Inflammatory Markers of Atherosclerotic Plaque Stabilization after Acute Coronary Event – Temporal Trends

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Objective

To evaluate the length of time required for atherosclerotic plaque stabilization in acute coronary syndromes (ACS), using inflammatory markers.

Methods

In this prospective study, C-reactive protein (CRP), fibrinogen, factor VIIIc, interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) levels were measured on admission, at discharge, and three and six months post-discharge in 40 patients with non-ST-segment elevation ACS (NSTE-ACS) and 40 healthy subjects.

Results

C-reactive protein levels were significantly higher on admission and at discharge, but not at three and six months post-discharge, compared with the control group. Fibrinogen levels remained unchanged, except at six months, when they were significantly lower than in the control group. Factor VIII-c did not differ from that of the control group on admission, but it was significantly higher at discharge, with no differences at three and six months. Interleukin-6 levels were significantly higher than in the control group in all time points. However, they declined significantly between discharge and three months. In no time point was TNF-α significantly different from that of the control group. Only IL-6 correlated significantly and independently with future cardiovascular events.

Conclusions

With respect to CRP and factor VIIIc, plaque stabilization is suggested in up to three months; IL-6 analysis suggests stabilization as from the third month, although it remained higher than that of the control group for up to six months. Only IL-6 showed prognostic value for further events within a year.

Key words

Inflammatory markers, stable angina, non-ST-segment elevation myocardial infarction.

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Acute coronary syndromes (ACS) – unstable angina and myocardial infarction – are caused mainly by rupture or erosion of atherosclerotic plaque, with subsequent thrombus formation. It is known that plaque instability, among other characteristics, is closely related to the inflammatory process[1-4].

Of the circulating inflammatory markers, C-reactive protein (CRP) is the most extensively studied; high CRP plasma levels are found in ACS patients and are associated with increased rates of future events[5]. On the other hand, interleukin-6, which prompts C-reactive protein production, is also elevated in ACS patients and is associated with worse prognosis, thereby reinforcing the role of inflammation in these syndromes[6]. Tumor necrosis factor-α (TNF-α), a cytokine produced mostly by activated macrophages, stimulates the synthesis of other cytokines[7]. Persistently increased TNF-α levels after acute myocardial infarction (AMI) are correlated with worse prognosis; however, little information is available on TNF-α kinetics in non-ST-segment elevation ACS[8]. Fibrinogen is an acute-phase protein directly involved in the coagulation cascade; increased fibrinogen levels are associated with higher risk of thrombotic events[9] and higher risk of events in ACS patients[10-14]. Finally, factor VIII-c, a procoagulant enzyme cofactor, is also involved in inflamation[15] and is a marker of ischemic heart disease[16-18].

Little has been published about how long it takes for stabilized coronary lesions to heal completely in patients with non-ST-segment elevation ACS. A study using cell adhesion molecules (CAMs) suggested that the inflammatory response might persist for up to six months following an acute coronary event[19].

The primary focus of this study was to evaluate the length of time required for atherosclerotic plaque to become stabilized in patients with non-ST-segment elevation ACS treated clinically, through the behavior of plasma inflammatory and coagulation markers levels.

Methods

Forty out of the 64 patients admitted to our hospital between September 2000 and May 2001 with unstable angina and no-ST-segment elevation AMI were included in the study (22 men; mean age 61 ± 12). Forty age- and sex-matched healthy volunteers served as the control group. Patients were followed-up over one year.

Inclusion criteria were chest pain lasting 24 hours, suggestive of myocardial ischemia of accelerated pattern, or a prolonged one (> 20 minutes), or with recurrent episodes at rest, or at minimal exertion, in addition to at least one of the following: (a) new or presumed new ECG changes (any of the following three characteristics): ST-segment depression ≥ 0.5 mm, transient ST-segment elevation (< 20 minutes) ≥ 1 mm, T-wave inversion ≥ 3 mm in two or more contiguous leads; (b) and raised levels of cardiac markers (CKMB ≥ 2X the upper limit of normal).

Exclusion criteria were persistent ST-segment elevation; secondary angina; history of percutaneous coronary intervention (PCI) or coronary artery bypass grafting (CABG) within the previous six months; left bundle branch block (LBBB) or pacemaker rhythm; fibrinolytic therapy in the last 48 hours; any inflammation, infection or neoplasic disease; regular or chronic use of anti-inflammatory drugs in the previous two months; AMI and stroke within the past six months; severe congestive heart failure (CHF) or cardiogenic shock; severe systemic disease; serum creatinine > 2.5 mg/dL; or surgery or trauma within the past thirty days. Patients who underwent CABG or PCI during the study were also excluded (nine patients were excluded on the basis of this criterion).

