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Smoking and FOS expression from blood leukocyte transcripts in patients with coronary artery disease

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ABSTRACT

Objective: Analysis of the leukocyte transriptome, in particular the Finkel-Biskis-Jinkins Osteosarcoma (*c-Fos*) gene, which has a prominent role in inflammation, provides new insights into atherosclerosis mechanisms. Although smoking is a major risk factor, the links between smoking status and coronary artery disease (CAD) remains unclear. We aimed to analyze the relationship between smoking status and *c-Fos* expression in circulating leukocytes of patients with CAD.
Methods: c-Fos expression was measured by RT-Q-PCR, from blood leukocytes of 239 consecutive patients after acute myocardial infarction (MI). The patients were asked about their smoking status and stratified into 3 groups: current smokers (CS) ($N = 85$), past smokers (PS) ($N = 78$) and never smokers (NS) ($N = 76$).
Results: NS had a higher risk profile including hypertension, and CS were younger than PS and NS (−13 years and 17 years respectively). There was only a trend towards lower CRP levels in NS and PS than in CS. The mean *c-Fos* transcript level was slightly higher in CS than in PS and NS (0.924 vs. 0.908 and 0.861 AU, respectively; $p = 0.005$). By univariate analysis, neither age, nor sex, nor CRP nor white blood cell count was associated with *c-Fos* transcript levels. By multivariate analysis, CS (vs. PS + NS) was the strongest predictor of the *c-Fos* transcript level, ($B = 0.042 \pm 0.014$, $p = 0.003$), even after adjustment for confounding factors (i.e. hypertension, chronic medication, family history of CAD, and prior MI).
Conclusion: Our work suggests that c-Fos transcript level in blood leukocyte could be considered a cumulative biomarker of smoking. As the *c-Fos* gene has been put forward as a new factor in the progression and severity of atherosclerosis, it could be considered a novel potential pathway of tobacco toxicity in coronary artery disease.

1. Introduction

Smoking is a major avoidable cause of cardiovascular disease worldwide [1] and was responsible for around 100 million deaths during the 20th century. This could rise to 1 billion deaths during the 21st century [2]. Smoking is associated with circulatory and vascular alterations, including atherosclerosis [3], endothelial dysfunction [4], altered blood lipid profile [5], increased platelet aggregation [6] and oxidative stress-associated biomarkers of inflammation [7]. However, the mechanisms by which smoking influences atherosclerosis, and in particular coronary artery disease (CAD), are still not completely understood.

Finkel-Biskis-Jinkins Osteosarcoma (*c-Fos*) stress-induced transcription factors, which respond to and integrate many signaling pathways, are involved in inflammation and pathogenic changes in atherosclerosis [8]. *c-Fos* has been shown to be strongly expressed in the leukocytes of patients with atherosclerosis and has been described as a better marker of atherosclerosis than CRP [8]. Moreover, circulating *c-Fos* transcripts have also been shown to provide additional insights into the biology of cardiovascular (CV) diseases, suggesting that *c-Fos* expression in the blood could serve as a sensitive marker to improve treatment outcome [9]. However, there are very few data on the impact of smoking on this key oncogene. Comprehensive gene expression profiles reveal that *c-Fos* is specifically expressed in the lung tissue of smokers [10]. In adult rat aortic VSMC and adventitial fibroblasts, exposure to nicotine, a major

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component of cigarette smoke, exerts growth-promoting effects on vascular cells through the enhancement of angiotensin-induced DNA synthesis and *c-Fos* expression [11].

In a prospective study, we aimed to analyze the relationship between smoking status and *c-Fos* mRNA expression in the blood leukocytes of patients with CAD.

2. Methods

2.1. Patients

All consecutive patients aged >18 years and hospitalized <24 h after symptoms onset for acute myocardial infarction (MI) in the coronary care unit of Dijon University Hospital between 1st March and 31st July 2009 were included. Patients with malignancy were excluded from the study. MI was defined by an increase in serum troponin I (2× upper limit of the hospital normal range) associated with symptoms of ischemia and/or characteristic electrocardiographic (ECG) signs. The present study complied with the Declaration of Helsinki and was approved by the ethics committee of University Hospital of Dijon. Each patient gave written consent before participation.

