Reticulated platelets predict cardiovascular death in acute coronary syndrome patients

Insights from the AMI-Florence 2 Study

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Summary
Reticulated platelets (RP) are newly-formed platelets with a greater mass, a residual amount of RNA and an increased prothrombotic potential. No studies investigating the association between RP and the risk of cardiovascular death in acute coronary syndrome (ACS) patients are available. In the frame of the AMI-Florence 2 study, we investigated RP in 229 (154 M/75 F) ACS patients (125 ST-elevation myocardial infarction [STEMI]; 104 Non-STEMI/Unstable Angina). RP were measured by using the Sysmex XE-2100 haematology analyzer and were expressed as the percentage of RP out of the total optical platelet count (immature platelet fraction; IPF) and as the percentage of RP highly fluorescent (H-IPF). At one-year follow-up, 22 out of 229 patients (9.6%) died from cardiovascular causes. Higher values of IPF (p=0.05) and H-IPF (p=0.006) were detected in dead compared to alive patients. A receiver operating characteristics curve analysis identified IPF ≥3.3% and H-IPF ≥0.9% as optimal cut-off values to predict cardiovascular death. At the multivariate model adjusted for the Global Registry of Acute Coronary Events (GRACE) risk score, the association between RP and cardiovascular death remained significant for both IPF [OR (95%CI) : 4.15 (1.24–13.91) p=0.02] and H-IPF [OR (95%CI): H-IPF 5.03 (1.38–18.38) p=0.01]. In conclusion, RP are independent predictors of cardiovascular death and may be useful in improving risk stratification for ACS patients. Future prospective studies to evaluate the role of RP in determining cardiovascular events are warranted.

Keywords
Acute coronary syndrome, cardiovascular death, platelet turn-over, reticulated platelets

Introduction
Platelets play a pivotal role in the occurrence of atherothrombotic events (1). The cornerstone of therapy in these conditions is represented by antiplatelets and the recent findings of the association between platelet hyperreactivity and prognosis in ACS (2, 3) have reinforced the major role of platelets in the pathophysiology of thrombotic events.

In the last years, an increasing interest on platelet size has been manifested. Mean platelet volume (MPV), the most widely used parameter to measure platelet size, has been reported to be associated with both platelet reactivity and clinical atherothrombotic events. Larger platelets are metabolically and enzymatically more active (4), thus having a greater prothrombotic potential (5). Furthermore, larger platelets are more often reticulated.

Reticulated platelets (RP) are newly-formed platelets with high granule content and a residual amount of megakaryocyte-derived mRNA (6). Detection of these platelets in the circulating blood reflects the increased platelet production from megakaryocytes in the bone marrow and hence the rate of platelet turnover (7).

Increased levels of RP have been found in various clinical conditions, in particular in patients with thrombocytopenia associated with peripheral destruction such as idiopathic thrombocytopenic purpura (ITP) and disseminated intravascular coagulation (DIC) (8, 9). Recently, increased levels of RP have been reported in patients with arterial thromboses including cerebrovascular disease, stent thrombosis and ACS (10-12). Moreover, RP have been demonstrated to be independent predictors of high on-treatment platelet reactivity (HPR) in ACS patients on dual antiplatelet therapy as well as in stable coronary artery disease (CAD) patients treated with aspirin and clopidogrel or with aspirin alone (13-16).

To the best of our knowledge, no studies investigating the ability of RP in predicting cardiovascular death in ACS patients are available. The aim of the present study was to evaluate the impact of RP on the occurrence of cardiovascular death in ACS patients after percutaneous coronary intervention (PCI).
Materials and methods

Study population

The study population comprised a group of 229 patients admitted to the Coronary Unit of the Careggi University Hospital, Florence, Italy from April 2008 to April 2009 enrolled on the frame of the Florence Acute Myocardial Infarction-2 (AMI-Florence 2) registry in the first year of recruitment.

The AMI-Florence Registry prospectively included all patients who arrived alive to the emergency departments of one of the six participating hospitals in the Florence health district with a suspected STEMI between March 2000 and February 2001.

All patients fulfilling the diagnostic criteria for acute or subacute (symptom onset ≤ 24 or > 24 hours, respectively) STEMI were screened for enrolment in the registry, without any exclusion criteria.

The AMI-Florence 2 registry is a second-wave survey of the AMI-Florence registry, and was extended to include all cases of suspected ACS arriving alive to any of the six hospitals, with no exclusion criteria.

