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Authors: Elliott P, Chambers JC, Zhang W, Clarke R, Hopewell JC, Peden JF, Erdmann J, Braund P, Engert JC, Bennett D, Coin L, Ashby D, Tzoulaki I, Brown IJ, Mt-Isa S, McCarthy MI, Peltonen L, Freimer NB, Farrall M, Ruukonen A, Hamsten A, Lim N, Froguel P, Waterworth DM, Vollenweider P, Waeber G, Jarvelin MR, Mooser V, Scott J, Hall AS, Schunkert H, Anand SS, Collins R, Samani NJ, Watkins H, Kooner JS

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Genetic Loci Influencing C-reactive Protein Levels and Risk of Coronary Heart Disease

Prof Paul Elliott, FMedSci*, **Dr John C. Chambers, PhD***, and **Dr Weihua Zhang, PhD**
Faculty of Medicine, Imperial College London, London, UK

Dr Robert Clarke, MD and **Dr Jemma C. Hopewell, PhD**
Clinical Trial Service Unit and Epidemiological Studies Unit, Oxford, UK

Dr John F. Peden, PhD
Department of Cardiovascular Medicine and The Wellcome Trust Centre for Human Genetics, University of Oxford

Dr Jeanette Erdmann, PhD
Universität zu Lübeck, Lübeck, Germany

Mr Peter Braund, MSc
Department of Cardiovascular Sciences, University of Leicester, Leicester, UK

Dr James C. Engert, MD
Departments of Medicine and Human Genetics, McGill University, Montreal, Quebec, Canada

Dr Derrick Bennett, PhD
Clinical Trial Service Unit and Epidemiological Studies Unit, Oxford, UK

Dr Lachlan Coin, PhD and **Prof Deborah Ashby, PhD**
Faculty of Medicine, Imperial College London, London, UK

Dr Ioanna Tzoulaki, PhD, Dr Ian J. Brown, PhD, and Dr Shahrul Mt-Isa, BSc
Faculty of Medicine, Imperial College London, London, UK

Prof Mark I. McCarthy, FMedSci
Oxford Centre for Diabetes, Endocrinology and Metabolism and Oxford NIHR Biomedical Research Centre, Oxford, UK

Prof Leena Peltonen, MD, PhD
Wellcome Trust Sanger Institute, Hinxton, Cambridge, UK

Prof Nelson B. Freimer, MD
Center for Neurobehavioral Genetics, UCLA, Los Angeles, USA

Corresponding authors: Prof Paul Elliott, FMedSci, MRC-HPA Centre for Environment and Health, Department of Epidemiology and Public Health, Imperial College London, St Mary's Campus, Norfolk Place, London W2 1PG, UK (p.elliott@imperial.ac.uk), and Prof Jaspal Kooner, FRCP, National Heart and Lung Institute, Imperial College London, Hammersmith Campus, Du Cane Road, London W12, UK (j.kooner@imperial.ac.uk)..

*These authors contributed equally

Author contributions

PE and JSK had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

PE, JCC, JS, JSK designed the study and wrote the report. JCC, WZ, RC (Clarke), JH, LC, JT, IJB, SMI, DA, AR carried out the statistical and biochemical analyses. MIM, LP, NBF, NL, PF, DMW, PV, GW, MRJ, VM provided genome wide data and analysis. RC (Clarke), JH, JFP, DB, JE, PB, JCE, MF, AH, ASH, HS, SSA, RC (Collins), NJS, HW provided analysis of SNP data for cases and controls.

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Dr Martin Farrall, FRCPath

Department of Cardiovascular Medicine and The Wellcome Trust Centre for Human Genetics, University of Oxford

Dr Aimo Ruukonen, MD, PhD

Institute of Diagnostics, University of Oulu

Prof Anders Hamsten, MD

Department of Medicine, Karolinska Institutet, Stockholm, Sweden

Dr Noha Lim, PhD

Genetics Division, GlaxoSmithKline, King of Prussia, PA, USA

Prof Philippe Froguel, MD

Faculty of Medicine, Imperial College London, London, UK

Dr Dawn M. Waterworth, PhD

Genetics Division, GlaxoSmithKline, King of Prussia, PA, USA

Dr Peter Vollenweider, MD and Dr Gerard Waeber, MD

Department of Medicine, CHUV University Hospital, Lausanne, Switzerland

Prof Marjo-Riitta Jarvelin, MD

Faculty of Medicine, Imperial College London, London, UK

Institute of Health Science and Biocenter Oulu, University of Oulu, and the National Institute for Health and Welfare, Oulu

Prof Vincent Mooser, MD

Genetics Division, GlaxoSmithKline, King of Prussia, PA, USA

Prof James Scott, FRS

Faculty of Medicine, Imperial College London, London, UK

Prof Alistair S. Hall, FRCP

Leeds Institute for Genetics and Therapeutics, University of Leeds, Leeds, UK

Prof Heribert Schunkert, MD

Universität zu Lübeck, Lübeck, Germany

Prof Sonia S. Anand, MD

Population Health Research Institute, Hamilton Health Sciences, and the Departments of Medicine and Clinical Epidemiology and Biostatistics, McMaster University, Hamilton, Ontario, Canada

Prof Rory Collins, FRCP

Clinical Trial Service Unit and Epidemiological Studies Unit, Oxford, UK

Prof Nilesh J. Samani, FMedSci

Department of Cardiovascular Sciences, University of Leicester, Leicester, UK

Hugh Watkins, FRCP

Department of Cardiovascular Medicine and The Wellcome Trust Centre for Human Genetics, University of Oxford

Prof Jaspal S. Kooner, FRCP

Faculty of Medicine, Imperial College London, London, UK

Abstract

Context: Plasma levels of C-reactive protein (CRP) are independently associated with risk of coronary heart disease, but whether CRP is causally associated with coronary heart disease or merely a marker of underlying atherosclerosis is uncertain.

Objective: To investigate association of genetic loci with CRP levels and risk of coronary heart disease.

