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The Role of Inflammatory Biomarkers in the Assessment of Coronary Artery Disease

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1. Introduction

Coronary artery disease (CAD) is the world's leading cause of illness and death. In 2005, approximately 7.6 million deaths were attributed to coronary artery disease, accounting for almost 13% of the total deaths (WHO, 2011). From a pathophysiological point of view the disease could be considered as a severe clinical manifestation of atherosclerosis (Mallat & Tedgui, 2001). The disruption of an atherosclerotic plaque with superimposed thrombosis had been identified as the main cause of acute coronary syndromes, including acute myocardial infarction (AMI), and sudden death (Gensini & Dilaghi, 2002; Shah, 2003).

The initially silent progression of arterial plaque, prompted by classic risk factors (including hypertension, diabetes mellitus, dyslipidaemia, age, stress, physical inactivity, dietary habits and cigarette smoking), is followed by a phase of acute or chronic progression toward an increasing degree of stenosis that eventually causes thrombosis (Gensini & Dilaghi, 2002; Fuster *et al*., 2005). Plaque disruption and/or endothelial activation represent the main trigger event for AMI, through exposure of plaque thrombogenic components to platelets and to clotting components of flowing blood. In this phase, haemostasis related risk factors and platelet status play a crucial role. However, current research supports the view of atherosclerosis as an inflammatory process that initiates and promotes lesion development to the point of acute thrombotic complications and clinical events. Thus, the time has come to embrace inflammation as a common pathway for atherogenic risk factors and for providing new opportunities for therapeutic intervention (Libby, 2003).

Acute myocardial infarction is a critical clinical presentation of coronary artery disease in many asymptomatic patients and often the event is fatal. Establishing the presence of coronary lesions either in asymptomatic patients or in symptomatic patients with acute or chronic chest pain can be a challenging task. Consequently, major clinical research efforts

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have been dedicated to the identification of patients at higher risk and to the diagnosis of coronary artery disease.

Angiography remains the undisputed standard in interventional cardiology. Succeeding breakthroughs for cardiac imaging from every branch of radiology technology offer remarkable views and are providing new insights into coronary pathology. Although angiography is a first-line test for coronary artery disease, particularly for screening symptomatic patients, the evaluation of asymptomatic individuals currently relies on the identification of risk factors (e.g., hypertension, dyslipidaemia, diabetes mellitus, stress and smoking habits) (Conroya *et al.*, 2003; Kotecha *et al.*, 2010). However, neither the absence of high-grade stenosis provided by imaging modalities such as angiography assure the lack of future cardiac events, nor the cardiovascular events are readily explained by cardiovascular risk factors (Fisher *et al.*, 2000; Kern, 2000; Hadamitzky *et al.*, 2009; Marwan *et al.*, 2009). The understanding of the cellular biology of the unstable plaque remains poorly known, and the crucial question is still the identification of the factor(s) that play a significant role in the plaque disruption.

Rupture of atherosclerotic plaque has been identified as the proximate event in the majority of cases of acute ischemic syndromes. Plaque rupture exposes thrombogenic components of the plaque, activating the clotting cascade and promoting thrombus formation. Future culprit lesions are difficult to identify, however, and angiographic assessment of stenosis severity is prone to underestimation. Compared with plaques that cause severe luminal stenosis, vulnerable plaques may cause relatively minor stenosis, although they account for more cases of rupture and thrombosis. Such unstable, vulnerable plaques may be associated with outward remodeling of the vessel. Because severely stenotic plaques are more likely to stimulate collateral circulation to the post-stenotic segment, plaque rupture and thrombosis at such sites may be clinically silent. Characteristic histomorphologic features of vulnerable plaques include a high lipid content, increased number of inflammatory cells, and extensive adventitial and intimal neovascularity. These cells are mostly monocyte-macrophages and they are probably recruited into the atherosclerotic plaques by adhesion molecules, especially intracellular adhesion molecule (ICAM)-1 and P-selectin, and chemokines such as monocyte chemoattractant protein (MCP)-1. Another potential avenue for the entry and recruitment of inflammatory cells inside the atherosclerotic lesion may be through the adventitial neovasculature, which is enhanced in atherosclerosis. In addition, other factors that may contribute to the recruitment of inflammatory cells and their activation in atherosclerosis include oxidized lipids, cytokines such as tumor necrosis factor alpha (TNF-α), increased angiotensin II activity, elevated arterial pressure, diabetes, chronic infections remote from the arterial wall, possible infectious organisms in the vessel wall and activation of the immune system (Shah, 2003). In addition, interaction between inflammatory cells, vascular smooth muscle cells, endothelial cells and extracellular matrix may contribute to the development of plaque and its rupture. TNF-α, mainly secreted by macrophages, influences many aspects of atherosclerosis by increasing the permeability of endothelial cells, promoting monocyte adhesion, inducing macrophage differentiation and probably promoting vascular calcification (Trion & van der Laarse, 2004). The calcification of arteries resembles the bone formation (Trion & van der Laarse, 2004). Activated monocytes produce TNF-α and other osteoinductive factors that stimulate the differentiation and mineralization of cardiovascular cells (Trion & van der Laarse, 2004).

New approaches claim that measurements of carotid intima-media thickness or coronary artery calcium obtained by non-invasive techniques can be used to identify vulnerable patients at a time when risk factor modification can slow or stop the atherosclerotic process. Nevertheless, the uncertainty about the functional significance of these markers in the unstable plaque context has not yet been overcome. The crucial questions still are the identification and characterization of the vulnerable plaque in hopes of identifying morphologic and physiological features that predict plaque rupture.

An increase body of literature associates plaque rupture with inflammatory mediators, such as tissue factors, cell adhesion molecules and cytokines, expressed by vascular and immune cells (Shah, 2003; Fuster *et al.*, 2005; Mauriello *et al.*, 2005; Armstrong *et al.*, 2006a).

White blood-cell count (WBC), the most widely available and inexpensive measure of systemic inflammation has been associated with cardiovascular mortality both in primary and secondary prevention settings. In apparently healthy individuals, a high white blood-cell count has been associated with increased cardiovascular mortality and incidence of cardiovascular disease, independently of traditional atherosclerotic risk factors (Folsom *et al.*, 1999; Margolis *et al.*, 2005; Shankar *et al.*, 2007). In a study of patients with acute coronary syndromes, higher baseline white blood-cell counts were associated with greater extent of coronary artery disease, lower thrombolysis in myocardial infarction (TIMI) flow and myocardial perfusion grades during coronary angiography in addition to a higher 6-month mortality independently of other risk factors including ST-segment deviation and troponin levels (Sabatine *et al.*, 2002a). A high neutrophil count and a low lymphocyte count may carry most of this increased risk, as reported for patients assessed for coronary artery disease by coronary angiography (Horne *et al.*, 2005).

Thus, for prognostic purposes, clinicians have focused on white blood-cell, neutrophils or downstream products such as C-reactive protein (CRP). Neutrophils, however, live only for hours and generate no memory of their engagement, which is carried out through inherited receptors that are similar in all hosts, while lymphocytes are long-lived cells that can survive for decades, and the lymphocyte repertoire is tailored for each individual. When mobilized in immune responses, lymphocytes undergo clonal burst, differentiate into distinct types of effector cells, and memorize information about the antigen (Bodi *et al*, 2008). Little is known regarding the role of lymphocytes, which play an important role in the control of the inflammatory system and in the pathophysiology of coronary disease (Bodi *et al*, 2008). Several studies had demonstrated that the adaptative immunity plays an important role in the pathogenesis of coronary artery disease (Blum & Yeganeh, 2003; Methe *et al.*, 2005; Han *et al.*, 2007; Packard *et al.*, 2009; Hansson, 2009). In the culprit lesions of patients with acute coronary syndromes the percentage of activated T lymphocytes is significantly increased, and experimental results (Caligiuri *et al.*, 2000) suggest the existence of antigenic stimuli in these lesions. These findings lead to the paradigm that the transition from a stable to an unstable plaque includes immunological activation and may be T cell-dependent (Steppich *et al.*, 2007).

The interactions between leucocytes, activated platelets and activated endothelial cells, mediated by P-selectin, E-selectin and ICAM-1 whose soluble forms can be released in circulation, associate with the initiation of arterial thrombus (Price & Loscalzo, 1999). The expression of ICAM-1 in activated endothelial cells and of P-selectin in activated platelets, seems to have key roles in binding and rolling of leukocytes along the activated endothelium, and in platelet aggregation and platelet-leukocyte adhesion.

The majority of soluble P-selectin appears to be derived from activated platelets, as its levels are correlated with other established platelet markers but not with endothelial markers (Price & Loscalzo, 1999). Stimulated platelets and immune cells express membrane proteins, such as integrins, CD40 and its ligand (CD40L) (Henn *et al.*, 2001), and eventually secrete products with pro-inflammatory properties, such as TNF-α, and also soluble forms of adhesion molecules. Clinical data relating soluble P-selectin, ICAM-1 and TNF-α to coronary disease are limited and have been derived primarily from randomized clinical trials, cross-sectional or retrospective studies in patients with acute coronary syndromes. While, increased levels of soluble P-selectin have been consistently associated with acute coronary syndromes (Ridker *et al.*, 2001), the measurement of sICAM-1 is not consensual. Prolonged high levels of sICAM-1 were associated with unstable angina and acute myocardial infarction (Mulvihill *et al.*, 2000), although a stronger predictive information for sICAM-1 could not be found (Haim *et al.*, 2002; Hartford *et al.*, 2006). Increases in TNF-α and in some of its soluble receptors were related to primary cardiovascular events and to mortality in heart failure subsequent to myocardial infarction (Ponthieux *et al.*, 2004; Valgimigli *et al.*, 2005).

