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Variations of the interleukin-6 promoter are associated with features of the metabolic syndrome in Caucasian Danes

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Abstract *Aims/hypothesis:* The cytokine interleukin 6 (IL-6) is an essential regulator of the acute phase response associated with insulin-resistant states including type 2 diabetes and obesity. Three polymorphisms at positions −597, −572, and −174 of the *IL6* promoter have been reported to influence *IL6* transcription. The aim of this study was to investigate whether the *IL6* promoter polymorphisms were associated with features of the WHO-defined metabolic syndrome and related quantitative traits in 7,553 Caucasian Danes. *Methods:* Using analysis of PCR-generated primer extension products by mass spectrometry we examined −597 G/A, −572 G/C, and −174 G/C *IL6* variants in the population-based Inter99 study cohort of middle-aged people (n=6,164) and in a group of type 2 diabetic patients (n=1,389). *Results:* The −174 G/C and −597 G/A polymorphisms were in strong linkage disequilibrium (R^2=0.95). In the Inter99 cohort the −174 G-allele was associated with insulin resistance (p<0.02) and dyslipidaemia (p<0.007) whereas the C-allele of the −572 polymorphism was associated with increased serum insulin release during an OGTT (p<0.0005). Composite genotype or haplotype analyses of all 3 *IL6* promoter variants showed associations with type 2 diabetes (p<0.002), obesity (p<0.02), and the metabolic syndrome (p<0.01). *Conclusions:* The present studies suggest that single-nucleotide polymorphisms and composite genotypes or haplotypes of the *IL6* promoter may be associated with several features of the metabolic syndrome in Caucasians.

Keywords Cytokine · IL-6 · Genetics · Insulin resistance · Metabolic syndrome · Obesity · Type 2 diabetes mellitus

Abbreviations F: Fasting · HDLC: HDL cholesterol · HOMA: Homeostasis model assessment · IL-6: Interleukin-6 · P: Plasma · S: Serum · SNP: Single-nucleotide polymorphism · WHO: World Health Organization

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Introduction

Interleukin-6 (IL-6) is a pleiotropic cytokine involved in the pathophysiology of various human diseases. It is secreted by different cell types including leukocytes and endothelial cells and has recently been shown to be released from muscle tissue and adipose cells [1–3]. IL-6 production is stimulated by tumour necrosis factor-α (TNF-α), interleukin-1, bacterial endotoxin, and catecholamines, and is suppressed by glucocorticoids and oestrogen [1, 4]. It is also known to be a potent stimulator of the hypothalamic–pituitary–adrenal axis in states of inflammation [1, 5]. Moreover, IL-6 stimulates the secretion of growth hormone and inhibits thyroid-stimulating hormone secretion [1, 5]. IL-6 signals are transmitted via a heterodimeric receptor complex consisting of a soluble interleukin-6 alpha subunit (IL-6R) and a signal-transducing subunit, gp130 [6].

IL-6 is an important regulator of the acute phase response that is associated with insulin-resistant states including type 2 diabetes [7–10]. Hence, higher circulating IL-6 levels have been demonstrated in obese subjects and type 2 diabetic patients, particularly in subjects also having features

of the metabolic syndrome [11, 12]. Moreover, subcutaneous administration of recombinant IL-6 to humans induces a dose-dependent increase in fasting plasma glucose and glucagon levels without affecting plasma insulin or C-peptide concentrations [13].

The pathogenic impact of *IL6* in insulin-resistant states is underscored by the effect of the functional *IL6* −174 G/C promoter polymorphism. The −174 G/C variant has been shown to influence the transcriptional regulation of *IL6*, and human −174 G-allele carriers exhibit higher plasma IL-6 levels compared with homozygous C-allele carriers [14], an effect which is modulated by age and gender [15, 16]. Although the results have been inconsistent, previous smaller studies of subjects of different ethnic origin have linked the −174 G/C variant to indices of obesity and insulin resistance. Hence, in Native Americans and Caucasians the GG genotype was associated with type 2 diabetes [17] whereas a recent Swedish study and another study in a French Canadian population showed that the C-allele was associated with indices of obesity [18, 19]. In Spanish populations the G-allele has been related to decreased insulin sensitivity and hyperglycaemia [20] and to alterations in serum lipids [21].

Recently, two other functional SNPs in the *IL6* promoter at positions −597 and −572 were identified [22, 23]. The −572 G/C SNP (denoted −634C/G in Ref. [22]) has been reported to be associated with progression of diabetic nephropathy in Japanese type 2 diabetic patients and to have an effect on IL-6 secretion capacity [22]. Furthermore, an in vitro study showed that the −572 G/C *IL6* polymorphism influenced gene expression levels after stimulation with interleukin-1β and dexamethasone [24]. It has also been shown that these three SNPs (−174, −572, −597) of the *IL6* promoter do not act independently in the regulation of *IL6* transcription [25–27]. In a study of lipopolysaccharide-stimulated IL-6 production by leucocytes it was demonstrated that leucocytes from the homozygous carrier of the GGG-haplotype (−597 GG, −572 GG and −174 GG) produced the highest amount of IL-6 [25].

