Evaluation of a Platelet Function Analyser (PFA-100®) in patients with a bleeding tendency

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Objective: To investigate pre-analytical variables and the diagnostic performance of the platelet function analyser (PFA-100®), a new device to test primary haemostasis in vitro by simulating platelet adhesion and aggregation under high shear stress.

Methods: Venous whole citrated blood is aspirated through a capillary towards an aperture of a collagen coated membrane containing either adenosine diphosphate (ADP) or epinephrine (EPI). The time needed for occluding this aperture by plug formation is called closure time (CT) and was assessed in 70 healthy subjects and 43 patients with a suspected mild bleeding disorder.

Results: The reference range for the PFA-100® was found to be 82–159 s for EPI-CT and 62.5–120.5 s for ADP-CT. Duplicate analyses revealed a mean coefficient of variations of 7.1% (EPI-CT) and 5.7% (ADP-CT). The EPI- and ADP-CT of blood samples collected in the evening were significantly longer (p = 0.002 and p = 0.004, respectively) than the CT of blood samples collected in the morning. Acetylsalicylic acid (100 mg, 300 mg or 500 mg) administered as a single dose or daily on 10 consecutive days resulted in a prolongation of the EPI-CT, whereas the ADP-CT was not affected. EPI-CT was more sensitive in detecting acetylsalicylic acid (ASA) ingestion than was the bleeding time (BT). Sensitivity and specificity of the PFA-100® to detect von Willebrand disease (vWD) were comparable to the results obtained with the BT.

Conclusion: The PFA-100® represents a simple and easy to use test for investigation of primary haemostasis. Limitations of the system are: special citrated whole blood has to be proceeded within 0.5 to 4 h after sampling, duplicate measurements are necessary, and the results differ between blood samples collected in the morning or in the afternoon. The data indicate that the test is sensitive to ASA intake and vWD. Its use is preferable to BT determination, because it is less invasive and more sensitive to abnormalities of primary haemostasis.

Keywords: PFA-100®, platelet disorders, von Willebrand disease, bleeding time, primary haemostasis

Introduction

The haemostatic process can be divided into three major steps: vasoconstriction, platelet adhesion and aggregation, and finally plasmin coagulation resulting in fibrin formation. Primary haemostasis involves platelet adhesion to subendothelial tissue (e.g. collagen) of the damaged vessel wall [1]. At high shear rates the von Willebrand factor (vWF) acts as an important adhesive protein for both adhesion and aggregation of platelets. vWF function is mediated by two platelet membrane receptors, glycoprotein (GP) Ib and GP IIb/IIIa, in a co-ordinated and synergistic man-
ner. Adhesion of platelets results in their activation and the release of different platelet activating mediators such as adenosine diphosphate (ADP) and thromboxane A2 (TXA2). Released ADP and TXA2 stimulate platelet aggregation leading to the build-up of an enlarging platelet plug – the primary haemostatic plug. Ultimately, the coagulation cascade is activated and a definitive secondary haemostatic plug is generated.

The traditional procedures to diagnose defects in primary haemostasis are bleeding time (BT) determination, measurement of vWF, and investigation of platelet aggregation in platelet rich plasma (PRP). Utility, predictive value for diseases of primary haemostasis, and reproducibility of the BT have been analysed critically [2], and investigation of platelet aggregation is a labour-intensive procedure. Recently, a new test method, the platelet function analyser (PFA-100®) has been introduced. It measures primary haemostasis in vitro by simulating in vivo platelet adhesion and aggregation under high shear stress [3]. Whole, citrated venous blood is aspirated through a capillary towards an aperture of a collagen coated membrane containing either adenosine diphosphate (ADP) or epinephrine (EPI). Time until plug formation is measured in seconds and called closure time (CT).

Two different cartridges containing the agonists epinephrine (EPI cartridge) and ADP (ADP cartridge), are used for the PFA-100® measurement.

The purpose of the present study was to investigate pre-analytical variables and the diagnostic performance of the PFA-100® analyser in an outpatient setting. Consecutive outpatients and patients with a previously diagnosed von Willebrand disease (vWD) or with a mild platelet disorder were studied in addition to 70 healthy subjects. Moreover, the influence of acetylsalicylic acid (ASA) on PFA-100® measurements was investigated among normal subjects.