2.2. Data collection

For each patient, data on demographics, risk factors (history of hypertension, diabetes, hyperlipidemia, obesity {defined as a body mass index (BMI) ≥ 30}), a family history of CAD [i.e. parental history of premature (<55 years for men and <65 years for women) CAD in first degree relative] and prior MI were prospectively collected. Clinical data, including hemodynamic parameters on admission, left ventricular ejection fraction (LVEF) and location of infarction were recorded. LVEF was determined by echocardiography at 2 ± 1 days after admission.

2.3. Angiographic data

The coronary angiography was performed via the femoral or radial approach. The number of diseased vessels (stenosis >50%) was determined. The angiograms were analyzed by 2 expert investigators blinded to the clinical and biological data. Among the study population ($n = 239$), 15(6%) patients did not undergo coronary angiography.

2.4. Biological data

Blood samples were drawn at admission [time from symptom onset to blood sampling: 240(160–579) min] to assess biological variables.

Homocysteine concentrations were determined by chemiluminescence on an Immulite 2000 analyzer (Diagnostic Products Corporation, Los Angeles, USA), C-reactive protein (CRP), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C) and triglyceride (TG) concentrations were measured on a Dimension analyzer (Dade Behring, Newark, NE) using immunonephelometry assay. Low-density lipoprotein cholesterol (LDL-C) levels were calculated using the Friedewald formula [12]. Plasma glucose concentrations [enzymatic method (glucose oxidase)] and creatinine levels were measured on a Vitros 950 analyzer (Ortho Clinical Diagnostics, Rochester, NY). The MDRD (Modification of the Diet in Renal Disease) level was calculated as $186.3 \times (\text{Cr}) - 1.154 \times (\text{age}) - 0.203 \times (0.742 \text{ if female}) \times (1.21 \text{ if African American})$ [13]. Glycated hemoglobin A1 C (HbA1c) was measured with ion exchange HPLC (Bio-Rad Laboratories, Richmond, CA). Creatine kinase (CK) peaks were assessed by sampling every 8 h during the first two days after admission (Dimension Vista Intelligent Lab System, Siemens). Plasma N-terminal pro B-type natriuretic peptide (NT-proBNP) was determined by ELISA with an Elecsys NT-proBNP sandwich immunoassay on Elecsys 2010 (Roche Diagnostics, Basel, Switzerland). Peak creatine kinase (CK) was assessed by sampling every 8 h during the first two days after admission (Dimension Vista Intelligent Lab System, Siemens).

2.5. mRNA extraction and Quantitative RT-PCR

Blood samples were salted-out in a lysing solution (Tris HCL pH 7.5; 50 mM; MgCl2 25 mM; NaCl 50 mM) to obtain a total leukocyte pellet. mRNA were obtained using a TRIZOL® (Invitrogen, Paisley, UK) extraction assay protocol on the leukocyte pellet, and reverse transcribed using Murine Moloney Leukemia Virus Reverse Transcriptase (Invitrogen, Paisley, UK).

The levels of the transcripts were quantified by the relative gene expression analysis method using Q-PCR technique, in duplicate at least two or three times. Briefly, for each amplification, aliquots of cDNA from 50 ng of total RNA were amplified by the Q-PCR technique on the Rotorgene 3000 (Corbett Research, Cambridge, UK), using the Quantifast SYBR Green PCR kit (Qiagen S.A., France) in a 25 μL reaction volume. PCR amplification was run on a Light Cycler 480 (Roche Diagnostics, France). The GAPDH gene was selected as the reference (normalizing) gene after testing the homogenous expression of three genes (GAPDH, B2M and ACTB) using geNorm software. Each gene sequence was amplified in triplicate for 45 cycles under standardized conditions and the results were expressed as the mean of the three Cycle Threshold (Ct) values. The reaction mixture contained 200 ng of each primer and the PCR cycle parameters were: 94° for 15 s, 60° for 1 min, 72° for 40 s. The sequences of the primers selected by the Primer3 software (Whitehead Institute for Biomedical Research) were tested for their specificity by obtaining the melting curves at the end of each PCR. The *c-Fos* specific primers were: forward 5′-GGA GGA CCT TAT CTG TGC GTG A-3′, and reverse 5′-GAA CAC ACT ATT GCC AGG AAC ACA-3′. The GAPDH specific primers were: forward 5′-CAT CTC TGC CCC CTC TGC T-3′, and reverse 5′-ACG CCT GCT TCA CCA CCT T-3′. The PCR efficiency was established at 0.95 by the standard curve. RT-PCR was normalized by measuring the average Ct ratio in the investigated genes and in the control gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

