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Association between the -174 *G/C* promoter polymorphism of the interleukin-6 gene and cardiovascular disease risk factors in Brazilian older women

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In worldwide studies, interleukin-6 (IL-6) is implicated in age-related disturbances. The aim of the present report was to determine the possible association of IL-6 -174 *C/G* promoter polymorphism with the cytokine profile as well as with the presence of selected cardiovascular risk features. This was a cross-sectional study on Brazilian women aged 60 years or older. A sample of 193 subjects was investigated for impaired glucose regulation, diabetes, hypertension, and dyslipidemia. Genotyping was done by direct sequencing of PCR products. IL-6 and C-reactive protein were quantified by high-sensitivity assays. General linear regression models or the Student *t*-test were used to compare continuous variables among genotypes, followed by adjustments for confounding variables. The chi-square test was used to compare categorical variables. The genotypes were consistent with Hardy-Weinberg equilibrium proportions. In a recessive model, mean waist-to-hip ratio, serum glycated hemoglobin and serum glucose were markedly lower in *C* homozygotes (P = 0.001, 0.028, and 0.047, respectively). In a dominant hypothesis, *G* homozygotes displayed a trend towards higher levels of circulating IL-6 (P = 0.092). Non-parametric analysis revealed that impaired fasting glucose and hypertension were findings approximately 2-fold more frequent among *G* homozygous subjects (P = 0.042 and 0.043, respectively). Taken together, our results show that the IL-6 -174 *G*-allele is implicated in a greater cardiovascular risk. To our knowledge, this is the first investigation of IL-6 promoter variants and age-related disturbances in the Brazilian elderly population.

Key words: Cytokine; Interleukin-6; Genetics; Elderly, Cardiovascular disease; Brazil

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INTRODUCTION

Inflammation is recognized as a central component of cardiovascular disease (CVD), even though the underlying regulation and molecular mechanisms remain unclear (1). Interleukin-6 (IL-6) is a pleiotropic cytokine involved in a range of immunological activities, especially the synthesis of acute-phase substances by the liver. As its receptor subunit (gp130) is widely expressed, deregulated high-level production of the cytokine combined with its agonistic soluble receptor (sIL-6R) may induce an undesired basal inflammatory state in many organs and thus cause non-communicable diseases (2). It has been reported that the common -174 *C/G* polymorphism in the promoter region of human IL-6 regulates its transcription *in vitro*, with the *G*

allele showing increased transcriptional activity both under basal conditions and in response to inflammatory stimuli such as lipopolysaccharides or IL-1 (3-5). However, data on the effects of this polymorphism on IL-6 levels *in vivo* have led to conflicting results. Most studies have shown that *G/G* individuals have an increased IL-6 mRNA expression (6) and higher circulating IL-6 levels (3,7), as would be expected from *in vitro* data. Conversely, other studies have reported no differences among genotypes or increased levels of the cytokine among *C/C*-carrying subjects (8,9). In addition to the issue of genotype-related expression levels, it is also recognized that an age-related increase in serum IL-6 levels takes place among men and women (10), beginning as early as at 30-40 years of age (11), being prevalent even in people devoid of any overt acute or chronic disease (12), becoming prominent in later life (13,14), and acting as a predictor of mortality independent of pre-existing morbidity (15).

By and large, elevated levels of IL-6 are associated with an increased risk of death from cardiovascular causes in the elderly. Elevated levels of C-reactive protein (CRP), an acute-phase reactant largely regulated by IL-6, are also independently associated with a long-term risk for CVD (16). Our underlying hypothesis is that IL-6 genotypes may predispose to risk factors for CVD (hypercholesterolemia, hypertriglyceridemia, hypertension, diabetes, and impaired glycemic homeostasis) which would then be accompanied by a chronic inflammatory disorder. The existence of linkage disequilibrium between -174 *C/G* polymorphism and other genotypes, revealed by Western-based population haplotyping studies (5,17), demonstrates that investigating solely the -174 *C/G* polymorphism is as informative as investigating the entire promoter haplotype. Because a large fraction of cardiovascular events occurs in people aged 60 years or older and the IL-6 promoter polymorphism has not been studied extensively among aged women, we examined the effects of the -174 *C/G* genotypes on CVD risk factors in a subset of subjects from the Elderly Health Promotion Project held in Brasília, Brazil.

