Serum C-Reactive Protein Concentration and Genotype in Relation to Ischemic Stroke Subtype

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Background and Purpose—C-reactive protein (CRP) has evolved as an inflammatory risk marker of cardiovascular disease. Several single-nucleotide polymorphisms at the CRP locus have been found to be associated with CRP levels. The aim of the present study was to investigate CRP levels and genetic variants in etiological subtypes of ischemic stroke.

Methods—The Sahlgrenska Academy Study on Ischemic Stroke (SAHLSIS) comprises 600 consecutive ischemic stroke cases (18 to 69 years) and 600 matched controls from western Sweden. Stroke subtypes were defined by the TOAST classification. Serum CRP levels were determined by a high-sensitivity immunometric assay.

Results—CRP levels were significantly higher for all ischemic stroke subtypes compared with controls, both in the acute phase and at the 3-month follow-up. After adjustment for traditional risk factors, CRP at follow-up was related to higher odds ratios (ORs) of overall ischemic stroke (OR, 1.25; 95% CI, 1.09 to 1.43) and large-vessel disease (OR, 1.48; 95% CI, 1.09 to 2.00). The CRP −286C>T>A, 1059G>C, and 1444C>T single-nucleotide polymorphisms showed significant associations with CRP levels. However, neither CRP genotypes nor haplotypes showed an association to overall ischemic stroke.

Conclusions—This is the first large study on CRP in different TOAST subtypes in a young ischemic stroke population. CRP levels differed between etiological subtypes of ischemic stroke both in the acute phase and at the 3-month follow-up. CRP at follow-up was associated with overall ischemic stroke and the large-vessel disease subtype. Genetic variants at the CRP locus were associated with CRP levels, but no association was detected for overall ischemic stroke.

Key Words: C-reactive protein • polymorphism • genetics • stroke, ischemic • stroke classification

Inflammation is important in ischemic stroke, both in the development of atherosclerosis and during the ischemic event.1-3 Human C-reactive protein (CRP) is an acute-phase reactant that is rapidly upregulated by inflammatory cytokines.4 In line with this concept, CRP is a sensitive indicator of inflammation and has also evolved as a marker of atherosclerosis.5 Several prospective studies have shown that a single measurement of CRP in serum is a predictor of first-ever2,3,6 as well as recurrent7-9 cerebrovascular events. Family and twin studies show that CRP levels are influenced by genetic factors, with a heritability of 40% to 50%.10-12 Several studies have reported an association between genotypes within the CRP gene and CRP levels.13-17

Ischemic stroke is a heterogeneous disease, and inflammatory pathways may have different impacts depending on underlying pathophysiological processes. Here we addressed the hypothesis that CRP levels differ by ischemic stroke subtypes. To this end, CRP levels were determined among participants in the Sahlgrenska Academy Study on Ischemic Stroke (SAHLSIS), for whom stroke subtype was classified by the Trial of Org 10172 in Acute Stroke Treatment (TOAST) criterion. Furthermore, we sought to confirm earlier findings of an association between single-nucleotide polymorphisms (SNPs) in the CRP gene and CRP levels and to investigate whether these genetic variants also show an association to ischemic stroke and/or any subtype.

Subjects and Methods

Study Population

SAHLSIS has been described in detail elsewhere.18 In short, it comprises 600 consecutive white patients <70 years of age presenting with acute ischemic stroke and 600 healthy white community controls, matched for age (±1 year), sex, and geographical residence area. All patients underwent neuroimaging. They were examined by a physician at both admission and the 3-month follow-up. Additional
diagnostic work-up was performed when clinically indicated.18 Stroke subtype was classified by 2 neurologists blinded to genotype and CRP level, according to 2 frequently used systems, OCSP and TOAST. OCSP is a classification used in population studies, whereas TOAST is designed for use in hospital-based cohorts that are investigated in greater detail to reveal underlying pathophysiological mechanisms. TOAST was chosen for the primary etiological subtype analyses, whereas OCSP provided information on the clinical extent of brain damage.19 Analyses by TOAST subtype included large-vessel disease (LVD), small-vessel disease (SVD), cardioembolic (CE), cryptogenic stroke, and other stroke. The latter group includes strokes of other determined etiology and cases for which >1 cause was identified or when the evaluation was cursory. Functional outcome after 3 months was assessed with the modified Rankin Scale. The study was approved by the ethics committee of Göteborg University. All participants gave written, informed consent. When patients were unable to communicate, his or her next-of-kin gave informed consent.