In the present large-scale investigation of Caucasian subjects we have examined if the −174 G/C, −572 G/C, and −597 G/A *IL6* promoter polymorphisms, which in previous studies have been shown to modulate *IL6* expression, separately or in combination are associated with features of the 1999 WHO-defined metabolic syndrome and related hormonal and metabolic quantitative traits.

Subjects, materials and methods

Subjects The study involved two groups of subjects: (1) a group of type 2 diabetic patients recruited from the outpatient clinic at Steno Diabetes Center, and (2) the Inter99 cohort.

The Inter99 cohort is a population-based randomized non-pharmacological intervention study for prevention of

Table 1 Genotype distribution and allele frequencies of the −174 G/C and −572 G/C *IL6* promoter polymorphisms of *IL6* in type 2 diabetic patients and glucose-tolerant Danish Caucasian subjects

	All		*p* value*	*p* value**	*p* value***	OR (95% CI)
	T2D	NGT				
−174 GC						
Number (M/W)	1,389 (843/546)	4,401 (2,053/2,348)				
GG	402 (29.9%)	1,246 (28.3%)				
GC	659 (47.4%)	2,133 (48.5%)				
CC	328 (23.6%)	1,022 (23.2%)	0.71	0.71	0.57	
C-allele %	47.3 (45.5–49.2)	47.5 (46.4–48.5)	0.93			1.03 (0.87–1.23)[a] 1.05 (0.89–1.23)[b]
−572 GC						
Number	1,361 (824/537)	4,382 (2,038/2,344)				
GG	1,233 (90.6%)	4,037 (92.1%)				
GC	123 (9.0%)	325 (7.4%)				
CC	5 (0.4%)	20 (0.5%)	0.02	1.0	0.008	
C-allele %	4.9 (4.1–5.7)	4.2 (3.7–4.6)	0.11			1.03 (0.33–3.26)[a] 1.40 (1.09–1.80)[b]

Data are number of subjects with each genotype (percentage of each group). *p* values for allele frequencies were calculated using Fisher's exact test

T2D, type 2 diabetes; NGT, normal glucose-tolerant; M, men; W, women; OR, odds ratio

**p* values for genotype distribution were obtained using regression analysis with adjustment for age and gender in a co-dominant (GG versus GC versus CC) model

***p* values for genotype distribution were obtained using regression analysis with adjustment for age and gender in a recessive (GG+GC versus CC) model

****p* values for genotype distribution were obtained using regression analysis with adjustment for age and gender in a dominant (GG versus GC+CC) model

[a]OR (95% CI) comparing GG+GC versus CC

[b]OR (95% CI) comparing GG versus GC+CC

Table 2 Clinical and biochemical characteristics of middle-aged normal glucose-tolerant (NGT) subjects when classified in accordance with their genotype of the −174 G/C and −572 G/C polymorphisms of the *IL6* promoter

NGT	−174 G/C						−572 G/C					
	GG	GC	CC	*p* value*	*p* value**	*p* value***	GG	GC	CC	*p* value*	*p* value**	*p* value***
N (M/W)	1,246 (601/645)	2,133 (1,022/1,111)	1,022 (430/592)				4,037 (1,847/2,190)	325 (179/146)	20 (12/8)			
Age (years)	45±8	45±8	46±8				45±8	44±8	42±8			
BMI (kg m^{-2})	25.5±3.9	25.6±4.1	25.2±4.2	0.04	0.02	0.92	25.5±4.1	25.3±3.8	24.7±2.2	0.27	0.37	0.13
WHR	0.84±0.09	0.85±0.08	0.84±0.08	0.67	0.41	0.56	0.84±0.08	0.85±0.08	0.85±0.07	0.82	0.68	0.72
P-glucose 0 min (mmol L^{-1})	5.3±0.4	5.3±0.4	5.3±0.4	0.68	0.99	0.42	5.3±0.4	5.3±0.4	5.2±0.7	0.26	0.18	0.54
iAUC-glucose (mmol L^{-1})	182±104	182±99	176±98	0.51	0.25	0.58	181±101	173±100	183±101	0.48	0.70	0.30
S-Insulin 0 min (pmol L^{-1})	38±24	38±23	36±22	0.15	0.05	0.35	38±23	38±24	38±25	0.94	0.90	0.93
iAUC–insulin (pmol L^{-1})	21,118±12,683	21,545±14,281	19,798±11,678	0.07	0.02	0.55	20,840±13,179	21,913±14,224	29,083±18,061	0.001	0.002	0.01
Insulinogenic index-insulin	31.0±19.0	31.6±21.4	29.2±16.8	0.20	0.08	0.69	30.6±19.5	32.9±22.4	48.6±34.4	<0.001	<0.001	0.004
S-cholesterol (mmol L^{-1})	5.4±1.1	5.4±1.0	5.4±1.0	0.35	0.48	0.35	5.4±1.0	5.4±1.1	5.4±0.9	0.64	0.47	0.45
S-HDL-cholesterol (mmol L^{-1})	1.44±0.40	1.45±0.39	1.50±0.41	0.27	0.15	0.24	1.46±0.40	1.41±0.37	1.40±0.54	0.36	0.45	0.18
S-triglycerides (mmol L^{-1})	1.20±0.91	1.19±1.06	1.14±0.70	0.66	0.88	0.43	1.18±0.94	1.20±0.96	1.31±0.80	0.65	0.38	0.92
HOMA insulin-resistance index	9.1±5.9	9.1±5.7	8.5±5.4	0.17	0.06	0.48	8.9±5.7	9.0±5.6	8.7±6.4	0.93	0.93	0.72

