

The effect of interleukin-6 C/G-174 polymorphism and circulating interleukin-6 on fibrinogen plasma levels

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Background and Objectives. C-reactive protein (C-RP) levels correlate with fibrinogen values and are predictive of coronary artery disease. Interleukin-6 (IL-6) strongly regulates the production of C-RP. A polymorphism (C/G-174) within the IL-6 gene has been shown to affect IL-6 gene expression and plasma concentrations.

Design and Methods. In 598 asymptomatic employees of a hospital in Southern Italy, we investigated the association between IL-6 C/G-174 gene variants and plasma fibrinogen levels.

Results. Subjects with IL-6 plasma levels >2.0 pg/mL had a higher body mass index (BMI) (24.5 ± 1.2) than subjects with IL-6 levels below this cut-off value (23.7 ± 1.2 ; $p = 0.005$). No association was found with sex, cigarette smoking, or alcohol consumption (p always >0.05). When the whole sample was analyzed according to the IL-6 C/G-174 polymorphism, there was no difference with respect to age, sex, alcohol consumption, cigarette smoking, total cholesterol, triglycerides, and body mass index. Median plasma fibrinogen levels, as well as carriers of plasma levels of IL-6 >2.0 pg/mL and C-RP >0.33 mg/L, were similar among subjects with different IL-6 genotypes. Similarly, no difference was observed when only carriers of plasma levels of IL-6 >2.0 pg/mL were analyzed, whereas in those with C-RP >0.33 mg/L IL-6 GG carriers had significantly lower plasma fibrinogen levels.

Interpretation and Conclusions. The investigation of the IL-6 C/G-174 polymorphism does not seem to be a useful tool for predicting raised plasma fibrinogen levels.

Key words: IL-6, C-reactive protein, fibrinogen, gene polymorphism

In the early 1980s, Meade *et al.* first reported that subjects who died from myocardial infarction had had at recruitment, 5 years earlier, significantly higher fibrinogen plasma levels than survivors or subjects who died from other causes.^{1,2} In the following years, other studies confirmed the association between raised plasma fibrinogen levels and myocardial infarction, extended it to stroke, and identified a series of factors that affect plasma fibrinogen concentrations.³⁻⁷ Age and lifestyle were found to account for about 30% of the between-individual variance of plasma fibrinogen levels⁸ whereas the within-individual variation accounted for up to 1/4 of the variability of plasma fibrinogen.⁹ The data so far available clearly support the importance of genetic determinants in the regulation of plasma fibrinogen concentrations, suggesting a major effect in youth.¹⁰⁻¹⁴ Fibrinogen values are strongly correlated with C-reactive protein (C-RP), an acute-phase reactant which is a marker for systemic inflammation.¹⁵ Elevated C-RP levels have been found to predict recurrent ischemia, myocardial infarction and sudden death among patients with angina pectoris and individuals with multiple risk factors for atherosclerosis.^{16,17} On the other hand, fibrinogen is itself an acute-phase reactant and interleukin-6 (IL-6) significantly raises its plasma concentrations.¹⁸

A polymorphism within the 5' flanking region of the IL-6 gene locus (C/G-174) has been reported to regulate gene transcription.¹⁹ In transient transfection studies, the G allele showed a higher expression rate of a reporter gene than the C allele. In addition, the G allele was associated with higher plasma IL-6 levels in normal individuals.¹⁹

These findings suggested that molecular variations of genes playing a role in the acute-phase reaction, such as IL-6, may exert a significant effect and their clinical impact should be taken into account in the regulation of plasma fibrinogen levels and their association with arterial thrombotic events.

In a large cohort of individuals from a general population, we investigated whether the IL-6C/G-174 polymorphism plays a role in the regulation of plasma fibrinogen levels.