2.6. Group definition

Patients were rigorously asked about their self-reported smoking status by structured interviews conducted at the time of their hospital admission. Current smokers and patients who had stopped smoking <3 months earlier were classified as current smokers. Patients who had stopped smoking ≥3 months earlier were classified as past smokers and those who had never smoked were classified as never smokers.

2.7. Statistical analysis

Continuous variables are presented as medians (25–75th percentile) or means ± SD, as appropriate or as proportions. For continuous variables, a Kolmogorov–Smirnov analysis was performed to test normality. One Way ANOVA was performed to compare the 3 groups (current, past and never smokers). Dichotomous variables, expressed as numbers (%), were compared by Chi-square tests for the 3 groups. For the comparison between 2 groups (univariate predictor of c-FOS level, Table 2), Pearson's or Spearman's rank correlation was used for continuous variables, and Student *t* test or Wilcoxon test was used for dichotomous variables. Multiple linear regression analysis was performed with the *c-Fos* transcript level as a dependent variable, to test the indepen-

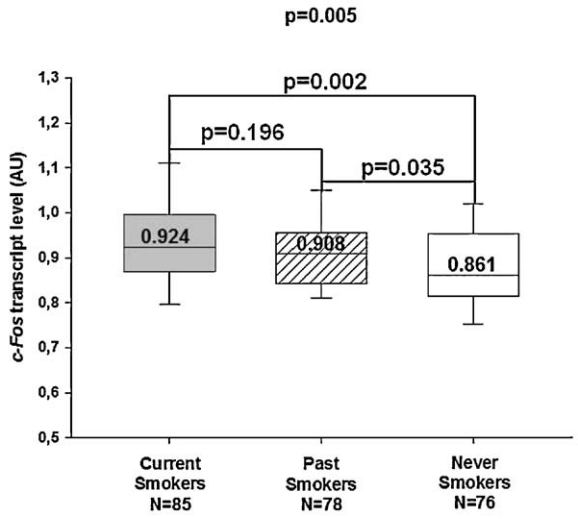

Fig. 1. *c-Fos* transcript level according to smoking status (AU). Patients who currently smoke or had stopped smoking <3 months were classified as current smokers ($n = 85$). Patients who had stopped cigarette consumption ≥3 months were classified as past smokers ($n = 78$) and those who had never smoked were classified as never smokers ($n = 76$).

dent predictors of FOS expression. Variables entered into the model were those with a significant relationship ($p < 0.05$) in univariate analysis. Given that only CS, but not PS or NS, is expected to be associated with the risk of MI, we focused our multivariate analysis on CS for more clinical relevance. Smoking was then entered as a dichotomous variable (current smoking vs. past or never smokers). In accordance with the Bonferroni correction, the alpha value was lowered to 0.02 to account for the number of comparisons being performed.

3. Results

3.1. Characteristics of the study population

During the inclusion period, 239 patients were included in the study. Baseline characteristics of the population according the smoking status are shown in Table 1. As expected, never smokers were the oldest and current smokers the youngest.

Furthermore, never smokers had a higher risk profile, including hypertension and hypercholesterolemia. CS were younger than PS and NS (−13 years and 17 years respectively) and more likely to be male. Cardiovascular drugs, except aspirin, were more frequently used in the PS and NS groups. Blood glucose levels were lower for CS. Kidney function as assessed by MDRD gradually deteriorated from current to never smokers, as age increased. CRP levels was similar for the 3 groups.

One major finding of our study was that the mean *c-Fos* transcript level in CS was markedly higher than that in PS and the difference was even greater for NS (0.924 [0.869–0.997] AU vs. 0.908 [0.841–0.955] AU and 0.861 [0.817–0.958] AU, respectively, $p = 0.005$) (CS vs. PS: $p = 0.196$; PS vs. NS: $p = 0.035$; CS vs. NS: $p = 0.002$) (Fig. 1).

