The pro- and anticoagulant role of blood-borne phagocytes in patients with acute coronary syndrome

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Summary
This study was performed to gain further insight in pro- and anticoagulant characteristics of leukocytes in acute coronary syndrome (ACS). For this purpose, patients presenting on the emergency department (ED) with anginal chest pain were included in this study. In peripheral blood, procoagulant tissue factor (TF) expression was measured in the different blood-borne phagocytes, i.e. neutrophilic granulocytes and the three different monocyte subsets based on expression of CD14 and CD16. Simultaneously, intracellular presence of platelet-(CD41) and/or endothelial cell-remnants (CD62e) was analysed in these different leukocyte subsets. Neutrophils showed a weak intracellular staining of CD62e and CD41 that increased with severity of ACS. Monocytes, and especially the classical (CD14++CD16-) and intermediate monocytes (CD14++CD16+) showed a clear and significant increase in intracellular CD41-staining after coronary damage. The different monocyte subsets showed an increase in expression of TF in severe ACS. Finally, it appeared that also neutrophils showed a significant increase in expression of TF on their membrane. In conclusion, this study showed an increased intracellular staining in blood-borne phagocytes for CD62e and CD41 in patients with ACS compared to non-cardiac related control patients. This indicates that at least in the acute phase of ACS phagocytosis of platelet and endothelial cell-remnants is increased. These data support the recent hypothesis that neutrophils protect against further thrombotic processes by clearing platelet and endothelial cell-remnants. In addition, this study shows that the different monocyte subsets are also involved in this process. Furthermore, both monocytes and neutrophils show increased TF expression in ACS.

Keywords
Platelets, endothelium, microparticles, phagocytosis, flow cytometry

Introduction
The acute coronary syndrome (ACS) is a clinical state induced by thrombosis following the rupture of an unstable atherosclerotic plaque. The atherosclerotic lesion triggers both platelet adhesion and fibrin formation, which leads to the formation of a growing thrombus. This process can occlude the blood vessel causing an acute myocardial infarction (AMI) (1). Activated platelets and procoagulant cell-derived microparticles are a common finding in patients with ACS (2). The activated platelets and microparticles represent a threat to the haemostatic balance, since they express both anionic phospholipids and tissue factor (TF), which could increase the risk for further thrombo-embolic events, including re-infarction.

Numerous studies show that activated platelets and microparticles interact with circulating leukocytes (3–5). Platelets and platelet-derived microparticles bind via P-selectin (CD62P) exposed on the surface of activated platelets to the leukocyte receptor, P-selectin glycoprotein ligand-1 (PSGL-1) (6, 7). This results in the formation of heterotypic aggregates. Recently, it was suggested that the heterotypic aggregates could represent an intermediate stage of an active clearance process, and contribute to limit the thrombogenicity of circulating activated platelets. Maugeri et al. showed that neutrophilic granulocytes are capable of clearing activated platelets from the blood by phagocytosis (8). They demonstrated this in patients with AMI using confocal laser microscopy (9) and hypothesised that their active removal by these phagocytes might represent a protective mechanism against thrombosis (8).
monocytes turned out to play an important role in the clearance of platelet remnants and procoagulant microparticles (10). Monocytes are known to be heterogeneous and recently it was demonstrated that three subsets could be determined based on CD14 (LPS receptor) and CD16 (Fcγ-receptor III) expression (11, 12). Next to the already described classical monocytes (CD14++CD16−, −90%) it appeared that the remaining fraction of monocytes (~10%) can be subdivided into intermediate monocytes (CD14++CD16+) and non-classical monocytes (CD14+CD16++) (12). In earlier studies of Ziegler-Heitbrock et al., it was presumed that the subset of CD16-positive blood monocytes exhibited features of tissue macrophages (13).

Recent investigations have shown that classical monocytes are mainly involved in phagocytosis, while intermediate and non-classical monocytes induce T-cell proliferation and -stimulation. In addition, the intermediate monocyte subset has also been reported to be pro-angiogenic and the main producer of reactive oxygen species (ROS) during homeostasis (11, 14, 15). The non-classical monocytes are found to have a patrolling behaviour (11, 16).

