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THE IMPACT OF BLEEDING HISTORY, VON WILLEBRAND FACTOR AND PFA-100® ON THE DIAGNOSIS OF TYPE 1 VON WILLEBRAND DISEASE: RESULTS FROM THE EUROPEAN STUDY MCMDM-1VWD

Giancarlo Castaman, Alberto Tosetto, Anne Goodeve, Augusto B Federici, Stefan Lethagen, Ulrich Budde, Javier Batlle, Dominique Meyer, Claudine Mazurier, Jenny Goudemand, et al.

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**THE IMPACT OF BLEEDING HISTORY, VON WILLEBRAND
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Key Words:	Von Willebrand disease , Von Willebrand factor , Inherited bleeding disorders , PFA-100 closure time, Bleeding score



For Peer Review

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8 **RESULTS FROM THE EUROPEAN STUDY MCMDM-1VWD**
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ABSTRACT

We evaluated the relationships of Platelet Function Analyzer (PFA)-100 with von Willebrand factor (VWF) levels and bleeding score (BS) within a multicenter project on Molecular and Clinical Markers for the Diagnosis and Management of type 1 von Willebrand disease (MCMDM-1VWD). PFA-100 closure time, either with epinephrine (EPI) or ADP-cartridges, was measured in 107 index cases, 105 affected and 71 unaffected family members, and 79 healthy controls. By regression analysis VWF levels were strongly related with both closure times, with a non-linear progression. In a multiple stepwise regression model, age- and sex-adjusted, PFA-100 ADP and VWF ristocetin cofactor activity (VWF:RCo) were independently associated with BS. Most of the variation of BS is predicted by PFA-100 ADP and VWF:RCo alone. In the subgroup of patients with subtle abnormalities of the multimeric pattern VWF was invariably reduced and closure time prolonged in almost all of them. Neither PFA-100 ADP nor EPI closure times appeared to significantly improve the diagnostic capability of VWF antigen (VWF:Ag) measurement. Thus, in an unselected population a normal PFA-100 would be useful to exclude VWD, but whether it could replace the more specific VWF assay in patients with significant mucocutaneous bleeding symptoms remains to be investigated prospectively.

Key words: von Willebrand disease – von Willebrand factor – inherited bleeding disorders – PFA-100 closure time – bleeding score

INTRODUCTION

The diagnosis of von Willebrand disease (VWD), the most frequent inherited bleeding disorder, is based on the presence of bleeding symptoms and abnormal laboratory tests suggesting a qualitative or quantitative defect of von Willebrand factor (VWF) (Castaman et al, 2003). While the use of a standardized bleeding score (BS) has recently gained a wide acceptance as a useful way to assess the clinical severity of the disorder in patients with type 1 VWD (Rodeghiero et al, 2005; Tosetto et al, 2006), there is still considerable uncertainty about the optimal use of laboratory tests. In the past, the bleeding time (BT) was considered useful for VWD screening, but nowadays it has lost importance because of its invasive nature and the wide availability of VWF assays to most clinical laboratories. BT has been replaced in many laboratories by the Platelet Function Analyzer (PFA-100™) closure time, a global test that mimics *in vitro* the BT by recording the time required to stop the flow of the patient's whole blood under high shear stress (Kundu et al, 1995). While the PFA-100 could be useful for screening severe forms of VWD (Fressinaud et al, 1998), it is not known whether it could be of value for diagnostic or prognostic purposes in patients with milder forms of VWD in comparison to other laboratory tests. In particular, two questions remain unresolved. Firstly, whether the addition of PFA-100 could improve the diagnostic efficiency of the current approach based on the quantitation of VWF and bleeding severity, particularly in subjects with borderline BS or secondly, in patients with mild VWD, possibly accounting for up to 60-70% of all VWD patients, where VWF level does not completely explain the severity of bleeding as measured by a quantitative BS (Tosetto et al, 2006). Thus, it would be useful to know whether abnormal PFA-100 closure times could add more information over VWF measurement to identify patients with a more severe VWD phenotype.

In this study we have evaluated the impact of the PFA-100 by assessing its relationship with BS and VWF level in a population of families with VWD enrolled in a multicenter European study (MCMDM-1VWD).

