

## RAPID COMMUNICATION

# Inflammation and circulating endothelial progenitor cells in patients with coronary artery disease and residual platelet reactivity

Antonio Eduardo P. Pesaro,<sup>I,II</sup> Carlos V. Serrano Jr,<sup>II</sup> Marcelo Katz,<sup>I</sup> Alexandre H. Campos,<sup>I</sup> Renato D. Lopes,<sup>III,IV</sup> Luciana C. Marti,<sup>I</sup> Herlon S. Martins,<sup>IV</sup> Rodrigo S. Sunahara,<sup>II</sup> Raul C. Maranhao,<sup>II</sup> José C. Nicolau<sup>II</sup>

<sup>I</sup>Albert Einstein Hospital, São Paulo/SP, Brazil. <sup>II</sup>Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo, Heart Institute, São Paulo/SP, Brazil. <sup>III</sup>Duke University Medical Center, Duke Clinical Research Institute, Durham, NC, USA. <sup>IV</sup>Federal University of São Paulo, Paulista School of Medicine, São Paulo/SP, Brazil. <sup>V</sup>Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo, São Paulo/SP, Brazil.

Email: antonioepp@einstein.br

Tel.: 55 11 2151-5408

## INTRODUCTION

Atherosclerotic plaque development and progression are associated with vascular inflammation, endothelial dysfunction, and platelet activation (1). Inflammatory mediators, such as C-reactive protein (CRP) and oxidized low-density lipoprotein (oxLDL), can trigger platelet activation and subsequent thrombus formation (2). In turn, activated platelets secrete cytokines, recruit monocytes, and release soluble CD40 ligand (sCD40L), which mediate vascular inflammation (3). An important determinant of endothelial dysfunction in patients with coronary artery disease (CAD) is the reduced number of endothelial progenitor cells (EPCs) (4). Interestingly, EPCs can inhibit platelet activation, while activated platelets modulate EPC proliferation (5).

Aspirin is widely used in the prevention of complications related to CAD. However, the mechanisms that promote high residual platelet reactivity (RPR) in aspirin-treated patients are still under debate (6,7). Because inflammation and endothelial dysfunction may possibly contribute to platelet activation and RPR, we sought to compare inflammatory markers and EPC levels between stable CAD patients treated with aspirin with and without RPR.

## METHODS

**Study design.** This was a substudy of a randomized trial testing lipid-lowering drugs that was performed from July 2006 to January 2009 in a single tertiary specialized cardiology hospital (ClinicalTrials.gov: NCT00474123). Details of the protocol have been published previously (8). In brief, all of the patients (n=83) met the following inclusion criteria: angiographically documented CAD, simvastatin treatment, and age <80 years. The exclusion criteria were the following: myocardial infarction or revascularization <3 months, ventricular systolic dysfunction, warfarin treatment, malignancy, inflammatory diseases, and renal/liver dysfunction.

Of the original 83 patients, 63 were on aspirin treatment (100 mg/day), presented complete data for hsCRP, sCD40L, oxLDL, and circulating EPCs, and were included in the present study. No patients were treated with clopidogrel within the four weeks before enrollment. Platelet aggregation was measured by the platelet function analyzer (PFA)-100 closure time (CT). Patients with a CT ≤150 seconds (sec) were considered as having RPR (9).

We compared baseline characteristics, inflammatory levels, and EPC counts between patients with and without RPR. This study was approved by the institutional research committee. All of the subjects gave written informed consent.

**Platelet aggregation using the PFA-100 assay.** Samples were collected in 3.2% sodium citrate (buffered, pH 5.5, Vacutainer, Becton Dickinson, UK) for platelet function tests. Platelet function was measured with the PFA-100 (epinephrine) point-of-care assay (Dade-Behring, Deerfield, IL, USA). We defined the upper normal limit for PFA-100 CT as 150 sec, in agreement with previous studies and the manufacturer's protocol recommendation (9).