C-reactive protein is a down-stream marker of inflammation produced in the liver. Its production appears to be regulated, during the acute phase response, by several cytokines, including TNF-α (Calabrò *et al.*, 2009). Though it was originally proposed as a nonspecific marker of inflammation, several reports suggest that CRP may play a direct pathophysiological role in the development and progression of atherosclerosis. Proposed mechanisms include induction of endothelial dysfunction, promotion of foam cell formation, inhibition of endothelial progenitor cell survival and differentiation, and activation of complement in atherosclerotic plaque intima and ischemic myocardium (Armstrong *et al.*, 2006b). CRP is a robust clinical marker because of its stability, reproducible results, and ease of assay (Armstrong *et al.*, 2006b). An increasing variety of literature has been propose CRP as a major cardiovascular risk factor. Elevated baseline concentrations of this acute phase-protein are associated with the risk of atherosclerotic events in general populations (Calabrò *et al.*, 2009) and show a predictor value in terms of cardiovascular risk associated with both primary and secondary prevention of coronary artery disease (Ikonomidis *et al.*, 2008).

Although a great pool of information concerning systemic inflammation markers has been so far collected in different cardiovascular conditions, the evolution of coronary syndromes is not well depicted.

An approach with multiple inflammatory markers might be of interest since different markers may enhance or initiate different and not always overlapping inflammatory pathways, leading to atherosclerosis and cardiac events and may indicate different stages of disease (Ikonomidis *et al.*, 2008). Therefore, the relationship of inflammatory molecules and cells with the coronary disease severity and extension, and with the physiological response of the cardiovascular system may evidence the underlying mechanisms of inflammation responsible for plaque instability.

The main objective of this study was to investigate several inflammatory markers in coronary artery disease. The circulating levels of CRP, sP-selectin, sICAM-1, TNF-α, and inflammatory blood cells were assessed as they express cell adhesion, cell activation and inflammation processes which are crucial in thrombosis and secondary tissue remodelling as the cause of ischemia and necrosis.

This combined evaluation in a well-defined group of patients that had undergone angiography to precisely define coronary artery phenotype, may reveal the relevant roles of those markers in coronary disease and in the processes involved in lesion vulnerability.

2. Materials and methods

2.1 Study groups

To achieve the proposed objective it is necessary to unveil the effect of coronary occlusion, ischemia, and necrosis. Therefore, patients with different stages of coronary artery disease were included in the study.

A total of 177 subjects (53 women and 124 men) were recruited at the Cardiology Service in Santa Marta Hospital (Lisbon, Portugal).

Among them, 65 patients with acute myocardial infarction constituted the acute myocardial infarction (AMI) group. Those patients were diagnosed with ST-elevation myocardial infarction (ST-element changes and creatine kinase >3 times normal; n=56) or non-ST-elevation myocardial infarction (creatine kinase >3 times normal and without ST-element changes; n=9). All AMI patients were enrolled in the first 24 hours of hospital admission, and were submitted to primary percutaneous transluminal coronary angioplasty as reperfusion therapy. The time period from the onset of chest pain to the intervention was less than 9 hours for the majority of AMI patients.

Fifty-five patients with angiographically confirmed coronary artery disease suffering from chest discomfort, were also enrolled in the study and constituted the CAD group.

A coronary control group (CC) was established. This group was constituted by twenty-nine age-matched patients with chest discomfort complain but without coronary artery disease confirmed by coronary angiography.

A reference group (REF) of 26 healthy non-smoking volunteers was also established to help on inflammation baseline interpretation. Inclusion criteria for reference controls was absence of any history of coronary disease, dyslipidaemia or hypertension, any mobility limiting conditions, life threatening diseases, or any other disease or condition that would impair compliance. These 26 volunteers were not submitted to coronary angiography.

Informed consent was obtained for all subjects enrolled, and the study was approved by the local Ethical Committee.

2.2 Criteria used for patient's selection

Exclusion criteria were age above 85, significant co-morbidities as peripheral artery disease or carotid artery disease, known antecedents of malignance or infectious diseases, chronic renal insufficiency and previous myocardial infarction in the last 5 years. Concurrent inflammatory disorders, malignant neoplasm or infection were also excluded.

All patients were clinically and biochemically characterized, by a battery of systemic indicators.

The patients' characterization was accomplished with anthropometric data (body mass index, waist perimeter and blood pressure) and biochemical data consisting of a battery of systemic indicators, such as glucose, haematocrit, albumin, triglycerides, total cholesterol, cholesterol of LDL and HDL. The cardiac function enzymes creatinine kinase and cardiac troponin T (cTnT) were also determined, and ventricle electrolytic regulation assessed by the determination of N-terminal pro B type natriuretic peptide (NT-proBNP).

Furthermore, detailed in-hospital data was registered, including: demographic (such as age and sex), and coronary risk factors data (such as, smoking, previous diagnostic of diabetes mellitus, hypertension and hyperlipidaemia) and personal history and family history of coronary artery disease. Current, in-hospital and after angioplasty medication was also recorded: insulin or other anti-diabetic drugs; antiplatelet and anti-aggregant drugs, such as aspirin, clopidrogel or glycoprotein IIb/IIIa receptor antagonists; β-blockers; ACE inhibitors and statins.

2.2.1 Definition of risk factors, clinical signs and syndromes

Diabetes was diagnosed on the basis of fasting plasma glucose concentration ≥7.0 mmol/l (126 mg/dl) or 2–h plasma glucose ≥11.1 mmol/l (200 mg/dl) or confirmed as clinically known and treated diabetes mellitus. Subjects were diagnosed hypertensive if they were documented to have systolic blood pressure ≥140 mmHg and/or diastolic blood pressure ≥90 mmHg or were already on anti-hypertensive therapy. Dyslipidaemia was identified in subjects who had been given lipid-lowering medication or having total serum cholesterol level ≥190 mg/dl or serum triglycerides ≥180 mg/dl.

Smoking was defined as the inhaled use of cigarettes, cigars or pipes in any quantity. Subjects who smoked within the previous 1 year were also defined as smokers.

Several inflammatory markers such as white blood-cell count, CRP and TNF-α levels have been extensively associated with cardiovascular disease risk (Ikonomidis *et al.*, 2008). In fact, several studies established cut-off values associated with cardiovascular risk and to the occurrence of adverse cardiovascular events for these biomarkers (Sabatine *et al.*, 2002a; Sabatine *et al.*, 2002b; Grau *et al.*, 2004; Ridker & Cook, 2004; Ikonomidis *et al.*, 2005; Ridker *et al.*, 2005).

Based on these documented limits values, we establish risk classes for white blood-cell count, CRP and TNF-α levels. For the white blood-cell counts the cut-off value (low/high risk) considered was $>10.1 \times 10^9$/l (Sabatine *et al.*, 2002a; Grau *et al.*, 2004). The classes of cardiovascular risk associated to CRP were considered as low and high risk based on the cut-off value of ≥3 mg/dl (Sabatine *et al.*, 2002b; Ridker & Cook, 2004; Ridker *et al.*, 2005). Finally, for the TNF-α concentrations the cut-off value considered for low/high risk was ≥3.61 pg/ml (Ikonomidis *et al.*, 2005).

2.2.2 Angiographic data

AMI and CAD patients were clinically characterized for the extension of coronary artery disease through the characterization of lesion morphology data to define the coronary stenosis, the number of diseased vessels, flow characteristics of responsible vessel such as the thrombolysis in myocardial infarction (TIMI) risk score (from 0-3 referring to the levels of coronary blood flow assessed during coronary angiography), lesion length and the presence of calcium and/or thrombi in the lesions. The number and type of stents (bare metal or drug eluting stent) positioned in patients undergoing coronary angiography were also recorded.

A coronary stenosis was considered clinically significant (high-grade) above 70% narrowing in the luminal diameter. Multivessel disease was defined when more than one major coronary artery presented high-grade stenosis. Patients were classified according to the class of stenosis based on TIMI risk score: TIMI 3 – patients having normal flow and complete perfusion; and TIMI < 3 – patients with some degree of occlusion. In the later the TIMI scores considered were TIMI 2 for flow partial perfusion and TIMI 0 for no perfusion.

Additionally, patients were divided into 2 subgroups according to the lesion length using a cut-off of 15 mm. Small lesions were considered ≤15 mm and large lesions >15 mm.

2.3 Study protocol

A longitudinal study was carried out in the AMI patients. Patients were assessed at hospital admission before the administration of IIb/IIIa inhibitors and coronary angioplasty intervention, 2 and 40 days after the initial intervention. At these three time-points biochemical parameters and circulating inflammatory markers were measured. CAD and CC patients were only assessed at hospital admission before percutaneous intervention.

2.4 Blood sampling and laboratory assays

Blood samples were drawn from all patients into pyrogen-free blood collection tubes without additives and immediately centrifuged (2500 rpm for 10 minutes). The serum was collected after centrifugation and aliquots were stored at -80ºC until analysis. Sample storage period did not exceed 6 months. Samples were thawed only once.

Lipid profile, glucose levels, albumin, creatinine kinase, troponin T, NT-proBNP, blood cells count and levels of high-sensitivity CRP were routinely measured on hospital.

Soluble concentrations of ICAM-1 and P-selectin were measured by enzyme-linked immunosorbent assays (ELISA) commercial kits (R&D Systems, Minneapolis, USA). The concentrations of TNF-α were assessed using a commercial available high sensitivity ELISA (R&D Systems, Minneapolis, USA). All the assays were performed on serum according to the manufacturer's recommendations. Each sample was measured in duplicate; the intra-assay variation among the duplicates for all samples was <10%.

