Mendelian Randomization Suggests No Causal Association Between C-reactive Protein and Carotid Intima-media Thickness in the Young Finns Study

To the Editor:

It is unclear whether C-reactive protein (CRP), a nonspecific marker of acute phase inflammatory response, is causally related to arterial intima-media thickness (IMT), a risk factor for coronary heart disease (CHD). Previous evidence from conventional observational studies is inconsistent and suggests that the association may be biased or confounded.1 According to the Mendelian randomization approach, the genetic variants in the CRP gene (CRP) may represent good instruments for CRP levels that are largely free from reverse causation bias and confounding.1 If the association between CRP and IMT is causal, then genetic variants in CRP should be related to IMT to the extent predicted by the magnitude of their association with average CRP levels.

We examined the causality between CRP and carotid IMT by determining haplotypes from genetic variants in CRP among 1609 individuals (768 men and 841 women) participating in the Cardiovascular Risk in Young Finns study.2 We genotyped 5 single nucleotide polymorphisms (SNPs) in the CRP gene: CRP-717A>G (rs 2794521); CRP-286C>T>A (rs3091244); CRP +1059G>C (rs1800947); CRP +1444T>C (rs130864); and CRP +1846G>A (rs1205). The SNPs were in Hardy-Weinberg equilibrium and strongly linked D’ values ranging from 0.98 to 0.99. After exclusion of rare haplotypes (frequency <1%), 5 haplotypes remained for analysis. We assessed serum high-sensitive CRP in 1980 (at age 3 to 18) and 2001 (at age 24 to 39), and carotid IMT in 2001 to 2002. Potential confounding factors measured included adult biological risk factors (body mass index, systolic and diastolic blood pressure, total, HDL, and LDL cholesterol, triglycerides), smoking, alcohol consumption, and occupational status.

Details of the methods and results are provided in the online supplement (available at http://atvb.ahajournals.org). Of the 55 associations between haplotypes and potential confounders, there was no strong evidence of any robust association between haplotypes and potential confounding factors (P<0.05 only for 2 associations). This finding supports the assumption that the haplotypes represent a nonconfounded estimate of CRP levels. In contrast, circulating CRP was associated with all biological risk factors and smoking.

We used instrumental variable methods to obtain estimates of the causal (unbiased and nonconfounded) association between circulating CRP and IMT.3 Although the ordinary least squares regression analysis suggested a positive association of CRP levels measured in adulthood and mean life course CRP levels (higher CRP levels associated with greater IMT), the instrumental variables analysis suggested inverse associations of all three of childhood, adulthood, and mean life course CRP levels (higher CRP levels associated with lower IMT), though these were imprecisely estimated (Table).

Thus, our Mendelian randomization analysis with a null association between CRP haplotypes and IMT provides no support for the hypothesis that circulating CRP would causally influence IMT in a healthy population of young adults. The few previous Mendelian randomisation studies used CRP genotypes rather than haplotypes, they genotyped a smaller number of SNPs than was done in this study, their assessment of circulating CRP was limited to adulthood, and instead of IMT, they were focused on risk factors, such as blood pressure4 or components of the metabolic syndrome,5 or CHD events.6 However, similar to our results, those studies found no evidence to causally link CRP to coronary outcomes. In a cohort of adults aged 65 or older, CRP genotypes were associated with cardiovascular events, but not with carotid IMT.7

Disclosures

None.

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Comparison of the Association of CRP with IMT Obtained From Ordinary Least Squares Linear Regression to That Obtained from the Instrumental Variables Analysis (in Which CRP Haplotypes Act as An Instrument for the Non-confounded and Unbiased Effect of CRP)

<table>
<thead>
<tr>
<th>Exposure</th>
<th>Age- and Sex-Adjusted Mean Difference in IMT (µm) Per Unit of Exposure (95%CI)</th>
<th>$P$ for Difference in effect Between the Two Analyses</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Ordinary Least Squares Linear Regression</td>
<td>Instrumental Variables Analysis</td>
</tr>
<tr>
<td>Per doubling of CRP concentration in childhood</td>
<td>1.07 (−1.49 to 3.62)</td>
<td>−10.35 (−23.92 to 3.21)</td>
</tr>
<tr>
<td>Per doubling of CRP concentration in adulthood</td>
<td>4.16 (1.35 to 6.97)</td>
<td>−7.50 (−25.27 to 10.27)</td>
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<tr>
<td>Per 1 SD increase in lifecourse CRP z-score</td>
<td>4.80 (0.40 to 9.20)</td>
<td>−13.02 (−34.11 to 8.06)</td>
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</tbody>
</table>
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Supplementary Information