To gain further insight into the clearance of cells involved in cardiovascular damage due to ACS, we studied the intracellular presence of platelets (or platelet-derived microparticles) by CD41-expression (platelet glycoprotein [GP] IIb) as well as fragments of stimulated endothelial cells by CD62e expression (endothelial selectin) in the different blood-borne phagocytes (neutrophils, monocytes, and monocytes). In previous studies of Ziegler-Heitbrock et al., it was presumed that the subset of CD16-positive blood monocytes play an important role in the clearance of cells involved in ACS. Simultaneously, we also investigated in the same patient population the expression of TF by neutrophils and monocytes.

Materials and methods

Patients

The study population (85 patients) consisted of patients presenting to the Emergency Department with a chief complaint of chest pain. Exclusion criteria were as follows: (i) recent (within 3 months) ACS, (ii) evidence of malignant disease, (iii) systemic inflammatory conditions and (iv) unwillingness to participate. The group consisted of 41 male and 44 female patients. Blood samples were obtained from all patients before any treatment was started and were processed within 6 hours (h) after admission. They were collected in heparin and EDTA tubes. The EDTA tubes were processed for flow cytometric analysis within 24 h. The heparin tube was centrifuged at 400 g for 15 minutes (min) at 4°C. Based on clinical findings, electrocardiography outcome and laboratory test results, the cardiologists classified the patient into one of the following categories: stable angina pectoris (AP), unstable angina pectoris (UAP) and AMI. The patients with non-cardial related causes of chest pain were classified as such. The study was performed according to the Helsinki Declaration and was approved by the local Ethics Committee of our hospital. We also obtained written informed consent from all the participants.

Clinical chemistry and haematology

All heparin-blood samples were tested for troponin T (TnT), c-reactive protein (CRP) and creatinine kinase (CK) on a Roche modular analytic system (Roche Diagnostics, Basel, Switzerland). A complete blood count, including an automated cell differentiation was performed in the EDTA-blood sample with a XE2100-hemocytometer (Sysmex Europe, Hamburg, Germany).

Immunocytochemical staining.

One hundred µl of EDTA-whole blood was incubated simultaneously with directly conjugated antibodies against CD45 [allophycocyanin(APC)-Cy7; clone 2D1; Becton & Dickinson, San Jose, USA (BD)], CD14 (APC; clone MoP9, BD), CD16 (Pe-Cy7; clone NPK15, BD) and TF (FITC, clone TF-5, Sanquin bv, Amsterdam, The Netherlands). The cells were incubated in the dark at room temperature (RT) for 15 min, after which the cells were washed with phosphate-buffered saline (PBS). Then the bound antibodies were fixed by adding 100 µl fixation reagent A (Caltag, Invitrogen, Carlsbad, CA, USA) to the cell pellet for 15 min at RT. This was followed by two PBS wash steps afterwards.

Then the cells were incubated with unconjugated anti-CD41 (10 µl; clone HlP8, BD) and/or anti-CD62e (10 µl; clone 68-5H11, BD) for 15 min at RT. This incubation resulted in a blocking of the epitopes of platelets or endothelial cells which adhere to the surface of the different leucocytes. The optimal concentration of antibody needed to block the epitopes was determined in experiments with increasing amounts of antibody (data not shown). This incubation step was followed by another washing step to remove unbound anti-CD41 and anti-CD62e.

The permeabilisation step was subsequently performed by adding 100 µl permeabilisation reagent B in the presence of one of the following fluorochrome-conjugated monoclonal antibodies: anti-CD62e (rPE; clone 68-5H11, BD) or anti-CD41a (PerCP-Cy5.5, clone HlP8, BD). Two washing steps with 2.0 ml PBS were preceded by 15 min incubation in the dark at RT. The cell-pellet was resuspended in 0.5 ml PBS, and then the sample was ready for flow cytometric analysis. As a negative control sample, from each patient a portion of the cell suspension was in the permeabilisation step incubated with directly conjugated (FITC, PE, and PerCP-Cy5.5) non-relevant isotype specific mouse immunoglobulins, next to membranous staining with CD45-APC-Cy7, CD14-APC and CD16-PE-Cy7 (all from BD). This was performed to reveal and to correct for background staining by the conjugated primary antibody.