MATERIAL AND METHODS

Subjects

Brasília is the administrative capital of the Brazilian federation, located in Central West Brazil. Sampling for the present study was based on preliminary genotyping of 30 subjects. A minimum series of 175 subjects was determined for a frequency of risk allele of 0.80, with a confidence level of 0.95 and an estimation error of 0.05 for a finite population. Therefore, cross-sectional analyses were performed using data obtained from 224 apparently healthy, female outpatients from the urban outskirts of the Brazilian Federal District, aged 60 years or older and recruited to undergo health screenings and intervention (medical, nutritional and/or pharmacological) for the prevention of CVD at the Hospital of Universidade Católica de Brasília. For recruitment purposes, public invitations through mass communication networks were broadcast early in 2005. Invitations were directed at aged women from the outskirts of Brasília invited to join a university research project involving health promotion actions and ambulatory follow-up. The project became known as the Elderly Health Promotion Project, and this is the first report of our activities in the project. The assessment on cardiovascular risk reported in this study was performed from April to October 2005. The study was approved by the institutional Research Ethics Committee (CEP/UCB). Participation was voluntary, and informed written consent was obtained from each participant in accordance with the principles of the Helsinki Declaration.

Clinical procedures

Laboratory tests included serum liver tests (aspartate aminotransferase, alanine aminotransaminase, and alkaline phosphatase), cytomegalovirus and Epstein-Barr IgM serology, and total blood count. Fasting serum determinations of glucose, glycated hemoglobin (HbA1c), triglycerides, total cholesterol, and fractions (low- and high-density lipoprotein) were also obtained. All tests were performed following routine clinical analysis. CRP was measured using a high-sensitivity turbidimetric assay (BioTécnica®, Varginha, MG, Brazil) with a sensitivity of 0.05 mg/L.

For IL-6 measurement, whole blood was collected into endotoxin-free tubes. Serum was separated from whole blood within 1 h of collection, stored in aliquots at -80°C and analyzed in batches using a specific enzyme-linked immunosorbent assay kit (GE Healthcare, Uppsala, Sweden). Samples for IL-6 quantification were analyzed in duplicate and their coefficient of variation (CV) were determined ($CV_{C/C}$ 11.7 *vs* 15.6; $CV_{G/C}$ 29.6 *vs* 26.3; $CV_{G/G}$ 66.4 *vs* 84.7). The minimum detectable dose was 0.10 pg/mL.

On the occasion of the clinical visit, blood pressure was measured after at least 10 min of rest in the sitting position. The blood pressure value of each subject was the mean of three physician-obtained measurements, recorded >3 min apart. Body mass index (BMI; weight (kg)/height (m^2)) and waist-to-hip ratio (WHR; waist circumference/hip circumference) were obtained for each patient. Body weight was measured to the nearest 0.1 kg with the subject using light clothing and without shoes and height was measured to the nearest 0.1 cm. Waist circumference was measured midway between the iliac crest and the lower costal margin, whereas hip circumference was measured at its maxi-

mum. Cases of active infection, inflammation or malignancies were assessed by semiological criteria based on medical examination assisted by laboratory data.

Hypertension was defined as systolic blood pressure ≥135 or diastolic blood pressure ≥85 mmHg, or current use of antihypertensive medication, whereas diabetes mellitus was characterized by fasting blood glucose ≥126 mg/dL, or current use of insulin or oral anti-diabetic drugs. Impaired glucose regulation encompassed cases of diabetes mellitus and any cases of fasting blood glucose ≥110 mg/dL. Hypertriglyceridemia was defined as triglycerides ≥150 mg/dL and hypercholesterolemia was defined as total cholesterol ≥200 mg/dL or low-density lipoprotein ≥130 mg/dL. Current use of anti-lipemic medication was considered to represent either entity. Dyslipidemia was defined as isolated or combined hypertriglyceridemia, hypercholesterolemia and high-density lipoprotein <50 mg/dL.

