

## The balance between pro- and anti-inflammatory cytokines is associated with platelet aggregability in acute coronary syndrome patients

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### Abstract

**Background:** Residual platelet reactivity (RPR) on antiplatelet therapy in ischemic heart disease patients is associated with adverse events. Clinical, cellular and pharmacogenetic factors may account for the variable response to antiplatelet treatment.

**Objective:** We sought to explore the interplay of multiple pro-inflammatory and anti-inflammatory cytokines with platelet function in patients with acute coronary syndrome (ACS) undergoing percutaneous coronary intervention (PCI) on dual antiplatelet therapy.

**Methods:** In 208 ACS patients undergoing PCI on dual antiplatelet therapy we measured platelet function by platelet aggregation with two agonists [1 mM arachidonic acid (AA) and 10  $\mu$ M ADP]. IL-1 $\beta$ , IL-1ra, IL-4, IL-6, IL-8, IL-10, IL-12, IP-10, IFN- $\gamma$ , MCP-1, MIP-1 $\alpha$ , MIP-1 $\beta$ , TNF- $\alpha$ , and VEGF levels were determined by using the Bio-Plex cytokine assay (Bio-Rad Laboratories Inc., Hercules, CA, USA). We defined patients with RPR those with platelet aggregation by AA  $\geq 20\%$  and/or ADP (10  $\mu$ mol)  $\geq 70\%$ .

**Results:** We documented a significant association between IP-10, IFN- $\gamma$ , IL-4 and RPR by both AA- and ADP-induced platelet aggregation after adjustment for age, sex, cardiovascular risk factors, ejection fraction, BMI, vWF and CRP. Patients with pro-inflammatory cytokines not compensated by anti-inflammatory cytokines had higher risk of RPR by both AA and ADP (AA: OR = 3.85, 95% CI 1.52–9.74; ADP: OR = 2.49, 95% CI 1.33–4.68) with respect to patients with balanced anti-/pro-inflammatory cytokines. Patients with anti-inflammatory response overwhelming pro-inflammatory response have lower risk of RPR (AA: OR = 0.55, 95% CI 0.28–1.06; ADP: OR = 0.47, 95% CI 0.26–0.87).

**Conclusion:** Our study provides new insights into the interplay of anti-/pro-inflammatory cytokines with platelet hyper-reactivity in high-risk patients.

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**Keywords:** Inflammation; Residual platelet reactivity; Anti-inflammatory cyto-chemokines; Pro-inflammatory cyto-chemokines; C-reactive protein; Von Willebrand factor

### 1. Introduction

Good evidence exists indicating that inflammation plays a crucial role in the processes influencing the development of ischemic events after percutaneous coronary intervention (PCI) [1]. Pro-inflammatory molecules are actively involved in the activation and migration of leukocytes to sites of

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vascular injury and inflammation, and may contribute to the release by activated cells of prothrombotic factors, which in turn may activate platelets and other cell types [2,3]. Platelets represent an important linkage between inflammation, thrombosis, and atherogenesis, as they are able to interact with leukocytes and endothelial cells with the participation of Von Willebrand factor (vWF) [4]. When activated, platelets coagulate with circulating leukocytes and after their adherence to the vascular wall, platelets also provide a sticky surface to recruit leukocytes on the vessel wall [5].

Clinical studies documented that a residual platelet reactivity (RPR) on antiplatelet therapy in patients with ischemic heart disease is associated with adverse clinical events [6–10].

Clinical, cellular and pharmacogenetic factors may account for the variable response to antiplatelet treatment [9,11–14].

The role of the inflammatory state in modulating the effect of antiplatelet therapy on platelet reactivity has been suggested by clinical studies demonstrating the high prevalence of RPR in the acute phase of disease [15–16], and that RPR is independently associated with routinely inflammatory markers [erythrocyte sedimentation rate (ESR) and leukocyte number] in acute coronary syndrome (ACS) patients [9]. However, the precise mechanisms by which the inflammatory state may influence platelet hyper-reactivity in high-risk cardiac patients on dual antiplatelet therapy have not yet been elucidated. In particular, no information is available about the dynamic relationship between the various anti-inflammatory and pro-inflammatory molecules in relation to platelet activation.