To confirm that intracellular CD41 (GPIIb)-positive reactivity is specifically derived from platelets, two other conjugated monoclonal antibodies (mAbs) against different platelet epitopes were used in a few samples. This was done using the same approach as described above by blocking surface-bound epitopes with unconjugated CD41 and CD42b (GP1bα; clone CLB-MB45, Cell Sciences, Canton, MA, USA). Unfortunately, no unconjugated form of the CD61 clone was available. For the intracellular staining the following antibodies/clones were used: CD41-PerCP-Cy5.5...
(clone HIP8, BD), CD42b-rPE (GPIIb; clone CLB-MB45; Sanquin bv) and CD61 (GPIIIa; clone RUU-PL7F12, BD).

The samples were analysed using flow cytometry (BD FACSCanTo flow cytometer; BD Biociences) using FACSDiva software (BD Biosciences). At least 30,000 CD45+ cells were collected for each sample.

### Gating procedure

For monocyte gating, the procedure described by Hristov et al. was adapted and used (17, 18). Briefly, a FSC/SSC plot was used to select intact leukocytes and to exclude dead cells and debris (Figure 1A). These gated leukocytes were plotted in a SSC/CD45 plot to identify and to gate the lymphocytes (Figure 1A). In a SSC/CD14 dotplot granulocytes selection and a preselection of CD14+/low+ cells was done (Figure 1A). This preselection of monocytes was back-gated in the SSC/CD45 dotplot and based on their location in this plot a new final monocyte-gate was drawn (Figure 1A). This monocyte population was used to differentiate the three different monocyte subsets in a CD14/CD16 dotplot (Figure 1A). Nomenclature of monocyte subsets followed the recommendation of the Nomenclature Committee of the International Union of Immunological Societies (12). Finally, based on these selected cell population the size of the fraction of TF, CD62e or CD41a-containing cells within the neutrophils as well as in classical, intermediate and non-classical monocytes (Figure 1B) was determined. The cut-off for positive intracellular staining was determined in the negative control sample by drawing a rectangular gate in the relevant fluorescence/CD45-APC-Cy7 dotplot, allowing not more than 3% positive events.

### Statistical analysis

Categorical variables are presented as numbers (percentages) of patients, and compared by Fisher’s exact test. Continuous variables are expressed as mean ± standard deviation for variables with a normal distribution and median (interquartile range) for skewed variables. Normality was tested by the Kolmogorov-Smirnov (KS) test. One-way analysis of variance (post-hoc Bonferroni test) was applied for variables with a normal distribution and the Kruskal-Wallis test was applied for skewed variables. All tests were two-sided, and p-values less than 0.05 were considered statistically significant.
Statistical analysis was performed using Microsoft Excel (Microsoft, Redmond, WA, USA) and GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA) software. The data are shown graphically by box-and-whiskers plots.

**Results**

Patients were grouped in four categories: ‘non-cardial related’ (NC), AP, UAP and AMI. In this last group ST-elevated as well as non-ST-elevated MIs were present.