2.5 Statistical analysis

Data were summarized as mean and standard deviation (SD) or median and quartiles for continuous variables and as proportions for categorical variables. Non-continuous variables were analysed using a 2x2 table and χ^2 test. Differences between the four study groups were compared using a general linear model analysis of variance (ANOVA) followed by post hoc procedures (Tukey and Scheffé tests) to identify differences. ANOVA adequacy was weighted by checking the variance homogeneity using Levene's test and by verifying the significance of F distribution with the Welch statistics. For the variables white blood-cell, neutrophils, lymphocytes and monocyte counts, sP-selectin, sICAM-1, CRP, total cholesterol, LDL-cholesterol, triglycerides, glucose, troponin T and NT-proBNP the homogeneity of variance was not reached, thus variables were logarithmic transformed before performing ANOVA analysis.

Associations between inflammatory mediators, and with risk factors, co-morbidities and angiographic data were evaluated using non-parametric Spearman correlations.

The study of the repeated measures of inflammatory parameters in AMI patients through time requires specific statistical methods as common analysis of variance or non-parametric correlations are inappropriate. The inflammatory parameters and mediators, CRP, sP-selectin, sICAM-1, TNF-α, and blood cell counts, were measured in day 0 at hospital admission before percutaneous transluminal coronary angioplasty, and repeated at day 2 and day 40 after the initial percutaneous transluminal coronary angioplasty in the same patient. Consequently, the observations are inter-correlated and classical statistical techniques do not account for this type of variability. For that reason, a non-linear regression

algorithm that accounts for the effect of repeated measures was applied, the linear mixed effects model. The procedure of lineal mixed effects models the concentrations of the inflammatory markers measured through time considering that measures for each patient were not independent (Twisk, 2006). Using this algorithm the concentrations of the inflammatory parameters CRP, sP-selectin, sICAM-1, TNF-α) and blood cell counts can be modelled as a response variable on time. This statistical model describes the longitudinal variations of each patient for each variable by calculating slopes and averages of the variables in each time point. Therefore, it allows to estimate the differences in average slopes between baseline (day 0) and the other time points, giving a measure of the variation of each blood marker over time. In order to apply the lineal mixed effects model, variables should comply with normality distribution. A logarithm transformation was used for the variables CRP, sICAM-1, white blood-cell, neutrophil and lymphocyte counts, while a square root transformation was used for monocyte counts, sP-selectin and TNF-α.
Values of $p<0.05$ (two-tailed) were considered statistical significant.
The calculations were performed using SPSS (version 19.0) and R (version 2.11.1) software.

3. Results

3.1 Demographic and clinical characteristics of the study groups

The demographic characteristics and clinical features of the study groups including risk factors, co-morbidities and previous-event medication intake are summarized in Table 1.
The age of subjects enrolled ranged between 27 and 81 years, having the three groups matching ages. There were no significant differences between AMI, CAD and CC subjects in body mass index (BMI), waist perimeter and blood pressure, except for sex ratio. As can be inferred from Table 1, AMI patients were mostly men (80%), while in CAD and CC groups the percentage of the masculine gender decreased (70% and 60%, respectively). The three groups also had similar prevalence of risk factors as diabetes, dyslipidaemia and hypertension, however smoking was more frequent in AMI patients.
Since coronary disease risk factors and co-morbidities were exclusion criteria in REF group selection, these subjects were not considered in this comparison.

	CC (n=29)	**CAD** (n=55)	**AMI** (n=65)
Sex, f/m	13/16	18/37	15/50
Age (y)	60±9	64±9	61±15
Body mass index (kg/m^2)	29±6	27±3	27±4
Waist perimeter (cm)	100±13	98±9	94±17
Systolic blood pressure (mm Hg)	140±20	152±23	125±22
Diastolic blood pressure (mm Hg)	77±10	78±10	73±15
Risk factors and co-morbidity			
Hypertension, n (%)	19 (66)	35 (63)	42 (64)
Smoking, n (%)	3 (10)	6 (11)	33 (50) [a,b]
Dyslipidaemia, n (%)	21 (72)	34 (61)	38 (58)
Diabetes, n (%)	7 (24)	16 (24)	12 (21)
Family history of CAD, n (%)	1 (3)	12 (21)	9 (14)

Table 1. Clinical characteristics of the studied groups.

Values are expressed as mean±SD, except otherwise indicated. [a] $p<0.05$ vs CTR group; [b] $p<0.05$ vs CAD group.
Medication intake in AMI group had also into account the medication before hospital admission and the in-hospital and follow-up treatments. There were no significant differences in the use of pre-event medication in AMI patients and patients with CAD or CC subjects (Table 2).

	CC (n=29)	**CAD** (n=55)	**AMI** (n=65)
Previous-event medication			
Aspirin, n (%)	9 (31)	23 (41)	20 (30)
ACE-inhibitor, n (%)	9 (31)	20 (36)	23 (35)
β-blockers, n (%)	7 (24)	15 (27)	11 (17)
Statins, n (%)	13 (45)	17 (48)	19 (29)

Table 2. Pre-event medication in the studied groups.

After admission, in-hospital medication for the AMI patients included aspirin (59%), β-blockers (52%), angiotensin-converting enzyme (ACE)-inhibitors (55%), statins (12%) and antiplatelet inhibitors (79%). During follow-up 80% of patients took antiplatelet inhibitors (clopidogrel and aspirin) in addition to the previous medication referred above. Furthermore, in the course of the angioplasty, stents were implanted in 86% of the patients, being 29% drug-eluting stents.

	CC (n=29)	**CAD** (n=55)	**AMI** (n=65)
Total cholesterol (mg/dl) *	165 (148 – 200)	158 (133 – 205)	191 (158 – 230)
LDL-cholesterol (mg/dl) *	111 (85.8 – 136)	102 (82.0 – 129)	128 (103 – 149)
HDL-cholesterol (mg/dl) *	40 (312 – 50)	42 (35 – 50)	38 (31 – 47)
Triglycerides (mg/dl) *	104 (65 – 137)	104 (68 – 130)	122 (59 – 154)
Haematocrit (%)	40 (36 – 43)	39 (36 – 42)	40 (37 – 44)
Glucose (mg/dl) *	111 (95 – 128)	115 (94 – 134)	139 (116 – 202)
Albumin (g/dl)	3.70 (3.40 – 3.90)	3.6 0 (3.20 – 4.00)	3.40 (3.10 – 3.70)
Troponin T (ng/ml)	<0.01 **	<0.01 **	0.34 [a,b] (0.08 – 1.74)
NT-proBNP (pg/ml)	71 (40 – 126)	102 (52 – 235)	275 [a,b] (137 – 1030)

Data are expressed as median and quartiles (lower quartile-upper quartile). * not compared (see text); ** values below detection limit; [a] $p<0.05$ vs CC group; [b] $p<0.05$ vs CAD group.

Table 3. Biochemical data of the studied groups.

Concerning biochemical data (as listed in Table 3) AMI patients at admission had high levels of troponin T and NT-proBNP. Lipid and glucose data obtained could not be directly compared as fasting-blood tests were only performed for REF individuals.

3.2 Angiographic features

Of the 151 subjects submitted to angiography (CC, CAD and AMI groups), 29 subjects (19%) had at least one episode of chest pain previous to the coronary angiography, whereas 122 (81%) were asymptomatic.

Lesion morphology data, based on angiography, in AMI and CAD patients was obtained for 94 patients (77%), for the remaining patients those data were not possible to be obtained from the hospital registries.

Multivessel disease was found in a total of 42 patients (45%). Analysis of angiographic complexity of the diseased vessels showed impaired flow (TIMI <3) in 44 patients (54%), and large lesions (>15 mm) also in 44 patients.

From all the patients analysed, it was possible to detect the presence of calcium within the lesions in 14 patients (13%) and the presence of thrombi in 25 patients (24%).

The resume of the angiographic data for the CAD and AMI patients is listed in Table 4.

Comparing the morphologic data of patients from the CAD and AMI groups, it is possible to verify that an impaired flow (TIMI <3) is more frequent in AMI patients than in CAD patients. Furthermore, AMI patients had more often thrombi in the lesions than the CAD patients (Table 4).

	CAD	**AMI**
Multivessel disease, n (%)	15 (42)	27 (47)
TIMI		
TIMI 0, n (%)	2 (8)	32 (58)
TIMI 1, n (%)	2 (8)	3 (5)
TIMI 2, n (%)	1 (4)	4 (7)
TIMI 3, n (%)	21 (81)	16 (29)
Impaired flux TIMI <3, n (%)	5 (19)	39 (71) [a]
Lesion length		
Small lesions, n (%)	11 (44)	18 (56)
Large lesion, n (%)	14 (38)	30 (63)
Lesions with calcium, n (%)	9 (20)	5 (8)
Lesions with thrombi, n (%)	2 (5)	23 (38) [a]

Data are expressed as number and percentages. [a] $p<0.05$ vs CAD group.

Table 4. Angiographic data of the patients studied.

3.3 Inflammatory mediators

The blood cell counts and concentration level of inflammatory mediators for the studied groups are presented in Table 5.

