bjh research paper

Variables influencing Platelet Function Analyzer-100^{TM} closure times in healthy individuals

Hannelore Haubelt, Christof Anders, Anette Vogt, Petra Hoerdt, Ulrich Theo Seyfert and Peter Hellstern

Institute of Hemostaseology and Transfusion Medicine, Academic City Hospital, Ludwigshafen, Germany

Summary

We investigated the relationship between platelet function analyzer (PFA-100^{TM}) closure times (CT) and bleeding time (BT), platelet aggregation (PA) induced by ADP, arachidonic acid, and collagen, blood cell counts, and von Willebrand factor (VWF) in 120 well-characterised healthy individuals. Pre-analytical and analytical conditions were standardised comprehensively. In a substantial number of cases the differences between duplicate measurements exceeded 15%. The reference range (5th and 95th percentiles) for CT with the collagen/epinephrine (CEPI) and the collagen/ADP (CADP) cartridge was 93–223 s and 64–117 s respectively. Re-examination of 11 individuals with CEPI-CT above the 95th percentile revealed considerable batch-to-batch variation of CEPI-CT. Males had significantly longer CADP than females ($P = 0{\cdot}002$). CEPI and CADP-CT measured PM were significantly longer than corresponding values determined AM ($P = 0{\cdot}003$ and $P < 0{\cdot}0001$ respectively). Blood group O was associated with greater CEPI and CADP-CT and lower VWF levels compared with non-O blood groups ($P = 0{\cdot}008$, $P = 0{\cdot}0003$ and $P < 0{\cdot}0001$ respectively). Linear regression analysis revealed association between CEPI-CT, CADP-CT and VWF ($P < 0{\cdot}0001$), but no relationship was found between CT and BT or between CT and PA. We conclude that VWF plasma levels modulate PFA-100^{TM} CT to a greater extent than platelet function. Establishment of reliable reference ranges and careful standardisation of pre-analytical and analytical conditions is a prerequisite for obtaining reliable PFA-100^{TM} results. Duplicate measurements are necessary.

Keywords: platelet function analyzer 100, reference ranges, bleeding time, platelet function tests, von Willebrand factor.

Received 21 April 2005; accepted for publication 20 June 2005

Correspondence: Peter Hellstern, MD, Institute of Hemostaseology and Transfusion Medicine, Academic City Hospital, D-67063 Ludwigshafen, Germany.
E-mail: peterhellstern@medicusnet.de

The platelet function analyzer, PFA-100^{TM}, manufactured by Dade-Behring, Liederbach, Germany, was developed to assess primary haemostasis *in vitro* using citrated whole blood (Kundu *et al*, 1995; Mammen *et al*, 1995; Harrison, 2004). The device simulates *in vivo* haemostatic plug formation under high shear flow by measuring the time required to occlude (closure time, CT) a collagen/epinephrine (CEPI) or collagen/ADP (CADP)-coated aperture inserted in a plastic membrane. Most clinical studies regard the PFA-100^{TM} as a simple and easy-to-use tool to detect platelet dysfunction and von Willebrand disease (VWD), and for monitoring treatment with desmopressin, von Willebrand factor (VWF) concentrates and antiplatelet agents (Mammen *et al*, 1998; Jilma, 2001; Favarolo, 2002; McKee *et al*, 2002). Conflicting findings from validation studies and in patients with primary haemostasis disorders have, however, raised concerns about the diagnostic performance of the device and its suitability for non-specialised laboratories. The CT depends strongly on VWF plasma levels (Favarolo, 2002), anticoagulant pH value and citrate concentration (Heilmann *et al*, 1997; Jilma, 2001; Favarolo, 2002), and on the time of day when blood is collected for analysis (Dalby *et al*, 2000; Wuillemin *et al*, 2002). Low platelet and leucocyte counts and low haematocrits may lead to prolonged CT (Kundu *et al*, 1996; Jilma, 2001; Favarolo,

  doi:10.1111/j.1365-2141.2005.05680.x

2002). Some studies performing duplicate analysis showed coefficients of variation above 10% (Heilmann *et al*, 1997; Mammen *et al*, 1998) or differences between duplicates exceeding 10 or even 15% in a substantial number of cases (Wuillemin *et al*, 2002). It would seem therefore that earlier statements that duplicate analysis is unnecessary in clinical routine have been premature (Mammen *et al*, 1998; Böck *et al*, 1999; Jilma, 2001). The vast majority of clinical studies dealing with the clinical significance of the PFA-100™ system derived their reference ranges from groups of fewer than 40 healthy individuals, and these were often only poorly characterised. This resulted in substantially differing references ranges between study groups (Jilma, 2001). As the upper limits of respective reference ranges are the cutoff levels required to calculate sensitivity, specificity and predictive values in clinical studies, it is not surprising that many (Fressinaud *et al*, 1998; Cattaneo *et al*, 1999; Dean *et al*, 2000; Schlammadinger *et al*, 2000; Cariappa *et al*, 2003; Posan *et al*, 2003) but not all (Wuillemin *et al*, 2002; Quiroga *et al*, 2004) trials found the PFA-100™ system to be sufficiently sensitive and superior to the bleeding time (BT) in detecting VWD.