PATIENTS AND METHODS

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Subjects. The “Molecular and Clinical Markers for the Diagnosis and Management of Type 1 von Willebrand disease (MCMDM-1VWD)” Study was a multicenter, EU funded, survey on type 1 VWD (see also <http://www.shef.ac.uk/euvwd/> for further details). Details about the selection of investigated families have been already published in detail (Tosetto et al, 2006; Goodeve et al, 2007). Briefly, 154 families from 9 European countries were enrolled, the recruitment criteria being the presence of an index case and at least one other affected family member, both previously diagnosed as having type 1 VWD on the basis of the criteria adopted at each Center. Within the families, subjects were classified by the enrolling Center as the index case (IC, subject who led to investigation) and affected or unaffected family member (AFM and UFM respectively) on the basis of reduced VWF levels and bleeding symptoms at time of investigation of the IC, as previously described (Tosetto et al, 2006). On the basis of multimeric and genotypic analysis performed in the MCMDM-1VWD study, subjects from the enrolled families were subsequently divided in three groups: subjects with abnormal multimers (Group 1), subjects with normal multimers and at least one candidate mutation (Group 2) and subjects with normal multimers and no mutation detected (Group 3) (Goodeve et al, 2007). A total of 1166 healthy controls, defined as subjects that never sought medical attention for a bleeding symptom, were also enrolled.

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Laboratory tests. Twenty ml of citrated blood were obtained from all investigated family members and normal controls, and platelet-poor plasma was immediately aliquoted and stored at -80°C for subsequent measurements. All the subjects were fasting and with no significant low hematocrit or platelet count. Plasma aliquots were sent on dry ice by overnight courier to the central laboratory in Vicenza, where VWF ristocetin cofactor activity (VWF:RCo) and VWF antigen (VWF:Ag) were measured, as previously described (Tosetto et al, 2006; Goodeve et al, 2007). PFA-100 closure time was performed in 5 Centers experienced with this test, immediately after

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3 venepuncture on citrated whole blood, according to the manufacturer instructions. A single batch
4 of PFA-100 ADP and epinephrine (EPI) cartridges was supplied free of charge by Dade Behring
5 (Marburg, Germany) and distributed among the Centers performing the assay.
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10 *Bleeding score.* The severity of bleeding symptoms in each investigated subject was assessed by
11 using a standardized bleeding questionnaire, as previously described (Tosetto et al, 2006).
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15 *Statistics.* All computations were performed using the Stata software package (College Station,
16 TX, USA). Variables influencing VWF:RCo and VWF:Ag levels were identified using a multiple
17 linear regression model (Breiman et al, 1984; Kleinbaum et al, 1987), using the whole MCMDM-
18 1VWD dataset. Univariate differences in VWF:RCo and VWF:Ag levels for different categories
19 were tested using the Kruskal-Wallis non-parametric test or chi-square statistic, as appropriate. We
20 computed positive and negative predictive values (PPV, NPV) and receiver-operator curves (ROC)
21 and their associated c-statistics to test the diagnostic capabilities of PFA-100. The c-statistics tests
22 the null hypothesis that two independent tests have a comparable area under the ROC curve,
23 which is a measure of the diagnostic efficiency of a test. One of the advantages of the c-statistics is
24 that several tests may be combined, so that the diagnostic efficiency of a set of tests may be
25 compared. In this study, we tested the hypothesis that the addition of PFA-100 may improve the
26 overall diagnostic efficiency of the usual diagnostic criteria (based on symptoms and VWF
27 measurements). For this purpose, we considered as “VWD affected” a subject fulfilling both the
28 following criteria: 1) diagnosis as affected by the enrolling Center (either IC or AFM) and 2) member of a
29 family showing complete segregation of the VWD phenotype with the *VWF* locus.. These criteria were the
30 same adopted for a previous study evaluating the predictive power of VWF measurement in this cohort
31 (Tosetto et al, 2006).
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RESULTS

Enrolled subjects and laboratory data. Table 1 reports the clinical characteristics of the investigated subjects for whom PFA-100 measurement (either ADP or EPI cartridges) was available. A total of 107/154 originally included index cases, 105/273 affected family members and 71/295 unaffected family members were tested. In addition a control group of 79 healthy subjects was also included. Blood group distribution showed a significant increase of O-blood group subjects in index cases and affected family members.

Normal range in control subjects. After excluding 3 and 5 outlier observations for EPI and ADP cartridges, respectively (defined as observation lying outside 3 standard deviations from mean), the upper 97.5 percentiles were 183.8 and 122 seconds for EPI and ADP cartridges.

PFA-100 in the MCMDM-1VWD families according to clinical status, multimeric pattern and linkage analysis. Figure 1 A reports the distribution of closure times in investigated subjects according to clinical status. Median values were similarly prolonged in index cases and affected family members, while median values of normal controls overlapped those of unaffected family members, even though some abnormal results were observed in the latter group. Subjects with abnormal multimers had on average significantly prolonged PFA-100 results compared to subjects with normal multimers with and without a VWF gene mutation ($P < 0.001$ for both EPI and ADP cartridges)(Figure 1 B). Subjects belonging to families with proven linkage had a more prolonged mean PFA-100 values compared to subjects with no or uninformative linkage ($P < 0.001$ for both EPI and ADP cartridges) (Figure 1 C).