Clinical management - In-hospital medications were used according to the medical approach (see Table 1). The assistant doctor also made decisions regarding invasive or conservative strategy and the need of coronary revascularization.

Blood samples - Peripheral venous blood samples were collected on admission, at hospital discharge, and three and six months after discharge. A single measurement was performed in healthy controls. All samples (patients and controls) were centrifuged, stored at -70 °C, and tested for high-sensitivity CRP, IL-6, TNF-α, fibrinogen and factor VIII-c in a single series at the end of the study.

Laboratory assessment - High-sensitivity CRP (hs-CRP) was determined by particle-enhanced immunoturbidimetric assay (COBAS INTEGRA 700; Roche Diagnostics). IL-6 and TNF-α were evaluated by ELISA (Enzyme-Linked Immunosorbent Assay- R&D Systems). Fibrinogen was measured using the automated Clauss method (Fibriquik, manufactured by Organon Teknika). Factor VIII activity was measured by determining the ability of the tested sample to correct the clotting time of factor VIII-deficient plasma (Organon Teknika).

Purpose - The primary purpose of this study was to indirectly evaluate the length of time required for atherosclerotic plaque stabilization in ACS patients (unstable angina and non-ST-segment elevation AMI), by measuring inflammatory markers (CRP, fibrinogen, factor VIII, IL-6, and TNF-α) during six months after hospital discharge. The secondary purpose was to correlate the plasma concentrations of these markers with the prognosis (risk of future cardiovascular events) in patients with non-ST-segment elevation ACS in up to one year of follow-up.

Statistical analysis - The sample size of forty patients was based on probabilistic properties of means and proportions estimators, because no *a priori* information is available for an estimation that takes into account the accuracy of final results. As the majority of inflammatory marker values were not normally distributed, data were expressed as median (25th-75th percentile); when there was a normal distribution, values were expressed as mean

± standard deviation.

To compare both groups, the Mann-Whitney test (non-parametric) was used for non-normally distributed values, while Student's unpaired t-test, Welch-corrected, was used for normally distributed values.

Categorical variables were expressed as number and percentage. Fisher's exact test was performed to compare these variables.

When the patient was his own control and thus acted as paired groups do, Wilcoxon's non-parametric paired test was used to compare the different time points analyzed. The same comparisons in the different time points were performed for normally-distributed markers using the paired t-test.

Correlation between cytokines and acute phase protein values was performed using a Spearman non-parametric correlation.

Every marker was compared with the composite endpoint of recurrent angina, readmission for angina, reinfarction or cardiovascular death after a one-year follow-up. In these analyses, the Mann-Whitney test (univariate analysis) and logistic regression (multivariate analysis) were used.

Group differences were considered statistically significant when $p \leq 0.05$ (two-tailed).

Ethical considerations - The project was approved by the Institutional Research Ethics Committee of the Faculdade de Medicina de São José do Rio Preto, São Paulo (Famerp) and the Faculdade de Medicina da Universidade de São Paulo (FMUSP). Informed consent was obtained from all patients.

Results

The characteristics of the study population are shown in Table 1. As may be noted, groups were paired according to age and gender. As expected, the case group showed a larger proportion of patients with specific risk factors and pre-treatments.

Figure 1 shows that median CRP levels were significantly higher in ACS patients, compared to control subjects, both on admission and at discharge, but not at three and six months. Median IL-6 levels declined significantly between discharge and three months. However, they remained significantly higher than those of the control group for up to six months (Fig. 2). Median TNF-α levels decreased significantly between three and six months after discharge. Yet, the levels compared with the controls did not reach statistical significance at any time of follow-up (Fig. 3). Change in patterns over time were also found for fibrinogen (Fig. 4), although no significant increase was found during follow-up compared with controls. Factor VIII-c levels were significantly higher at discharge, compared with controls, but not at three and six months (Fig. 5).

