Effect of DDAVP on Platelet Activation and Platelet-Derived Microparticle Generation

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Abstract

Background The way by which 1-deamino-8-D-arginine vasopressin (DDAVP) acts on platelets remains unclear. Data from the literature tend to show that there is no definite effect on platelet activation, but recent work has suggested that a subtype of platelets, activated by the combined action of collagen and thrombin, was triggered by DDAVP. Moreover, platelet microparticles (PMPs), which have been shown to be procoagulant, have rarely been studied in this context. The goal of this study was to analyze the effects of DDAVP on PMPs' release through platelet activation.

Methods Fifteen out of 18 consecutive patients undergoing a therapeutic test with DDAVP were included. They were suffering from factor VIII deficiency or from von Willebrand disease. The expression of P-selectin and PAC-1 binding on platelets and the numbers of circulating PMPs were evaluated ex vivo before and after DDAVP infusion. Peripheral blood was collected on CTAD to limit artifactual platelet activation.

Results DDAVP induced a significant decrease of platelet counts and volume. Only small changes of P-selectin expression and PAC-1 binding were observed. Considering PMPs, two populations of patients could be defined, respectively, with (120%, $n = 6$) or without (21%, $n = 7$) an increase of PMPs after DDAVP. The decrease in platelet counts and volume remained significant in the group of responders.

Conclusion This study shows that DDAVP induces the generation/release of PMPs in some patients with factor VIII deficiency and von Willebrand disease 1 hour after DDAVP infusion.

Keywords
- blood platelets
- platelet activation
- platelet function
- platelet-derived microparticles
- deamino D-arginine vasopressin

Introduction

Desmopressin, also called DDAVP (1-deamino 8-D-arginine vasopressin), is a synthetic derivative of the peptidic hormone vasopressin. In the early 1980s, DDAVP's efficiency and relative harmlessness in hemophilia A and von Willebrand disease (VWD) led to its use to control bleeding in these hemorrhagic disorders. However, the important variation of interindividual responses to the infusion of DDAVP requires that a test is performed before using DDAVP as therapeutic agent. DDAVP induces an increased plasmatic discharge of von Willebrand factor (VWF), factor VIII (FVIII), and tissue plasminogen activator from the Weibel–Palade bodies (WPB) of endothelial cells. Moreover, DDAVP may induce the expression of other components of WPB such as P-selectin, and, thus, could contribute to P-selectin–mediated platelet adhesion on endothelial cell surfaces expressing the P-selectin ligand 1,[1–4] yet without any clinical

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impact in the absence of endothelial damage. However, the way by which DDAVP interacts with platelets remains obscure. DDAVP acts on vasopressin receptors, present on platelets,[5] but current knowledge about DDAVP does not favor a direct interaction with platelets. Published reports based on the capacity of DDAVP to activate or potentiate the expression of glycoproteins are inconsistent.[5–7] Indeed, some authors did not find any in vivo or in vitro effect of DDAVP. Conversely, Balduini et al[8] reported on the sensitization of platelets by DDAVP, while other authors observed an increased platelet expression of P-selectin or glycoprotein Ib.[9,10] Horstman et al reported on the formation of platelet-derived microparticles (PMPs) and the initiation of a procoagulant state.[11] More recently, Pearson et al[12] observed that DDAVP did not increase normal platelet coagulation functions (thromboelastography, Multiplate and Sonoclot) in vitro. Besides, Swieringa et al[13] showed that in vivo administration of DDAVP caused an increase in collagen-dependent thrombus formation and phosphatidylserine exposure. Of note, the effects of DDAVP on coagulation were also successfully examined in microfluidics by Ogiwara et al.[14] This method, using microchips coated with collagen or collagen/thromboplastin (PL-chips, T-TAS), allowed to assess the effect of VWF and FVIII in VWD patients who had received DDAVP. The lack of clear evidence of any action of DDAVP on platelet surface glycoproteins prompted us to turn to a different approach to study the effects of DDAVP on platelets in vivo, by analyzing the discharge of PMPs before (T0) and 1 hour after (T1) DDAVP infusion in patients requiring this test. Microparticles, which are a type of extracellular vesicles (EVs), bud from activated or apoptotic cells and are known to be procoagulant in physiologic or pathologic situations.[15,16] PMPs are a common type of blood microparticles, representing 30 to 90% of global circulating microparticles originating from platelets or megakaryocytes.[17,18] Moreover, we analyzed the exposure of P-selectin and activated GPIIb–IIIa (PAC-1 binding) on the platelets' surface at T0 and T1.