Design, setting and participants: We first carried out a genome-wide association (n=17,967) and replication study (n=14,747) to identify genetic loci associated with plasma CRP concentrations. Data collection took place between 1989 and 2008 and genotyping between 2003 and 2008. We carried out a Mendelian randomisation study of the most closely associated SNP in the *CRP* locus and published data on other *CRP* variants involving a total of 28,112 cases and 100,823 controls, to investigate the association of *CRP* variants with coronary heart disease. We compared our finding with that predicted from meta-analysis of observational studies of CRP levels and risk of coronary heart disease. For the other loci associated with CRP levels, we selected the most closely associated SNP for testing against coronary heart disease among 14,365 cases and 32,069 controls.

Main outcome measure: Risk of coronary heart disease.

Results: Polymorphisms in five genetic loci were strongly associated with CRP levels (% difference per minor allele): SNP rs6700896 in *LEPR* (-14.7% [95% Confidence Interval {CI}], -17.5 – -11.9, $P=1.6\times 10^{-21}$), rs4537545 in *IL6R* (-10.8% [95% CI, -13.8 – -7.7], $P=5.1\times 10^{-11}$), rs7553007 in *CRP* locus (-20.7% [95% CI, -23.5 – -17.9], $P=3.3\times 10^{-38}$), rs1183910 in *HNF1A* (-13.6% [95% CI, -16.4 – -10.6], $P=1.2\times 10^{-17}$) and rs4420638 in *APOE-CI-CII* (-21.8% [95% CI, -25.4 – -18.1], $P=2.1\times 10^{-25}$). Association of SNP rs7553007 in the *CRP* locus with coronary heart disease gave odds ratio (OR) 0.98 (95% CI, 0.94 – 1.01) per 20% lower CRP. Our Mendelian randomisation study of variants in the *CRP* locus showed no association with coronary heart disease: OR 1.00 (95% CI, 0.97 – 1.02) per 20% lower CRP, compared with OR 0.94 (95% CI, 0.94 – 0.95) predicted from meta-analysis of the observational studies of CRP levels and coronary heart disease (Z-score -3.45, $P<.001$). SNPs rs6700896 in *LEPR* (OR 1.06 [95% CI, 1.02 – 1.09] per minor allele), rs4537545 in *IL6R* (OR 0.94 [95% CI, 0.91 – 0.97]) and rs4420638 in the *APOE-CI-CII* cluster (OR 1.16 [95% CI, 1.12 – 1.21]) were all associated with risk of coronary heart disease.

Conclusions: The lack of concordance between the effect on coronary heart disease risk of *CRP* genotypes and CRP levels argues against a causal association of CRP with coronary heart disease.

INTRODUCTION

Coronary heart disease (CHD) is the leading cause of death worldwide.¹ Inflammation plays a key role in the pathogenesis of CHD at every stage from initiation, to progression and rupture of the atherosclerotic plaque.² C-reactive protein (CRP), an acute phase protein synthesised primarily by the liver, is currently the most widely used biomarker of inflammation.³ Observational studies have consistently demonstrated that higher plasma levels of CRP are associated with higher risk of CHD^{4,5}, and measurement of CRP has been advocated as a means of improving risk prediction.⁶ There is considerable interest in establishing whether CRP has a causal role in CHD, or whether CRP is merely a marker of underlying atherosclerosis.^{7,8} While previous studies have addressed this question, it is unclear if they included a sufficient number of cases to have adequate statistical power to confirm or refute associations of CHD with genetically determined differences in CRP levels.⁸ Resolution of this question will improve understanding of inflammatory mechanisms in atherosclerosis.

The “Mendelian randomisation” concept has been used to investigate possible causal relationships of an intermediate trait (such as CRP levels) with disease, taking advantage of the random allocation of alleles at conception.⁹ If the intermediate trait is causally linked to disease, then genetic variants influencing the trait should also influence disease risk.¹⁰ Mendelian randomisation studies should be unaffected by confounding from environmental factors, e.g., smoking, and reverse causation bias, i.e., where the disease itself (atherosclerosis) influences the trait (CRP levels).⁹

The aims of the present study were to conduct a genome-wide association study to identify common genetic loci that influence CRP levels, and use the concept of Mendelian randomisation to improve understanding of the possible causal relationship of CRP levels with CHD.

DESIGN AND METHODS

Study design and rationale

The study involved four inter-related components:

- i. a genome-wide association and replication study to identify genetic loci associated with CRP levels, and at each locus, selection of the most closely associated SNP;
- ii. a Mendelian randomisation study of CHD risk for the most associated SNP in the *CRP* locus in our data, and published data on *CRP* variants, to assess the potential causal association of CRP with CHD;
- iii. comparison of the finding from the Mendelian randomisation study with that predicted from meta-analysis of the relationship of CRP levels with CHD from observational studies;
- iv. a genetic association study of CHD for the most associated SNPs in genetic loci outside the *CRP* locus, using the concept of CRP as an intermediate phenotype¹⁰ to identify putative pathways linking inflammation with CHD.

Population cohorts

Genome-wide and replication study for CRP—Genome-wide association to identify variants related to CRP levels, measured using high sensitivity assays, was carried out in 17,967 participants from five studies: the London Life Sciences Population study (LOLIPOP: N=5,502), a population based cohort of European white and Indian Asian men and women, aged 35-75 yrs, living in West London, UK¹¹ (data collection 2001 – 2007, genome-wide genotyping 2003 – 2008); the 1966 Northern Finnish Birth Cohort (NFBC: N=4,761), a prospective birth cohort of persons born in 1966 in the two northernmost provinces of Finland^{12,13} (data collection 1997 – 1998, genotyping 2007 – 2008); the Lausanne Cohort (CoLaus: N=5,226), a cross-sectional study of a random sample of European men and women, aged 35–75 yrs, living in Lausanne, Switzerland¹⁴ (data collection 2003 – 2006, genotyping 2006 – 2007); the Genetic Epidemiology of Metabolic Syndrome study (GEMS: N=1,781), a case-control study of dyslipidaemic cases (age 20–65 years) matched with normolipidaemic controls by sex and recruitment site¹⁵ (data collection 2003 – 2006, genotyping 2006 – 2007); and the Data from an Epidemiological Study on the Insulin Resistance syndrome study (DESIR: N=697), a longitudinal French general population cohort of persons aged 30-64 years recruited through the French social security system¹⁶ (data collection 1994 – 2004, genotyping 2006 – 2007). Replication of SNPs associated with CRP, identified in the genome-wide association study, was performed in a further 14,747 LOLIPOP participants who were not included in the genome-wide association study and were free of known CHD.