Data are presented as mean (±SD)
M, Men; W, women; P, plasma; S, serum; iAUC, incremental area under the curve after an OGTT
**p* values comparing genotype effect in a co-dominant (GG versus GC versus CC) model were obtained after adjustment for age, gender and BMI (*p* values for BMI were adjusted only for age and gender)
***p* values comparing genotype effect in a recessive (GG+GC versus CC) model were obtained after adjustment for age, gender and BMI (*p* values for BMI were adjusted only for age and gender)
****p* values comparing genotype effect in a dominant (GG versus GC+CC) model were obtained after adjustment for age, gender and BMI (*p* values for BMI were adjusted only for age and gender)

cardiovascular disease done at the Research Centre for Prevention and Health involving 6,514 Caucasian subjects (6,164 with OGTT data), 4,568 with normal glucose tolerance (NGT), 508 with impaired fasting glycaemia (IFG), 707 with impaired glucose tolerance (IGT), 256 with screen detected diabetes mellitus, and 125 with known type 2 diabetes. Details of this cohort have been reported previously [28]. Some subjects were not included in the analyses because of missing genotype data and only subjects with genotype data are presented in the tables.

The case-control study of type 2 diabetes involved all NGT subjects from the Inter99 cohort and 1,389 unrelated type 2 diabetic patients recruited from the outpatient clinic at Steno Diabetes Center, Copenhagen and the Research Centre for Prevention and Health through the Inter99 study. Diabetes was diagnosed in accordance with the 1999 World Health Organization criteria [29]. The basic characteristics of type 2 diabetic patients were: mean age (±SD) 57±11 years, age at clinical diagnosis 51±11 years, BMI 29.6±5.3 kg m^{-2} and HbA_1c 7.8±1.7%. Patients with diabetes due to known chronic pancreatitis, haemochromatosis, severe insulin resistance, maturity-onset diabetes of the young (MODY), and maternally inherited diabetes and deafness (MIDD), patients with a family history of first degree relatives with type 1 diabetes, patients with insulin requirement within the first year after diabetes diagnosis, or patients with a fasting serum C-peptide level ≤150 pmol L^{-1} at the time of recruitment were excluded in the present study from the category of clinically defined type 2 diabetes.

In the genotype-quantitative trait studies only NGT Caucasian subjects from the Inter99 cohort were included.

Also, the Inter99 participants were evaluated for the metabolic syndrome according to WHO criteria [29]; those who had none of the components of the metabolic syndrome were qualified as control subjects.

In the case-control study of obesity, we stratified the Inter99 cohort into two groups according to BMI: 2,581 lean subjects with a BMI ≤25.0 kg m^{-2} (1,002 men and 1,579 women) and 1,009 obese subjects with a BMI >30.0 kg m^{-2} (501 men and 508 women).

All study participants were Danish Caucasians by self-report. The studies were approved by the Ethical Committee of Copenhagen and oral and written consent was obtained in accordance with the Helsinki Declaration II.

Anthropometric measurements Body weight was measured to the nearest 0.1 kg with subjects wearing only light indoor clothing without shoes. Waist circumference was measured midway between the iliac crest and the lower costal margin, and hip circumference was measured at its maximum. Blood pressure was measured after at least 5 min rest in a sitting position. The mean of two to three systolic and diastolic blood pressures was calculated and used in the analyses.

Biochemical measurements Blood samples for analyses of biochemical variables were drawn in the morning after an overnight fast. Plasma glucose, serum-specific insulin [excluding des(31, 32)- and intact proinsulin], plasma triglycerides, HDL-cholesterol, serum total cholesterol, and urinary albumin and creatinine were analysed using Steno Diabetes Center standard methods. The insulinogenic index was calculated as fasting serum insulin (pmol L^{-1}) subtracted from 30-min post-OGTT serum insulin (pmol L^{-1}) and divided by 30-min post-OGTT plasma glucose (mmol L^{-1}). HOMA-IR was calculated as fasting plasma glucose (mmol L^{-1}) multiplied by fasting serum insulin (pmol L^{-1}) and divided by 22.5.

