P-selectin (CD62P) is an α-granule membrane protein of mass 140 kDa that is translocated and expressed on the plasma membrane during platelet activation and degranulation.5–7 Although P-selectin has been identified on endothelial cells, it is becoming clear that the majority of, if not all, soluble P-selectin in the plasma (sP-selectin) arises from the platelet.8–10 Expression of P-selectin on the platelet surface on stimulation is an irreversible phenomenon,11 with the main function being to mediate adhesion of activated platelets to monocytes and neutrophils.12,13 While sP-selectin levels are quantified by enzyme-linked immunosorbent assay (ELISA), platelet membrane P-selectin requires detection and quantification with flow cytometry or whole platelet ELISA.14,15 Nevertheless, it has been suggested that sP-selectin levels are a more reliable marker of in vivo platelet activation than membrane P-selectin (defined by flow cytometry).16,17 Platelet surface P-selectin and sP-selectin in the plasma have been studied as potential markers of platelet activation in various disease conditions, such as ischemic heart disease, rheumatic mitral stenosis, and atrial fibrillation (AF).14,15,18 A number of cross-sectional studies in AF have provided conflicting reports, with some studies demonstrating increased platelet surface P-selectin15,19,20 and sP-selectin21 levels in AF, especially in patients with embolic and preembolic status (eg, spontaneous echo contrast and/or left atrial thrombus), and with others failing to do so.

Despite the common use of these 2 approaches, few studies have compared platelet P-selectin (pP-selectin) and sP-selectin in the same group(s) of patients and controls. Thus, the roles of whole or total pP-selectin and sP-selectin in both health and disease states are unexplored. Hence, the devel-
opment of an assay to measure pP-selectin may potentially be useful in further exploring the concept of platelet function/dysfunction. In addition, it is unclear whether the amount of P-selectin in each platelet varies in health and disease and whether the amount of P-selectin in each platelet is altered by antithrombotic therapy. In this latter respect, there are reports that platelet α-granule secretion in response to ADP is independent of the lipoxygenase- and cyclooxygenase-dependent metabolites of arachidonic acid and aspirin and therefore may not decrease ADP-induced P-selectin expression on platelets or sP-selectin.

The aim of the present study was to further investigate the role of platelets in AF and the influence of antithrombotic therapy. To achieve this we set out to develop and then apply a new assay to detect the absolute amount of P-selectin per platelet (ie, pP-selectin). The target population of subjects with AF (who are at risk of thrombosis) was compared with healthy controls, and whole pP-selectin was compared with other indices of platelet activity, ie, plasma levels of sP-selectin and β-thromboglobulin.

Subjects and Methods

Development of Platelet Lysate Assay

Platelet Preparation

We developed an assay to quantify the amount (mass) of P-selectin in a defined number or concentration of whole platelets (ie, pP-selectin). A blood sample (approximately 5 mL) was drawn from the antecubital vein into plastic tubes with 3.2% sodium citrate. Platelet-rich plasma (PRP) was obtained by centrifugation within 1 hour of collection, at room temperature at 1000 rpm for 10 minutes; the volume was recorded, and a cell count was performed. The PRP was then centrifuged at 2000 rpm for 20 minutes to obtain a clear citrated supernatant, that is, platelet-free plasma (PFP), which was stored at −70°C. The platelet pellet was washed twice in PBS and resuspended gently in PBS to give a concentration of 1×10^8 platelets per milliliter (generally 1 to 3 mL).

Optimization of Detergent

The optimum concentration of the detergent Triton X-100 (Sigma/Aldrich) to lyse the platelets was defined in a series of experiments in which 0.5 mL of platelet suspension was mixed with 0.5 mL of different concentrations of Triton, ranging from 20% to 0.01% vol/vol in saline. The negative control was an aliquot of platelets incubated with saline. Hence, 5×10^7 platelets were present in the lysate vial that had a volume of 1 mL. The suspension was aspirated manually several times, without foaming, to produce a platelet lysate, which was then incubated at 37°C for 20 minutes, followed by centrifugation in a microfuge at 15 000 rpm for 5 minutes. sP-selectin was then determined in the supernatant by ELISA (see below).