Mendelian randomisation and genetic association studies with CHD—Variants related to CRP were tested for association with CHD among 14,365 CHD cases and 32,069 controls. The participating studies comprised the Precocious Coronary Artery Disease study (PROCARDIS: N=8,328), a case-control study of premature CHD before age 66 years¹⁷ (data collection 1999 – 2006, genotyping 2008); the International Studies of Infarct Survival (ISIS: N=3,624), comprising men aged 30–54 yrs and women aged 30–64 yrs, with non-fatal MI, and their spouse controls^{18,19} (data collection 1989 – 1992, genotyping 2008); the British Heart Foundation Family Heart Study comprising individuals with myocardial infarction or coronary revascularisation before the age of 66 years and at least one first-degree relative with premature CHD, who were also studied as part of the Wellcome Trust Case Control Consortium (WTCCC: N=3,249 to 4,863)²⁰ (data collection 1998 – 2006, genotyping 2006 – 2008); the German MI Family Studies (GerMIFS I: N=2,519; GerMIFS II: N=2,520, comprising persons with myocardial infarction before the age of 60 years and at least one first-degree relative with premature CHD, and matched controls^{20,21} (data collection 1996 – 2008, genotyping 2006 – 2008); the INTERHEART study, a multinational case-control study of persons presenting with first myocardial infarction (N=4,043)²², (data collection 1999 – 2003, genotyping 2008); and the LOLIPOP study (N=20,475) (data collection 2001 – 2007, genotyping 2008).

Genotyping

Genome-wide association scans were performed using the Affymetrix 500K mapping array, the Illumina 317K array and Perlegen Sciences customised arrays. To combine data across genotyping platforms, imputation was done using a Hidden Markov Model algorithm implemented in MACH v1.0 software (Center for Statistical Genetics, University of Michigan, MI, USA) (LOLIPOP) or IMPUTE v0.5.0 (Genetics Software Suite, University of Oxford, UK) v0.5.0 (other studies), and phased haplotypes from HapMap build35, dbSNP build 125. For the European datasets, the HapMap CEU sample was used for reference haplotypes; Indian Asian datasets were imputed based on a combination (mixed) of HapMap populations. Imputed SNPs with minor allele frequency (MAF)<.01, or low quality score ($r^2 < 0.30$ in MACH, or information score <0.5 in IMPUTE) were removed. This left ~1.4 million directly genotyped or imputed autosomal SNPs per participant with data available in all samples. Genotyping for replication testing and for evaluation against CHD was performed using KASPar (LOLIPOP), Sequenom (INTERHEART, PROCARDIS), TaqMan (ISIS, WTCCC), or Affymetrix mapping arrays (GerMIFS1&2, WTCCC).

Statistical analyses

Genome-wide association and replication study for CRP—Genome-wide SNP associations for CRP were tested in multiple linear regression analyses, using an additive genetic model. CRP levels were log transformed to achieve approximate normal distribution, and analysed as a quantitative trait with adjustment for age and sex (analysis of residuals showed good adherence to normality assumptions). To account for heterogeneity in population structure, principal components derived from EIGENSTRAT v2.0 (Reich Lab, Harvard University, MA, USA) were included as covariates in age, sex adjusted analyses for CoLaus, GEMS, NFBC, and LOLIPOP Indian Asian Illumina analyses (the number of principal components included varied from 4 to 10, depending on the population structure of the specific cohort). For other LOLIPOP data sets genomic control factors were used to correct for any inflation. No principal components were included for DESIR as the population was recruited from a geographically restricted area. Statistical software used for genome-wide associations comprised SNPTTEST (Genetics Software Suite, University of Oxford, UK) v1.1.3 (DESIR), v1.1.4 (CoLaus, GEMS, NFBC); and MACH2QTL (Center for Statistical Genetics, University of Michigan, MI, USA) v.1.0 (LOLIPOP). Results of the separate genome-wide association studies were combined using weighted Z-scores, and a

fixed rather than random effects model to maximise discovery, since random effects estimates are associated with larger variance. QQ plots showed good adherence to null expectations (Lambda for combined data = 1.0625). We used $P < 5 \times 10^{-8}$ to designate genome-wide significance, taking account of the ~ 1 million independent tests for common variants across the genome.²³ For five genetic loci associated with CRP at genome-wide significance, we selected the single most closely associated SNP (i.e., smallest P -value) for replication against CRP.

By use of QUANTO24 v1.2 for quantitative traits, we estimated that the genome-wide association study had 80% power to detect SNPs associated with 0.2% of population variation in CRP levels, or an 11% difference in CRP levels per allele copy, at MAF 30% and genome-wide level of significance ($P < 5 \times 10^{-8}$).

Mendelian randomisation study of genetic variants in the CRP locus and CHD

—We analysed relationship of dbSNP rs7553007 in the *CRP* locus, the SNP most strongly associated with CRP levels in our data, for association with CHD risk using logistic regression under an additive genetic model, as part of a Mendelian randomisation study. To identify published data on the relationship of *CRP* variants with CHD, two electronic databases (Medline and EMBASE) were searched up to and including November 2008 for all prospective studies (including cohort, nested case-control and case-cohort studies), and case-control studies, with no threshold sample size. For the search, the MeSH terms ‘C-reactive protein’ and ‘polymorphism, single nucleotide’ or ‘polymorphism, genetic’ or ‘haplotype’ in combination with ‘coronary disease’ or ‘heart disease’, were used and the search was limited by the terms ‘human’ and ‘English language’. We also scanned reference lists of previous reports. Eight studies of 18 cohorts were identified examining. Two studies^{25,26} examining nine cohorts reported results for a single SNP (dbSNP rs1130864); for these studies, odds ratios (ORs) were reported under a recessive model comparing homozygotes (TT) for the minor allele with CT/CC genotype, and mean effect on CRP was obtained from published ratio of geometric means (1.21).^{25,26} For the remaining studies^{8,27,28,29,30,31}, we selected dbSNP rs1205 on the basis of MAF > 0.1 and largest per allele effect size on CRP levels (−0.35 mg/L) reported in the study of *CRP* variants and CRP levels by Verzilli et al.³² We used per allele OR of rs1205 with CHD where available^{27,29} or where this could be estimated directly from the data^{8,31}; otherwise ORs were estimated from averaging published effect sizes for minor allele homozygotes (TT) and heterozygotes (CT) compared to wild type (CC).^{28,30} We estimated standard errors of the effect sizes of *CRP* variants on CHD from the reported 95% confidence intervals (CIs), assuming normality. Estimated ORs for *CRP* variants on CHD were converted to a common scale by standardising to 20% lower CRP, i.e., the approximate effect per minor allele of rs7553007 on CRP levels. Results were combined across studies by SNP, and across the three SNPs weighted by the inverse of variance. We used 95% CIs and assessed heterogeneity with standard χ^2 statistics, expressed as I^2 , the proportion of variability between studies due to heterogeneity.³³ In the absence of heterogeneity, we used a fixed effects model.