Genotyping Genomic DNA was isolated from human leucocytes using standard methods [30]. The genotyping method used for detection of the *IL6* −174, −572, −597 promoter variants (rs1800795, rs1800797, rs1800796) was a chip-based matrix-assisted laser-desorption/ionisation time-of-flight (MALDI-TOF) mass spectrometric (DNA MassARRAY) analysis of PCR-generated primer extension products as described by Buetow et al. [31]. Sequences of amplification and extension primers used in the genotyping assay are available on request from the corresponding author. The genotyping success rates of the mass spectrometry-based method for these three SNPs were >97%, and among 89 replicate samples there were no mismatches.

Statistical analysis Linkage disequilibrium was estimated as R^2, where R^2=1 for complete linkage and R^2=0 for no linkage. R^2 was calculated as described at http://www.ekstroem.com. Fisher's exact test was used to test for significance of differences in allele and haplotype distributions. Regression analyses with adjustments for age and gender were used to test for significance of differences in genotype frequencies and composite genotypes between cases and control subjects in the case-control studies of type 2 diabetes and the metabolic syndrome.

Phenotypic differences between the genotype groups among NGT subjects were tested with a general linear model including gender and genotype as fixed factors and age and BMI as covariate factors. For genotype-quantitative trait studies normal distribution of the residuals was verified and if appropriate logarithmically transformed. A p value less than 0.05 was considered significant. All analyses were done using Statistical Package for Social Sciences (SPSS) for Windows, version 12.0 (http://www.spss.com).

Results

The −174 G/C and −597 G/A variants were in tight linkage disequilibrium (R^2=0.95). Hence, only data for the −174 G/C and −572 G/C polymorphisms will be presented in the single-variant association studies. Genotype distributions in both the total Inter99 cohort and in the combined type 2 diabetes groups were in Hardy–Weinberg equilibrium for the −174 G/C and −597 G/A polymorphisms whereas the genotype distribution of the −572 G/C polymorphism did not obey Hardy–Weinberg equilibrium among control subjects. Also 410 subjects were independently genotyped using a modified allele-specific genotyping method, as

Table 3 Genotype distribution and allele frequencies of −174 GC and −572 GC promoter polymorphisms of the *IL6* gene according to the 1999 WHO-defined metabolic syndrome components among subjects from the Inter99 cohort

	Control subjects	Impaired glucose regulation	Insulin resistance	Hypertension	Dyslipidaemia	Indices of obesity	Microalbuminuria	One or more components of the metabolic syndrome	The metabolic syndrome as a whole
−174 GC									
Definition	Subjects having no components of the metabolic syndrome	IFG, IGT and T2D	Fasting serum, insulin above the highest quartile in the Inter99 cohort	Treatment for hypertension and/or BP ≥140/90 mmHg	Treatment for dyslipidaemia and/or s. triglycerides >1.7 mmol L^{-1} and/or HDLC <0.9 mmol L^{-1} in men; HDLC <1.0 mmol L^{-1} in women	BMI >30 kg m^{-2} and/or WHR >0.90 in men, WHR >0.85 in women	Urinary albumin/creatinine ratio ≥30 mg g^{-1}		1999 WHO definition
N (M/W)	1,661 (494/1,167)	1,524 (908/616)	1,532 (855/677)	2,423 (1,443/980)	1,543 (995/548)	2,566 (1,741/825)	168 (75/93)	4,377 (2,522/1,855)	1,277 (866/411)
GG (%)	474 (28.5)	434 (28.5)	430 (28.1)	673 (27.8)	447 (29.0)	717 (27.9)	39 (23.2)	1,235 (28.2)	359 (28.1)
GC (%)	771 (46.4)	756 (49.6)	782 (51.0)	1,221 (50.4)	796 (51.6)	1,275 (49.7)	93 (55.4)	2,172 (49.6)	659 (51.6)
CC (%)	416 (25.0)	334 (21.9)	320 (20.9)	529 (21.8)	300 (19.4)	574 (22.4)	36 (21.4)	970 (22.2)	259 (20.3)
C-allele%	48.3 (46.6–50.0)	46.7 (44.9–48.5)	46.4 (44.6–48.2)	47.0 (45.6–48.4)	45.2 (43.5–47.0)	47.2 (45.8–58.6)	43.8 (43.8–54.5)	47.0 (45.9–48.0)	46.1 (44.2–48.0)
p Value*	–	0.15	0.02	0.05	0.007	0.29	0.07	0.07	0.03
p Value**	–	0.17	0.03	0.10	0.008	0.49	0.35	0.09	0.06
p Value***	–	0.42	0.36	0.25	0.49	0.28	0.10	0.38	0.27
−572 GC									
N (M/W)	1,654 (493/1,161)	1,525 (914/611)	1,518 (846/672)	2,411 (1,434/977)	1,549 (999/550)	2,554 (1,733/821)	167 (73/94)	4,367 (2,514/1,853)	1,273 (867/406)
GG (%)	1,518 (91.8)	1,387 (91.0)	1,385 (91.2)	2,204 (91.4)	1,422 (91.8)	2,346 (91.9)	159 (95.2)	3,997 (91.5)	1,170 (91.9)
GC (%)	128 (7.7)	132 (8.7)	127 (8.4)	201 (8.3)	117 (7.6)	199 (7.8)	7 (4.2)	352 (8.1)	99 (7.8)
CC (%)	8 (0.5)	6 (0.4)	6 (0.4)	6 (0.2)	10 (0.6)	9 (0.4)	1 (0.6)	18 (0.4)	4 (0.3)
C-allele%	4.4 (3.7–5.0)	4.7 (4.0–5.5)	4.6 (3.8–5.3)	4.4 (3.7–5.1)	4.4 (3.8–5.0)	4.2 (3.7–4.8)	2.7 (1.0–4.4)	4.4 (4.0–4.9)	4.2 (3.4–5.0)
p value*	–	0.63	0.89	0.36	0.55	0.64	0.30	0.90	0.61
p value**	–	0.75	0.68	0.17	0.53	0.38	0.90	0.64	0.32