ACS was diagnosed according to criteria established by the European Society of Cardiology (17); briefly, acute myocardial infarction (MI) was defined as typical rise and gradual fall of troponin, or more rapid rise and fall of CK-MB, defined as > 99% of normal levels (troponin T >0.05 ng/ml; CK-MB >10 ng/ml), with at least one of the following: acute onset of typical ischaemic chest pain; some Q waves in V1-V3, 30 ms Q waves ≥1 mm in two contiguous leads; ST-segment elevation or depression ≥2 mm leads , ≥0.2 mV in V1-V3, >0.1 mV in other leads. Unstable angina was defined as a history of new-onset, more frequent, more persistent or rest episode of chest pain, without typical changes of myocardial enzymes and with ECG evidence of myocardial ischaemia (transient ST segment displacement >0.1 mV during chest pain).

All patients underwent coronary angiography performed by the Judkins’ technique and PCI. Before PCI, all patients received a loading dose of 500 mg of acetylsalicylic acid (ASA) and 300 mg of clopidogrel, followed by 100 (15.9%) or 325 mg (84.1%) of ASA daily and 75 mg of clopidogrel daily. The use of glycoprotein (GP)IIb/IIIa inhibitors was at discretion of the operator and was administered as a percentage (%) of the total optical platelet count (immature platelet fraction; IPF) which indicates the rate of platelet production, and as the percentage of platelets, within the immature platelet fraction, with a major amount of m-RNA thus highly fluorescent (highly fluorescent immature platelet fraction; H-IPF). Finally, a third parameter that represents the absolute immature platelet count (IPC) can also be obtained.

The complete blood count was measured using an automated cell-counting machine (Beckman Coulter ACTdiff2, Milan, Italy).

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The complete blood count was measured using an automated cell-counting machine (Beckman Coulter ACTdiff2, Milan, Italy).

Reticulated platelets

Recently, a fully automated method that uses blood cell counter for the quantification of reticulated platelets has been developed (21). RP were measured by using the Sysmex XE-2100 haematology analyser (Sysmex, Kobe, Japan). Briefly, the flow cytometric determination of RP uses a proprietary fluorescent dye containing polymethine and oxazine. These two dyes penetrate the cell membrane staining the m-RNA in RP. The stained cells are passed through a semiconductor diode laser and the resulting forward scatter light and fluorescent intensity were measured. A computer algorithm (Sysmex IPF Master) applies a pre-set gate to separate mature platelets (blue dots) and RP (green dots). RP were expressed as a percentage (%) of the total optical platelet count (immature platelet fraction; IPF) which indicates the rate of platelet production, and as the percentage of platelets, within the immature platelet fraction, with a major amount of m-RNA thus highly fluorescent (highly fluorescent immature platelet fraction; H-IPF). Finally, a third parameter that represents the absolute immature platelet count (IPC) can also be obtained.

The average coefficients of variation (CV) for IPF and H-IPF were 10.6% and 18.8%, respectively.

Light transmission aggregometry

Turbidimetric platelet aggregation (PA) was used to measure agonist-induced PA. Whole blood samples were centrifuged for 10 minutes (min) at 250 x g to obtain platelet-rich plasma (PRP). PRP was stimulated with 10 µM ADP (Mascia Brunelli, Milan, Italy) and with 1 mmol arachidonic acid (AA) (Sigma-Aldrich, Milan, Italy) using an APACT 4 aggregometer (Helena Laboratories Italia S.P.A, Milan, Italy).
PA according to Born’s method was evaluated considering the maximal percentage of platelet aggregation in response to different stimuli (ADP-PA and AA-PA) after 10 min.

Statistical analysis
Statistical analysis was performed using the SPSS (Statistical Package for Social Sciences, Chicago, IL, USA) software for Windows (Version 18.0). Values are presented as median and interquartile range (IQR).

A correlation analyses for non-parametric (Spearman’s Rho) data was performed to establish relationships between IPF, H-IPF and haematological parameters.

The Mann-Whitney test for unpaired data was used for comparison between two groups. Dichotomous variables were compared by Chi² test. A receiver-operating characteristic curve (ROC) analysis was used to determine the ability of basal IPH, H-IPF and MPV in predicting cardiovascular death at 12 months. The optimal cut-off point was calculated by determining the basal IPF, H-IPF and MPV that provided the greatest sum of sensitivity and specificity.