Participants
The participants were from the Cardiovascular Risk in Young Finns Study, an on-going multicentre follow-up study of cardiovascular risk factors in Finnish children and adolescents. The original sample was 4,320 White children and adolescents aged 3, 6, 9, 12, 15, and 18 years randomly chosen in five areas of Finland from the national register. The baseline examination was conducted in 1980, with the participation rate being 83%, ie 3,596 of those invited. Re-examinations have included follow-ups in 1983, 1986, 1989, 1992 and 2001. At the latest follow-up, the participants had reached 24 to 39 years of age. From the 2,283 participants of this follow-up, we excluded those with missing CRP in 1980 (n=138), CRP values >10 mg/L in 1980 or 2001 (n=107), history of recent infection (n=98), chronic rheumatic disease (n=25), lactating women and pregnant women in 2001(n=46), as well as those with missing data on genetic variants and IMT (n=260). Thus, the participants of this study comprised 1,609 individuals (768 men and 841 women) with full information about 5 genetic variants of the CRP gene, circulating CRP levels in 1980 and 2001, and IMT in 2001. This study was conducted according to the guidelines of the Helsinki declaration and the study protocol was approved by local ethics committees. All participants gave their informed consent.

CRP Genotyping
We genotyped 5 SNPs in the CRP gene [CRP-717A>G (rs 2794521); CRP-286C>T>A (rs3091244); CRP+1059G>C (rs1800947); CRP+1444T>C (rs1130864); and CRP+1846G>A (rs1205)] using the ABI Prism 7900HT Sequence Detection System for both PCR and allelic discrimination (Applied Biosystems, Foster City, CA). For SNP CRP+1059G>C a commercial kit from Applied Biosystem was used (Assay On Demand, C_177490_10 CRP). The other SNPs were genotyped using Assays By Design from Applied Biosystems under standard conditions, with the exception of the triallelic tagSNP, which was genotyped as described previously, except for the genotype calling which was done manually from the PCR run component tab.
Measurement of C-Reactive Protein

We assessed serum high-sensitive CRP in 1980 (childhood) and 2001 (adulthood) using an automated analyzer (Olympus AU400, Olympus, USA) and a highly sensitive turbidimetric immunoassay kit (CRP-UL-assay, Wako Chemicals, Neuss, Germany). Detection limit of the assay was 0.06 mg/L. The inter-assay coefficient of variation was 3.33% at the mean level of 1.52 mg/l (n=116) and 2.65% at the mean level of 2.51 mg/l (n=168).

Measurement of Carotid IMT

Ultrasound studies, carried out in 2001-2002, were performed using Sequoia-512 ultrasound mainframes (Acuson, Mountain View, CA, USA) to measure carotid IMT. The measurement has previously been described in detail.\textsuperscript{3,4} In brief, the image was focused on the posterior (far) wall of the left carotid artery. A minimum of four measurements of the common carotid far wall were taken approximately 10 mm proximal to the carotid bifurcation to derive mean carotid IMT. The between-visit coefficient of variation of IMT measurements was 6.4%.

Assessment of Potential Confounding Factors

All potential confounding factors were assessed in 2001, except for socioeconomic position in childhood or adolescence, which was assessed in 1980. Physical measurements of weight (kilograms) and height (mm) were obtained to calculate body mass index (BMI=weight in kg/ height\textsuperscript{2} in m). Systolic (SBP) and diastolic (DBP) blood pressure (mm Hg) were measured with a random zero sphygomanometer (Hawksley & Sons Ltd, West Sussex, UK) in a sitting position after at least five minutes rest. Readings were performed at least three times on each participant and the average was used in the statistical analysis. Standard enzymatic methods were used for serum total cholesterol, HDL cholesterol and triglycerides. LDL cholesterol concentration was calculated using the Friedewald formula for subjects with <4 mmol/L triglycerides. All blood samples were taken after an overnight fast and analyzed in duplicate in the same laboratory. Information on smoking (current smoker vs not) and alcohol consumption (units per week) was obtained by questionnaire. One unit of alcohol (12 g) was equal to a glass of wine, a single 4 cl shot of spirits or a 33 cl bottle of beer. Socioeconomic position in childhood or adolescence was assessed by parental occupational status (1=manual; 2=lower-grade non-manual; and
Where socioeconomic position differed between parents, data on the parent with the higher occupational status/education were used. The participants' own adult socioeconomic position was measured through occupational status and categorised as for parental SEP indicators.³

**Statistical Analysis**

The three variables of circulating CRP used in analyses were age- and sex-standardized z-scores of log CRP in childhood, in adulthood and across the life course (i.e., the average of age- and sex-standardized z-scores of log CRP in childhood and adulthood). It is necessary to limit the number of variables used to model the effect of haplotype on CRP in order to ensure these variables are strongly correlated with CRP, and thereby avoid problems of bias due to 'weak instruments'.⁶,⁷ In agreement with previous studies,⁸ we therefore excluded rare haplotypes (frequency<1%) and chose to use a model for the haplotype-CRP association that assumes each of a participant's two haplotypes contributes additively to his/her value of CRP. As the interactions we tested between sex and these haplotypes on all of the indicators of CRP (all p>0.52) and the interactions between sex and the CRP indicators on IMT (all p>0.09) gave no strong evidence of meaningful interactions analyses were based on a combined sample of men and women. We used age- and sex-adjusted least square regression analysis to assess the association of haplotypes with risk factors (i.e., BMI, systolic and diastolic blood pressure, HDL- and LDL-cholesterol, triglycerides, smoking, alcohol consumption, parental and own socioeconomic position score).