We re-evaluated PFA-100™ testing and investigated those factors influencing CEPI and CADP-CT in a larger number of well-characterised healthy individuals under comprehensively standardised conditions. As there is a lack of data on the relationship between PFA-100™ CT and more specific platelet function tests, we also measured platelet aggregation (PA) induced by ADP, arachidonic acid (AA) and collagen.

Materials and methods

Selection of healthy subjects and study design

We included 120 apparently healthy individuals (60 males and 60 females aged between 19 and 63 years). Sixty-three individuals were voluntary blood donors from the local blood donation centre. The remaining 57 volunteers were students or members of hospital staff. Individuals were excluded when they had taken aspirin, anti-inflammatory drugs, antibiotics or other drugs with potential to influence platelet function within 10 days prior to study, or had donated blood or blood components within 10 days prior to blood collection. Allowed drugs were oral contraceptives (OC) and thyroid gland hormones. Individuals reporting a history of bleeding on a standardised questionnaire were also excluded. The following data were registered: age, gender, body weight, height, ABO blood group, blood donors or non-donors, smoking habits, all drugs including OC, intake of garlic within 24 h prior to blood collection and time of blood collection.

Blood collection and processing

After a 30-min rest in a sitting position, blood samples were drawn from the antecubital vein using 21-gauge butterfly needles between 8:30 and 11:00 AM. A second blood collection was carried out in half of the individuals (30 males and 30 females) on the same day between 2:00 and 5:00 PM. A small lunch and soft drinks were allowed between the AM and PM blood collection. Platelet activation was minimised by using a light tourniquet, short and moderate phlebostasis, smooth blood flow, plastic tubes (Sarstedt, Nuembrecht, Germany) and immediate gentle mixing with anticoagulant. The first 3 ml were collected into plastic tubes containing potassium EDTA and used for blood cell counts. Blood for PA testing and for determining fibrinogen and VWF collagen-binding assay (VWF:CBA) was drawn into S-Monovettes™ from Sarstedt, containing 0·106 mol/l unbuffered trisodium citrate (9 parts of blood to 1 part of 0·106 mol/l citrate), followed by a 3·8-ml S-Monovette™ containing 0·38 ml of 0·129 mol/l buffered sodium citrate for PFA-100™ analysis.

Platelet rich plasma (PRP) was obtained by 10-min centrifugation at $164 \times g$. Platelet poor plasma (PPP) was prepared by centrifugation at $3500 \times g$ for 10 min. The platelet count of PRP was adjusted to 250×10^9/l by adding PPP to PRP. Aliquots of 0·5 ml of PPP were snap-frozen and stored at −70°C. When required for further analysis (VWF:CBA), samples were thawed at 37°C for 10 min.

Assays

Except for the determination of VWF:CBA, blood processing, BT and all assays were performed by one experienced medical technician.

BT was carried out by the Ivy technique using a Surgicutt™ device from International Technidyne (Edison, NJ, USA). CEPI and CADP-CT were determined in duplicate (channels 1 and 2) between 30 min and 1 h after blood collection, using one single lot each of CEPI and CADP cartridges and a PFA-100™ instrument from Dade-Behring (Liederbach, Germany). Another lot of CEPI and CADP cartridges was used to re-examine those individuals with CEPI-CT above the 95th percentile. Device and cartridges were handled in accordance with manufacturer' s instructions. Measurements were repeated on the same channel of the PFA-100™ instrument if CT exceeded 300 s. The shorter CT was used for further analysis. For CT values greater than 300 s, a value of 300 s was arbitrarily assigned for further statistical analysis.

The PA tests were run on an APACT-4 aggregometer from Labitec (Arensburg, Germany), between 1 and 2 h after blood collection. PRP samples were stimulated by adding 25 µl of ADP or AA to 225 µl of PRP to obtain final concentrations of 5 µmol/l of ADP and 500 µg/ml of AA. Five microlitre of collagen fibrils from equine tendon were added to 245 µl of PRP to achieve a final concentration of 1 µg/ml. ADP and AA were purchased from Progen (Heidelberg, Germany) and collagen from Nycomed (Linz, Austria). Aggregations were recorded for 7 min. The difference in light transmission on a linear scale between PRP and PPP was set to 100% to calculate the maximum changes in light transmission due to PA. Any observed disaggregation was also recorded.