Relationship of PFA-100 with VWF:RCo and VWF:Ag. Figure 2 shows that by regression analysis VWF levels (either VWF:RCo or VWF:Ag) were both strongly related with the PFA-100 closure times (either with ADP or EPI), with a non-linear progression

Relationship of bleeding score with PFA-100 and VWF levels. In a multiple stepwise regression

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3 model, adjusted for age and sex, both PFA-100 ADP and VWF:RCo were independently associated
4 with bleeding score with a similar magnitude (*t* statistics for regression coefficients 2.92 and 2.4
5 respectively for PFA-100 ADP and VWF:RCo) (Figure 3). PFA-100 EPI and VWF:Ag were
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9 discarded from the model, suggesting that most of the variation of bleeding score is predicted by
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11 PFA-100 ADP and VWF:RCo alone. The regression model showed that both the third and fourth
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13 quartiles of PFA-100 ADP closure times were associated with higher bleeding scores (mean
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15 increase in the third and fourth quartiles vs. the first quartile: 2.2 and 2.9 respectively, $p=0.02$ and
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17 $p=0.007$), even for normal VWF:RCo values (Figure 2).

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21 *PFA-100 and VWF levels in families with/without linkage between VWF locus and phenotype or with*
22 *multimeric abnormalities.* Since PFA-100 closure times were associated with bleeding symptoms (as
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24 expressed by the bleeding score) independently from VWF levels, we evaluated whether isolated
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26 prolongation of PFA-100 was differently distributed in the MCMDM-1VWD families. Isolated
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28 prolongation of the PFA-100 was defined as a closure time in the highest two quartiles (hence,
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30 above the median value: 124 sec for ADP and 181 sec for EPI cartridges respectively) in the
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32 presence of VWF levels in the highest two quartiles (above the median value of 55 IU/dL for
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34 VWF:RCo). Isolated prolongation of the PFA-100 closure time was present in 4.2% of individuals
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36 belonging to families in linkage vs. 16.5% of the families not showing segregation ($p=0.005$).
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38 There were no individuals with subtle abnormalities of the multimeric pattern (group 1) showing
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40 isolated prolongation of the PFA-100 closure time, vs. 12.3% of group 2 and 13.4% of group 3
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42 ($p=0.001$).

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49 *Additional diagnostic value of PFA-100 for the diagnosis of VWD.* The NPV and PPV were calculated
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51 using the above defined normal ranges in reference limits in patients considered affected using the
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53 above mentioned criteria. Assuming a 0.1% prevalence of clinically relevant VWD, both PFA-100
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55 EPI and ADP closure times showed a very high NPV but a very low PPV (100% for both PFA-
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3 100 EPI and ADP and 1.34 and 1.16%, respectively). Using a c-logistic model, we subsequently
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5 tested whether addition of PFA could add to the diagnosis of VWD. Although PFA-100 EPI and
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7 ADP closure times were associated with VWD independently from VWF:RCo levels, neither EPI
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9 or ADP added to a model based on measurement of VWF:RCo and Bleeding Score, either
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11 measured as a quantitative or qualitative trait (above/below the normal range).
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16 17 **DISCUSSION** 18 19

