Platelet activation and reactivity in the convalescent phase of ischaemic stroke

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Summary
The study was aimed at the evaluation of blood platelet activation and reactivity in patients in the convalescent phase of stroke (n=58) and controls matched in respect to risk factors of vascular pathology (n=55). Both groups were treated daily with acetyl salicylic acid (ASA), 150 mg/day. Using flow cytometry, the expressions of P-selectin and the active GP IIb/IIIa receptor, as well as the fraction of platelet-derived microparticles (PMPs) and total platelet aggregates (Ag), were evaluated in non-stimulated platelets and in platelets stimulated in vitro by thrombin, thrombin receptor activating peptide (TRAP) or ADP. The expression of P-selectin in non-stimulated platelets was found to be significantly (p=0.04) lower in stroke patients. In parallel, these patients manifested a significantly (p=0.0008) higher proportion of PMPs and a lowered (p=0.003) proportion of Ag, as compared to the controls. In the stroke patients the increased expressions of P-selectin and active GP IIb/IIIa in TRAP- or ADP-activated cells were less pronounced (p<0.01), while the increments in PMP fraction remained higher (p<0.05). Our results may indicate that chronic platelet activation develops in patients in the convalescent phase of stroke and the process of PMP generation prevails over blood platelet degranulation and aggregation. This shift may be particularly unfavourable due to the procoagulative and proatherosclerotic properties of PMPs, accompanied by their decreased sensitivity to the action of antiplatelet drugs.

Keywords
P-selectin, platelet-derived microparticles, cerebrovascular disease

Introduction
Increased blood platelet activation is related to various pathological conditions, including cardiovascular (1–4), metabolic disorders (diabetes, hyperlipidaemia) (5, 6) and other inflammatory diseases, such as connective tissue diseases and atherosclerosis (7, 8). The consecutive measurements of platelet activation have been performed in the acute and convalescent phases of ischaemic stroke, and various markers of platelet activation have been assessed. Flow cytometric measurement in whole blood represents a useful tool in such evaluations but, unfortunately, it remains difficult to implement in clinical practice due to its high cost and elaborate sample preparation. The expression of platelet membrane glycoproteins presented during degranulation, adhesion and aggregation on the platelet surface provide useful markers of platelet activation. The activation markers most frequently employed for investigatory purposes include the expressions of P-selectin (CD62P) and of CD63 (as markers of the secretion from alpha granules and lysosomal granules, respectively), the fraction of monocyte-platelet complexes, the expression of the active form of the GPIIb/IIIa receptor (a marker of platelet predisposition to aggregation) and the expression of CD40L, which is the ligand for CD40 receptor on endothelial cells, lymphocytes B and macrophages (9–12). Attention has also been paid to fractions of platelet-derived microparticles (PMPs), as significant markers of platelet activation, which plays a pivotal role in the system of clotting and fibrinolysis (13). Studies evaluating platelet activation in the acute and convalescent phases of stroke apparently determined the sequence of alterations in platelet activation, but their results still remain equivocal and have instituted a wide-ranging discussion on the platelet activation profile following an acute cerebral ischaemic incident. A number of authors have confirmed an increased platelet activation in the first days after stroke (8, 14–18), and during the subsequent subacute and chronic phases (17–19). Another group of investigators challenged the presence of increased activation of circulating platelets during the three months following a stroke and suggested that platelet activation returned to normal in that time (16, 17, 20, 21). It remains to be clarified whether the distinct platelet activation profile in after-stroke patients represents a sequel, or a cause, of stroke. However, the studies quoted above

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failed to determine the effect of agonists on after-stroke platelet reactivity. Accordingly, we decided to test the expression of P-selectin, the expression of activated GPIIb/IIIa, the percentage of PMPs and the percentage of platelet aggregates in non-stimulated platelets (activation) and platelets activated under in vitro conditions with various agonists: ADP, TRAP or thrombin (reactivity) using whole blood flow cytometry. The aim of the present study was therefore to assess platelet activation and reactivity in after-stroke patients and to compare the results with those obtained in the matched control subjects. We hypothesised that platelet activation and reactivity in convalescent stroke patients differ from those observed in the at-risk control group.