DNA analysis

Total DNA was isolated from peripheral blood according to standard procedures. The -174 *G/C* polymorphism in the promoter of the human IL-6 gene (rs1800795) was determined by direct sequencing of a polymerase chain reaction product. A 628-bp region was amplified using a pair of specific primers: 5'-GAACACAGAAGAACTCAGATG ACTGG-3' (sense) and 5'-AGGAGTTCATAGCTGGGCTC CTGGAG-3' (antisense), which flank the polymorphism. The reaction tubes contained 100 ng DNA, 10 mM Tris-HCl, pH 9.2, 25 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 20 pmol of each primer, 0.5 µg purified chicken albumin, and 1 unit *Taq* DNA polymerase (Phoneutria®, Belo Horizonte, MG, Brazil) in a final volume of 50 µL. After 1 min of hot start at 80°C and an initial denaturation for 2 min at 94°C, the amplifications were carried out for 36 cycles of 40 s at 94°C, 45 s at 64°C, and 50 s at 72°C followed by a final 5-min extension at 72°C. Each polymerase chain reaction product was directly sequenced on an ABI PRISM 3700 DNA analyzer (Applied Biosystems, Foster City, CA, USA), using the 5'-GCCTCAGAGACATCTCCAGTCC-3' primer. Each sequence obtained was examined using the Staden software package (MRC, Cambridge, UK), and confirmed by visual inspection.

Statistical analysis

Violation of Hardy-Weinberg equilibrium was tested using the Fisher exact test. The Kolmogorov-Smirnov test was used to determine the distribution of data from continuous variables. Whenever appropriate, data were reported as means ± SD. General linear regression models or the Student-*t* test were used to compare means of continuous variables (levels of fasting glycemic markers, blood pressure, white blood cell count, lipids, liver markers, CRP, and IL-6) across genotypes using dominant, co-dominant and recessive comparison models. *Post hoc* comparisons were made using the Tukey test for pairwise comparisons. Phenotypic differences between genotypes were also tested for influence of co-variables by adjustment for confounding factors (age and BMI) by ANOVA.

Association of IL-6 genotypes with categorical variables representing CVD risk factor (states of impaired glucose regulation, diabetes, hypertension, and dyslipidemia) was investigated by the χ^2 test. All analyses were performed employing the Statistical Package for Social Sciences (SPSS) for Windows (version 10.0). A P value < 0.05 was considered to be significant.

RESULTS

Data from 31 individuals enrolled in the clinical screening were eliminated from the databank due to exclusion reasons described as follows: 17 for unavailability for laboratory testing, 12 for current use of anti-inflammatory drugs, and 2 for cytomegalovirus or Epstein-Barr reactivity. No subjects were excluded based on the criteria of active infection, inflammation or malignancy. Therefore, 193 subjects fulfilling the inclusion criteria were included.

The prevalence of the IL-6 -174 *G* and *C* alleles was 78.9 and 21.1%, respectively. The genotypes were consistent with Hardy-Weinberg equilibrium proportions (61.9% *G/G*, 34.0% *C/G*, 4.1% *C/C*; P > 0.05). The clinical and laboratory characteristics of the subjects analyzed are summarized in Table 1. In a co-dominant model, no significant differences in age, BMI, fasting glucose levels, HbA1c levels, serum lipids, liver marker concentration, leukocyte count, or CRP were observed between subjects carrying the three genotypes. Nonetheless, the mean WHR was significantly reduced in subjects carrying the -174 *C/C* genotype compared to carriers of the *G/G* (P = 0.004) and *C/G* (P = 0.001) genotypes, concurring with a recessive model of genotype effect. In agreement, homozygous carriers of the *C* allele also had significantly lower levels of fasting serum glycated hemoglobin and glucose compared to carriers of the *G* allele (Table 1). To explore a dominant hypothesis, analysis combining the *C/G* and *C/C* genotypes in one group was also performed. In this scenario, the mean WHR and average glycemic markers did not differ significantly between the *G* homozygotes and the *C* carriers. By contrast, a tendency towards higher serum IL-6 levels was observed among *G* homozygotes compared to carriers of the *C* allele (P = 0.092).