Therefore, aim of the present study was to explore the interplay of multiple pro-inflammatory and anti-inflammatory cytokines with platelet function in patients with ACS undergoing PCI on dual antiplatelet therapy.

## 2. Materials and methods

### 2.1. Study population

In the framework of an ongoing project aimed to investigate the prevalence and the clinical implications of a RPR in patients with ACS undergoing PCI on dual antiplatelet therapy, we investigated 208 patients in relation to the presence or the absence of RPR by AA defined as platelet aggregation  $\geq 20\%$  (92/208).

All patients undergoing primary PCI received a clopidogrel loading dose (73 received 600 mg and 135 received 300 mg) followed by a daily dose of 75 mg. All patients received unfractionated heparin 70 IU/kg during the procedure and acetylsalicylic acid i.v. 500 mg followed by a daily dose of 100–325 mg by oral route.

Acute myocardial infarction (AMI) was diagnosed as an increase in creatine kinase MB isoenzyme at least twice the upper normal limits (3.6 ng/mL), and/or elevated cardiac Troponin I (cTnI) ( $>0.15$  ng/mL) levels with at least

one of the following: acute onset of prolonged ( $\geq 20$  min) typical ischemic chest pain; ST-segment elevation of at least 1 mm in two or more contiguous electrocardiographic leads or ST-depression of  $\geq 0.5$  mm, 0.08 s after the J point in  $\geq 2$  contiguous leads, or T waves inversion  $>1$  mm in leads with predominant R waves. All patients underwent coronary angiography performed by the Judkins' technique and PCI if indicated.

Unstable angina was defined as angina pain at rest fulfilling Braunwald's IIIb criteria with transient significant ischemic ST-segment or T-wave changes, or both, without evidence of myocardial damage.

Patients were considered to have hypertension if they had been diagnosed as hypertensives according to the guidelines of European Society of Hypertension/European Society of Cardiology [17] or were taking antihypertensive drugs. Dyslipidemia was defined according to the third report of the National Cholesterol Education Program (NCEP III) [18] and diabetes in agreement with the American Diabetes Association [19].

The exclusion criteria included history of bleeding diathesis, platelet count  $\leq 100,000/\text{mm}^3$ , hematocrit  $\leq 30\%$ , creatinine  $\geq 4.0$  mg/dL, and glycoprotein (Gp) IIb/IIIa inhibitors use.

Informed written consent was obtained from all patients and the study was approved by the local Ethical Review Board. The investigation conforms with the principles outlined in the Declaration of Helsinki [20].

### 2.2. Experimental procedure

Venous blood samples anticoagulated with 0.129 M sodium citrate (ratio 9:1) were taken from each patient 24 h after PCI. Whole venous blood was also collected in tubes without anticoagulant.

Whole-blood specimens were centrifuged for 10 min at  $250 \times g$  to obtain platelet-rich plasma (PRP). As previously described [9] PRP was stimulated with 2 and 10  $\mu\text{M}$  ADP (Mascia Brunelli, Milan, Italy) and with 1 mM arachidonic acid (AA) (Sigma–Aldrich, Milan, Italy) using a APACT 4 aggregometer (Helena Laboratories Italia s.p.a., Milan, Italy). The coefficient of variation of AA-PA and ADP-PA were 5.8% and 6.8%, respectively.

Citrated and serum samples were centrifuged at room temperature ( $1500 \times g$ ) for 15 min. The supernatants were stored in aliquots at  $-80^\circ\text{C}$  until assays.

Von Willebrand factor (vWF:Ag) was measured by an in-house ELISA, based upon a commercial rabbit anti-human VWF polyclonal antibody (DAKO, Copenhagen, Denmark) as first and second antibody.

Interleukin-1 $\beta$  (IL-1 $\beta$ ), interleukin 1 receptor antagonist (IL-1ra), interleukin-4 (IL-4), interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-10 (IL-10), interleukin-12 (IL-12), interferon-inducible protein (IP-10), interferon- $\gamma$  (IFN- $\gamma$ ), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein 1- $\alpha$  (MIP-1 $\alpha$ ),

macrophage inflammatory protein 1- $\beta$  (MIP-1 $\beta$ ), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), and vascular endothelial growth factor (VEGF) levels were determined by using the Bio-Plex cytokine assay (Bio-Rad Laboratories Inc., Hercules, CA, USA) according to manufacturer's instructions.