Design and Methods

Subjects

After approval from the local Ethics Committee, the study was carried out according to the Principles of the Declaration of Helsinki; informed consent was obtained from 1,056 apparently healthy employees of the Casa Sollievo della Sofferenza Hospital, S. Giovanni Rotondo, Southern Italy. Plasma aliquots for IL-6 plasma level determinations and/or DNA for the evaluation of the IL-6 C/G-174 polymorphism were not available for 458 of them. Thus, from January 1995 to October 1996, 598 employees (22-65 years old) were enrolled. All were Caucasians, none was the offspring of a consanguineous marriage and all their parents and grandparents had been born in the same region. The male/female ratio of the sample was 0.72 (men=250; 41.8%), (women=348; 58.2%). Subjects not analyzed were entirely comparable with those included within the study as far as concerns age, sex, BMI, and alcohol and smoking habits.

Blood was collected by venipuncture between 9-11 am after 12-15 hours of fasting. Platelet-free plasma obtained by centrifugation (2,000g × 10 min at room temperature) was immediately divided into aliquots of 500 µL in plastic tubes (NUNC, Roskilde, Denmark) and frozen at -70°C until assayed. A detailed clinical summary with emphasis on personal and family history for angina pectoris, myocardial infarction, ischemic stroke, and peripheral arterial disease was obtained from all subjects by specially trained staff, employing a previously validated questionnaire²⁰ prepared according to the World Health Organization questionnaire for cardiovascular disease. In addition to questions about symptoms of ischemic heart disease, peripheral vascular disease and previous vascular surgery, information concerning stroke history and risk factors, diabetes mellitus, arterial hypertension, drug use, alcohol consumption and smoking habits was obtained. The subject was defined as having hypertension if he or she was being treated with hypertensive drugs at the time of examination or had a systolic blood pressure greater than 140 mmHg and/or a diastolic blood pressure greater than 90 mmHg in the sitting position on at least three different occasions. Subjects with a positive history of diabetes mellitus or with fasting blood glucose levels higher than 7.0 mmol/L were considered diabetics. Alcohol drinkers and smokers were defined as never/past consumers or current consumers. The demographic characteristics of the study sample analyzed as a whole and stratified according to sex are shown in Table 1.

Biochemical variables

The concentrations of total cholesterol and triglycerides were detected enzymatically with commercially available reagents (Roche, Milan, Italy). Both the reagent and the apparatus (CoA Data 2000) for the measurement of the fibrinogen were from Boehringer-Mannheim, Milan, Italy. Reference pooled normal plasma from 216 apparently healthy male and female volunteers (29-70 years old) who had been instructed to avoid any med-

Table 1. Clinical characteristics of the whole sample.

Variable	All (n=598)	Women (n=348)	Men (n=250)	p*
Age (years) median (range)	36 (22-66)	35 (22-65)	37 (23-65)	n. s. ¹
Alcohol consumers % (n)	53.2 (318)	37.6 (131)	74.8 (187)	<0.001
Smokers % (n)	24.2 (145)	19.5 (68)	30.8 (77)	<0.002
C-RP > 0.33 mg/L % (n)	8.0 (48)	8.3 (29)	7.6 (19)	n. s.
IL-6 > 2.0 pg/mL % (n)	37.6 (225)	37.4 (130)	38.0 (95)	n. s.
Total cholesterol mg/dL mean (SD)	185.4 (1.2)	184.3 (1.2)	186.9 (1.2)	n. s.
Triglycerides mg/dL mean(SD)	95.1 (1.7)	80.9 (1.6)	118.9 (1.8)	<0.001
BMI Kg/m ² mean (SD)	24.0 (1.2)	23.2 (1.2)	25.1 (1.1)	<0.001
Bb A-455 allele % (n)	36.0 (215)	36.9 (108)	34.8 (87)	n. s.

*Chi-squared test for categorical and Student's t-test (¹Mann-Whitney) for continuous variables were used. Skewed variables (see methods) were log-transformed and geometric means (antilogged SD) are indicated.

ication for at least 1 week, was prepared and stored under the same conditions applied to the samples from the study subjects. The intra- and inter-assay coefficients of variation of fibrinogen did not exceed 8%.

