Mechanisms of Cerebrovascular Events as Assessed by Procoagulant Activity, Cerebral Microemboli, and Platelet Microparticles in Patients With Prosthetic Heart Valves

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Background and Purpose—Cerebrovascular events (CVE) in patients with prosthetic heart valves (PHV) have remained a severe and frequent complication despite oral anticoagulation with or without aspirin. We studied the possible pathophysiological involvement of platelet-derived microparticles (PMP) as a contributing factor for the increased incidence of CVE in patients with PHV.

Methods—We compared in a retrospective, case-control study the clinical outcome after the implantation of the PHV with several different independent morphological and functional methods, including simultaneous transcranial Doppler monitoring of both middle cerebral arteries, PMP detection by flow cytometry with use of platelet-specific antibodies, coagulation markers, and determination of the procoagulant activity by Russell’s viper venom time, a phospholipid-dependent coagulation assay.

Results—Eight of 26 patients with PHV had 9 CVE during 136 person-years of observation. Transcranial Doppler monitoring revealed an increased frequency of microembolic signals recorded over a 30-minute period in patients with CVE (75 ± 25; median, 55; range, 27 to 248) compared with those without CVE (23 ± 12; median, 7; range, 0 to 153; P < 0.05) or with control subjects (P < 0.001). Flow cytometry analysis showed increased levels of PMP in patients with compared to those without CVE (4.1 ± 0.6% versus 2.4 ± 0.4% of all fluorescence-positive events gated; P < 0.05). Increased procoagulant activity was documented by the shortened Russell’s viper venom time expressed as an increased level of platelet equivalents per microliter of plasma in patients compared with control subjects (24.7 ± 14.9%; P < 0.01). Subgroup analysis revealed that patients with CVE had a higher excess of platelet equivalents per microliter of plasma than patients without CVE in relation to the controls (68.7 ± 36.7%; P < 0.05). Mildly elevated thrombin–antithrombin III complexes (2.9 ± 0.7; median, 2.3; normal, < 2.0 µg/L) suggested incompletely suppressed thrombin formation, and fibrin generation (fibrinopeptide A) was in the upper normal range (2.1 ± 0.2; median, 1.8; normal, < 2.0 ng/mL), despite adequate anticoagulation (INR = 3.6 ± 0.1).

Conclusions—Our data show increased microembolic signals, platelet microparticles, and procoagulant activity in symptomatic patients with PHV and provide a potential pathophysiological explanation of CVE. (Stroke. 1998;29:1770-1777.)

Key Words: cerebral embolism • coagulation • heart valve prosthesis • platelets • procoagulant

The incidence of thromboembolic events in patients with prosthetic heart valves (PHV) is in a range of 1% to 5% per year despite oral anticoagulation, which is thought to be optimal with an INR of 3.0 to 4.5. In most cases (approximately 85%) the cerebral circulation is involved.1-4 Approximately 75 000 mechanical PHV are annually implanted worldwide.5 As the risk is continuous and cumulative, several thousand strokes occur every year in this patient population, and approximately 5% to 10% of them are fatal.5,6

Several studies have tried to define the optimal range of anticoagulant treatment, optimizing between thromboembolic and bleeding complications. The results demonstrate a U-shaped curve with an optimal therapeutic level of INR 3.5.1 In addition, combinations with antiplatelet agents at different dosages have been studied, but in the most pertinent studies the annual incidence of thromboembolic events has remained at 2% to 3%.4

Further studies should therefore focus on the analysis of the pathophysiological mechanisms leading to cerebrovascular events (CVE) in patients with PHV. Transcranial Doppler ultrasonography is able to detect signals representing microemboli in the basal cerebral arteries.7-11 Recent studies12-14 have shown conspicuously high frequencies of such high-intensity transient signals (HITS) in patients with PHV. The...
debate over whether these HITS are formed elements (eg, platelet aggregates) or gaseous bubbles resulting from cavitation is still not settled,\textsuperscript{12,13} and their clinical significance is disputed.