3.2. Determinants of c-Fos transcript level

All of the parameters listed in Table 1 were tested for their relationship with the *c-Fos* transcript level, and the results are presented in Table 2. The *c-Fos* transcript level gradually increased with HbA1c and homocysteine levels ($p = 0.125$ and $p = 0.168$ respectively). Neither blood lipids (LDL-cholesterol, triglyceride, HDL-cholesterol or total cholesterol), nor systemic inflammation markers (leukocyte count or CRP) were significantly associated with the *c-Fos* transcript level.

In contrast, *c-Fos* transcript levels tended to be higher in hypertensive patients ($p = 0.188$). Interestingly, patients with a family history of CAD or prior MI had a lower *c-Fos* transcript level ($p = 0.070$ and $p = 0.064$, respectively). No clinical parameter was significantly associated with the biomarker. The *c-Fos* transcript level was slightly lower in patients under ACE inhibitors ($p = 0.174$) or beta blockers ($p = 0.067$), which could explain the lower level of *c-Fos* in patients with prior MI. The frequency of diabetes in patients with prior MI was similar to that in patients without prior MI (9(31%) vs. 48(19%), $p = 0.110$). Moreover, the levels of homocysteine and HbA1c were similar in patients with and without prior MI (14.6 ± 6.8 μmol/L vs. 13.6 ± 6.3 μmol/L, $p = 0.440$ and 6.2 ± 1.1 vs. 6.1 ± 1.2, $p = 0.572$, respectively).

We further performed linear regression analyses to test the predictors of *c-Fos* transcript levels. Four variables showed a significant association with *c-Fos* transcript levels by univariate analysis ($p < 0.05$) (i.e. current smoking, family history of CAD, prior MI, treatment with ACE inhibitors or beta blockers. In order to avoid multicollinearity between prior MI and chronic treatment with ACE inhibitors or beta blockers, only prior MI was introduced into the model. The multivariate model showed that only smoking status and a family history of CAD remained independent predictors of the *c-Fos* transcript level ($\beta = 0.042$, SE = 0.014, $p = 0.003$ and $\beta = -0.036$, SE = 0.015, $p = 0.016$, respectively; $R^2 = 0.075$). Current smoking was the strongest predictor of elevated levels of *c-Fos* transcripts ($p = 0.003$). Given the major difference in risk factors among the 3 smoking status groups, in particular for age, gender, hypertension, diabetes and hypercholesterolemia, we performed a series of additional analyses by including each of these variables in the multivariate linear regression model. These risk factors were included as forced individual variables, with prior MI, a familial history of CAD and current smoking as covariates. Neither age ($p = 0.202$), female gender ($p = 0.697$), hypertension ($p = 0.292$), diabetes ($p = 0.862$) nor hypercholesterolemia ($p = 0.397$) were significantly associated with FOS. These additional analyses did not alter the strength of the predictive value of smoking on the *c-Fos* transcript levels. Patients on chronic therapy with ACEi or Betablockers showed a trend towards lower levels of FOS ($p = 0.174$ and $p = 0.067$, respectively (Table 2)). Treatments such as ACEi or betablockers may therefore have affected FOS transcript levels. In a comprehensive univariate linear regression analysis, we tested treatments with ACEi or betablockers for their relationship with FOS levels. The use of betablockers or ACEi was associated with only a trend towards lower levels of FOS ($\beta = -0.032$, SE = 0.017, $p = 0.057$; $\beta = -0.017$, SE = 0.017, $p = 0.310$). Only the use of the combined variable [betablocker or ACEi] was significantly associated with lower FOS transcript levels ($\beta = -0.032$, SE = 0.015, $p = 0.027$). When included in the multivariate model including familial history and smoking, this combined variable was not independently associated with FOS transcript levels ($\beta = -0.025$, SE = 0.015, $p = 0.089$). Prior MI was not included in this model to avoid multicollinearity.

4. Discussion

Our prospective study in patients with CAD shows for the first time a strong association between *c-Fos* gene transcript levels in blood leukocytes and smoking. Our data further suggest that this association is strikingly independent of classical inflammation markers such as CRP or white blood cells (WBC). *c-Fos is* a transcription factor involved in many signaling pathways, and given its prominent role in atherosclerosis severity, our study suggests that it may be implicated in a novel pathophysiological mechanism that underlies the biological effects of smoking.