**Patients and methods**

**Healthy subjects**

Seventy healthy subjects (35 women, 35 men) without a history of a bleeding disorder were included in the study. Their median age was 26 years (range: 21–58 years). They had not been taking ASA, nonsteroidal anti-inflammatory drugs or any other medication (except oral contraception) during the 10 days prior to the first examination and during the study period.

These 70 healthy subjects were divided randomly into 7 groups of 10 individuals each. Forty subjects (groups 1–4) received ASA: the individuals of groups 1 and 2 received a single dose of 100 mg and 500 mg ASA, respectively, once daily on 10 consecutive days. PFA-100® analysis and BT measurement were performed prior to ASA ingestion and 24 h thereafter. The subjects of groups 3 and 4 ingested 100 mg and 300 mg ASA, respectively, once daily on 10 consecutive days. PFA-100® analysis in subjects of groups 3 and 4 was performed on day 1 prior to ASA ingestion, and on days 10, 12, 15 and 20. Individuals of group 4 had an additional PFA-100® measurement on day 2 before intake of the second dose of 300 mg ASA. The blood samples of the subjects of group 5 were analysed with the PFA-100® 0.5, 1 and 4 h after blood collection. The individuals of group 6 had blood collection in the morning (between 8 and 11 am) and a second blood collection in the afternoon (between 3:45 and 5:45 pm) used for PFA-100® measurement. The subjects of group 7 had one single blood collection and PFA-100® measurement. Blood cell count and vWF antigen (vWF:Ag) were assayed in all healthy subjects.

**Patients**

Thirty-one otherwise healthy patients referred to our outpatient clinic for investigation of a suspected mild bleeding disorder were included into the study. They had not been taking ASA, nonsteroidal anti-inflammatory drugs or any other medication (except oral contraception) during the 10 days prior to the first examination and during the study period. Their median age was 26 years (range: 21–58 years).

**Diagnosis in these 12 patients had been previously established based on a personal bleeding history and based on the tests mentioned above. BT and blood cell count were reassessed in these 12 patients, platelet aggregation was reinvestigated in the 4 patients with platelet disorders and PFA-100® analysis was performed in all.

All healthy subjects and patients gave informed consent. The study was approved by the local ethics committee.

**Blood samples**

Blood was drawn by clean venipuncture from an antecubital vein with a 19-gauge butterfly needle. Blood was collected in the morning between 8 and 11 am (in group 6, blood was collected in addition between 3:45 and 5:45 pm) into plastic syringes (Monovette®, Sarstedt, Nümbrecht, Germany). First, an EDTA blood sample (4 ml) was drawn for blood cell count, followed by two 3.8 ml tubes containing 0.38 ml of 0.129 mol/l buffered sodium citrate (pH 5.5) for PFA-100® analysis, and finally by a 10-ml tube containing 1 ml of 0.106 mol/l trisodium citrate for determination of vWF:Ag. The blood samples designed for PFA-100® analysis were stored at room temperature for 1 hour (except for group 5) before measurement.

**PFA-100®**

The PFA-100® (Dade Behring, Düdingen, Switzerland) is composed of a microprocessor-controlled device and single-use test cartridges. The test cartridges consist of a sample reservoir, a capillary and a membrane coated with 2 mg equine type I collagen and either 10 mg epi-
nephrine bitartrate (EPI cartridge) or 50 mg adenosine 5'-diphosphate (ADP cartridge). Blood is pipetted into the reservoir and aspirated through a capillary with a diameter of 200 µm with constant negative pressure resulting in high shear forces (5000–6000 s⁻¹). The capillary ends in a membrane aperture with a diameter of 150 µm. Platelets adhere at the aperture where they are activated by the collagen and then aggregate. The two agonists epinephrine and ADP enhance aggregation. Finally, a platelet plug occludes the aperture and blood flow stops. The time measured in seconds from the beginning of the test until formation of an occluding platelet plug is called closure time (CT). If an occluding platelet plug does not form after 300 s, the analysis is stopped. All subjects were tested with both reagents (EPI and ADP) and double measurements were performed each time.

Other assays
Whole blood count was performed on a STKS-Beckmann Coulter. vWF:RCof and vWF:Ag levels were measured as described [5]. BT was measured using the retractable Simplate® II R-device (Organon Teknika, Durham, N.C.). Platelet aggregation in PRP upon stimulation with ADP, collagen, arachidonate and ristocetin was tested under standardised conditions as previously described [4].