Correlations between acute-phase proteins and cytokines- There were significant correlations between CRP and IL-6 levels measured on admission ($r = 0.5$; $p < 0.001$), at discharge ($r = 0.6$; $p < 0.001$), and after three months ($r = 0.4$; $p < 0.007$); between CRP and fibrinogen levels measured on admission ($r = 0.4$; $p = 0.003$) and at discharge ($r = 0.4$; $p = 0.003$); and between CRP and TNF-α levels measured on admission ($r = 0.3$; $p = 0.024$) and at discharge ($r = 0.4$; $p = 0.009$).

No significant correlations between CRP and factor VIIIc were found at any time during follow-up.

Clinical events and inflammatory marker levels during a one-year follow-up- At the end of a follow-up period of one year, there were 45 cardiovascular events (32 recurrent cases of angina without hospitalization, four deaths, eight readmissions for angina, and one non-fatal AMI). Of the inflammatory markers analyzed, only IL-6 levels on admission correlated significantly and independently with the composite endpoint, as shown in Tables 2 and 3.

Discussion

CRP - This study showed that CRP levels were high in patients with unstable angina or non-ST-segment elevation AMI. Liuzzo *et al*.[20] demonstrated that 65% of the patients had CRP levels ≥ 3 mg/L on admission, similar therefore to our findings, in which 62.5% of the patients had CRP levels ≥ 4 mg/L (median CRP for controls) at the time of admission. On the other hand, elevated CRP plasma levels on admission were found in 65% of the patients, higher than those described by Biasucci et al[21] (49%), which may suggest a higher risk population in the present study.

Following hospital discharge, O'Malley et al[22] did not find significant differences in the CRP levels of patients and controls after three months of follow-up, which is similar to our findings. To our knowledge, no comparative data is available in the literature that analyzes CRP levels after three months of follow-up in patients with non-ST-segment elevation ACS.

Different studies have emphasized the usefulness of CRP measurement during acute coronary syndrome as a marker for future cardiac events[12,20,23,24]. However, this correlation has not been found by others[22,25-28], which is again similar to our results.

IL-6 - Biasucci et al[6] measured IL-6 levels in 38 patients with unstable angina and in 29 with stable angina. Median IL-6 levels were 5.25 pg/mL in patients with unstable angina and below the detection limit (< 3 pg/mL) in patients with stable angina. Likewise and using the same methodology, we found median IL-6 levels of 6.15 pg/mL on admission in the case group with non-ST-segment elevation; in the control group, consisting of healthy subjects, median levels were

Table 1 – Characteristics of the Study Population

	Cases	Controls	p value
Number of patients	40	40	
Age (years, mean ± SD)	61 ± 12	65 ± 10	0.11
Gender (M/F)	22/18	22/18	1.0
Risk factors; number of patients (%):			
-Family history of CAD	18 (45)	0	<0.001
-Dyslipidemia	14 (35)	9 (22.5)	0.069
-Diabetes	10 (25)	3 (7.5)	0.033
-Hypertension	38 (95)	14 (35)	<0.001
-Cigarette smoking	14 (35)	2 (5)	<0.001
Past history; number of patients (%):			
-Previous MI > six months	11 (27.5)	0	
-Stable angina	2 (5)	0	
-Unstable angina	2 (5)	0	
-CABG > six months	10 (25)	0	
-PTCA > six months	4 (10)	0	
Previous medication (%):			
-Beta-blocker	14 (35)	1 (2.5)	<0.001
-ACEI	15 (37.5)	4 (10)	0.007
-ASA	16 (40)	1 (2.5)	<0.001
-Calcium antagonist	11 (27.5)	1 (2.5)	0.003
-Vastatin	6 (15)	1 (2.5)	0.108
-Digitalis	5 (12.5)	0	0.054
-Diuretics	12 (30)	7 (17.5)	0.293
-Nitrate	6 (15)	0	0.025
In-hospital medication (%):			
-Beta-blocker	39 (97.5)	0	
-ACEI	39 (97.5)	0	
-ASA	40 (100)	0	
-Calcium antagonist	22 (55)	0	
-Vastatin	19 (47.5)	0	
-Diuretics	16 (40)	0	
-Nitrate	29 (72.5)	0	
Admission diagnosis (%):			
-Unstable angina	34 (85)	0	
-Non-ST-segment elevation AMI	6 (15)	0	

CAD - coronary artery disease; AMI - acute myocardial infarction; MR - myocardial revascularization; PTCA - percutaneous transluminal coronary angioplasty; ACEI - angiotensin-converting enzyme inhibitor; ASA - aspirin; ST - standard deviation; n - number; CABG - coronary artery bypass graft.