**hsCRP, sCD40L, and oxLDL.** Serum hsCRP was assessed with a latex microparticle-enhanced immunoturbidimetric assay (BN II analyzer, Dade Behring, Deerfield, IL, USA). The minimal detectable concentration of hsCRP was 0.2 mg/L. For the other markers, commercial ELISAs detecting sCD40L (R&D Systems, Minneapolis, MN, USA) and oxLDL (Mercodia, Winston Salem, NC, USA) were used. The detection limits of the ELISAs were as follows: sCD40L 15.6 pg/mL (intra-assay variability not available); oxLDL, 0.3 U/L (intra-assay variability 6.1%).

**Endothelial progenitor cells.** The detection of EPCs was performed as previously described (10). In brief, 100 µL of mononuclear cells derived from peripheral blood was immunostained with monoclonal antibodies against human CD31/FITC, CD3/PE-Cy5, CD19/PE-Cy5, CD34/PE-Cy7, CD14/APC Cy-7 (BD Pharmingen, UK), VEGFR2/PE (R&D System, Minneapolis, MN, USA), and the appropriate isotype control antibodies (BD Pharmingen, UK). EPCs were negative for CD3, CD19, and CD14 and positive for CD31, CD34, and VEGFR2.

At least 70,000 events were acquired per tube by flow cytometry (FACSARIA, BD Biosciences, UK). The analysis was performed using FACSDIVA software (BD Biosciences, UK).

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No potential conflict of interest was reported.

**Statistical analysis.** Continuous variables were expressed as the mean ( $\pm$  standard deviation [SD]) or median (25<sup>th</sup>, 75<sup>th</sup> percentile). Differences were analyzed with a 2-sample *t* test when the distribution was normal; otherwise, we used the Mann-Whitney U test. The  $\chi^2$  test was used for categorical variables. A value of  $P < 0.05$  was considered significant. Analyses were performed using STATA/SE 9.2 (Stata Corp LP, USA). Spearman's rank test was performed to assess correlations.

**RESULTS**

The baseline characteristics, including lipids and metabolic profiles, of the 63 patients are shown in Table 1. The mean ( $\pm$ SD) age was  $63 \pm 9$  years. Forty patients (64%) were male, and 36 (57%) were classified as having RPR. The median (25<sup>th</sup>, 75<sup>th</sup>) PFA-100 CT was 202 (165, 300) sec in patients without RPR vs. 101 (86, 120) sec in patients with RPR ( $p < 0.001$ ). The median (25<sup>th</sup>, 75<sup>th</sup>) LDL cholesterol levels were similar and in the normal range in patients both without and with RPR (103 [89, 117] vs. 99 [85, 126] mg/dL,  $p = 0.99$ ), as were triglycerides (118 [85, 151] vs. 136 [92, 163] mg/dL,  $p = 0.86$ ) and hemoglobin A1c (5.9% [5.7%, 6.5%] vs. 6% [5.7%, 6.3%],  $p = 0.73$ ).

As demonstrated in Figure 1, inflammatory markers and the circulating levels of EPCs were similar in patients with and without RPR. Inflammatory marker and EPC levels were not associated with RPR (sCD40L, hazard ratio [HR] 0.99, 95% confidence interval [CI] 0.99–1.0,  $p = 0.75$ ; oxLDL, HR 1.0, 95% CI 0.99–1.02,  $p = 0.94$ ; hsCRP, HR 1.02, 95% CI 0.89–1.17,  $p = 0.71$ ; and EPCs, HR 1.03, 95% CI 0.97–1.1,  $p = 0.30$ ). Additionally, no correlation was noted between PFA-100 and inflammatory markers or EPC values (Figure 2).

**DISCUSSION**

Our study did not identify an association between inflammatory markers or circulating EPCs and RPR in

patients with stable CAD treated with low-dose aspirin. Additionally, no correlation was noted between PFA-100 and inflammatory markers or EPC values.