Table 1
Patients characteristics. *n*(%), mean ± SD or median (IQR).

	Current smokers, *N* = 85	Past smokers, *N* = 78	Never smokers, *N* = 76	*p*
Risk factors				
Age, years	55 ± 10	68 ± 13	72 ± 12	<0.001
Female	13(20)	6(8)	40(52)	<0.001
Hypertension	27(32)	42(54)	48(63)	<0.001
Obesity	16(19)	16(21)	19(26)	0.619
Diabetes	15(18)	11(14)	22(29)	0.056
Hypercholesterolemia	29(34)	39(50)	36(47)	0.089
Family history of CAD	28(33)	20(26)	21(34)	0.566
Prior MI	6(7)	10(13)	8(11)	0.467
Clinical data				
LVEF, %	54 ± 12	54 ± 12	54 ± 12	0.128
Systolic blood pressure, mmHg	138 ± 30	139 ± 28	141 ± 31	0.815
Diastolic blood pressure, mmHg	82 ± 19	82 ± 19	78 ± 17	0.429
Heart rate, b/min	76 ± 21	76 ± 15	82 ± 20	0.053
Anterior wall location	27(31%)	27(35%)	31(42%)	0.349
Chronic treatments				
ACE inhibitor	11(12)	21(27)	21(28)	0.033
Statin	13(15)	26(33)	23(30)	0.017
Aspirin	15(18)	15(19)	13(17)	0.955
Betablocker	10(12)	18(23)	26(34)	0.002
Fibrate	3(4)	0(0)	6(8)	0.032
Oral antidiabetic drug	8(9)	8(10)	15(20)	0.086
Biological data				
Time to sampling, min	230(140–530)	240(169–639)	206(170–560)	0.267
Glucose, mmol/L	6.5(5.5–8.0)	7.2(6.0–8.5)	7.2 (6.2–10.0)	0.017
HbA1c, %	5.7(5.5–6.1)	5.7(5.5–6.2)	5.8(5.5–6.3)	0.394
MDRD, mL/min	86.0(70.9–100.4)	73.0(56.8–89.9)	66.4(51.6–77.7)	<0.001
CRP, mg/L	4.6(2.9–9.75)	5.0(2.9–11.9)	6.0(2.9–24.9)	0.468
Homocysteine, μmol/L	12(9–18)	12(9–15)	12(9–16)	0.421
HDL-cholest, g/L	0.43(0.32–0.49)	0.42(0.36–0.50)	0.45(0.38–0.53)	0.129
Triglycerides, g/L	1.40(0.93–2.11)	1.35(0.89–1.87)	1.15(0.80–1.80)	0.120
LDL-cholest, g/L	1.24(0.97–1.52)	1.22(0.97–1.51)	1.21(0.93–1.46)	0.730
Total-cholest, g/L	1.99 ± 0.51	1.97 ± 0.45	1.97 ± 0.52	0.978
Leukocyte count, $10^3/mm^3$	11.0(9.3–13.6)	9.3(7.8–11.4)	10.1(8.6–12.6)	<0.001
CK peak, IU/L	692.5(207.5–1639.5)	473.5(159.3–1430.0)	541.5(224.0–1828.5)	0.397
Angiographic data	*N* = 84	*N* = 73	*N* = 67	
Number diseased vessels				0.009
0	1(1%)	4(5%)	2(3%)	
1	41(49%)	27(37%)	28(42%)	
2	34(40%)	21(29%)	16(24%)	
3 or LM	8(9%)	21(29%)	21(31%)	

ACE, inhibitor: angiotensin-converting enzyme inhibitor; CAD, coronary artery disease; CK: creatine kinase; CRP, C-reactive protein; LM, left main; LVEF, left ventricular ejection fraction; MDRD, Modification of the Diet in Renal Disease; MI, myocardial infarction; CAD, coronary artery disease; MI, myocardial infarction; LVEF, left ventricular ejection fraction; STEMI: ST segment elevation MI.

