardised times), which means that the mechanism of the association remains uncertain. We hypothesise that the IL-6 −174C allele increases baseline plasma CRP via an influence on IL-6 gene transcription and the consequent effects of differing levels of that cytokine on CRP gene expression. However, in the absence of IL-6 levels, alternative explanations, such as linkage disequilibrium between this polymorphism and others either within the IL-6 gene affecting IL-6 protein function rather than concentration, or within other genes neighbouring IL-6 that directly affect CRP gene transcription in other ways, cannot be ruled out. Further studies will therefore be necessary to clarify the mechanism of the association.

Somewhat surprisingly, we observed an apparent dominant effect of the C allele on baseline plasma CRP levels, which potentially invokes a threshold effect of IL-6 gene transcription on CRP levels. This does not diminish the likelihood of our result being correct: there are many examples of genetic effects on quantitative traits that are not co-dominant (for example, the association between the MTHFR C677T mutation and plasma homocysteine levels, which has been observed in over 5000 cases of cardiovascular disease and controls [23]). We typed a polymorphism in the IL-6 rather than the CRP gene: although some polymorphisms in the CRP gene have been described [24], none appear to be such good candidates as regulatory variants when compared with IL6 −174 G/C whose importance has been demonstrated in vitro [5,22]. However, in view of the current results, further investigations
of the effect of haplotypes at both the IL-6 and CRP genes on baseline plasma levels of CRP would be of interest.

When robust associations such as that we describe between genetic polymorphisms and hypothesised novel cardiovascular risk factors such as baseline CRP can be identified, genotyping of such polymorphisms together with measurement of the novel factor in studies involving large numbers (several thousands) of cases of disease and healthy controls could potentially assist in determining whether such novel factors cause CHD. If the novel factor were causal, the polymorphism might be expected to show association with disease that would be of a magnitude commensurate with the sizes of the associations between polymorphism and risk factor, and between risk factor and disease. Moreover, since the differences in the risk factor were genetically determined, such differences in risk would be free of confounding. Our study thus also provides strong justification for the typing of this polymorphism together with plasma CRP measurement in suitable large case-control studies of cardiovascular events.

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References