We evaluated inflammation by measuring the plasma levels of hsCRP, oxLDL, and sCD40L which are biomarkers that have traditionally been correlated with atherosclerotic activity (11–13). Despite the previous use of aspirin, 57% of our patients presented with RPR. Previous studies have also reported a high RPR prevalence in patients treated with aspirin, but RPR may differ depending on the test applied (14,15). We performed a PFA-100, an assay with high sensitivity that is extensively used to identify RPR (15,16). Moreover, RPR detected by PFA-100 may identify patients with a higher cardiovascular risk (17).

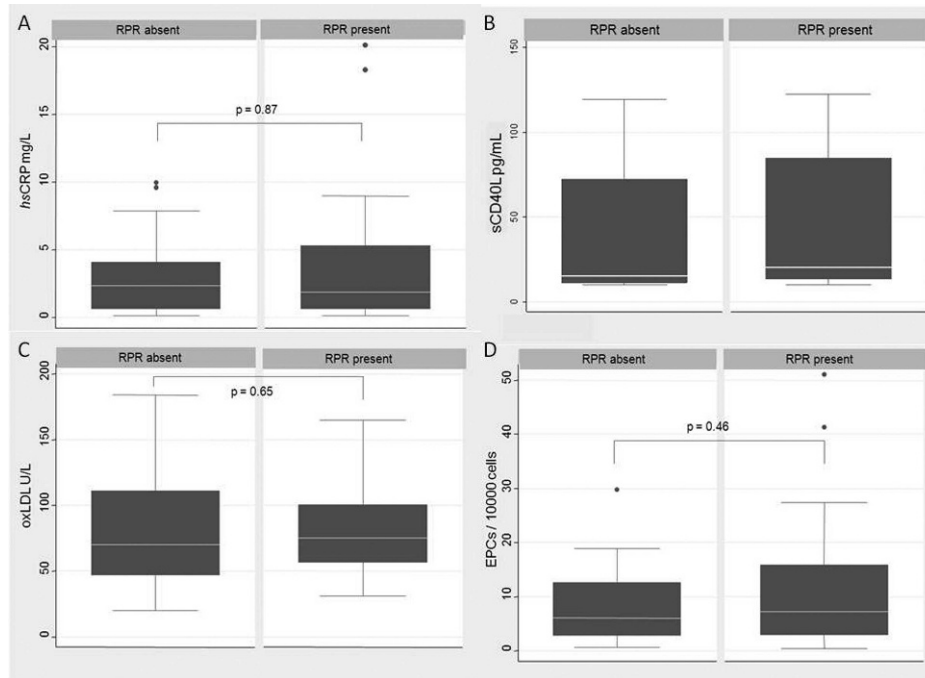
We hypothesized that RPR could be related to inflammation and endothelial dysfunction. Our hypothesis was supported by several in vitro studies demonstrating inflammatory mechanisms activating platelets, which in turn increase atherothrombotic activity (18). OxLDL can activate platelets by acting on the scavenger receptor CD36 (19). Similarly, sCD40L can trigger platelet aggregation and is correlated with plasma P-selectin and urinary thromboxane levels (20,21). Activated platelets can induce the secretion of chemokines in various cells of the vascular wall (3). Platelet factor-4 attracts monocytes, promoting their differentiation into macrophages (22). In addition, CD40L expressed by activated platelets acts on endothelial cells, increasing the release of interleukin-8 and monocyte chemoattractant protein-1 (23).