C-reactive protein (CRP) was assessed by a high-sensitivity assay on a BN II nephelometer (Dade Behring, Marburg, Germany) allowing detection of values as low as 0.17 mg/L.

### 2.3. Residual platelet reactivity

For selecting study population we defined patients with RPR those patients with platelet aggregation induced by AA  $\geq 20\%$ ; in addition, we studied ADP-induced platelet aggregation and we considered residual platelet reactivity to ADP. According to literature [21,22] and studies from our group [9,10] for RPR by ADP we chose a cut-off value of 70%.

### 2.4. Statistical analysis

Statistical analysis was performed with SPSS (Statistical Package for Social Sciences, Chicago, USA) software for Windows (Version 11.5). Values are presented as median and range.

As the parameters investigated had a non-Gaussian distribution, log-transformed values for cyto-chemokines, CRP and vWF were used in the analyses, and back transformed for data presentation.

After logarithmic transformation the *t*-test for unpaired data was used for comparisons between single groups. Furthermore, the evaluation of the relationship between pro-, anti-inflammatory cytokines, CRP, vWF and platelet aggregation by 1 mM AA or by 2 or 10  $\mu$ M ADP was performed by Pearson's correlation test and *p* values were adjusted by Bonferroni's correction for multiple comparisons.

To perform the multivariate analysis, logistic regression was used with RPR as the dependent variable and with the parameters differently (*p* < 0.20) distributed between patients with and without RPR as the independent variables. Clinical characteristics (age, sex, cardiovascular risk factors) were included in the logistic regression analysis in a model in which vWF, CRP and cyto-chemokine levels were added as continuous variable. Variables that resulted not to be associated with the outcome were removed from the final most parsimonious regression model through a backward selection algorithm in a model in which all parameters were added simultaneously.

High concentrations of pro-inflammatory markers were defined as the highest tertile of the distribution of the cyto-chemokines. Low concentrations of anti-inflammatory markers were defined as the lowest tertile of the distribution of these cyto-chemokines. The odds ratio for having RPR according to cyto-chemokine tertiles was estimated by multivariate logistic regression analysis, using RPR as dependent variable, cyto-chemokines as independent variables, and

age, sex, BMI, traditional cardiovascular risk factors, ejection fraction CRP and vWF as covariates.

In order to evaluate the role of the balance between anti-inflammatory and pro-inflammatory cytokines, we categorized patients into three groups: group 1 patients have a balanced anti-/pro-inflammatory cytokines, i.e. pro-inflammatory and anti-inflammatory cytokines in the same tertiles; group 2 patients have the pro-inflammatory cytokines not compensated by the anti-inflammatory cytokines, i.e. pro-inflammatory cytokines in the third tertile and anti-inflammatory cytokines in the first or second tertiles; group 3 patients in which the anti-inflammatory response overwhelms the pro-inflammatory cytokines, i.e. pro-inflammatory cytokine in the first or second tertiles and anti-inflammatory cytokines in the third tertile.

All odds ratios (OR) are given with their 95% confidence interval. All probability values are two-tailed, with values of less than 0.05 considered statistically significant.

## 3. Results

### 3.1. Relationship between 1 mM AA-induced platelet aggregation and cyto-chemokine levels

The evaluation of the relationship between pro-, anti-inflammatory cytokines and platelet aggregation by 1 mM AA, performed by Pearson's correlation tests, showed that IP-10 and IL-6 serum levels, but not other pro-inflammatory cytokines (IL-1 $\beta$ , IL-8, IL-12, TNF- $\alpha$ , MCP-1, MIP-1 $\alpha$ , MIP-1 $\beta$ , IFN- $\gamma$  and VEGF), were mildly, but significantly, related with platelet aggregation (IP-10: *r* = 0.30, *p* < 0.001; IL-6: *r* = 0.28, *p* < 0.05). Serum levels of anti-inflammatory cytokines IL-4 and IL-10 were inversely related with platelet aggregation by AA (IL-4: *r* = -0.28, *p* < 0.05; IL-10: *r* = -0.20, *p* = 0.14).

