Unraveling the Directional Link between Adiposity and Inflammation: A Bidirectional Mendelian Randomization Approach

Paul Welsh, Eliana Polisecki, Michele Robertson, Sabine Jahn, Brendan M. Buckley, Anton J. M. de Craen, Ian Ford, J. Wouter Jukema, Peter W. Macfarlane, Chris J. Packard, David J. Stott, Rudi G. J. Westendorp, James Shepherd, Aroon D. Hingorani, George Davey Smith, Ernst Schaefer, and Naveed Sattar

Division of Cardiovascular and Medical Sciences (P.W., P.W.M., C.J.P., D.J.S., J.S., N.S.), University of Glasgow, Glasgow G12 8QQ, United Kingdom; Cardiovascular Research Laboratory, Lipid Metabolism Laboratory (E.P., E.S., S.J.), Jean Mayer United States Department of Agriculture Human Nutrition Research Center on Aging, Tufts University, Boston, Massachusetts 02111; Robertson Centre for Biostatistics (M.R., I.F.), University of Glasgow, Glasgow G12 8QQ, United Kingdom; Department of Pharmacology and Therapeutics (B.M.B), University College Cork, Cork, Ireland; Departments of Gerontology and Geriatrics (A.J.M.d.C., R.G.J.W.) and Cardiology (J.W.J.), Leiden University Medical Centre, 2300 RA Leiden, The Netherlands; Division of Population Sciences (A.D.H.), Department of Epidemiology and Public Health, University College London, London WC1E 6BT, United Kingdom; and Medical Research Council Centre for Causal Analyses in Translational Epidemiology (G.D.S.), Department of Social Medicine, University of Bristol, Bristol BS8 1TH, United Kingdom

Context: Associations between adiposity and circulating inflammation markers are assumed to be causal, although the direction of the relationship has not been proven.

Objective: The aim of the study was to explore the causal direction of the relationship between adiposity and inflammation using a bidirectional Mendelian randomization approach.

Methods: In the PROSPER study of 5804 elderly patients, we related C-reactive protein (*CRP*) single nucleotide polymorphisms (SNPs) (rs1800947 and rs1205) and adiposity SNPs (*FTO* and *MC4R*) to body mass index (BMI) as well as circulating levels of CRP and leptin. We gave each individual two allele scores ranging from zero to 4, counting each pair of alleles related to CRP levels or BMI.

Results: With increasing *CRP* allele score, there was a stepwise decrease in CRP levels (P for trend $<$ 0.0001) and a 1.98 mg/liter difference between extremes of the allele score distribution, but there was no associated change in BMI or leptin levels ($P \geq 0.89$). By contrast, adiposity allele score was associated with 1) an increase in BMI (1.2 kg/m^2 difference between extremes; P for trend 0.002); 2) an increase in circulating leptin (5.77 ng/ml difference between extremes; P for trend 0.0027); and 3) increased CRP levels (1.24 mg/liter difference between extremes; P for trend 0.002).

Conclusions: Greater adiposity conferred by *FTO* and *MC4R* SNPs led to higher CRP levels, with no evidence for any reverse pathway. Future studies should extend our findings to other circulating inflammatory parameters. This study illustrates the potential power of Mendelian randomization to dissect directions of causality between intercorrelated metabolic factors. **(*J Clin Endocrinol Metab* 95: 93–99, 2010)**

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Abbreviations: BMI, Body mass index; CRP, C-reactive protein; HWE, Hardy-Weinberg equilibrium; MR, Mendelian randomization; SNP, single nucleotide polymorphism.

Recent research efforts have focused on identifying genetic polymorphisms associated with vascular disease end-points as well as intermediate phenotypes associated with higher vascular risk (1, 2). Germline genetic variation is generally unrelated to lifestyle, socioeconomic, and environmental variables that generate confounding in conventional epidemiological approaches (3–5). Single nucleotide polymorphisms (SNPs) in the C-reactive protein (CRP) gene have been identified that influence CRP concentration (6). For instance, on meta-analysis, *CRP* SNP rs1800934 (G > C) and rs1205 (C > T) variants are associated with a per allele decrease of 0.35 mg/liter (95% confidence intervals, 0.41, 0.28 mg/liter) and 0.38 mg/liter (95% confidence intervals, 0.50, 0.25 mg/liter) of circulating CRP, respectively (6). More recently, SNPs in *FTO* and *MC4R* have been described that are robustly associated with body mass index (BMI) (1). For instance, the rs9939609 (T > A) variant in *FTO* is associated with a 0.36 kg/m^2 (range, 0.34–0.46 kg/m^2) per-allele increase in BMI (7). Similar but quantitatively weaker results have been described for *MC4R* SNP rs17782313 (T > C) being associated with BMI (8). Both of these *FTO* and *MC4R* SNPs may increase BMI specifically by increasing appetite and calorific intake (9–12).