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21 In the last years, many efforts have been undertaken for a more stringent and objective
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23 diagnosis of VWD (Sadler & Rodeghiero, 2005). These efforts include the evaluation of new
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25 diagnostic tests (such as the PFA-100 closure time) but also the re-evaluation of time-honoured
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27 procedures such as the bleeding history, and they resulted in new, promising approaches to VWD
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29 diagnosis (Rodeghiero et al, 2005; Tosetto et al, 2006). The BT has been traditionally used as a
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31 challenging test for primary hemostasis, but the test is invasive, is influenced by the operator and
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33 by device-dependent reproducibility and does not help in distinguishing among the various types
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35 of defects of primary hemostasis (Cattaneo, 2004). Therefore, the test has been replaced in many
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37 laboratories by the Platelet Function Analyzer (PFA-100™) closure time (Kundu et al, 1995). The
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39 two tests were compared in the diagnosis of VWD in several studies with similar design. Well-
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41 characterized VWD patients underwent both BT and PFA-100 testing, and the sensitivities of the
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43 two tests were compared (specificity being fixed for all studies at about 97%) (Fressinaud et al,
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45 1998; Cattaneo et al, 1999; Schlamadinger et al, 2000; Nitu-Whalley et al, 2003). Although
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47 performed in small series of patients, these studies observed comparable sensitivities, with a
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49 pooled estimate around 90% for PFA-100 (92% and 90% for the EPI and ADP cartridges
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51 respectively) that was significantly higher than the sensitivity of the bleeding time (61%). The
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3 sensitivity of PFA-100 was even higher in subjects with severe or variant VWD (e.g., type 3 or
4 2A). On the contrary, the test appears to be somewhat insensitive and of poor utility in subjects
5 with borderline VWF levels (Posan et al, 2003; Quiroga et al, 2004).
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10 The use of PFA-100 for screening purposes was subsequently tested in non-selected series of
11 patients referred for haemostatic evaluation, with reported sensitivities of PFA-100 for type 1
12 VWD ranging from 61.5% to 71% in the two studies (Podda et al, 2007; Quiroga et al, 2004).
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17 In this study we have evaluated the diagnostic impact of the PFA-100 and its relationship with
18 VWF level and BS in a population of families diagnosed with VWD type 1 in a multicenter
19 European study (MCMDM-1VWD). Index cases and affected family members had significantly
20 more prolonged PFA-100 closure times compared to unaffected family members (Figure 1 A). The
21 results of PFA-100 were associated with the presence of abnormal multimers and proven linkage
22 with VWF gene mutation (Figure 1 B and 1 C). A strong relationship was found with VWF levels
23 for both cartridges (Figure 2), while both PFA-100 ADP and VWF:RCo were independently
24 associated with bleeding score (Figure 3). The degree of prolongation with PFA-100 ADP
25 correlated with higher bleeding scores, even in presence of normal VWF:RCo values.
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38 Isolated prolongation of the PFA-100 closure time was present in 4.2% of individuals
39 belonging to families in linkage vs. 16.5% of the families not in linkage ($p=0.005$). This suggests
40 that in families in linkage PFA-100 prolongation is mostly associated with a true VWD, as
41 suggested by the high likelihood of finding a VWF gene mutation (Goodeve et al, 2007), while in
42 families not in linkage other disorders (e.g, mild platelet function disorders) could be present.
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45 Furthermore, it also suggests that the families could have been mainly selected on the basis of
46 their bleeding symptoms. This is also supported by the fact that no individuals with subtle
47 abnormalities of the multimeric pattern and thus almost all with a proven VWF mutation (group 1)
48 had isolated prolongation of the PFA-100 closure time compared to 12.3% of subjects in group 2
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3 and 13.4% of group 3 ($p=0.001$).
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5 It has been demonstrated that the haplotype(s) of platelet glycoprotein polymorphisms
6 influences the bleeding severity of patients with VWD (Kunicki et al, 2004) and furthermore that
7 true inherited platelet function disorders may coexist in patients with VWD , thus complicating the
8 definitive diagnosis of VWD (Daly et al, 2009). At present, however, it is not known whether
9 platelet glycoprotein haplotypes can modulate PFA-100 results independently from the presence of
10 reduced VWF levels.
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19 Despite the evidence of an independent role of PFA-100 ADP in influencing the BS and the
20 correlation with VWF levels, the role of PFA-100 in the diagnostic work-up of patients with
21 suspected VWD type 1 remains however uncertain. Assuming a prevalence of clinically significant
22 VWD around 0.1%, the positive predictive value of an abnormal PFA-100 would be around 1%,
23 while the negative predictive value would be 100%. Of course, this figure is biased by the selection
24 criteria and the small number of cases included and results from larger populations of unselected
25 cases should be available for a more reliable estimate. Thus, while in an unselected population a
26 normal PFA-100 would be useful to rule out VWD, it is doubtful whether it could replace the
27 more specific VWF assay in patients with significant mucocutaneous bleeding symptoms. This is
28 also suggested by the findings that adding PFA-100 EPI or ADP closure times does not
29 significantly add to the information given by VWF:RCo.
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45 Thus, until validation of the role of this test in prospective studies becomes available, a careful
46 approach to the patients and their clinical indexes is advised to achieve a sound diagnosis, which
47 could be particularly relevant for the prevention of and the optimal treatment of hemorrhagic
48 events in VWD patients (Rodeghiero et al, 2009).
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LEGEND TO FIGURES

Figure 1.

A) Distribution of the closure times with ADP- and EPI-cartridges among the investigated subjects according to clinical status. Median within each group and the 97.5 percentile of normal reference group are reported.

B) Distribution of the closure times with ADP- and EPI-cartridges among the investigated subjects according to presence of an abnormal or normal multimeric pattern.