Fibrinogen plasma levels, total cholesterol and triglycerides were determined as described elsewhere.²⁰ C-reactive protein was assayed by rate nephelometry (N latex CRP mono; Behring Institute, Scoppitto, L'Aquila, Italy) according to the manufacturer's recommendations. Because of the reduced precision in the low range of the assay (0.175 to 0.33 mg/L), a cut-off value of 0.33 mg/L was employed as the lower limit of detection. IL-6 plasma levels were determined by means of an immunoenzymometric assay (IL-6 EASIATM; BioSource Europe S.A. Nivelles, Belgium). According to the manufacturer's recommendations, the minimum detectable concentration (2 pg/mL) was employed as cut-off value. Intra- and inter-assay coefficients of variation of C-reactive protein and IL-6 were 2.5% and 5.1%, 3.8% and 5.7%, respectively.

Genetic analysis

Blood samples were collected and DNA extracted according to standard protocols.²⁰ Fibrinogen B β -chain -455 G/A polymorphism was evaluated as previously reported.²⁰ IL-6 C/G-174 polymorphism was investigated by means of polymerase chain reaction (PCR) and subsequent Nla III restriction enzyme analysis. Briefly, PCR was carried out on 50 µL volume samples, in a Perkin Elmer-Cetus thermal cycler. Each sample contained 0.5 µg of genomic DNA, 15 pmoles of each primer, 100 mM of dNTP, 10 mmol/L Tris HCl pH 8.3, 50 mmol/L KCl, 1.5 mmol/L MgCl₂, and 1 U thermostable Taq polymerase. The 30 cycles consisted of steps at 95°C for 60 sec, at 58°C for 50 sec and at 72°C for 100 sec. Then, 20 µL volumes of the amplification products were digested for 2.5 hrs at 37°C with 2 U of the Nla III restriction enzyme. The fragments were fractionated by 4% agarose-gel electrophoresis, and visualized under UV light.

Statistical analysis

All the analyses were performed according to the Statistical Package for Social Science (SPSS 6.0.1 for Apple Macintosh). Plasma fibrinogen, body mass index (BMI), cholesterol and triglycerides levels were logarithmically transformed to allow for the use of parametric tests. C-RP and IL-6 data were entered as categorical variables using 0.33 mg/L and 2.0 pg/mL as cut-off values, respectively. Differences in baseline characteristics between sexes were evaluated by the Student's t-test and Mann-Whitney U test for continuous variables and by the χ^2 test for discrete variables. The allelic frequencies were estimated by gene counting, and genotypes were scored. The numbers observed for each fibrinogen genotype were compared with those predicted in a population by the Hardy-Weinberg equilibrium using a χ^2 test. Plasma fibrinogen means in different categories were evaluated by the Student's t-test. Differences between IL-6 C/G-174 genotypes and different categorical variables were analyzed by the chi-squared test; univariate analysis of variance was employed for continuous variables. Differences between different genotypes were evaluated by Scheffe's test. General factorial ANOVA models evaluated factors related to plasma fibrinogen concentrations as well as the possibility of interactions by creating interaction terms. For all data, statistical significance was established as a *p* value <0.05.

Results

Fibrinogen

A close correlation between fibrinogen plasma levels and a series of variables was observed. Pearson's correlation coefficients showed a significant association with age ($r=0.278$; $p<0.001$), cholesterol ($r=0.271$; $p<0.001$), BMI ($r=0.212$; $p<0.001$), and triglycerides ($r=0.143$; $p<0.001$). In the whole sample (Table 2), mean plasma levels of fibrinogen were higher in women, and in carriers of the A allele. On the other hand, plasma fibrinogen concentrations were lower in alcohol drinkers. When analyzed according to median values, plasma fibrinogen levels were higher in individuals >36 years old, total cholesterol >187 mg/dL, triglycerides >88 mg/dL, and BMI >23.9 kg/m². Likewise, significantly higher levels were found in subjects with C-RP plasma levels >0.33 mg/dL, and IL-6>2.0 pg/mL (Table 2).

