Association of the IL6-174(G/C) polymorphism with C-reactive protein concentration after weight loss in obese men

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ABSTRACT. Elevated plasma concentration of C-reactive protein has emerged as an important predictor of future cardiovascular diseases and metabolic abnormalities in apparently healthy individuals. Obese individuals tend to have elevated C-reactive protein concentrations. Weight loss induces a change in this protein, and single nucleotide polymorphisms in regulating genes might affect this change, since C-reactive protein concentration is known to be approximately 40-50% heritable. Our aim was to study the association between the *IL6* -174(G/C), *IL1B* +3954(C/T) and *CRP* +1059(G/C) single nucleotide polymorphisms, and CRP concentrations in obese men during a weight reduction program. We genotyped 72 obese men who had participated in a weight reduction program. Their C-reactive protein concentrations, interleukin-6 levels and fat mass were determined at two time points: at baseline and after weight reduction (after 2 months). After weight reduction, the mean weight loss was 14.3 kg. Median C-reactive protein concentrations decreased, after weight reduction, from 1.72 to 1.22 mg/l ($p < 0.02$). The baseline C-reactive protein concentration did not differ between the *IL6*-174(G/C) genotypes, but after weight loss, concentrations differed ($p = 0.03$ Kruskal-Wallis test); the highest concentration was found in the CC genotype (CC 1.01 versus GG 1.93 mg/l, $p = 0.007$ ANOVA *post-hoc* test). This change in concentration was associated with the *IL6*-174(G/C) genotype ($p = 0.01$, Kruskal-Wallis test), being least in the CC genotype. The other single nucleotide polymorphisms studied were not associated with CRP concentrations. Our results show that, at baseline, there is no difference in C-reactive protein concentrations among the different *IL6*-174(G/C) genotypes, but after weight loss the CC genotype is associated with highest C-reactive protein concentrations, resulting from the fact that C-reactive protein seems not to decrease with weight loss in this genotype.

Keywords: obesity, weight loss, C-reactive protein, interleukin-6

Obesity is a worldwide epidemic. It is a risk factor for many chronic diseases; coronary heart disease, type 2 diabetes and hypertension. Weight reduction induces favourable changes in blood lipoproteins, blood pressure, insulin and C-reactive protein (CRP) concentrations, i.e. components of insulin resistance syndrome. Obese individuals tend to have elevated CRP concentrations, but these decrease with weight loss. [1-3] Even slightly elevated baseline CRP concentrations have been associated with increased risk of cardiovascular diseases in men [4] and type 2 diabetes in healthy middle-aged women and men [5, 6].

The acute-phase protein CRP is mainly produced by the liver in response to interleukin-6 (IL-6) and interleukin-1β (IL-1β). IL-6 is a pleiotropic cytokine produced by almost all nucleated cells, the most prominent of them being activated monocytes/macrophages. A recently discovered source of IL-6 is adipose tissue, which has been argued to produce about 25-30% of the systemic IL-6 *in vivo* [7]. IL-6-knock-out mice develop mature-onset obesity and have low energy expenditure [8]. IL-1β in turn, is a proinflammatory cytokine produced by many cell types, including monocytes and Kupffer cells in liver, and is known to enhance CRP transcription [9]. Since basal values for CRP seem to be highly heritable (~40-52%), [10-12] it is very likely that polymorphisms in genes controlling CRP expression affect these basal values. Single nucleotide polymorphisms (SNPs) of several genes have been shown to be

associated with CRP levels. The *IL1B* gene SNP+3954 T allele was associated with CRP concentrations in patients with cardiac symptoms, [13] and the C allele in healthy blood donors [14]. An association between the *CRP* gene SNP+1059 C allele and lower baseline CRP concentrations was found in initially healthy American men [15] and healthy male blood donors [16]. Studies on CRP and the *IL6* gene SNP-174 have been strikingly contradictory [11, 17-19].

The aim of this study was to investigate the effect of *IL1B* +3954, *CRP* +1059 and *IL6*-174 genetics on CRP concentrations in obese men participating in a weight reduction program. We performed association studies between these SNPs and CRP in obese, but otherwise healthy, middle-aged men, at baseline (0 months) and after weight reduction (2 months later).