C) Distribution of the closure times with ADP- and EPI-cartridges among the investigated subjects according to linkage status.

Figure 2. Relationship of the closure times with ADP- and EPI-cartridges with VWF:RCo and VWF:Ag.

Figure 3. Relationship of the closure times with ADP-cartridge and bleeding score and VWF:RCo levels. In a multiple stepwise regression model, adjusted for age and sex, both PFA-100 ADP and VWF:RCo were independently associated with bleeding score. PFA-100 EPI and VWF:Ag were discarded from the model, suggesting that most of the variation of bleeding score is predicted by PFA-100 ADP and VWF:RCo alone. The regression model showed that both the third and fourth quartiles of PFA-100 ADP closure times were associated with higher bleeding scores (mean increase in the third and fourth quartiles vs. the first quartile: 2.2 and 2.9 respectively, $p=0.02$ and $p=0.007$).

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For Peer Review

Table I. Clinical parameters in enrolled subjects, as classified by the participating Centers (index case, IC; affected family member, AFM; unaffected family members, UFM)

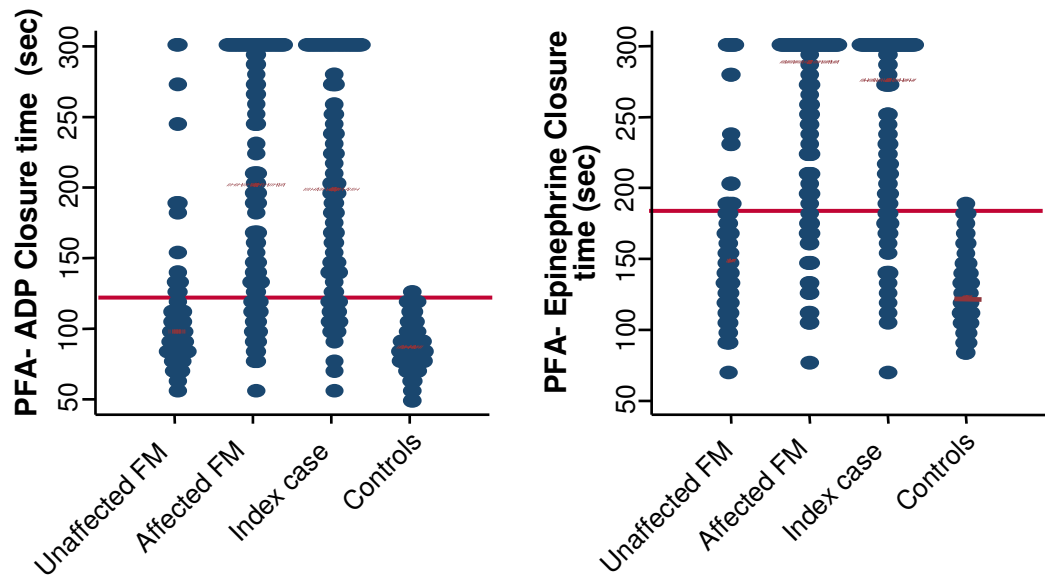
	IC (n=107)	AFM (n=105)	P*	UFM (n=71)	Controls (n=79)	P†
Females (%)	73 (68.2)	57 (54.2)	0.03	39 (54.9)	52 (65.8)	0.17
Mean age, years (range)	35.9 (1-80)	34.2 (3-85)	0.50	39.1 (9 – 75)	41.2 (14 – 78)	0.41
Blood group O (%)	71 (66.3)	64 (60.9)	0.41	33 (46.4)	23 (29.1)	0.02
Mean VWF:RCo, IU/dL (±SD)	33.7 (25.6)	33.1 (30.6)	0.87	90.2 (30.7)	90.8 (26.4)	0.89
Mean VWF:Ag, IU/dL (±SD)	36.5 (22.1)	36.4 (25.7)	0.77	98.1 (32.2)	96.7 (29.3)	0.97
Mean FVIII:C, IU/dL (±SD)	50.3 (25.7)	53.4 (33.4)	0.47	109.7 (54.1)	(not applic.)	-
Group 1 (Abnormal multimers)	43 (40.2 %)	56 (53.3 %)		0 (-)	-	
Group 2 (NM, mutation)	37 (34.5 %)	20 (19.2 %)	0.03	8 (11.2 %)	-	
Group 3 (NM, no mutation)	27 (25.3 %)	29 (27.6 %)		63 (88.8 %)	-	