Platelet and leucocyte counts, mean platelet volume (MPV) and Hb were determined on an ADVIA analyzer from Bayer Vital (Fernwald, Germany). We measured VWF:CBA in duplicate, using an enzyme immunoassay from Progen.

Statistical analysis

The D'Agostino-Pearson omnibus test for normality was used to analyse the values. As not all values were normally distributed, data are given as medians, 5th and 95th percentiles or as medians and ranges (minimum–maximum). The 97·5th percentiles were also calculated from the 120 CEPI-CT and CADP-CT values determined AM. Percentages of differences between duplicate measurements of all CEPI and CADP-CT ($n = 180$ each) were calculated as follows: [(CT1−CT2)/average] × 100. The mean ± SD and the numbers of differences >10% and 15% were used to characterise imprecision. To check whether variability was related to the magnitude of measurement, percentages of differences between duplicate measurements were plotted against the average of both measurements (Blant & Altman, 1986).

Paired and unpaired data were compared using the Wilcoxon matched pairs test and the Mann–Whitney *U*-test respectively. Differences in the proportions of smokers, individuals with blood group O, blood donors and garlic consumers were checked by Fisher's exact test. Any two-tailed probability below 0·05 was considered significant.

Spearman rank correlation coefficients were calculated using the data from all individuals when examined at AM ($n = 120$) and checked by subsequent multiple linear regression analysis. Log-transformed data were used if log-transformation resulted in a better adjustment to normal distribution according to the D'Agostino-Pearson omnibus test. A correlation matrix was constructed to discover any multicolinearity between parameters. Two-tailed $P < 0·01$ were regarded as statistically significant.

Analyses were conducted using InStat for Windows software, version 3.0, and GraphPad software, version 4.02 (GraphPad Software Inc, San Diego, CA, USA). Blant–Altman plots without regression line were created using GraphPad software, version 4.02 (GraphPad Software Inc.).

Results

Baseline data for all individuals, men and women are shown in Table I. Females and males did not differ in age or in the proportion of smokers, whole blood donors, blood group O and garlic consumption. As expected, females had significantly lower body mass indices than males ($P = 0·0005$). Age was significantly associated with body mass index ($r = 0·39$, $P < 0·0001$; data not shown). The statement of all included individuals that they had taken neither aspirin nor any other drug potentially influencing platelet function for 10 days prior to blood collection was confirmed by AAPA, which was within the normal range (AM values) and showed no disaggregation in all individuals (data not shown).

Duplicate analysis of PFA-100™ CT showed a mean difference of 0·05 ± 10·2% for the CEPI cartridge and of 1·8 ± 9·5% for the CADP cartridge. However, 76 of 180 CEPI-CT differences exceeded 10% and 21 of 180 exceeded 15%. The respective data for CADP-CT were 48 of 180 greater than 10% and 19 of 180 greater than 15%. Blant–Altman plots revealed similar imprecision of duplicate measurements over the full measuring range (Fig 1). CEPI-CT determined on channels 1 and 2 of the PFA-100™ equipment were not significantly different ($P = 0·818$), whereas CADP-CT measured on channel 1 were slightly greater than respective values obtained on channel 2 (medians, 86 s vs. 85 s; $P = 0·002$).

CEPI-CT, CADP-CT, BT, PA induced by ADP, AA and collagen, platelet and leucocyte count, MPV, Hb, fibrinogen and VWF:CBA determined in the 120 individuals are shown in Table II. The reference range (5th and 95th percentiles) of CEPI-CT and CADP-CT determined AM was 93–223 s and 65–117 s respectively. The 97·5th percentiles for CEPI-CT and CADP-CT were 254 s and 129 s respectively. Ten (8·3%) of the 120 subjects had CEPI-CT greater than 200 s. The

Table I. Baseline data of 120 healthy individuals.

	All	Females	Males	*P*-value (F *versus* M)
Age (years)	38·5 (19–63)	38·5 (19–63)	38·5 (19–60)	0·981
Female/male ratio	60/60			
Body mass index (kg/m^2)	25 (17–35)	26 (18–35)	23 (17–35)	**0·0005**
Smokers/non-smokers	24/96	10/50	14/46	0·494
Donors/non-donors	63/57	29/31	34/26	0·464
Blood group O/non-O	52/68	21/39	32/28	0·069
OC users/non-users		25/35		
Garlic consumers/ non-consumers*	28/92	14/46	14/46	1·0

Data are expressed as median value (minimum–maximum).
F, females; M, males.
*Within 24 h prior to blood collection.