Blood Sampling and CRP Assay
Among stroke cases, blood sampling was performed within 10 days of the stroke event and at the 3-month follow-up. On both occasions, as well as in controls, venous blood samples were drawn between 8:30 and 10:30 AM after an overnight fast. High-sensitivity CRP (hs-CRP) was analyzed in serum by a solid-phase chemiluminescent immunometric assay on Immulite 2000 (Diagnostic Products Corp, Los Angeles, Calif) with the manufacturer’s reagents as directed. The analytical sensitivity was 0.1 mg/L, and the intra-assay coefficient of variation was, on average, 3.4%.

Genotyping
Based on results from earlier studies4-17 as well as relevant databases, the CRP −732T>C, −286C>T>A, 1059G>C; and 1444C>T SNPs (dbSNP reference IDs: rs2794521, rs3091244, rs1800947, and rs1130864, respectively) were selected to capture genetic variation at the CRP locus. Genotyping was performed by 5’ nuclease (TaqMan) assays. Assays for the 3 diallelic SNPs were custom designed (supplemental Table I, available online at http://stroke.ahajournals.org), whereas the triallelic variant was analyzed as described.15 Amplifications were carried out on the Dual 96-well GeneAmp PCR system 9700 (Applied Biosystems), and fluorescence was read on an ABI PRISM 7900HT sequence detector system (Applied Biosystems). A subset (10% of the samples) was reanalyzed for each SNP, and all results were consistent. Genotyping was performed blinded to case/control status.

Statistics
Differences in characteristics between cases and controls were examined with the χ2 test for proportions and with Student’s t test for continuous variables. Because the distribution of CRP values was positively skewed, nonparametric tests were used. ANOVA and Tukey’s post hoc test were used to test for differences in lognormally transformed CRP levels by TOAST subtype. The association between CRP concentration and case/control status was investigated with binary logistic regression adjusted for age, sex, hypertension, smoking status, diabetes mellitus, hyperlipidemia, and waist-hip ratio (WHR). In this model, CRP levels were lognormally transformed and used as a continuous variable. Reported odds ratios (ORs) were scaled to estimate the ORs associated with an increase of 1 SD (1.105 mg/L) in CRP concentration. Similar models were used to study the association between single polymorphisms and case/control status. In all single-locus analyses on CRP level, additive and dominant or recessive models were considered. The genetic model showing the strongest association to CRP level was used in single-locus analyses on case/control status. In ischemic stroke subtype regression models, the whole control population was used. Data were analyzed with SPSS 12.0.1, and statistical analyses were performed in a 2-tailed fashion. P<0.05 was considered significant.

Allele frequencies were derived from genotype data, and deviations from Hardy-Weinberg equilibrium were tested. Haplotype frequencies, geometric means, and pairwise linkage disequilibrium (LD) coefficients, D’, were estimated with the use of THESIAS software.20 Haplotype analyses combining all polymorphisms were also performed with THESIAS. The method uses a stochastic-EM algorithm for likelihood maximization and allows for simultaneous estimation of haplotype-phenotype association parameters. Covariate-adjusted ORs were estimated for each haplotype by comparison to a reference haplotype, represented by the most frequent allele for each SNP.

Missing Values
The number of individuals with missing values was as follows: diabetes 2, smoking 3, hypertension 9, hyperlipidemia 56, WHR 54, CRP −732T>C genotype 1, CRP 1059G>C genotype 1, CRP levels in controls 2, and CRP levels in acute stroke 24. In 48 patients, serum levels at follow-up were missing because of intervening death (7), technical difficulties (17), or unwillingness to take part in the follow-up examination or provide blood samples (24). In the regression models, missing values for lnCRP and WHR were replaced by the mean value, and for categorical variables, dummy variables were introduced.