Circulating leukocytes, which are exposed to the systemic environment including risk factors, are directly involved in low-grade inflammation related to atherosclerosis, may be useful to improve phenotype of the patient. Furthermore, using new techniques it is possible to explore the leukocyte transriptome to assess the systemic consequences of CV risk factors, including smoking [14]. A recent large community-based cohort study demonstrated the clinical correlates of gene expression, and the results were consistent with the hypothesis that leukocyte inflammatory transcripts play a role in the pathogenesis of CAD and its risk factors [15].

4.1. c-Fos and atherosclerosis

The *c-Fos* gene is rapidly activated in the presence of different stimuli, resulting in a mitogenic effect, which has been shown in many proliferative processes, particularly during embryonic development and in some types of tumor [16,17]. The *c-Fos* gene is also known to regulate the growth, differentiation, and migration of a variety of vascular and non-vascular cells, and to regulate matrix degradation [18]. A panel of genes, including *c-Fos*, that were differentially expressed in microarray analysis, was clearly able to identify acute ischemic stroke patients from healthy subjects [19]. Immunohistological analyses have shown that *c-Fos* expression characterizes the initial phases of the destabilization of unstable atherosclerotic carotid plaques [20]. Moreover, *c-Fos* is highly expressed and collocated with macrophage CD14+ immunoreactivity in human coronary artery plaque, suggesting its potential involvement in pathogenesis [8]. *c-Fos* overexpression may also promote early atherosclerotic coronary lesions by causing the proliferation of smooth muscle cells of the media [21]. Interestingly, an association between early atherosclerosis and *c-Fos* transcription was found in arterial biopsies of prenatal infants and infants with mothers who smoked [22].

4.2. c-Fos and smoking

Our study, which was supported by multivariate adjustments that included many demographic and biological variables, showed for the first time that in CAD patients, *c-Fos* transcript levels were associated with smoking status, independently of classical markers of inflammation, such as CRP or WBC. These findings are consistent with data from the literature, which show that conventional markers of inflammation are not significantly higher in smokers than in non-smokers. In a large contemporary randomized prospective clinical trial of current smokers, smoking intensity was not associated with CRP levels, and smoking cessation did not reduce CRP levels [23]. The effect of smoking reported in the present study seems specifically targeted to the blood

Table 2
Univariate predictors of *c-Fos* transcript levels (AU).

		Median	IQR	*r*	*p*
Risk factors					
Age				+0.005	0.943
Female	Yes	0.900	0.837–0.969		0.703
	No	0.900	0.838–0.980		
Hypertension	Yes	0.891	0.827–0.957		0.188
	No	0.906	0.848–0.993		
Obesity	Yes	0.900	0.584–0.970		0.980
	No	0.897	0.835–0.980		
Diabetes	Yes	0.909	0.840–0.970		0.993
	No	0.891	0.837–0.981		
Hypercholesterolemia	Yes	0.925	0.839–0.987		0.274
	No	0.891	0.836–0.963		
Family history of CAD	Yes	0.891	0.805–0.953		0.070
	No	0.907	0.848–0.990		
Prior MI	Yes	0.891	0.824–0.925		0.064
	No	0.908	0.840–0.987		
Clinical data on admission					
LVEF				+0.007	0.919
Systolic blood pressure				−0.083	0.205
Diastolic blood pressure				−0.093	0.158
Heart rate				0.073	0.268
Anterior wall	Yes	0.910	0.847–0.984		0.326
	No	0.834	0.691–0.975		
Chronic treatments					
ACE inhibitor	Yes	0.886	0.823–0.943		0.174
	No	0.908	0.845–0.982		
Statin	Yes	0.909	0.848–0.973		0.796
	No	0.895	0.836–0.979		
Aspirin	Yes	0.900	0.855–0.946		0.775
	No	0.901	0.836–0.991		
Betablocker	Yes	0.893	0.814–0.935		0.067
	No	0.909	0.846–0.989		
Fibrate	Yes	0.979	0.821–1.055		0.226
	No	0.897	0.838–0.975		
Oral antidiabetic	Yes	0.900	0.837–0.978		0.397
	No	0.925	0.838–0.979		
Biological data					
Onset to sampling				+0.084	0.213
Glucose				+0.003	0.969
HbA1c				+0.106	0.125
MDRD				+0.067	0.302
CRP				+0.012	0.852
Homocysteine				+0.098	0.168
HDL-cholest				+0.066	0.329
Triglycerides				−0.015	0.829
LDL-cholest				−0.004	0.949
Total-cholest				−0.004	0.952
CK peak				+0.009	0.895
NT-proBNP				+0.038	0.568
Leukocyte count				−0.019	0.711
Angiographic data					
Number of diseased vessels					0.465
	0	0.848	0.811–0.908		
	1	0.889	0.825–0.979		
	2	0.909	0.856–0.991		
	3 or LM	0.899	0.837–0.958		