Materials and methods

Selection of stroke patients and controls

The sample size was a priori evaluated using standard statistical criteria for the estimation of sample size and statistical power. As far as we expected to reveal at least 30% differences between the groups with a significance at least 1% and statistical power of at least 90%, taking into an account that the natural variability is at the most 30% and the ratio of case to control sample size equals 1, the estimated minimum sample size is 32 experimental subjects and 32 matched controls.

The inclusion criteria were: patient’s age between 45 and 75 and at least three months having passed since the first ischaemic stroke. The stroke was confirmed by the clinical examination, computed tomography (CT) or magnetic resonance imaging (MRI) scans and patient history. The study included patients whose neuromaging confirmed an ischaemic stroke in the blood supply region of the anterior circulation (Total Anterior Circulation Stroke [TACS] and Partial Anterior Circulation Stroke [PACS]) according to the Oxfordshire Community Stroke Project (OSCP) Classification (22), were burdened with at least two concomitant cardiovascular disease risk factors including hypertension (defined as a systolic blood pressure ≥140 mm Hg and diastolic blood pressure ≥90 mm Hg) or antihypertensive treatment or/and hypercholesterolaemia (blood cholesterol level ≥5.17 mM or statin treatment), smoking (current or recent) or obesity (body mass index [BMI] >30 kg/m²). All the subjects received 150 mg/day acetylsalicylic acid (ASA) as a secondary stroke prophylaxis. All those patients with a history of intracranial haemorrhage, diabetes, heart disease (atral fibrillation, coronary disease, myocardial infarction, unstable angina, patent foramen ovale or valvular heart disease), transient ischaemic attack (TIA) during the convalescent phase of a stroke, any history of trauma or surgery within the preceding three months, any history of a haematologic disorder, deep venous thrombosis or alcohol and/or drug abuse were excluded from the study. Patients with stroke within the region of the posterior circulation or with a stroke of potential lacunar etiology (POCS and LACS, respectively, according to the OCSP Classification) and embolic etiology and all subjects with a carotid artery stenosis exceeding 70% on colour-Doppler ultrasonography, were also excluded. Additional exclusion criteria at study entry included an abnormal glucose level (fasting glucose level >7 mM), abnormal platelet count, abnormal liver function, renal insufficiency, acute or chronic inflammatory disease or history of connective tissue disease. None of the patients included in the study had received anticoagulants or antiplatelet drugs, other than ASA and/or non-steroid anti-inflammatory drugs (NSAIDs) during the previous three months.

Based on the medical records at the Department of Neurology, we reviewed the available medical records of 523 subjects who had earlier been treated for ischaemic stroke within the previous 12 months. Then, using the inclusion and exclusion criteria specified above, we found a group of 108 subjects potentially suitable for inclusion in our program. Of these patients, 81 gave their informed consent and agreed to participate in our study. Initially, based on these patients’ histories and the results of the supplementary examinations, 62 patients who had not shown any additional exclusion criteria formed the population of the stroke subjects studied.

The matched controls were recruited from the General Practice outpatient clinic in Poznan, Poland. They were examined simultaneously in a pair-wise manner, and according to the same protocol as the stroke subjects (the concurrent control group). Out of the target population of 215 individuals (without a stroke incident) belonging to the same general population as the stroke patients, a source population of 111 subjects was chosen, which served for the further selection of 60 age-, gender- and risk factor-matched subjects who had never experienced a TIA and/or stroke. All the controls received 150 mg ASA per day for seven days preceding blood sampling.

Both groups fulfilled the critical a priori estimates of the minimum sample size and we collected laboratory data for all the selected patients and controls.

We succeeded in obtaining reliable flow cytometry and laboratory data (without the outliers as decided on Cook’s distances and 4 sigma analysis) resulting in the final selection of 58 patients and 55 controls.

The study protocol was approved by the local ethics committee, and informed consent was obtained from all study subjects.