Increased shear rates at the PHV can activate platelets\textsuperscript{15} and generate platelet-derived microparticles (PMP). Such generated platelet fragments provide negatively charged surfaces and have a potent procoagulant activity.\textsuperscript{16} The phospholipids facilitate the assembly of the prothrombinase complex and may accelerate the coagulation cascade and thrombin formation several thousandfold,\textsuperscript{17} while multiple receptors (platelet glycoprotein IIb/IIIa and GP Ib) on PMP function as cross-links for mixed thrombi.\textsuperscript{18}

We therefore hypothesized that the CVE might result from an increased procoagulant activity mediated by PMP generated at the PHV and analyzed the clinical course after implantation of the PHV in relation to several independent morphological and functional parameters. Microembolic signals in both middle cerebral arteries (MCAs) were measured by transcranial Doppler ultrasound, and PMP were detected and quantified by flow cytometry using 2 well-characterized platelet-specific monoclonal antibodies directed against 2 different platelet glycoproteins. Procoagulant activity was determined by the Russell’s viper venom (RVV) time (RVVT), and thrombin and fibrin generation were assessed by the currently available methods.

### Subjects and Methods

#### Patients and Controls

Twenty-six patients (aged 61.5±1.8 years; 14 women and 12 men) with PHV gave their informed consent to participate in this retrospective case-control study. The study was approved by the Ethics Committee of the Medical Faculty of the University of Bern (Switzerland).

The 26 patients examined were referred to us at our request from cardiologists in the catchment area of our hospital. (None of our group ordinarily take care of patients with PHV.) There were no special selection criteria other than the following: patients having a PHV with an interval between valve implantation and study enrollment of at least 3 months and patients not having a major cerebrovascular event before PHV implantation. Patients with major CVE prior to the PHV implantation were excluded.

Patients were classified into the following 3 groups: A, those with proven cerebrovascular events; B, those with nonspecific transient neurological symptoms, such as vertigo, blurred vision, or recurrent amnestic; and C, those without cerebrovascular events. Classification was based on the patient’s history and the clinical and neuropsychological examination by the same neurologist (M.S.) and performed before further examination.

Cardiovascular risk factors (hypertension, smoking, diabetes, hyperlipidemia, and atrial fibrillation) were distributed similarly in the patients with and without CVE. Patient characteristics and data concerning the PHV implantation are summarized in Table 1. Patients who suffered from cerebral infarction or transient ischemic attacks (TIAs) were extensively evaluated clinically, radiologically, and with blood analysis, including cerebrovascular ultrasound and transesophageal echocardiography studies, to exclude causes of platelet activation other than the PHV.

Five patients were analyzed in part in a previous preliminary study, in which markers of plasmatic coagulation and Doppler microemboli counts were measured.\textsuperscript{19} The patients agreed to be reexamined according to this more elaborate protocol.

All patients were on a regimen of oral anticoagulant therapy with either acenocoumarol (n=4) or phenprocoumon (n=22), with a target INR of between 3.0 and 4.0.\textsuperscript{0} Actual mean INR was 3.6±0.1 throughout the observation period. Further medication consisted of ACE inhibitors (11 patients), diuretics (10 patients), digoxin (7 patients), calcium antagonists (5 patients), \(\beta\)-blockers (7 patients), nitrates (3 patients), amiodarone (2 patients), antibiotics (2 patients), oral antidiabetics (1 patient) or insulin (1 patient), low-dose estrogen replacement therapy (2 patients), benzodiazepines (2 patients), and thyroxine substitution (2 patients). The distribution of the medication