Platelet Number Dose-Response Analysis

The relationship between the number of platelets lysed by 0.1% Triton and the concentration of sP-selectin in the lysate supernatant was examined by dose-response analysis. In this experiment, PRP was resuspended to 2×10^7 cells per mL and was two thirds serially diluted, leaving a 0.5-mL aliquot. This aliquot was incubated with 0.5 mL 0.1% Triton for 20 minutes at 37°C, followed by microfugation at 15 000 rpm for 5 minutes as described above. P-selectin was determined in supernatant by ELISA.

ELISA for sP-Selectin

Reagents and recombinant human P-selectin (as a standard) were obtained from R&D Systems (UK) Ltd. Levels of sP-selectin were detected in a 1/5 dilution of plasma in PBS. P-selectin was assessed in an undiluted aliquot of the platelet lysate supernatant. The intra-assay coefficient of variation of the ELISA was 2.8% at 60 ng/mL, 3.4% at 30 ng/mL, 3.8% at 6 ng/mL, and 5.0% at 3 ng/mL (n=64). The interassay coefficient of variation of the ELISA was 6.7% at 60 ng/mL, 7.8% at 30 ng/mL, 8.5% at 6 ng/mL, and 7.8% at 3 ng/mL (n=13). The lower limit of sensitivity of the assay was 0.8 ng/mL.

Subjects

The study group comprised 122 patients with nonrheumatic AF lasting at least 6 weeks before examination, confirmed electrocardiographically on at least 2 occasions, and 23 normal, healthy subjects.

Patients with AF were divided into 3 subgroups: (1) 34 patients with AF on no antithrombotic therapy; (2) 30 patients with AF who were established on aspirin (75 to 325 mg/d; n=30); and (3) 58 patients with AF established on dose-adjusted warfarin (international normalized ratio range, 2.0 to 3.0). Patients with AF on no antithrombotic therapy predominantly consisted of patients with persistent AF who were found to have AF routinely by the general practitioner and referred for consideration of antithrombotic therapy and cardioversion.

We excluded patients with hematologic, renal (creatinine >200 mg/dl), hepatic (liver enzymes >2 times the upper limit of normal), inflammatory, and neoplastic disorders and those who suffered recent (<4 weeks) myocardial infarction or stroke because these disorders might influence platelet activation and the level of the markers. Patients who used nonsteroidal anti-inflammatory drugs, corticosteroids, or hormone replacement therapy regularly and those on heparin were also excluded. Furthermore, patients with rheumatic mitral valvular disease, prosthetic cardiac valves, and acute AF precipitated by thyrotoxicosis or any acute infection were excluded. The study was approved by the City Hospital ethics committee, and written, informed consent was obtained from each participant.

The healthy control group consisted of 23 normal subjects (7 men and 16 women; mean [SD] age, 74 [9] years; range, 60 to 92 years) who were recruited from healthy hospital staff, relatives of the patients, or attending the hospital for routine cataract surgery. The subjects were nonsmokers with no clinical evidence of vascular, metabolic, neoplastic, or inflammatory disease by careful history, examination, and routine laboratory tests. These subjects were normotensive and in sinus rhythm.

Plasma Markers

Blood samples were drawn from an antecubital vein with atraumatic venipuncture. Citrated evacuated tubes (Vacutainers) were used for the collection of samples for P-selectin levels, and Vacutainers containing citrate, theophylline, adenosine, and dipyridamole were used for plasma β-thromboglobulin levels. pP-selectin was measured as described above. Blood samples for β-thromboglobulin were immediately placed on ice, and plasma was separated within 1 hour of collection. Aliquots were stored at −70°C to allow batch analysis. β-Thromboglobulin levels were determined by ELISA (Asserachrom β-thromboglobulin, Diagnostica Stago).

