



C-reactive protein and its role in metabolic syndrome: mendelian randomisation study

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Summary

Background Circulating C-reactive protein (CRP) is associated with the metabolic syndrome and might be causally linked to it. Our aim was to generate estimates of the association between plasma CRP and metabolic syndrome phenotypes that were free from confounding and reverse causation, to assess the causal role of this protein.

Methods We examined associations between serum CRP concentration and metabolic syndrome phenotypes in the British Women's Heart and Health Study. We then compared these estimates with those derived from a mendelian randomised framework with common CRP gene haplotypes to generate unconfounded and unbiased estimates of any causal associations.

Findings In a sample of British women, body-mass index (BMI), systolic blood pressure, waist-to-hip ratio, serum concentrations of HDL cholesterol and triglycerides, and insulin resistance were all associated with plasma CRP concentration. CRP haplotypes were associated with plasma CRP concentration ($p < 0.0001$). With instrumental variable analyses, there was no association between plasma CRP concentration and any of the metabolic syndrome phenotypes analysed. There was strong evidence that linear regression and mendelian randomisation based estimation gave conflicting results for the CRP–BMI association ($p = 0.0002$), and some evidence of conflicting results for the association of CRP with the score for insulin resistance ($p = 0.0139$), triglycerides ($p = 0.0313$), and HDL cholesterol ($p = 0.0688$).

Interpretation Disparity between estimates of the association between plasma CRP and phenotypes comprising the metabolic syndrome derived from conventional analyses and those from a mendelian randomisation approach suggests that there is no causal association between CRP and the metabolic syndrome phenotypes.

Introduction

Raised concentrations of circulating C-reactive protein (CRP) are associated with an increased risk of the metabolic syndrome^{1,2} and cardiovascular disease.³ Though the mechanism is unclear, some suggest^{4–7} that CRP is causally linked to the development of the metabolic syndrome, cardiovascular diseases, and type 2 diabetes mellitus. Potential mechanisms of action involve CRP eliciting pro-inflammatory responses through the mediation of cytokines, adhesion molecules, other signalling molecules, or endothelial nitric oxide.⁸ The results of recent studies, however, suggest that plasma CRP is not a pathological agent.^{9–12}

However, observed epidemiological associations between plasma CRP and health outcomes might be affected by reverse causation or confounding.^{13,14} For example, the positive relation between plasma CRP and obesity might be the result of the up-regulation of cytokines, such as tumour necrosis factor α (TNF α), as a result of obesity (ie, reverse causation), rather than an effect of CRP on obesity.¹⁵ Furthermore, confounding environmental factors, such as smoking and socioeconomic position, could contribute substantially to observed associations.^{13,14} The method of mendelian randomisation overcomes these problems since the association between a disease and a genetic polymorphism that serves as a proxy for an environ-

mentally modifiable exposure is not generally susceptible to reverse causation or confounding.¹⁶ This technique is analogous to a randomised clinical trial, but one in which the random assignment to genotype takes place at conception.¹⁷

Variation in CRP is associated with the concentration of circulating CRP,^{18–20} and therefore genetic variation in CRP might predispose to the metabolic syndrome and cardiovascular disorders.⁵ Using the paradigm of mendelian randomisation, our aim was two-fold: 1) to examine the association between certain CRP haplotypes and the phenotypes of the metabolic syndrome, and 2) to obtain unbiased estimates of the effect of circulating CRP on these metabolic outcomes, together with the degree of uncertainty of these effects.

Methods

Participants

We did a mendelian randomisation study, using data obtained for the British Women's Heart and Health Study.¹⁴ For that study, between 1999 and 2001, women aged 60–79 years were randomly selected from 23 British towns, and were interviewed, examined, and completed medical questionnaires. At baseline, blood samples were taken after a minimum 6-h fast and used to assess insulin resistance (homoeostasis model assessment [HOMA-R]; calculated from fasting

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insulin and glucose concentrations), and to measure concentrations of triglycerides, HDL cholesterol, and CRP. Blood pressure, height, and weight (used to calculate body-mass index [BMI]), and waist and hip circumferences were measured with standard procedures. CRP was assayed in citrated plasma with a high-sensitivity immunonephelometric assay on a ProSpec protein analyser (Dade-Behring, Milton Keynes, UK).