	REF (n=25)	**CC** (n=29)	**CAD** (n=55)	**AMI** (n=65)
Blood cell count				
White blood-cells ($x10^9$/l)	5.36 (5.06 – 6.26)	6.81 (5.74 – 8.56)	6.32 (5.39 – 7.88)	11.3 [a,b,c] (7.69 – 13.9)
Neutrophils ($x10^9$/l)	3.00 (2.49 – 3.59	3.54 (3.20 – 4.19)	4.08 [a] (2.94 – 4.95)	8.34 [a,b,c] (5.61 – 11.2)
Lymphocytes ($x10^9$/l)	2.02 (1.71 – 2.26)	2.03 (1.69 – 3.32)	1.77 (1.38 – 2.30)	1.54 (1.24 – 2.24)
Monocytes ($x10^9$/l)	0.37 (0.28 – 0.51)	0.42 (0.28 – 0.58)	0.50 (0.34 – 0.67)	0.60 [a,b,c] (0.42 – 0.81)
Inflammatory markers				
CRP (mg/dl)	<0.32 **	0.66 (0.31 – 0.74)	0.43 (0.18 – 1.29)	0.69 [a] (0.34 – 1.39)
TNF-α (pg/ml)	0.37 (0.12 – 1.14)	1.17 (0.73 – 2.26)	1.57 [a] (0.75 -2.69)	1.57 [a] (0.57 – 2.47)
sP-selectin (ng/ml)	84 (93 – 119)	68 [a] (41 – 83)	53 [a] (42 – 82)	83 (54 – 116)
sICAM-1 (ng/ml)	214 (190 – 246)	247 (204 – 316)	220 (200 – 237)	248 (218 – 258)

Data are expressed as median and quartiles (lower quartile-upper quartile). ** values below detection limit; [a] $p<0.05$ vs REF group; [b] $p<0.05$ vs CC group; [c] $p<0.05$ vs CAD group.

Table 5. Inflammatory mediators in the four study groups.

The results revealed that the circulating counts of white blood-cells and neutrophils in AMI patients at hospital admission were increased relative to the other groups (REF, CC and CAD). Monocyte counts were also increased in AMI patients relative to a normal baseline situation (REF group). The circulating levels of CRP and TNF-α also showed the same trend (Table 5). Furthermore, in CAD patients the neutrophils counts and the TNF-α concentrations were also increased in comparison to REF subjects (Table 5).

Opposite, the concentrations of sP-selectin were significantly decreased in CAD and CC subjects in comparison to the REF subjects (Table 5).

The relationships of white blood-cells, neutrophils, monocytes, lymphocytes counts, inflammatory mediators (CRP, TNF-α, sP-selectin and sICAM-1) and cardiac markers (NT-proBNP and troponin T) concentrations, were assessed using non-parametric Spearman correlations. Several significant associations were found.

Relevant positive correlations were verified between CRP and ICAM-1($r=0.415$, $p<0.001$), neutrophil ($r=0.378$, $p<0.001$) and monocyte counts ($r=0.437$, $p<0.001$). sICAM-1 also showed positive associations with white blood-cell counts ($r=0.342$, $p=0.004$) and TNF-α concentrations ($r=0.415$, $p<0.001$).

Apart from the relationship between the number of neutrophils and monocytes in blood ($r=0.464$, $p<0.001$), neutrophils and monocytes were also associated to the levels of cardiac function markers troponin T and NT-proBNP ($r=0.526$ and $r=0.315$, $p<0.001$, respectively for neutrophils; and $r=0.438$ and $r=0.356$, $p<0.001$, respectively for monocytes). CRP was also significantly associated to the levels of troponin T and NT-proBNP ($r=0.626$ and $r=0.470$, $p<0.001$, respectively).

Given the importance of risk factors and co-morbidities in the disease evolution, hypertension, dyslipidaemia, smoking habits and diabetes were also tested for Spearman correlations with inflammatory markers. Relevant correlations were only verified for sP-selectin and sICAM-1 with cardiovascular risk factors. sP-selectin showed a negative correlation with hypertension ($r=-0.379$, $p<0.001$) and a positive correlation with smoking ($r=0.381$, $p<0.001$). Furthermore, sICAM-1 was also positively associated to smoking ($r=0.373$, $r<0.001$).

Importantly, levels of P-selectin were also negatively associated with age ($r=-0.399$, $p<0.001$). In fact, subjects with age above 65 had lower levels of sP-selectin (55 ng/ml) compared to younger (≤65 years) subjects (78 ng/ml).

As mentioned previously the enrolled subjects were classified in risk classes white blood-cell count, CRP and TNF-α levels. The frequency of each class in the studied population is resumed in Table 6.

	CC	CAD	AMI	Total Population
WBC risk score				
Low risk (≤10.1x10^9/l), n (%)	28 (97)	50 (89)	24 (36)	127 (72)
High risk (>10.1x10^9/l), n (%)	1 (3)	1 (2)	33 (50)	35 (20)
CRP risk score				
Low risk (<3 mg/dl), n (%)	22 (76)	38 (68)	51 (77)	135 (76)
High risk (≥3 mg/dl), n (%)	1 (3)	3 (5)	5 (8)	9 (5)
TNF-α risk score				
Low risk (>3.61 pg/ml), n (%)	19 (66)	20 (36)	35 (53)	89 (72)
High risk (≥3.61 pg/ml), n (%)	3 (10)	2 (4) [a]	4 (6)	9 (5) [a]

Data are expressed as number and percentages.

Table 6. Distribution of the enrolled subjects in the white blood-cell (WBC), CRP and TNF-α risk classes.

3.3.1 Longitudinal variations

The blood cell counts, the concentrations of inflammatory mediators and other biochemical markers, in AMI patients assessed at hospital admission, 2 and 40 days after percutaneous transluminal coronary angioplasty intervention are presented in Table 7.

In the overall an increasing trend of the measured concentrations of sICAM-1 and TNF-α through time was observed, while a decreasing one was observed for white blood-cell and neutrophil counts. Monocyte counts and CRP concentration showed an initial increase. To assess the significance of these changes through time a regression model (linear mixed effects model) was applied. As mentioned before (see section 2.5 Statistical analysis), appropriate transformations of variables had to be applied. Therefore, the results from the

	AMI patients		
	Day 0	**Day 2**	**Day 40**
Biochemical characterization			
Total cholesterol (mg/dl)	191 (158 – 230)	162 (136 – 192)	141 [a] (127 – 180)
LDL-cholesterol (mg/dl)	128 (103 – 149)	105 (78 – 122)	81 [a] (70 – 106)
HDL-cholesterol (mg/dl)	38 (31 – 47)	38 (27 – 47)	35 [a] (29 – 41)
Triglyceride (mg/dl)	122 (59 – 154)	95 (90 – 110)	112 [a] (79 – 175)
Haematocrit (%)	40 (37 – 44)	39 [a] (34 – 42)	42 [c] (39 – 45)
Glucose (mg/dl)	139 (116 – 202)	121 (102 – 151)	102 [a] (93 – 135)
Albumin (g/dl)	3.40 (3.10 – 3.70)	3.19 [a,b,c] (3.00 – 3.50)	3.90 (3.60 – 4.20)
Troponin T (ng/ml)	0.34 [b,c] (0.08 – 1.74)	2.40 [a,b,c] (1.70 – 4.10)	<0.01 **
NT-proBNP (pg/ml)	275 [b,c] (137 – 1030)	1324 [a,b,c] (519 – 2955)	611 [a,b,c] (354 – 1009)
Blood cell counts			
White blood-cells ($x10^9$/l)	11.3 [a,b,c] (7.69 – 13.9)	8.48 [a,c] (6.31 – 10.3)	6.68 (5.37 – 7.33)
Neutrophils ($x10^9$/l)	8.34 [a,b,c] (5.61 – 11.2)	5.32 [a,b,c] (4.05 – 6.37)	3.82 [a] (3.12 – 4.85)
Lymphocytes ($x10^9$/l)	1.54 (1.24 – 2.24)	1.79 (1.25 – 2.51)	1.93 (1.54 – 2.23)
Monocytes ($x10^9$/l)	0.60 [a,b,c] (0.42 – 0.81)	0.74 [a,b,c] (0.62 – 0.91)	0.48 (0.40 – 0.58)
Inflammatory markers			
CRP (mg/dl)	0.69 [a] (0.34 – 1.39)	3.49 [a,b,c] (1.52 – 6.92)	0.34 (0.32 – 0.70)
TNF-α (pg/ml)	1.57 [a] (0.57 – 2.47)	1.57 (0.75 – 2.24)	2.10 (1.06 – 3.15)
sP-selectin (ng/ml)	83 (54 – 116)	67 (48 – 79)	70 (62 – 103)
sICAM-1 (ng/ml)	248 (218 – 258)	281 (238 – 309)	298 (224 – 400)

Data are expressed as median and quartiles (lower quartile-upper quartile). ** values below detection limit; [a] $p<0.05$ vs REF group; [b] $p<0.05$ vs CC group; [c] $p<0.05$ vs CAD group (for comparison see Table 5).

Table 7. Biochemical characterization and inflammatory mediators in the AMI patients at the three time-points: hospital admission (Day 0), two (Day 2) and 40 days (Day 40) after percutaneous transluminal coronary angioplasty intervention.

obtained longitudinal models had into account these transformations (as can be observed in Figures 1 and 2). However, for results considerations purposes those transformations will not be further mentioned in the text.

The association between white blood-cells, neutrophils, monocytes and lymphocytes and time were significant ($p<0.05$) (see Figure 1).

Higher white blood-cell, neutrophil and monocyte counts were observed in the acute phase of AMI (Table 5). The high white blood-cell and neutrophil counts at admission significantly decrease in the following weeks (Figure 1A and 1B), reaching values similar to those observed in CAD patients and in control subjects (Tables 5 and 7).

By the contrary, slightly low lymphocyte counts were observed in the acute phase of myocardial infarction, although that difference was not significant (Table 5). Those low levels were maintained after percutaneous transluminal coronary angioplasty (day 2) and increased with patient's stabilization (Figure 1C and Table 7).

Monocyte counts reach the highest value at day 2 (Figure 1D), decreasing in the following days (Day 40). At that time-point, the monocyte counts were similar to the counts verified in CAD, CC and REF groups (Tables 5 and 7).