Cumulative survival curves were constructed by the Kaplan-Meier method, and the log-rank test was used to assess statistical differences between survival curves.

To examine the association between RP and cardiovascular death, we performed an univariate and multivariate logistic regression analysis. In order to test the independent association between IPF, H-IPF and cardiovascular death, we performed a multiple logistic regression analysis adjusted for an established risk score which has been extensively validated such as the Global Registry of Acute Coronary Events (GRACE) risk score (which includes age, heart rate, systolic blood pressure, creatinine, killip class, elevated cardiac enzymes, ST-segment deviation, cardiac arrest at admission). All odds ratios (OR) are given with their 95% confidence interval (CI).

P<0.05 was considered to be statistically significant.

Results
Demographic and clinical characteristics of the study population are presented in Table 1. Significant positive correlations between IPF, H-IPF and MPV in the whole population were detected (IPF and MPV r=0.67, p=0.0001; H-IPF and MPV r=0.58, p<0.0001). Furthermore, IPF, H-IPF and MPV showed significant, although weak, and negative correlations with platelet count (IPF r= -0.29, p<0.0001; H-IPF r= -0.26, p<0.0001; MPV r= -0.38 p<0.0001).

Table 1: Clinical characteristics of patients investigated.

<table>
<thead>
<tr>
<th></th>
<th>Overall group (n=229)</th>
<th>12 month follow-up CV death (n=22)</th>
<th>12 month follow-up no CV death (n=207)</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years ^</td>
<td>76 (39–98)</td>
<td>87 (63–98)</td>
<td>75 (38–96)</td>
<td>0.001</td>
</tr>
<tr>
<td>Male gender, n (%)</td>
<td>154 (67.2)</td>
<td>13 (59.1)</td>
<td>141 (68.1)</td>
<td>0.2</td>
</tr>
<tr>
<td>Diabetes, n (%)</td>
<td>57 (24.9)</td>
<td>9 (40.9)</td>
<td>48 (23.2)</td>
<td>0.1</td>
</tr>
<tr>
<td>Smoking, n (%)</td>
<td>69 (30.1)</td>
<td>2 (9.1)</td>
<td>67 (32.4)</td>
<td>0.3</td>
</tr>
<tr>
<td>Hypertension, n (%)</td>
<td>131 (57.2)</td>
<td>15 (68.1)</td>
<td>116 (56.0)</td>
<td>0.4</td>
</tr>
<tr>
<td>Dyslipidaemia, n (%)</td>
<td>88 (38.4)</td>
<td>6 (27.3)</td>
<td>82 (39.6)</td>
<td>0.4</td>
</tr>
<tr>
<td>Renal failure*, n (%)</td>
<td>13 (5.6)</td>
<td>2 (9.0)</td>
<td>11 (5.3)</td>
<td>0.3</td>
</tr>
<tr>
<td>STEMI, n (%)</td>
<td>125 (54.6)</td>
<td>13 (59.0)</td>
<td>112 (54.1)</td>
<td>0.6</td>
</tr>
<tr>
<td>ACE-inhibitors, n (%)</td>
<td>60 (26.2)</td>
<td>8 (36.4)</td>
<td>52 (25.1)</td>
<td>0.07</td>
</tr>
<tr>
<td>Beta-blockers, n (%)</td>
<td>40 (17.5)</td>
<td>3 (13.6)</td>
<td>37 (17.9)</td>
<td>0.1</td>
</tr>
<tr>
<td>Statins, n (%)</td>
<td>47 (20.5)</td>
<td>2 (9.1)</td>
<td>45 (21.7)</td>
<td>0.06</td>
</tr>
<tr>
<td>Glycoprotein IIb/IIIa, n (%)</td>
<td>111 (48.5)</td>
<td>5 (22.7)</td>
<td>106 (51.2)</td>
<td>0.01</td>
</tr>
<tr>
<td>Killip Class ^</td>
<td>1 (1–3)</td>
<td>2 (1–3)</td>
<td>1 (1–2)</td>
<td>0.0001</td>
</tr>
<tr>
<td>One vessel treated, n (%)</td>
<td>73 (31.9)</td>
<td>4 (18.2)</td>
<td>69 (33.3)</td>
<td>0.03</td>
</tr>
<tr>
<td>Two vessels treated, n (%)</td>
<td>63 (27.5)</td>
<td>6 (27.3)</td>
<td>57 (27.5)</td>
<td>0.42</td>
</tr>
<tr>
<td>Three vessels treated, n (%)</td>
<td>93 (40.6)</td>
<td>12 (54.5)</td>
<td>81 (39.1)</td>
<td>0.04</td>
</tr>
<tr>
<td>In-hospital Grace risk score^</td>
<td>154 (47–260)</td>
<td>198 (129–260)</td>
<td>152 (47–244)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>6-month Grace risk score^</td>
<td>132 (37–206)</td>
<td>165 (115–206)</td>
<td>127 (37–203)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Drug eluting stent, n (%)</td>
<td>158 (69)</td>
<td>16 (72.7)</td>
<td>142 (68.6)</td>
<td>0.2</td>
</tr>
</tbody>
</table>