Results
CRP Levels in Ischemic Stroke
For overall ischemic stroke as well as for TOAST subtypes, CRP levels were higher during the acute phase compared with 3-month follow-up (Table 1). At both time points, there was a significant difference in CRP between subtypes (P<0.001 acutely and P<0.05 at follow-up). In the acute stage, CRP levels were highest in CE stroke, whereas the LVD group showed higher CRP levels at follow-up compared with all other subtypes. Compared with controls, overall ischemic stroke and all subtypes showed significantly higher CRP levels both in the acute phase and at follow-up (Table 1). Results from binary logistic-regression analyses are shown in Figure 1. In univariate analyses, CRP showed significant associations to overall ischemic stroke and to all subtypes at both time points. After adjustment for traditional risk factors, these associations remained significant for acute-phase CRP levels. However, at follow-up, only the association to overall ischemic stroke, other stroke, and LVD remained.

CRP Genotypes in Relation to Ischemic Stroke and CRP Levels
Genotype frequencies are shown in supplemental Table II (available online at http://stroke.ahajournals.org). All genotype distributions were consistent with Hardy-Weinberg equilibrium. No significant association was detected between any SNP and overall ischemic stroke. Further analysis in subgroups below and above the median of 58 years did not alter this result. The unadjusted ORs of ischemic stroke among subjects homozygous for the −732C, −286C, 1059G, and 1444T allele were 1.00 (95% confidence interval [CI], 0.65 to 1.54), 1.08 (95% CI, 0.86 to 1.36), 1.14 (95% CI, 0.81 to 1.61), and 1.10 (95% CI, 0.75 to 1.61), respectively. Subtype analyses revealed an association between the 1059 G>C SNP and CE stroke, with an increased risk for C allele carriers (OR, 2.2; 95% CI, 1.26 to 3.97, P=0.006). This association was independent of the covariates indicated earlier, and it also remained after inclusion of CRP in the model (adjusted OR, 1.31; 95% CI, 1.01 to 1.70 for CRP and OR, 2.5; 95% CI, 1.41 to 4.63 for the 1059C allele). No significant associ-
SNPs showed associations to CRP levels: the CRP/H11002/286C/H11022T/A (CC versus other allelic combinations,
CRP/H110050.75 mg/L [95% CI, 0.64 to 0.88], P<0.001),
1059G/H11022C (GG versus GC/CC, CRP/H110051.38 mg/L [95%
CI 1.09 to 1.75], P<0.05), and 1444C/H11022T (CC versus
CT+TT, ΔCRP=0.80 mg/L [95% CI, 0.69 to 0.94], P<0.01). These associations were also observed in cases (data
not shown). No association to CRP levels was detected for the
CRP/H11002732 T/H11022C SNP (P=0.6).

<table>
<thead>
<tr>
<th>Table 1. Risk Factors and CRP Levels in Controls, Overall Ischemic Stroke and Ischemic Stroke Subtype</th>
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<tbody>
<tr>
<td><strong>Control, n=600</strong></td>
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<tr>
<td>Mean age, y (SD)</td>
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<td>Male sex, n (%)</td>
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<td>Hyperlipidemia, n (%)</td>
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<td>CRP, mg/L</td>
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| **Median (IQR-range)** | **3-Months follow-up** | **Median (IQR-range)** | **LVD indicates large-vessel disease; SVD, small-vessel disease; CE, cardioembolic stroke.**
| Differences between cases and controls were examined with the χ² test for proportions and with Student’s t test or Mann-Whitney U test (CRP) for continuous variables. *P<0.05; †P<0.01; ‡P<0.001.

In controls, 3 SNPs showed associations to CRP levels: the CRP−286C>T>A (CC versus other allelic combinations, ΔCRP=0.75 mg/L [95% CI, 0.64 to 0.88], P<0.001), 1059G>C (GG versus GC+CC, ΔCRP=1.38 mg/L [95% CI 1.09 to 1.75], P<0.05), and 1444C>T (CC versus CT+TT, ΔCRP=0.80 mg/L [95% CI, 0.69 to 0.94], P<0.01). These associations were also observed in cases (data not shown). No association to CRP levels was detected for the CRP−732 T>C SNP (P>0.6).

