

Original investigation

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An interaction between the interleukin-6 -174G>C gene variant and urinary protein excretion influences plasma oxidative stress in subjects with type 2 diabetes

Jeffrey W Stephens*^{1,2}, Steven J Hurel², Jayshree Acharya¹ and Steve E Humphries¹

Address: ¹Centre for Cardiovascular Genetics, British Heart Foundation Laboratories, Royal Free & University College London Medical School, 5 University Street, London WC1E 6JF, United Kingdom and ²Department of Diabetes & Endocrinology, UCL Hospitals, Mortimer Street, London W1T 3AA, United Kingdom

Email: Jeffrey W Stephens* - rmhajst@ucl.ac.uk; Steven J Hurel - s.hurel@ucl.ac.uk; Jayshree Acharya - rmhajay@ucl.ac.uk; Steve E Humphries - rmhaseh@ucl.ac.uk

* Corresponding author

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Abstract

Background: Microalbuminuria and subsequent progression to proteinuria and nephropathy is associated with increased oxidative stress, increased inflammatory cytokines and increased cardiovascular (CVD) risk. The common functional IL-6 -174G>C gene variant is also associated with elevated levels of inflammatory cytokines and CVD risk.

Methods: The aim of this study was to examine the association between the IL-6 -174G>C gene variant with plasma total antioxidant status (TAOS) in 552 subjects with type 2 diabetes in relation to urinary protein excretion.

Results: In subjects free from CVD, there was a significant interaction between urinary protein excretion (normoalbuminuria/ microalbuminuria/proteinuria) and the -174C allele (compared to -174GG) in determining plasma TAOS (p value for interaction = 0.03). In the -174C allele carriers there was a significant association between plasma TAOS and urinary protein excretion: normalalbuminuria v microalbuminuria v proteinuria: $44.30\% \pm 11.32$ vs. $39.74\% \pm 14.83$ vs. $37.93\% \pm 16.42$, ANOVA $p = 0.025$. In those with CVD, no interaction or association was observed with the -174C allele ($p = 0.246$).

Conclusion: The IL-6 -174G>C gene variant is associated with differences in plasma oxidative stress in response to altered protein excretion in subjects with type 2 diabetes.

Background

Interleukin-6 (IL-6) is a multifunctional inflammatory cytokine expressed in many tissues [1,2]. The IL-6 -174G>C functional gene variant has previously been stud-

ied in relation to the risk of coronary heart disease (CHD) [3], juvenile onset chronic arthritis [4] and primary Sjogren's syndrome [5]. The promoter region of this gene contains many functional sites including a multiple

response element (-173 to -145), which is responsive to interleukin-1, tumour necrosis factor- α , nuclear factor- κ B (NF κ -B) and glucocorticoids [6]. In addition there is a variable run of A and T bases (-257 to -276) which may affect promoter strength [4,6]. Increased IL-6 is associated with numerous adverse effects on the cardiovascular system, including increased synthesis of reactive oxygen species (ROS) via NADPH oxidase [7], and hence increased oxidative stress. Previous studies have shown the -174C allele to be associated with prospective CHD risk and hypertension [3], and in an environment of increased stress is associated with elevated plasma IL-6 levels [8,9].

Microalbuminuria is a marker of vascular dysfunction in subjects with type 2 diabetes mellitus (Type 2 DM), however whether it plays a causal role in cardiovascular disease (CVD) remains to be clarified [10-12]. Microalbuminuria and subsequent progression to proteinuria and nephropathy is associated with increased oxidative stress [13], inflammatory cytokines [14] and increased CVD risk [12]. Previous studies have also shown that functional gene variants in the IL-6 gene may influence progression from microalbuminuria to overt proteinuria and subsequent renal failure [15], where CVD is the major cause of mortality [16]. *In vitro* studies have shown that IL-6 stimulates mesangial cell proliferation and matrix production, two of the core features of diabetic glomerulosclerosis [17,18]. Moreover, urinary levels of IL-6 are elevated in patients with diabetic nephropathy [19].

Therefore IL-6, urinary protein excretion and oxidative stress appear to be closely associated in their common outcome of CVD and within this cluster of phenotypes there may be important genotypic-phenotypic (gene-environment) interactions. From the above studies we would hypothesise that the -174C allele would be associated with higher oxidative stress and to modulate urine protein excretion. The aim of this study was to examine the association and potential interaction between the IL-6 -174G>C functional gene variant and urinary protein excretion, with oxidative stress in subjects with Type 2 DM. Since oxidative stress is increased in the presence of atherosclerosis [7], we reasoned that it would also be essential to study the association after grouping the subjects by CVD status.

