Original Article

Effects of the Interaction between Interleukin-6 −634C/G Polymorphism and Smoking on Serum C-Reactive Protein Concentrations

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Smoking and interleukin-6 (IL-6) are major factors in inflammation. The aim of this study was to investigate whether or not the *IL6* −634C/G polymorphism (rs1800796) and its interaction with smoking influence serum C-reactive protein (CRP) concentrations. The subjects were 347 Japanese male employees of a transit company. CRP and conventional cardiovascular risk factors were evaluated. *IL6* −634C/G polymorphisms were genotyped by allelic discrimination using fluorogenic probes and the 5′ nuclease assay. The mean values of CRP were significantly higher in current smokers than in nonsmokers after adjustment for age, body mass index (BMI), systolic blood pressure, total cholesterol, log triglycerides, high-density lipoprotein cholesterol (HDL-C), fasting glucose, and drinking habit ($p=0.011$). Comparison of three genotypes revealed significant interaction between smoking and the *IL6* −634C/G genotype manifested by CRP concentrations ($p=0.007$) after the adjustments cited above. After stratification by smoking status, CRP differed significantly among *IL6* −634C/G genotypes groups in nonsmokers ($p=0.010$, p for trend $=0.007$), whereas no significant difference was found in current smokers. Comparison between −634C/C and C/G+G/G groups revealed also a significant interaction between smoking and the *IL6* −634C/G genotype ($p=0.007$). These findings suggest that the impact of the −634G allele on CRP elevation is greater in nonsmokers than in current smokers. Since gene-environment interactions have been insufficiently examined, further studies are required to clarify their effect on inflammation, including CRP elevation. (*Hypertens Res* 2007; 30: 593–599)

***Key Words*: C-reactive protein, smoking, interleukin-6, gene polymorphism, interaction**

Introduction

Atherosclerosis is now generally accepted as an inflammatory disorder in the arterial wall (*1*), and the C-reactive protein (CRP) level is a strong predictor of cardiovascular events (*2*–*5*). The influences of lifestyle and genetic factors on CRP, and their interactions, are therefore important.

The synthesis of CRP in the liver is mainly under the control of interleukin-6 (IL-6) (*6*). Smoking is known to increase IL-6 (*7*, *8*). Many trials, including ECAT (*9*) and the MONICA study (*3*) have documented increased CRP concentrations in smokers.

There have been several reports on the relationships between *IL6* gene polymorphisms and CRP. One study has reported that the presence of the C allele of the *IL6* −174G/C polymorphism was significantly associated with higher CRP concentrations in 98 hypertensive probands and their families

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Table 1. Patient Characteristics According to Smoking Status

	Nonsmoker (n=182)	Current smoker (n=165)	p value*
Age (years)	49.7±5.1	48.0±4.6	0.004
BMI (kg/m^2)	23.9±2.4	23.6±2.9	0.24
SBP (mmHg)	126.4±15.8	119.1±15.8	<0.0001
DBP (mmHg)	82.9±10.9	76.8±10.6	<0.0001
Total cholesterol (mg/dL)	204.8±29.2	201.0±33.2	0.27
Triglycerides (mg/dL)	108 (73–153)	123 (88–188)	0.011
HDL-C (mg/dL)	55.8±14.8	51.1±12.1	0.002
Fasting glucose (mg/dL)	100.3±13.5	97.0±8.5	<0.001
Uric acid (mg/dL)	5.7±1.1	5.8±1.2	0.38
Drinker (%)	81.9	77.6	0.32
CRP (mg/dL)	0.036 (0.020–0.071)	0.051 (0.026–0.092)	0.013

Variables are presented as mean±SD, median (interquartile range) for skewed variables, or percentage. *Student's unpaired t-test, the Wilcoxon rank-sum test, or the χ^2 test. BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HDL-C, high-density lipoprotein cholesterol; CRP, C-reactive protein.

with or without hypertension (n=588) (*10*). Another study has failed to show a significant association of *IL6* −174C/G polymorphism with CRP concentration in 160 coronary artery disease patients (*11*). On the other hand, a significant association of the −174G allele with increased levels of CRP in 290 type 2 diabetes patients (*12*) has been reported. These inconclusive results may suggest that the −174C/G polymorphism could influence underlying diseases and conditions. Furthermore, in young and healthy subjects carrying the −174C allele, those who smoked had higher leukocytes, lymphocytes, and monocytes than those who did not smoke (*13*). Thus, gene-environment interactions in the pathogenesis of inflammation should also be elucidated.