Because of the possible association of IL-6 expression, glycemic levels and WHR with variables such as age and adiposity, differences between genotypes were inves-

tigated by adjusting to these confounding factors. In brief, all significant differences described so far resisted adjustment for age and BMI, while no other difference in biochemical or clinical quantitative traits became significant after correction (data not shown). CRP levels showed nonsignificant variance in either model, adjusted or not to age and adiposity. This homogeneity persisted after additional adjustment to markers of liver status (aspartate aminotransferase, alanine aminotransaminase and alkaline phosphatase).

In order to study the association of -174 *G/C* polymorphism with clinically established risk parameters for CVD, we performed non-parametric analysis to evaluate the difference in distribution of hypercholesterolemia, hypertriglyceridemia, hypertension, diabetes, and impaired glucose regulation among genotypes. Due to the low number of homozygous individuals for the *C* allele, the genotype was merged with the heterozygous subjects for the categorical analysis. According to this dominant model (*GG vs GC + CC*), *G* homozygous subjects were roughly twice as much affected by impaired fasting glucose metabolism and hypertension compared to the *C*-carrier counterparts (Table 2). In the present study, we did not observe a trend towards an association between genotypes and serum lipid disturbances.

DISCUSSION

Increasing evidence suggests that low-grade inflammation could be one of the determinants of an unbalanced glucose handling capacity and blood pressure disorders (18,19). The implication of IL-6 promoter polymorphisms in prominent transcriptional effects on the gene (3) makes these variations credible candidates for association with chronic disorders.

The aim of the present study was to determine a possible association of the IL-6 -174 *C/G* promoter polymorphism with the amount of cytokine released in the serum of older female adults from the Brazilian population, as well as with the presence of selected biochemical or clinical features representative of cardiovascular risk in this age stratum. Regardless of any age-related up-regulation of plasma IL-6 levels, the data presented here suggest a tendency to greater *in vivo* release of the cytokine among aged individuals carrying the IL-6 -174 *G/G* genotype. Thus, an underlying lesson is that it may be important to take into account IL-6 genotypes whenever comparisons of cytokine production are performed between different age strata.

We suggest that the quantitative findings presented so far may be of physiological relevance. The association between *G* homozygosis and CVD-prone conditions (im-

Table 1. Clinical and biochemical data as a function of the -174 *G/C* genotypes of the IL-6 gene in 193 older Brazilian women.

	G/G (N = 119)	*G/C* (N = 66)	*C/C* (N = 8)	P (*G/G vs C/G vs C/C*)*	P (*G/G vs C/G + C/C*)**	P (*C/C vs C/G + G/G*)***
Age (years)	68.8 ± 6.2	67.4 ± 5.3	66.1 ± 3.4	0.185	0.067	0.313
Body mass index (kg/m^2)	26.9 ± 4.0	27.0 ± 4.2	28.1 ± 3.1	0.753	0.781	0.453
Waist-to-hip ratio	0.88 ± 0.08	0.90 ± 0.08	0.79 ± 0.28	0.001	0.721	0.001
Fasting glucose (mg/dL)	105.2 ± 32.2	106.7 ± 31.7	91.4 ± 8.2	0.121[a]	0.989[a]	0.047[b]
Fasting glycated hemoglobin (%)	5.6 ± 0.6	5.7 ± 0.6	5.2 ± 0.5	0.083	0.854	0.028
Total cholesterol (mg/dL)	230.0 ± 45.2	230.0 ± 45.2	216.6 ± 37.9	0.544	0.736	0.355
HDL cholesterol (mg/dL)	61.0 ± 9.9	61.9 ± 10.6	63.1 ± 9.8	0.279	0.506	0.623
LDL cholesterol (mg/dL)	138.8 ± 39.9	138.5 ± 40.3	127.0 ± 32.8	0.332	0.797	0.416
Triglycerides (mg/dL)	149.2 ± 72.0	152.9 ± 64.6	131.2 ± 72.9	0.703	0.900	0.442
Systolic blood pressure (mmHg)	137.5 ± 26.0	140.7 ± 28.7	126.2 ± 29.2	0.336	0.688	0.206
Diastolic blood pressure (mmHg)	82.1 ± 16.6	81.4 ± 13.9	76.2 ± 16.8	0.594	0.589	0.327
Aspartate aminotransferase (U/L)	25.4 ± 7.4	25.2 ± 7.4	25.5 ± 7.2	0.980	0.874	0.940
Alanine aminotransferase (U/L)	20.3 ± 12.5	19.8 ± 9.6	19.8 ± 7.4	0.947	0.742	0.924
Alkaline phosphatase (U/L)	194.6 ± 74.7	189.2 ± 74.7	171.1 ± 69.7	0.648	0.502	0.423
White blood cell count (x 10^9/mL)	4.5 ± 1.7	4.5 ± 1.7	4.3 ± 1.0	0.933	0.995	0.721
C-reactive protein (mg/L)	2.2 ± 2.8	2.4 ± 3.2	2.2 ± 2.0	0.926[a]	0.870[b]	0.699[b]
Fasting interleukin-6 (pg/mL)	1.16 ± 3.84	0.39 ± 1.36	0.00 ± 0.00	–	0.092[b]	–