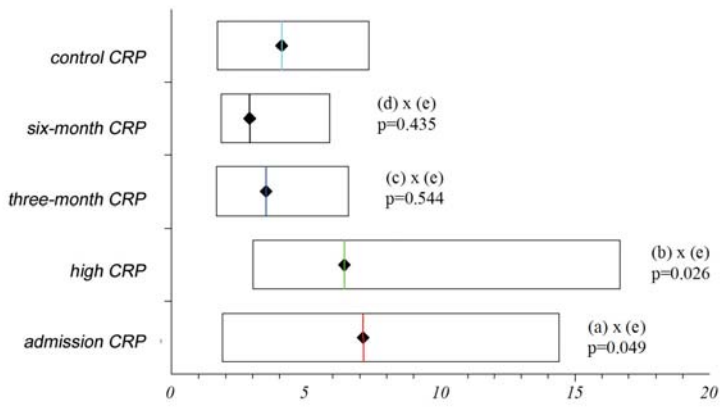

***Fig. 1** – Median (25th-75th percentile) CRP levels (mg/L) of the case group compared with the control group at the four time points analyzed during follow-up.*

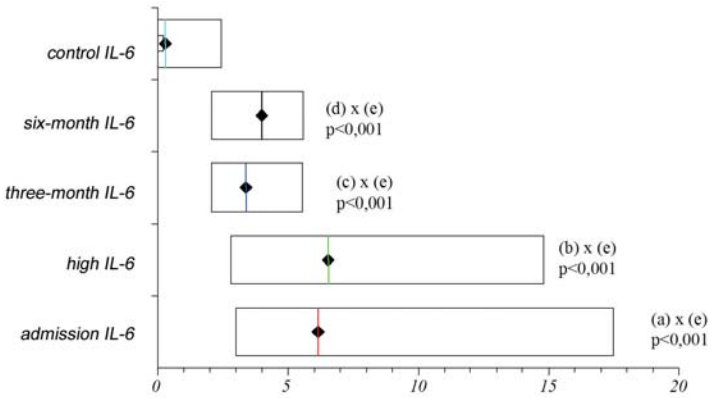

Fig. 2 *– Median (25th-75th percentile) IL-6 levels (pg/mL) of the case group compared with the control group at the four time points analyzed during follow-up.*

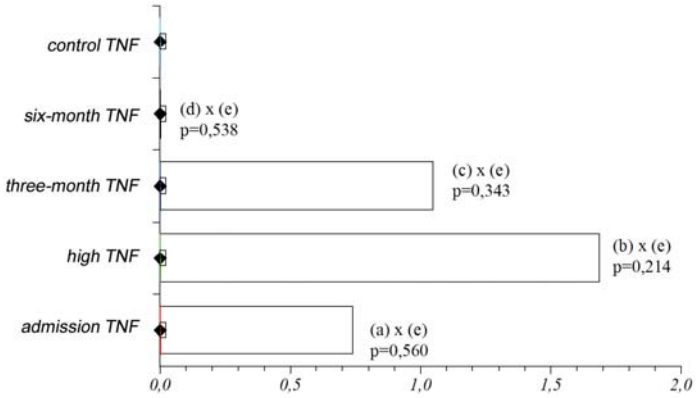

Fig. 3 *– Median (25th-75th percentile) TNF-α levels (pg/mL) of the case group compared with the control group at the four time points analyzed during follow-up.*

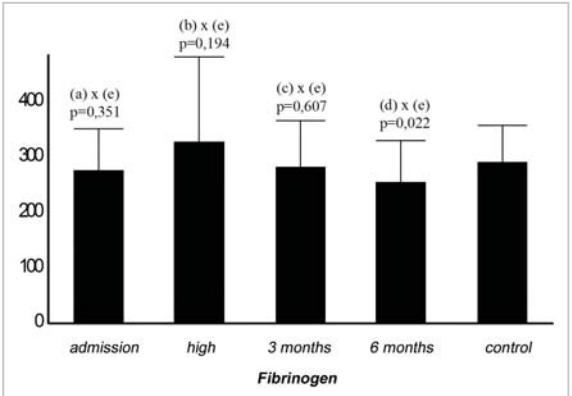

Fig. 4 *– Mean ± SD fibrinogen levels (mg/dL) of the case group compared with the control group at the four time points analyzed during follow-up.*