Table 3 (continued)

	Control subjects	Impaired glucose regulation	Insulin resistance	Hypertension	Dyslipidaemia	Indices of obesity	Microalbuminuria	One or more components of the metabolic syndrome	The metabolic syndrome as a whole
p value***	–	0.41	0.88	0.89	0.48	0.60	0.14	0.95	0.70

Data are number of subjects with each genotype (percentage of each group) and allele frequency in percent with 95% confidence interval. Cases were defined according to the WHO criteria [29]
M, Men; W, women *p values compare genotype distributions in a co-dominant (GG versus GC versus CC) model using regression analysis with adjustment for gender and age between cases and control subjects having no single component of the metabolic syndrome
**p values compare genotype distributions in a recessive (GG+GC versus CC) model using regression analysis with adjustment for gender and age between cases and control subjects having no single component of the metabolic syndrome
***p values compare genotype distributions in a dominant (GG versus GC+CC) model using regression analysis with adjustment for gender and age between cases and control subjects having no single component of the metabolic syndrome

described elsewhere [27], and no genotyping mismatches were found (data not shown). Also in this material the −572 G/C polymorphism did not obey Hardy–Weinberg equilibrium. The distributions of all composite genotypes obeyed Hardy–Weinberg equilibrium.

The −174 G/C polymorphism In the type 2 diabetes case-control study there were no significant differences between genotype or allele frequencies of the −174 G/C variant in 1,389 type 2 diabetic patients and 4,401 glucose-tolerant subjects from the Inter99 cohort (Table 1). Similarly, when the control subjects were confined to the 1,464 age-matched and gender-matched glucose-tolerant control subjects, no association between the *IL6* promoter variant and type 2 diabetes could be demonstrated (data not shown). Repeated analysis using a subgroup consisting of the obese type 2 diabetic and glucose-tolerant subjects with BMI above the median did not reveal any association between the −174 G/C variant and type 2 diabetes (data not shown).

In the study of 4,401 glucose-tolerant subjects from the Inter99 cohort, subjects carrying the G-allele (GG and GC carriers) of the −174 *IL6* variant had statistically higher BMI (p=0.02) compared with CC carriers (Table 2). Furthermore, a contribution of the −174 variant to BMI was also found in the Inter99 cohort by comparing the frequencies of the G-allele carriers of the variant to CC homozygous carriers between lean (BMI≤25 kg m^{-2}) and obese (BMI>30 kg m^{-2}) subjects (p=0.008) (data not shown). A borderline significant genotype effect on post-OGTT serum insulin (incremental $AUC_{0–120\ min}$) was found among glucose-tolerant subjects from the Inter99 cohort (Table 2). There was no genotype effect on quantitative estimates of plasma glucose levels, insulin resistance as measured by the HOMA insulin-resistance index, or fasting serum lipid profiles (Table 2).

In the case-control study of the metabolic syndrome and the −174 G/C polymorphism we found differences between the allele frequencies and genotype distributions in cases with the metabolic syndrome and control subjects having no components of the metabolic syndrome (Table 3). Furthermore, examination of the individual components of the WHO-defined metabolic syndrome demonstrated an association with insulin resistance and dyslipidaemia (Table 3).

The −572 G/C polymorphism The case-control study of type 2 diabetes revealed an association with type 2 diabetes. However, the NGT control group was, as already mentioned, not in Hardy–Weinberg equilibrium (Table 1) and the association with diabetes might well be spurious. We found no genotype effect of this variant on BMI or the metabolic syndrome (Tables 2 and 3). However, in the genotype-quantitative trait study of the glucose-tolerant subjects in the Inter99 cohort this polymorphism had a significant impact on post-OGTT serum insulin release as estimated by the incremental $AUC_{0–120min}$ and the insulinogenic index for insulin (Table 2). Homozygous carriers for the mutant allele (CC) also had higher serum insulin and C-peptide levels after oral glucose stimulation

at 30 min during the OGTT (data not shown). Among 672 IGT subjects and 491 IFG subjects from the Inter99 cohort the same phenomenon was observed (data not shown).