### Table 1. Characteristics of Patients With PHV

<table>
<thead>
<tr>
<th></th>
<th>Group A (n=8)</th>
<th>Group B (n=4)</th>
<th>Group C (n=14)</th>
<th>Total (n=26)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>59.6±2.4</td>
<td>55.0±4.1</td>
<td>64.4±2.7</td>
<td>61.5±1.8</td>
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<tr>
<td>Male/female</td>
<td>4/4</td>
<td>2/2</td>
<td>6/8</td>
<td>12/14</td>
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<tr>
<td>Indication</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aortic</td>
<td>5 (62.5)</td>
<td>1 (25.0)</td>
<td>8 (57.1)</td>
<td>14 (53.9)</td>
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<tr>
<td>Mitral</td>
<td>2 (25.0)</td>
<td>1 (25.0)</td>
<td>1 (7.1)</td>
<td>4 (15.4)</td>
</tr>
<tr>
<td>Combined</td>
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<td>2 (50.0)</td>
<td>5 (35.7)</td>
<td>8 (30.8)</td>
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<tr>
<td>Type of PHV</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>St Jude Medical</td>
<td>4 (50.0)</td>
<td>4 (100)</td>
<td>12 (85.7)</td>
<td>20 (76.9)</td>
</tr>
<tr>
<td>Björk-Shiley</td>
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<td>...</td>
<td>3 (21.4)</td>
<td>5 (19.2)</td>
</tr>
<tr>
<td>CarboMedics</td>
<td>2 (25.0)</td>
<td>...</td>
<td>2 (14.3)</td>
<td>4 (15.4)</td>
</tr>
<tr>
<td>Observation time, y</td>
<td>41.2</td>
<td>14.8</td>
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<td>136.0</td>
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<tr>
<td>Mean, per patient</td>
<td>5.2</td>
<td>3.7</td>
<td>5.7</td>
<td>5.2</td>
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<td>CVE (No. of occurrences)</td>
<td>TIA (1)</td>
<td>Vertigo (3)</td>
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<td>...</td>
</tr>
<tr>
<td></td>
<td>Recurrent TIA (4)</td>
<td>Amnesia (1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>PRIND (1)</td>
<td>Visual disturbances (2)</td>
<td></td>
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</tbody>
</table>

Values are mean±SEM or the absolute (and relative) number of patients in the category.

Group A indicates patients with CVE; group B, patients with nonspecific transient neurological symptoms; group C, patients without CVE.
was similar in the patients with and without CVE except for aspirin, which was given to 4 of 12 patients with CVE or nonspecific neurological symptoms and 1 of 14 patients with no CVE. One patient with CVE was on additional diprydamole.

Control subjects were 26 healthy, age- and sex-matched volunteers who were examined according to the protocol on the same day as the patient. Except for 1 subject with well-controlled, mild hypertension on an ACE inhibitor, none of the control subjects took any medication.

Transcranial Doppler Ultrasonography

Studies were performed according to a protocol described elsewhere. Briefly, patients were examined under standard conditions in a supine position. The recording, performed with a 2-channel transcranial Doppler system (Multi-Dop X/TCD 7, Firma DWL, Elektronische Systeme GmbH) equipped with a specially developed software for automated emboli detection, was started after the subjects had rested for at least 20 minutes in a quiet room. The Doppler frequency spectra of both MCAs were recorded simultaneously and continuously during 30 minutes. HTIs were identified by their short (<0.1 second), transient, unidirectional, high-amplitude signal with a narrow spectrum and counted by 3 different methods: (1) visually (on the monitor displaying the fast Fourier transform of the color-coded Doppler spectra), (2) acoustically (by continuous on-line observation by an examiner using headphones), and (3) computer-assisted (with a special software). The detected HTIs counts in the left and right MCAs were added, thus reflecting the total over the registered 30 minutes.