In contrast, few clinical studies have investigated the association between inflammation or endothelial dysfunction and platelet hyper-aggregation. Dotsenko et al. have demonstrated a correlation between increased platelet aggregation and hsCRP (24). In patients with acute coronary syndromes, Gori et al. tested the association between several inflammatory markers and platelet aggregation and found an association only with IFN-gamma and IL-4 (25). Two

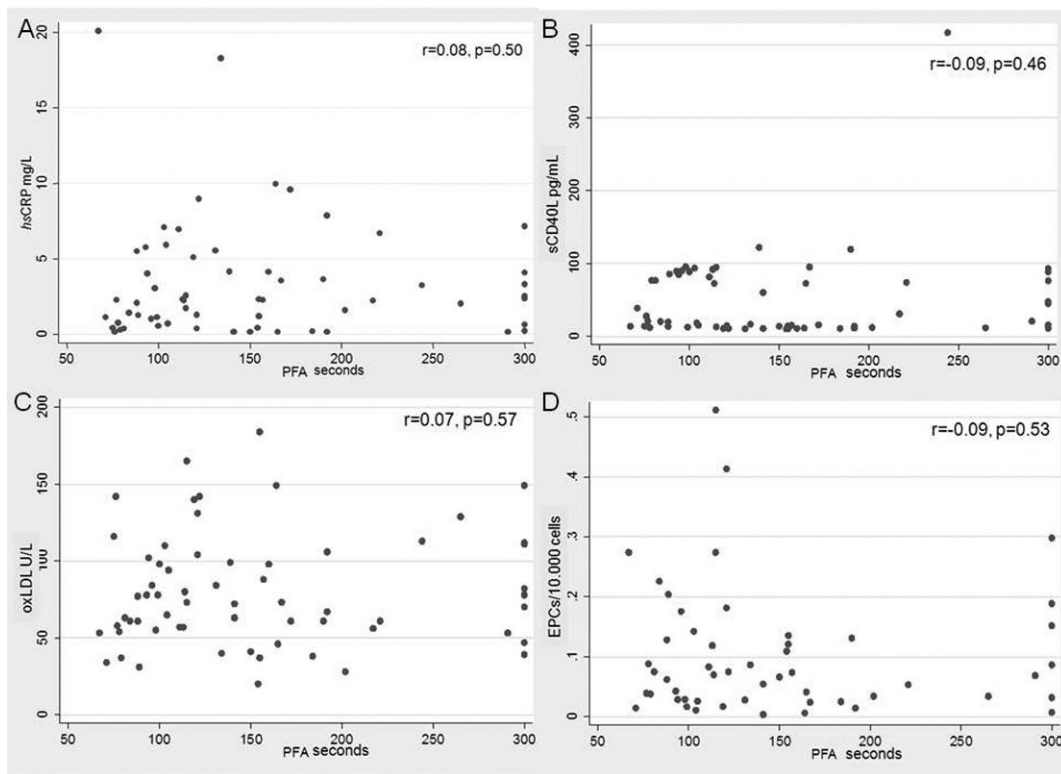
**Table 1 - Baseline patient characteristics according to the PFA-100 CT value.**

	Patients w/out RPR PFA-100 CT >150 sec (n = 27)	Patients w/RPR PFA-100 CT ≤150 sec (n = 36)	p-value
Men, n (%)	18 (67)	22 (63)	0.76
Mean age, in years ( $\pm$ SD)	63.3 $\pm$ 10.2	63.1 $\pm$ 8.92	0.94
Body mass index, kg/m <sup>2</sup> ( $\pm$ SD)	27.8 $\pm$ 2.7	28.3 $\pm$ 3.5	0.54
<b>Coronary risk factors, n (%)</b>			
Diabetes mellitus	11 (44)	19 (53)	0.51
Hypertension	19 (81)	30 (83)	0.85
Habitual smoker	3 (11)	8 (22)	0.25
<b>Concomitant medication, n (%)</b>			
ACE inhibitor or AT-1 receptor blocker	23 (86)	33 (93)	0.42
Aspirin	27 (100)	36 (100)	
Clopidogrel	0	0	
Simvastatin	27 (100)	36 (100)	
<b>Metabolic parameters, median (IQR)</b>			
TG, mg/dL	118 (85–151)	136 (92–163)	0.86
Total cholesterol, mg/dL	171 (156–195)	172 (157–203)	0.59
LDL-C, mg/dL	103 (89–117)	99 (85–126)	0.99
HDL-C, mg/dL	45 (39–51)	43 (38–48)	0.96
Glucose, mg/dL	102 (90–113)	110 (95–125)	0.13
HbA1c, %	5.9 (5.7–6.5)	6 (5.7–6.3)	0.73