Data are reported as means ± SD.
*P values for comparison of differences between genotypes in a co-dominant model (*GG vs GC vs CC*) using ANOVA or the [a]Kruskal-Wallis test. **P values for comparison of differences between genotypes in a dominant (*GG vs CG + CC*) model using the unpaired Student *t*-test or [b]Mann-Whitney test. ***P values for comparison of differences between genotypes in a recessive (*GG + CG vs CC*) model using the unpaired Student *t*-test or [b]Mann-Whitney test.

paired fasting glucose and hypertension) is consistent with a dominant model of genotype effect. On the other hand, the relationship of *C* homozygosis with lower WHR, HbA1c and serum glucose suggests that a recessive model of genotype effect is advantageous at clinical level. Regardless of the exact model, our results, taken together, assign a determining effect to the *G* allele. Previous investigations with Western populations also consider the -174 *G* allele as the risk allele. The largest meta-analysis on genetics of type 2 diabetes among Caucasians published to date provides evidence for an association of the disorder with the *G/G* genotype (20). In Pima Indians (21) and in Native American and Spanish (22) admixed populations, the *G* allele was also associated with type 2 diabetes. Fernandez-Real and colleagues (23) found higher blood HbA1c in carriers of the *G* allele, while other studies found that carriers of the *G*-variant allele had lower glucose handling capacity in insulin sensitivity assays (6,24). The present study also reveals a tendency to an increased frequency of the hypertensive phenotype among *G/G* homozygotes. To the authors' knowledge, blood pressure might be influenced by IL-6 expression but a smaller body of evidence is supportive for an association between hypertension and low-grade inflammation in the female gender (25). Thus, the observed association may represent an accentuation of the phenotype due to the age stratum of the sample.

The lack of association between genotypes and other markers of the acute phase response (namely CRP and peripheral leukocyte count) suggests that clinical outcomes of IL-6 levels may derive from endocrine rather than immunological effects of this pleiotropic cytokine. The mechanisms by which the *G* allele might cause an increased WHR are unknown, but this allele might act as a triggering agent of the dose-dependent lipolytic effect of IL-6 on peripheral storages (26) driving to fat mobilization toward the abdominal compartment. According to a vicious cycle hypothesis, IL-6 secreted from the abdominal adipose tissue in a non-inflammatory condition (27) would flow by venous drainage directly into the liver and drive a persistent increase in hepatic triglyceride secretion (28,29), intensifying the phenotype.