*Renal insufficiency defined by creatinine levels above 2.0 mg/dl. ^= CV death vs. no CV death. ^ Values are expressed as median and (range).
**Table 2: Laboratory parameters of patients investigated.**

<table>
<thead>
<tr>
<th></th>
<th>Overall group (n=229)</th>
<th>12 month follow-up CV death (n=22)</th>
<th>12 month follow-up no CV death (n=207)</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td>IPF (%)*</td>
<td>2.9 (1.9–4.1)</td>
<td>3.7 (2.4–5.0)</td>
<td>2.8 (1.9–4.1)</td>
<td>0.05</td>
</tr>
<tr>
<td>H-IPF (%)*</td>
<td>0.8 (0.5–1.2)</td>
<td>1.1 (0.9–1.5)</td>
<td>0.8 (0.5–1.2)</td>
<td>0.006</td>
</tr>
<tr>
<td>IPC (10⁹/l) *</td>
<td>6.1 (4.2–8.2)</td>
<td>6.6 (5.2–10.7)</td>
<td>5.9 (4.1–8.2)</td>
<td>0.08</td>
</tr>
<tr>
<td>MPV (f/l)*</td>
<td>11.1 (10.5–11.9)</td>
<td>11.3 (10.6–12.5)</td>
<td>11.1 (10.5–11.9)</td>
<td>0.09</td>
</tr>
<tr>
<td>Platelets (x10³/µl)*</td>
<td>224 (184–270)</td>
<td>224 (188–309)</td>
<td>221 (184–268)</td>
<td>0.64</td>
</tr>
<tr>
<td>Haemoglobin (g/dl)*</td>
<td>13.7 (12.3–14.8)</td>
<td>11.7 (10.7–13.6)</td>
<td>13.7 (12.6–14.9)</td>
<td>0.01</td>
</tr>
<tr>
<td>Haematocrit (%)*</td>
<td>40.8 (36.5–43.5)</td>
<td>35.6 (32.5–38.8)</td>
<td>41 (37.0–43.7)</td>
<td>0.002</td>
</tr>
<tr>
<td>Leucocyte number (x10³/µl)*</td>
<td>9.4 (7.30–12.50)</td>
<td>11.8 (9.34–15.57)</td>
<td>9.2 (7.27–12.35)</td>
<td>0.01</td>
</tr>
<tr>
<td>Red blood cells (x10⁶/µl)*</td>
<td>4.53 (4.16–4.90)</td>
<td>4.18 (3.55–4.66)</td>
<td>4.56 (4.24–4.92)</td>
<td>0.02</td>
</tr>
<tr>
<td>AA-PA (%)</td>
<td>14 (9–19)</td>
<td>17 (12–35)</td>
<td>14 (9–18)</td>
<td>0.02</td>
</tr>
<tr>
<td>ADP-PA (%)</td>
<td>52 (62–67)</td>
<td>62 (33–74)</td>
<td>52 (32–67)</td>
<td>0.41</td>
</tr>
<tr>
<td>Creatinine (mg/dl)</td>
<td>1.0 (0.80–1.48)</td>
<td>1.10 (0.97–1.79)</td>
<td>1.0 (0.80–1.40)</td>
<td>0.16</td>
</tr>
<tr>
<td>cTnI peak values (ng/ml)</td>
<td>33.37 (6.40–91.06)</td>
<td>34.47 (12.16–205.09)</td>
<td>33.37 (6.08–88.98)</td>
<td>0.18</td>
</tr>
<tr>
<td>CK-MB peak values (ng/ml)</td>
<td>54.30 (19.30–165.80)</td>
<td>74.80 (19.10–276.42)</td>
<td>54.00 (19.15–158.35)</td>
<td>0.51</td>
</tr>
</tbody>
</table>