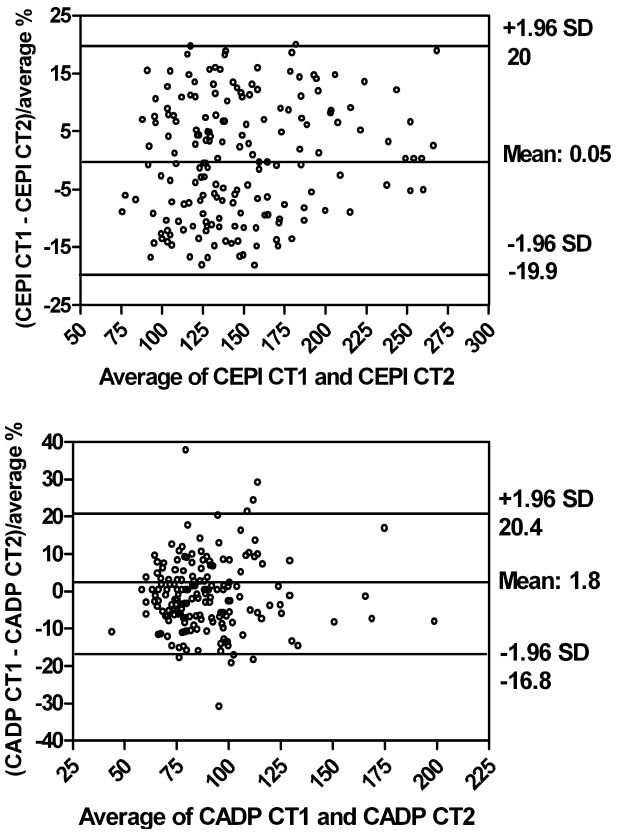

Fig 1. Bland–Altman plots of duplicate measurements of collagen/epinephrine (CEPI) and collagen/ADP (CADP) platelet function analyser (PFA-100™) closure times (CT) in 120 individuals determined AM ($n = 120$) and PM ($n = 60$).

results obtained in 11 individuals with CEPI-CT exceeding the 95th percentile, using two different lots of CEPI and CADP cartridges, respectively, are shown in Table III. CEPI-CT measured with the second lot (579568) were significantly shorter ($P = 0{\cdot}018$), but CADP-CT, BT, PA and VWF:CBA were not significantly different between the two measurements.

Males had significantly longer CADP-CT than females ($P = 0{\cdot}002$). CEPI and CADP-CT measured PM were significantly longer than corresponding values determined AM ($P = 0{\cdot}003$ and $<0{\cdot}0001$ respectively). BT, MPV and leucocyte counts also increased significantly from AM to PM, and PA induced by ADP, AA or collagen dropped significantly during the day. Blood group O was significantly associated with greater CEPI-CT and CADP-CT and with lower VWF:CBA levels compared with non-O blood groups ($P = 0{\cdot}008$, $P = 0{\cdot}0003$ and $P < 0{\cdot}0001$ respectively). No significant differences between blood group O and non-O individuals or between males and females were found with respect to BT and PA. We noted no significant differences in any assay results between whole blood donors and non-donors (data not shown). OC users had significantly stronger collagen PA values than OC non-users ($P = 0{\cdot}027$) and garlic consumption resulted in a significant impairment of ADPPA ($P = 0{\cdot}014$; data not shown).