Laboratory Studies

Blood was drawn from patients and controls by a specially trained technician through clean venipuncture from an antecubital vein under controlled venous stasis of 60 mm Hg for maximally 45 seconds. Previous studies have shown normal values for fibrinopeptide A and thrombin–antithrombin III complexes for this technique. The Monovette system (Sarstedt) was used. A total of 45 mL of blood was drawn. The first 5 mL was discarded, then 5 parts blood was added to 1 part acid citrate dextrose (citric acid 38 mMol/L, sodium citrate 74.8 mMol/L, dextrose 124 mMol/L; pH 5.0) and prepared for flow cytometry and RVVT (see below); 9 mL blood was added to 1 mL citric acid (110 mMol/L) with the inhibitors theophylline (15 mMol/L), adenosine (3.7 mMol/L), dipyridamole (0.62 mMol/L), pH 5.0 (CalBiochem), for the measurement of theophylline (15 mmol/L), adenosine (3.7 mmol/L), dipyridamole (0.62 mMol/L), pH 5.0 (CalBiochem), for the measurement of HITS counts in the left and right MCAs were added, thus reflecting the total over the registered 30 minutes.

Flow Cytometry

Platelet-rich plasma (PRP) was prepared immediately after venipuncture by centrifugation with 800g for 2 minutes at room temperature. Platelets were counted (Coulter Counter, Coulter Electronics Ltd, or Sysmex K-1000, Toa Medical Electronics) and adjusted to 300 000/mL with sterile-filtered (0.2-μm pore size, Schleicher and Schuell) and degased PBS. Platelets were fixed within 10 minutes after venipuncture by adding paraformaldehyde (ratio 1:1) in PBS, pH 7.4, to achieve a final concentration of 0.5%. The fixation step was early because several monoclonal antibodies have been reported to induce vesiculization. For analysis by flow cytometry, the following monoclonal antibodies (Mab) were used: Mab 6D1, directed against GP Ib, and Mab 7H2, directed against GP IIIa (both kindly provided by Dr. B.S. Gmelin, from Dako); Mab 7H2 was then added without and Mab against P-selectin respectively CD62P (Serotec) in order to include a marker of platelet activation. We also used monoclonal antibodies directed against a red cell antigen (Glycoporphin-A, Serotec) and a leukocyte antigen (CD44, Dako) to obtain data on microparticles potentially derived from the other cell lines. Nonspecific mouse IgG1 (Dako) and PBS instead of the primary antibody were used as negative controls. Incubations with the first, GP-specific antibody (Mab 6D1, 7H2), or aCD62, aGlycoporinin, and aCD44, were performed for 45 minutes at a final concentration of 10 μg/mL, which is definitely above the saturation level. Fluorescein isothiocyanate (FITC)-labeled goat anti-mouse antibody (Fab’) fragment of affinity-isolated goat anti-mouse immunoglobulins, final concentration 1.5 μg/mL, for anti-CD62 and anti-CD44, was added without washing (so as not to lose any microparticles) and incubated for another 45 minutes. All steps were performed at room temperature. After a 20-fold dilution with PBS (typically, 100 mL sample was diluted with 1.9 mL buffer), the samples were analyzed on a Becton-Dickinson FACScan equipped with 15-mW air-cooled argon laser (Becton-Dickinson), and 10 000 gated events per sample were analyzed with Cell Quest software (Becton-Dickinson). Standard beads of different sizes (2, 0.5, 0.1, and 0.01 μm) were used to calibrate the system (Polysciences Inc). PMP were defined by their size and their specific positive fluorescence for platelet glycoprotein and quantified as described by Warkentin et al. Briefly, the number of GP Ib- or GP IIIa–positive particles of <87 relative fluorescence units was expressed as percentage of the total number of GP Ib- or GP IIIa–positive particles gated. This cutoff value was derived from the analysis of PRP of 25 normal donors, in accordance with the findings of Warkentin et al. This threshold (87 fluorescence units) separates optimally the well-defined platelet population from microparticles, whereas nonspecific binding of the control mouse Mab sets a much lower fluorescence margin.