Local ethics committee approvals were obtained for the British Women's Heart and Health Study along with UK multicentre ethical approval. At time of baseline assessment of this study, women were asked for written informed consent to review their medical records and for permission to undertake anonymised genetic tests relating to cardiovascular disease on stored blood.

Procedures

On the basis of published work,^{18–20} indicating their involvement in the regulation of plasma CRP concentrations, we selected the following common genetic variants of CRP for assessment: rs2794521 (GÆ C, 5' flanking region, AF449713 position 1009), rs1800947 (GÆ C, synonymous change at codon 188, AF449713 position 2667), rs1130864 (GÆ A, 3' untranslated region, AF449713 position 3014), and rs1205 (CÆ T, 3' flanking/untranslated region, AF449713 position 3872).

KBiosciences, Essex, UK, genotyped single nucleotide polymorphisms (SNPs) for us with their KASPar system, which is a competitive allele specific PCR SNP genotyping system that uses FRET quencher cassette oligos.

Statistical analysis

We used linear regression analyses to assess the association of log plasma CRP concentration with insulin resistance (log HOMA-R), concentrations of log triglyceride and HDL cholesterol, systolic blood pressure, BMI, and waist-to-hip ratio.

We tested the Hardy-Weinberg equilibrium at each SNP locus on a contingency table of observed-versus-predicted genotypic frequencies with an exact test. We quantified linkage disequilibrium estimates by D' and r^2 values calculated with Stata (version 9.1).

We constructed haplotypes with the genetic data analysis program SIMHAP, which we also used to examine the associations between common CRP haplotypes and plasma CRP concentration. We derived the mean plasma CRP concentration by common CRP haplotype from the regression coefficients with confidence intervals estimated by SIMHAP.

We used instrumental variable methods²¹ to obtain estimates of the association between CRP and the continuous outcomes implied from mendelian randomisation, since approximate methods used previously^{16,22} to derive estimates do not extend easily to situations of more than two common haplotypes. Briefly, an instrument is a variable that is associated with an exposure of interest, and is only related to the outcome being studied through the exposure of interest.²² As such, it is not affected by either confounding or reverse causation that may distort the directly assessed association between the exposure of interest and the outcome. Hence, as instrumental variables, CRP haplotypes associated with plasma CRP act as non-confounded and non-biased markers for lifetime CRP concentration. We used these instruments to estimate the causal effect of a doubling in CRP concentration on the metabolic syndrome components. As a result of the incorporation of imprecision that surrounds both the estimates of gene (CRP) variation/outcome and exposure of interest (CRP)/outcome associations, we anticipated wide CIs. We used the most likely haplotype assignment for each woman in analyses, and did sensitivity analysis for the estimated effect of haplotypes if posterior probabilities for haplotype reconstruction fell below 0.9.

The association between serum CRP concentration and CRP variation and binary potential confounding variables was assessed with χ^2 tests. For serum CRP concentration, we used the three-tiered categorisation that we used for the main analysis—ie, CRP category low (<1 mg/L), medium (1–3 mg/L), and high (>3 mg/L). For CRP variation, we generated three groups on the basis of haplotypic carriage. We derived a socioeconomic position score (range 0–10) from ten indicators of socioeconomic position across life course