* tests differences between IC and AFM as defined by enrolling Centers

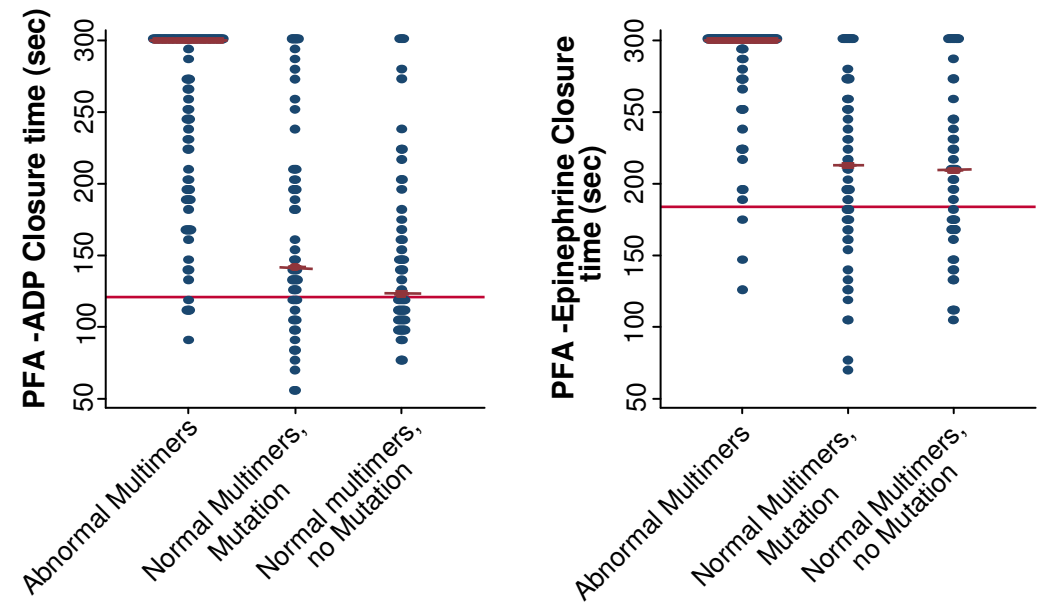
† tests differences between controls and UFM as defined by enrolling Centers