Russell’s Viper Venom Time

Procoagulant activity was determined by the dilute RVVT, a sensitive, phospholipid-dependent coagulation assay. To 100 μL of pooled, ultracentrifuged (2000g for 20 minutes, followed by 15 000g for 20 minutes) and filtered (0.2-μm pore size) plasma from 12 healthy donors, we added 100 μL RVV solution (1 μg/mL) (Pen-tapharm AG, with high purity grade) and 20 μL of a PMP-containing solution the assay was then started by recalcification (100 mL of 20 mMol/L CaCl2). Coagulation times were recorded with use of an ST4 instrument (Diagnostics Stago). To quantitate procoagulant activity, a standard curve was obtained with frozen-thawed platelets as a source for PMP, as proposed by Warkentin et al. This standard was prepared by freezing platelets (300×10^9/L) twice in liquid nitrogen for 20 minutes, followed by thawing (37°C). Final concentrations ranged from 50 000 down to 1 single platelet equivalent of frozen-thawed platelets per microliter of plasma. The RVVTs were then expressed as platelet equivalents per microliter of plasma.

Different concentrations of platelets were measured in addition to platelet-poor plasma (PPP) and high-speed PPP (HS-PPP). PPP was prepared by centrifugation of the whole blood with 2 000 for 20 minutes and HS-PPP by an additional centrifugation with 15 000g for 20 minutes, followed by filtration with a 0.2-μm filter.
Statistical Analysis
Values were analyzed by the Student t test and Wilcoxon-Mann test for paired data, the Mann-Whitney test for unpaired data, and ANOVA, where appropriate. For multiple testing, the Bonferroni-Holm test was used. Differences were considered significant at \( P<0.05 \). Values are given as mean±SEM; in some instances the median values were analyzed as well, as indicated.

Results
Clinical Course
The total accumulated observation time of the 26 patients evaluated was 136 patient-years. Time span between PHV insertion and enrollment in the study was 5.2±4.6 years (range, 8 months to 20 years). In patient group A, the following 9 CVE had occurred in 8 patients: 3 completed strokes (cerebrovascular infarctions [CVIs]), 1 prolonged reversible ischemic neurological deficit (PRIND), 1 single TIA, and 4 recurrent TIAs (2 or more events), corresponding to an incidence of 6.6 per 100 patient-years for all CVE and of 2.9 per 100 patient-years for definite stroke (CVI and PRIND). In group B, 4 patients complained of nonspecific transient neurological symptoms such as recurrent attacks of vertigo (3 occurrences); recurrent amnesias (1 occurrence); and visual disturbances, such as blurred or flickering vision (2 occurrences). All symptoms occurred exclusively after the valve replacements and at least 3 months before laboratory workup and transcranial Doppler monitoring, despite a level of oral anticoagulation considered to be therapeutically optimal for these patients (INR, 3.0 to 4.5). Extensive clinical, laboratory, and ultrasonographic evaluation in the patients with CVE did not disclose any other sources of thromboemboli or other acute illnesses that may induce platelet activation or PMP generation. Fourteen patients had neither CVE nor nonspecific transient neurological symptoms during the observation time (group C).

Doppler Emboli Monitoring
The majority of patients with PHV (81%) showed a strikingly high frequency of HITS, with a wide range (48±12 emboli per 30 minutes; median, 24; range, 0 to 248). Control subjects had no HITS \((P<0.001)\).

The subgroup analysis of the patients in relation to their clinical events revealed 75±25 HITS (median, 55; range, 27 to 248) in the group of patients with CVE (group A, n=8); 83±41 HITS (median, 63; range, 14 to 191) in the group of patients with nonspecific transient neurological symptoms (group B, n=4); and in sharp contrast, 23±12 HITS (median, 7; range, 0 to 153; \( P<0.05 \)) in patients without CVE (group C), which is a 3- to 4-fold lower emboli count (Figure 1).

