Combined effects of smoking and interleukin-6 and C-reactive protein genetic variants on endothelial function, inflammation, thrombosis and incidence of coronary artery disease

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Tobacco smoking is an established risk factor for coronary artery disease (CAD) and overwhelming data indicate that smoking fuels the fire of inflammation and thrombosis, which in turn play a key role in the initiation and progression of atherosclerotic process [1]. Moreover, smoking impairs endothelial function via the suppression of nitric oxide promoting thus further the atherosclerotic mechanisms [1]. Two single nucleotide polymorphisms, the −174 G/C polymorphism on interleukin-6 (IL-6) gene promoter (rs1800795) and the 3872 A/G polymorphism on the 3′ flanking region of C-reactive protein (CRP) gene (rs1205) have been found to being associated with altered expression of these inflammatory molecules and studies have explored their role in the risk for CAD [2,3]. However, no study so far has examined a possible combined effect of rs1205 with smoking on the incidence of CAD, while only few have addressed an analogous question for rs1800795 [4–8]. Moreover, to best of our knowledge, least data exist concerning the synergistic role of the aforementioned polymorphisms with smoking in endothelial function and no study has focused on such an effect on the expression of several inflammatory and coagulation markers [6]. Therefore, in the present study we investigated the synergistic effect of smoking with the specific gene variants of rs1800795 and rs1205 on endothelial function, on the expression of IL-6, tumor necrosis factor-a (TNF-a), high sensitivity CRP (hsCRP), fibrinogen, sC40L and D-dimers as well as on the risk for CAD.

Our study consisted of 646 subjects of single Caucasian origin (285 smokers), who were submitted to our department due to stable angina pectoris symptoms. After undergoing thorough clinical examination, all participants were subjected to coronary angiography in our department. Participants were categorized as CAD patients determined by a >50% stenosis in one of three coronary arteries or major branches or as controls if they had a normal coronary angiogram. Smoking status was clearly determined by a special detailed questionnaire, with smokers being characterized those currently smoking >1 cigarette per day for at least 1 year and non-smokers those with no history of tobacco use the last 5 years before stratification and with history of <100 cigarettes during lifetime. Subjects referring themselves as intermittent smokers or not giving full and persuasive background of their smoking habits were excluded from the study. The existence of hypertension, diabetes mellitus II and dyslipidemia was based on current guidelines for the diagnosis of the diseases. Exclusion criteria were: age >75 years, chronic heart failure with an ejection fraction < 45%, renal or liver abnormalities, diabetes type I, malignancies of any type, existence of any inflammatory, infectious or thrombophilic disease and documentation of deep vein thrombosis, pulmonary embolism, stroke or myocardial infarction (MI) (for CAD patients) in the last 6 months before hospitalization. Informed consent was obtained from each patient and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki, while also being approved by the institution's human research committee. The authors of this manuscript have certified that they comply with the Principles of Ethical Publishing in the International Journal of Cardiology.

The rs1800795 and rs1205 were determined with appropriate genotype techniques, as we have previously described [9,10]. IL-6 (pg/ml), TNF-a (pg/ml) and sC40L (ng/ml) were assessed with an enzyme linked immunosorbent assay (ELISA) (R&D Systems, Bender MedSystems), while hsCRP (mg/l) (CRP reagent, Dade Behring) and D-dimers (μg/l) (Innovance D-Dimer, Siemens) with immunonenphelometric methods. Fibrinogen (mg/dl) was determined with the Clauss method (Multifibren U, Siemens). Endothelial function was measured with flow mediated dilation (FMD), as previously described [11].

Continuous variables are presented as mean and standard deviation if are normally distributed or median and interquartile range (75th percentile–25th percentile) if their distribution is skewed. Student's *t* test was implemented to test for differences between the various characteristics among cases and controls, where applicable, or Mann–Whitney test was used if the assumptions of *t*-test were not met. Chi square test was used for comparisons between categorical variables, using the homozygotes of the common allele as baseline variable. Simple and multiple logistic regression analysis were used for the estimation of the crude and adjusted odds ratio between the polymorphisms' carriers and the several clinical outcomes of the study. The choice of the confounders was based on the published literature about the topic. P value of <0.05 was considered to indicate statistical significance. Assuming a 1:1 ratio between smoking groups, a frequency of 0.7 for the major allele of rs1205 (G allele) and a frequency of 0.4 for the minor allele of rs1800795 (C allele) according to previous studies, our a priori power calculation indicated that we would need a total sample of at least 572 subjects in order to detect a 55% increase in the risk for CAD for the main key variables, G carriers vs AA for rs1205 and C carriers vs GG for rs1800795 with a power of 80% and statistical significance of a = 0.05. Our sample was powered enough (95%) to detect standardized differences of 0.25 (25%) for IL-6, hsCRP, FMD, fibrinogen values between controls' and patients' group with a significance level a = 0.05. All statistical calculations were performed using SPSS (version 18.0; SPSS, Chicago, IL).