The C allele of the *IL6* −174G/C polymorphism is common among Caucasians but extremely rare among East Asians (*14–17*). However, the G allele of the *IL6* −634C/G polymorphism is common among East Asians, and this genotype is significantly related to recurrent pregnancy loss (*14*) and to the loss of bone mineral density (*18*). The −634G allele was significantly more common in patients with macroalbuminuria than in those with normoalbuminuria among type 2 diabetic patients, and a study reported that the −634G allele was associated with elevated production and secretion of IL-6 by peripheral blood mononuclear cells *in vitro* (*19*).

Thus, the *IL6* −634C/G polymorphism may affect inflammation and, both in itself and through its interaction with smoking, may affect CRP concentrations. The aim of this study was to investigate whether or not the *IL6* −634C/G polymorphism and its interaction with smoking influence serum CRP concentrations in healthy Japanese male workers.

Methods

Subjects

The subjects were transit company employees (1,255 men and 94 women aged 35 to 60 years) who had an annual health checkup between April 2003 and March 2004. We used a self-administered questionnaire including items on clinical history, family history, smoking, and alcohol consumption. The questionnaire was distributed to the subjects prior to the checkup and was collected at the checkup. Answers to the questionnaire and written informed consent to view health checkup data were obtained from 413 men and 5 women (response rate: men 32.9%, women 5.3%). A total of 71 subjects were excluded for the following reasons: women (n=5; because of sample size), history of hypertension (n=34), history of dyslipidemia (n=17), history of diabetes (n=18), history of coronary disease or stroke (n=9), or blood samples not analyzed (n=8). Finally, we analyzed 347 male employees who had no history of cancer and no past or present systemic inflammation such as active chronic arthritis.

This study was conducted with written informed consent from all subjects and approved by the institutional ethics board for epidemiological studies of the Hokkaido University Graduate School of Medicine.

Data Collection

Subjects were classified as either current smokers or nonsmokers, with the latter group including both never- and ex-smokers. Drinkers were defined as those who consumed alcohol once a week or more.

Anthropometric measures (height, body weight, and waist and hip circumferences) were recorded by a standardized protocol. Body mass index (BMI) was calculated as weight (kg)/height (m)^2. Resting blood pressure was measured in the sitting position using an automated blood pressure monitor (BP-103iII, Omron Colin, Tokyo, Japan) following at least 5 min of rest.

Blood samples were drawn from the antecubital vein of the seated subject with minimal tourniquet use after a 12-h fast.

Table 2. Patient Characteristics According to Interleukin-6 −634C/G Genotype

	Interleukin-6 −634C/G genotype			*p* value*
	C/C (*n*=202)	C/G (*n*=128)	G/G (*n*=17)	
Age (years)	48.7±5.4	49.1±5.2	50.2±4.6	0.44
BMI (kg/m^2)	23.8±2.8	23.6±2.3	23.4±2.9	0.61
SBP (mmHg)	122.8±15.9	122.3±16.6	128.5±16.6	0.33
DBP (mmHg)	80.0±11.5	80.0±11.0	80.8±9.3	0.96
Total cholesterol (mg/dL)	203.4±32.3	201.3±28.3	210.8±38.4	0.48
Triglycerides (mg/dL)	119.0 (84–175)	112.0 (83–156)	94.0 (55–157)	0.26
HDL-C (mg/dL)	53.7±13.8	53.1±13.9	55.6±13.4	0.76
Fasting glucose (mg/dL)	98.7±12.2	98.6±10.6	100.0±9.5	0.89
Uric acid (mg/dL)	5.8±1.3	5.7±1.0	5.9±1.3	0.66
Current smoker (%)	48.5	45.3	52.9	0.77
Drinker (%)	80.7	76.6	94.1	0.21
CRP (mg/dL)	0.037 (0.021–0.083)	0.044 (0.024–0.076)	0.050 (0.026–0.125)	0.56

Variables are presented as mean±SD, median (interquartile range) for skewed variables, or percentage. *Analysis of variance (ANOVA), the Kruskal-Wallis test, or the χ^2 test. BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HDL-C, high-density lipoprotein cholesterol; CRP, C-reactive protein.