### 3.2. Relationship between CRP, vWF levels and 1 mM AA-induced platelet aggregation

No significant association between CRP serum levels and AA-induced platelet aggregation was found.

A significant association between vWF levels and AA-induced platelet aggregation (*r* = 0.30, *p* < 0.001) was documented.

### 3.3. Residual platelet reactivity (RPR) by AA and cyto-chemokine, CRP and vWF levels

Age, diabetes and vWF levels were significantly associated with RPR by AA (Table 1).

IL-6, IP-10, IFN- $\gamma$  and vWF levels were significantly higher in patients with RPR than in patients without RPR (Table 1). IL-4 and IL-10 levels were significantly lower in RPR patients than in patients without RPR detected by AA (Table 1). No significant difference in CRP levels between

Table 1

Clinical and laboratory characteristics according to the presence or the absence of residual platelet reactivity by AA-PA (AA-platelet aggregation  $\geq 20\%$ )

|                                   | With RPR (n = 92)     | Without RPR (n = 116) | p      |
|-----------------------------------|-----------------------|-----------------------|--------|
| Age                               | 70.6 (68.2–72.1)      | 64.9 (65.1–68.5)      | 0.001  |
| Sex (M/F)                         | 62/30                 | 90/26                 | 0.182  |
| Smoking habit, n (%)              | 38 (41.3)             | 52 (44.6)             | 0.610  |
| Hypertension, n (%)               | 52 (56.5)             | 67 (57.8)             | 0.220  |
| Diabetes, n (%)                   | 32 (34.8)             | 25 (22.4)             | 0.020  |
| Dyslipidemia, n (%)               | 39 (42.4)             | 56 (46.6)             | 0.790  |
| Ejection fraction $\leq 40\%$ (%) | 37 (40.2)             | 42 (36.2)             | 0.630  |
| Von Willebrand factor (%)         | 240.5 (91–755)        | 191 (76–854)          | 0.010  |
| CRP (mg/L)                        | 23.2 (4.6–437.0)      | 20.3 (1.5–422.0)      | 0.563  |
| IL-1 $\beta$ (pg/mL)              | 32.0 (7.2–623.1)      | 31.0 (7.8–102.1)      | 0.720  |
| IL-6 (pg/mL)                      | 256.1 (34.1–3541.7)   | 186.7 (1.6–5147.7)    | 0.0001 |
| IL-12 (pg/mL)                     | 64.3 (23.4–1849.4)    | 76.7 (21.6–656.8)     | 0.229  |
| TNF- $\alpha$ (pg/mL)             | 493.6 (220.3–12087.7) | 452.4 (219.8–1536.9)  | 0.184  |
| IFN- $\gamma$ (pg/mL)             | 661.4 (186.5–2219.7)  | 552.2 (136.6–1043.7)  | 0.007  |
| IP-10 (pg/mL)                     | 4156 (344–55603)      | 2892 (249–32254)      | 0.0001 |
| IL-1ra (pg/mL)                    | 922.9 (227–82463)     | 968.9 (185–126849)    | 0.580  |
| IL-4 (pg/mL)                      | 66.4 (30.4–163.2)     | 75.2 (34.0–144.2)     | 0.009  |
| IL-10 (pg/mL)                     | 26.1 (3.3–364.8)      | 38.0 (6.5–745.2)      | 0.024  |
| MCP-1 (pg/mL)                     | 657.9 (186.2–5976.9)  | 624.2 (155.7–31305.4) | 0.895  |
| MIP-1 $\alpha$ (pg/mL)            | 78.6 (28.7–729.3)     | 89.5 (20.0–1793.6)    | 0.511  |
| MIP-1 $\beta$ (pg/mL)             | 1098.1 (273–19984)    | 1164.9 (282–14782)    | 0.725  |
| IL-8 (pg/mL)                      | 259.6 (44.5–42021)    | 281.9 (27.4–16940.6)  | 0.240  |
| VEGF (pg/mL)                      | 845.1 (45.0–3281.2)   | 718.5 (2.0–7191.5)    | 0.335  |

Data are expressed as % or as median and range.

patients with RPR and patients without RPR was found (Table 1).