We also investigated the three-way associations between *CRP* genetic variants, CRP levels, and CHD in prospective studies which reported all three sets of data in the same cohorts^{25,26,27,30}; we did not include retrospective (case-control) studies since these could be biased by treatment effects.³⁴

Comparison of the result of the Mendelian randomisation study with meta-analysis of the CRP-CHD relationship—We compared the result from our Mendelian randomisation study with that predicted from a meta-analysis of the observational studies of CRP levels and CHD.^{5,8,26,35,36,40,50-72} This was obtained from a systematic review of the CRP-CHD relationship published by Shah et al.⁵, updated with all studies published

from August 2007 until November 2008. For the search, the MeSH terms ‘C-reactive protein’ and ‘CRP’ in combination with ‘coronary’, ‘coronary heart disease’, ‘CHD’ and ‘CVD’ were used, and the search was limited by the terms ‘human’ and ‘English language’. Studies in which total mortality was the only outcome reported were excluded; if more than one paper was published on the same cohort/ population the most recent one was used in the meta-analysis. Five new population studies were identified from four reports.^{8,26,35,36} Risk ratios for CHD associated with CRP levels (logarithmically transformed) were extracted from each study. Two studies were excluded: one³⁷ which cited risk ratios per unit increase in CRP (i.e., not logarithmically transformed) and one³⁸ which did not provide CIs for the association of CRP with CHD. The studies reported risk ratios based on different comparisons of CRP (tertiles, quintiles, or quartiles), or as differences in risk for a given increase in CRP; these were converted to common unit of 1 standard deviation (SD) change. The risk ratio per SD change was converted to per 20% lower CRP by multiplying coefficients (and 95% CIs) on the logarithmic scale by -0.223 assuming 20% reduction in CRP is equivalent to a $0.223SD$ reduction in log CRP. Multivariate adjusted risk ratios, controlled for conventional cardiovascular risk factors, were used when available; two studies reported risk ratios adjusted for age, and age and smoking only.^{39,40} Because of significant heterogeneity, random effects meta-analysis was used to combine the risk ratios from the individual studies. The overall OR for CHD was used to test the observed vs. predicted association of *CRP* variants with CHD, standardised to 20% lower CRP.

Genetic association of variants outside the CRP locus and CHD—The relationships with risk of CHD of the most associated SNP in the four genetic loci other than the *CRP* locus in our data, were analysed by logistic regression under an additive genetic model. Results were combined across studies by SNP, using a fixed effects model weighted by the inverse of variance, as there was no significant heterogeneity. By use of the Genetic Power Calculator⁴¹, we estimated that the genetic association study with CHD had 80% power to detect an OR of 1.04 per allele copy at $P < 0.05$ and MAF 30%.

With the exception of the genome-wide association analyses described above, all statistical analyses were done with STATA v10 (Statacorp LP, TX, USA). A significance level of $P < 0.05$ was used; all tests were two-sided.

Ethics approval was obtained locally for each of the participating cohorts for analyses of genetic markers of cardiovascular disease risk. No additional ethics approvals were required for this study.

RESULTS

Genome-wide association and replication study for CRP

We found 160 SNPs to be associated with CRP levels at $P < 5 \times 10^{-8}$, distributed in the following five loci: *LEPR* (GeneID 3953; GenBank NC_000001.9, region 65658906 to 65875410), *IL6R* (GeneID 3570, GenBank NC_000001.9, region 152644293 to 152706812), *CRP* (GeneID 1401, GenBank NC_000001.9, region 157951003 to 157948703), *HNF1A* (GeneID 6927, GenBank NC_000012.10, region 119900932 to 119924698) and *APOE-C1-CII* (GeneID 348, 341, 344, GenBank NC_000019.8, region 50100879 to 50104490). A Manhattan plot of results from the combined analysis of genome-wide association data is shown in Figure 1. Genomic context and P -values for the most associated SNP at each of the five loci are shown in Table 1. The association of these SNPs with CRP was confirmed in replication testing (all $P < 10^{-10}$); for all five SNPs, the minor allele was associated with reduced levels of CRP (Table 1). For the most associated SNP in the *CRP* locus (rs7553007), CRP levels were lower by 21% (95% CI, -23.5 — -17.9) per minor allele. For the other four SNPs, per minor allele differences in CRP levels ranged

from -10.8% (95% CI, -13.8—7.7) for dbSNP rs4537545 in *IL6R* to -21.8% (95% CI, -25.4—-18.1) for dbSNP rs4420638 in the *APOE-CI-CII* cluster (Table 1).

In the replication study (LOLIPOP), the percent variance of CRP explained ranged from 0.2% (dbSNP rs11839910 in *HNF1A*) to 1.3% (rs7553007 in the *CRP* locus). SNP rs4420638 in the *APOE-CI-CII* cluster was strongly associated with total cholesterol (0.18 [95% CI, 0.13–0.22] mmol/L per minor allele), LDL cholesterol (0.16 [95% CI, 0.12–0.2] mmol/L), triglycerides (0.11 [95% CI, 0.07–0.16] mmol/L) and HDL cholesterol (-0.03 [95% CI, -0.04—0.02] mmol/L) (Table 2). SNP rs1183910 in *HNF1A* was also associated with total cholesterol (0.03 [95% CI, 0.00–0.06] mmol/L per minor allele), LDL cholesterol (0.03 [95% CI, 0.01–0.05] mmol/L) and HDL cholesterol (0.01 [95% CI, 0.00–0.02] mmol/L). The remaining three SNPs were not significantly related to plasma lipids or with any of the other phenotypes tested (Table 2).