These SNPs, which are now widely accepted to be robustly associated with the phenotypes outlined, may help provide insight into the causal relationships between obesity and CRP that cannot be addressed by traditional observational approaches (13, 14). Specifically, it has been widely reported in cross-sectional studies that CRP is strongly associated with BMI, waist circumference, and waist-hip ratio (15). Indeed, recent prospective evidence shows this to be a relationship independent of physical fitness (16). In explaining this relationship, it is usually assumed that endocrinologically active visceral adipose tissue releases proinflammatory cytokines such as IL-6 and TNFα into the circulation, resulting in a low-grade hepatic acute phase response and, hence, CRP production (17). Although this mainstream belief is reasonable, it is also possible that CRP or generalized inflammation itself may exacerbate deposition of metabolically active fat deposits (18); in this regard, the potential physiological roles of CRP are still being debated (19–21). The two directions of causality are not necessarily mutually exclusive, and there may be an element of both pathways playing a role in a potential positive feedback loop, but, as far as we are aware, this possibility lacks detailed and direct study. Intercorrelation between adiposity and inflammation precludes elucidation of the casual direction of the relationship using conventional epidemiological tools (22).

In the PROSPER (PROspective Study of Pravastatin in the Elderly at Risk) study of 5804 older men and women (23), we have measured both CRP and adiposity-associated genetic variants (*FTO* and *MC4R*) in blood samples taken at baseline, and we have used the principles of Mendelian randomization (MR) (3) to explore the nature and causal direction of the links between CRP and adiposity. Although an elderly cohort (70–82 yr old), where it might be expected that illness leading to lower BMI is a stronger phenomenon than in younger cohorts (24, 25) and the associations of common genetic variants with BMI may be weaker, we also have measures of leptin in PROSPER, an adipokine that correlates well with percentage fat mass (26). Finally, the use of an elderly population allows us to examine whether *FTO* and *MC4R* are associated with adiposity in the elderly as they are in younger populations (9–12, 27).

Subjects and Methods

Participants

The protocol of PROSPER (23) and the methods and outcomes of the main trial have been published (28).

Between December 15, 1997, and May 7, 1999, we screened 23,770 individuals and randomized 5804 from Scotland, Ireland, and The Netherlands. Men and women aged 70–82 yr were recruited if they had either preexisting vascular disease (coronary, cerebral, or peripheral) or raised risk of such disease because of smoking, hypertension, or diabetes. Their plasma total cholesterol had to be between 4.0 and 9.0 mmol/liter and their fasting triglyceride concentrations less than 6.0 mmol/liter. All subjects were given a clinical examination at baseline before randomization, which included drawing of venous blood samples, measurement of weight, height, and BMI (weight/height^2) among other clinical parameters by health professionals. The institutional ethics review boards of all centers approved the protocol, and all participants gave written informed consent. The protocol was consistent with the Declaration of Helsinki. Participants were randomly assigned to receive either pravastatin 40 mg daily or matching placebo. All data were processed and analyzed at the Robertson Centre for Biostatistics, University of Glasgow (Glasgow, UK).

Genotyping

Genotyping of *FTO* rs9939609 [intronic nucleotide substitution T > A; reported minor allele frequency, 0.44 (http://www.ncbi.nlm.nih.gov/projects/SNP/)] and *MC4R* rs17782313 [nucleotide substitution (188kb downstream of the melanocortin 4 receptor gene) T > C; reported minor allele frequency, 0.38] as well as *CRP* rs1800947 (nucleotide substitution G > C; allele change CTG > CTC; protein position 184 residue change Leu > Leu; reported minor allele frequency, 0.08) and rs1205 (untranslated region-3′nucleotide change C > T; reported minor allele frequency, 0.46) nucleotide changes were carried out using real-time PCR with TaqMan SNP Genotyping Assays from Applied Biosystems (Foster City, CA) in the collaborating laboratory at Tufts University (Boston, MA) (6–8).