Regarding the association between *G* homozygotes and aspects of an unbalanced glucose regulation, it is known that insulin attenuates several stimulatory effects of IL-6 (30,31). Thus, the lack of a significant insulin action, as found in type 2 diabetes, would lead to prolonged detrimental effects of the cytokine (32). In addition, it has been demonstrated that increased levels of IL-6 impair insulin signaling in models using human cells, such as adipocytes and hepatocytes, by a mechanism that involves at least in part an up-regulation of the inhibitory suppressor of cytokine signaling-3 (33,34). Again, according to a vicious cycle hypothesis, genetically prone producers of IL-6 such as *G/G* individuals may be predisposed to intensification of an insulin-resistant state initiated by inappropriate food intake and life style.

The present results are in contrast to reports showing a significant relationship between the *G/G* genotype and different lipidemia-related (serum lipids and BMI) phenotypes (23,35). We have no simple explanation for this discrepancy. Obviously, any apparent disparity in results might be attributable in part to interethnic variation owing to the remarkable Brazilian genetic admixture (36), as well as to food intake habits not investigated here. As stated before, no other inflammatory markers differed among genotypes, in contrast to reports based on western elderly populations. Regarding total (Table 1) and differential (not shown) white blood cell counts, described elsewhere as genotype-influenced factors (23), no higher or lower counts

Table 2. Frequency distribution of the IL-6 -174 *G/C* genotypes in different conditions that represent cardiovascular risk factors, according to a dominant model.

Condition	Frequencies*		χ^2	P
	G/G (N = 119)	*G/C* + *C/C* (N = 74)		
Blood pressure				
Normotensive	13.4	24.3	3.72	0.043
Hypertensive	86.6	75.7		
Ratio	6.5	3.1		
Glucose regulation				
Regular	67.2	79.7	3.54	0.042
Impaired	32.8	20.3		
Ratio	0.5	0.2		
Non-diabetics	84.7	89.3	0.83	0.363
Diabetics	15.3	10.7		
Ratio	0.2	0.1		
Cholesterolemia				
Normocholesterolemic	24.4	20.3	0.44	0.509
Hypercholesterolemic	75.6	79.7		
Ratio	3.0	3.9		
Triglyceridemia				
Normotriglyceridemic	49.6	56.8	0.94	0.332
Hypertriglyceridemic	50.4	43.2		
Ratio	1.0	0.8		
HDL cholesterol				
≥50 mg/dL	77.3	77.0	0.00	0.964
<50 mg/dL	22.7	23.0		
Ratio	0.3	0.3		
Lipidemia				
Normolipemic	14.3	12.2	0.18	0.674
Dyslipemic	85.7	87.8		
Ratio	6.0	7.2		

*Frequencies are expressed as percentage within the genotype.

could be associated with either hereditary model under our conditions. Also in disagreement with the literature (37), no variance in CRP levels was observed among genotypes. Although increased IL-6 levels were not paralleled by CRP levels in the various genotypes, the present study yields the assumption that baseline levels of the cytokine are more predictive of cardiovascular events than levels of the acute-phase reactant.

The present study has limitations. We have not been able to genotype other potentially functional (coding and non-coding) variations in the IL-6 locus so that such interference could be ruled out. Since the study was carried out on a female series, the authors are reticent in extrapolating conclusions to male counterparts, since the literature demonstrates significant gender-dependent predisposition to increased IL-6 serum levels in aging women, but not in men (38,39).

To our knowledge, this is the first investigation of whether the IL-6 promoter variants -174 *G/C* are linked to age-related metabolic disturbances in the Brazilian elderly population. Our results suggest that this functional polymorphism influences circulating IL-6 levels, and indicate a contribution of the *G* allele to a glucose-intolerant and a hypertensive state as well to an increased WHR in postmenopausal women. The present study does not support associations with further age-related disorders. Whether this polymorphism is a risk factor for obesity or type 2 diabetes could be estimated only in prospective population-based studies. In conclusion, we demonstrated that the -174 *C/G* promoter polymorphism of the IL-6 gene is unequivocally associated with several features consistent with cardiovascular risk in apparently healthy elderly Brazilian females. Immunogerontological avenues of investigation on the effect of cytokine genes and levels in diseases of aging are appropriate for this population.

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