Mendelian randomisation study of genetic variants in the *CRP* locus and CHD

Figure 2 shows the results of the Mendelian randomisation experiment of CHD with variants in the *CRP* locus: SNP rs7553007 (our data) and published data for SNPs rs1130864 and rs1205 for 18 cohorts. SNP rs7553007 was not significantly associated with CHD; estimated OR was 0.98 (95% CI, 0.94–1.01) per 20% lower CRP. For rs1130864, OR was 1.00 (95% CI, 0.86–1.15) and for rs1205, OR was 1.03 (95% CI, 0.99–1.07). There was no association of CHD with *CRP* variants (per 20% lower CRP) when results for all three SNPs were combined, OR 1.00 (95% CI, 0.97–1.02) (Figure 2).

In the three-way comparison of *CRP* genetic variants, CRP levels, and CHD risk reported in prospective studies, there was significant association of *CRP* variants with CRP levels, CRP levels with CHD, but not *CRP* variants with CHD (Figure 3).

Comparison of the result of the Mendelian randomisation study with meta-analysis of the *CRP*-CHD relationship

The meta-analysis of observational studies of CRP levels and CHD gave a predicted OR 0.94 (95% CI, 0.94–0.95) per 20% lower CRP (Figure 4). This is significantly different to the estimated effect on CHD (OR 1.00) of genetically determined differences in CRP levels obtained from our Mendelian randomisation study ($Z=-3.45$, $P<0.001$).

Genetic association of variants outside the *CRP* locus and CHD

Minor alleles of dbSNP rs6700896 in *LEPR* and rs4420638 in *APOE-CI-CII* cluster were associated with significantly increased risk of CHD (OR 1.06 [95% CI, 1.02–1.09] and 1.16 [95% CI, 1.12–1.21] respectively), SNP rs4537545 in *IL6R* with a decreased risk of CHD (OR 0.94 [95% CI, 0.91, 0.97]), while SNP rs1183910 in *HNF1A* was not significantly associated with CHD (Figure 5). The effects of SNPs rs6700896 in *LEPR* and rs4420638 in the *APOE-CI-CII* cluster on CHD were in the opposite direction to that predicted from the relationship of CRP levels with CHD (Figure 4); for rs4420638 the finding was consistent with the effects on blood lipid concentrations (Table 2).

COMMENT

The present genome-wide association study confirms the associations of common genetic variants in the *LEPR*, *IL6R*, *CRP*, *HNF1A* loci and *APOE-CI-CII* cluster with CRP levels. However, minor allele of SNP rs7553007 and other variants in the *CRP* locus included in our Mendelian randomisation study were not associated with CHD risk.

The variants included in our Mendelian randomisation study are associated with ~20% lower CRP levels³², corresponding to 6% reduction in CHD risk predicted by the meta-analysis of observational studies of CHD risk in relation to differences in CRP levels. The lack of association with CHD of genetic variants in the *CRP* locus suggests that the observational data linking CRP levels and CHD may be confounded by association with other CHD risk factors, or reflect a secondary inflammatory response associated with atherosclerosis ('reverse causation'), rather than indicating a causal relationship.

Our analysis of SNP rs7553007 with CHD risk includes more cases than all previously published studies of *CRP* variants and CHD combined^{8,25,26,27,28,29,30,31}, yielding a total of over 28,000 cases and 100,000 controls for the Mendelian randomisation study. The largest previous study, of three cohorts in Copenhagen, included 6,545 cases.⁸ In addition to rs1205 included in our Mendelian randomisation study, it provides data on the tri-allelic SNP rs3091244 and SNP rs3093077, both having variants with larger effects on CRP levels (20-30%) than rs1205; however, both variants are rare, and neither was associated with CHD in the Copenhagen study.⁸

The JUPITER trial recently reported a benefit of treatment with rosuvastatin on CHD risk among men and women with elevated CRP levels (2.0 mg/L or higher) and with low-density lipoprotein (LDL) cholesterol levels below 130 mg/dL (3.4 mmol/L).³⁴ In the treatment group, there was 54% reduction in rates of myocardial infarction compared with placebo; LDL levels were reduced by 50% and CRP levels by 37%. Although the JUPITER trial demonstrates the benefits of statin therapy in people with LDL levels below current treatment threshold, the results may simply reflect the benefits of lipid-lowering therapy in people who would not currently be considered for pharmacotherapy, rather than the benefits of CRP-lowering per se.⁴⁴

Investigation of genetic variants underlying an intermediate phenotype (such as CRP) has been advocated as a means of discovering new disease susceptibility loci.¹⁰ In our study, minor alleles of SNPs rs6700896 in *LEPR* and rs4420638 in the *APOE-CI-CII* cluster showed significantly increased risk of CHD. However, both variants were associated with reduced levels of CRP, suggesting that the links with CHD are not mediated by CRP. *LEPR* has not previously been reported to increase risk of CHD, though associations of variants in the *APOE-CI-CII* cluster with CHD have been observed.^{45,46} While the association of genetic variation in *APOE-CI-CII* with CHD can be explained in large part by its effects on blood lipids, this is not the case for SNP rs6700896 in *LEPR*, which was not associated with major CHD risk factors. SNP rs6700896 is located in intron 19 of *LEPR*, the gene encoding the leptin receptor, a member of the class I cytokine receptor family.⁴⁷ *LEPR* is expressed in the hypothalamus and vascular endothelial cells⁴⁸, and *LEPR* signalling has a role in appetite control, weight regulation, glucose homeostasis, blood pressure regulation and angiogenesis.^{47,48,49} The minor allele of SNP rs4537545 in *IL6R* was also associated with reduced CRP levels, and reduced risk of CHD. SNP rs4537545 in *IL6R* is in high LD ($r^2=0.96$ in the HapMap CEU reference population) with a non-synonymous SNP (rs8192284, Asp358Ala) associated with increased *IL6R* expression, and alterations of *IL6R* membrane binding⁵⁰, providing a potential mechanism linking rs4537545 to biological function. Further studies will be needed to confirm *LEPR* and *IL6R* as new susceptibility loci for CHD.