Clinical assessment and laboratory investigations

All the subjects were assessed clinically in detail. Information regarding vascular risk factors (smoking, alcohol intake, family history) and medications was collected. The physical and neurologi- cal examination, blood pressure measurement and ultrasound colour Doppler carotid and vertebral arteries assessment were performed on all subjects by the same examiner [M.L.]. The exclusion criterion of an extracranial carotid stenosis >70% was diagnosed if the peak systolic velocity (PSV) in the internal carotid artery (ICA) was >2.1 m/s and the end diastolic velocity (EDV) in the ICA was >1.1 m/s, or the ratio of PSV in the common carotid artery to that in the ICA was >4.0 (23). Height and weight assessments were car-

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ried out to establish a precise BMI. A full clinical assessment was supplemented by laboratory investigations, including complete blood count, biochemical tests and routine cardiac analysis with 12-lead echocardiogram (ECG). In patients suspected of a cardioembolic stroke etiology, transthoracic +/- transesophageal echocardiography was additionally performed to exclude cardioembolism.

Blood samples

Blood samples were obtained from the after-stroke patients within one year, but not earlier than three months (90 days), after the stroke. In the control subjects blood samples were obtained after seven days of ASA-intake period. In both groups fasting blood samples were always obtained between 08.00 a.m. and 09.00 a.m. to avoid the influence of circadian variations. The blood samples of 4.5 ml were withdrawn with a 19-gauge needle by direct, single venepuncture of the antecubital vein, without pressure, in a sitting position, into a tube with 0.105 M buffered sodium citrate anticoagulant (Becton Dickinson, Plymouth, UK) and very gently mixed. An additional aliquot of 2.6 ml blood was drawn into a tube with EDTA potassium salt (Sarstedt Monovette, Sarstedt, Germany) to perform a blood count. For the biochemical tests, 10 ml blood were drawn into a tube (Sarstedt Monovette) and allowed to clot. All the samples were coded and blind-analysed by technicians.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Stroke patients (n=58)</th>
<th>Controls (n=55)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years ≥ 60</td>
<td>61 (53–70)</td>
<td>59 (52–67)</td>
<td>0.06</td>
</tr>
<tr>
<td>BMI, kg/m² ≥ 26</td>
<td>26 (24.1–28.7)</td>
<td>25.2 (21.8–27.7)</td>
<td>0.16</td>
</tr>
<tr>
<td>Males, n (%)</td>
<td>26 (44.8)</td>
<td>28 (50.9)</td>
<td>0.56</td>
</tr>
<tr>
<td>Hypertension, n (%)</td>
<td>32 (55.2)</td>
<td>36 (65.5)</td>
<td>0.37</td>
</tr>
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<td>Hypercholesterolaemia, n (%)</td>
<td>44 (75.8)</td>
<td>34 (61.8)</td>
<td>0.11</td>
</tr>
<tr>
<td>Smoking, n (%)</td>
<td>19 (32.8)</td>
<td>18 (32.7)</td>
<td>0.99</td>
</tr>
<tr>
<td>Family history of stroke, n (%)</td>
<td>12 (20.7)</td>
<td>7 (14.6)</td>
<td>0.08</td>
</tr>
<tr>
<td>Medications</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACE inhibitors, n (%)</td>
<td>30 (51.7)</td>
<td>26 (47.3)</td>
<td>0.78</td>
</tr>
<tr>
<td>β-blockers, n (%)</td>
<td>5 (8.6)</td>
<td>12 (21.8)</td>
<td>0.09</td>
</tr>
<tr>
<td>Statins, n (%)</td>
<td>39 (67.2)</td>
<td>27 (49.1)</td>
<td>0.31</td>
</tr>
<tr>
<td>Antidepressants, n (%)</td>
<td>6 (10.3)</td>
<td>4 (7.3)</td>
<td>0.59</td>
</tr>
<tr>
<td>Stroke location</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PACS, n (%)</td>
<td>48 (82.8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TACS, n (%)</td>
<td>10 (17.2)</td>
<td></td>
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</tr>
</tbody>
</table>

Values are expressed as median values and 25th to 75th percentile ranges.