Laboratory variables

All analyses were performed on blood samples drawn at baseline, before participants received study medicine. CRP was measured by automated particle-enhanced immunoturbidimetric assay (Roche UK, Welwyn Garden City, UK) (29). The relevant laboratory participates in a national external quality control for high-sensitivity CRP. The method has a lower limit of sensitivity of 0.1 mg/liter and interassay and intraassay coefficients of variation of 3%. Leptin was measured by an in-house RIA validated thoroughly against the commercially available Linco Research Co. (St. Charles, MO) assay (30). The intra- and interassay coefficients of variation were below 7% and below 10%, respectively. The detection limit of the assay was 0.5 ng/ml. Samples were processed blinded to their identity.

Statistical analysis

The distributions of CRP and leptin were positively skewed; therefore, a logarithmic transformation was used. Observed genotype frequencies were compared with those expected under Hardy-Weinberg equilibrium (HWE) using a χ^2 test. Baseline characteristics were compared between the levels of SNPs, or allele scores from a test for trend to give a *P* value, and where a significant ($P < 0.05$) trend was found using allele scores, pairwise comparisons between individual data points were calculated between a score of 0 and the other levels of the score. Relationships between log CRP, log leptin, and BMI were assessed through the use of the Pearson correlation coefficient. To increase the power of our observations, we assigned every individual a score based on genotype of *MC4R* and *FTO*, such that AA = 0 (most common genotype), AB = 1, and BB = 2 (least common genotype). By then combining scores for *MC4R* and *FTO*, every individual had an overall allele score ranging from zero (most common) to 4 (least common). This process was repeated for *CRP* SNPs so that each individual was assigned an allele score ranging from zero to 4. The assumption of linear disequilibrium was tested between the *CRP* SNPs and adiposity SNPs, respectively. Where any evidence of linear disequilibrium existed, a sensitivity analysis was carried out to validate the simple allele score model, weighting the allele score according to the effect size of each SNP, using the β coefficient from a regression analysis.

Results

As a continuous variable, log CRP levels were positively correlated with BMI after adjusting for age, sex, and smoking status (r = 0.252; $P < 0.0001$). After similar adjustment, log leptin was positively correlated with BMI as well as log CRP (r = 0.658, $P < 0.0001$; and r = 0.303, $P < 0.0001$, respectively).

Considering the SNPs of interest, the polymorphism frequency is displayed in Table 1. None of the four SNPs showed strong evidence of departure from HWE: rs1800447, HWE = 0.043, $P = 0.84$; rs1205, HWE = 3.615, $P = 0.06$; *FTO*, HWE = 1.790, $P = 0.18$; and *MC4R*, HWE = 0.463, $P = 0.50$. The *CRP* SNPs rs1800947 and rs1205 were in moderate (31) linkage disequilibrium: $r^2 = 0.36$, $P < 0.0001$; whereas *FTO* and *MC4R* were not in linkage disequilibrium: $r^2 = 0.00096$, $P = 0.94$.

As expected, *CRP* polymorphisms rs1800947 and rs1205 were associated with circulating levels of CRP (1.76 and 1.2 mg/liter difference in circulating levels, respectively, comparing homozygotes; both *P* for trend <0.0001) (Supplementary Table 1, published as supplemental data on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org). However, neither polymorphism showed any association with BMI (*P* for trend ≥ 0.78) or with leptin (*P* for trend ≥ 0.69). There was weak evidence of an association for the *FTO* variant with higher BMI (0.4 kg/m^2 comparing homozygotes; *P* for trend = 0.06), and there was some evidence of an association of *FTO* with leptin (1 ng/ml difference in levels comparing homozygotes; *P* for trend = 0.02) (Supplementary Table 1). The *MC4R* variant showed a somewhat more robust association with BMI (0.6 kg/m^2 comparing homozygotes; *P* for trend = 0.0017) and weak evidence of an association with leptin (1.3 ng/ml difference in levels comparing homozygotes; *P* for trend = 0.06) (Supplementary Table 2). Both *FTO* and *MC4R* showed associations with circulating levels of CRP (0.34 mg/liter difference in circulating levels for both SNPs comparing homozygotes; both *P* for trend ≤ 0.04).

Allele score data

We analyzed the allele scores for *CRP* SNPs and adiposity SNPs to increase the power of our observations.