Our study polymorphisms were not in LD and did not deviate from Hardy–Weinberg equilibrium (rs1800795: $X^2 = 3.33$, p = 0.64, rs1205: $X^2 = 0.37$, p = 0.61) [12]. The polymorphic allele of rs1800795 had a frequency of 0.57, while this of rs1205 had a frequency of 0.62. Demographic data of the study participants as well as the crude odds

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Table 1
Demographic characteristics of study participants and the crude effect of polymorphisms on the risk for CAD.

	Non-smokers (n = 361)		Smokers (n = 285)		p[d]
	CAD (n = 197)	Controls (n = 164)	CAD (n = 150)	Controls (n = 135)	
Age[a] (years)	63 (10.5)	57.2 (10.2)	63.2 (9.6)	56.9 (12)	0.92
Males[a]	177 (89.8)	94 (57.3)	100 (66.7)	89 (65.9)	0.018
Hypertension[a]	126 (63.9)	103 (62.8)	117 (78)	52 (38.5)	0.46
Diabetes[a]	56 (28.4)	27 (16.5)	52 (34.7)	17 (12.6)	0.78
Dyslipidemia[a]	126 (63.9)	97 (59.1)	116 (77.3)	52 (38.5)	0.47
History Of MI[a]	58 (29.4)	–	50 (33.3)	–	0.67
Family history of CAD[a]	86 (43.7)	36 (22)	57 (0.38)	22 (16.3)	0.1
F.M.D. (%)[b]	4.16 (2.56)	6.32 (2.76)	3.35 (2.57)	6.22 (3.15)	0.052
hsCRP (mg/l)[b]	2.19 (0.71)	1.17 (0.49)	2.33 (0.79)	0.94 (0.54)	0.75
IL-6 (pg/ml)[b]	2.67 (1.27)	1.51 (0.57)	3.35 (1.4)	1.31 (0.54)	0.006
TNF-a (pg/ml)[b]	5.58 (1.05)	2.1 (0.67)	6.49 (1.26)	1.71 (0.6)	0.056
Fibrinogen (mg/dl)[b]	446.2 (133.3)	360.8 (84.1)	449 (136.5)	398.9 (112.5)	0.13
sCD40L (ng/ml)[b]	2.21 (1.94)	0.74 (1.83)	2.03 (1.88)	1.05 (2.44)	0.38
D-dimers (μg/l)[b]	388.3 (305.3)	268.2 (230)	314.5 (411.9)	314.5 (172.9)	0.57
rs1800795 genotype distribution[a]					0.002
GG	109 (55.3)	64 (39)	36 (24)	67 (49.6)	
GC	76 (38.6)	72 (43.9)	71 (47.3)	57 (42.2)	
CC	12 (6.1)	28 (17.1)	43 (28.7)	11 (8.2)	
rs1205 genotype distribution[a]					0.023
GG	61 (31)	88 (54.9)	75 (50)	20 (14.8)	
AG	94 (47.7)	72 (42.7)	60 (40)	74 (54.8)	
AA	42 (21.3)	4 (2.4)	15 (10)	41 (30.4)	
		Crude ORs[c]		Crude ORs[c]	
rs1800795 on CAD					
C carriers vs GG (recessive)		0.52 (0.34–0.8), p = 0.002		3.1 (1.8–5.2), p < 0.001	
CC vs G carriers (dominant)		0.3 (0.15–0.6), p = 0.001		4.6 (2.2–9.3), p < 0.001	
rs1205 on CAD					
A carriers vs GG (recessive)		2.6 (1.7–4), p < 0.001		0.2 (0.1–0.3), p < 0.001	
AA vs G carriers (dominant)		10.8 (3.7–30), p < 0.001		0.2 (0.1–0.4), p < 0.001	