Flow Cytometry
The number of PMP, as quantified from freshly drawn, carefully standardized and fixed PRP, was dramatically increased by 70.1% in patients with documented CVE (group A, n=8) compared to those without CVE (group C, n=14): 4.1±0.6% with CVE and 2.4±0.4% without CVE, using Mab 7H2; \( P<0.05 \). The latter group of patients did not differ from the healthy control subjects (2.2±0.2%). In addition, the patients with nonspecific transient neurological symptoms (group B, n=4) did not show significant differences in the number of PMP compared with control subjects or patients without CVE.

Considering all patients together, the number of PMP was slightly higher compared with the controls, but it did not reach significance (2.9±0.3% compared with 2.2±0.2% using anti-GP IIIa and 2.2±0.2% compared with 2.0±0.2% using anti-GP Ib [both NS]; Figure 2). The Mab directed against GP IIIa appeared more sensitive for the analysis of PMP, therefore distinguishing better between the 3 patient groups and the control group.

The binding to P-selectin or CD62P on PMP surfaces was low and similar in patients and controls (0.3% of all events gated). Microparticles derived from red cells and leukocytes were present at very low concentrations as well (0.4±0.08 in patients compared with 0.2±0.02% in controls for glycoporphin-A [NS] and 0.1% in both groups for CD44).
**Coagulation Markers**

To detect increased thrombin or fibrin generation or fibrinolysis, prothrombin fragment 1+2, thrombin–antithrombin III complex, fibrinopeptide A, D-dimer, and β-thromboglobulin were measured. Prothrombin fragment 1+2, D-dimer, and β-thromboglobulin were measured. Prothrombin fragment 1+2, D-dimer, and β-thromboglobulin were within normal ranges (see Table 2), whereas the thrombin–antithrombin III complex levels (2.9±0.7 µg/L; median, 2.3; normal, <2.0) and fibrinopeptide A levels (2.1±0.2 ng/mL; median, 1.8; normal, <2.0) were slightly elevated despite oral anticoagulation. One patient, however, showed extremely high levels of prothrombin fragment 1+2 (2.42 nmol/L), thrombin–antithrombin III complex (17.86 µg/L), and fibrinopeptide A (3.62 ng/mL); tragically, he developed a CVI 1 day after testing. 

There were no significant differences when the results of the different clinical subgroups (groups A, B, and C) were analyzed (Table 2).

**Procoagulant Activity (RVVT)**

Patients had a significantly higher amount of (frozen-thawed) platelet equivalents per microliter of plasma compared with control subjects (P<0.01; Figure 3), which is also expressed in a significant shortening of the RVVT. These differences appeared most prominent in diluted samples with low platelet concentrations. When the platelets, but not the PMP, were removed by differential centrifugation (PPP), the effect could still be observed, whereas it disappeared after the elimination of PMP by high speed centrifugation and filtration of the plasma (HS-PPP) (Figure 3). The excess of platelet equivalents (comparable to whole platelets and control subjects) was most prominent in the group of patients with CVE (group A) (+68.7±36.7%; P<0.05) compared to the patients with nonspecific transient neurological symptoms (group B) or those without CVE (group C), where no significant differences compared to the controls could be found (Figure 3, insert).

**Discussion**

Ischemic CVE in patients with PHV may have 2 causes: (1) the level of anticoagulation is unintentionally too low or (2) thrombogenic mechanisms are not sufficiently suppressed by the reduction of coagulation factors during adequate coumadin prophylaxis. A number of well-designed and well-conducted studies have shown that it is possible to reduce (but not completely suppress) the incidence of CVE by actually achieving the targeted INR in a high percentage of the prothrombin times measured. However, the precise pathophysiology of thromboembolism in patients with PHV is still not established. A thrombogenic role has been attributed to activation of platelets or plasma coagulation on foreign surfaces, incomplete “wash out” of blood from valves, and increased shear rates with secondary platelet activation.