Data are expressed as the mean  $\pm$  SD, median (interquartile range [IQR]), or n (%). RPR, residual platelet reactivity; PFA-100 CT, platelet function analyzer-100 closure time; ACE, angiotensin-converting enzyme inhibitor; AT, angiotensin; TG, triglyceride; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; HbA1c, hemoglobin A1c.



**Figure 1** - The levels of (A) high-sensitivity C-reactive protein (*hsCRP*), (B) soluble CD40 ligand (*sCD40L*), (C) oxidized low-density lipoprotein (*oxLDL*), and (D) endothelial progenitor cells (*EPCs*) in patients without and with residual platelet reactivity (*RPR*). The data are presented as box plots displaying the mean, 25th and 75th percentiles and the minimum, maximum, and outlying values. In Figure 1-B, one outlier was excluded.



**Figure 2** - Correlations between the platelet function analyzer (PFA)-100 and the levels of (A) high-sensitivity C-reactive protein (*hsCRP*), (B) soluble CD40 ligand (*sCD40L*), (C) oxidized low-density lipoprotein (*oxLDL*), and (D) endothelial progenitor cells (*EPCs*).

small studies have demonstrated a correlation between sCD40L, CRP, and IL-8 and heightened thrombogenicity in patients undergoing coronary angioplasty (26,27). Conversely, in patients with stable CAD, a larger study did not find an association between circulating sCD40L levels and RPR (28).

In our study, the EPC levels did not differ between patients with and without RPR. We found EPC levels comparable to previous findings in patients with stable CAD (29). EPCs are reduced in CAD patients and are inhibited by atherosclerotic inflammatory agents, such as oxLDL and CRP (31,32). The relationship between EPCs and RPR has been poorly investigated. Daub et al. demonstrated that oxLDL-activated platelets may reduce EPC numbers (2). Alternatively, chemokine stromal cell factor-1, which is derived from activated platelets, may increase EPC levels (33). Finally, Abou-Saleh et al. showed that EPCs bind to and inhibit platelet activation via prostacyclin modulation (5).

Thus, despite previous in vitro evidence linking platelet activation to atherosclerotic inflammation, when we translated this assumption to human subjects, we did not find an association between inflammatory markers or circulating EPCs and RPR in patients with stable CAD treated with aspirin.

### Study limitations

This was a subgroup analysis of a trial investigating lipid-lowering agents (8). The sample size and power were primarily estimated based on the main outcome of the original trial. However, our main results were corroborated by the absence of a correlation between PFA-100 CT and inflammatory markers or EPC values. Additionally, we used a single platelet function assay; it is possible that our results may have been different if a different platelet test had been used.

This investigation demonstrated no association between inflammatory markers or circulating EPCs and RPR in patients treated with aspirin. Thus, low-grade inflammation and endothelial dysfunction do not affect the responsiveness to aspirin in patients with stable CAD. Our findings highlight the importance of better understanding the underlying mechanism of RPR and the uncertainties inherent to this field of study.

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### AUTHOR CONTRIBUTIONS

Pesaro AE and Campos AH were responsible for study design, acquisition, analysis and interpretation of data, manuscript draft and approval of the manuscript final version. Serrano Jr CV and Nicolau JC were responsible for study design, analysis and interpretation of data, manuscript draft and approval of the manuscript final version. Katz M and Lopes RD were responsible for analysis and interpretation of data, manuscript draft and approval of the manuscript final version. Marti LC, Martins HS, Sunahara RS and Maranhao RC were responsible for acquisition, analysis and interpretation of data, review of the manuscript draft and approval of the manuscript final version.

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