For details of SIMHAP, see <http://www.genepi.com.au/project/simhap>

	CRP category (mean, 95%CI)			p*	Change in phenotype for a doubling in plasma CRP concentration or ratio of geometric means for a doubling in plasma CRP	p†
	Low (<1 mg/L)	Medium (1–3 mg/L)	High (>3 mg/L)			
BMI (kg/m ²)	25.3 (25.1 to 25.5)	27.6 (27.3 to 27.8)	29.6 (29.3 to 30.0)	<0.0001	1.05 (0.95 to 1.14)	<0.0001
Systolic blood pressure (mm Hg)	143.8 (142.2 to 145.40)	147.5 (146.0 to 148.9)	149.6 (148.1 to 151.1)	<0.0001	1.37 (0.84 to 1.89)	<0.0001
Waist-to-hip ratio	0.79 (0.79 to 0.80)	0.82 (0.82 to 0.83)	0.84 (0.84 to 0.84)	<0.0001	0.0113 (0.0099 to 0.0126)	<0.0001
HDL cholesterol (mmol/L)	1.79 (1.76 to 1.82)	1.66 (1.63 to 1.68)	1.54 (1.52 to 1.57)	<0.0001	−0.06 (−0.07 to −0.05)	<0.0001
Triglycerides (mmol/L)‡	1.41 (1.37 to 1.44)	1.70 (1.66 to 1.75)	1.88 (1.83 to 1.93)	<0.0001	1.08 (1.07 to 1.09)	<0.0001
HOMA-R‡	1.39 (1.34 to 1.44)	1.68 (1.62 to 1.74)	1.94 (1.86 to 2.02)	<0.0001	1.09 (1.07 to 1.10)	<0.0001

*One way ANOVA analysis across clinically relevant CRP categories. †Basic linear regression of metabolic syndrome components by logged plasma CRP concentration. ‡Log transformed so geometric mean presented.

Table 1: Associations between plasma CRP concentrations and components of the metabolic syndrome

	Major homozygote	Heterozygote	Minor homozygote	Minor allele frequency (SE)	p
rs1800947 (n=3218)	2797 (87%; CC)	410 (13%; CG)	11 (0.3%; GG)	0.07 (0.0031)	0.3979
rs1130864 (n=3218)	1543 (48%; GG)	1387 (43%; AG)	288 (9%; AA)	0.31 (0.0057)	0.3604
rs1205 (n=3218)	1452 (45%; CC)	1443 (45%; CT)	323 (10%; TT)	0.32 (0.0058)	0.2126

Data are number of samples assessed (%; genotype).

Table 2: Allelic frequencies for CRP SNPs

as previously described,¹⁴ and assessed the score for differences by category via one way analysis of variance. We considered the following binary variables as confounding factors: smoking (current smokers vs never or previous smokers), alcohol consumption (≤ 2 vs > 2 alcoholic drinks per day), use of hormone replacement therapy (HRT; ever vs never), and physical activity (< 2 h vs ≥ 2 h moderate or vigorous activity per week).²³

See [Lancet Online](#) for webappendix, webtables 1 and 2

See the webappendix for a more detailed explanation of some of the statistical analyses done.

Role of the funding source

The sponsor of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Results

Of 4286 individuals in the British Women’s Heart and Health Study sample, 405 had insufficient blood taken for assays and eight refused consent to use their stored blood. Of the remaining 3873 samples, we genotyped 3597 (arithmetic mean across three polymorphic loci) for polymorphic loci. Removal of missing data points across the three SNP loci yielded complete genotypic datasets for 3218 women (75% of total sample). For all variables, other than concentration of plasma CRP, there were no meaningful differences between these women and those excluded because of missing data. 615 individuals excluded as a result of missing data had a detectable difference in plasma CRP concentration (mean CRP [95%CI] for included and excluded groups: 0.84 [0.81–0.88] and 0.78 [0.71–0.86], respectively; $p=0.0269$). There were strong linear associations between log plasma CRP concentrations and all components of the metabolic syndrome (HOMA-R, concentrations of triglycerides and HDL cholesterol, systolic blood pressure, BMI, and waist-to-hip ratio), with no evidence of a threshold effect for associations (table 1). These associations did not differ between included and excluded individuals (data not shown).

In this sample of British women, SNP rs2794521 was monomorphic and not analysed further. We noted minor allele frequencies for SNPs rs1800947, rs1130864, and rs1205 of 0.07, 0.31, and 0.32, respectively (table 2). We included these SNPs in analyses, and all had genotypic distributions consistent with Hardy-Weinberg