**Table 1: Baseline characteristics of the study groups.**

<table>
<thead>
<tr>
<th></th>
<th>NC (n=11)</th>
<th>AP (n=31)</th>
<th>UAP (n=26)</th>
<th>AMI (n=17)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age* years</td>
<td>57 (31 – 76)</td>
<td>66 (41 – 90)</td>
<td>71 (29 – 93)</td>
<td>78 (32 – 96)</td>
<td>0.31</td>
</tr>
<tr>
<td>Sex (male) %</td>
<td>6 55%</td>
<td>14 45%</td>
<td>10 38%</td>
<td>11 65%</td>
<td>0.37</td>
</tr>
<tr>
<td>Body Mass Index kg/m²</td>
<td>27.4 ± 5.2</td>
<td>29.2 ± 7.7</td>
<td>24.8 ± 5.0</td>
<td>23.6 ± 4.0</td>
<td>0.34</td>
</tr>
<tr>
<td>History of CVD %</td>
<td>3 27%</td>
<td>18 58%</td>
<td>17 65%</td>
<td>10 59%</td>
<td>0.19</td>
</tr>
<tr>
<td>History of PCI %</td>
<td>4 36%</td>
<td>13 42%</td>
<td>10 38%</td>
<td>5 29%</td>
<td>0.86</td>
</tr>
<tr>
<td>Coronary risk factors</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypertension %</td>
<td>2 18%</td>
<td>23 74%</td>
<td>17 65%</td>
<td>9 53%</td>
<td>0.0103</td>
</tr>
<tr>
<td>Diabetes mellitus %</td>
<td>2 18%</td>
<td>7 23%</td>
<td>6 23%</td>
<td>0 0%</td>
<td>0.20</td>
</tr>
<tr>
<td>Dyslipidaemia %</td>
<td>4 36%</td>
<td>16 52%</td>
<td>10 38%</td>
<td>8 47%</td>
<td>0.72</td>
</tr>
<tr>
<td>Current smoking %</td>
<td>2 18%</td>
<td>8 26%</td>
<td>8 31%</td>
<td>3 18%</td>
<td>0.74</td>
</tr>
<tr>
<td>Laboratory parameters on admission</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Platelet count ** *10⁹/L</td>
<td>263 ± 106</td>
<td>248 ± 74</td>
<td>270 ± 103</td>
<td>263 ± 84</td>
<td>0.82</td>
</tr>
<tr>
<td>WBC count ** *10⁹/L</td>
<td>8.4 ± 4.0</td>
<td>9.6 ± 3.6</td>
<td>11.3 ± 4.3</td>
<td>10.3 ± 3.2</td>
<td>0.16</td>
</tr>
<tr>
<td>Total cholesterol mmol/L</td>
<td>4.8 ± 0.9</td>
<td>4.7 ± 1.1</td>
<td>5.0 ± 1.2</td>
<td>4.8 ± 0.9</td>
<td>0.32</td>
</tr>
<tr>
<td>HDL-cholesterol mmol/L</td>
<td>1.4 ± 0.5</td>
<td>1.3 ± 0.7</td>
<td>1.2 ± 0.4</td>
<td>1.3 ± 0.4</td>
<td>0.91</td>
</tr>
<tr>
<td>LDL-cholesterol mmol/L</td>
<td>2.8 ± 1.0</td>
<td>2.7 ± 0.8</td>
<td>3.0 ± 1.0</td>
<td>2.4 ± 0.7</td>
<td>0.39</td>
</tr>
<tr>
<td>Triglycerides mmol/L</td>
<td>1.5 ± 0.6</td>
<td>1.5 ± 0.9</td>
<td>1.7 ± 0.6</td>
<td>1.1 ± 0.4</td>
<td>0.20</td>
</tr>
<tr>
<td>Glucose mmol/L</td>
<td>6.0 ± 1.2</td>
<td>7.3 ± 2.2</td>
<td>7.5 ± 2.6</td>
<td>7.3 ± 1.7</td>
<td>0.24</td>
</tr>
<tr>
<td>Creatinine μmol/L</td>
<td>76 (51 – 588)</td>
<td>79 (45 – 178)</td>
<td>84 (55 – 294)</td>
<td>94 (51 – 408)</td>
<td>0.10</td>
</tr>
<tr>
<td>AST U/L</td>
<td>20 (15 – 39)</td>
<td>22 (12 – 202)</td>
<td>23 (12 – 170)</td>
<td>28 (52 – 127)</td>
<td>0.11</td>
</tr>
<tr>
<td>ALT U/L</td>
<td>27 (15 – 39)</td>
<td>20 (11 – 94)</td>
<td>23 (11 – 232)</td>
<td>25 (16 – 108)</td>
<td>0.76</td>
</tr>
<tr>
<td>β-GT U/L</td>
<td>29 (16 – 120)</td>
<td>39 (13 – 107)</td>
<td>42 (14 – 231)</td>
<td>43 (11 – 195)</td>
<td>0.84</td>
</tr>
<tr>
<td>Alkaline phosphatase U/L</td>
<td>70 (36 – 101)</td>
<td>79 (41 – 144)</td>
<td>83 (54 – 378)</td>
<td>73 (53 – 190)</td>
<td>0.24</td>
</tr>
<tr>
<td>TnT** μg/L</td>
<td>0.02 (0.01 – 0.12)</td>
<td>0.01 (0.01 – 0.08)</td>
<td>0.01 (0.01 – 0.26)</td>
<td>0.3 (0.10 – 2.50)</td>
<td>p&lt;0.0001</td>
</tr>
<tr>
<td>CRP** mg/L</td>
<td>5.0 (3.0 – 19.0)</td>
<td>3.5 (1.0 – 8.0)</td>
<td>9.0 (0.8 – 91.0)</td>
<td>12.0 (2.0 – 456.0)</td>
<td>0.42</td>
</tr>
<tr>
<td>CK** U/L</td>
<td>96 (20 – 228)</td>
<td>83 (28 – 145)</td>
<td>98 (39 – 139)</td>
<td>207 (36 – 709)</td>
<td>0.0007</td>
</tr>
<tr>
<td>Medication on admission</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aspirin %</td>
<td>4 36%</td>
<td>16 52%</td>
<td>11 42%</td>
<td>8 47%</td>
<td>0.81</td>
</tr>
<tr>
<td>ACEI %</td>
<td>1 9%</td>
<td>12 39%</td>
<td>11 42%</td>
<td>7 41%</td>
<td>0.24</td>
</tr>
<tr>
<td>CCB %</td>
<td>0 0%</td>
<td>6 19%</td>
<td>3 12%</td>
<td>4 24%</td>
<td>0.31</td>
</tr>
<tr>
<td>β-blocker %</td>
<td>4 36%</td>
<td>19 61%</td>
<td>13 50%</td>
<td>11 65%</td>
<td>0.40</td>
</tr>
<tr>
<td>Statin %</td>
<td>4 36%</td>
<td>15 48%</td>
<td>10 38%</td>
<td>8 47%</td>
<td>0.83</td>
</tr>
<tr>
<td>anti-platelet drug (not aspirin) %</td>
<td>3 27%</td>
<td>7 23%</td>
<td>6 23%</td>
<td>9 53%</td>
<td>0.12</td>
</tr>
</tbody>
</table>