C-RP and IL-6

When the sample was analyzed according to C-RP levels, compared to subjects carrying C-reactive protein levels <0.33 mg/L, subjects whose levels were above this cut-off value turned out to be more often older (41.5±10.4 vs. 37.7±9.4 years [$p=0.017$]), overweight (BMI: 26.0±1.2 vs. 23.8±1.2 [$p=0.003$]), hypercholesterolemic (197.8±1.3 vs. 184.4±1.2 mg/dL [$p=0.036$]), and carriers of high plasma levels of fibrinogen (352.7±1.2 vs. 274.2±1.2 mg/dL [$p<0.001$]). Alcohol consumers more frequently had C-RP levels <0.33 mg/L (5.3% vs. 11.1%; $p=0.015$).

Table 2. Fibrinogen (mg/dL) levels according to clinical characteristics.

Variable	no	n	yes	n	<i>p</i> *
Age >36 years	276.6 (1.2)	302	292.8 (1.2)	296	<0.001
Men	289.5 (1.2)	348	266.8 (1.2)	250	<0.001
Alcohol consumers	289.9 (1.2)	280	271.2 (1.2)	318	<0.001
Smokers	280.9 (1.2)	453	276.4 (1.2)	145	n.s.
C-RP >0.33 mg/dL	274.2 (1.2)	550	352.7 (1.2)	48	<0.001
IL-6 >2.0 pg/mL	274.4 (1.2)	373	289.1 (1.2)	225	0.002
Total cholesterol >187 mg/dL	268.8 (1.2)	305	291.7 (1.2)	293	<0.001
Triglycerides >88 mg/dL	272.2 (1.2)	304	287.9 (1.2)	294	<0.001
BMI >23.9 kg/m ²	272.7 (1.2)	303	287.3 (1.2)	295	0.001
Bb A-455 allele	275.8 (1.2)	382	287.3 (1.2)	215	0.014

*Student's t-test. Plasma fibrinogen levels were log-transformed and geometric means (antilogged SD) are indicated. For continuous variables, median values were used as the cut-off.

Individuals with IL-6 plasma levels >2.0 pg/mL had a higher BMI (24.5±1.2) than subjects carrying IL-6 levels below this cut-off value (23.7±1.2; $p=0.005$). No association was found with sex, cigarette smoking, or alcohol consumption (p always >0.05).

When compared to normotensive subjects, hypertensive individuals more often had IL-6 plasma levels >2.0 pg/mL (50.0% vs. 36.0%; $p=0.029$) and C-RP levels >0.33 mg/L (17.2% vs. 6.9%; $p=0.004$). Finally, a significant relationship between IL-6 and C-RP plasma levels was observed: 31 out of 48 subjects with C-RP levels >0.33 mg/L also had IL-6 levels >2.0 pg/mL ($p<0.001$).

IL-6 C/G-174 gene polymorphism

Genotype frequencies of the IL-6 C/G-174 gene polymorphism in the whole sample were 7.9% for the CC polymorphism ($n=47$; 95% CI=5.6-10.2), 36.6% for CG ($n=219$; 95% CI=32.7-40.5), and 55.5% for GG ($n=332$; 95% CI=51.5-59.5). These frequencies and the calculated allele frequencies (C: 26.2% [95% CI=23.7-28.7]; G: 73.8% [95% CI=71.3-76.3]) did not differ from those predicted from the Hardy-Weinberg equilibrium.

When the whole sample was analyzed according to the IL-6 C/G-174 polymorphism, there was no difference with respect to age, sex, alcohol consumption, cigarette smoking, total cholesterol, triglycerides, and body mass index. Median plasma fibrinogen levels did not differ among subjects with the different IL-6 genotypes (Table 3). Likewise, carriers of plasma levels of IL-6 >2.0 pg/mL and C-RP >0.33 mg/L were equally distributed among different IL-6 C/G-174 genotypes (Table 3).

The effects of the IL-6 C/G-174 polymorphism, IL-6 and C-RP levels on fibrinogen concentrations were further analyzed using a factorial ANOVA, which took into account a series of confounding variables. In this model, age ($p<0.001$), sex ($p<0.001$), C-RP >0.33 mg/L ($p<0.001$), BMI ($p=0.007$), total cholesterol ($p=0.004$), fibrinogen G/A-455 ($p=0.038$) polymorphism, and alcohol consumption

Table 3. Clinical characteristics according to IL-6 C/G-174 genotypes.