Simple linear regression analysis revealed a significant association between CEPI-CT and CADP-CT ($r = 0{\cdot}61$; $P < 0{\cdot}0001$), between CEPI-CT and VWF:CBA ($r = -0{\cdot}44$; $P < 0{\cdot}0001$) and between CADP-CT and VWF:CBA ($r = -0{\cdot}47$; $P < 0{\cdot}0001$; Table IV). In contrast, CEPI-CT and CADP-CT did not correlate significantly with BT or PA. The BT correlated significantly with ADPPA and collagen PA, and PA induced by ADP, AA and collagen correlated significantly with each other. Platelet and leucocyte counts, MPV and fibrinogen did not influence CT significantly. Results from simple linear regression analysis were predominantly confirmed by subsequent multiple linear regression (Table III). Results from linear regression analysis were also confirmed by comparing individuals having CEPI-CT or CADP-CT above the respective 90th percentiles ($n = 12$ each) with the remaining 108 healthy subjects. Individuals with CEPI-CT above the 90th percentile had significantly longer CADP-CT (medians, 101 s vs. 85 s, $P = 0{\cdot}001$) and lower VWF:CBA levels (medians, 83·5 U/dl vs. 114·5 U/dl; $P = 0{\cdot}012$) than the remaining persons. When individuals with CADP-CT above the 90th percentiles were compared with the remaining 108 subjects, significant differences in CEPI-CT (medians, 175 s vs. 135 s; $P = 0{\cdot}008$) and VWF:CBA levels (medians, 69 U/dl vs. 117 U/dl; $P < 0{\cdot}0001$) were found. In contrast, BT and PA values in subjects with CEPI-CT or CADP-CT above the respective 90th percentiles did not differ significantly from respective values found in individuals with CT below the 90th percentiles. No significant differences could be found between the 14 individuals with BT above the 95th percentile and/or any PA below the respective 5th percentile and the other 106 subjects (CEPI-CT medians, 140 s vs. 138 s, $P = 0{\cdot}673$; CADP-CT medians, 88 s vs. 82 s, $P = 0{\cdot}603$). The 95th percentiles of CEPI and CADP-CT in the 106 individuals with BT below the 95th percentile and/or PA values above the respective 5th percentile were 218 and 124 s, respectively, which was very similar to the 223 s (CEPI-CT) and 117 s (CADP-CT) observed in the total cohort of 120 individuals (Table II).

Discussion

Most previous studies have suggested that the PFA-100™ system is a reliable and useful screening tool for assessing primary haemostasis (Kundu *et al*, 1995; Mammen *et al*, 1995, 1998; Jilma, 2001; Favarolo, 2002; McKee *et al*, 2002; Harrison, 2004). This however contrasts with reports on the device's limitations including poor reproducibility (Wuillemin *et al*, 2002), low specificity (Harrison *et al*, 2002) and low sensitivity to detect type 1 VWD (Favarolo, 2002; Quiroga *et al*, 2004) or mild platelet disorders (Favarolo, 2002; Wuillemin *et al*, 2002; Posan *et al*, 2003; Quiroga *et al*, 2004). Furthermore, the diagnostic performance of PFA-100™ testing may be affected by many factors that influence CEPI and CADP-CT, among

Table II. Collagen/epinephrine (CEPI) and collagen/ADP (CADP) platelet function analyzer (PFA-1700™) closure times (CT), bleeding time, platelet aggregation (PA) induced by ADP (ADPPA), arachidonic acid (AAPA) and collagen (collagen PA), blood cell counts, mean platelet volume (MPV), Hb, fibrinogen and von Willebrand factor collagen binding assay (VWF:CBA) in 120 healthy subjects.

Parameter	All (AM) ($n = 120$)	Males (AM) ($n = 60$)	Females (AM) ($n = 60$)	BGO (AM) ($n = 52$)	BG non-O (AM) ($n = 68$)	AM ($n = 60$)	PM ($n = 60$)	*P*-value M *versus* F*	*P*-value *BGO *versus* non-O	*P*-value AM *versus* PM†
PFA-100 CEPI-CT (s)	138 (93–223)	140 (94–253)	131 (96–216)	150 (99–243)	134 (87–242)	133 (97–196)	140 (105–245)	0·091	**0·008**	**0·003**
PFA-100 CADP-CT (s)	83 (65–117)	88 (67–126)	78 (64–113)	90 (67–127)	79 (61–108)	80 (66–117)	90 (74–167)	**0·002**	**0·0003**	**<0·0001**
Bleeding time (min)	4 (2·5–6)	4 (2·5–6)	4 (2·5–6)	4 (2–6)	4 (2·5–6)	4 (2·5–6)	5 (2·5–8·5)	0·744	0·902	**<0·0001**
ADPPA (% light transmission)	70 (46–112)	70 (40–112)	72 (46–112)	75 (52–114)	68 (39–107)	72 (41–114)	61 (30–105)	0·844	0·335	**0·003**
AAPA (% light transmission)	64 (37–96)	65 (41–95)	73 (33–110)	68 (44–106)	71 (34–100)	71 (31–110)	60 (14–101)	0·097	0·797	**0·008**
Collagen PA (% light transmission)	64 (33–94)	62 (32–93)	67 (33–99)	62 (43–95)	66 (31–96)	68 (33–99)	59 (10–102)	0·152	0·262	**0·009**
Platelet count/nl	271 (197–354)	253 (201–340)	279 (201–360)	269 (189–352)	271 (197–362)	254 (205–352)	258 (199–337)	0·064	0·405	0·762
MPV (fl)	8·7 (7·6–10·8)	8·5 (7·5–11·1)	8·8 (7·6–10·3)	8·5 (7·4–10·8)	8·8 (7·6–10·9)	8·7 (7·5–10·8)	9·8 (7·6–12)	0·098	0·340	**0·0004**
Leucocyte count ($\times 10^9$/l)	6·1 (4·2–9·9)	6·1 (4·1–10·6)	6·1 (4·2–8·8)	6·0 (4·1–11·3)	6·1 (4·3–9·1)	5·9 (4·1–9·4)	6·2 (4·7–10)	0·899	0·987	**<0·0001**
Hb (g/dl)	13·6 (11·6–15·7)	14·2 (12·9–15·8)	13·0 (11·4–14·5)	13·8 (11·7–15·7)	13·5 (11·5–15·7)	13·4 (11·6–16·7)	13·1 (11·1–15·2)	**<0·0001**	0·363	0·199
Fibrinogen (g/l)	2·9 (2·1–3·9)	2·8 (2·1–3·7)	3·0 (2·1–4)	2·9 (2·1–3·9)	2·9 (2·2–3·9)	2·7 (2·1–3·7)	2·7 (2·1–3·6)	0·143	0·626	0·067
VWF:CBA (U/dl)	110 (58–191)	107 (52–191)	113 (68–226)	90 (50–167)	124 (75–233)	106 (54–163)	106 (58–173)	0·312	**<0·0001**	0·609