Data are mean value ± SD, n % or median (interquartile range). CVD= cardiovascular disease, PCI= percutaneous coronary intervention, AST= aspartate aminotransferase, ALT= alanine aminotransferase, β-GT= gamma-glutamate transferase, TnT= troponin T, CRP= C-reactive protein, CK= creatine kinase. ACEI= angiotensin-converting enzyme. CCB= calcium-channel blocker.
Table 1 summarises the demographic, clinical and biochemical characteristics of the different patient categories enrolled in this study. There were no statistically significant differences in the patients’ characteristics (except for the presence of hypertension) among the four groups.

CRP levels were higher in ACS patients (UAP as well as AMI), but this increase was not significant.

The flow cytometric assay was performed on a blood sample drawn at the emergency department (ED) before any treatment was started. The time from onset of symptoms to start of the analysis ranged from 1 to 8 h. Some patients were using various combinations of drugs such as beta blockers and nitrates already before admission to the ED (see Table 1).

Neutrophils and intermediate monocytes are increased in ACS patients

The fraction of lymphocytes showed no significant difference between the different patient categories. As shown in Figure 2A, patients with an AMI had a significant higher relative fraction of neutrophils as compared to patients with non-cardiac related complaints. In addition, there were no significant differences in the fraction of total monocytes between the different patient groups (Figure 2A). However, when looking in detail at the different monocyte subsets (Figure 2B), a significant decrease in the relative fraction of classical monocytes was found in patients with stable AP and UAP when compared to the NC group. On the other hand, the fraction of intermediate monocytes (CD14++CD16+) was significantly increased in patients with UAP or AMI as compared to patients with non-cardiac related. Finally, there was also a significant difference in intermediate monocytes between patients with stable AP and AMI.

TF expression in monocytes and neutrophils is elevated in ACS patients

When analysing the cell surface-bound TF expression on the neutrophils, it became clear that the expression was significantly increased in patients with ACS (UAP and AMI) (Figure 3A).

TF expression on the surface of classical monocytes ranged between the different study groups from 60.7% (30.7-72.8%) to 70.0% (41.4%-84.9%) with no statistical differences. However, the intermediate and non-classical monocytes showed a statistical significant increase between patients with stable AP and AMI (p<0.05 and p<0.001, respectively).

Increased phagocytic function of the different blood-borne phagocytes in ACS

It turned out that neutrophils showed a weak intracellular staining with anti-CD41, which increased in patients with ACS. This increase was statistically significant between the NC group and pa-

![Figure 2: Relative fractions of the different leukocyte subsets in the different patient categories. The neutrophils as well as the intermediate monocytes shows a significant increase when the cardiovascular damage is more severe. *p<0.05, **p<0.001 and ***p<0.0001.](https://www.thrombosis-online.com)
...tients with AMI. Also, a significant increase was found in patients with an AMI when compared to those with a stable AP.