Table 1: Baseline characteristics of after-stroke patients and controls.

Flow cytometry studies

The flow cytometry measurement procedures were performed as described previously (24, 25), following a modification of the platelet activation procedure, using the antibodies and other materials for flow cytometry purchased from Becton Dickinson (San Jose, CA, USA). Briefly, the procedures were performed on whole blood immediately after sampling. In order to limit spontaneous platelet activation all samples were tenfold diluted in FACS-Flow immediately after withdrawal. Three portions of 100 μl each were supplemented with agonists: 5 μM ADP (30 s, room temperature [RT]) (Sigma Aldrich, St Louis, MO, USA), 8 μM TRAP-14 (4 min, RT) (Sigma Aldrich) or 0.15 IU/ml human thrombin (4 min, RT) (Dade Behring, Marburg, Germany) with 2.5 mM glycyl-L-prolyl-L-arginyl-L-proline (GPRP, Sigma Aldrich) added to inhibit thrombin-induced fibrin clot formation. Immediately after platelet activation, the samples were rediluted tenfold with FACS-Flow to retard further activation. In order to test the non-activated population of circulating platelets (F0), a separate blood sample was left unstimulated and diluted with FACS-Flow.

Subsequently, the diluted samples were immediately transferred to a mixture of staining antibodies (anti-CD61/PerCP – representing the gating antibody directed against human GPⅡbα, PAC-1/FITC, anti-CD62/PE) and stained simultaneously for 20 minutes at RT in the dark. Then the labelling procedure was interrupted by a sample fixation in 10-fold diluted CellFix solution (phosphate buffered fixative containing 10% formaldehyde and 1% sodium azide) resulting in a final fixative concentration of 1%. The samples prepared were stored until cytometric analysis at a temperature of +4°C to +6°C. The fluorescence of 5,000 platelets was measured with a FACS Calibur instrument (Becton Dickinson). The fractions of the specific fluorescence-positive platelets were obtained after the subtraction of non-specific mouse IgG1 binding platelets. The increase in platelet reactivity was defined as the difference between percentages of CD62P- or PAC-1-positive platelets following stimulation with agonists (ADP, TRAP or thrombin) and of CD62P- or PAC-1-positive platelets in the population of resting platelets not subjected to the effects of the agonists. The extent of forward light scatter (FSC) vs. side light scatter (SSC) was employed to evaluate the fractions of platelet-derived microparticles (PMPs) and aggregates (Ag). Flow cytometric measurements were performed at the Flow Cytometry Unit in the Institute of Pediatrics, Medical University of Lodz, Poland.

The within-assay reproducibility of flow cytometric evaluation (Pearson’s variability coefficient) was as follows: 0.5–4% for aggregates, 0.8–3% for PMPs, and 0.7–5% for the percentages of CD62P-, PAC1- and CD61-positive platelets.

Statistical analysis

The data are presented as median and 25th–75th percentile range. A normal distribution of the tested parameters was verified using the Shapiro-Wilk test. We used the Chi² test for comparison of clinical
characteristics between the after-stroke and control groups. Significance of intergroup differences was evaluated using the Mann and Whitney non-parametric U test. Associations between the examined parameters were determined using the Spearman rank and Kendall τ tests. The relation between the differences in the activation and reactivity of platelets and the time which elapsed after stroke was evaluated using non-parametric linear regression (β coefficient). The null hypothesis stating no differences between groups or no relation between the variables was rejected for significance at p<0.05.

**Results**

**Demographics**

Clinical data on the patients in the convalescent phase after an ischaemic stroke (98–323 days) and individuals from the control group are presented in Table 1. No significant differences were disclosed in the clinical parameters characterising these two groups.