At the logistic regression analysis, pro-inflammatory (IL-6, IP-10 and IFN- $\gamma$ ) and anti-inflammatory cytokines (IL-4 and IL-10) were significantly associated with RPR after adjustment for age, sex, cardiovascular risk factors, ejection fraction, BMI, CRP and vWF (Table 2).

### 3.4. Residual platelet reactivity (RPR) by AA and cyto-chemokine balance profile

Patients with two or three pro-inflammatory cytokines (IP-10, IL-6, IFN- $\gamma$ ) in the higher tertile of their distribution were significantly more likely to have RPR (OR = 1.95, 95% CI 1.10–3.44,  $p = 0.04$ ) than patients in the other tertiles, after adjusting for all covariates.

Patients with 1–2 anti-inflammatory cytokines (IL-4 and IL-10) in the higher tertile had significantly decreased risk of

having RPR (OR = 0.40, 95% CI 0.22–0.72,  $p = 0.002$ ) than patients in the other tertiles after adjusting for all covariates.

Patients with pro-inflammatory cytokines not compensated by the anti-inflammatory cytokines, i.e. pro-inflammatory cytokines in the third tertile and anti-inflammatory cytokines in the first and second tertiles (group 2) had significantly higher risk of having RPR (OR = 3.85, 95% CI 1.52–9.74,  $p = 0.004$ ) with respect to patients with balanced anti-/pro-inflammatory cytokines (group 1), i.e. pro-inflammatory anti-inflammatory and cytokines in the same tertiles, whereas patients with anti-inflammatory response overwhelming the pro-inflammatory cytokines (group 3), i.e. pro-inflammatory cytokines in the first or second tertiles and anti-inflammatory cytokines in the third tertile, had lower, but not significant, risk of RPR by AA (OR = 0.55, 95% CI 0.28–1.06,  $p = 0.073$ ) (Fig. 1).

### 3.5. Relationship between ADP-induced platelet aggregation and cyto-chemokine levels

The evaluation of the association between pro, anti-inflammatory markers and platelet aggregation by 2 and 10  $\mu\text{M}$  ADP, performed by Pearson's correlation test showed that IP-10 and IL-6 serum levels, but not other pro-inflammatory cytokines (IFN- $\gamma$ ; IL-1 $\beta$ , IL-8, IL-12, TNF- $\alpha$ , MCP-1, MIP-1 $\alpha$ , MIP-1 $\beta$ , and VEGF) were mildly but significantly related with 2  $\mu\text{M}$  ADP platelet aggregation (IP-10:  $r = 0.32$ ,  $p < 0.001$ ; IL-6:  $r = 0.28$ ,  $p < 0.05$ ) and 10  $\mu\text{M}$  ADP platelet aggregation (IP-10:  $r = 0.39$ ,  $p < 0.001$ ; IL-6:  $r = 0.29$ ,  $p < 0.01$ ).

Table 2

Odds ratio for having RPR by AA in relation to cyto-chemokine levels (multivariate logistic regression analysis)

|                    | Odds ratio <sup>a</sup> | 95% CI     | p value |
|--------------------|-------------------------|------------|---------|
| Ln(IL-6)           | 1.80                    | 1.09–2.98  | 0.022   |
| Ln(IP-10)          | 2.31                    | 1.42–3.76  | 0.001   |
| Ln(IFN- $\gamma$ ) | 34.8                    | 4.34–278.4 | 0.001   |
| Ln(IL-4)           | 0.014                   | 0.007–0.27 | 0.004   |
| Ln(IL-10)          | 0.55                    | 0.36–0.84  | 0.006   |

<sup>a</sup> Adjusted for adjustment for age, sex, cardiovascular risk factors, ejection fraction, BMI, CRP and vWF.