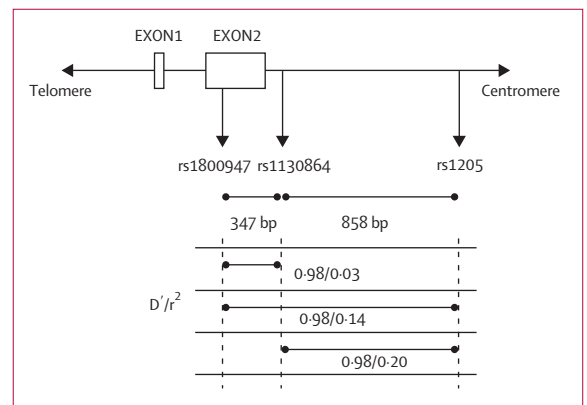


Figure 1: Diagrammatic representation of the CRP region, showing relative positions of informative variants

equilibrium. Pairwise linkage disequilibrium was assessed; D'/r^2 values between variant pairs were 0.98/0.03 (rs1800947 and rs1130864), 0.98/0.14 (rs1800947 and rs1205), and 0.98/0.20 (rs1130864 and rs1205; figure 1). There were four three-SNP haplotypes with a frequency of more than 5%, of which haplotypes CAC and GGT were associated with highest and lowest plasma CRP values, respectively (table 3).

We assessed the association between potentially confounding variables and plasma CRP concentrations (webtable 1) and CRP haplotypes (webtable 2). High concentrations of plasma CRP were associated with smoking, reduced physical activity, daily alcohol intake, and poor socioeconomic position. We assessed the relation between CRP variation and potential confounding variables with groups based on the observed association between CRP haplotypes and plasma CRP concentration (table 3). By contrast with plasma CRP concentrations (webtable 1), CRP haplotype groups were not associated with potential confounding variables (webtable 2).

	Estimated frequency (SE)	Plasma CRP (mg/L) (geometric mean, 95%CI)
CGC	0.37 (0.006)	1.81 (1.66–1.96)
CGT	0.26 (0.005)	1.70 (1.58–1.83)
CAC	0.30 (0.006)	2.03 (1.90–2.18)
GGT	0.07 (0.003)	1.39 (1.23–1.56)

Global ANOVA for differences in CRP concentration by haplotype $p < 0.0001$. Haplotypes CAT, GGC, GAC, GAT excluded from table because of inferred frequencies of $< 1\%$.

Table 3: Common haplotypes for the CRP region

	Change with doubling of CRP concentrations (linear regression)	Change with doubling of CRP concentration (instrumental variables)	p*
BMI (kg/m ²)	1.04 (0.94 to 1.14)	-0.44 (-1.34 to 0.46)	0.0002
Systolic blood pressure (mm Hg)	1.4 (0.9 to 1.9)	-0.9 (-5.3 to 3.5)	0.3003
Waist-to-hip ratio	0.011 (0.099 to 0.013)	0.005 (-0.007 to 0.016)	0.2388
HDL cholesterol (mmol/L)	-0.064 (-0.073 to -0.055)	0.006 (-0.072 to 0.084)	0.0668
Triglycerides (mmol/L)†	1.08 (1.07 to 1.09)	0.99 (0.92 to 1.08)	0.0313
HOMA-R†	1.09 (1.07 to 1.10)	0.94 (0.84 to 1.07)	0.0139

*Test of equality of linear regression and instrumental variables estimates. †Ratios of geometric means by a doubling in plasma CRP concentration. Instrumental variables are two-stage least squares estimates with p values to compare between these and ordinary linear regression estimates obtained from Durbin-Wu-Hausman test;²³ results were similar with two other instrumental variable estimators and corresponding tests.

Table 4: Comparison of associations between CRP and other variables estimated by linear regression and with instrumental variables (with CRP haplotypes as instruments)

We incorporated the four most common haplotypes identified in instrumental variable analyses (n=3206 women). 12 women were excluded from this set of analyses because they carried haplotypes with a frequency of less than 1%. We did not do a sensitivity analysis at this stage, incorporating the uncertainty of haplotype assignment, since all probabilities for haplotypic inference were 0.99 or more. Table 4 shows the results of the instrumental variable analysis and comparisons with the direct associations estimated by ordinary linear regression.²⁴ The first-stage F statistic was 13.7 for HOMA-R (reflecting the smaller sample) and in excess of 15 for the other outcome variables, indicating that CRP variation had sufficient instrument strength to avoid the problem of so-called weak instruments (see webappendix).²⁵

With the instrumental variable approach, we noted no association between plasma CRP concentration and any of the metabolic syndrome phenotypes assessed. The point estimates were closer to the null than those from ordinary linear regression; however, as anticipated, the CIs from the instrumental variables were much wider. There was strong evidence that the two methods of estimation gave conflicting results for the CRP–BMI association (p=0.0002), and some evidence of conflicting results for the association of CRP with HOMA-R score (p=0.0139), triglyceride concentration (p=0.0313), and HDL-cholesterol values (p=0.0668). There was no strong evidence of difference in the estimates of the associations with systolic blood pressure or waist-to-hip ratio.