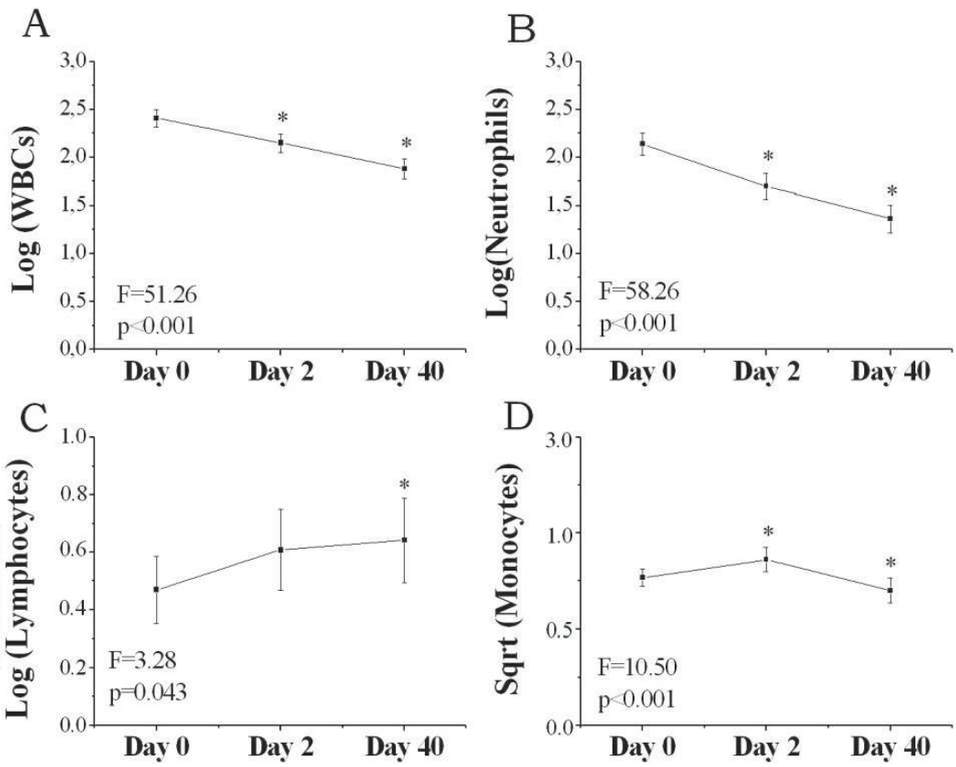

Fig. 1. Longitudinal variations of inflammatory cell counts, white blood-cell (WBC; A), neutrophils (B), lymphocytes (C) and monocytes (D) in AMI patients at hospital admission, two (Day 2) and 40 days (Day 40) after percutaneous transluminal coronary angioplasty intervention. * $p<0.05$ vs AMI patients at Day 0.

The levels of sP-selectin in AMI patients were characterized by a decrease after PCTA followed by an increase to day 40 (Figure 2A). The association between sP-selectin and time was significant ($p=0.003$). Serum levels of this soluble adhesion molecule at inclusion were

remarkably elevated than 48 h later (p=0.001). After that abrupt decrease, sP-selectin levels seem to slightly increase, reverting to the levels observed at hospital admission.
An increasing trend of sICAM-1 to day 40 was observed (Table 7) but the association of sICAM-1 with time was not significant (p=0.085; see Figure 2B).
The levels of CRP were increased in AMI at admission (Table 5), and further increase at day 2 reverting to significantly low levels after 40 days (p<0.05), as verified in Figure 2C. Those concentration levels were not significantly different from those verified in a normal non-inflammatory situation – REF group (Tables 5 and 7).
The serum levels of TNF-α increased from day 0 to day 40 (Figure 2D). TNF-α levels were remarkably elevated through time (p<0.001). TNF-α concentrations were higher at day 2 than at admission (day 0) and continue to increase until day 40 (p<0.001 relative to day 0).

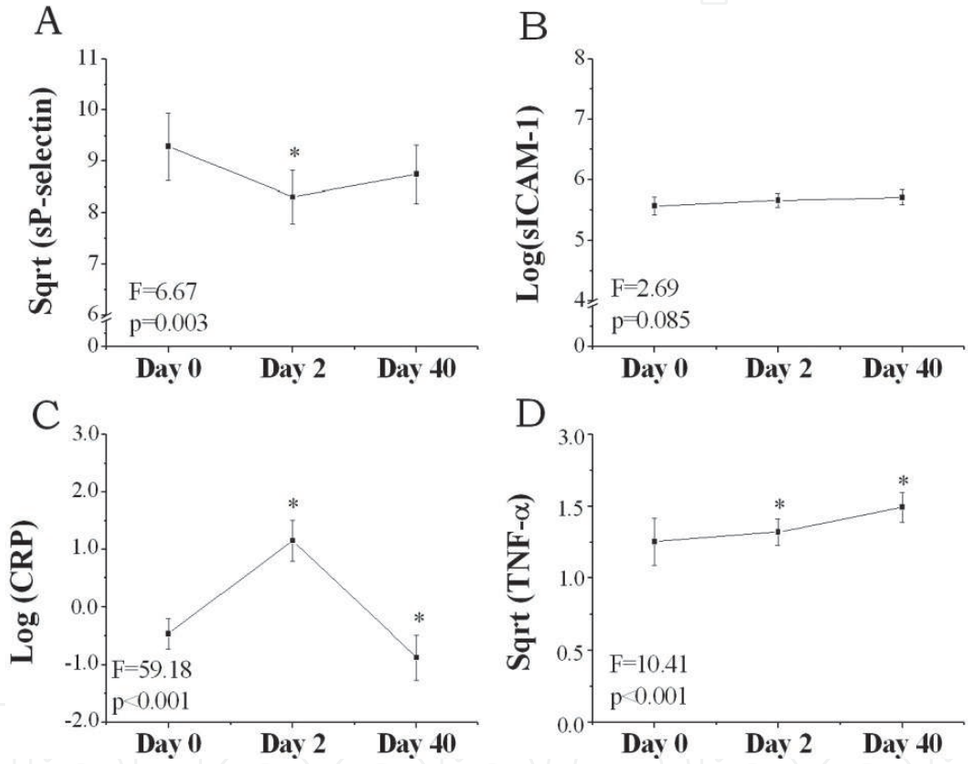

Fig. 2. Longitudinal variations of systemic concentrations of sP-selectin (A), sICAM-1 (B), CRP (C) and TNF-α (D) in AMI patients at hospital admission, two (Day 2) and 40 days (Day 40) after percutaneous transluminal coronary angioplasty intervention. * p<0.05 vs AMI patients at Day 0.

3.3.2 Medication influence

To assess the influence of previous-event medication on inflammatory mediators, subjects that did not take medication before enrolment were compared to the remaining patients that had prescribed medication. Only sP-selectin and sICAM-1 concentrations were significantly affected by the previous-event drug intake (p=0.002 and p=0.014, respectively). Subjects without pre-event medication had higher levels of these molecules (94±32 ng/ml and 280±96 ng/ml, respectively) than those with prescribed aspirin, ACE-inhibitors, β-blockers or statins (61±22 ng/ml and 235±58 ng/ml, respectively).

To further evaluate the possible influence of drug therapy in serial changes of the inflammatory markers, the pre-event, in-hospital and follow-up medication data were categorized to type (antiplatelet inhibitors, ACE-inibitors, β-blockers and statins) and added to the linear mixed effects models as co-variables. None of the drugs administered to the patients before or after admission significantly influenced the neutrophil and monocyte counts, and the CRP, TNF-α or sICAM-1 concentrations (data not shown). However, the sP-selectin variations over time were significantly influenced by pre-event ACE-inibitors ($p<0.001$) and β-blockers ($p=0.019$) intake. Patients that received β-blockers or ACE-inibitors showed minor serial changes and low levels of sP-selectin opposite to those that were not taking those drugs. The same trend was verified for the white blood-cell and lymphocytes counts longitudinal variations with the pre-event and β-blockers ($p=0.024$ and $p=0.25$, respectively) intake. AMI patients that received β-blockers showed minor serial changes and low levels of white blood-cell and lymphocytes counts compared to those that were not taking those drugs.

3.4 Relationships between angiographic features and inflammatory mediators

The existence of associations or variations between the levels of inflammatory mediators and the characterization of lesion morphology was also tested, including the number of diseased vessels (single versus multivessel disease), lesion length (small ≤15 mm versus large >15 mm lesions), TIMI risk score and the presence of calcium and thrombi.

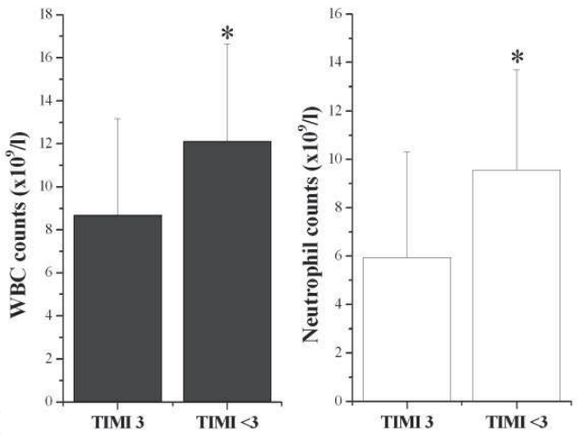

Fig. 3. Variations of white blood-cell (WBC) and neutrophil counts in the two classes of stenosis based on TIMI risk score (TIMI 3 and TIMI <3). * $p<0.05$ vs normal flux TIMI 3.

The neutrophil counts were negatively correlated to the TIMI risk score classes ($r=-0.503$, $p<0.001$) and positively with the presence of thrombi in lesions ($r=0.424$, $p<0.001$). In fact, higher counts of neutrophils (and also white blood-cell) were observed in patients with impaired coronary flux (TIMI <3) than in patients with normal flux (Figure 3). The same tendency of increased levels was verified in patients with thrombi in lesions (Figure 4).