Methods

Subject population

Patients were recruited from the University College Diabetes and Cardiovascular Study (UDACS). This comprises of 1020 consecutive subjects recruited from the diabetes clinic at University College London Hospitals NHS Trust (UCLH) between the years 2001-2. All patients had diabetes according to WHO criteria [20]. No subjects requiring renal dialysis were recruited. Analyses was confined to

Caucasian subjects with Type 2 DM (605, of whom 569 were successfully genotyped for the IL-6 -174G>C gene variant and 552 had plasma total antioxidant status (TAOS) and urine protein excretion measured). Analysis was therefore confined to 552 subjects. Ethical approval was obtained from UCL/UCLH ethics committee.

Identification and classification of endpoints

Microalbuminuria was defined as an albumin:creatinine ratio (ACR) of greater than 2.5 mg/mmol in men and 3.5 mg/mmol in women. Proteinuria was defined as an ACR > 30 mg/mmol.

Subjects were categorised by the presence/absence of clinically manifest CVD. CVD was recorded if a patient had one or more of CHD, peripheral vascular disease (PVD) or cerebrovascular disease (CbVD). The presence of CHD was recorded if any patient had positive coronary angiography or angioplasty, coronary artery bypass, a positive cardiac thallium scan or exercise tolerance test, documented evidence of myocardial infarction or symptomatic/treated angina. The presence of PVD was recorded in any patient with absent peripheral pulses and abnormal lower limb doppler pressures or an abnormal lower limb angiogram, previous angioplasty or limb by-pass graft. CbVD was recorded if a patient had been investigated for symptoms or signs consistent with a cerebrovascular accident and had a brain CT showing any evidence of infarction (diffuse/localised) or haemorrhage. Those with either CHD or PVD or CbVD were designated as having CVD. Subjects who were asymptomatic for CHD/CbVD/PVD or had negative investigations were categorised as having no CVD.

Measurement of plasma TAOS

Plasma TAOS was measured by a photometric microassay previously described by Sampson *et al* [21]. The TAOS of plasma was determined by its capacity to inhibit the peroxidase-mediated formation of the 2,2-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid (ABTS⁺) radical. In the assay, the relative inhibition of ABTS⁺ formation in the presence of plasma is proportional to the antioxidant capacity of the sample. Therefore, there are two arms to the assay, a control arm and test arm. In the control arm phosphate buffered saline (PBS) is used instead of plasma. The assay is performed in a 96 well ELISA plate using 2.5 μ l of plasma. The difference in absorbance (control absorbance minus test absorbance) divided by the control absorbance (expressed as a percentage) was used to represent the percentage inhibition of the reaction. Plasma TAOS is therefore inversely related to oxidative stress (the higher the oxidative stress, the lower the TAOS). The inter assay coefficient of variation (CV) was 14.1%, and the intra assay CV was 4.3%.

IL-6 -174G>C genotyping

Genomic leukocyte DNA was extracted by salting out from 5 ml EDTA blood samples. Genotypes were determined using polymerase chain reaction amplification (PCR) with published primers and conditions [3,22]. Digest products were resolved by micro-titre array diagonal gel electrophoresis (MADGE) [23] and confirmed by two independent technicians blind to subject outcome, with discrepancies resolved by repeat genotyping.

Statistical analysis

Analysis was performed using SPSS (version 10.1, SPSS Inc., Chicago). Data are reported for those individuals amongst whom high-throughput genotyping, plasma TAOS and urine protein excretion measurement was successful. Results are presented as mean \pm standard deviation or median (interquartile range). Deviations from Hardy-Weinberg equilibrium were considered using chi-squared tests. Deviations from Hardy-Weinberg equilibrium were considered using chi-squared tests. Hardy-Weinberg equilibrium gives the expected genotype distribution based on the observed frequency of the rare allele (q) and common allele (p) as $p^2+2pq+q^2$, where p^2 is the predicted frequency for homozygosity of the common allele, q^2 is the predicted frequency for homozygosity of the rare allele and $2pq$, the heterozygotes. These frequencies are expected provided the sample is drawn from a population with random mating and no strong selection. Deviations from the expected frequencies may suggest selection bias.