**Results**

**General aspects**
We first analysed the blood samples of the 70 healthy subjects. The normal range for the PFA-100® CT was established at 82–159 s for EPI and 62.5–120.5 s for ADP. Duplicate analysis revealed a coefficient of variation (mean ±SD) of 7.1% ± 7.7 (EPI-CT) and 5.7% ± 7.3 (ADP-CT). However, 16% (EPI-CT) and 9% (ADP-CT) of the duplicates showed a difference of more than 15% of their means. No differences were found between the EPI-CT or the ADP-CT, measured 0.5 h, 1 h and 4 h after blood sampling (data not shown), showing the blood samples to be stable at room temperature between 0.5 and 4 h. The EPI- and ADP-CT of the blood samples collected in the evening (125 s [83.5–221.0 s] and 84.8 s [58.0–116.5 s]) were significantly longer (p = 0.002 and p = 0.004) than the CT of the blood samples collected in the morning (105 s [80.0–184.5 s] and 73.3 s [59.5–101.5 s]).

No difference in EPI-CT (p = 0.75) or ADP-CT (p = 0.7) was observed between men and women, and no correlation was found between age and EPI-CT (rs = –0.12) or ADP-CT (rs = –0.10). Correlation of CT with vWF:Ag, measured in a subset of 50 normal subjects was r = –0.38, p <0.0001 for EPI-CT and r = –0.66, p <0.0001 for ADP-CT.

**Influence of ASA on PFA-100® CT in healthy volunteers**
None of the healthy subjects had prolonged EPI-CT or ADP-CT before ASA intake. Neither a single dose of ASA nor ASA intake for 10 consecutive days had any effect on ADP-CT.

**Influence of a single dose of ASA on EPI-CT**
EPI-CT was significantly prolonged 24 hours after a single ASA dose of 100 mg (p = 0.004), of 300 mg (p = 0.002), and of 500 mg (p = 0.002). Prolongation of EPI-CT was more pronounced among the individuals taking 300 mg or 500 mg ASA than among those taking 100 mg: 10/10 subjects (300 mg ASA) and 9/10 subjects (500 mg ASA), had EPI-CT above the normal range as compared to 3/10 subjects in the group with 100 mg ASA (figure 1).

**Influence of daily ASA ingestion during 10 consecutive days on EPI-CT**
One hundred mg ASA or 300 mg ASA daily for 10 days prolonged EPI-CT significantly in both groups (p = 0.002). Mean EPI-CT at day 10 were 218 s and 289 s with 100 mg ASA and 300 mg ASA, respectively. Two days after the last ASA intake (on day 12), EPI-CT was still prolonged, whereas on days 15 and 20, it was again within the normal range (figure 2).
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**Influence of daily ASA ingestion during 10 consecutive days on EPI-CT.** The EPI-CT was measured in 10 healthy subjects each before ASA intake (day 1) and after 10 days (day 10) of ingestion of 100 mg (squares) or 300 mg (circles) of ASA and thereafter on days 12, 15 and 20. Squares and circles denote mean values, vertical bars denote SEM.

**Table 1**
Number of individuals with prolonged EPI-CT, ADP-CT and bleeding time (BT) among patients with von Willebrand disease (vWD), with a mild platelet function disorder (PD) or among patients with no abnormal laboratory findings (normal).

<table>
<thead>
<tr>
<th>Type</th>
<th>Total number</th>
<th>EPI-CT</th>
<th>ADP-CT</th>
<th>BT</th>
</tr>
</thead>
<tbody>
<tr>
<td>vWD Type 3</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>vWD Type 1</td>
<td>7</td>
<td>5</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>PD</td>
<td>17</td>
<td>2</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>normal</td>
<td>17</td>
<td>2</td>
<td>0</td>
<td>6</td>
</tr>
</tbody>
</table>

**Table 2**
Negative predictive value (NPV) and positive predictive value (PPV) of the PFA-100® and the bleeding time (BT) to detect von Willebrand disease or mild platelet disorder.

<table>
<thead>
<tr>
<th></th>
<th>NPV</th>
<th>PPV</th>
</tr>
</thead>
<tbody>
<tr>
<td>EPI-CT</td>
<td>58% (15/26)</td>
<td>60% (3/5)</td>
</tr>
<tr>
<td>ADP-CT</td>
<td>57% (17/30)</td>
<td>100% (1/1)</td>
</tr>
<tr>
<td>BT</td>
<td>61% (11/18)</td>
<td>54% (7/13)</td>
</tr>
</tbody>
</table>

NPV and PPV were calculated on the basis of 31 consecutive outpatients with a bleeding tendency.