Patients and Methods

Patients

This prospective study, approved by the institutional ethical board, was conducted in accordance with the Declaration of Helsinki on patients with medical follow-up in the Hemophilia Treatment Center at Nantes University Hospital. All patients provided written informed consent. Patients treated with antiplatelet agents were excluded from the study. Patients who were followed up for FVIII deficiency (mild hemophilia or hemophilia carriers with FVIII deficiency) or VWD and who received DDAVP in the context of a therapeutic test were eligible. We investigated 18 patients, 9 men and 9 women (median age: 35 years, range: 18–67). Ten patients had a VWD, 5 had mild hemophilia A, and 3 women were hemophilia A carriers with low levels of FVIII. Patients were administered 0.3 µg/kg of DDAVP (Ferring Pharmaceutical, Saint-Prex, Lausanne, Switzerland) intravenously over 30 minutes in 50 mL 0.9% NaCl as per current recommendations.

Peripheral blood was sampled before (T0) and 1 hour after DDAVP infusion (T1) to take into account the possible short lifespan of PMPs. Blood was drawn by venipuncture, using a high-caliber needle (21-gauge) and vacuum plastic tubes, without venostasis (tourniquet was released promptly). The first few milliliters of blood drawn were discarded. Blood was gently mixed with the anticoagulant.

Methods

von Willebrand Factor and FVIII Levels

Assays for VWF and FVIII were performed on citrated plasma as part of the DDAVP protocol using aggregometry and an immunoturbidimetric test, respectively. VWF antigen was assayed for patients with VWD and FVIII activity for patients with hemophilia.

Platelet Microparticles

Plasma samples were prepared for PMP's analysis following the protocol recommended by the International Society on Thrombosis and Hemostasis (ISTH).[19] Blood samples were collected on CTAD (sodium citrate solution, theophylline, adenosine, and dipyridamole) Greiner Bio-One tubes (Greiner Bio-One France, Les Ulis, France), to prevent platelet activation. All samples were submitted to two successive centrifugations at 2,500 *g* for 15 minutes at 20°C immediately after sampling. The plasma was then stored at −80°C as recommended. PMPs analyses were conducted on a Navios flow cytometer (Beckman Coulter, Miami, FL) and data were processed using the Kaluza software (Beckman Coulter). Annexin V-FITC/7-AAD, CD31-PE, and CD41-PC5 antibodies (all from Beckman-Coulter) were used as PMP markers. This protocol allows to detect MPs from platelets ($CD41^+$ $CD31^+$ annexin V) and from endothelial cells ($CD41^-$ $CD31^+$ annexin V), but we focused our analyses on PMPs. All manipulations involved the inhibition of coagulation pathways by heparin sodium, since calcium present within buffer solutions can activate clotting which would interfere with PMP analysis according to Iversen et al.[20] Megamix-calibrated beads were used as recommended by the ISTH.[19] The 0.3- to 1-µm window was the most accurate for the detection of PMPs. It allows to assess rather large EVs, confirming that they are indeed microvesicles and not exosomes. PMP counts were evaluated at T0 and T1. Briefly, 5 µL of platelet poor plasma was added to 3 µL of annexin V-FITC, 3 µL of CD41-PC5, 5 µL of CD31-PE, and 5 µL of heparin sodium (10 U/mL). The sample was complemented with 935 µL of phosphate buffered saline (PBS) and incubated at room temperature for 1 hour in the dark. Ultimately, 500 µL of each sample was transferred to a TrueCount (BD Biosciences, San Jose, CA) tube containing a precise number of calibrated fluorescent beads. After analysis in flow cytometry, where the calibrated beads are gated on a region that does not interfere with PMPs, the amount of PMPs per microliter in each sample (i.e., annexin-positive and platelet marker–positive events) was determined by the equation: [Number of PMPs counted in 300 seconds × Number of fluorescent beads in the TrueCount tube]/[Number of fluorescent beads counted over 300 seconds].

Compensations had been established for this protocol using Compbeads (BD Biosciences; ►Supplementary Fig. S1 [online only]).