Figure 1. OR and 95% CI of ischemic stroke and different stroke subtypes per 1-SD increase in CRP concentration in the acute phase (1A and 1B) and at 3-month follow-up (2A and 2B). A, Univariate analysis and (B) multivariate analysis adjusted for age, sex, diabetes mellitus, smoking, hypertension, hyperlipidemia, and WHR. Crypt indicates cryptogenic stroke. *P<0.05, †P<0.01, ‡P<0.001.
CRP Haplotypes in Relation to Ischemic Stroke and CRP Levels

All SNPs were in high and significant LD with each other (D’>0.9). Consequently, only 5 haplotypes occurred with a frequency >1%, and they accounted for 99% of the chromosomes in the current sample. Unadjusted haplotypic ORs for overall ischemic stroke with corresponding 95% CI were estimated by comparison to the haplotype combining the most frequent allele for each SNP.

CRP in Relation to the Clinical Extent of Infarction and Outcome After 3 Months

CRP levels were higher in cases compared with controls during the acute phase, indicating a general inflammatory response to the ischemic event. In support of this notion, cases with clinical presentation indicating an extensive infarct (total anterior circulation infarcts, TACIs) displayed the highest CRP during the acute phase (18.5 mg/L compared with 3.5 and 3.3 mg/L in partial anterior circulation infarcts, PACIs, and lacunar infarcts, LACIs, respectively; P<0.001). At the 3-month follow-up, there were no differences in CRP levels among OCSP groups (P>0.6). Cases with an unfavorable functional outcome after 3 months (modified Rankin Scale score of 3 to 6) had higher CRP levels both in the acute phase and at follow-up compared with those with a favorable outcome (score 0 to 2; 10.1 versus 2.8 mg/L, P<0.001 acutely and 3.2 versus 2.1 mg/L, P<0.01, at follow-up). Exclusion of individuals who died before follow-up did not alter these results.

Discussion

In this case-control study of patients with ischemic stroke before the age of 70 years, we found independent associations to elevated CRP serum levels in the acute phase and at the 3-month follow-up. Analysis by etiological subtype according to TOAST criteria showed associations for all subtypes during the acute stage. However, at follow-up, there was a strong association between CRP and LVD, whereas no significant association was detected for SVD, CE stroke, or cryptogenic stroke. High CRP levels, both acutely and after 3 months, were also associated with an unfavorable functional outcome at follow-up. We also found associations between polymorphisms within the CRP gene and CRP levels in accord with earlier studies. However, we did not detect any association between the genetic variants and overall ischemic stroke.

The association between CRP levels and overall ischemic stroke is in agreement with a growing body of evidence that CRP is a predictor of cerebrovascular events. Recently, a systematic review including a meta-analysis was published on the association between CRP level and stroke in the general population. This study identified 2 cross-sectional studies and 5 prospective studies. All of these, except 1 prospective study with a relatively short follow-up, showed an association between CRP and stroke. In most studies, stroke/ischemic stroke was defined on the basis of self-report and/or medical history, and none of the studies differentiated between subtypes of ischemic stroke.

In SAHLSIS, all patients were assessed by a neurologist experienced with stroke and were classified with regard to subtype of ischemic stroke according to TOAST criteria. All 4 main subtypes showed a significant association with CRP levels at the 3-month follow-up. However, after adjustment for traditional risk factors, this association remained for LVD only. In LVD, the most common underlying pathophysiological event is likely to be rupture of an atherosclerotic plaque. The present result is therefore in line with earlier studies showing increased CRP levels in patients with myocardial infarction, as well as with results from a small study showing that follow-up CRP levels predict future ischemic events in transient ischemic attack and stroke patients with intracranial large-artery occlusive disease. The finding is also corroborated by recent experimental data showing that CRP is involved in the atherosclerosis process.
The lack of association between CRP and SVD contrasts recent results from the Rotterdam Scan Study, showing that higher CRP levels were associated with the presence and progression of white matter lesions. However, it is of note that the present population was younger, and we did not assess asymptomatic white matter lesions. In line with our results, no significant association between CRP and the presence of lacunar infarcts was observed in the Rotterdam study.