**Platelet data**

The mean percentage of CD61 expression was similar in the after-stroke patients (99.1%) and the controls (99.3%), confirming that the cells analysed in flow cytometry were, in fact, platelets. Not all the analysed parameters of blood platelet activation manifested the traits of a normal distribution. In non-stimulated platelets the subpopulation of PMPs was greater in the post-stroke group (Fig. 1A). The fraction of platelet aggregates, and the percentage of platelets expressing P-selectin, were significantly lower in the post-stroke patients (Figs. 1B and 2A). There were no significant differences between the groups in the fractions of platelets showing the activated form of GPIIb/IIIa receptor (Fig. 2B).

Significant differences were also disclosed in platelet reactivity in post-stroke patients as compared to control individuals. In the post-stroke group a smaller increase in the surface expression of P-selectin and in the percentage of activated GPIIb/IIIa receptor was observed after activation with TRAP or ADP, as compared to the control group (Table 2). No significant differences were detected either in aggregate subpopulations after stimulation with any of the agonists or in the expression of the assessed surface antigens in thrombin-activated platelets.

The post-stroke patients manifested a faint, but significant, negative relationship between the expression of P-selectin on non-stimulated platelets and the proportion of PMPs following stimulation with agonists (CD62P F0 vs. PMPs TB: τ = –0.38, p=0.003; CD62P F0 vs. PMPs TRAP: τ = –0.26, p=0.047; CD62P F0 vs. PMPs ADP: τ = –0.28, p=0.036). Likewise, a significant negative relationship was demonstrated between the proportion of PMPs and the proportion of aggregates in the post-stroke group. This was most pronounced following the stimulation with ADP: PMPs ADP vs. Ag ADP: τ = –0.6, p=0.000009. In the control group these associations were not significant.

Analysis of the data showed that the time which elapsed after stroke was related to the platelet activation and reactivity. We showed that in the population of unstimulated platelets the time elapsing was paralleled by an increased proportion of PMPs (β = 0.26; p=0.0006) and a decreased proportion of platelet aggregates (β = –0.17; p=0.018). Reductions were also noted in the increments of PAC-1 binding following the stimulation with ADP (β = –0.23; p=0.0008), and in the increments in CD62P expression following the stimulation with either TRAP (β = –0.25; p=0.0005) or ADP (β = –0.19; p=0.009).

**Discussion**

Analysis of blood platelet function using whole blood flow cytometry seems to be the most appropriate method for evaluating platelet ac-
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Figure 2: The fractions of CD62P-positive platelets (A) and the fractions of PAC-1-positive platelets (B) in controls and convalescent stroke subjects. Results refer to non-stimulated platelets. Symbols represent individual values; bold horizontal lines represent median values; thin horizontal lines represent 25th and 75th percentiles.

Table 2: Differences in platelet reactivity between after-stroke patients and controls.

<table>
<thead>
<tr>
<th>Platelet parameter</th>
<th>Stroke patients (n=58)</th>
<th>Controls (n=55)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Δ PMPs TB</td>
<td>0.7 (0.1–1.0)</td>
<td>0.3 (0.1–0.5)</td>
<td>0.012</td>
</tr>
<tr>
<td>Δ PMPs ADP</td>
<td>0.6 (0.2–1.0)</td>
<td>0.4 (0.2–0.7)</td>
<td>0.041</td>
</tr>
<tr>
<td>Δ CD62p TRAP</td>
<td>74.5 (62.7–85.3)</td>
<td>86.3 (80.6–90.1)</td>
<td>0.001</td>
</tr>
<tr>
<td>Δ CD62p ADP</td>
<td>42.1 (24.9–53.8)</td>
<td>49.7 (37.6–65.6)</td>
<td>0.007</td>
</tr>
<tr>
<td>Δ PAC-1 TRAP</td>
<td>60.8 (31.9–74.7)</td>
<td>70.9 (55.5–75.2)</td>
<td>0.003</td>
</tr>
<tr>
<td>Δ PAC-1 ADP</td>
<td>51.5 (35.3–65.1)</td>
<td>66.9 (52.6–76.6)</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Values are expressed as median values and 25th to 75th percentile ranges. Δ PMPs TB – increment in the fraction of platelet-derived microparticles after stimulation with thrombin (0.15 IU/ml, 4 min, RT); Δ PMPs ADP – increment in the fraction of platelet-derived microparticles following stimulation with ADP (5 μM, 30 s, RT); Δ PAC-1 TRAP – increment in the fraction of PAC-1-positive platelets after stimulation with TRAP (8 μM, 4 min, RT); Δ PAC-1 ADP – increment in the fraction of PAC-1-positive platelets after stimulation with ADP; Δ CD62p TRAP – increment in the fraction of CD62P-positive platelets after stimulation with TRAP; Δ CD62p ADP – increment in the fraction of CD62P-positive platelets following stimulation with ADP.