**Influence of ASA on BT**

Twenty tested healthy individuals had a normal BT before ingesting a single dose of ASA (310 s [165–435 s]). Twenty-four hours after 100 mg ASA intake, 2/10 individuals had prolonged BT (560 s and 900 s) and 3/10 subjects had prolonged EPI-CT, none showing both measurements prolonged. Among the 10 individuals taking 500 mg ASA, 5 showed a pathological BT (530 s [500–900 s]) whereas 9 had a prolonged of EPI-CT 24 hours later.

**Patients**

Of the 31 patients referred to our outpatient clinic for investigation of a suspected mild bleeding disorder, one patient was found to suffer from vWD type 1 and in 13 patients a mild platelet function disorder was diagnosed based on platelet aggregation studies in PRP. In the remaining 17 patients, six had a prolonged BT; however, no other laboratory abnormality was found explaining the mild bleeding tendency. These 31 patients were included in the analysis of the NPV and the PPV of the PFA-100®. In addition, 8 patients with previously diagnosed vWD (2 patients with vWD type 3 and 6 patients with vWD type 1) and 4 patients with a known mild platelet disorder were reinvestigated and included together with the 31 patients into the PFA-100® sensitivity/specificity analysis.

The EPI-CT was prolonged in 5/31 newly investigated patients (in the patient with vWD type 1, in 2 patients with a mild platelet disorder and in 2 patients without any pathological findings) and also in 6 of the 8 patients with previously diagnosed vWD, but in none of the 4 patients with a known mild platelet disorder. The ADP-CT was prolonged in the vWD patient of the 31 newly investigated patients and in 4/8 previously diagnosed vWD patients, but in 0/4 patients with a previously diagnosed mild platelet disorder. These results are summarised together with the results of the BT in table 1.

**NPV and PPV of the PFA-100®**

The NPV and PPV to detect any abnormality (vWD or mild platelet disorder) were calculated on the basis of 31 newly investigated consecutive outpatients and are given in table 2. Since only one patient had vWD, we did not calculate the NPV/PPV for vWD or platelet disorder separately.

**Sensitivity and specificity of the PFA-100®**

Sensitivity and specificity to detect vWD or a mild platelet disorder was calculated on the basis of all 43 patients. The results are given in table 3.

**Table 3**
Sensitivity and specificity of the PFA-100® and the bleeding time (BT) to detect von Willebrand disease (vWD) or mild platelet function disorder (PD).

<table>
<thead>
<tr>
<th></th>
<th>vWD</th>
<th>PD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>sensitivity</td>
<td>specificity</td>
</tr>
<tr>
<td>EPI-CT</td>
<td>78% (7/9)</td>
<td>88% (30/34)</td>
</tr>
<tr>
<td>ADP-CT</td>
<td>56% (5/9)</td>
<td>100% (34/34)</td>
</tr>
<tr>
<td>BT</td>
<td>75% (6/8*)</td>
<td>65% (22/34)</td>
</tr>
</tbody>
</table>

Sensitivity and specificity were calculated on the basis of 31 consecutive outpatients with a bleeding tendency and on the 12 patients who were reinvestigated. * BT was not interpretable in two patients.
Discussion

The in vivo bleeding time (BT) test is known to be an imprecise, impractical and invasive method [2, 4]. The PFA-100® test, by contrast, provides an excellent means of obtaining similar information in a reliable and reproducible manner [6]. In the present study, we have investigated pre-analytical variables as well as the diagnostic performance of the PFA-100® analyser in an out-patient setting.

The PFA-100® test results have been found to depend on the pH and on the sodium citrate concentration of the anticoagulant [7]. We, therefore, used for all PFA-100® measurements the same buffered sodium citrate anticoagulant. First, we established a normal range based on the results obtained in 70 healthy subjects. The observed values for EPI-CT (82–159 s) and for ADP-CT (62.5–120.5 s) are comparable to the reference ranges found in other studies and to those provided by the manufacturer [3, 8]. No correlations were found between age or gender and EPI-CT or ADP-CT. Duplicate analysis revealed an acceptable mean coefficient of variation. However, since a respectable number of duplicate analyses showed differences of more than 15%, we decided to perform duplicate analyses for routine use of the PFA-100®. In agreement with other investigators, we found the CT results to be stable within the time frame of 0.5 to 4 h after blood sampling [6, 7]. Nevertheless, since we found the CTs to be significantly shorter in the morning than in the evening, this should be taken into account when interpreting PFA-100® test results.