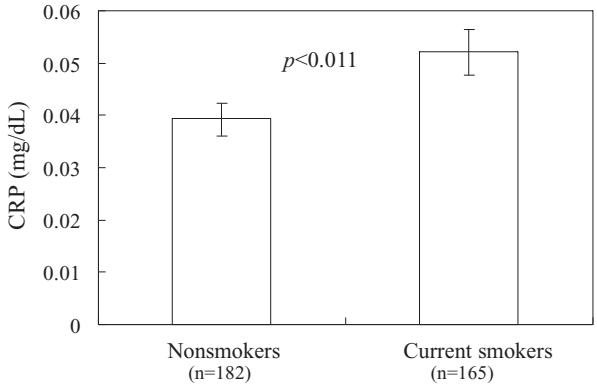

Fig. 1. *Adjusted mean (±SEM) concentrations of CRP according to smoking status. Using general linear model (GLM) univariate analyses adjusted for age, BMI, systolic blood pressure, TC, log TG, HDL-C, fasting glucose, and drinking habit, adjusted mean (±SEM) values of log CRP were back-transformed.*

Total cholesterol (TC), triglyceride (TG), uric acid (UA), and glucose levels were measured by enzymatic methods. The high-density lipoprotein cholesterol (HDL-C) level was measured by a direct method. CRP was measured by nephelometry, with a latex particle–enhanced immunoassay (N Latex CRP II; Dade Behring, Tokyo, Japan). The assay could detect 0.004 mg/dL of CRP. Undetectable CRP values were recorded as 0.002 mg/dL.

Genomic DNA was extracted from peripheral blood lymphocytes using the EZ1 DNA blood kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. *IL6* −634C/G gene polymorphisms were determined by the TaqMan polymerase chain reaction (PCR) method using a Minor Groove Binder (MGB) probe as described previously (*20*). To detect a polymorphism of *IL6* −634C/G (rs1800796), two MGB probes were prepared: a C allele–specific probe, 5′-FAM-CAACAGCCCCTCACAG-MGB-3′, and a G allele–specific probe, 5′-VIC-CAACAGCCGCTCACAG-MGB-3′. Each of the reporters was quenched by MGB, which was typically located at the 3′ end. The primers for PCR of the promoter region including the −634C/G polymorphism of *IL6* were as follows: forward, 5′-GGATGGCCAGGCAGTT CTA-3′; reverse, 5′-CCAGTCATCTGAGTTCTTCTGT GTT-3′. The reaction mixture contained approximately 40 ng of template DNA, 5.0 μL of TaqMan Universal PCR master mixture, and 0.3 μL of 40× assay mixture in a volume of 10 μL. Real-time PCR was performed on a 7500 real-time PCR System (Applied Biosystems, Foster City, USA) using a procedure consisting of incubation at 50°C for 2 min and 95°C for 10 min, followed by 50 cycles of denaturation at 92°C for 15 s and annealing/extension at 60°C for 1 min. FAM and VIC fluorescence levels of the PCR products were measured at 60°C for 1 min, resulting in the clear identification of the three genotypes of the *IL6* promoter region on a two-dimensional graph.

Statistical Analysis

The subjects were categorized according to smoking status (current smoker or nonsmoker) and *IL6* −634C/G genotype (C/C, C/G, or G/G). The data were presented as means±SD or as median values (and interquartile ranges) for variables with skewed distributions or percentages, and the data were compared among groups using Student's unpaired *t*-test, analysis of variance (ANOVA), the Wilcoxon rank-sum test, the Kruskal-Wallis test, or the χ^2 test.