It is intriguing that no such a phenomenon was observed in the control group, in which a positive correlation was proved, although not one of statistical significance. From the clinical point of view, this phenomenon appears disquieting, since PMPs exhibit their own specific reactivity, which can only marginally be restricted by antiplatelet drugs, and the role of which in the coagulation process manifests several aspects (30–34).

In the after-stroke group, but not in the controls, the negative association demonstrated between the fraction of PMPs and ag-

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ggregates may also point to a shift in the activation processes toward sequestration of microvesicles, rather than toward aggregation. The formation of PMPs seems to become intensified with the time elapsing after a stroke. Obviously, our evaluation of platelet reactivity over time was performed indirectly since we did not conduct any prospective sequential appraisals, but the trends reported herein are consistent with our other findings.

The results documenting a significantly lower binding of PAC-1 following stimulation with TRAP or ADP in after-stroke patients, as compared to the control group, corroborate previously reported results. As suggested by McCabe et al., in the course of ongoing platelet stimulation following a cerebral ischaemic incident, the binding of PAC-1 becomes lower, possibly due to the lower accessibility of binding sites, which might have become blocked by fibrinogen (18). Although intriguing, such an explanation does not fully correspond to the present findings. Instead, in our study we have demonstrated that, following a stroke, the reduced binding of PAC-1 has been accompanied by lowered fractions of (homo- and heterologous) platelet aggregates. Results similar to ours had earlier been reported by Singh et al., who analysed patients with unstable angina pectoris and confirmed an augmented fraction of microplatelets, lower expression of CD62P and the absence of differences in the expression of an active GPIIb/IIIa receptor (33). Also, the findings revealed by Serebruany et al. are very consistent with our data in that the authors have demonstrated the lowered expression of P-selectin and of the active GPIIb/IIIa complex in a group of after-stroke patients administered aspirin, as compared to patients using no secondary prophylaxis (20).

Otherwise, our results differ from previously published studies reporting an increased expression of P-selectin in the convalescent phase of stroke. Marquardt et al. showed that the elevated expression of CD62P and the absence of differences in the expression of an active GPIIb/IIIa receptor (33). Also, the findings revealed by Serebruany et al. are very consistent with our data in that the authors have demonstrated the lowered expression of P-selectin and of the active GPIIb/IIIa complex in a group of after-stroke patients administered aspirin, as compared to patients using no secondary prophylaxis (20).

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Overall, the outcomes of this study support the theory on the heterologous characteristics of platelet activation in after-stroke

What is known about this topic?
- Inconsistent data on P-selectin expression after stroke.
- No previous data on platelet reactivity in the convalescent phase of stroke assessed with flow cytometry.
- Only a few data on platelet-derived microparticles in ischaemic stroke.

What does this paper add?
- Assessment of platelet reactivity in response to the agonists: ADP, TRAP and thrombin in the convalescent phase of stroke.
- Our work confirms the shift of platelet reactivity more toward microparticle formation than toward both degranulation and aggregation processes. This shift may be particularly unfavourable due to the procoagulative and proatherosclerotic properties of PMPs, accompanied by their reduced reactivity to antithrombotic drugs.
patients and may indicate first, that platelet activation develops in patients in the convalescent phase of stroke, and second, that the phenomenon of PMP separation prevails over those of degranulation and aggregation. This shift may be particularly unfavourable due to the proatherothrombotic properties of PMPs and their decreased susceptibility to antiplatelet drugs.

Acknowledgement
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References