Composite genotype and haplotype studies of the −597 G/A, −572 G/C and −174 G/C polymorphisms of the IL6 promoter To identify *IL6* promoter haplotypes in Danish Caucasian subjects we initially genotyped 410 subjects (type 2 diabetic patients and glucose-tolerant subjects) using allele-specific primers for PCR as described by Terry et al. [27]. We found only four haplotypes (AGC, GGG, GCG, and GGC) among these 820 chromosomes (data not shown). After genotyping of the three *IL6* promoter SNPs in all Inter99 and type 2 diabetes subjects it was possible to assign specific haplotypes and composite genotypes to a total of 6,916 subjects who had genotype data of all −597, −572 and −174 SNPs. In eight subjects we were not able to assign any specific haplotypes and composite genotypes. These eight subjects and subjects with missing genotyping data in one or more SNPs were excluded from the present haplotype analyses.

The studies of the composite genotypes among type 2 diabetic patients and glucose-tolerant subjects from the Inter99 cohort showed an association with type 2 diabetes for AGC/GCG (p=0.002) (Table 4). Moreover, the frequency of the GCG haplotype was higher among type 2 diabetic subjects compared with the glucose-tolerant subjects (Table 4). The AGC/GGG composite genotypes were more frequent among cases of the metabolic syndrome and the AGC/AGC composite genotypes were more frequent among control subjects without any features of the metabolic syndrome (Table 5). No association of haplotypes with the metabolic syndrome was found. Finally, in the obesity case-control study the frequency of the GGG haplotype was higher among obese subjects and the AGC haplotype was more frequent among lean subjects (Table 6).

Discussion

In this study we investigated whether polymorphisms of the *IL6* promoter (−597 G/A, −572 G/C and −174 G/C), which in previous studies have been shown to modulate *IL6* expression, are, separately and/or together, associated with features of the WHO-defined metabolic syndrome or related quantitative traits. Overall we demonstrate that single-nucleotide polymorphisms and composite genotypes and haplotypes of the *IL6* promoter are associated with several features of the metabolic syndrome.

The genotype-quantitative trait interaction study in 4,401 middle-aged glucose-tolerant subjects suggested that the −174 G/C but not the −572 G/C *IL6* promoter polymorphism contributes to the interindividual variation in BMI. Furthermore, in the obesity case-control study comparing haplotypes of the investigated three polymorphisms of the *IL6* promoter, we found the AGC haplotype to be more frequent in the lean group with BMI ≤25 kg m^{-2} than among obese subjects with BMI >30 kg m^{-2}, whereas the GGG haplotype was more frequent among obese subjects.

The mechanisms by which the *IL6* promoter polymorphisms might cause an increase in BMI are unknown, but it might be because of an effect on insulin resistance in

Table 4 The distribution of composite genotypes and haplotype frequencies of the *IL6* promoter in a group of type 2 diabetic patients and a group of glucose-tolerant Danish Caucasian subjects with genotype data for all −597, −572 and −174 SNPs

	T2D	NGT	OR (95% CI)	p value
Composite genotypes				
Number (M/W)	1,313 (797/516)	4,181 (1,951/2,230)		
GGG/GGG	322 (24.5%)	1,020 (24.4%)	1.00 (0.84–1.19)	1.00
AGC/AGC	298 (22.7%)	913 (21.8%)	1.04 (0.87–1.24)	0.65
AGC/GGG	543 (41.4%)	1,827 (43.7%)	0.87 (0.74–1.01)	0.06
GGG/GCG	54 (4.1%)	154 (3.7%)	1.19 (0.82–1.72)	0.37
AGC/GCG	68 (5.2%)	156 (3.7%)	1.73 (1.23–2.43)	0.002
AGC/GGC	12 (0.9%)	45 (1.1%)	0.88 (0.41–1.92)	0.75
GGG/GGC	11 (0.8%)	44 (1.1%)	0.85 (0.37–1.94)	0.70
GCG/GCG	5 (0.4)	18 (0.4%)		
GGC/GCG	0	3 (0.1%)		
GGC/GGC	0	1 (0.0%)		
Haplotypes				
AGC% (95% CI)	46.4 (44.5–48.3)	46.3 (45.2–47.4)		0.77
GGG% (95% CI)	47.7 (45.8–49.6)	48.6 (47.5–49.7)		0.41
GCG% (95% CI)	5.0 (4.2–5.8)	4.2 (3.8–4.6)		0.06
GGC% (95% CI)	0.9 (0.5–1.3)	1.3 (0.9–1.3)		0.33