The intracellular staining for CD41 in monocytes increased significantly with increasing severity of ACS. Where patients without ACS (NC and AP) have only median of 3.0% (range 1.8-3.6%) and 7.2% (4.0-10.6%), respectively, of classical monocytes positive for CD41, this subset increased significantly to 9.1% (6.6-17.4%) in UAP and 16.0% (12.3-47.1%) in AMI. In intermediate monocytes, this trend continued to a certain degree. A higher amount of cells intracellularly positive for CD41 was seen in the intermediate subset: 28.3% (14.0-52.0%), 21.2% (15.0-35.0%), 43.2% (25.4-58.6%) and 66.4% (44.9-98.3%) for NC, AP, UAP and AMI, respectively, with significant differences between AP vs UAP (p<0.05) and AP vs AMI (p<0.0001). The pattern of non-classical monocyte platelet remnant uptake was similar to intermediate monocytes but with a decreased overall intracellular staining (16.2% (10.4-18.9%) in NC, 12.3% (8.9-18.7%) in AP, 21.2% (16.0-25.6%) in UAP and 23.6% (13.9-57.9%) in AMI); significant changes were seen in AP vs UAP (p<0.05) and AP vs AMI (p<0.01; Figure 3 B).

Intracellular staining for CD62 in the neutrophils was low, and no statistical significant difference was found between the different patient groups. The fraction of classical monocytes with an intracellular staining for CD62e was also small in patients with no ACS (NC and AP). In patients with UAP an increase was seen [4.1% (2.3-32.8%)]. Patients with stable AP (p<0.05) as well as UAP (p<0.05) showed a significant increase compared to the NC group. In the intermediate or non-classical monocyte subpopulation no statistical differences were found between the different study groups. However, the overall positive cell amount was increased (Figure 3 B).

**Intracellular platelet-epitope staining**

Other monoclonal antibodies against platelet-specific antigenic epitopes were tested to confirm intracellular presence of platelet-remnants. When stained simultaneously with CD41, CD61 and CD42b, the fraction of neutrophils positive for CD41 was similar in size with that staining for CD61 (Figure 4). In addition, it turned out that this concerned the same cells, which are positive...
for these two markers. For CD42b a small decreased fraction of neutrophilic granulocytes were positive for intracellular platelet remnants when compared with CD41 and CD61. For the monocytes and the intermediate monocytes the same results were found (data not shown). Surface-bound epitopes of platelets were blocked with a mixture of unconjugated anti-CD41 and anti-CD42b.

**Discussion**

It is now widely recognised that atherosclerosis is a specific example of a chronic inflammatory response in which leukocytes play a major role (19). Inflammatory changes and activation of immune cells are also involved in the acute phase of the coronary artery disease (CAD) (20). The present study further elucidates the involvement of immune cells in ACS and the preceding stages.

In ACS several processes find place in the microvasculature of the myocardium, which can lead to profound systemic consequences. First of all, due to accumulation of biological substances in the vessel wall of the coronary arteries an atherosclerotic plaque develops. Disruption of this plaque leads to exposure of TF and procoagulant cell surfaces to the flowing blood stream, which trigger and propagate the coagulation cascade. Another consequence of the ruptured coronary artery is activation of circulating blood cells and platelets and formation of microparticles. TF positive cells and microparticles adhere to the thrombotic surface and stimulate further thrombus growth. Possible protective mechanisms to limit these procoagulant processes is clearance of procoagulant cellular material including activated platelets, endothelial cells and their remnants or released microparticles (8). In this study we investigated the expression of TF on the different circulating phagocytic cells in control subjects and in patients during the acute phase of ACS. Next to this, we also examined the clearance of blood cell derived substances by the different circulating phagocytic cells.