Power Calculations and Statistical Analysis

At the outset of this study, there was no existing literature from which to hypothesize a difference in pP-selectin, and therefore a formal power calculation was not possible. Nevertheless, in the cross-sectional aspect (23 cases with AF versus 23 healthy controls), we have power to detect a difference of at least two thirds of an SD of 0.8. Therefore, because we have far more AF patients, our power is greater. Comparing the 3 AF subgroups, with a minimum of 30 subjects per group, we have power to detect a difference of half of an SD under the same criteria. Therefore, because we have recruited in excess of this number, our power is also greater.

Results are expressed as mean with SD or as median with interquartile range for the normally distributed data and skewed data, respectively. Data between patients and controls were analyzed by t-test and 1-way ANOVA or Mann-Whitney U-test and Kruskal-Wallis
test, with Tukey post hoc analysis as appropriate. Correlations were performed by the Pearson correlation method on logged data. All statistical calculations were performed on a microcomputer with the use of a commercially available statistical package (Minitab release 12, Minitab Inc). A value of $P < 0.05$ was considered statistically significant.

Results

Lysate Assay Development

Figure 1 shows the mean (full range) of sP-selectin derived from $5 \times 10^7$ platelets from 5 healthy subjects. Concentrations of Triton ranging from 0.1% to 5% provided a plateau of P-selectin. At low Triton levels (eg, 0.01%), we presume that there is insufficient detergent to lyse all the platelets, so that the spun supernatant is effectively free of platelet lysate. At high concentrations of Triton (eg, 10% to 20%), we presume that falling levels of sP-selectin are due to destruction/denaturing of the antibodies in the ELISA (ie, poisoning) by the detergent. Thus, we chose 0.1% as the standard concentration of Triton in all experiments. The dose-response curve (Figure 2), pooled from experiments with platelets from 4 healthy subjects, indicates that $0.5$ to $1.25 \times 10^8$ platelets (ie, in the middle of the curve) provides sufficient P-selectin signal to allow accurate estimations. Therefore, PRP was used at a cell (PRP) concentration of $1 \times 10^8$ cells per milliliter. With the lower limit of sensitivity of 0.8 ng/mL, the minimum number of platelets that can be reliably detected is $5 \times 10^4$ cells per microtiter well, ie, the mass of P-selectin derived from $1 \times 10^6$ cells per milliliter of PRP.

Clinical Study

Clinical characteristics, including patient demography and associated medical conditions and risk factors of the study population, are shown in Table 1. There were no significant differences in age or sex ratio between patients and controls and the various subgroups of AF. The prevalence of the majority of clinical cardiovascular risk factors or diseases did not differ significantly between various subgroups of patients with AF, although patients with AF on warfarin therapy had significantly higher prevalence of ischemic heart disease ($P < 0.01$) and history of stroke/transient ischemic attack ($P = 0.01$) compared with AF patients on no antithrombotic therapy. Patients with AF on dose-adjusted warfarin therapy had fairly good control of their international normalized ratio (mean, 2.6; SD 0.7).

<table>
<thead>
<tr>
<th>TABLE 1. Clinical Characteristics of Patients With AF</th>
</tr>
</thead>
<tbody>
<tr>
<td>All Patients With AF</td>
</tr>
<tr>
<td>No. of patients</td>
</tr>
<tr>
<td>Age, y</td>
</tr>
<tr>
<td>Male/female</td>
</tr>
<tr>
<td>Hypertensive/normotensive</td>
</tr>
<tr>
<td>Diabetics/nondiabetics</td>
</tr>
<tr>
<td>Cardiac failure/no cardiac failure</td>
</tr>
<tr>
<td>Smoker/nonsmoker</td>
</tr>
<tr>
<td>CVA/no CVA</td>
</tr>
<tr>
<td>IHD/no IHD</td>
</tr>
</tbody>
</table>

CVA indicates cerebrovascular accident; IHD, ischemic heart disease.