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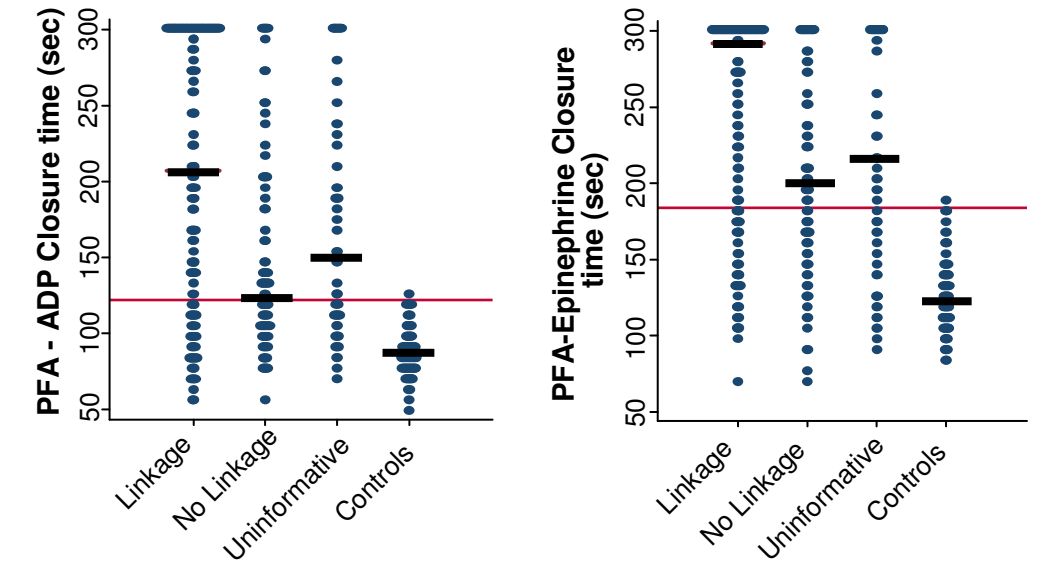
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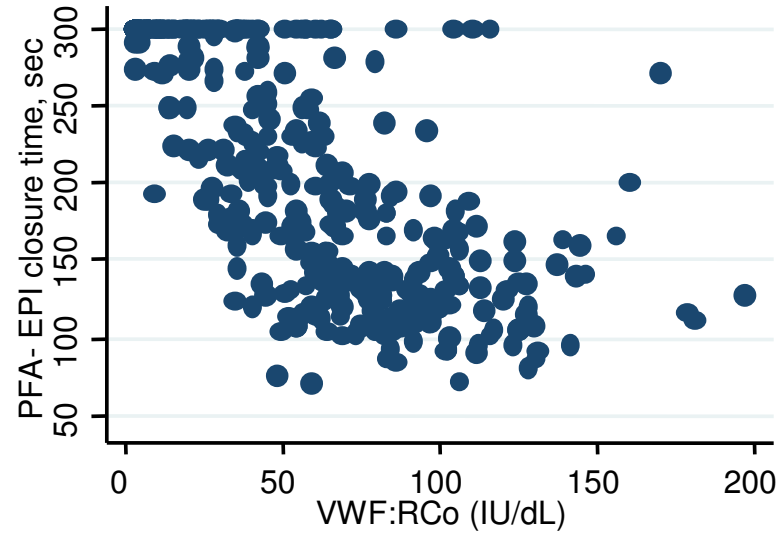
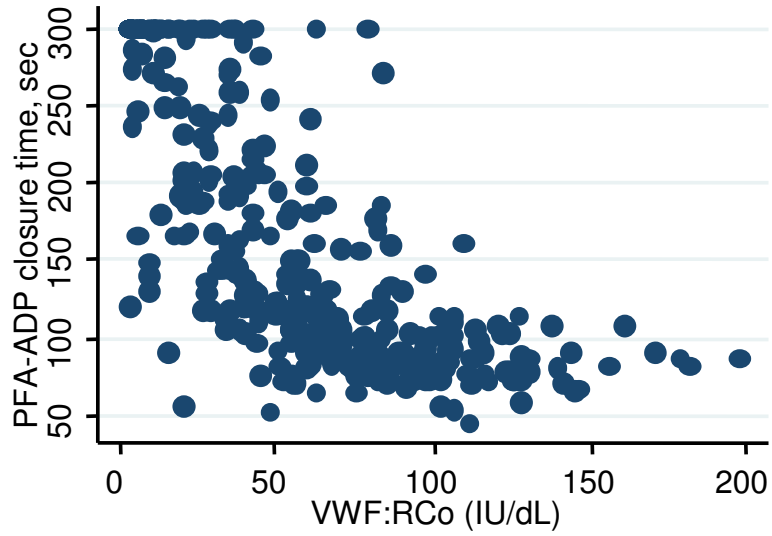
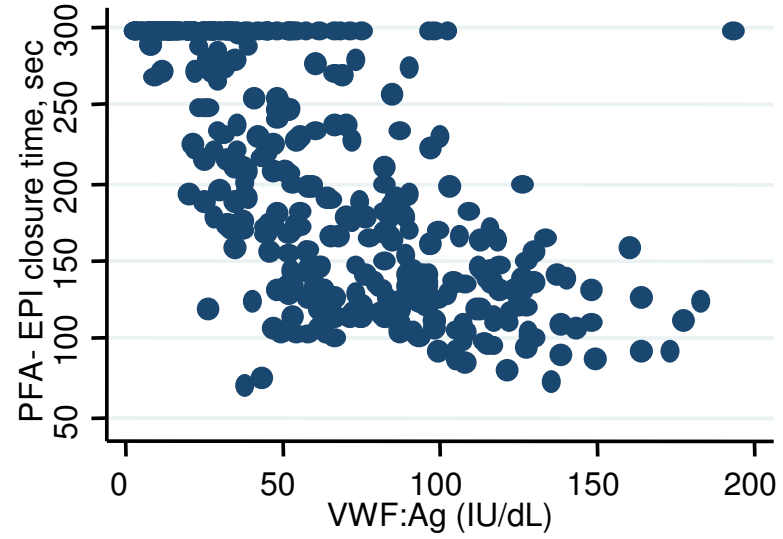
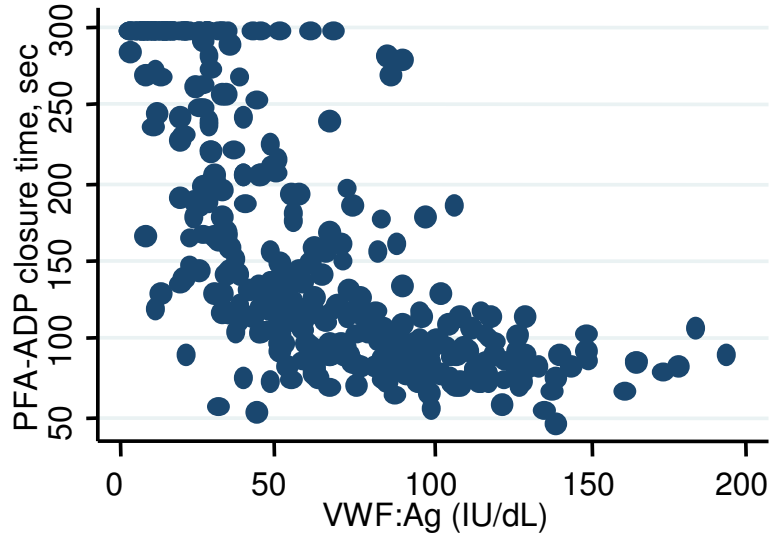