Our study has limitations. Because of the relatively small effect of common genetic variants in *CRP* locus on CRP levels, large sample size is needed to detect associations with CHD. In order to combine data across studies in our Mendelian randomisation study, we assumed a common effect on risk of CHD from different variants in the *CRP* locus, by standardising to a common difference on the log CRP scale. This approach is valid to the extent that the

hypothesised effect of *CRP* variants on CHD risk reflects circulating levels of CRP. Though we found no association of *CRP* variants with CHD risk, it is not possible to exclude a small effect, despite the large sample size. However, we can effectively exclude the size of association predicted from observational data on the relationship of CRP levels to CHD. The Mendelian randomisation approach makes a number of assumptions concerning possible causality. These include the potential for pleiotropic effects of the genetic variants under study (or variants in high LD with them) giving an alternative pathway to CHD, or confounding through associations with disease risk factors.⁹ Neither rs7553007 or the other two variants included from the *CRP* locus correlate with CHD risk factors^{8,26}, satisfying an important condition for a valid Mendelian randomisation experiment.⁹

In summary, our Mendelian randomisation study of over 28,000 cases and 100,000 controls found no association of variants in the *CRP* locus and CHD, arguing against a causal role for CRP in atherosclerosis. Moreover, this study suggests that development of therapeutic strategies targeting specific reductions in plasma levels of CRP are unlikely to be fruitful.

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PROCARDIS. Investigators: RC, MF, Anuj Goel, AH, Simon C Heath, G Mark Lathrop, JFP, Udo Seedorf, Ann-Christine Syvänen, Giovanni Tognoni, H.W. Principal investigator for project: HW. Principal investigators for collection centre: RC, AH, US, GT. Genotyping: SCH, GML, A-CS. Quality control: SCH. Data analysis: MF, AG, JFP. Project management: JFP, HW. See www.procardis.org for full membership of PROCARDIS consortium. We are grateful to the participants and to the medical and nursing staff who assisted in this project. This work was funded by the British Heart Foundation, EC Sixth Framework Programme (LSHM33 CT- 2007- 037273), AstraZeneca AB and the Knut and Alice Wallenberg Foundation.

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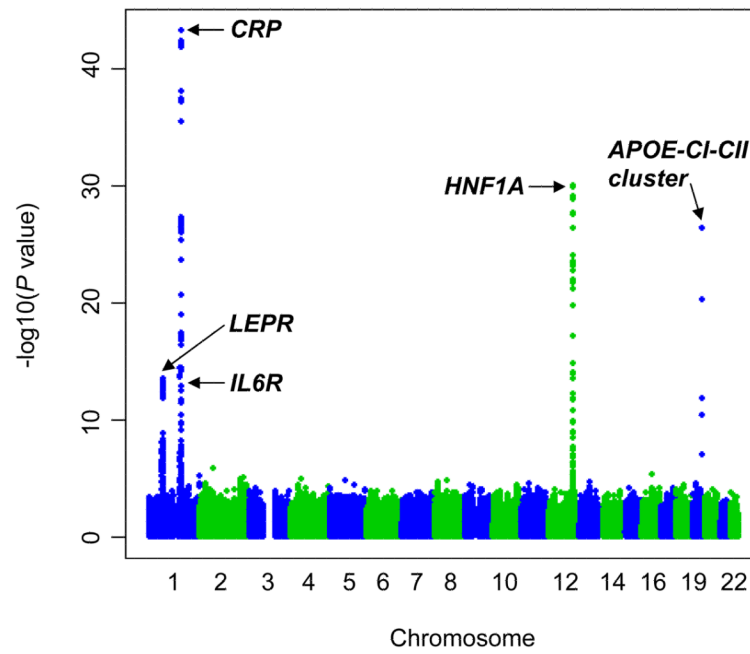


Figure 1. Manhattan plot of results from the combined analysis of genome-wide association data (9 cohorts)

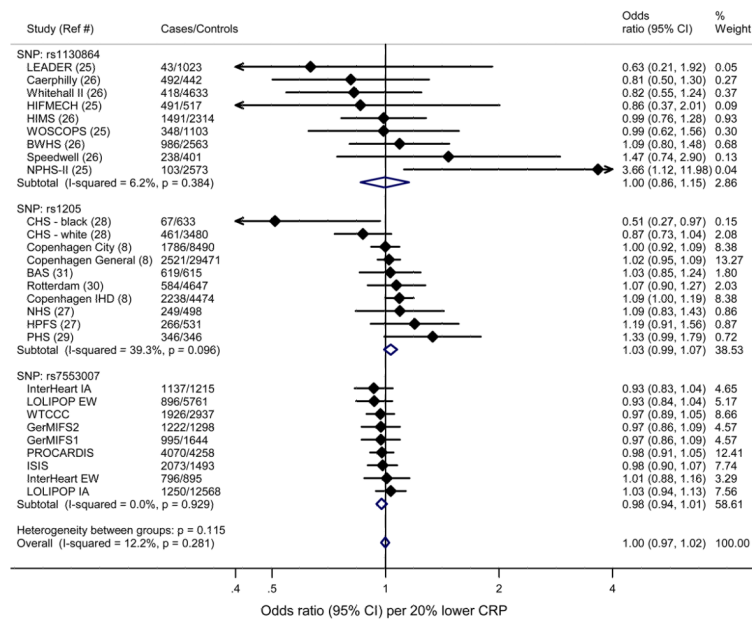
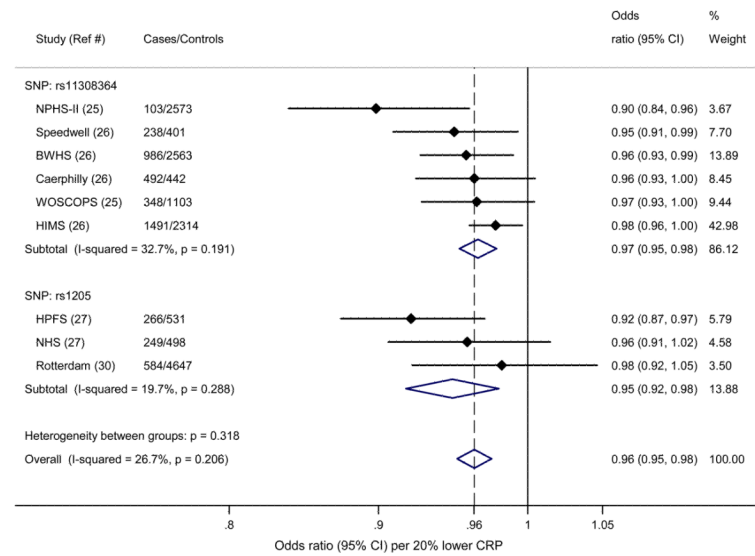
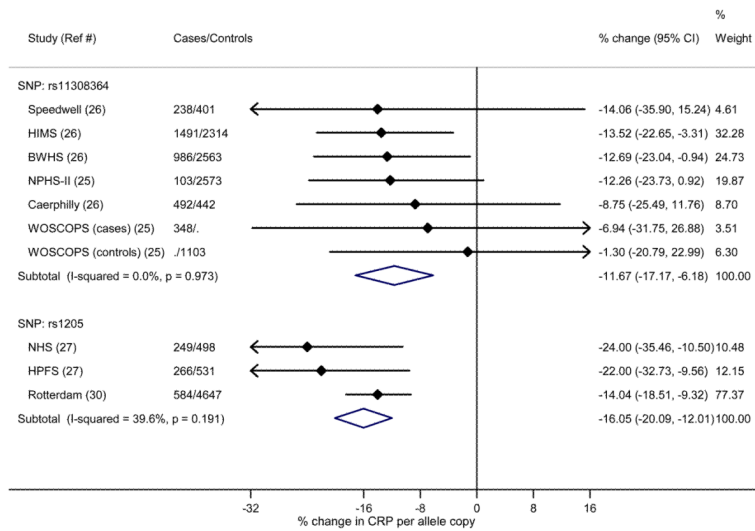