METHODS

The trial on weight reduction and maintenance consisted of 3 phases and lasted for 31 months [20]. However, in this report we analysed only the first phase of the study, weight reduction (WR). The WR phase was a 2-month program of a very-low-energy diet (VLED; 2MJ/day) for 8 weeks (Nutrilett, Leiras Oy, Turku, Finland). Before the WR phase, the participants were on a low-energy diet (5 MJ/day) for 1 week. Weekly WR group meetings, supervised by a nutritionist, were arranged for the participants. The participants were assessed before and after WR, the assessment time points being 0 and 2 months from the start of the study.

Subjects

The inclusion criteria for the men were age 35-50 years, body mass index (BMI) 30-40 kg/m^2 and waist circumference over 100 cm. Users of regular medication, smokers or physically active subjects (leisure-time exercise > twice weekly) were excluded. Persons were also excluded if resting blood pressure was > 160/105 mmHg, fasting serum cholesterol > 8 mmol/L, triglycerides > 4 mmol/L or blood glucose 6.7 mmol/L in screening examinations [20]. Suspected binge eaters were excluded as a function of the BITE questionnaire [21]. The ethical committee of Pirkanmaa Hospital District approved the study. Written informed consent was obtained from the participants.

Ninety obese, but otherwise clinically healthy, middle-aged men participated in the study and completed the WR, but DNA samples were only available from 72 men. Subjects did not have any serious diseases during the program.

Body composition

Body weight was measured with a high-precision scale after an overnight fast (F150S-D2, Sartorius, Göttingen, Germany). Body density was measured by underwater weighing, as described previously [22]. Body composition was calculated from the body density by a two-component model [23].

Laboratory measurements

The plasma CRP concentrations were analyzed by particle-enhanced, immunonephelometry, using the Dade Behring N High Sensitivity CRP on the Dade Behring Nephelometer II (Dade Behring, Marburg, Germany). The lower detection limit for CRP was 0.16 mg/l (0.016 mg/dl) [24]. Blood plasma IL-6 concentrations were measured using an enzyme-linked immunosorbent assay (ELISA; CLB, PeliKine Compact human IL-6 ELISA kit, Amsterdam, The Netherlands). The sensitivity of the assay was 0.2-0.4 pg/ml. Serum insulin was determined using radioimmunoassay-based method (Pharmacia, Uppsala, Sweden). Blood glucose was assessed by the glucose dehydrogenase method (Roche Ltd). Waist circumference was measured midway between the lowest rib and the iliac crest.

Genotyping of the *CRP* gene polymorphism at the nt position +1059 (exon 2) and the *IL1B* gene polymorphic site at the nt position +3954 (exon 5) was performed using the ABI PRISM 7000 Sequence Detection System for both PCR and allelic discrimination (Applied Biosystems, CA, USA). A commercial kit from Applied Biosystems was used for *CRP* +1059 detection (Assay On Demand, C_177490_10 CRP, Applied Biosystems, CA, USA), which corresponds to the NCBI SNP database rs number 1800947. For *IL1B* +3954 primers and probes were designed based on the NCBI SNP database rs number 1143634. Genotyping of the *IL6* gene polymorphism at the nt position –174G/C, rs number 1800795, was performed using the ABI PRISM 7000 Sequence detection system with designed primers and probes.

Statistical analyses

CRP concentrations ≥ 10 mg/L, indicating clinically relevant inflammatory conditions, were excluded. Spearman rank correlation coefficients were used to quantify the relation between metabolic variables. Between different time points, the t-test for dependent samples was used for the detection of difference in mean values (log values for CRP). Parametric ANOVA or ANCOVA was used to test significant associations between IL-6, BMI, fat mass, CRP and genotypes at individual time points. For this, CRP was first log-transformed and then back-transformed to a normal scale. For CRP, the non-parametric Kruskal-Wallis test and the Mann-Whitney U-test were also used where mentioned. Statistical calculations were performed using Statistica software (ver, Win.6, StatSoft, Inc, Tulsa, OK, USA). The genotype frequencies did not deviate from the Hardy-Weinberg equation. In genetic analyses, individuals having CRP concentration ≥ 10 mg/L at any time point (0 and 2 months later) were excluded. Therefore n = 72 at 0 and 2 months.

RESULTS

Changes in weight, fat mass and metabolic variables during the study

The metabolic characteristics of the participants during the study are shown in *table 1*. The mean weight loss was 14.3 kg, of which 10.1 kg was fat (70.6%). Median CRP decreased by 0.50 mg/L (29.1%, $p < 0.02$, t-test). Insulin concentrations decreased by 5.7 mU/L, 39.9%, $p < 0.001$, t-test). IL-6 concentrations did not change during WR.