BG, blood group; M, males; F, females.
Medians (5th and 95th percentiles).
*Mann–Whitney test.
†Wilcoxon matched pairs test.

Table III. Collagen/epinephrine (CEPI) and collagen/ADP (CADP) platelet function analyzer (PFA-100™) closure times (CT), bleeding time, platelet aggregation (PA) induced by ADP (ADPPA), arachidonic acid (AAPA) and collagen (collagen PA) and von Willebrand factor collagen binding assay (VWF:CBA) in 11 of the 12 subjects with CEPI-CT values exceeding the 90th percentile at the first examination.

Parameter	First examination	Second examination	*P*-value*
PFA-100 CEPI-CT (s)	253 (197–269)	161 (110–271)	0·018
Lot no.	579524	579568	
PFA-100 CADP-CT (s)	101 (78–115)	96 (84–127)	0·464
Lot no.	569635	569673	
Bleeding time (min)	4·5 (3–6)	4 (2·5–5·5)	0·105
ADPPA (% light transmission)	72 (53–112)	81 (60–123)	0·320
AAPA (% light transmission)	75 (33–91)	67 (50–110)	0·320
Collagen PA, % light transmission	67 (31–88)	67 (31–112)	0·203
VWF:CBA (U/dl)	85 (48–149)	83 (52–150)	0·820

Values are given as median (range)
*Wilcoxon matched pairs test

them pH and citrate concentration of the anticoagulant (Jilma, 2001; Favarolo, 2002), time of day (Dalby *et al*, 2000; Wuillemin *et al*, 2002), ABO blood group (Jilma-Stohlawetz *et al*, 2001; Lippi *et al*, 2001; Moeller *et al*, 2001), haematocrit, platelet and leucocyte counts (Jilma, 2001; Favarolo, 2002) and VWF plasma levels (Jilma, 2001; Favarolo, 2002; Chakroun *et al*, 2004). Although 0·106 mol/l and 0·129 mol/l buffered citrate anticoagulant are both recommended by the manufacturer, the lower citrate concentration results in substantially shorter CT (Jilma, 2001; Favarolo, 2002). The majority of clinical studies have based reference ranges for CEPI-CT and CADP-CT on fewer than 40, often poorly characterised healthy individuals, although the reliable calculation of sensitivity, specificity and predictive values of PFA-100™ CT for detecting VWD or platelet dysfunction depend decisively on the upper limits of respective reference ranges. Hence, it is not surprising that the 95th percentiles of normal ranges established in clinical trials using 0·129 mol/l buffered citrate as anticoagulant ranged from 150 to 202 s for CEPI-CT and from 101·5 to 151 s for CADP-CT (Jilma, 2001; Wuillemin *et al*, 2002; Harrison, 2004). This study therefore re-investigated reference ranges and factors influencing PFA-100™ CT in a larger number of well-characterised healthy individuals under standardised conditions.