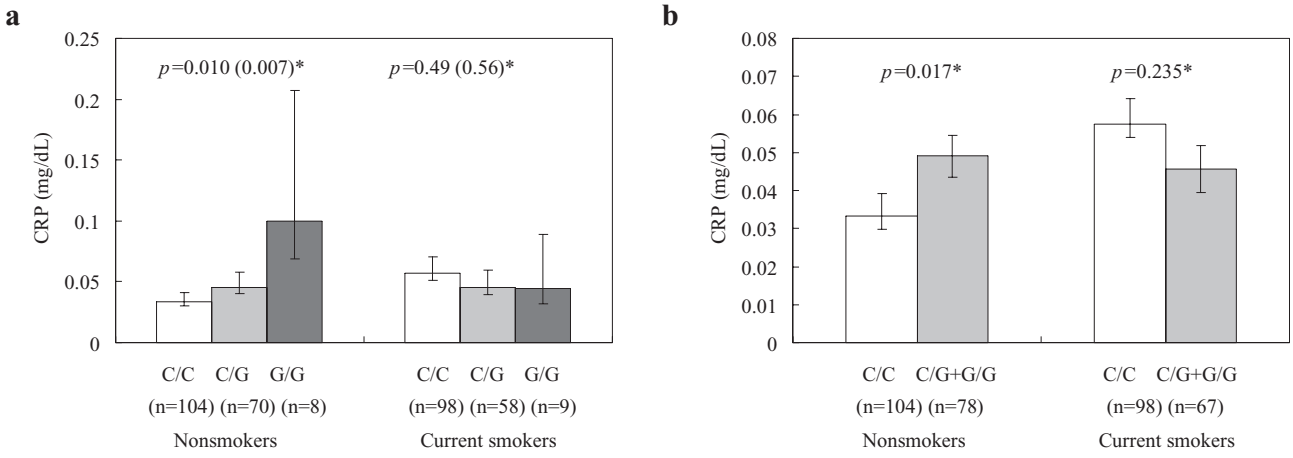

Fig. 2. *a: Adjusted mean (±SEM) concentrations of CRP in relation to smoking status and interleukin-6 −634C/G genotype (comparing all three genotypes). Using general linear model (GLM) univariate analyses adjusted for age, BMI, systolic blood pressure, TC, log TG, HDL-C, fasting glucose, and drinking habit, adjusted mean (±SEM) values of log CRP were back-transformed. *p value for difference (p for trend). b: Adjusted mean (±SEM) concentrations of CRP in relation to smoking status and interleukin-6 −634C/G genotype (C/C vs. C/G+G/G). Using GLM univariate analyses adjusted for age, BMI, systolic blood pressure, TC, log TG, HDL-C, fasting glucose, and drinking habit, adjusted mean (±SEM) values of log CRP were back-transformed. *p value for difference.*

Because of its skewed distribution, the mean log-transferred CRP was compared between current smokers and nonsmokers using general linear model (GLM) univariate analyses adjusted for age, BMI, systolic blood pressure, TC, log TG, HDL-C, fasting glucose, and drinking habit. Next, a GLM was employed to evaluate the significant contributions of smoking status and genotype interaction to CRP levels, after adjustment for the possible confounders named above. The back-transformed means±SEM of log-transferred CRP in each group are presented in the results and the figures.

A p value of <0.05 was considered statistically significant. All analyses were conducted using the SPSS software package version 14.0 for Windows (SPSS Inc., Chicago, USA).

Results

The characteristics of the groups according to smoking status are shown in Table 1. TG and CRP were significantly higher in current smokers than in nonsmokers. Age, systolic and diastolic blood pressure, HDL-C, and fasting glucose were significantly lower in current smokers than in nonsmokers.

The characteristics of the groups according to *IL6* −634C/G genotypes are shown in Table 2. None of the variables, including CRP, differed significantly among the genotypes. The distribution of the genotypes was in Hardy-Weinberg equilibrium.

Figure 1 shows the back-transformed adjusted means±SEM values of log CRP according to smoking status. The back-transformed adjusted mean values of log CRP in nonsmokers and current smokers were 0.039 and 0.052 mg/dL, respectively ($p=0.011$).

Comparison of the three genotypes revealed a significant interaction between smoking and the *IL6* −634C/G genotypes, as manifested by CRP concentrations ($p=0.007$, Fig. 2a). After stratification by smoking status, CRP was found to differ significantly among the *IL6* −634C/G genotype groups among nonsmokers after adjustment for age, BMI, systolic blood pressure, TC, log TG, HDL-C, fasting glucose, and drinking habit ($p=0.010$, p for trend$=0.007$), whereas no significant differences among genotype groups were found in current smokers ($p=0.49$, p for trend$=0.56$).