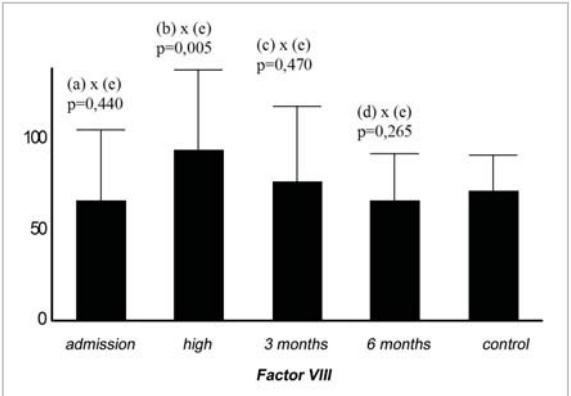

Fig. 5 *– mean ± sd factor VIII levels (%) of the case group compared with the control group at the four time points analyzed during follow-up.*

also below detection limit. These findings support the hypothesis that detectable levels of IL-6 are related to atherosclerotic plaque instability, suggesting that this is not independent of myocardial necrosis, since this cytokine is also detectable in the absence of raised levels of creatine kinase or troponin T[6].

That study[6] found a significant correlation between IL-6 and CRP levels ($p = 0.013$) measured on admission. In the present study, a significant correlation between both markers was obtained not only on admission ($p = 0.001$) but also at discharge and at three months of follow-up. These findings corroborate the hypothesis that, in the presence of ACS, increased levels of acute-phase proteins are a result of cytokine inflammatory pathways.

Table 2 – Inflammatory Markers (admission) Correlation with Events* in a One-year Follow-up – Univariate Analysis

Inflammatory marker	with event (n=25)	without event (n=55)	p-value	95% CI
CRP (median, 25th-75th percentile)	7.82, 1.73-12.21	4.74, 1.71-7.84	0.288	–1.21-5.26
IL-6 (median, 25th-75th percentile)	5.87, 2.37-13.90	1.32, 0-4.55	< 0.001	1.8- 5.87
TNF-α (median, 25th-75th percentile)	0, 0-1.24	0, 0-0	0.644	0-0
Fibrinogen (mean ± SD)	272.1± 67.9	288.6 ± 73.1	0.337	–50.6-17.5
Factor VIII (mean ± SD)	73.8 ± 42.8	66.9 ± 23.7	0.354	–7.9-21.7

**Events = recurrent angina, readmission, myocardial infarction, death; CRP = C-reactive protein; IL-6 = interleukin-6; TNF = tumor necrosis factor.*

Table 3 – Inflammatory Markers (admission) Correlation with Events* in a One-year Follow-up – Multivariate Analysis

Inflammatory marker	OR	p value
CRP	-0.570	0.569
IL-6	2.190	0.029
TNF-α	-0.757	0.449
Fibrinogen	-1.431	0.152
Factor VIII-c	0.520	0.603

**Events = recurrent angina, readmission, myocardial infarction, death; CRP = C-reactive protein; IL-6 = interleukin-6; TNF = tumor necrosis factor.*

With regard to IL-6 evolution after discharge, O'Malley et al[22] found that, compared to controls, unstable patients showed significantly higher IL-6 levels on admission, followed by a drop in three months, at which time IL-6 levels in both groups became similar. In our study, although the number of patients with elevated IL-6 levels in the case group decreased significantly between discharge and at three months, this number remained significantly higher than that of the control group in up to six months of follow-up. These differences may be explained by the fact that, in the O'Malley and et al study, the unstable angina population was at lower risk if we take into account that median IL-6 level on admission (3±3 pg/ml^{-1}) was half of that obtained on admission in our study. It could be also speculated that the persistently elevated levels observed in our patients may be related to reactivation of the inflammatory process.

As for the prognosis, it was recently demonstrated that high IL-6 levels on admission in patients with non-ST-segment elevation ACS is a good marker of future events[7,29,30]. Accordingly, in the present study no significant and independent correlation was found between IL-6 levels on admission and cardiovascular events up to one year of follow-up.

TNF-α - Little data is available regarding TNF-α levels in non-ST-segment elevation ACS. Simon et al[31] compared patients with unstable angina with healthy subjects and found no significant difference between both groups. Similarly, in the present study TNF-α levels in the case group were not significantly different from those of the control group in any of the periods studied. This might be justified by Bazaran et al findings that demonstrated that plasma TNF-α levels in patients with AMI and unstable angina peaked within six hours and disappeared after 24 hours from symptoms onset. In our analysis, TNF-α levels on admission were higher compared with the control group, although not significantly. Indeed, TNF-α plasma half-life is short, and baseline levels are low in most patients[32].