ACE, inhibitor: angiotensin-converting enzyme inhibitor; CAD, coronary artery disease; CK, creatine kinase; CRP, C-reactive protein; LVEF, left ventricular ejection fraction; MDRD, Modification of the Diet in Renal Disease; MI, myocardial infarction.

leukocyte transcriptome such as *c-Fos*. The results of the multivariate analysis also showed that the lower use of treatments such as betablocker or ACEi does not explain, or only partly explains the elevated levels of FOS in the CS group. There was a gradual increase in expression according to smoking status. Past smokers had a significantly higher level of *c-Fos* transcript than never smokers but a lower level than that in current smokers. This reversible relationship is consistent with the known reversible CV risk associated with tobacco use and suggests that the *c-Fos* transcript level could be considered a cumulative biomarker of exposure to cigarette smoke. The underlying mechanism of the high levels of *c-Fos* induced by smoking remains unclear. Cigarette smoke is a complex mixture of >4000 chemical substances that are distributed in particulate and gaseous phases. It has been difficult to identify the pathophysiologically relevant components of cigarette smoke. Nicotine combustion products, the major psychoactive component in tobacco smoke, could promote the activation of genes such as *c-Fos*, further promoting other genes involved in smooth muscle cell proliferation and/or apoptosis. Nicotine-induced *c-Fos* immunostaining has been reported in rat brain [24], and the co-administration of nicotine and Ang II induced DNA synthesis and *c-Fos* expression in adult rat aortic VSMC and adventitial fibroblasts [11]. Whether smoking is also associated with increased expression of pro-apoptosis genes, such as p53 or p16Ink4a, in such high risk patients remains to be determined.

4.3. Study limitation

This study suffers from the usual limitation of non-randomized studies in that it determined correlations rather than causal relationships. Moreover, no assessment of smoking intensity or carbon monoxide levels was performed. However, the main strengths of this work are the use of a prospective design and a comprehensive analysis of a large number of confounding variables to analyze factors that affect the circulating leukocyte transcriptome. Moreover, we did not assess either smoking intensity or biological markers of exposure to tobacco smoke. Because biomarkers of exposure to cigarette smoke, such as cotinine levels, are highly specific to tobacco products, previous epidemiologic studies have used biomarkers of exposure to cigarette smoke to verify the accuracy of self-reported smoking. Quantitative biomarker data provide significantly more detailed and objective information on tobacco smoke exposure than does smoking history. It has been shown that self-reported smoking history markedly underestimates exposure to cigarette smoke in high risk patients [25]. A strong association between systemic inflammatory response and cotinine levels to measure second-hand exposure [26], or active smoking [27] has also been reported. However, both smokeless tobacco and nicotine replacement therapy, which may have been used by the high risk patients of our study, can raise nicotine levels, potentially confounding the interpretation of markers of exposure to cigarette smoke. Moreover, there is no gold standard to measure exposure to cigarette smoke, and, historically, self-reported smoking remains the standard for comparison in the real world setting.

5. Conclusion

The results of our investigation offer insights into cigarette-smoke related pathophysiologic processes by determining for the first time that smoking status is significantly associated with elevated levels of circulating *c-Fos* transcripts. If these findings are confirmed in larger prospective studies, our work suggests that the level of c-Fos transcripts could be considered as a biomarker of cumulative exposure to tobacco smoke. As *c-Fos* has been implicated in the progression and severity of atherosclerosis, it could be considered a potential pathway for tobacco toxicity in coronary artery disease.

Conflict of interest

No conflicts of interest to disclose that could inappropriately influence the present work.

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