The concentrations of sP-selectin were negatively correlated to the TIMI score ($r=-0.554$, $p=0.041$). No further correlations of inflammatory markers and angiographic features were observed, except for the AMI patients' longitudinal variations.

Considering the presence of calcium in lesions, the lymphocyte counts were lower in calcified lesions than in lesions without calcium (Figure 5).

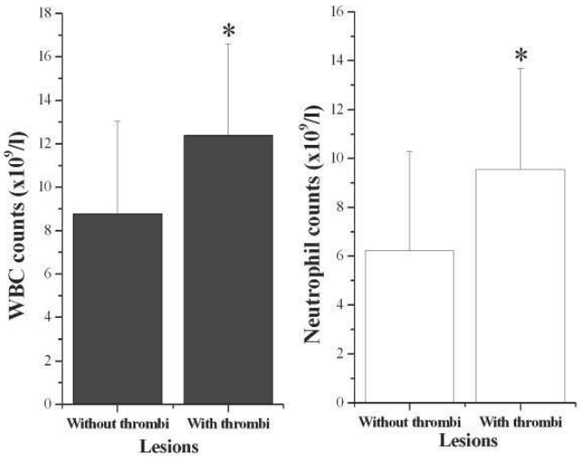

Fig. 4. Variations of white blood-cell (WBC) and neutrophil counts in the presence or absence of thrombi in the lesions. * p<0.05 vs lesions without thrombi.

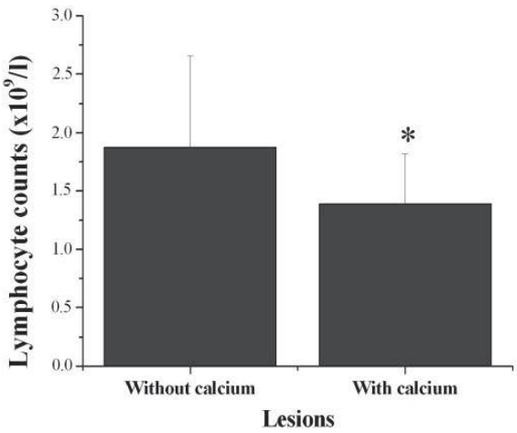

Fig. 5. Variations of lymphocytes counts in the presence or absence of calcium in the lesions. * p<0.05 vs lesions without calcium.

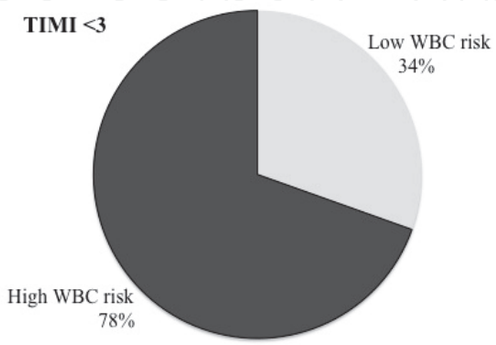

Fig. 6. Distribution of subjects with impaired flux (TIMI <3) through the white blood-cell (WBC) risk classes of cardiovascular events (low/high risk based on the cut-off level of >10.1x10⁹/l).

Examining the longitudinal variations of inflammatory mediators and the angiographic features, no associations were verified for serial changes of white blood-cells, neutrophils, sP-selectin and sICAM-1 ($p>0.05$; data not shown).
In what concerns the disease extension, the concentrations of CRP over time were positively correlated with multivessel disease ($p=0.026$). While, the monocyte counts and TNF-α serial changes were correlated to the lesion length ($p=0.043$ and $p=0.38$, respectively).
The risk classes of cardiovascular events based on white blood-cell and TNF-α cut-off levels were also tested for possible associations to the lesion morphology data.
No correlations were found to the CRP risk class with the lesion morphology data.
By the contrary, the white blood-cell risk class was negatively correlated with the TIMI score classes ($r=-0,439$, $p<0.001$) and positively with the presence of thrombi in lesions ($r=0.460$, $p<0.001$). The distribution of patients within the TIMI score classes differs in the low versus high white blood-cell risk classes. As can be observed in Figure 6, among patients with impaired flux (TIMI <3), there were a higher number of patients with high WBC risk ($>10.1x10^9/l$) than patients with low WBC risk ($p<0.001$). The same tendency is verified for the presence of thrombi in lesions ($p<0.001$; see Figure 7).

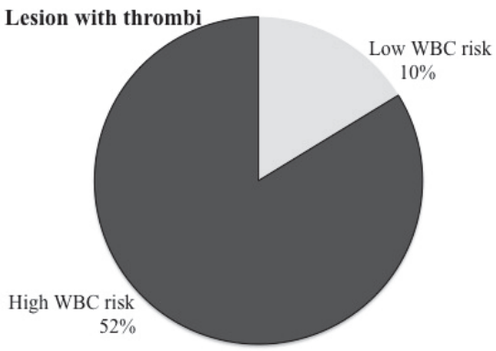

Fig. 7. Distribution of subjects with thrombi in lesions through the white blood-cell (WBC) risk classes of cardiovascular events (low/high risk based on the cut-off level of $>10.1x10^9/l$).

Furthermore, among patients with calcified lesions there is also a higher frequency of patients with high TNF-α risk (≥3.61 pg/ml) than patients with low TNF-α risk (see Figure 8), although the difference did not reach significance ($p=0.08$).

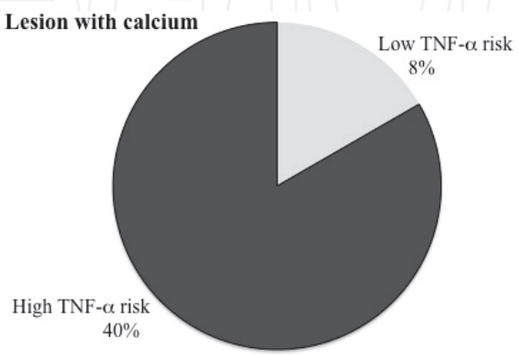

Fig. 8. Distribution of subjects with thrombi in lesions through the TNF-α risk classes of cardiovascular events (low/high risk based on the cut-off level of (≥3.61 pg/ml).

4. Discussion

It is now widely recognized that inflammation plays a critical role in plaque destabilization and vulnerability and that is a key-event in coronary artery disease (Alam *et al.*, 2004). In the last decades, important information about the pathophysiology and mechanism of coronary artery disease and acute myocardial infarction had been extensively studied (Davies, 2000; Frangogiannis *et al.*, 2002; Libby, 2003; VanWijik *et al.*, 2003; Alam *et al.*, 2004; Fichtlscherer *et al.*, 2004; Kumar *et al.*, 2004; Wiviott *et al.*, 2004; Jefferson *et al.*, 2005; Armstrong *et al.*, 2006a; Armstrong *et al.*, 2006b; Libby, 2008; Skyschally *et al.*, 2008). However, the understanding of the interactions between inflammatory markers and between the different cell types involved in those processes remains relatively unexplored, especially in human populations. Angiography is a first-line test for coronary artery disease, particularly for screening symptomatic patients. The evaluation of asymptomatic individuals relies on the identification of risk factors. However, neither the absence of stenosis provided by angiography assure the lack of future cardiac events, nor the cardiovascular events are readily explained by cardiovascular risk factors (Fisher *et al.*, 2000; Kern, 2000; Hadamitzky *et al.*, 2009; Marwan *et al.*, 2009). Therefore, non-invasive estimation of coronary disease risk is important to screening both symptomatic and asymptomatic patients. Furthermore, the understanding of the cellular biology of the unstable plaque remains poorly known, and the crucial question is still the identification of the factor(s) that play a significant role in the plaque vulnerability.

A multi-parameter approach was used in the present study, which allowed a better understanding of the complex relationships between the studied markers that meant to capture different stages of the inflammatory response involved in the different phases of coronary artery disease evolution and lesion progression.

4.1 Inflammatory markers in CAD

White blood-cell count, the most widely available and inexpensive measure of systemic inflammation has been associated with cardiovascular mortality both in primary and secondary prevention settings. In apparently healthy individuals, a high white blood-cell count has been associated with increased cardiovascular mortality and incidence of coronary artery disease, independently of traditional atherosclerotic risk factors (Ikonomidis et al., 2008). In acute myocardial infarction two different inflammatory processes can be considered: the coronary arterial inflammation that leads to the pathogenesis of acute myocardial infarction; and the myocardium inflammation after the acute phase that leads to ventricular remodeling and cardiac repair (Cheng *et al.*, 2005). The immune cells can be related to both processes.

The increase in total white blood-cells occurring in AMI patients is considered as an expression of acute-phase reaction (Dragu *et al.*, 2008; Bodi *et al.*, 2008), reflecting the infiltration of leukocytes into the necrotic tissue in response to ischemia and reperfusion. Neutrophils are the first leukocytes to be found in damaged myocardial area (Yu *et al.*, 2009). The prognostic role of leucocytes is supported by observations from thrombolysis trials that identified leukocyte count as a predictor of short- and long-term adverse clinical outcomes, whereas elevated neutrophil count is significantly associated with myocardial infarct extension and the early development of congestive heart failure (Yu *et al.*, 2009).

Monocytes infiltrate the infarct zone where they appear to orchestrate the cardiac repair and remodeling process through a complex cascade involving cytokines and growth factors secretion (Dragu *et al.*, 2008). Their number is reported (Bodi *et al.*, 2008) to increase 2 to 3

days after the acute episode. The elapsed time between neutrophils and monocytes responses was observed in the present study, as monocyte count peak was only reached at day 2 in AMI patients.