Allele frequencies are shown with the 95% confidence interval. To assess the differences between CVD and no CVD patients, two-sided T-tests were performed on normally distributed data or after appropriate transformation (log or square root). Analysis of variance (ANOVA) was used to assess the association between genotypes /urinary protein excretion and plasma TAOS, followed by a comparison made after combining the GC and CC groups as the C allele has previously been shown to have a dominant effect on CVD risk [3,9,24] and plasma IL-6 [8]. This provided an ANOVA p-value between groups and a p-value for the linear relationship between groups. The relationships between baseline parameters and plasma TAOS were tested by Spearman rank correlation co-efficient. An ANOVA was also performed to test the association between genotype and TAOS after adjustment for the potential confounders using multiple regression analysis to obtain a residual. For the analysis of the combined effect of genotype and urinary protein excretion on plasma TAOS, subjects were grouped by genotype (GG and GC/CC). ANOVA was then performed separately within each genotype group with urinary protein excretion (normoalbuminuria/ microalbuminuria/proteinuria) as the independent and plasma TAOS as the

dependent variable. The interaction between genotype and urinary protein excretion in determining plasma TAOS was performed using linear regression where the combined effects were compared to the individual effects of these variables. In all cases a P value of less than 0.05 was considered statistically significant.

Results

The baseline characteristics of the subjects grouped by CVD status are summarized in table 1. Of the 552 subjects, 188 had CVD (141 CHD, 45 PVD and 37 CbVD) and 364 no CVD. The genotype distribution was in Hardy-Weinberg equilibrium ($\chi^2 = 0.019$, $p = 0.998$) with a G allele frequency of 0.652 [0.62–0.68] and C allele frequency of 0.348 [0.32–0.38]. In the whole group, no significant association was seen between genotype and plasma TAOS (GG: 42.26% [13.20] v GC: 41.91% [13.15] v CC: 43.39% [13.70], ANOVA $p = 0.720$), and urinary protein excretion (normoalbuminuria/microalbuminuria/proteinuria $p = 0.558$) and CVD status, $p = 0.985$.

Plasma TAOS was independent of pharmacotherapy, but correlated positively with plasma HDL-cholesterol, and negatively with triglyceride, glucose and HbA_{1c} (correlation co-efficient $r = 0.12, -0.15, -0.11$ and -0.10 respectively; all $P < 0.05$). After adjustment for these variables, the above results remained unchanged (for genotype and plasma TAOS, $p = 0.704$). No association was observed between genotype and baseline lipid measures, blood pressure, C-reactive protein and modes of therapy (anti-hypertensive, oral hypoglycaemic, statin and aspirin).

Subjects without CVD

In subjects without CVD, no association was seen between genotype and plasma TAOS (GG: 43.23% [12.40] v GC 41.79% [13.30] v CC 42.59% [14.14], ANOVA $p = 0.614$, after adjustment $p = 0.645$) or between urinary protein excretion and TAOS (normoalbuminuria 43.55% [11.92] v microalbuminuria 41.54% [14.03] v proteinuria 40.00% [14.82], ANOVA $p = 0.194$, after adjustment $p = 0.284$). However in those with the -174C allele, as shown in figure 1, there was a significant association between higher urinary protein excretion and lower plasma TAOS (ANOVA $p = 0.025$, linearity between groups, $p = 0.008$). This remained significant after adjustment for age, triglyceride, HDL-C, glucose and HbA_{1c} (ANOVA $p = 0.031$). No such differences were observed in those subjects homozygous for the -174G allele. The interaction between urinary protein excretion and genotype on plasma TAOS was statistically significant, $p = 0.032$ (after adjustment $p = 0.029$).

When subjects with normoalbuminuria were compared to those with microalbuminuria and/or proteinuria, the -174C allele was still associated with variation in plasma