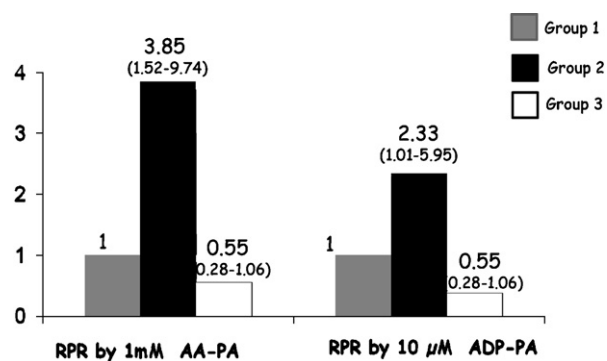


Fig. 1. Odds ratio for having residual platelet reactivity (RPR) by 1 mM AA or 10 μM ADP according to the pro-inflammatory and anti-inflammatory cytokine balance: group 1: balanced pro-/anti-inflammatory cytokines; group 2: pro-inflammatory cytokines not compensated by anti-inflammatory cytokines; group 3: anti-inflammatory response overwhelming the pro-inflammatory response.

Anti-inflammatory cytokines IL-4 and IL-10 serum levels were inversely related with 2 μM ADP platelet aggregation (IL-4:  $r = -0.26$ ,  $p < 0.05$ ; IL-10:  $r = -0.26$ ,  $p < 0.05$ ) and with 10 μM ADP-PA platelet aggregation (IL-4:  $r = -0.27$ ,  $p < 0.05$ ; IL-10:  $r = -0.28$ ,  $p < 0.05$ ).

### 3.6. Relationship between CRP, vWF levels and ADP-induced platelet aggregation

No significant association between CRP serum levels and ADP-induced platelet aggregation was found.

Table 3

Clinical and laboratory characteristics according to the presence or the absence of residual platelet reactivity by 10 μM ADP-PA (ADP-platelet aggregation  $\geq 70\%$ )

|                                   | With RPR (n = 68)    | Without RPR (n = 140) | p      |
|-----------------------------------|----------------------|-----------------------|--------|
| Age (year)                        | 68.3 (65.7–70.8)     | 68.3 (66.9–69.8)      | 0.093  |
| Sex (M/F)                         | 45/23                | 107/33                | 0.219  |
| Smoking habit (%)                 | 25 (36.8)            | 68 (48.6)             | 0.307  |
| Hypertension (%)                  | 45 (66.2)            | 86 (61.4)             | 0.977  |
| Diabetes (%)                      | 20 (29.4)            | 25 (17.9)             | 0.047  |
| Dyslipidemia (%)                  | 28 (41.2)            | 66 (47.1)             | 0.602  |
| Ejection fraction $\leq 40\%$ (%) | 41 (60.3)            | 58 (41.4)             | 0.006  |
| Von Willebrand factor (%)         | 232.5 (76.0–854.0)   | 221 (82.0–835.0)      | 0.036  |
| CRP (mg/L)                        | 23.2 (4.6–437.0)     | 21.5 (1.5–422.0)      | 0.743  |
| IL-1β (pg/mL)                     | 35.4 (7.4–96.1)      | 29.1 (7.2–623.1)      | 0.335  |
| IL-6 (pg/mL)                      | 256.6 (1.6–3541.7)   | 188.9 (34.1–5147.7)   | 0.003  |
| IL-12 (pg/mL)                     | 60.3 (21.6–1849.4)   | 76.7 (24.8–600.5)     | 0.234  |
| TNF-α (pg/mL)                     | 499.7 (219.8–1490.9) | 469.0 (259.5–12087.7) | 0.515  |
| IFN-γ (pg/mL)                     | 667.2 (277.0–1345.5) | 568.6 (136.6–2219.7)  | 0.015  |
| IP-10 (pg/mL)                     | 4957.3 (610–55603)   | 3238.3 (249–43005)    | 0.0001 |
| IL-1ra (pg/mL)                    | 987.8 (186–15476)    | 929.5 (185–126849)    | 0.528  |
| IL-4 (pg/mL)                      | 67.5 (30.4–102.2)    | 72.5 (30.4–163.2)     | 0.123  |
| IL-10 (pg/mL)                     | 26.4 (5.1–263.9)     | 37.8 (3.3–745.2)      | 0.063  |
| MCP-1 (pg/mL)                     | 618.1 (186.2–5976.9) | 649.6 (155.7–31305.4) | 0.998  |
| MIP-1α (pg/mL)                    | 89.2 (19.9–1793)     | 72.9 (28.7–729.3)     | 0.317  |
| MIP-1β (pg/mL)                    | 1172.8 (343.0–8596)  | 1142.5 (273.0–19984)  | 0.281  |
| IL-8 (pg/mL)                      | 237.4 (54.6–21373.2) | 295.2 (27.4–42021)    | 0.234  |
| VEGF (pg/mL)                      | 720.5 (2.0–3142.5)   | 738.2 (2.7–7191.5)    | 0.605  |

Data are expressed as % or as median and range.