Table 1
Anthropometric and metabolic characteristics at baseline (month 0) and after weight reduction phase (WR) (2 months from the beginning)

Variable	0 month n = 77-78	2 months n = 78	P (t-test)[a]
Weight (kg)	105.8 (103.7-108)	91.5 (89.4-93.6)	p < 0.001
BMI (kg/m^2)	32.9 (32.3-33.4)	28.4 (27.8-29.0)	p < 0.001
Fat mass (kg)	37.4 (35.7-39.0)	27.3 (25.6-29.1)	p < 0.001
Fat-free mass (kg)	68.5 (67.2-69.8)	64.1 (62.9-65.4)	p < 0.001
Waist (cm)	113 (111-114)	98 (96-100)	p < 0.001
Glucose (mmol/l)	5.06 (4.96-5.16)	4.76 (4.66-4.85)	p < 0.001
Insulin (mU/l)	14.3 (13.0-15.6)	8.6 (7.7-9.5)	p < 0.001
CRP (mg/l)	1.72 (1.02-2.94)	1.22 (0.58-2.48)	p < 0.02
IL-6 (pg/ml)	2.66 (2.25-3.07)	2.65 (2.19-2.62)	p = n.s.

Values are expressed as means (95% CI), except for CRP which is expressed as median (25-75%).
[a] T-test for dependent samples.

Table 2 shows the correlation coefficients between CRP, BMI, fat mass, IL-6 and insulin. At baseline, CRP concentrations correlated significantly with insulin and BMI (r = 0.43 and 0.46, respectively, p < 0.001). After weight reduction, CRP correlated with BMI, fat mass and insulin (r = 0.34, 0.31 and 0.35 respectively, p < 0.01).

Fat mass and biochemical variables changes in relation to interleukin and CRP genotypes

IL6-174(G/C) genotyping identified 16 G/G homozygotes, 39 G/C heterozygotes and 25 C/C homozygotes. *Table 3* shows the study variables according to IL6 genotype at baseline and after WR (CRP levels ≥ 10 were excluded from the table). At baseline, there were no differences between the genotypes, but after weight loss a difference was apparent (p = 0.03, Kruskal-Wallis test). *Post-hoc* analysis showed that the CC genotype had significantly higher concentrations than GG (p = 0.007, ANOVA Fisher LSD-test with log-transformed values). The result remained significant after adjustment for fat mass (p = 0.006) and after the Bonferroni correction (p = 0.02).
The change in CRP was also significantly associated with the IL6-174(G/C) genotype (Kruskal-Wallis test, p = 0.01). Compared to other genotypes, the CC genotype had the smallest decrease in C-reactive protein levels.
CRP +1059(G/C) genotyping revealed 75 G/G homozygotes and 7 G/C heterozygotes, but no (0) C/C homozygotes. At baseline, the GC heterozygotes had significantly lower CRP levels than the GG homozygotes (median 1.02 *versus* 1.81 mg/L, p = 0.01, Mann Whitney U-test). The result remained significant after adjustment for fat mass and age (p < 0.01). No other statistically significant associations were found between CRP, IL-6 concentrations or fat mass and *CRP* +1059(G/C) genotypes (data not shown). *IL1B* +3954(C/T) genotyping identified 43 C/C homozygotes, 33 C/T heterozygotes and 6 T/T homozygotes, but no association between +3954 genotypes and CRP, IL-6 concentration or fat mass was found.

DISCUSSION

The aim of this study was to examine the genetic regulation of blood CRP concentrations in 72 obese individuals before and after a WR program. As IL-6 is produced by adipose tissue, we studied the effect of the *IL6* promoter region polymorphism -174(G/C), which is known to have an effect on the transcription of the gene, on CRP concentrations in these individuals. Also, based on our previous results on the impact of *IL1B* +3954 and *CRP* +1059 [14, 16] on CRP concentrations, the effect of these polymorphisms was analyzed as well.
The median CPR concentration in these middle-aged men before weight loss was higher than reported for healthy, middle-aged, Finnish male blood donors (1.80 versus 0.71 mg/L, respectively, BMI not known) [14] Previously,