No significant variations were observed in lymphocytes count in the four groups studied. However, in AMI patients the slight decrease in lymphocytes number at the onset of the acute event was emphasized in the longitudinal study where their number significantly increased to 40 days. Lymphopenia was previously described in the literature (Bodi *et al.*, 2008). The reason for the fall in T lymphocytes numbers and activity is not yet completely understood (Takeshita *et al.*, 1997), but several authors proposed that could result from a self-protective response in face of an overshoot of pro-inflammatory cytokines and other products with tissue-damage potential (Steppich *et al.*, 2007; Elenkov *et al.*, 2005). A massive lymphocyte apoptosis is proposed as the underlying mechanism (Bodi *et al.*, 2008). This theory gained more attention since hyper-inflammation was disregarded as the body primary response in acute stress situations, such as sepsis (Hotchkiss & Karl, 2003). Severe lymphopenia occurs in the initial stages of sepsis, but as the acute stress conditions evolves the lymphocyte counts return to normal levels (Bodi *et al.*, 2008).

During the acute event important feedback mechanism may be triggered to protect the organism from an "overshoot" of systemic pro-inflammatory cytokines and other products with tissue-damage potential by activated macrophages (Elenkov *et al.*, 2005). As a consequence of those feedback mechanisms no systemic T lymphocytes activation would happen (Steppich *et al.*, 2007), which could partially explain the low counts of lymphocytes verified at AMI onset. After acute myocardial infarction, myocardial necrosis releases or exposes normally sequestered antigenic constituents that may cause activation and proliferation of lymphocytes (Cheng *et al.*, 2005), returning to the normal levels as verified in our study.

After ischemia/reperfusion injury, leukocyte sequestration and the release of cytokines, such as TNF-α, may occur. Injured myocardium (Dawn *et al.*, 2004), several extra-cardiac tissues and immune system activation (Chiu *et al.*, 2005), proved contributing to circulating TNF-α levels. Increasing levels of this pro-inflammatory cytokine after infarct, as measured in AMI patients, has been reported previously (Bauriedel *et al.*, 2003; Barbaux *et al.*, 2001; Blancke *et al.*, 2005). This increasing suggests a continuous systemic inflammatory stimulation that can trigger and/or amplify local inflammatory responses related to ischemia/reperfusion injury (Barbaux *et al.*, 2001). Though a cytoprotective role for TNF-α has also been suggested (Blancke *et al.*, 2005; Zirlik *et al.*, 2007). The reported influence of TNF-α in the upregulation of adhesion molecules is evidenced by its positive association with sICAM-1.

No significant changes were verified in sICAM-1 levels in CAD and CC patients relative to healthy volunteers (REF group). Also, the levels of this adhesion molecule remained unchanged in AMI patients over 40 days after the acute event. Previous medication intake was found to exert no influence on sICAM-1 levels at admission. Also, in-hospital and follow-up medication had no influence on serial changes of this adhesion molecule. However, sICAM-1 levels were positively correlated with white blood-cell counts, suggesting ongoing inflammatory response in coronary artery disease patients that may favor the adhesion of inflammatory cells at injured site of lesions (Mulvihill *et al.*, 2000; O'Malley *et al.*, 2001). Some of the reported data demonstrated increases in circulating sICAM-1 for the first month after the acute event (O'Malley *et al.*, 2001; Haim *et al.*, 2002; Hartford *et al.*, 2006), plausibly reflecting in specific conditions ICAM-1 expression at the

endothelial surface (Mulvihill *et al.*, 2000). Although a stronger predictive information for sICAM-1 could not yet be found (Haim *et al.*, 2002; Hartford *et al.*, 2006).

Similar values of sP-selectin between coronary artery disease patients and controls, as verified in the present study, had already been reported in literature (Barbaux *et al.*, 2001; Blancke *et al.*, 2005; Khare *et al.*, 2005).

In this study, a negative association between soluble P-selectin levels and age was observed, similar to that referred by Barbaux *et al.* (2001). Those authors called the attention for a complex relation between P-selectin and coronary artery disease dependent on age, which could be related to the different effects of P-selectin according to the stage of progression of atherosclerosis. The soluble form of P-selectin could bind to leukocytes via PSGL-1 without triggering their subsequent recruitment on the vascular surface, which limits the excessive activation and extravasation of leukocytes. Therefore, high levels of sP-selectin may be beneficial in some situations by protecting against inflammatory reactions (Barbaux *et al.*, 2001). This may explain the unexpectedly higher levels of sP-selectin found in REF group similar to the levels of AMI patients and higher than the levels observed in CAD patients. In fact, subjects from REF group were younger than those in AMI and CAD groups, 40% of which had more than 65 years old. Similar sP-selectin values between coronary artery disease patients and controls had already been described in literature (Barbaux *et al.*, 2001; Khare *et al.*, 2005).

Furthermore, during the myocardial infarction, sP-selectin levels may be influenced by an intricate network of processes involving inflammatory stimulus of injured myocardium and vascular wall, and administrated medication. The serial changes of sP-selectin shortly after AMI clearly evidenced the fall of activated platelets in consequence of massive anti-platelet and anti-thrombotic therapeutic measures during intervention. The results of the changes of sP-selectin over time evidence a significant influence of medication. Shimomura *et al.* (1998) reported sP-selectin changes in AMI patients at admission and after reperfusion therapy similar to ours. Currently used therapies, such as ACE-inhibitors and β-blockers effectively counteract heightened platelet activation and aggregability (Bauriedel *et al.*, 2003) resulting in decreased circulating levels of P-selectin (Cha *et al.*, 2004; Xiao *et al.*, 2004) as observed in this work. In fact, decreased sP-selectin levels were also found in CAD patients and CC subjects, which had a long-term therapy history.

C-reactive protein (CRP) is considered by many authors as one of the most suitable candidates as nontradicional risk factors, since it meets most of the criteria to be a useful indicator in cardiovascular diseases (Calabrò *et al.*, 2009). Elevated baseline concentrations of CRP are associated with the risk of atherosclerotic events in general populations and show a predictive value in terms of secondary prevention, both in patients with chronic stable angina and acute coronary syndromes (Calabrò *et al.*, 2009). The prognostic significance of CRP has also been shown in apparently healthy adults without cardiovascular disease (Ridker *et al.*, 2003; Dansesh *et al.*, 2004; Ridker & Cook 2004). Our results are therefore consistent with previous variations of this acute-phase reactant described in literature (Fang *et al.*, 2004; Li *et al.*, 2005; Hartford *et al.*, 2006).

Leukocytes and released cytokines may contribute to ischemia/reperfusion injury by interacting with endothelial cells (Xu *et al.*, 2006), linking the thrombotic and inflammatory responses (Libby & Simon, 2001). Thus, temporal and sequential association of events orchestrated by both inflammatory cells, e.g. white blood-cells, monocytes, neutrophils and lymphocytes, and inflammatory markers, such as TNF-α, CRP, sICAM-1, and sP-selectin seem to be crucial in the initiation of inflammatory responses after ischemia/reperfusion injury.

4.2 Inflammatory markers and angiographic features

There is a substantial interest in research and in clinical practice in the development and application of new biomarkers for risk stratification in patients with acute coronary syndromes.

In particular, strategies combining multiple biomarkers that may reflect diverse pathophysiological contributors to the onset and complications of acute events are appealing as an approach to improve risk assessment and effective therapy. Numerous studies have demonstrated independent associations between levels of various inflammatory markers and the presence of angiographically documented coronary artery disease (Sabatine *et al.*, 2002a). Evidences have been established for improved risk stratification using white blood-cells, B-type natriuretic peptide, high-sensitivity CRP, and troponin T, to mention a few, alone or in combination (Cavusoglu *et al.*, 2006; Sanchis *et al.*, 2004; James *et al.*, 2006). However, the relative importance of the various inflammatory markers with coronary disease is still scarce. Comparative evaluation of newer markers is necessary to assess these candidates for integration into present strategies concerning the evaluation of the strongest candidates in order to guide further development as well as potential clinical application.

Acute phase proteins, adhesion molecules and cytokines have appeared among the potential candidate biomarkers of inflammation based upon prognostic performance in studies in patients with coronary artery disease (Armstrong *et al.*, 2006a; Armstrong *et al.*, 2006b; Armstrong *et al.*, 2006c).

In our study various clinical, biochemical and inflammatory markers were correlated with angiographic findings and risk scores. White blood-cells, neutrophils, sP-selectin, sICAM-1, CRP and TNF-α, were found to be associated either with risk scores for stenosis and TIMI or with the presence of calcium and thrombi in lesions. It was also found that high neutrophil and white blood-cell counts were correlated to high-grade stenosis and to the presence of thrombi. In addition, increased risk score based on white blood-cells counts was strongly correlated with high-risk TIMI score.

White blood-cells counts have been associated with the lesion coverage and magnitude of coronary artery disease (Cavusoglu *et al.*, 2006), lower TIMI flow and myocardial perfusion grades during coronary angiography (Sabatine *et al.*, 2002a). Furthermore, white blood-cell count was strongly associated to multivessel coronary artery disease (Cavusoglu, *et al.* 2006). In unstable angina patients the baseline of white blood-cells count proved to be predictive of unfavorable clinical outcomes being highly significant for death within 30 days to six months (Sabatine *et al.*, 2002a). In addition, establishing levels of white blood-cells counts, contributed to improved risk stratification. In patients with low white blood cell counts the predictive of mortality ranged from 1.5% (25th percentile) to 3.6% among patients with an intermediate white blood-cells count (25th to 75th percentiles), to 5.1% among patients with a high white blood-cell count (75th percentile). So far, no association between white blood cells count and new or recurrent myocardial infarction or rehospitalization for acute coronary syndromes could be established.