Therefore, we have taken a stepwise approach to analyze the thrombogenic mechanisms in 26 patients. To potentially identify subclinical events and therefore possibly a subgroup of patients particularly at risk for strokes, we have evaluated all patients and a control group for cerebrovascular microembolic signals (HITS) with transcranial Doppler monitoring. We have tried to analyze the procoagulant activity by simultaneously quantifying the circulating platelet membrane microparticles as well as their functional procoagulant impact on the RVVT, a clotting test particularly sensitive to the presence of phospholipids.

Our clinically observed CVE rate of 6.6 per 100 patient-years and the rate of definitive stroke of 2.9 per 100

![Figure 3](https://via.placeholder.com/150)

**Figure 3.** Increased procoagulant activity in patients with PHV. The RVVT was determined with use of PRP at different platelet concentrations in addition to PPP and HS-PPP, ie, decreasing concentrations of platelet membranes and membrane fragments, respectively, as a source of phospholipids. Filled bars represent patients and hatched bars controls. Values are the mean±SEM of frozen-thawed platelet equivalents per microliter of plasma. Insert, The excess of platelet equivalents (platelet equivalents of patients compared with controls) is shown in the different clinical subgroups (group A, patients with CVE; group B, patients with nonspecific transient neurological symptoms; and group C, patients without CVE) (P<0.05).
patient-years is in the range found in the literature.\textsuperscript{4} If other transient and less specific symptoms are included as well, the rate is in the upper range (11.0 per 100 patient-years). Possible reasons include a relatively high number of patients with mitral valve replacements (n=4) and double valve replacements (aortic and mitral, n=8) known to be at higher risk for thromboembolic complications and a scrutinized neurological evaluation (MS), which may have detected discrete but relevant symptoms other studies might have missed.

The patients with CVE had significantly higher HITS counts during Doppler monitoring compared with the asymptomatic patients. It appears possible that these patients constantly embolize into the cerebral circulation platelet aggregates generated at the site of the PHV by the previously mentioned mechanisms. Other compositions of the embolic particles appear less likely, considering the results of the coagulation studies, which showed no thrombin or fibrin generation. Mixed thrombotic materials at this high embolization rate would be expected to result in positive tests for fibrin formation and/or fibrinolysis (fibrinopeptide A, D-dimer). Recent studies suggest that at least part of HITS represent air bubbles generated by the cavitation effect just behind the valve leaflets.\textsuperscript{21} However, these air bubbles are of a size unable to cause strokes. Because air-fluid interfaces activate platelets, this might be another mechanism generating platelet aggregates.\textsuperscript{24-28} Microbubbles may also become stabilized, probably by being coated with membrane fragments (from platelets and other cells) generated at the PHV. Otherwise, it is difficult to understand how bubbles caused by the cavitation effect of PHV should persist until their arrival in the MCA.

Almost all of these HITS, despite occurring at high frequency, are asymptomatic. Thus, postulated aggregates likely disaggregate in the cerebral microcirculation,\textsuperscript{29} embolize into clinically silent areas,\textsuperscript{30} or are too small to generate ischemic injury. The stabilized bubbles may also disaggregate in the periphery, as may be the case with HITS composed of particulate materials. All control subjects had HITS counts of 0, whereas even the asymptomatic PHV patients had a mean count of approximately 20 HITS. It remains to be determined whether their increased rate identifies these patients to be at risk for future strokes. Additional important determinants may include size of the emboli (which cannot be estimated by Doppler analysis), their detailed composition, and most importantly, the ability to dissolve the emboli rapidly.