ASA had no effect on ADP-CT, whereas the EPI-CT was significantly prolonged, as shown by others [6, 9–12]. Because the ASA-induced cyclooxygenase inhibition is only detected by the EPI cartridge, the PFA-100® test provides a means of discriminating between this acquired defect and other inherited platelet disorders. We found a clear dose- and time-dependent effect of ASA on EPI-CT with more pronounced prolongation among individuals taking 300 or 500 mg of ASA as compared to those taking 100 mg, or among those taking ASA for 10 days as compared to those taking only a single dose of the drug. Five days after having stopped ASA intake, the EPI-CTs were again in the normal range. These findings on the influence of ASA on PFA-100® test results are in agreement with earlier studies [10, 13–15]. The BT was less sensitive than the EPI-CT, being prolonged only in 50% of the individuals 24 hours after taking 500 mg ASA, whereas EPI-CT was prolonged in 90%. Our study indicates a lower sensitivity of the BT as compared to the EPI-CT towards an ASA-induced platelet disorder and shows that the PFA-100® system is a less invasive alternative to the BT in the diagnosis of this acquired platelet aggregation defect.

The PFA-100® has been extensively investigated in well-defined patient populations. One of the purposes of the present study was to investigate the test system in patients sent to our department for work-up of a possible mild bleeding diathesis. The EPI-CT and/or ADP-CT were prolonged in 11 out of the 43 investigated patients, whereas the BT was prolonged in 18/43 patients. The PFA-100® test revealed an abnormal result in only 2 out of the 17 patients with normal vWF and normal platelet aggregation, whereas the BT was falsely positive in 6/17 patients. Seven of the 9 vWD patients were detected with the PFA-100®, whereas the BT was prolonged in 6 patients. Our results show a good performance of the PFA-100® for the diagnosis of patients with vWD and basically agree with those published by others [16–18], although we found a slightly lower sensitivity.

Among the 17 patients found to have a mild platelet function disorder, only two had a prolonged EPI-CT, whereas 6 had prolonged BT. This would mean that neither test is a suitable screening test for this abnormality, however, BT is somewhat more sensitive than the PFA-100® test. The main problem for the interpretation of this result is that we do not know the clinical importance of the laboratory abnormality denoted “mild platelet function disorder” based on abnormal aggregation results. It is known that conventional BT is not predictive of surgical blood loss in random patients undergoing surgical intervention. It is not known, however, whether aggregation tests would predict surgical bleeding or whether they detect even minor abnormalities of platelet aggregation not clinically relevant for in vivo haemostasis. Only large prospective studies comparing platelet aggregation and PFA-100® test results in subjects undergoing defined haemostatic challenge, e.g. by surgery, will ultimately answer this question.

Other investigators have shown that the PFA-100® is influenced by the haematocrit and the platelet count, but not by oral anticoagulation or heparin [10, 13–15], nor by coagulation disorders due to deficiencies of the clotting factors VIII, IX, or XI [17]. Moreover, the test appears not to depend strongly on plasma fibrinogen, since a patient with congenital afibrinogenemia only exhibited slightly prolonged CTs [6]. In contrast, the PFA-100® test reveals pathological results in various platelet disorders, such as Glanzmann’s thrombasthenia, the Bernard Soulier syndrome, storage pool disease, or the Hermansky Pudlak syndrome [6]. Moreover, evaluation of the performance of the PFA-100® test is under investigation in a variety of other clinical settings such as therapeutic monitoring of patients with vWD receiving desmopressin or assessment of GP IIb/IIIa blockade during percutaneous coronary intervention [19–21].

In conclusion, the PFA-100® device represents an interesting test for primary haemostasis. The
system is simple and easy to use. However, the user should know the limitations of the device, such as: defined, buffered citrated whole blood can be used within 0.5 to 4 h after sampling, low haematocrit (<30%) and low platelet count (<100 G/l) may result in prolongation of the CTs, duplicate measurements are necessary, the results differ between blood sampled in the morning or in the afternoon. Our study shows that the system is sensitive to ASA intake and vWD. It is preferable to the BT, because it is less invasive and more sensitive to abnormalities of primary haemostasis.

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References


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