*One-way ANOVA or $\chi^2$ test, as appropriate.
### Table 2. Hematologic Parameters in Healthy Controls and Patients With Persistent AF on No Antithrombotic Therapy

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Healthy Subjects (n=23)</th>
<th>AF on No Therapy (n=34)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood platelet count, ×10^9/L</td>
<td>270±49</td>
<td>253±67</td>
<td>0.51</td>
</tr>
<tr>
<td>β-Thromboglobulin, IU/mL</td>
<td>61 (46–90)</td>
<td>81 (55–119)</td>
<td>0.09</td>
</tr>
<tr>
<td>sP-selectin, ng/mL</td>
<td>36±9</td>
<td>37±10</td>
<td>0.37</td>
</tr>
<tr>
<td>pP-selectin, ×10^3 ng/platelet</td>
<td>180 (77–1033)</td>
<td>101 (35–275)</td>
<td>0.03</td>
</tr>
</tbody>
</table>

β-Thromboglobulin and pP-selectin are expressed as median (interquartile range); sP-selectin is expressed as mean±SD.

### Cross-Sectional Analyses

In the entire cohort, the amount of P-selectin in the lysate of 10⁶ platelets was in the order of 10 to 1000 ng. This therefore equates to approximately 10 to 1000 fg or 0.01 to 1 pg per platelet. However, there was considerable variation both within and between groups.

pP-selectin was significantly lower among patients with AF on no antithrombotic therapy than among healthy controls (Table 2). AF patients on aspirin had the lowest median pP-selectin levels compared with other AF patients (Table 3). sP-selectin and β-thromboglobulin levels were similar between healthy controls and patients with AF who were not taking antithrombotic therapy (Table 3). Furthermore, sP-selectin and β-thromboglobulin levels were similar in various subgroups of patients with AF.

### Correlations

There was a significant positive correlation between pP-selectin and sP-selectin in healthy controls (Pearson r=0.47, P=0.03) but a significant negative correlation (r=−0.43, P=0.03) in AF patients on no antithrombotic therapy (Figures 3 and 4). There were no significant correlations between sP-selectin and β-thromboglobulin in healthy controls or any of the subgroups of patients with AF.

### Discussion

We have developed a method of platelet lysis to quantify the absolute amount of P-selectin in platelets and applied it to a particular condition, ie, AF, finding that abnormalities in the absolute levels of pP-selectin may be present in these patients. To our knowledge, this is the first report to systematically quantify the mass of P-selectin per platelet both in healthy controls and in patients with AF with the use of the principle of cell lysis. Moreover, the influence of antithrombotic therapy on the pP-selectin levels has not been previously studied.

Study of thrombogenesis in AF has predominantly concentrated on various coagulation markers. Of the platelet markers investigated in the present study, β-thromboglobulin is released from the platelet α-granule and reflects platelet activation. P-selectin is a component of the membrane of the α-granules of platelets and is expressed on the surface during their degradation after platelet activation. sP-selectin in the plasma is thought to arise predominantly from platelets, with a minimal contribution from endothelial cells, suggesting that sP-selectin is likely to reflect platelet activation.

Studies of plasma platelet markers in AF have yielded inconsistent results: some have found no difference in β-thromboglobulin compared with normal subjects, whereas other found increased levels. Similarly, sP-selectin levels were no different in patients with AF compared with normal subjects in some studies but were increased in others. Hence, the role(s) of platelets in AF and their contribution to thromboembolism is unclear. Moreover, the inconsistent benefits of aspirin compared with warfarin in patients with AF at risk of thromboembolic events further support the view that platelet activation may have a lesser role to play than coagulation markers in AF. This view is further supported by the present findings, which show lack of

### Table 3. Hematologic Parameters in Different Subgroups of Patients With AF

<table>
<thead>
<tr>
<th>Parameter</th>
<th>AF on No Therapy (n=34)</th>
<th>AF on Aspirin (n=30)</th>
<th>AF on Warfarin (n=58)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood platelet count, ×10^9/L</td>
<td>253±67</td>
<td>269±67</td>
<td>263±68</td>
<td>0.68</td>
</tr>
<tr>
<td>β-Thromboglobulin, IU/mL</td>
<td>81 (55–119)</td>
<td>86 (61–121)</td>
<td>79 (41–103)</td>
<td>0.36</td>
</tr>
<tr>
<td>sP-selectin, ng/mL</td>
<td>37±10</td>
<td>33±8</td>
<td>33±12</td>
<td>0.21</td>
</tr>
<tr>
<td>pP-selectin, ×10^3 ng/platelet</td>
<td>101 (35–275)</td>
<td>43 (11–141)</td>
<td>225 (74–648)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

β-Thromboglobulin and pP-selectin are expressed as median (interquartile range); sP-selectin is expressed as mean±SD.