Then, comparison between −634C/C and C/G+G/G groups revealed a significant interaction between smoking and *IL6* −634C/G genotypes, as manifested by CRP concentrations ($p=0.007$, Fig. 2b). After stratification by smoking status, CRP was found to differ significantly between two genotype groups among nonsmokers after adjustment for age, BMI, systolic blood pressure, TC, log TG, HDL-C, fasting glucose, and drinking habit ($p=0.017$), whereas no significant differences between two genotype groups were found among current smokers ($p=0.235$).

Discussion

In this study, smoking status was related to CRP concentration in whole subject analysis, but *IL6* −634C/G polymorphism was not. However, significant interaction was found between smoking and the *IL6* −634C allele, thus affecting CRP concentrations. The *IL6* −634C/G polymorphism was significantly associated with CRP concentration in nonsmok-

ers but not in current smokers.

The synthesis of CRP in the liver is mainly under the control of IL-6 (*6*). Therefore, a genotype or haplotype that influences IL-6 production could affect CRP concentrations.

Ota *et al*. (*18*) speculated that a decrease in bone mineral density found in carriers of the −634G allele, but not in subjects with the −634C allele, was due to effects of transcriptional activation that is caused by the presence of the G allele, since IL-6 is known to stimulate osteoclast development. Those with the −634G variant had a lower risk of abortion (*14*). We attribute this to increased IL-6 production in the −634G variant, because low serum concentrations of IL-6 during early pregnancy in women with recurrent pregnancy loss were found to be associated with unsuccessful pregnancy outcome (*21*). The −634G allele was significantly more common in patients with macroalbuminuria than in patients with normoalbuminuria among type 2 diabetic patients, and the study reported that the −634G allele was associated with elevated production and secretion of IL-6 by peripheral blood mononuclear cells *in vitro* (*19*).

Meanwhile, the −634C allele (denote −572G/C in Refs. *22–24*) is less frequent in Caucasians (*22–24*). In Caucasians, the −634C allele was associated with increased serum insulin release during an oral glucose tolerance test (*23*). The −634C allele induced higher gene expression levels than the G allele after stimulation with interleukin-1β and dexamethasone, and the C allele was related to higher serum CRP levels in Caucasian postmenopausal women (*22*). However, an *in vitro* study found that the −634C/G allele was not associated with IL-6 production by leukocytes after lipopolysaccharide stimulation (*25*).

Several studies among Caucasians have shown that the combination of polymorphisms in the promoter region of *IL6* affects its gene expression (*25–27*). But, as mentioned above, the −174C allele is extremely rare and the −634C allele is common in Japanese, whereas in Caucasians the −174C allele is relatively frequent and the −634C allele is less frequent. Tanaka *et al*. speculated that racial variation of the −634C/G polymorphism affects IL-6 production, since there are such racial differences in allelic distribution (*28*).

In young and healthy subjects who carry the *IL6* −174C allele, those who smoked had higher levels of leukocytes, lymphocytes, and monocytes than those who did not smoke (*13*). If the −634C/G polymorphism had had the same effect as the −174G/C polymorphism on inflammation, the CRP concentrations in current smokers with the −634G allele would have been higher than in nonsmokers with the −634C allele. However, the authors of the −174G/C polymorphism study did not explain the mechanism underlying the *IL6* −174C allele's enhancement of inflammation in smokers (*13*). Therefore, we can only speculate as to the possible reason for the difference in the −634G allele's effect between smoker and nonsmokers. A possible reason for the lack of a −634C/G genotype effect on CRP concentrations in current smokers is that continuous smoking, which stimulates inflammation, could reduce the −634G allele's effect on CRP concentration; the −634G allele may increase CRP concentration when smoking does not stimulate inflammation. Also, glutathione S-transferase (GST) M1 and P1, phase II detoxification enzymes, were related to increased risk of smoking-related diseases (*29*, *30*) and to the metabolism of many carcinogenic compounds in tobacco smoke (*31*). It has been reported that the GSTM1 and GSTP1 gene polymorphisms are related to inflammation (*32*). Thus, smokers' detoxification enzyme gene polymorphisms may affect the −634G allele's influence on inflammation in smokers.