A significant association between vWF levels and platelet aggregation by 2 μM ADP ( $r = 0.21$ ,  $p < 0.05$ ) and 10 μM ADP platelet aggregation ( $r = 0.22$ ,  $p < 0.05$ ) was documented.

### 3.7. Residual platelet reactivity (RPR) by ADP and cyto-chemokine, CRP and vWF levels

Sixty-eight patients of 208 (32.7%) had RPR (ADP-induced platelet aggregation  $\geq 70\%$ ) by 10 μM ADP and 16 of 208 (7.6%) patients by 2 μM ADP. As the 16 patients found with RPR by 2 μM ADP had RPR also by 10 μM ADP, we report below the results obtained with 10 μM ADP.

In addition to diabetes and vWF also IL-6, IP-10 and IFN-γ serum levels significantly differed according to RPR (Table 3). No significant difference in CRP levels between patients with RPR and patients without RPR was found (Table 3).

At the multiple logistic regression analysis, after adjustment for all potential confounders, in addition to diabetes, also IL-4, IFN-γ, IP-10 but not IL-6 levels remained significant and independent predictors of RPR detected by 10 μM ADP (Table 4).

### 3.8. Residual platelet reactivity (RPR) by ADP and cyto-chemokine balance profile

Patients with two or three pro-inflammatory cytokines (IP-10, IL-6 and IFN-γ) in the highest tertile were significantly more likely to have RPR (OR = 2.49, 95% CI 1.33–4.68,

Table 4  
Odds ratio for having RPR by 10  $\mu$ M ADP in relation to cyto-chemokine levels (multivariate logistic regression analysis)

|                    | Odds ratio <sup>a</sup> | 95% CI      | <i>p</i> value |
|--------------------|-------------------------|-------------|----------------|
| Ln(IP-10)          | 2.51                    | 1.70–3.72   | 0.001          |
| Ln(IFN- $\gamma$ ) | 2.51                    | 1.70–3.72   | 0.001          |
| Ln(IL-4)           | 0.07                    | 0.017–0.295 | 0.001          |

<sup>a</sup> Adjusted for adjustment for age, sex, cardiovascular risk factors, ejection fraction, BMI, CRP and vWF.

$p=0.005$ ) than patients in the other tertiles, after adjustment for all covariates.

Patients with 1–2 anti-inflammatory cytokines (IL-4 and IL-10) in the higher tertile had significantly decreased risk of having RPR (OR = 0.47, 95% CI 0.26–0.87,  $p=0.015$ ) than patients in the other tertiles after adjustment for all covariates.

Patients with pro-inflammatory cytokines not compensated by the anti-inflammatory cytokines, i.e. pro-inflammatory cytokines in the third tertile and anti-inflammatory cytokines in the first and second tertiles (group 2) had significantly higher risk of having RPR (OR = 2.33, 95% CI 1.01–5.95,  $p=0.048$ ) with respect to patients with balanced anti-/pro-inflammatory cytokines, i.e. pro-inflammatory anti-inflammatory and cytokines in the same tertiles (group 1). Patients with anti-inflammatory response overwhelming the pro-inflammatory response, i.e. pro-inflammatory cytokines in the first or second tertiles and anti-inflammatory cytokines in the third tertile (group 3) had significantly lower risk of RPR (OR = 0.37, 95% CI 0.18–0.74,  $p=0.005$ ) (Fig. 1).

#### 4. Discussion

This study provides new insights into the association between inflammation and residual platelet reactivity on antiplatelet treatment, which was previously found to be related to the occurrence of clinical events in high-risk patients such as ACS patients undergone PCI on dual antiplatelet agents [9,15,16].

In the present study, by using extensive array of cytokines and other inflammatory markers, we demonstrated a modulatory effect of the interplay between pro- and anti-inflammatory molecules on platelet function in patients with a marked endothelial, leukocyte and platelet activation, such as those with ACS undergone PCI.