Platelet Count and Mean Platelet Volume

Platelet count and mean platelet volume (MPV) were measured on EDTA-anticoagulated samples by impedancemetry using an automated hematology analyzer XN10 (Sysmex, Kobe, Japan).

P-Selectin and PAC-1

To assess CD62P expression, 5 µL of whole blood was added to 10 µL of PE-conjugated anti-CD62P, 10 µL of FITC-conjugated anti-CD41, and 80 µL of PBS. A second tube was prepared in parallel for each sample where anti-CD62P was replaced by a murine-irrelevant IgGk (BD Biosciences) as isotypic control. After 15 minutes of incubation in the dark, 1 mL of PBS was added and suspensions directly processed for flow cytometry according to Fouassier et al.[21] At least 10,000 platelets were acquired at low speed. A platelets' gate was drawn based on light scatter properties and FITC-CD41 fluorescence to assess platelet numbers. Data were recorded as mean fluorescence intensity (MFI) and as percentage of platelets expressing CD62P.

A similar whole blood no-wash no-lysis procedure was applied using PAC-1 to assess the fibrinogen-binding conformation of the platelet fibrinogen receptor with 20 µL of FITC-conjugated PAC-1 (BD Biosciences), 10 µL of PE-conjugated anti-CD49b, and 70 µL of PBS. A murine IgMk (BD Biosciences) was used as isotypic control.

Statistics

To appreciate the effect of DDAVP on PMPs, paired samples were compared using the nonparametric Wilcoxon's signed-rank test (MedCalc, Ostend, Belgium). Subgroups were compared using Kruskal–Wallis or Mann–Whitney tests (R forge, MedCalc). Statistical significance was considered for p values less than 0.05.

Results

von Willebrand Factor and FVIII Levels Increase after DDAVP Infusion

These parameters were available for 17 patients (9 VWD patients and 8 patients with hemophilia). As expected, these factors were increased by mean ratios of, respectively, 3.3 (range: 2.1–5) and 3.1 (range: 2.1–4.8) in patients with VWD and hemophilia A at T1 compared with T0 (►Fig. 1).

PMPs Do Not Increase in all Patients after DDAVP Infusion

PMP levels could be assessed for 15 of the 18 patients included and two more samples had to be excluded because of mishandling of preanalytical procedures (aberrant data or centrifugation issues). Therefore, 13 patients were tested.

The median PMP level before DDAVP injection was 1,594 (1st–3rd quartiles: 1,102–1,858) PMPs/µL and increased up to 1,796 (1,404–2,722) after injection. This represents a median increase of 37% of PMPs after DDAVP usage, yet not significant ($p = 0.21$). However, the results were quite heterogeneous with some patients showing a clear increase on PMPs after DDAVP, while others had stable levels. We had previously estimated the coefficient of variation (CV) at 17% in our methodological conditions (data not shown), a value consistent with the literature.[20] Accordingly, an increase of more than 558 PMPs (twice 17% of the mean PMP level) allowed to divide the population into two subgroups. Patients with an increase in PMPs level greater than 558/µL after the injection of DDAVP were considered responders ($N = 6$) and those with a decrease or stability of PMPs levels at T1 were considered nonresponders ($N = 7$; ►Fig. 2). In the group of responders, a median increase of PMPs levels of 120% was observed, from 1,348/µL (range: 1,056–1,658) to 2,962/µL (range: 2,327–3,733). The average ratio of increase (PMPs after DDAVP/PMPs before DDAVP) was 2.35 (range: 1.38–4.41). In the group of nonresponders, there was an average apparent decrease of PMPs (21%) from 1,858/µL (range: 1,328–2,544) to 1,524/µL (range: 1,321–1,752). Of note, the increase in plasma levels of FVIII and VWF was similar in responders and nonresponders.