Table 1: Baseline differences in subjects by CVD status

Trait	No CVD (n = 364)	CVD (n = 188)	P Value
Age (years)	65.5 (11.3)	69.6 (9.6)	<0.001
Duration (years)+	8 (4–15)	10 (5.5–17)	0.008
Systolic blood pressure (mmHg)*	141 (128–153)	139 (129–154)	0.340
Diastolic blood pressure (mmHg)*	81 (74–87)	76 (71–83)	0.004
Body mass index (kg/m ²)*	29.17 (26.23–32.58)	29.08 (26.13–31.97)	0.262
HbA _{1c} (%)*	7.6 (6.6–8.9)	7.4 (6.5–8.7)	0.336
Creatinine (mmol/l)*	88 (76–103)	99 (84–122)	<0.001
CRP (mg/l)*;	1.73 (1.06–3.18)	1.80 (0.99–3.34)	0.578
TAOS	42.50 (13.01)	41.83 (13.61)	0.578
Sex (F/M)	165/199 (45.3/54.7%)	57/131 (30.5/69.5%)	0.001
Normo/micro/proteinuria	201/128/35 (55.2/35.3/9.5%)	83/75/30 (44.1/39.9/16.0%)	0.019
Genotype distribution	155/165/44 (42.6/45.3/12.1%)	80/85/23 (42.6/45.2/12.2%)	0.999
ACEI (No/Yes)	197/167 (54.0/46.0%)	79/109 (42/58%)	0.008
Aspirin (No/Yes)	215/149 (59.2/40.8%)	50/138 (26.7/73.3%)	<0.001
Insulin (No/Yes)	280/84 (76.8/23.2%)	130/58 (69.1/30.9%)	0.052
Statin (No/Yes)	289/75 (79.3/20.7%)	85/103 (45.2/54.8%)	<0.001

Mean (SD) or Median (Interquartile range) shown, *Log transformed, +Square root transformed. Analysis performed by two-sided T-tests after appropriate transformation. χ^2 test were used to compare groups.

Table 2: Genotype distribution by CVD status and urinary protein excretion

-174G>C genotype	No CVD			CVD		
	Normoalbuminuria	Microalbuminuria	Proteinuria	Normoalbuminuria	Microalbuminuria	Proteinuria
GG	91 (25.0%)	50 (13.7%)	14 (3.8%)	35 (18.6%)	31 (16.5%)	14 (7.4%)
GC	89 (24.5%)	58 (15.9%)	18 (4.9%)	41 (21.8%)	33 (17.6%)	11 (5.9%)
CC	21 (5.8%)	20 (5.5%)	3 (0.8%)	7 (3.7%)	11 (5.9%)	5 (2.7%)
C allele frequency	0.326 (0.28–0.37)	0.383 (0.32–0.44)	0.343 (0.23–0.45)	0.331 (0.26–0.40)	0.367 (0.29–0.44)	0.350 (0.23–0.47)

The percentage in brackets is the percentage of the subject in each cell with respect to CVD/No CVD. The -174C allele frequency is shown with the 95% confidence interval in brackets.

TAOS (p interaction = 0.02, after adjustment p = 0.016). When subjects with frank proteinuria were excluded from the analysis, those subjects with the -174C allele and microalbuminuria still had the lowest plasma TAOS (p value for interaction = 0.01, after adjustment p = 0.023).

Subjects with CVD

In subjects with CVD, no association was seen between genotype and plasma TAOS (GG: 40.39% [14.50] v GC 42.37% [12.84] v CC 44.91% [12.98], ANOVA p = 0.332, after adjustment p = 0.319) or between urinary protein excretion and TAOS (normoalbuminuria 42.73% [12.84] v microalbuminuria 41.84% [13.53] v proteinuria 39.36% [15.87], ANOVA p = 0.510, after adjustment p = 0.615). In contrast to those without CVD, no interaction was seen between genotype and urinary protein excretion or plasma TAOS by genotype (p = 0.246, after adjustment p = 0.303).

Conclusion

Although there was no overall significant influence of the IL-6 -174G>C variant on plasma TAOS or on urinary protein excretion, this study provides evidence of an impact of this common variant in the IL-6 gene, on the relationships between an intermediate phenotype (urinary protein excretion) and oxidative stress. Carriage of the -174C allele increased the likelihood of a nephropathy-associated increase in oxidative stress in subjects with Type 2 DM. This effect was confined to subjects free from clinically manifest CVD and no such interaction was observed in those subjects with CVD. This may be explained by the fact that in the presence of established CVD, ROS formation is increased as a result of the atherosclerotic process [7] and this overwhelms the modest effect of genotype. Plasma TAOS was not however significantly different between subjects with and without CVD in our study, possibly due to the fact that those with CVD are being treated