	CC n=47	CG n=219	GG n=332
Age (years) median (range)	39 (29-61)	36 (22-65)	36 (22-65)
Male sex % (n)	25.5 (12)	44.7 (98)	42.2 (140)
Alcohol consumers % (n)	44.7 (21)	58.0 (127)	51.2 (170)
Smokers % (n)	31.9 (15)	21.0 (46)	23.5 (78)
C-RP >0.33 mg/dL % (n)	6.4 (3)	8.7 (19)	7.8 (26)
IL-6 >2.0 pg/mL % (n)	42.6 (20)	40.6 (89)	34.9 (116)
Fibrinogen mg/dL mean (SD)	293.5 (1.2)	276.3 (1.2)	280.2 (1.2)
Total cholesterol mg/dL, mean (SD)	185.3 (1.2)	183.5 (1.2)	186.7 (1.2)
Triglycerides mg/dL mean(SD)	90.3 (1.7)	92.9 (1.7)	97.2 (1.7)
BMI Kg/m ² mean (SD)	23.3 (1.2)	24.0 (1.2)	24.1 (1.2)

Skewed variables (See Design and Methods) were log-transformed and geometric means (antilogged SD) are indicated. *p* values were >0.05 in all cases.

($p = 0.048$) independently and significantly predicted plasma fibrinogen levels. No significant interaction between fibrinogen values and IL-6 >2.0 pg/mL ($p = 0.084$), and the IL-6 C/G-174 polymorphism ($p = 0.464$) was found.

When the analysis was performed in the subset of individuals with IL-6 plasma levels >2.0 pg/mL ($n = 225$) using a factorial ANOVA, C-RP >0.33 mg/L ($p < 0.001$), sex ($p < 0.001$), age ($p = 0.004$), BMI ($p = 0.026$), and alcohol consumption ($p = 0.032$) were significantly related and total cholesterol ($p = 0.060$) approached a significant level. No significant relationship between fibrinogen values and IL-6 plasma levels ($p = 0.245$), and the IL-6 C/G-174 polymorphism ($p = 0.244$) was found.

Among the 48 individuals with C-RP levels >0.33 mg/L, subjects carrying the IL-6 GG genotype ($n = 26$) had significantly lower plasma fibrinogen levels (antilogged mean [SD]: 326.9 [1.2] mg/dL) than those with IL-6 CG or CC genotype (372.6 [1.2]; $p = 0.013$), whereas no significant difference in plasma IL-6 levels was observed.

Discussion

Prospective epidemiological studies have indicated an independent and significant association of plasma fibrinogen with the development of arterial ischemic episodes, myocardial infarction and stroke.⁶ Plasma levels of fibrinogen rise acutely in response to infection, injury, or other trauma.²¹ Raised concentrations of C-reactive protein have been found to predict recurrent ischemia, myocardial infarction and sudden death among patients with multiple risk factors for coronary artery disease and angina pectoris.¹⁶⁻¹⁷ In the ECAT study, raised levels of C-reactive protein at baseline consistently predicted myocardial infarction or sudden death over the 3 years of follow-up.¹⁷ In the present study, personal, environmental and genetic factors significantly affected plasma fibrinogen levels. In addition, we have confirmed that regardless of the presence of bio-

chemical and lifestyle variables, such as age, obesity, total cholesterol, smoking and alcohol habits, one of the major determinants of fibrinogen values was the elevated levels of C-RP. Increased C-RP concentrations reflect the inflammatory condition of the vascular wall. C-RP is the major acute-phase reactant and is widely used as an indicator of an inflammatory state in humans. IL-6 is a strong regulator of a broad array of acute-phase genes and production of C-RP is stimulated by IL-6.²² In the present setting, C-RP concentrations were associated with BMI, IL-6 levels and alcohol consumption. C-RP and IL-6 have been correlated with each other,²³ have been found to be associated with several markers of endothelial dysfunction and IL-6 has been shown to induce endothelial activation and release of adhesion molecules, such as ICAM-1.²⁴ Thus, it is conceivable that raised C-RP levels, as a surrogate for IL-6, are a non-specific but very sensitive marker of inflammatory response to injury. Vascular injury is an inflammatory and proliferative event, possibly enhanced by smoking and/or activation of the immune system (infection, immune disorders).^{25,26}