With regard to acute CRP levels, highest levels were observed for CE stroke. Analysis by OCSIP group showed the highest levels in TACIs, suggesting that a more intense inflammatory response is related to infarct size. In accordance with our results, an association between acute CRP and infarct size has been reported. Because CE stroke was most prevalent in the TACHI group, it is possible that the cardiac condition resulting in an embolic episode contributes to an increased CRP level. Also in accordance with previous findings, an elevated CRP value in the acute phase was associated with functional outcome at 3 months. Despite the fact that follow-up CRP levels were unrelated to OCSIP type, it is important to note that increased CRP levels at follow-up also showed a significant association with unfavorable outcome. A tentative explanation may be that persistent activation of inflammation, or an underlying inflammatory pathophysiological process, has a negative influence on recovery.

This study also investigated the effect of 4 different polymorphisms on CRP levels. Associations were observed for the −286C>T>A, 1059G>C, and 1444C>T SNPs, which is in line with earlier data. Haplotype analysis showed that the clade of haplotypes associated with low CRP levels corresponded to the C variant of the triallelic −286C>T>A and, in line with previous findings, the H4 haplotype carrying both the −286C and the 1059C allele was associated with the lowest levels of CRP. Moreover, the H2 haplotype carrying the −286T and 1444T alleles was associated with high CRP levels. Interestingly, recent data suggest the triallelic −286C>T>A promoter SNP as being a functional variant.

Although replicating earlier findings of an association between CRP polymorphisms and levels in the present population from western Sweden, we did not detect any association between genetic variants and overall ischemic stroke. Analysis by subtype showed a significant and independent association for the 1059G>C SNP and CE stroke. Unexpectedly, an increased risk was observed for the allele that is associated with low CRP levels (1059C), and the association remained after adjustment for CRP levels. One possible explanation might be that the 1059G>C SNP is a marker of another genetic variant associated with a pathophysiological process related to CE stroke, such as atrial fibrillation. However, this observation may be a chance finding and needs replication.

This study has some limitations. First, pharmacological therapy may potentially influence CRP levels. Although hyperlipidemia was included in the multivariate analysis, confounding effect of lipid-lowering therapy on CRP concentrations cannot be completely excluded. A higher proportion of cases (33%) compared with controls (7.5%) were receiving statins or other lipid-lowering drugs, and this disproportion was most evident in the LVD group (42%). However, if anything, this would underestimate the difference in CRP levels between groups. In contrast, the number of subjects receiving hormone replacement therapy was similar in cases and controls (6%). Second, our protocol did not include history with regard to recent infections and/or inflammatory events. Third, blood samples were taken over a period of 10 days after incident stroke. However, there were no significant differences with regard to median time of sampling in relation to stroke onset, between either TOAST or OCSIP subtypes (data on file). Finally, case and control ascertainment may influence results via selection bias. However, in our study, patients were consecutively recruited when they arrived at the hospital. The stroke admission rate to hospital in Sweden is high, and because the early case fatality rate in ischemic stroke is low, especially for the age group studied here, it is unlikely that this type of bias had any major influence on our results. The control group was recruited by random sampling from the general population in the same geographical areas as patients, which diminished the possibility of spurious results due to population stratification.

In conclusion, the present study reports significant differences in CRP levels between different ischemic stroke etiological subtypes. An independent association between high CRP levels at the 3-month follow-up and overall ischemic stroke in the LVD group was observed. Although both individual SNPs and haplotypes were associated with CRP levels, none of the genetic variants showed an association with overall ischemic stroke. The observation of an association between the 1059G>C SNP and CE stroke is intriguing, and if confirmed, it will require further study of haplotype blocks in the CRP genomic region.

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Disclosures

None.

References

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