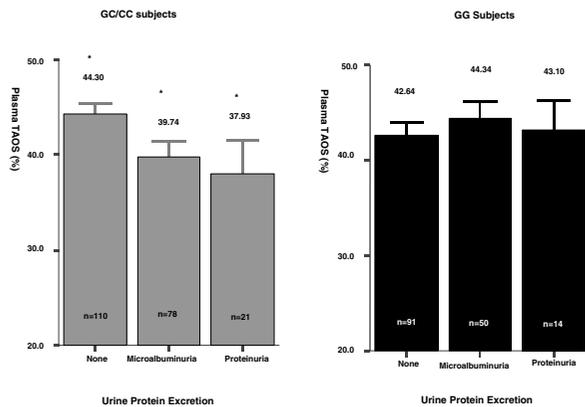


Figure 1

Plasma TAOS by genotype and urinary protein excretion in subjects free from CVD. Footnote: Mean (\pm standard error) shown. Numbers of subjects are shown at the base of each column. In those without CVD, the interaction between genotype (GG v GC/CC) and urinary protein excretion in determining plasma TAOS was significant ($p = 0.032$). *In the GC/CC subjects, there was a significant difference in plasma TAOS by urinary protein excretion (normoalbuminuria/microalbuminuria/proteinuria), ANOVA between groups $p = 0.025$ (as expected the linear association in TAOS between these groups was significant $p = 0.008$ with normoalbuminuric subjects having the highest TAOS and those with proteinuria the lowest). In GG Subjects no such difference was observed, (ANOVA between groups, $p = 0.399$)

with agents such as statins and aspirin, which may be overwhelming the genotype effect.

The -174C allele has been associated with increased plasma IL-6 under conditions of stress and with CHD risk [3,8,9]. High levels of plasma IL-6 have also been shown to be an independent predictor of mortality in patients with renal impairment, prior to starting dialysis treatment [25]. We did not observe a decrease in plasma TAOS associated with the -174C allele, but rather an interaction with urinary protein excretion.

Glomerular basement thickening, increased accumulation of extracellular matrix and expanded mesangium are common features of diabetic nephropathy [17,18]. Previous *in vitro* studies have shown that IL-6 may stimulate mesangial cell proliferation. High levels of urinary IL-6 have also been noted in patients with diabetic nephropathy [19]. As described, increased oxidative stress and CVD risk is associated with nephropathy, renal failure [12] and elevated plasma IL-6. Therefore in a patient with the -174C allele and early/established nephropathy, the addi-

tive effects may be to increase plasma IL-6 and subsequent ROS generation. Interestingly, ROS may also promote IL-6 gene expression by activating the NF κ -B binding site, 100 base pairs downstream from the -174G>C variant [26].

This study provides an example of a genotypic-phenotypic (gene-environment) interaction. 'Genotype-phenotype interaction' implies that in combination, the risk associated with a particular gene variant in a particular environment is more than the effects of each independently. From a mechanistic viewpoint, interaction suggests that at the molecular level, the effect or by product of the phenotypic insult modifies the molecular function of the gene product under observation or *vice-versa*. In this study we provide evidence that a common gene variant in the IL-6 -174 gene increases the likelihood of a nephropathy-associated increase in oxidative stress in subjects with Type 2 DM. The study of common 'genotype-phenotype' or 'gene-environment interactions' not only provides a useful means to improve our understanding of disease at the molecular level but also allows specific targeting of advice and therapies to high risk individuals (those with high-risk genotype in a high-risk environment). From the results described, this might involve targeting microalbuminuria and proteinuria more aggressively in those patients who carry the -174C allele, for example with anti-inflammatory agents.

Authors' contributions

JWS, SJH and SEH are the lead investigators of the UDAC study. Subject recruitment was performed by JWS and SJH. JA is the curator of the study DNA. DNA extraction and preparation was performed by JWS and JA. Genotyping and plasma TAOS measurements were performed by JWS. Data analysis was performed by JWS, SJH and SEH.

All authors have read and approved the manuscript for publication

Competing interests

None declared.

Abbreviations

CVD = Cardiovascular disease

CHD = Coronary heart disease

PVD = Peripheral vascular disease

CbVD = Cerebrovascular disease

TAOS = Total antioxidant status

IL-6 = Interleukin-6

Type 2 DM = Type 2 diabetes

ANOVA = Analysis of variance

UDACS = University College London diabetes and cardiovascular disease study

UCL = University College London

WHO = World Health Organisation

CV = Co-efficient of variation

MADGE = Microtitre array diagonal gel electrophoresis

ROS = Reactive oxygen species

CRP = C-reactive protein

ACEI = Angiotensin converting-1 enzyme inhibitor

HbA_{1c} = Glycated haemoglobin

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