Recently, a functional polymorphism within the 5' flanking region of the IL-6 gene locus was identified.¹⁹ Carriers of the GG genotype had higher IL-6 levels at rest and, in constructs expressed in HeLa cells, the G allele showed a significantly higher luciferase activity.¹⁹ The possibility of an inter-individual and genetically determined difference in basal and post-stimulus IL-6 levels suggested that this polymorphism may play a role in the regulation of inflammatory processes, synthesis of acute-phase reactants, such as fibrinogen, and in the susceptibility to myocardial infarction. Plasma fibrinogen levels did not differ among patients with the different genotypes of the IL-6 C/G-174 polymorphism. Furthermore, the number of subjects with basal circulating levels of IL-6 >2.0 pg/mL were similar. A limitation of the present study is the sensitivity of the assay for IL-6 measurement that would affect the probability of detecting a real difference (type II error). Since the size of the study allowed, with a sufficient statistical power, detection of a difference of 14 mg/dL in plasma fibrinogen values between subjects carrying the IL-6 GG genotype and subjects without, lower significant differences would have been undetected. Similarly, among subjects with different IL-6 genotypes, the importance of a difference lower than 11.3% in those with IL-6 plasma levels above 2.0 pg/mL would be understated. When the association of a series of variables with plasma fibrinogen levels was investigated in a multivariate analysis, the effect of C-RP was not affected by either IL-6 concentrations or by the IL-6 C/G-174 polymorphism. Similar findings were observed among subjects with IL-6 plasma levels >2.0 pg/mL. The present data confirm that C-RP may be a stronger predictor of plasma fibrinogen concentrations, whereas they did not support a predictive role for the IL-6 C/G-174 polymorphism, unless among subjects with high C-RP levels. However, since findings from stratifications performed

may be influenced by the low numbers, in the subgroups analyzed, these differences may just reflect the play of chance. A series of *in vitro* studies showed that IL-6 has a direct effect on transcription of the fibrinogen α , β , and γ genes.²⁷⁻²⁹ In addition, besides IL-6, other cytokines, such as IL-1 and IL-11,³⁰ also contribute to the regulation of the acute-phase response and in animal models there is evidence of an IL-6-independent regulation of the C-RP gene.³¹

We conclude that evaluation of the IL-6 C/G-174 polymorphism does not provide additional information useful for identifying subjects with higher plasma fibrinogen levels to that provided by the assessment of circulating acute-phase reactant, i.e. C-RP.

Contributions and Acknowledgments

MM: was the principal investigator responsible for the conception and design, analysis and interpretation of data, drafting the article and revising it critically for important intellectual content, and final approval of the version to be submitted; AB: was deeply involved in analysis and interpretation of data, drafting the article and revising it critically for important intellectual content, and final approval of the version to be submitted; GC was involved in analysis and interpretation of data, drafting the article and revising it critically for important intellectual content, and final approval of the version to be submitted; DC: was deeply involved in analysis and interpretation of data, drafting the article, and final approval of the version to be submitted; EG was involved in conception and design, and interpretation of data, drafting the article and revising it critically for important intellectual content, and final approval of the version to be submitted; GdiM was deeply involved in conception and design, drafting the article and revising it critically for important intellectual content; and final approval of the version to be submitted. The order of authorship is a joint decision of the co-authors. The order of authorship (i.e., the criteria for the order in which their names appear) was: importance of the contribution to the realization of the whole study from the conception and design to the approval of the final version of the manuscript.

Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

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Potential implications for clinical practice

Routine measurements of IL-6 plasma levels or evaluation of the IL-6 C/G-174 polymorphism do not add further clinical information to that that obtained from plasma fibrinogen and CRP evaluation.

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