The main model presented above assumed an additive effect of each haplotype on log CRP. The pattern of residuals was similar for all pairs of haplotypes, indicating a good statistical fit (see webappendix). However, to incorporate the arguable biological plausibility of analysis by diplotype, we did a further sensitivity analysis, fitting a model that allowed a different log CRP concentration for each of the ten possible pairs of haplotypes (diploypes). This analysis gave similar results to the main analysis (webtable 3). The p values were similar, or somewhat smaller than those noted above, though the corresponding F statistics

were too small to exclude the possibility of bias due to weak instruments (5.4 for HOMA-R, 6.0–6.2 for other metabolic syndrome variables).

Discussion

Our findings show that plasma CRP concentrations are associated with all major components of the metabolic syndrome, as previously reported.¹ However, results of analyses that used the genetic determinants of plasma CRP as instruments to enable a non-confounded and unbiased assessment of the role of CRP in the metabolic syndrome phenotypes suggest that these associations are not causal.

If CRP was a causal factor in the metabolic syndrome, we would have noted similar associations when analysing plasma CRP and metabolic syndrome outcomes with conventional regression as when incorporating information from the non-confounded, genetically determined variation in plasma CRP concentration. If CRP does not determine key components of the metabolic syndrome, but is simply predictive of them because of reverse causality or confounding, the suitability of CRP as a potential therapeutic target for the prevention of metabolic disorders and related chronic disease becomes doubtful.^{4–6}

A limitation of our work relates to the fairly small change in circulating CRP concentrations attributable to genotypic variation and consequently the limited precision of our estimates of the non-confounded effect of circulating CRP concentrations. Much of the previous published work that has implicated circulating CRP as a cause of common disease has shown graded relations between circulating CRP and phenotypic outcome across broad categories of circulating CRP.²⁶ Findings of previous studies¹⁰ indicate that the 33rd and 66th percentiles of circulating CRP differ by a factor of about 2.5. In this study, the greatest absolute effect of CRP haplotype on plasma CRP concentration noted was smaller. Together with the fairly low frequency of the haplotype having the largest effect on circulating CRP, this factor contributes to wide CIs around the estimates of the effect of a doubling in the concentration of CRP predicted by instrumental variable analysis.

See [Lancet Online](#) for webtable 3

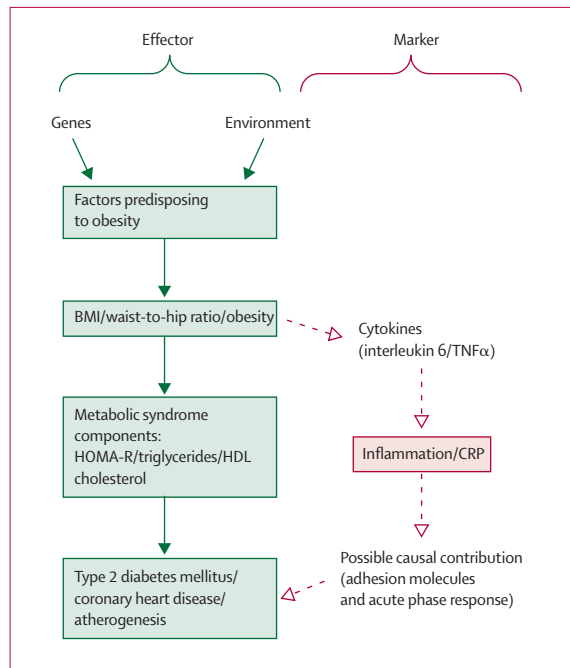


Figure 2: Possible associations between circulating CRP concentrations and metabolic syndrome