Figure 2. Results of Mendelian randomisation experiment of rs7553007 in the *CRP* locus (present study), with rs1130864 and rs1205 from published studies, with CHD, among 28,112 cases and 100,823 controls. Effects are given as Odds Ratios (95% Confidence Intervals) per 20% lower CRP



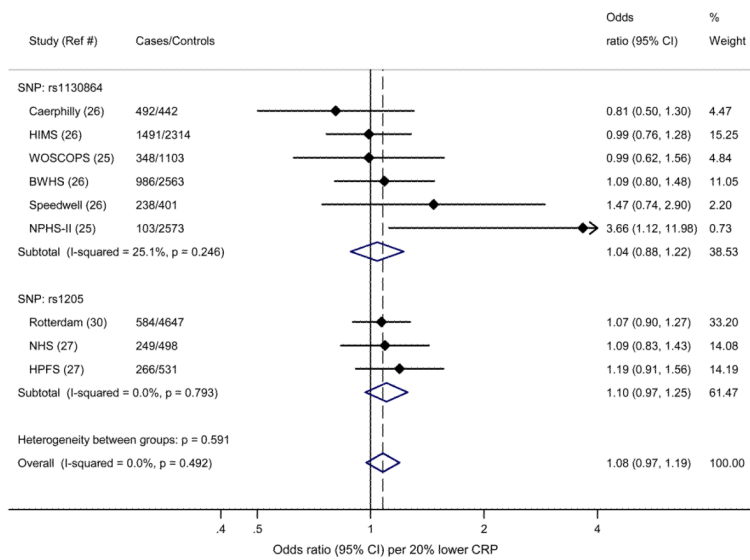


Figure 3.

Meta-analysis of associations of SNPs rs1130864 and rs1205 with CRP levels, CRP levels with CHD, and SNPs with CHD, in prospective studies where all three analyses have been reported. (a) Associations of SNPs rs1130864 and rs1205 with CRP levels. Effects are given as % change (95% Confidence Intervals) per minor allele (rs1205) and per major allele (rs1130864). (b) Associations of CRP levels with CHD. Effects are given as Odds Ratios (95% Confidence Intervals) per 20% lower CRP. (c) Associations of SNPs rs1130864 and rs1205 with CHD. Effects are given as Odds Ratios (95% Confidence Intervals) per 20% lower CRP

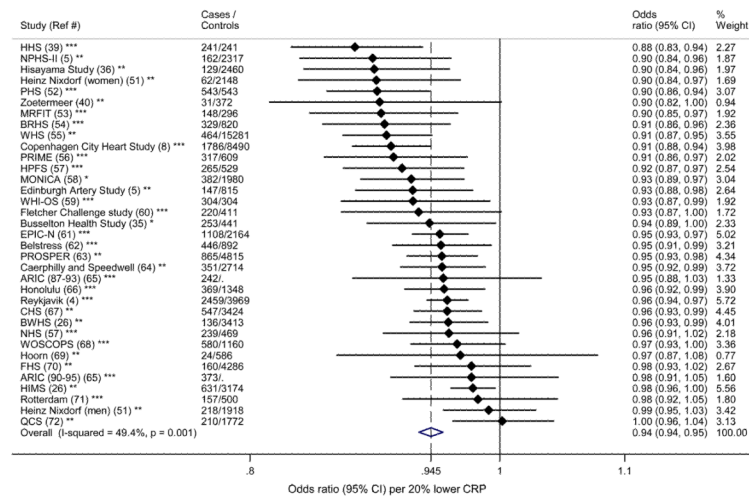


Figure 4. Meta-analysis of the relationship of CRP levels with CHD from prospective observational studies. Effects are given as Odds Ratios (95% Confidence Intervals) per 20% lower CRP
Footnote to Figure 4.

Random effects estimate presented in Figure, fixed effects estimate: OR 0.951 (95% CI, 0.945-0.957). For cohort studies, number of ‘controls’ represents the number of event-free individuals. Number of controls was not available for ARIC (Ref 65).