We attached great importance to obtaining a representative sample of normal individuals who had not taken any drugs that might impair platelet function within 10 days prior to examination. In addition to careful exploration of medical history, we excluded intake of aspirin or other drugs influencing platelet thromboxane synthesis by measuring AAPA, which showed no significant inhibition in any individual. To avoid haemodilution or haemoconcentration because of contemporary blood or blood component donation, only non-donors or subjects who had not donated within 10 days preceding blood collection were included. Baseline data showed that females and males did not differ significantly in age, blood group distribution, proportion of whole blood donors and smoking habits.

Table IV. Simple linear regression (Spearman rank correlation coefficients, level of significance $P < 0·01$); collagen/epinephrine (CEPI) and collagen/ADP (CADP) platelet function analyzer (PFA-100™) closure times (CT), bleeding time, platelet aggregation (PA) induced by ADP (ADPPA), arachidonic acid (AAPA) and collagen (collagen PA), blood cell counts, mean platelet volume (MPV), Hb, Fibrinogen and von Willebrand factor collagen binding assay (VWF:CBA) determined AM in 120 healthy subjects.

	CEPI-CT	CADP-CT	ADPPA	AAPA	Collagen PA	Platelet count	MPV	Leucocyte count	Fibrinogen	VWF:CBA
Bleeding time	NS	NS	$r = 0·21$, $P = 0·009$	$r = 0·21$, $P = 0·009$	NS	NS	NS	NS	NS	NS
CEPI-CT		$r = 0·61$*, $P < 0·0001$	NS	NS	NS	NS	NS	NS	NS	$r = -0·44$, $P < 0·0001$
CADP-CT			NS	NS	NS	NS	NS	NS	NS	$r = -0·47$*, $P < 0·0001$
ADPPA				$r = 0·51$, $P < 0·0001$	$r = 0·59$*, $P < 0·0001$	NS	NS	NS	NS	NS
AAPA					$r = 0·66$*, $P < 0·0001$	NS	NS	NS	NS	NS
Collagen PA						NS	NS	NS	NS	NS
Platelet count							$r = -0·31$*, $P < 0·0001$	$r = 0·29$*, $P < 0·0001$	NS	NS
MPV								NS	NS	NS
Leucocyte count									$r = 0·32$*, $P < 0·0001$	NS
Fibrinogen										NS

*Confirmed by multiple regression analysis.

In agreement with previous work (Wuillemin *et al*, 2002), our duplicate analyses revealed relative differences between duplicates exceeding 10% or even 15% in a substantial number of cases, with similar imprecision over the full range of measurement. These results strongly indicate that duplicate testing is required if reliable results are to be obtained, contradicting statements that single determinations are sufficient (Mammen *et al*, 1998; Böck *et al*, 1999; Jilma, 2001). We found no marked differences between CT values determined in channels 1 and 2 of the PFA-100™ device, which agrees with previous observations (Mammen *et al*, 1998).

We confirmed former findings that PFA-100™ CT are not influenced by age, smoking and oral contraceptive use and not associated with haematocrit, platelet and leucocyte counts and MPV in healthy individuals (Böck *et al*, 1999; Sestito *et al*, 1999; Jilma, 2001; Favarolo, 2002; Wuillemin *et al*, 2002). The upper limit of the reference range for CADP-CT (95th and 97·5th percentiles) found in this study agreed well with former findings (Jilma, 2001; Wuillemin *et al*, 2002; Harrison, 2004), but the 95th and 97·5th percentiles for CEPI-CT were greater than observed in any previous study (Table II). Re-examination of 11 individuals with CEPI-CT above the 90th percentile using another CEPI cartridge batch demonstrated that this was because of batch-to-batch variation (Table III), although previous examinations had failed to reveal significant variations from batch to batch (Mammen *et al*, 1995, 1998; Harrison *et al*, 1999). Unlike in some other studies (Böck *et al*, 1999; Sestito *et al*, 1999; Wuillemin *et al*, 2002), we observed longer CT in males, with a significant difference for CADP-CT. There is presently no conclusive explanation for this finding.

We found no differences in CT between whole blood donors and non-donors. This contradicts two previous studies observing a substantial greater percentage of prolonged CEPI-CT and CADP-CT in platelet donors than in controls (Jilma-Stohlawetz *et al*, 2001; Harrison *et al*, 2004). Jilma-Stohlawetz *et al* (2001) attributed this, at least in part, to decreased platelet thromboxane B_2 formation and frequent platelet donation, although controls showed significantly lower thromboxane B_2 levels than donors. Our subjects donated whole blood only at intervals varying between 2 and 6 months, and all individuals included in this study had normal BT and PA induced by ADP, AA and collagen. Thus, intake of aspirin or other drugs influencing platelet function could largely be excluded.