Data are number of subjects with each composite genotype (percentage of each composite genotype). The odds ratio (OR) (with 95% CI) was calculated as the risk of type 2 diabetes for the specific composite genotypes relative to all other composite genotypes. p values for composite genotype distributions were calculated using regression analysis. Distributions of composite genotype obeyed Hardy–Weinberg equilibrium. Haplotype frequencies are given with 95% CI. p values for haplotype frequencies were calculated using Fisher's exact test. T2D, type 2 diabetes; NGT, normal glucose-tolerant; M, men; W, women

Table 5 The distribution of the *IL6* promoter composite genotypes and haplotype frequencies according to the 1999 WHO-defined metabolic syndrome among the subjects from the Inter99 cohort

	Cases of the metabolic syndrome	Controls without any features of the metabolic syndrome	OR (95% CI)	*p* value
Composite genotypes				
Number (M/W)	1,248 (843/405)	1,580 (476/1,104)		
GGG/GGG	302 (24.2%)	389 (24.6%)	0.91 (0.74–1.10)	0.29
AGC/AGC	240 (19.2%)	372 (23.5%)	0.82 (0.67–1.01)	0.06
AGC/GGG	573 (45.9%)	651 (41.2%)	1.25 (1.05–1.48)	0.01
GGG/GCG	42 (3.4%)	59 (3.7%)	0.90 (0.57–1.41)	0.64
AGC/GCG	55 (4.4%)	67 (4.2%)	1.03 (0.68–1.56)	0.90
AGC/GGC	13 (1.0%)	20 (1.8%)	0.71 (0.33–1.54)	0.38
GGG/GGC	17 (1.4%)	14 (0.9%)	1.76 (0.80–3.88)	0.16
GCG/GCG	3 (0.2%)	8 (0.5)		
GGC/GCG	2 (0.2%)	0		
GGC/GGC	1 (0.1%)	0		
Haplotypes				
AGC% (95% CI)	44.9 (43.0–46.9)	46.9 (45.5–48.6)		0.14
GGG% (95% CI)	49.5 (47.6–51.5)	47.5 (45.8–49.2)		0.14
GCG% (95% CI)	4.2 (3.4–5.0)	4.5 (3.8–5.2)		0.65
GGC% (95% CI)	1.4 (0.9–1.9)	1.1 (0.7–1.5)		0.33

Data are number of subjects with each composite genotype (percentage of each composite genotype). The odds ratio (OR) (with 95% CI) was calculated as the risk of the metabolic syndrome for the specific composite genotypes relative to all other composite genotypes. *p* values for composite genotype distributions were calculated using regression analysis. Distributions of composite genotype obeyed Hardy–Weinberg equilibrium. Haplotype frequencies are given with 95% CI. *p* values for haplotype frequencies were calculated using Fisher's exact test

M, men; W, women

combination with the anabolic effect of a slightly increased serum insulin level after an oral glucose load among GG carriers of the −174 G/C SNP [1, 5]. An ex-vivo functional study [25] demonstrated that the GGG haplotype of the *IL6* promoter produces more IL-6 than other promoter haplotypes. Whether carriers of the GGG haplotype also secrete more IL-6 under physiological in-vivo conditions [26] is, however, unknown.

Although several in-vitro and in-vivo studies have examined the physiological and pathophysiological effects of IL-6, the role of this cytokine in the induction of metabolic disorders such as insulin resistance and obesity is still unsettled. Thus, it has been suggested that elevation of circulating IL-6 levels contributes to insulin resistance, type 2 diabetes [8, 10–12, 32–34], whereas other studies are consistent with an enhancing effect of IL-6 on glucose and lipid metabolism [3, 35, 36]. Also, animal models

Table 6 The distribution of the *IL6* promoter composite genotypes and haplotype frequencies according to obesity among subjects from the Inter99 cohort

	Obese	Lean	OR (95% CI)	*p* value
Composite genotypes				
Number (M/W)	1,009 (501/508)	2,581 (1,002/1,579)		
GGG/GGG	254 (25.2%)	614 (23.8%)	1.06 (0.89–1.16)	0.50
AGC/AGC	191 (18.9%)	609 (23.6%)	0.77 (0.64–0.92)	0.005
AGC/GGG	462 (45.8%)	1,088 (42.2%)	1.17 (1.00–1.35)	0.04
GGG/GCG	32 (3.2%)	104 (4.0%)	0.77 (0.51–1.16)	0.22
AGC/GCG	47 (4.7%)	103 (4.0%)	1.17 (0.81–1.67)	0.40
AGC/GGC	8 (0.8%)	37 (1.4%)	0.66 (0.28–1.53)	0.33
GGG/GGC	13 (1.3%)	22 (0.9%)	1.58 (0.78–3.19)	0.20
GGC/GGC	0	0		
GGC/GCG	1 (0.1%)	2 (0.1%)		
GCG/GCG	1 (0.1%)	2 (0.1%)		
Haplotypes				
AGC% (95% CI)	44.6 (42.2–46..8)	47.4 (46.0–48.8)		0.03
GGG% (95% CI)	50.3 (48.1–52.5)	47.3 (45.9–48.7)		0.02
GCG% (95% CI)	4.1 (3.2–5.0)	4.1 (3.6–4.6)		0.95
GGC% (95% CI)	1.1 (0.6–1.6)	1.2 (0.9–1.5)		0.81