Table 2
Spearman rank correlation coefficients and p-values between CRP and metabolic variables at baseline, after 2 months from the start, and between changes (Δ 0-2 months)

Metabolic variable	0 month n = 78	2 months n = 78	Δ 0-2 months n = 73-74
BMI	0.46	0.34	0.13
	p < 0.001	p < 0.01	p = 0.28
Fat mass	0.18	0.31	-0.13
	p = 0.12	p < 0.01	p = 0.26
Insulin	0.43	0.35	0.19
	p < 0.001	p < 0.01	p = 0.11
IL-6	0.23	0.20	0.36
	p = 0.06	p = 0.08	p < 0.01

Table 3
The genotype effect of *IL6*-174 (G/C) on fat mass, body mass index, CRP and IL-6 concentration before and after weight reduction (0 and 2 months from the start), and on their changes (Δ 0-2)

IL6-174(G/C)	0 month	P	2 months	P	Δ 0-2	P
Fat mass (kg)						
GG	34.0	0.05	24.2	0.11	-29.6	0.70
GC	36.8		27.1		-26.9	
CC	39.7		29.5		-26.5	
CRP (mg/L)						
GG	1.34	0.19	1.01	0.03	-9.52	0.01
GC	1.91		1.12		-45.44	
CC	1.82		1.93		-0.24	
IL-6 (pg/mL)						
GG	2.40	0.76	2.61	0.97	+2.98	0.59
GC	2.91		2.79		+3.61	
CC	2.74		2.72		+4.58	
BMI						
GG	31.9	0.31	27.6	0.47	-13.64	0.99
GC	33.1		28.6		-13.63	
CC	32.9		28.4		-13.64	

For normally distributed data (fat mass, IL-6 and BMI) ANOVA statistics are used and mean values are presented, for skewed data (CRP) Kruskal-Wallis test is used and median values are presented. At 0 and 2 months n = 72: 13 GG, 34 GC and 25 CC. Δ 0-2 values are calculated using following formula: (2 months-0 month)/0 month*100.

Pieroni *et al*. reported higher median CRP concentrations for obese than for non-obese French men (1.16 versus 0.40 mg/L) [25] Thus, elevated CRP concentrations seem to be associated with an obesity phenotype. After weight loss, we found a significant decrease in CRP concentrations. Decreases in CRP after weight reduction have been reported before in obese subjects (women [1-3, 26], men [27]), and elevated CRP has been reported in association with long-term body weight variability in Japanese men [28]. Even slightly elevated baseline CRP concentrations have been associated with increased risk of cardiovascular diseases [4].

We found an association between the *IL6* promoter region polymorphism –174(G/C) and CRP concentration after weight loss. CC homozygotes had significantly higher CRP values than GG homozygotes, and the change in concentration was least in CC homozygotes. Higher baseline CRP has been previously associated with the C allele in a family study of 588 subjects (mean age 49, female-:male 1:1) [11], and in healthy men [17]. In contrast to these studies, an opposite effect was found in postmenopausal women [18]. No association could be found in a study of asymptomatic hospital employees in Italy [19] or in a study of patients with coronary heart disease [29].

However, the plasma IL-6 concentrations did not change with weight loss and did not differ between -174(G/C) genotypes at any time point. Similar results have been previously reported by Wood *et al*. [30], where diet-induced weight loss did not change IL-6 concentrations and by Qi *et al*. [31], where IL-6 polymorphisms were not associated with plasma IL-6 concentrations in the control population of a diabetes study. Blood concentration of IL-6 does not necessarily reflect the effective concentration of IL-6. The lack of an association between *IL6* genetics and IL-6 blood levels, could, for example, be due to different kinetics (e.g. production/receptor binding) in people with different genotypes, i.e. the blood concentration reflects only the steady-state concentration.

The limitation of this study is the small number of subjects; there may be certain significant differences between the genotype groups that we were unable to detect due to the low power of the study. However, the power to detect the difference in CRP between the *IL6*-174(G/C) genotypes at two months was 0.77. Additionally, the strength of our study was the underwater weighing of body density. This is the "gold standard" for fat mass assessment.

In conclusion, weight loss induces a significant decrease in CRP concentration in obese men. The *IL6* -174(G/C) genotype is associated with CRP concentration after weight loss in obese men, CC homozygotes having the highest values. When weight is reduced, the reduction in CRP concentration is least in these individuals.

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