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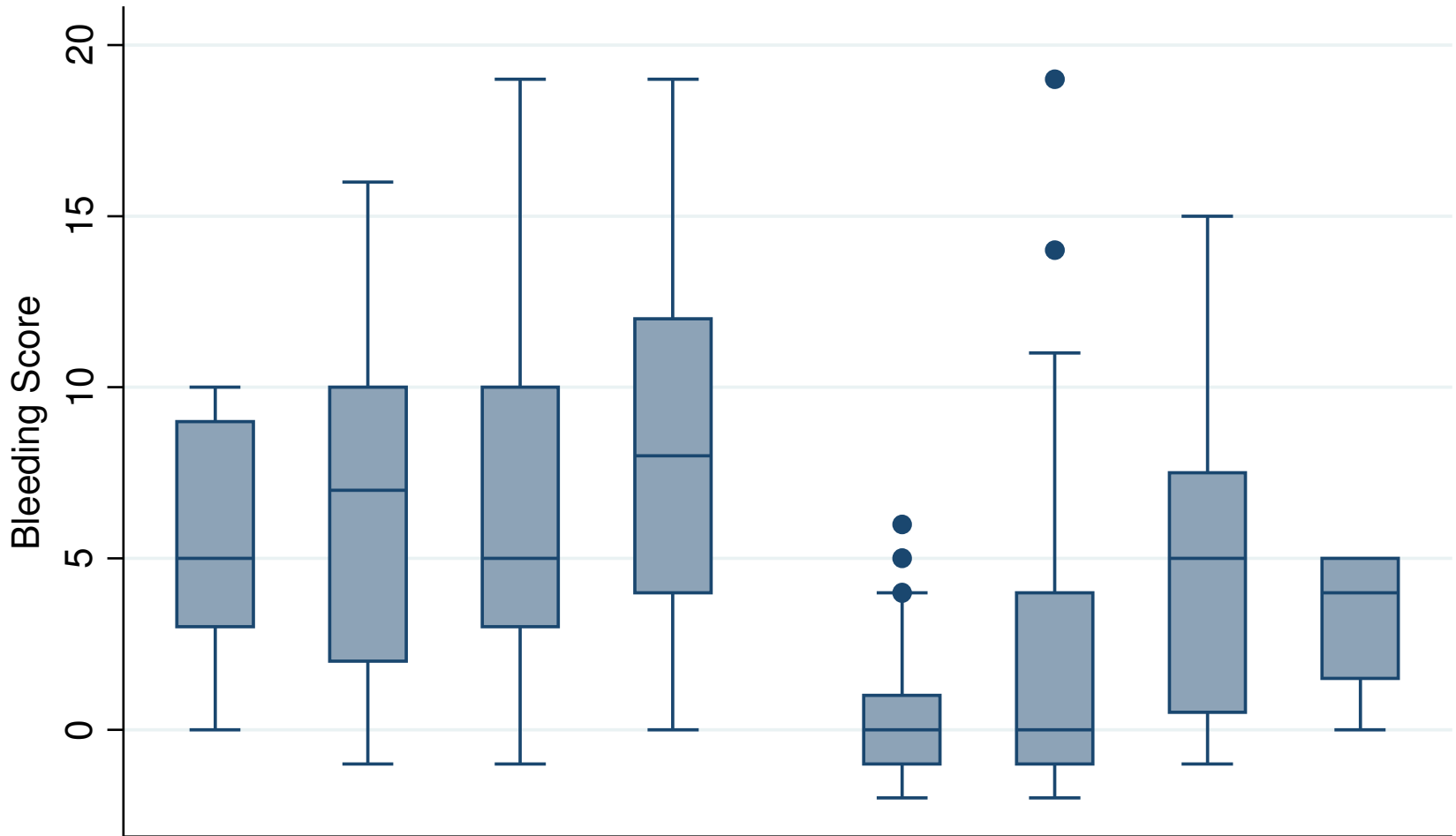


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PFA- ADP
Closure time

VWF:RCo

3-54 IU/dL (lower 2 quartiles)

55-197 IU/dL (upper 2 quartiles)

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