A second limitation is that we made several assumptions in the instrumental variables approach. For the application of this approach to be valid in this situation, the assumptions behind mendelian randomisation must hold. Pleiotropy in the instance of the *CRP* gene needs to be absent and canalisation—ie, the presence of compensatory adaptation as a result of early life systemic imbalance—unlikely to have arisen. Furthermore, the difficulty of weak instruments^{25,27} (the absence of strong correlation between, in this instance, *CRP* haplotypes and the intermediate phenotype, plasma CRP) needs to be avoided. Although pleiotropy and canalisation are difficult to assess directly, they have not been previously identified in the instance of the *CRP* gene. Furthermore, our use of a parsimonious model of the gene–CRP association and checking of the strengths of this model should have avoided known limitations of instrumental variable techniques in the presence of weak instruments. This model assumes that a woman's two haplotypes have an additive effect on her log CRP concentration. As shown in sensitivity analyses, such a model provided a good fit to the data.

Last, there are potential caveats with respect to the nature of the population sample more generally. Individuals excluded from our analysis on the grounds of missing genotype data had lower plasma CRP concentrations than those included. However, this difference was small and the relations between plasma CRP and components of the metabolic syndrome were consistent in those with and without complete data. Our study is of European women only, and therefore results

might not be generalisable to men or to other ethnic groups. However, the weight of evidence suggests that although some overall sex differences might exist, these are unlikely to affect *CRP*/plasma CRP and plasma CRP/metabolic syndrome component associations, and thus our overall conclusions.^{15,19,20} Also, although artificial genotype/phenotype association might be the result of population stratification in particular circumstances,²⁸ the low proportion of non-white individuals in this sample (16 of 4286; 0.4%) reduces this possibility (results of re-analyses are not materially altered by the exclusion of those individuals categorised as non-white).

So, is circulating CRP a determinant or a marker of the metabolic syndrome? There is increasing evidence that BMI and other indices of obesity are strong determinants of plasma CRP concentration.^{29–31} Increased systemic inflammation (with increased concentration of a related marker, such as CRP) might therefore be an indicator of pre-obese and obese states as a result of increased adiposity and consequent up-regulation of the cytokines interleukin 6 and TNF α . As summarised in figure 2, this situation might account for the lack of a direct link between *CRP* haplotypes and components of the metabolic syndrome. Notably, we have assessed the role of circulating CRP not tissue-specific CRP concentrations, which could theoretically be aetiologically important, but unrelated (or inversely related) to circulating CRP. A similar situation may explain the lack of concordance between the effects of measured extracellular superoxide dismutase (EC-SOD) and of a genotype relating to higher circulating EC-SOD³² and should not be dismissed.³³

While pathways in which CRP are involved are hypothetically linked to further disease related factors,⁸ it may be the largely unexplained links between these and common disease that are ultimately of pathogenic importance. Circulating concentrations of CRP might merely be marking the presence of metabolically related disease risk factors. Our findings add to the evidence that CRP is not causal in the pathology of the metabolic syndrome components⁹ and is not a suitable target for direct therapeutic intervention.^{13,14}

Contributors

G Davey Smith thought of the study aim, and developed it with input from N J Timpson, D A Lawlor, R M Harbord, A T Hattersley, and S Ebrahim. N J Timpson undertook the analyses with R M Harbord, wrote the first draft of the report, and coordinated further drafts. D A Lawlor is a co-director of the British Women's Heart and Health Study, provided analysis advice, and together with I N M Day and S Ebrahim obtained funds for DNA extraction. R M Harbord supervised the statistical analysis. T R Gaunt oversees the primary DNA bank. I N M Day is the principal applicant on a grant that funds DNA extraction and genotyping in the British Women's Heart and Health Study and supervised the DNA extraction. L J Palmer supervised the SIMHAP analyses. S Ebrahim is the director of the British Women's Heart and Health Study and contributed to obtaining funds for DNA extraction and genotyping. G D O Lowe obtained funds for undertaking the CRP assays and supervised these assays. A Rumley undertook the CRP assays. All authors helped to write the report.

Conflict of interest statement

We declare that we have no conflict of interest.

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