TABLE 1. Frequency of *CRP* and adiposity SNPs in PROSPER cohort

	FTO rs9939609 (n = 5724)			*MC4R* rs17782313 (n = 5748)		
Genotype	**TT**	**TA**	**AA**	**TT**	**TC**	**CC**
CRP rs1800947						
GG	1116	1330	401	1734	986	140
GC	875	1137	344	1425	806	134
CC	192	247	82	319	182	22
	FTO rs9939609 (n = 5734)			*MC4R* rs17782313 (n = 5759)		
CRP rs1205						
CC	987	1255	367	1602	890	127
CT	962	1156	354	1470	876	136
TT	239	308	106	413	212	33

Data for CRP and leptin levels as well as BMI were summarized and analyzed by allele score for both *CRP*- and adiposity-associated SNPs (Table 2). *CRP* allele score demonstrated a decrease in CRP levels with each increment in allele score. Indeed, participants with a score of 4 (n = 20) had on average a 2 mg/liter lower circulating CRP level compared with those with a score of zero (n = 2615) [3.45 (SD ± 3.02 mg/liter) *vs.* 1.47 mg/liter (SD ± 2.67 mg/liter) *P* for trend < 0.0001]. However, the *CRP* SNP allele score demonstrated no association with BMI (*P* for trend = 0.89) or leptin (*P* for trend = 0.98). Given linkage disequilibrium between the *CRP* SNPs, we performed a sensitivity analysis to validate the simple allele score approach for these *CRP* SNPs. Each allele was weighted by its β-coefficient in a regression analysis. Using this approach, the *p* for trend across weighted *CRP* allele score for CRP levels was < 0.0001 (directly comparable to simple allele count in Table 2). Likewise, weighted *CRP* allele scores for BMI and leptin gave nearly identical results as per simple allele scores (data not shown).

Adiposity-associated allele score was associated with on average a 1.2 kg/m^2 difference comparing zero (n = 1310) to 4 (n = 46) [26.62 kg/m^2 (SD ± 4.14 kg/m^2) *vs.* 27.89 kg/m^2 (SD ± 4.25 kg/m^2); *P* for trend = 0.002], and the score was also associated with on average an increase of 5.77 ng/ml in leptin levels [12.77 ng/ml (SD ± 2.42 ng/ml) *vs.* 18.54 (SD ± 2.06); *P* for trend = 0.0027], although this was mainly due to a large increase in levels for a score of 4 (n = 46) *vs.* 3 (n = 422). Adiposity allele score was additionally associated with CRP levels, such that those with a score of 4 relative to zero had on average a 1.2 mg/liter higher circulating CRP [2.91 mg/liter (SD ± 3.07 mg/liter) *vs.* 4.15 mg/liter (SD ± 2.64 mg/liter); *P* for trend = 0.002].

Discussion

Using a contemporary approach that exploits SNPs related to two different parameters enabled us to tease out causal directions between CRP and adiposity. This approach shows clearly that those genetic variants which lead to higher BMI are associated with increased CRP and leptin levels, whereas genetic variants that give rise to elevated CRP levels do not, in this study, predict higher BMI or leptin. Given that the genetic variants are theoretically unrelated to confounding factors and are not subject to reverse causality, our observations are less likely to be confounded by other factors, such as social class or smoking, which would be the case in conventional epidemiological approaches (3–5). Our work extends genetic epidemiological studies showing that SNPs associated with elevated circulating levels of CRP are not associated with insulin resistance or diabetes risk (32). Together with the present findings, this suggests that increased circulating CRP is a consequence of an adverse metabolic profile and is not a cause of it.

A further interesting finding in this study is that the *FTO* and *MC4R* variants are associated with adiposity, not just in the very young (9, 11) and the middle-aged (10, 27) but also in elderly participants at elevated vascular risk in the present study. This finding suggests that these associations may be remarkably robust throughout life. Recent evidence has suggested that these polymorphisms may be associated with higher body fat due to an associated increased calorific intake (9–12). This is a lifestyle pattern that one may anticipate remains evident throughout life on the population scale. That noted, the associations between *FTO* and *MC4R* and BMI are perhaps weaker in the present population than reported previously

TABLE 2. CRP, BMI, and leptin distribution by CRP and adiposity allele score

	CRP (mg/liter)	BMI (kg/m^2)	Leptin (ng/ml)
Low CRP polymorphisms			
0 (n = 2615)	3.45 (3.02)	26.82 (4.24)	13.32 (2.44)
1 (n = 2033)	3.15 (2.98)[a]	26.87 (4.16)	13.35 (2.41)
2 (n = 894)	2.40 (3.19)[a]	26.72 (4.13)	13.13 (2.51)
3 (n = 197)	2.02 (3.03)[a]	26.80 (4.26)	13.76 (2.35)
4 (n = 20)	1.47 (2.67)[a]	27.60 (4.87)	13.29 (2.67)
P value for trend	<0.0001	0.89	0.98
P value adjusted for country	<0.0001	0.88	0.93
High adiposity polymorphisms			
0 (n = 1310)	2.91 (3.07)	26.62 (4.14)	12.77 (2.42)
1 (n = 2431)	3.06 (3.09)	26.72 (4.18)	13.15 (2.44)
2 (n = 1522)	3.18 (3.02)[a]	27.07 (4.24)[a]	13.75 (2.44)[a]
3 (n = 422)	3.35 (3.05)[a]	26.92 (3.99)	13.87 (2.43)
4 (n = 46)	4.15 (2.64)[a]	27.89 (4.25)[a]	18.54 (2.06)[a]
P value for trend	0.002	0.002	0.0027
P value adjusted for country	0.0013	0.0014	0.0019