Just as TNF-α levels did not rise significantly compared with the control group at any time point of follow-up, neither was a correlation found between TNF-α levels and future events by the present study. Cusack et al[30] also found no difference regarding inflammatory marker levels among patients with unstable angina who subsequently experienced a major coronary event .

On the other hand, Ridker et al[33], analyzing a sample of patients included in the CARE trial (Cholesterol And Recurrent Events), demonstrated that plasma TNF-α levels were elevated several months after AMI among subjects at high risk for recurrent coronary events. Blood samples were collected on an average of nine months after AMI, suggesting that the higher risk for recurrent coronary events associated with TNF-α is not merely the result of a transient marker increase following coronary occlusion. These findings also suggest that subclinical persistent instability can be detected by presence of inflammatory markers, such as TNF-α. Nevertheless, it is worth noting that the origin of persistently elevated TNF-α levels among high risk subjects (post-AMI) remains unknown[33].

Fibrinogen - Becker et al[34] demonstrated an initial drop in fibrinogen levels within the first 12 to 24 hours, exceeding baseline levels in 96 hours. This result is consistent with fibrinogen levels measured on admission in the present study, when lower levels were found, compared to those of the control group, which increased up to discharge. In sum, fibrinogen levels during the acute phase seem to peak between three to five days, returning gradually to baseline values after resolution of the inflammation[35].

In the present study, fibrinogen levels in the case group were not significantly different from those of the control group during hospitalization or at three months of follow-up; however, at six months of follow-up, mean fibrinogen level was significantly lower in case subjects than in control subjects, suggesting a negative rebound effect,

which may be related to an exacerbation of fibrinogen uptake after the acute episode. To our knowledge, that is no study that analyzes fibrinogen profile up to six months of follow-up.

With regard to the prognostic value of fibrinogen levels after an acute coronary event, some authors describe this inflammatory marker as useful[10-14,34], while others do not[36-38], as is the case of this study.

Factor VIII-c - Olinic et al[17] found factor VIIIc activity to be significantly higher ($p < 0.01$) in 17 patients with unstable angina than in 10 healthy control subjects. In our analysis, factor VIIIc activity in the case group was similar to that of the control group on admission and significantly higher at discharge, returning to normal levels at three months of follow-up. Similarly, al-Nozha et al[16] demonstrated progressive and significant elevation in factor VIIIc activity in patients with unstable angina between eight hours and five days of evolution.

In the present analysis, no correlation was found between factor VIIIc and future events in ACS. In fact, there is no evidence in the literature regarding the prognostic value of increased factor VIIIc in ACS.

Study limitations - Firstly, the lack of correlation between the inflammatory markers analyzed and future events, with the exception of IL-6, should be interpreted cautiously, due to the small number of patients studied. Furthermore, in this study, patients who underwent revascularization procedures at any time during the follow-up period were excluded so that the natural course of inflammation could be documented in non-ST-segment elevation ACS. In most studies, such patients are included in this population, which might affect the results. Secondly, routine laboratory measurements of inflammatory markers, especially cytokines, present a host of technical difficulties, partly due to their short plasma half-lives and the presence of blocking factors[39], resulting in differences when comparing several studies. Additionally, thus far no standard and widely used methodology is available, and there are several kits to measure a single product, such as high-sensitivity CRP. The relevance of this issue was underscored by Roberts et al[40], who analyzed four high-sensitivity CRP methods and demonstrated differences among then in assessing a healthy population. Finally, troponin T was not measured in these patients, because this inflammatory marker was not available at the time the study was made.

Conclusion

Indirect temporal analysis of plaque stabilization following acute coronary event varies according to the inflammatory markers analyzed:

1. When taking CRP and factor VIII into account, plaque stabilization is suggested in up to three months.

2. IL-6 analysis suggests stabilization as from the third month of follow-up, remaining at these levels, though elevated compared to controls in up to six months of follow-up.

3. Fibrinogen and TNF-α were not useful markers in evaluation of plaque stabilization, since they did not increase significantly compared with the control group.

As for the prognosis, among the inflammatory markers analyzed, only IL-6 correlated significantly and independently with the development of future cardiovascular events.

Potencial Conflict of Interest

No potential conflict of interest relevant to this article was reported.

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