Several cytokines are involved in the inflammatory processes, have overlapping, antagonistic, and synergic effects on many cell types and up-regulate and down-regulate the production of other cytokines and inflammatory mediators [23,24]. For these reasons, it is crucial to evaluate a full profile of Th1 and Th2 pro-inflammatory and anti-inflammatory cytokines to obtain a more complete and precise picture of evolving interaction between platelets and leukocytes.

The acute coronary syndromes are characterized by an acute inflammatory response in which high levels of pro-inflammatory markers [25–27] and low levels of the anti-inflammatory cytokine IL-10 were documented [28,29].

Inflammatory mediators are able to activate different cell types such as monocytes and polymorphonuclear cells, and to induce the production of prothrombotic and chemotactic factors by these cells [25], which in their turn, determine the activation of platelets. Upon activation, platelets are able to bind to leukocytes and endothelial cells and promote the release of prothrombotic and pro-inflammatory factors, leading to a subsequent leukocyte attachment to injured endothelium [5,30].

On the other hand, activated platelets have been implicated not only in thrombosis, but also in inflammatory reactions and immune response; several studies have demonstrated the capability of activated platelets to induce an inflammatory reaction in cells of the vascular wall, leading to a subsequent cellular activation and production of prothrombotic and chemotactic factors [31].

A link between the hemostatic and the inflammatory system is represented by vWF, which provides an adhesive component for PMNs and monocytes [32].

Present findings of the relationship between inflammation and platelet aggregation are in keeping with clinical studies showing the high prevalence of RPR in the acute phase of the disease [15,16] and with the observation that in the ACS patients the leukocyte count and ESR are associated with an increased risk of having RPR despite dual antiplatelet therapy [9].

In this study we have documented a significant role of IP-10 in influencing platelet reactivity. IP-10 is a chemoattractant protein which promotes T cell adhesion to endothelial cells and it is a peculiar characteristic of human Th1 response [33]. Atherosclerosis may be considered mainly a Th1-driven disease. Furthermore, in vitro studies demonstrated that Th1 cells are able to induce the synthesis of monocyte tissue factor, which may determine the thrombin formation and subsequent platelet activation [34].

In our study we have documented an inverse relationship between anti-inflammatory cytokines (IL-10 and IL-4) levels and RPR. IL-10 has multifaced anti-inflammatory properties, including inhibition of the prototypic inflammatory transcription factor nuclear factor kappa B and promotion of phenotypic switch of lymphocytes into Th2 phenotype [35]. Moreover, clinical studies demonstrated that elevated IL-10 levels are predictive of a better clinical outcome after acute coronary syndromes, and abrogate the increased risk associated with elevated CRP levels [28,29].

We have found no association between platelet reactivity and the cyto-chemokines IL-1 $\beta$ , IL-1ra, IL-8, IL-12, MCP-1, MIP-1 $\beta$ , TNF- $\alpha$ , and VEGF. This finding may stem from the different half lives of the cyto-chemokines measured and from the intricate feedback among cytokines.

#### 4.1. Study limitations

Our study has some limitations. First, our study is a cross-sectional study, therefore, we are not able to demonstrate a causal relationship between inflammatory state and platelet reactivity. Second, we investigated the serum levels of cytokines, but the actual interaction platelets/leukocytes would have been obtained by the measurement of circulating platelet monocyte-aggregates and would have provided a further insight into the mechanisms involved in the different response to antiplatelet drugs [36]. Third, in this study we do not provide data about the relation between RPR and adverse ischemic events.

The possible modulation of the anti-inflammatory effects of ACE inhibitors and statins may have influenced the inflammatory state of our patients, nevertheless the large use of these drugs in our study group does not allow us to explore the interaction among platelet activation, inflammatory state, and the above-mentioned drugs.

In conclusion, our study documented that a pro-inflammatory state, not counteracted by an anti-inflammatory response, influences platelet hyper-reactivity in ACS patients on dual antiplatelet treatment. Present findings suggest that further studies, looking at the platelet activation during the acute phase of disease, have to consider that any intervention addressed to reduce inflammatory burden may also influence platelet activation.

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