We used instrumental variable methods to obtain estimates of the causal (non-confounded and unbiased) association between circulating CRP and IMT.⁹,¹⁰ The two-stage age- and sex-adjusted least squares to fit the instrumental variables models included (1) a comparison of the instrumental variable estimates to those from ordinary linear regression using the Durbin form of the Durbin-Wu-Hausman statistic (main analyses) and (2) an examination of F-statistics from the first-stage regressions to evaluate the strength of the instruments (all F-statistics were greater than 10 (range 10.37 to 18.39) indicating sufficient strength to ensure the validity of instrumental variable methods in these data).⁹ All analyses were performed with SAS statistical software, version 9.1 (SAS Institute, Cary, USA), except instrumental variable regression analysis which was performed with Stata, version 8.0 (Stata Institute, Texas, USA).
Supplementary Results

Median CRP was 0.21 (IQR 0.11-0.53) mmol/L in childhood and 0.65 (IQR 0.30-1.52) mmol/L in adulthood. Mean IMT was 0.58 (SD 0.09) mm. Of the 55 associations between haplotypes and potential confounders or mediators there was no strong evidence of any robust associations (Table I) suggesting no association between haplotypes and potential confounding factors. In contrast, all biological risk factors were associated with circulating CRP and, except for HDL cholesterol, with IMT (Table II).
<table>
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<tr>
<th>Haplotype</th>
<th>BMI, kg/m²</th>
<th>SystBP, mm Hg</th>
<th>DiastBP, mm Hg</th>
<th>Total cholesterol, mmol/L</th>
<th>HDL-c, mmol/L</th>
<th>LDL-c, mmol/L</th>
<th>Triglycerides, mmol/L</th>
<th>Smoking % current</th>
<th>Alcohol intake, units/week</th>
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</table>

*1=manual, 2=lower-grade non-manual, 3=upper-grade non-manual
TABLE II. Age- and Sex-adjusted Linear Regression Analysis of the Associations of Biological, Behavioural and Socioeconomic Risk Factors with CRP and Carotid IMT.

<table>
<thead>
<tr>
<th>Risk factor</th>
<th>Change per doubling of adult CRP concentration</th>
<th>Change per 1 SD increase in lifecourse CRP z-score</th>
<th>Change per 1 mm increase in Intima-media thickness</th>
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</thead>
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<td></td>
<td>B</td>
<td>P</td>
<td>B</td>
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<td>BMI (kg/m$^2$)</td>
<td>1.15 (1.03 to 1.27)</td>
<td>$&lt;0.0001$</td>
<td>1.53 (1.34 to 1.72)</td>
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<td>Systolic BP (mm Hg)</td>
<td>1.46 (1.08 to 1.84)</td>
<td>$&lt;0.0001$</td>
<td>1.73 (1.13 to 2.32)</td>
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<tr>
<td>Diastolic BP (mm Hg)</td>
<td>1.54 (1.22 to 1.86)</td>
<td>$&lt;0.0001$</td>
<td>1.70 (1.19 to 2.22)</td>
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<td>Total cholesterol (mmol/L)</td>
<td>0.11 (0.08 to 0.14)</td>
<td>$&lt;0.0001$</td>
<td>0.12 (0.07 to 0.17)</td>
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<td>LDL-cholesterol (mmol/L)</td>
<td>0.06 (0.04 to 0.09)</td>
<td>$&lt;0.0001$</td>
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<td>HDL-cholesterol (mmol/L)</td>
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<td>Triglycerides (mmol/L)</td>
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<td>$&lt;0.0001$</td>
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<tr>
<td>Current smoking</td>
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<td>0.02 (0.00 to 0.04)</td>
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<td>Alcohol intake (unit per week)</td>
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<td>0.03 (-0.39 to 0.44)</td>
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<td>0.37</td>
<td>-0.01 (-0.04 to 0.03)</td>
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<tr>
<td>Own SEP score</td>
<td>-0.01 (-0.03 to 0.02)</td>
<td>0.66</td>
<td>-0.01 (-0.05 to 0.03)</td>
</tr>
</tbody>
</table>
Acknowledgements

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