We demonstrate that the fraction of neutrophils is associated with the severity of ACS, indicating a possible involvement in the acute process. In addition, we see that neutrophil TF expression positively correlates with the extent of cardiac damage. Expression of TF on neutrophilic granulocytes is still a matter of debate (21, 22). Yet, Maugeri et al. recently demonstrated the presence of a significant amount of TF-mRNA in neutrophils after stimulation with P-selectin, which led to translocation of TF onto the cell surface in a fraction of neutrophils (23). In our study we found in AMI patients TF expression in about 10-20% of the total neutrophils. An explanation for this finding could be that in vivo older and younger cells differ in their capacity to react to stimuli and/or to synthesise endogenous TF. The hypothesis is that TF is not con-

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**Figure 4:** A) Neutrophils stained positive for the three different mAbs against platelet epitopes; CD41 (GPIIb, left), CD42b (GPIIbα, middle) and CD61 (GPIIIa, right). B) FMO’s (Fluorescence-minus-one) were used for proper compensation and threshold determination. C) The neutrophils that are positive for CD61, CD42b or CD41+ are the same cells.
What is known about this topic?

- Activated platelets and procoagulant derived microparticles are a common finding in patients with acute coronary syndrome (ACS).
- Activated platelets can be cleared by phagocytosis by neutrophilic granulocytes.
- In the acute phase of an acute myocardial ischaemia tissue factor (TF) activity as well as absolute number of microparticles are low.

What does this paper add?

- Next to monocytes, it appears that also neutrophils express TF in severe cardiovascular damage.
- There is an increased intracellular presence of endothelial and/or thrombocytic epitopes in phagocytic cells (monocytes and granulocytes) of patients with ACS when compared to healthy persons and patients with angina pectoris.
- Phagocytic cells (monocytes but also granulocytes) play an important role in the clearance of platelet- and endothelial cell remnants (microparticles).

Phagocytes contribute to the clearance of platelets or endothelium.

Activated platelets and procoagulant derived microparticles are a common finding in patients with acute coronary syndrome (ACS).

Activated platelets can be cleared by phagocytosis by neutrophilic granulocytes.

In the acute phase of an acute myocardial ischaemia tissue factor (TF) activity as well as absolute number of microparticles are low.

Neutrophilic granulocytes are capable of phagocytosing platelet remnants. They state that neutrophils represent an innborn clearance system, which in normal conditions removes the excess activated platelets, maintaining blood fluidity. In addition to the data of Maugeri et al., we show that neutrophils, also the circulating monocytes, and especially the classical and intermediate monocytes, are involved in the clearance of procoagulant cellular material. It is thus possible that monocytes are recruited to a vascular lesion for a first clearance of the damage. In line with this hypothesis, we found high expression of intracellular CD41 antigen in intermediate monocytes, suggesting that these cells are most active in the clearance of activated platelets.

Overall, we show that circulating phagocytes have internalised platelet (anti-CD41) and endothelial (anti-CD62e) cell remnants. These data are in line with the results of Maugeri et al. (8) and strongly suggest a protective mechanism to prevent further thrombotic processes.

Note that CD62e is only expressed on activated endothelial cells, and this study only measures this subpopulation of the phagocytised endothelial cell material.

A restriction of this study is that the group of patients which turned out to have non-cardial related chest pain contains a few patients with low increased inflammatory responses (CRP levels are low) and altered phagocytic activity (e.g. pneumonia).

We notice that the gating strategy used to identify the different monocyte subpopulations in this study fails to precisely distinguish monocytes from natural killer cells and granulocytes. Recent studies described proper monocyte gating which reduces spill-over of natural killer cells and granulocytes into the intermediate and non-classical monocyte gate (15, 32). For future studies we recommend to use a pan-monocyte marker for precise monocyte gating.
In conclusion, this study investigates procoagulant and phagocytic activity of monocytes and neutrophils in the different stages of coronary disease in patients and in non-cardiac related control patients. There is much evidence that phagocytes play a prominent role in procoagulant processes. This study confirms that TF expression on both monocytes and neutrophils is increased in AMI. In addition, this study supports the recent hypothesis that phagocytes play a prominent role in clearance of cells in the acute phase of coronary disease. We demonstrate that phagocytic clearance of platelet and endothelial material is increased in both neutrophils and monocytes in AMI. The involvement of neutrophils was already described. Here we add that monocytes, and especially classical and intermediate monocytes, are also actively involved in this process. From this we learn that next to their procoagulant involvement, circulating monocytes and neutrophils are also actively involved in protective, probably antithrombotic, mechanisms. Further studies are needed to explore the exact mechanisms that trigger this protective response. We speculate that inhibition of procoagulant activity of phagocytic cells and stimulation of their protective mechanisms are potential new targets for antithrombotic therapy.

Conflicts of interest
None declared.

References