Data are number of subjects with each composite genotype (percentage of each composite genotype). The odds ratio (OR) (with 95% CI) was calculated as the risk of obesity for the specific composite genotypes relative to all other composite genotypes. *p* values for composite genotype distributions were calculated using regression analysis. Distributions of composite genotype obeyed Hardy–Weinberg equilibrium. Haplotype frequencies are given with 95% CI. *p* values for haplotype frequencies were calculated using Fisher's exact test

indicate a complex involvement of IL-6 in body composition and glucose metabolism. Wallenius et al. [37] reported that *IL6*-deficient mice ($IL6^{-/-}$ mice) develop maturity-onset obesity with an expansion predominantly of subcutaneous fat tissue. Moreover, obesity in these mice is partly reversed by a low dose of IL-6 replacement. Nine-month-old $IL6^{-/-}$ mice have increased fasting plasma glucose levels. It has been proposed that the mechanisms underlying these abnormalities might involve stimulation of energy expenditure and inhibition of appetite at the CNS level [37]. Conversely, another recent study showed that $IL6^{-/-}$ mice did not develop type 2 diabetes or obesity, despite higher serum glucose levels, after a glucose-tolerance test in fat-fed mice [38]. Also intravenous injection of recombinant IL-6 into rats induced an increased level of plasma glucose and plasma glucagon, depletion of hepatic glycogen, and a compensatory increase in the plasma insulin level [39], as well as increased serum triglyceride and NEFA levels [40].

This complexity apparently also applies to the −174 G/C polymorphism of *IL6*. According to some previous investigations, the −174 G-allele is considered the risk allele. In Pima Indians [41] and in Native Americans and Spanish Caucasians [17] the G-allele was reported to be associated with type 2 diabetes. Other studies found that carriers of the −174 G-variant allele had a lower insulin-sensitivity index, as estimated from a frequently sampled IVGTT and minimal model analyses [20], and lipid abnormalities [21]. In contrast, results from a recent study of Finnish subjects showing lower energy expenditure and reduced whole body insulin sensitivity, as estimated by a euglycaemic hyperinsulinaemic clamp among −174 G/C carriers compared with subjects with the G-allele [42], suggest the C-allele is the risk allele.

In the present study we failed to show any impact of the *IL6* −174 G/C variant on type 2 diabetes or quantitative traits related to fasting serum lipids, hypertension and insulin resistance in the general glucose-tolerant population of Inter99. However, analysis of specific components of the WHO-defined metabolic syndrome as dichotomous traits revealed a higher prevalence of insulin resistance and dyslipidaemia among *IL6* −174 G-allele carriers.

The apparent disparity in results relating to the −174 promoter variant between previous studies and the present study might partly be related to population stratification and inter-ethnic variation in the allele frequency. Furthermore, other ethnic specific polymorphisms in the *IL6* promoter which might influence *IL6* transcription by complex interactions determined by various haplotypes might also help explain the discrepancy between studies [27]. Similarly, a gender-specific effect of *IL6* transcription cannot be excluded [15, 16]. Finally, previous studies examined the −174 promoter variant in small study samples, whereas the present study supporting an effect of the −174 G-allele on key features of the metabolic syndrome was undertaken in a relatively large population-based and homogeneous study of middle-aged people.

To the best of our knowledge, studies of the potential relationships between the −572 G/C polymorphism of *IL-6* and features of the metabolic syndrome or related quantitative traits have not previously been reported. In this study we observed higher serum insulin levels during an OGTT and a higher insulinogenic index among −572 C-allele carriers compared to non-C-carriers. The latter finding might indicate an influence of the −572 G/C polymorphism on insulin release after an oral glucose load. It is noticed that increased insulinaemia among C-allele carriers did not translate into alterations in glycaemia.

The results of the composite genotype and haplotype analyses are intriguing and suggest that the rare composite genotype AGC/GCG is associated with type 2 diabetes and the common AGC/GGG composite genotype is associated with the metabolic syndrome. If replicated it would be relevant to measure the circulating levels of interleukin-6 in subjects who are carriers of these at-risk composite genotypes in an attempt to gain further insights into the pathogenic mechanisms involved.

The present study has more limitations. We have not been able to measure the plasma IL-6 concentration of the involved subjects or to genotype other potentially functional variations in the *IL6* promoter locus such as the polymorphic A_nT_n tract [27]. Also, a possible LD of the three SNPs of the *IL6* promoter with other functional coding or non-coding variants in the region can not be excluded. Finally, we have performed multiple comparisons without performing Bonferroni corrections. Obviously, the present findings are explorative in nature and validation studies are needed before any more definite conclusions can be drawn.

In conclusion, single-nucleotide polymorphisms and composite genotypes or haplotypes of the *IL6* promoter may be associated with several features of the metabolic syndrome in Caucasians.

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