High neutrophil counts were also associated to high cardiovascular risk (Horne *et al.*, 2005). In the current study, the correlation of neutrophils with acute event manifestations, especially occlusive stenosis and the presence of thrombi in lesions, suggest their involvement in the coagulation management. Neutrophils are large cells that may accumulate in microvasculature after myocardial infarction. They can produce dramatic pathological anomalies as they adhere to capillary endothelium preventing reperfusion

(Bodi *et al.*, 2008). Neutrophils that are recruited to the thrombosis region may be trapped in cloth releasing, during degranulation, myeloperoxidase. Myeloperoxidase is in fact an abundant leukocyte lysosomal enzyme. It was found to be elevated in culprit lesions that have fissured or ruptured in patients with sudden death from cardiac causes. Numerous lines of evidence suggest mechanistic links between myeloperoxidase and both inflammation and cardiovascular disease, therefore linking neutrophils to acute coronary syndromes and highlighting its potential and usefulness for risk stratification among patients with chest pain (Brennan *et al.*, 2004).

Our concurrent results also evidence the ability of white blood-cells and neutrophils as inflammatory entities to predict the presence of angiographic coronary disease and in particular the acute event. The negative correlation of sP-selectin with the class of TIMI (flow from 0 to 3) reinforced the value of combining multiple pathophysiological contributors in the evaluation of angiographic coronary disease. High values of s-Pselectin are linked to limitations in flow. The sP-selectin is an adhesion molecule involved in cell-platelet aggregation and adhesion (Blann *et al.*, 2003; Armstrong *et al.*, 2006c). The verified synchronized rise of sP-selectin, white blood-cells and neutrophils count may be useful to further improve patients' evaluation and prognosis.

Also high CRP levels were associated to stenosis. Inflammation, atherosclerotic plaque rupture or myocardial necrosis, are mechanisms responsible for elevated CRP levels in the circulation. The potential use of CRP in patients' diagnosis and in risk stratification of patients with coronary disease has been largely studied. Although the use of CRP as a prognostic marker still remains controversial (Packard & Libby, 2008). In fact, our results indicated an inverse association of CRP levels with stenosis what may suggest that CRP increases may be governed by other mechanisms. Results may also express the non-relevance of CRP levels immediately after the acute event. Actually, the delay of approximately 48-h after the acute event for CRP increases is well documented in the literature (Fang *et al.*, 2004; Hartford *et al.*, 2006). Biomarkers studied in our work were measured at patient's admission, what for myocardial infarction patients correspond to a maximum of 6-h after acute event. Our findings also pointed out for a positive correlation of high-risk CRP score with sICAM-1, suggesting that endothelial activation may influence CRP expression rather than coronary stenosis.

On the other hand, the presence of calcium in the culprit lesion was associated to higher lymphocytes count, indicating that these cells may express the activity of the atherosclerotic plaque, as mineralized lesions usually indicate more stabilized plaques (Fischer *et al.*, 2000). As referred previously the moderate and non-significant decrease of lymphocytes at the acute phase of myocardial infarction is expressed in the longitudinal study carried out in our work. Lymphocytes are long-lived cells that memorize specialized information about the antigen pool at the individual level (Bodi *et al.*, 2008). Therefore, lymphocytes may keep information about the plaque composition and consequently be associated with the mechanisms of plaque formation during the life span of the individual. These findings are in line with current knowledge on coronary atherosclerotic plaque burden. Plaque burden, and not stenosis severity, was a more important marker of disease. Also, the prognosis of coronary artery disease is more closely related to atherosclerosis plaque stability than the extent of a particular stenosis. The lesion vulnerability is thought to be associated to the plaque composition (Fischer *et al.*, 2000). Lesions contain a lipid core intertwined by fibrous tissue that contributes to the disarrangement of intimal structure. In addition to the presence of macrophages and smooth muscle cells, lymphocytes and monocytes have been identified

in the sub-endothelial region close to the lipid core. The continuous accumulation of extracellular lipids and cell debris promote the atheroma growth with prominent fibrous connective tissue and intimal thickening. Eventually the lipid core or other areas of the arterial wall may calcify and the lesions may present fissures, hematomas and thrombi. Lesions with high fibrotic content are usually more occlusive and ultimately may progress to complete occlusion without participation of acute plaque rupture. Therefore, the susceptibility to rupture is not strictly linked to significant stenosis. Also, the acute coronary syndromes are associated with plaque disruption and associated flow-limiting thrombus that may not be caused by of a non-obstructive plaque.

The extent of coronary atherosclerosis, rather than the severity of stenosis, may be the most important predictor of death due to acute myocardial infarction or sudden cardiac death (Schmermund *et al.*, 1997). The quantification of atherosclerotic burden has become vital to proper risk stratification, especially in the intermediate risk population (Mieres *et al.*, 2005). Established noninvasive methods of evaluating CAD, such as stress testing, generally identify only patients with advanced atherosclerotic disease leading to a flow-limiting coronary stenosis and myocardial ischemia (Greenland & Gaziano 2003; Rumberger *et al.*, 2005). More recently published studies demonstrate a high sensitivity of coronary artery calcium for the presence of coronary artery disease but a lower specificity for obstructive coronary artery calcium depending on the magnitude of the coronary artery calcium (Budoff & Gul, 2008). Coronary calcification is a marker of atherosclerosis that can be quantified with the use of cardiac coronary tomography and it is proportional to the extent and severity of atherosclerotic disease. Coronary artery calcium was found to be a stronger independent predictor of future events than a sum of all of the conventional risk factors combined (Kennedy *et al.*, 1998). Based on multiple observational studies, patients with increased plaque burdens (increased coronary artery calcium) are approximately ten times more likely to suffer a cardiac event over the next 3–5 years (Budoff & Gul, 2008). Opposite, Bauer and coworkers (Bauer *et al.*, 2009) combining angiographic findings of calcified and non-calcified plaque burden and stenosis severity and the myocardial perfusion imaging finding of ischemia proposed that non-calcified plaque burden is a better predictor of the finding of myocardial ischemia at stress myocardial perfusion imaging than are calcium score and degree of stenosis.

A variety of inflammatory factors including the actions of inflammatory cells are thought to play an important role in plaque stability and calcification (Morrow *et al.*, 2008). Vascular calcification is a prominent feature of atherosclerosis but the mechanisms underlying calcification are still unclear. Vascular smooth muscle cells are currently considered to be responsible for the formation of vascular calcifications. Cytokines play an important role in regulation of vascular smooth muscle cells growth and differentiation.

In fact, in our work a strong positive association of TNF-α with lesion length was found. In addition high TNF-α levels (≥3.16 pg/ml) were associated with high calcium percentage in plaques. TNF-α was also found to be augmented at patients' admission (coronary artery disease and myocardial infarction) and progressively increase to 40 days in the infarction evolution. These findings suggest a dual role of TNF-α in coronary artery disease. On one hand TNF-α was associated with vascular calcification, possibly expressing a role in the stabilization of the plaque, and on the other hand TNF-α was related to the inflammatory process after myocardial infarction.

This pleiotropic nature of TNF-α is documented in literature (Trion & Laarse, 2004; Lencel *et al.*, 2010). TNF-α influences many aspects of atherosclerosis by increasing the permeability

of endothelial cells, promoting monocyte adhesion, inducing macrophage differentiation, and promoting foam cell formation. TNF-α is also a regulator of bone formation. In both intima and media, calcification resembles bone formation. This cytokine has indeed been shown to stimulate *in vitro* the expression by vascular smooth muscle cells key enzymes of the mineralization process inducing the calcification of collagen fibrils. TNF-α can also trigger the differentiation of vascular smooth muscle cells and/or mesenchymal stem cells into osteoblast-like cells, by expressing specific transcription factors, eventually leading to formation of a bone-like tissue (Lencel *et al.*, 2010).

5. Conclusion

Reported results support the concept of a differential response of inflammatory markers in coronary artery disease. In acute events the inflammatory response and the interactions between the inflammatory markers observed influenced both the clinical outcome and the vascular remodeling that persist after clinical stabilization as given by the interplay of the studied inflammatory mediators.

The inflammatory multimarker approach used and the differential response observed can contribute to a better assessment of the disease evolution and therapeutic plans.

Only the simultaneous assessment of several markers, as innovatively done in the present study, can give a valuable contribution to the understanding of their importance in coronary artery disease and in the evolution of acute myocardial infarction.

This study was useful both in research and clinical practice approaches. Combining inflammation assessment together with angiographic findings helped unravelling non-invasive markers for the disease.

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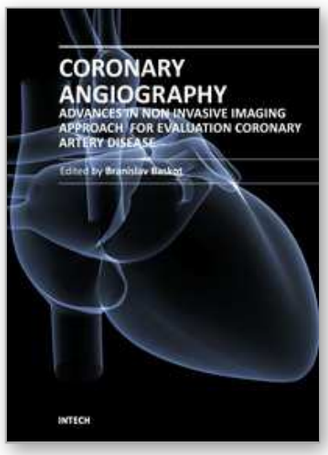

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In the intervening 10 years tremendous advances in the field of cardiac computed tomography have occurred. We now can legitimately claim that computed tomography angiography (CTA) of the coronary arteries is available. In the evaluation of patients with suspected coronary artery disease (CAD), many guidelines today consider CTA an alternative to stress testing. The use of CTA in primary prevention patients is more controversial in considering diagnostic test interpretation in populations with a low prevalence to disease. However the nuclear technique most frequently used by cardiologists is myocardial perfusion imaging (MPI). The combination of a nuclear camera with CTA allows for the attainment of coronary anatomic, cardiac function and MPI from one piece of equipment. PET/SPECT cameras can now assess perfusion, function, and metabolism. Assessing cardiac viability is now fairly routine with these enhancements to cardiac imaging. This issue is full of important information that every cardiologist needs to now.

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