*Case-cohort **Cohort ***Nested case-control

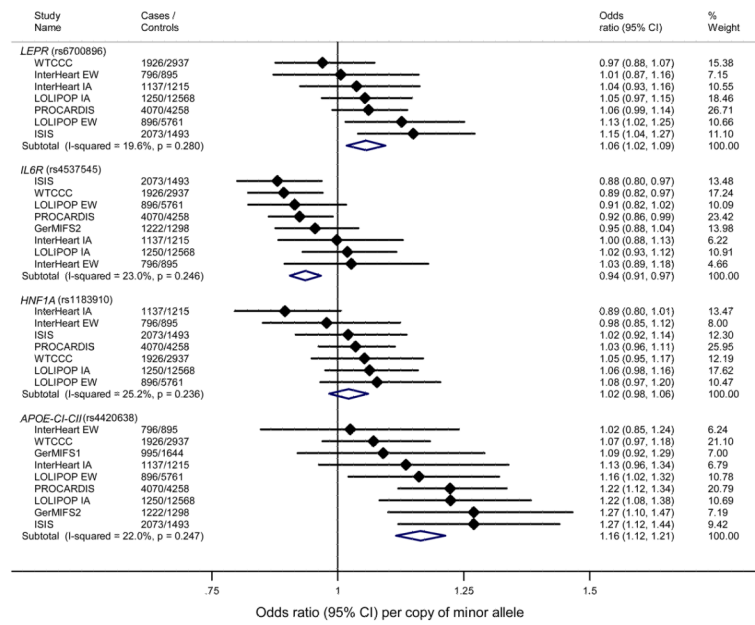


Figure 5. Associations of SNPs in *LEPR* (rs6700896), *IL6R* (rs4537545), *HNF1A* (rs1183910) loci and *APOE-C1-C2* (rs4420638) cluster in the genetic association study with CHD. Effects are given as Odds Ratios (95% Confidence Intervals) per copy of minor allele

Table 1

Genomic context[‡], alleles, minor allele frequency and association test results for most associated SNP at each locus. Effect size is change in CRP (% , 95% Confidence Interval) per copy of minor allele, under an additive genetic model

	SNP				
	rs6700896	rs4537545	rs7553007	rs1183910	rs4420638
Genomic context					
Chromosome	1	1	1	12	19
Position	65862370	152685503	157965173	119905190	50114786
Locus	<i>LEPR</i>	<i>IL6R</i>	<i>CRP</i>	<i>HNFA</i>	<i>APOE-CI-CII</i> cluster
Alleles					
Reference	C	C	G	C	A
Minor	T	T	A	T	G
Minor allele frequency*					
Europeans	0.38	0.43	0.33	0.32	0.19
Indian Asians	0.46	0.31	0.29	0.39	0.12
Genome-wide association					
<i>P</i> value	3.1×10^{-14}	1.8×10^{-14}	7.6×10^{-44}	1.2×10^{-30}	4.5×10^{-27}
Replication					
CRP effect, % [‡]	-14.7	-10.8	-20.7	-13.6	-21.8
95% CI	-17.5 to -11.9	-13.8 to -7.7	-23.5 to -17.9	-16.4 to -10.6	-25.4 to -18.1
<i>P</i> value	1.6×10^{-21}	5.1×10^{-11}	3.3×10^{-38}	1.2×10^{-17}	2.1×10^{-25}

* Minor allele frequency from people genotyped in replication sample.

[‡] dbSNP build 36.3 (dbSNP b126).

[‡] Based on geometric mean CRP (1.93 mg/L) in replication cohort, estimated absolute CRP effects (mg/L) are: for rs6700896, -0.28; for rs4537545, -0.21; for rs1183910, -0.26; for rs4420638, -0.42.

Table 2

Relationships of most associated SNPs in the five genetic loci influencing CRP levels, with cardiovascular risk factors in replication sample (LOLIPOP), from regression analyses adjusted for age, sex and ethnic group, under an additive genetic model. Data presented as unit change (continuous traits) or odds ratio (diabetes) per copy of minor allele

Cardiovascular risk factors	SNP (Locus)									
	rs6700896 (LEPR)	P	rs4537545 (IL6R)	P	rs7553007 (CRP)	P	rs1183910 (HNF1A)	P	rs4420638 (APOE-CLII)	P
Weight (kg)	-0.35 (-0.74 to 0.04)	0.08	0.01 (-0.39 to 0.42)	0.93	-0.06 (-0.48 to 0.36)	0.79	0.08 (-0.32 to 0.47)	0.68	0.11 (-0.43 to 0.65)	0.68
Body mass index (kg/m ²)	-0.06 (-0.19 to 0.08)	0.45	-0.08 (-0.21 to 0.06)	0.30	-0.03 (-0.18 to 0.11)	0.68	-0.02 (-0.16 to 0.11)	0.77	0.02 (-0.16 to 0.21)	0.77
Systolic BP (mm Hg)	-0.01 (-0.5 to 0.47)	0.98	0.28 (-0.22 to 0.79)	0.26	0.24 (-0.28 to 0.76)	0.36	0.19 (-0.3 to 0.68)	0.44	-0.05 (-0.72 to 0.62)	0.89
Diastolic BP (mm Hg)	0.03 (-0.25 to 0.32)	0.79	0.19 (-0.11 to 0.48)	0.21	-0.08 (-0.38 to 0.23)	0.65	0.06 (-0.23 to 0.35)	0.65	0.00 (-0.39 to 0.40)	0.97
Cholesterol (mmol/L)	0.01 (-0.02 to 0.04)	0.45	0.00 (-0.04 to 0.03)	0.99	-0.03 (-0.07 to 0.00)	0.08	0.03 (0.00 to 0.06)	0.02	0.18 (0.13 to 0.22)	7.5×10 ⁻¹⁷
Triglycerides (mmol/L)	-0.01 (-0.05 to 0.02)	0.58	-0.04 (-0.07 to 0.00)	0.05	-0.01 (-0.05 to 0.02)	0.71	-0.03 (-0.06 to 0.01)	0.23	0.11 (0.07 to 0.16)	3.0×10 ⁻⁷
HDL cholesterol (mmol/L)	0.00 (-0.01 to 0.01)	0.75	0.00 (-0.01 to 0.01)	0.34	0.00 (-0.01 to 0.01)	0.72	0.01 (0.00 to 0.02)	0.03	-0.03 (-0.04 to -0.02)	1.7×10 ⁻⁵
LDL cholesterol (mmol/L)	0.01 (-0.01 to 0.04)	0.21	0.00 (-0.03 to 0.03)	0.69	-0.03 (-0.06 to 0.00)	0.08	0.03 (0.01 to 0.05)	0.008	0.16 (0.12 to 0.20)	4.7×10 ⁻²⁰
Diabetes (OR)	1.01 (0.92 to 1.10)	0.90	1.01 (0.92 to 1.10)	0.89	1.03 (0.94 to 1.14)	0.48	1.04 (0.95 to 1.13)	0.39	0.92 (0.81 to 1.04)	0.19