As already noted previously (Dalby *et al*, 2000; Wuillemin *et al*, 2002), PFA-100™ CT and BT increased significantly from morning to afternoon (Table II). We also demonstrated a concomitant increase in BT, MPV and leucocyte count and a marked decrease in PA induced by ADP, AA and collagen. Previous small studies examining PA in 10–25 healthy individuals had lacked statistical power and yielded inconclusive or contradictory results (Tofler *et al*, 1987; Brezinski *et al*, 1988; Haus *et al*, 1990; Jovicic & Mandic, 1991; Malyszko *et al*, 1994). Our data suggest that the time of day when blood is collected for determining PFA-100™ CT, PA, MPV and leucocyte count or when BT is measured has to be kept within a narrow range.

Comparison between our blood group O and non-O individuals and regression analysis supported previous conclusions that PFA-100™ CT more significantly reflect VWF activity than platelet function. Most clinical studies in patients with VWD and platelet disorders had proven the good sensitivity of the PFA-100™ system to detect VWD (Fressinaud *et al*, 1998; Cattaneo *et al*, 1999; Dean *et al*, 2000; Schlammadinger *et al*, 2000; Jilma, 2001; Favarolo, 2002; Cariappa *et al*, 2003; Posan *et al*, 2003) but a lower diagnostic performance for diagnosing mild platelet dysfunction (Favarolo, 2002; Wuillemin *et al*, 2002; Posan *et al*, 2003; Quiroga *et al*, 2004). A direct comparison between our blood group O and non-O individuals revealed significantly greater CEPI and CADP-CT and lower VWF:CBA values in subjects with blood group O, whereas BT and PA was not significantly different between both groups. We confirmed a significant linear correlation between PFA-100™ results and VWF and between CEPI-CT and CADP-CT (Fressinaud *et al*, 1998; Cattaneo *et al*, 1999; Jilma-Stohlawetz *et al*, 2001; Favarolo, 2002; Wuillemin *et al*, 2002; Chakroun *et al*, 2004; Table IV). The BT correlated significantly with ADPPA and AAPA and the PA values correlated significantly with each other. However, we did not observe any correlation between PFA-100™ CT and BT or between PFA-100™ and PA. Previous work had, however, demonstrated a significant linear correlation between PFA-100™ results and BT in patients with VWD or platelet dysfunction (Francis *et al*, 1999; Posan *et al*, 2003). Studies directly comparing PA in PRP and PFA-100™ CT are rare. Mammen *et al* (1998) found a good concordance between CT and PA induced by ADP, collagen and AA in patients with VWD or platelet dysfunction. However, comparisons between the two methods in patients taking aspirin showed a poor relationship (Gum *et al*, 2001).

In accordance with others (Rahmann & Billington, 2000), we noted significantly lower ADPPA in garlic consumers. We could also confirm an increased PA induced by collagen in OC users (Miller *et al*, 1995; Norris *et al*, 1996). However, the influence of oestrogen-containing OC on platelet function remains a controversial issue (Saleh *et al*, 1995).

This study had several significant limitations. Although we tried to get a representative group of healthy males and females, we were unable to avoid bias because of different proportions of blood group O individuals in our subgroups. We are confident that our normal subjects were more meticulously characterised than in any previous study. Nevertheless, we cannot completely rule out that some individuals were taking drugs that influenced platelet function at the time of blood collection. None of our subjects had impaired PA indicating platelet dysfunction. However, more sensitive assays such as platelet thromboxane formation might have revealed individuals consuming antiplatelet drugs. The lack of significant linear correlations between PFA-100™ CT and some other variables, particularly BT and PA, may be caused by the

low differences between minimum and maximum values seen in normal individuals. As the correlation coefficients depend strongly on the ranges covered by the single values, inclusion of values outside normal ranges might have resulted in substantially higher correlation coefficients.

In conclusion, the clinical performance of the PFA-100™ system is critically dependent on carefully standardised pre-analytical and analytical conditions and establishing reference ranges derived from a sufficient number of well-characterised individuals. Duplicate testing is an essential requirement to obtain reliable results. That CT test results may vary from batch to batch must also be taken into consideration. Like BT, PA, leucocyte count and MPV, PFA-100™ CT are markedly influenced by the time of day when blood is collected. VWF plasma levels, ABO blood group and possibly gender are additional variables influencing PFA-100™ CT. The significant association between PFA-100 CT and VWF plasma levels and the lack of correlation between CT and PA in healthy individuals suggests that PFA-100™ results more significantly reflect VWF activity than platelet function. The use of the PFA-100™ system as a screening test for primary haemostasis outside specialised laboratories cannot be recommended.

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