*p = CV death vs no CV death. * Values are expressed as median and interquartile range (IQR). IPF: Immature Platelet Fraction; H-IPF: Highly Immature Platelet Fraction; IPC: Immature Platelet Count; MPV: Mean Platelet Volume; ADP-PA: platelet aggregation induced by ADP 10 µmol; AA-PA: platelet aggregation induced by AA 1 mmol; CK-MB: Creatine Kinase MB; cTnI: Cardiac Troponin I.

**Figure 1:**
Receiver-operating characteristic curve for Immature Platelet Fraction (IPF).
At one-year follow-up, 22 out of 229 patients (9.6%) had died from cardiovascular causes.

With regard to cardiovascular risk factors, medications and procedural characteristics, patients who died during the follow-up were significantly older, presented a lower utilisation of the anti-GPIIb/IIIa agents, a higher Killip class, a higher prevalence of three vessel disease and a higher GRACE risk score (in-hospital and at six months), as compared to patients who remained alive (Table 1).

As reported in Table 2, IPF and H-IPF values were higher in patients who died compared to those who remained alive [median (interquartile range [IQR]): IPF 3.7% (2.4-5.0) vs 2.8% (1.9-4.1) p=0.05; H-IPF: 1.1% (0.9-1.5) vs 0.8% (0.5-1.2) p=0.006, respectively], while MPV and IPC values did not significantly differ between the two groups [median (IQR): MPV f/l: 11.3 (10.6-12.5) vs 11.1 (10.5-11.9) p= 0.10; IPC , 10^9/l 6.6 (5.2-10.7) vs 5.9 (4.1-8.2) p=0.08].

Among haematological parameters, patients who died presented significant lower values of haemoglobin, haematocrit, red blood cells and a significantly higher number of leukocytes compared to patients who remained alive (Table 2).

The ROC analysis demonstrated that IPF and H-IPF, but not MPV or IPC were able to distinguish between dead or alive patients at 12-months of follow-up [area under the curve (95%CI): IPF 0.63 (0.51-0.75) p=0.02 ; H-IPF 0.68 (0.57-0.78) p=0.0006; MPV 0.62 (0.48-0.76) p=0.07; IPC 0.61 (0.50-0.72) p=0.08] (Figure 1 and Figure 2).

Hence, IPF ≥ 3.3% and H-IPF ≥ 0.9% were identified as optimal cut-off values to predict cardiovascular death at 12-months of follow-up, by providing a sensitivity of 63.6% (95%CI 40.7% to 82.8%) and a specificity of 61.8% (95%CI 54.8% to 68.5%) for IPF and a sensitivity of 77.3% (95%CI 54.6 % to 92.2 %) and a specificity of 56.0 % (95%CI 49.0% to 62.9%) for H-IPF.

By using IPF and H-IPF cut-off values, a significantly higher percentage of patients with IPF ≥ 3.3% and/or H-IPF ≥ 0.9% was present among those who died compared to those patients who remained alive (Table 3). Kaplan Meier curves for cardiovascular death according to elevated levels of IPF and H-IPF significantly diverged (Figure 3).

In order to evaluate the influence of RP (IPF and H-IPF) on the occurrence of cardiovascular death we performed a logistic regression analysis.

In the univariate model IPF ≥ 3.3% and/or H-IPF ≥ 0.9% were significantly associated with the risk of cardiovascular death [OR (95%CI): IPF 2.83 (1.14-7.06) p=0.02; H-IPF 4.29 (1.40-13.1) p=0.01].

In the multivariate model, after adjustment for the in-hospital GRACE score, the association between RP and cardiovascular death remained significant for both IPF and H-IPF [OR (95%CI): IPF 4.15 (1.24-13.91) p=0.02; H-IPF 5.03 (1.38-18.38) p=0.01].
Discussion

In this study we demonstrate, for the first time, that RP are independent predictors of cardiovascular death at 12-months of follow-up in patients with ACS who underwent PCI.