*Kruskal-Wallis test or 1-way ANOVA, as appropriate. Tukey post hoc analysis revealed a significant difference in pP-selectin levels between AF patients on aspirin and AF patients on warfarin, the former being significantly lower (P<0.05).
significant platelet activation, as expressed by sP-selectin and β-thromboglobulin, in patients with AF compared with healthy controls. Furthermore, a study of platelet activation in AF found that levels of sP-selectin in patients with persistent AF were no different from those in healthy controls.\(^\text{21}\) In contrast, patients with permanent AF had significantly higher plasma sP-selectin levels. Our study group of AF patients on no antithrombotic therapy comprised patients with relatively newly diagnosed AF referred by general practitioners for consideration of antithrombotic therapy and cardioversion. Thus, this group could perhaps be mainly composed of subjects with persistent AF, which may account for the lack of platelet activation, in agreement with the previous observations.\(^\text{21}\)

We found that pP-selectin demonstrated a wide range, both in healthy controls and in patients with AF. Furthermore, pP-selectin was significantly lower among AF patients on no antithrombotic therapy than among healthy controls. The results from this study suggest that even though P-selectin is expressed on the surface of platelets in AF, the absolute pP-selectin level is low. Furthermore, whether the absolute amount of P-selectin within a platelet reflects platelet activation is unclear. The finding of lower pP-selectin in AF than in healthy controls could potentially be explained in 2 ways. First, activated platelets in AF result in the fusion of α-granules with the platelet surface, followed by degranulation. This results in the expression of P-selectin on the platelet surface and possible release into the plasma. While the amount of P-selectin depleted may be great enough to result in significant decrease in pP-selectin, it may not be high enough to make a significant difference to the larger plasma reserve of sP-selectin. This is further supported by the finding that pP-selectin showed a negative significant correlation with sP-selectin in patients with AF and not in healthy controls. Therefore, after platelet activation, P-selectin may be depleted in the platelets and may increase in the plasma. Second, it is theoretically possible that P-selectin, which is expressed on the surface of the platelet on activation, changes its configuration and is detected by the specific antibody during flow cytometry. Since we used the ELISA that detects sP-selectin to detect pP-selectin, it is feasible that the antibody has detected only granule P-selectin, not recognizing the membrane P-selectin. This concept is also supported by Semenov et al,\(^\text{4}\) who demonstrated that the polyclonal antibody they used in their study to detect sP-selectin in a platelet had lower reactivity toward membrane P-selectin. Furthermore, 90% of the P-selectin in the lysate is accounted for by the membrane P-selectin and 10% by the sP-selectin in the platelet granules membrane.\(^\text{4}\)

Aspirin appears to decrease the amount of pP-selectin in patients with AF in comparison to those on adjusted-dose warfarin. Although previous studies\(^\text{22}\) have suggested a cyclooxygenase-independent mechanism for the production of P-selectin, our results demonstrate otherwise. Whether pP-selectin is a product of pathways involving cyclooxygenase remains to be seen. Importantly, aspirin has previously been shown to have no significant effect on sP-selectin levels.\(^\text{28–30}\)

In conclusion, we have developed a novel assay for the assessment of the amount of P-selectin in a platelet and have applied it to a disease known to have a prothrombotic component (ie, AF). The significance of pP-selectin needs to be studied in greater detail, especially with prospective interventional studies.

**Acknowledgments**

This study was supported by the City Hospital Research and Development program for the Hemostasis, Thrombosis, and Vascular Biology Unit. Dr Kamath is supported by a nonpromotional research fellowship from Sanofi-Winthrop.

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Platelet P-Selectin Levels in Relation to Plasma Soluble P-Selectin and β-Thromboglobulin Levels in Atrial Fibrillation
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Stroke. 2002;33:1237-1242
doi: 10.1161/01.STR.0000013739.82306.7F
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

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