Data are presented as mean (SD). CRP and leptin were analyzed on a log scale and back-transformed for presentation.

[a] Significant difference ($P < 0.05$) compared to allele score of 0 (unadjusted).

in younger populations, although this is somewhat speculative, and requires confirmation in larger general population studies. Lesser effect of *FTO* and *MC4R* on adiposity in later life could be expected at older ages because comorbidities are a more important influence on BMI in the elderly. In this study, associations of SNPs with leptin generally validated our findings with regard to BMI.

The bidirectional MR approach we have used here illustrates a useful method for exploring the direction of causality between two tightly correlated biological pathways. Both casual and reverse causality pathways can be examined (33). Clearly, scientific and clinical experiments examining the consequences of exogenous administration of inflammatory factors (34) are possible, but such experiments are generally short-term, whereas *in vitro* studies are not easily extrapolated to *in vivo* conditions. By contrast, genetic variants are stable over the life course, not (theoretically) subject to external confounding or reverse causation (5), and data from large populations can be obtained inexpensively.

Some limitations and strengths of the study require consideration. Pleiotropy of genetic variants is a potential source of residual confounding in MR studies unless all biological pathways have been examined and excluded as potential sources of bias or been fully adjusted for (3). *FTO* and *MC4R* could have pleiotropic effects on other metabolic systems independent of their influence on BMI, and as such we cannot exclude the possibility that these genetic variants influence circulating levels of CRP independently of their effects on BMI. However, the effects on downstream risk factors that are thought to be causally influenced by BMI, such as blood pressure (35), metabolic risk factors (36), and bone mineral density (37) are as predicted from the observational associations of BMI with these phenotypes. Furthermore, when multiple genetic variants are associated with a downstream phenotype to the degree predicted by their influence on BMI, although they are unlikely to have the same pleiotropic effects, this provides evidence against spurious associations, as has been demonstrated with respect to BMI and bone mineral density (38).

We combined genetic information from separate *CRP* SNPs; a previous meta-analysis showed that both rs1800947 and rs1205 were independently related to CRP concentrations (6). In common with our study, variant alleles for both the rs1800947 and rs1205 SNPs were associated with lower CRP levels in this meta-analysis (6), which reassures that our results are externally valid. Although the two measured *CRP* SNPs are in moderate linkage disequilibrium, they maintain independent effects on CRP levels, and our sensitivity analysis, as above, validated the use of a simple allele score approach. Although BMI is a rather indirect indicator of adiposity, especially in the elderly, we used leptin as a supplementary biochemical measure of percentage fat mass and were reassured to note that this parameter was also associated with the adiposity allele score, although it must be noted that the strength of the association of leptin with the allele score was largely driven by leptin levels in the double homozygote (allele score 4) group. Finally, although we only measured CRP and not other markers of inflammation, much of the relevant literature has concentrated on CRP as a possible causal agent in vascular and metabolic disease (19–21), whereas other recent work diminishes this possibility (32, 37, 39–41). Our findings now extend these observations by showing that CRP does not lead to adiposity *per se* but is caused by it. This observation in turn casts further doubt on CRP as a causal agent in insulin resistance or type 2 diabetes (32).

In conclusion, using a bidirectional genetic approach that limits confounding, our data support the hypothesis that elevated CRP levels are generated by greater adiposity, with no evidence that elevations in CRP levels *per se* contribute to fat deposition. This extends findings showing that elevated circulating CRP levels do not cause insulin resistance (32). Clearly, future studies should extend our findings to other inflammation parameters. Finally, the future use of this methodology could likewise help to disentangle directions of associations between many other risk pathways.

Acknowledgments

Address all correspondence and requests for reprints to: Prof. Naveed Sattar, British Heart Foundation Glasgow Cardiovascular Research Centre, University of Glasgow, 126 University Place, Glasgow G12 8TA, Scotland, United Kingdom. E-mail: nsattar@clinmed.gla.ac.uk.

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