RP are immature and newly-produced platelets which have a greater mass and are both metabolically and enzymatically more active than smaller platelets (4, 5). They have a greater prothrombotic potential and they aggregate more rapidly by collagen, having higher levels of intracellular thromboxane A2 as well as of procoagulant surface protein such as P-selectin and GPIIb/IIIa (22-24). Previous studies demonstrated that platelet turnover is accelerated in certain clinical settings, including cardiac surgery, MI, stent thrombosis and ischaemic stroke (10-12, 25) and that cardiovascular risk factors such as smoking habit and diabetes (26-28) are able to influence the number of RP.

Our finding is in line with our previous study reporting that the presence of a high rate of platelet turnover in ACS patients is an independent predictor of poor response to dual antiplatelet therapy (13), possibly due to the increased reactivity and to the presence of uninhibited cyclooxygenase (COX)-1 and COX-2 activity (29). In fact, aspirin irreversibly inhibits COX-1 and, as platelets are anucleate, COX-1 remains inhibited for the 7- to 10-day lifespan of a platelet. Newly formed platelets with intact COX activity can be detected as soon as 4-6 h after aspirin ingestion (30). Indeed, both these mechanisms can be ascribed in determining the increased risk of 12-month cardiovascular death in ACS patients with a high percentage of RP.

In our study, RP (IPF and H-IPF) strongly correlate with MPV but in a ROC curve analyses, RP represent a more specific and sensitive biomarker in predicting cardiovascular death compared to MPV.

Elevated MPV has been previously associated with acute MI and long-term mortality and with markers of platelet activity, such as platelet aggregation, thromboxane biosynthesis and expression of adhesion molecules (31).

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Table 3: Patients’ distribution according to IPF and H-IPF cut-off.

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<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td>IPF ≥3.3%, n (%)</td>
<td>93 (40.6)</td>
<td>14 (63.6)</td>
<td>79 (38.2)</td>
<td>0.02</td>
</tr>
<tr>
<td>H-IPF ≥0.9%, n (%)</td>
<td>108 (47.2)</td>
<td>17 (77.3)</td>
<td>91 (43.9)</td>
<td>0.003</td>
</tr>
<tr>
<td>IPF ≥3.3% and H-IPF ≥0.9%</td>
<td>87 (38.0)</td>
<td>14 (63.6)</td>
<td>73 (35.3)</td>
<td>0.01</td>
</tr>
</tbody>
</table>

p* = CV death vs no CV death. IPF: Immature Platelet Fraction; H-IPF: Highly Immature Platelet Fraction.

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Figure 3: Kaplan-Meier curves for cardiovascular death according to elevated levels of IPF (A) and H-IPF (B).

Log-rank; p<0.05
An increase in MPV has been noted in a number of patient groups with known CAD risk factors, such as smoking habit, diabetes mellitus, obesity, hypertension and hypercholesterolaemia (32-36).

A recent meta-analysis conducted on 3,000 subjects corroborated previous data about MPV and cardiovascular disease, by reporting that elevated MPV is associated with increased mortality and restenosis following MI and angioplasty (31).

In our study, the lack of a significant association between high MPV values and cardiovascular mortality can be due to the relative low number of patients investigated.

Moreover, MPV is a measure of platelet size but not all large platelets are reticulated platelets.

The rate of thrombopoiesis is reflected not only by platelet size but also by the amount of megakaryocyte-derived mRNA which is functional, and determines the ability of newly released platelets to produce membrane and secretory proteins such as tissue factor, GPIIb/IIIa and platelet factor 4 (37-38).

It is conceivable that IPF and H-IPF, taking into account not only platelet size but also the amount of megakaryocyte-derived mRNA, represent a more specific parameter of platelet activation compared to MPV.

Until a few years ago RP were determined only by flow cytometry using fluorescent RNA-staining dyes, but this method lacks standardisation and there is a significant variability in the various published protocols (39).

Recently a new method for the automated measurement of RP in peripheral whole blood was developed (21): it is simple to obtain, easy to interpret and routinely measured by automated cell counters allowing RP determination a standardised and reproducible methodology easy to perform as well as MPV.

In conclusion, we found that reticulated platelets are significantly associated with an increased risk of cardiovascular death in ACS patients. This result underlies the role of an increased platelet turnover in the presence of more active and aggressive platelets which are associated with a worse prognosis. The measurement of RP might help clinicians, at least in high risk condition such as ACS, to detect patients at higher risk of adverse event in the follow-up in order to intensify secondary prevention therapies.

Conflicts of interest
None declared.

References