Abbreviations. CAD: coronary artery disease, F.M.D.: flow mediated dilation, hsCRP: high sensitivity CRP, IL-6: interleukin-6, MI: myocardial infarction, OR: odds ratio, TNF-a: tumor necrosis factor-a.
Data are presented as:
[a] Absolute frequencies (relative freq.).
[b] Mean (standard deviation) or median (interquartile range).
[c] Odds ratio (confidence intervals).
[d] Values refer to comparisons between non-smokers and smokers.

ratio of the various genotypes on the risk for CAD in the various groups are presented in Table 1. We have found a statistically significant negative correlation of minor C allele of rs1800795 on the risk for CAD in non-smokers (OR: 0.42, CI: 0.26–0.68), while we observed the opposite effect of the specific allele on the risk for CAD in the smoking group compared to GG homozygotes (OR: 1.59, CI: 1.256–2.933), even after adjustment for age, sex, hypertension, dyslipidemia and diabetes (Table 2). Impressively, we have found a similar effect for the major G allele of rs1205. More specifically, the G allele demonstrated a negative association with the risk for CAD in non-smokers (AA vs G carriers: OR: 11.12, CI: 3.82–32.31), whereas a contradictory positive association with the incidence of CAD was found in smokers (AA vs G carriers, OR: 0.33, CI: 0.16–0.69), even after adjustment for age, sex, hypertension, dyslipidemia and diabetes (Table 2). Moreover, when the interaction term between smoking and gene allele variables was included in the multiple logistic regression analysis, a statistical significant p-value was revealed ($p < 0.001$). Since genotype itself, after adjustment for major risk factors for CAD, and smoking alone, corrected for the genotype and the major cofounders of CAD, were not related with the incidence of CAD (Table 2), we further examined whether the aforementioned combined effect of certain genotypes with smoking on the risk for CAD could be attributed to inflammatory and thrombotic processes. Importantly, both polymorphisms were related with altered inflammatory and thrombotic profile as well as endothelial function in both study groups. In particular, we observed a constant stimulating effect of the C allele of rs1800795 on all inflammatory markers of the study in both groups compared to GG homozygotes (*smokers*: TNF-a: 5.39 ± 2.41 vs 2.57 ± 1.82, IL-6: 3.09 ± 577.2 vs 319.4 ± 308.6) in non-smokers ($p \leq 0.004$ for all), while an opposite effect was demonstrated among smokers, where the G allele carriers had importantly higher values of TNF-a (4.89 ± 2.55 vs 2.37 ± 1.62), IL-6 (2.7 ± 1.52 vs 1.47 ± 0.89), hsCRP (1.9 ± 0.95 vs 0.97 ± 0.7), fibrinogen (442.2 ± 134.4 vs 365.4 ± 76.8) ($p < 0.001$ for all) and significantly impaired endothelial function (4.8 ± 2.9 vs 5.71 ± 3.04, $p = 0.003$) (Fig. 1).

Our results indicate an important synergistic effect of smoking with the minor allele of rs1800795 (C allele) and the major allele of rs1205 (G allele) on the risk for CAD. Moreover, we showed an important combined effect of smoking with the aforementioned polymorphisms on endothelial function, which is the initial and requisite step toward atherosclerotic mechanisms. The composite effect of the C allele of rs1800795 could be in part be explained by its striking impact on fibrinogen expression, since the effect on inflammatory markers (hsCRP, IL-6, TNF-a) was not different between the study groups. The detrimental impact of the C allele on FMD and on D-dimers in smokers could be an additive novel pathway through which this synergistic action could be explained. Only few studies have focused on such an interaction between smoking and gene variants of rs1800795, with results mainly analogous to our findings [4–8]. Similar to rs1800795, smoking was found to exert a synergistic effect with G allele of rs1205 on the risk for CAD in the smoking group, while this allele was presented to act protectively in non-smokers. This discrepancy could be largely explained by the significantly enhanced expression of the inflammatory markers (TNF-a, IL-6, hsCRP) as well as of fibrinogen in G carriers compared to AA homozygotes in smokers and the simultaneous down-regulating effect of the specific allele compared to AA homozygotes on TNF-a, hsCRP, fibrinogen and D-dimers in non-smokers. In addition, the important association of