PMP counts were significantly elevated in the subgroup of patients with CVE but not in asymptomatic patients. In sharp contrast to the HITS quantified by Doppler ultrasound, the PMP can be observed in the healthy control population as well; namely, at a level of approximately 2%, which is similar to that in the asymptomatic patients. The low PMP level in controls may represent background noise or an insignificant systemic level, or it may result from sample collection. This observation may reflect a threshold level for thromboembolic complications; however, the almost invariably observed shortened RVVT suggests an increased prothrombinase activity in the plasma of all (symptomatic and asymptomatic) PHV patients. Therefore, very small membrane fragments may have been missed by the higher threshold of flow cytometry. One might hypothesize that the larger microparticles are more thrombogenic. In addition, other qualitative differences of membrane microparticles may also contribute to their procoagulant role in these patients.\textsuperscript{31-35} Preliminary results of our group suggest that the orientation of the platelet receptor, and perhaps of the phospholipid bilayer of microparticles as well, is different depending on the mechanism of their formation.\textsuperscript{34} Therefore, “inside-out” vesicles may provide negatively charged phospholipids to a much greater extent than those that retain the nonactivated orientation. Moreover, in contrast to the platelets, the loss of the aminophospholipid-translocase activity in the PMP may lead to permanent loss of the lipid asymmetry.\textsuperscript{35} The fact that we found no significant levels of P-selectin- (or CD62P-) positive PMP or platelets might suggest a predominantly mechanical generation rather than the activation-induced vesiculation. Increased levels of PMP have also been reported in patients with a vascular pathology,\textsuperscript{36-38} favoring turbulent flow and thus high shear rates as the mechanism for their generation. The levels of β-thromboglobulin were not increased in our patients and do not support significant platelet release in vivo. On the other hand, reduced levels of PMP (ie, the inability to vesiculate) are associated with a bleeding disorder, Scott syndrome.\textsuperscript{33,39} The latter biological model provides the evidence that a physiological level of PMP appears to be required for normal hemostasis. This interpretation fits well with our findings that the control subjects had no HITS at all but had a constant level of approximately 2% of PMP in their circulation.

It is well established that negatively charged phospholipids serve as a template for the tenase/prothrombinase complex of plasmatic coagulation.\textsuperscript{16,40,41} This can be demonstrated most sensitively by the RVVT, which is shortened by the presence of phospholipids.\textsuperscript{22} In our hands, as little as the equivalent of 3 frozen and thawed platelets per microliter was sufficient to accelerate the RVV clotting time detectably. RVVT were substantially shortened in patients compared with control subjects, even though the activity of prothrombin and Factors VII, IX, and X was reduced to less than one fifth the normal values because of coumadin therapy.

The fact that the relative shortening of the RVVT in patients compared with controls increases continuously when the platelet count is lowered from 100×10\textsuperscript{9}/L to 5×10\textsuperscript{9}/L indicates that it is indeed the PMP rather than activated platelets that are responsible for the effect. The argument is further strengthened by the observation that the difference disappears after high-speed centrifugation and ultrafiltration (HS-PPP) to eliminate PMP. The latter method interestingly leads to an even slightly longer RVVT in patients, which might be explained with their lower content of vitamin K–dependent coagulation factors (Figure 3).

Interestingly, we found no evidence that the CVE itself induced a persistent procoagulant state: there was no inverse correlation between the parameters measured and the time elapsed between the CVE and the individual analysis.

Taken together, our data provide a potential pathophysiological model for the thromboembolic events that occur in patients with PHV and may help to explain the incomplete
efficiency of therapeutic oral anticoagulation: Elevated concentrations of PMP may be a risk factor and may decrease the threshold for thromboembolic complications; under careful oral anticoagulation only minimal, if any, β-thromboglobulin release, thrombin and fibrin generation, or fibrinolysis can be observed. Symptomatic patients, however, may have partic-

ularly elevated procoagulant activity provided by their PMP or may have additional congenital or acquired risk factors, which then lead to macroscopic emboli formation and cerebrovascular events. A prospective study with a larger and well-defined sample of patients is therefore needed to further elucidate this hypothesis.

It will be interesting to further evaluate platelet inhibitors as adjunct to anticoagulant therapy, particularly those that inhibit activation-induced vesiculation. Even if they will not inhibit mechanically generated PMP, they may inhibit the shear-induced activation and influence the further buildup of PMP or enhance their dissolution in the cerebral circulation, thus reducing their deleterious effects.

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