After comparing constructs of the 5′ flanking region in a luciferase reporter vector transiently transfected in HeLA cells, Fishman *et al*. (*26*) reported that the −174C construct showed lower expression than the −174G construct. After stimulation with lipopolysaccharide or IL-1, expression from the −174C construct did not significantly change after 24 h, whereas expression from the 174G construct increased significantly. Terry *et al*. (*27*) showed that more than one of the *IL6* promoter polymorphic sites was functional, but when the −174G/C polymorphism was considered alone, variants containing a C allele showed lower expression than the G/G genotype. Thus, these studies provide evidence of an enhancement in IL-6 transcription of IL-6 associated with the G-allele, whereas the −634G/C genotype had no transcriptional evidence.

However, as previously described, the results of studies on the association of the *IL6* −174G/C genotype with CRP concentrations were inconclusive (*10–12*). Furthermore, the association between the genotype and plasma IL-6 concentrations is also controversial. Fishman *et al*. showed that the mean plasma IL-6 concentration was lower in individuals with the −174C/C genotype than in those with the −174G/C or −174G/G genotypes (*26*). On the other hand, Jones *et al*. reported that abdominal aortic aneurysm patients with the −174G/G genotype had lower plasma concentrations of IL-6 than those with the −174G/C or −174C/C genotypes (*33*). Meanwhile, Brull *et al*. found no association between the genotype and baseline plasma IL-6 levels in 127 patients undergoing coronary artery bypass graft surgery (CABG), although, 6 h after CABG, peak IL-6 levels were significantly higher in those with genotype −174C/C compared with −174G allele carriers (*34*).

These discrepancies may occur because baseline diseases and conditions influence the genotype's effects on IL-6 transcription. Thus, since gene-environment interactions in chronic diseases are very important (*35*), we should investigate these interactions.

Furthermore, it has been reported that the haplotype of the *IL6* gene is associated with hypertension (*28*). Therefore, more haplotype studies of the *IL6* gene are needed to elucidate the effects on inflammation.

In general, CRP concentrations increase as cigarette consumption increases (*36*). Among smokers in present study, the back-transformed adjusted mean values of log CRP in

those who smoked ≥20 cigarettes per day was significantly higher than that among smokers of ≤19 cigarettes per day. But, the −634C/G genotype did not significantly affect CRP concentration among smokers of ≥20 cigarettes per day or smokers of ≤19 cigarettes per day (data not shown). Further studies are needed to clarify whether or not the interaction between the amount of tobacco consumption and *IL6* genotypes affects CRP concentration.

We believe that this type of findings on the relationships between cytokine polymorphisms and inflammation will lead to tailor-made treatment. For example, a −634C carrier with mild dyslipidema may have to take statins as early as possible because statins attenuate systemic inflammatory activity (*37*).

The present study has several limitations. First, the IL-6 concentrations were not measured. DeMichele *et al*. insisted that many studies failed to find an association between the *IL6* −174C/G polymorphism and spot serum IL-6, since there is tremendous intraindividual variation in IL-6 levels, even in nonpathological circumstances (*38*). Therefore, we evaluated the CRP level because of its popularity and measurement stability. Second, we hypothesize that the −634G allele, by increasing *IL6* gene transcription, raises serum CRP concentrations. However, linkage disequilibrium between this polymorphism and others that affect IL-6, other cytokines, or CRP gene transcription directly could influence serum CRP concentrations. Further studies are needed to clarify the mechanism underlying this association. Third, the smaller sample size of subjects with the −634G/G allele might be responsible for the nonsignificant relationship of −634C/G polymorphisms to CRP level among subjects overall or smokers. Fourth, the restriction of the current study to Japanese males aged 35 to 60 years means that additional work will be required to confirm these findings and to extend this work to older and younger men, women, and non-Japanese populations.

In summary, we observed significant interaction between smoking and the *IL6* −634C/G polymorphism, which is common among Japanese, and this interaction influenced CRP concentrations. The *IL6* −634G allele was significantly associated with CRP concentrations in nonsmokers but not in current smokers. Our results suggest that the impact of the −634G allele on CRP elevation is greater in nonsmokers than in current smokers. Since the gene-environment interaction has been insufficiently examined, further studies are required to clarify the gene-environment interactions affecting inflammation, including elevated CRP.

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