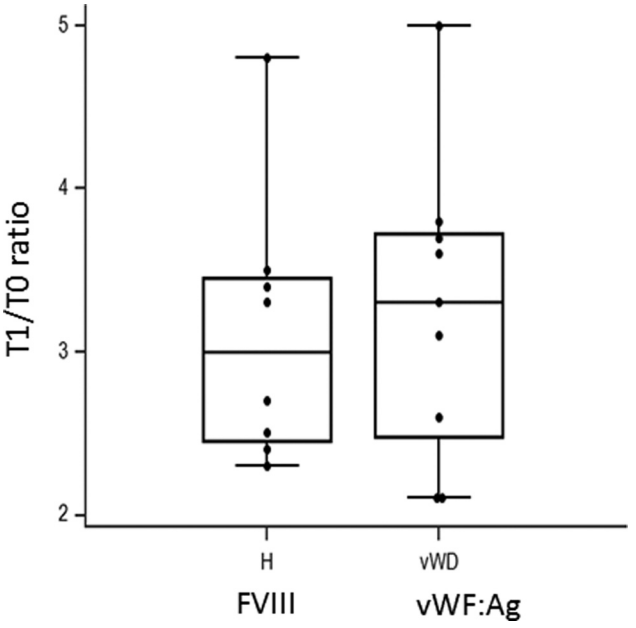

Fig. 1 Effect of 1-desamino-8-D-arginine vasopressin (DDAVP) on the release of factors. Ratios (T1/T0) of FVIII in patients with hemophilia (H, $n = 8$) and von Willebrand factor antigen (VWF:Ag) in patients with von Willebrand disease (VWD, $n = 9$).

Platelet Count and MPV Are Modified Differently in Responders and Nonresponders

These parameters were available for 18 patients. There was a decrease in platelet count between T0 and T1. The median platelet count was 279×10^9/L (1st–3rd quartiles: 243–322) at T0 and decreased significantly to 251×10^9/L (1st–3rd quartiles: 238–305) at T1 ($p = 0.0004$; ►Fig. 3, top). Analyses of subgroups (responders and nonresponders) disclosed a significant decrease in the responders group ($p = 0.03$) but not in nonresponders ($p = 0.06$). Yet, there was no difference

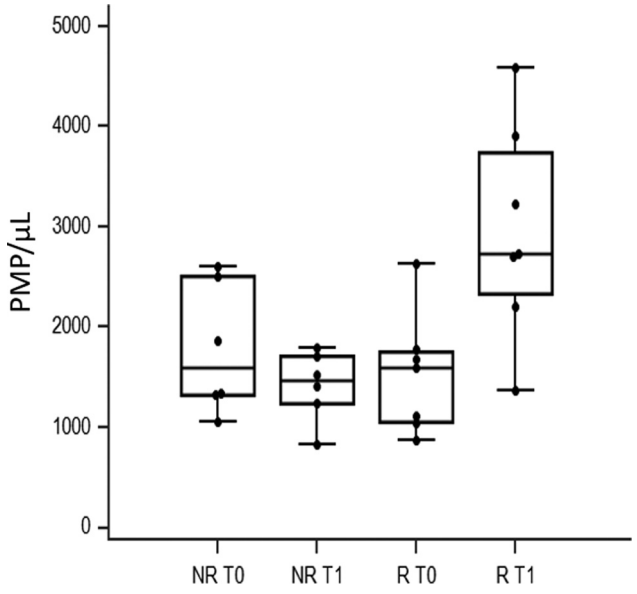

Fig. 2 Effect of 1-desamino-8-D-arginine vasopressin (DDAVP) on the release of platelet microparticle (PMP). $CD41^{+}$ PMPs at the two time points in nonresponders (NR, $n = 6$) and responders (R, $n = 7$).

in hematocrit between T0 and T1 ($p = 0.25$ and $p = 0.96$, respectively), excluding a potential effect of hemodilution induced by DDAVP.

There was also a significant decrease in median MPV between T0 and T1: 10.6 fL (1st–3rd quartiles: 10.25–11.2) at T0 and 10.2 fL (1st–3rd quartiles: 9.9–11) at T1 ($p = 0.01$). This significance was retained in the responders group ($p = 0.035$) but not in nonresponders ($p = 0.28$; ►**Fig. 3** bottom).

P-Selectin and PAC-1-Labeled Platelets and Expression Level Vary after DDAVP Infusion

P-selectin expression and PAC-1 binding were measured to assess, respectively, α-granule secretion and agonist-induced GPIIb–IIIa activation after DDAVP infusion. Expression at T0 was consistent with our usual standards.[21] P-selectin expression was significantly decreased at T1 compared with T0, with a mild yet significant mean decline in MFI (3,543 vs. 3,320; $p = 0.029$), whereas there was a 28% decrease of labeled platelets which failed to reach statistical significance ($p = 0.061$; ►**Fig. 4**). Conversely, the percentage of platelets

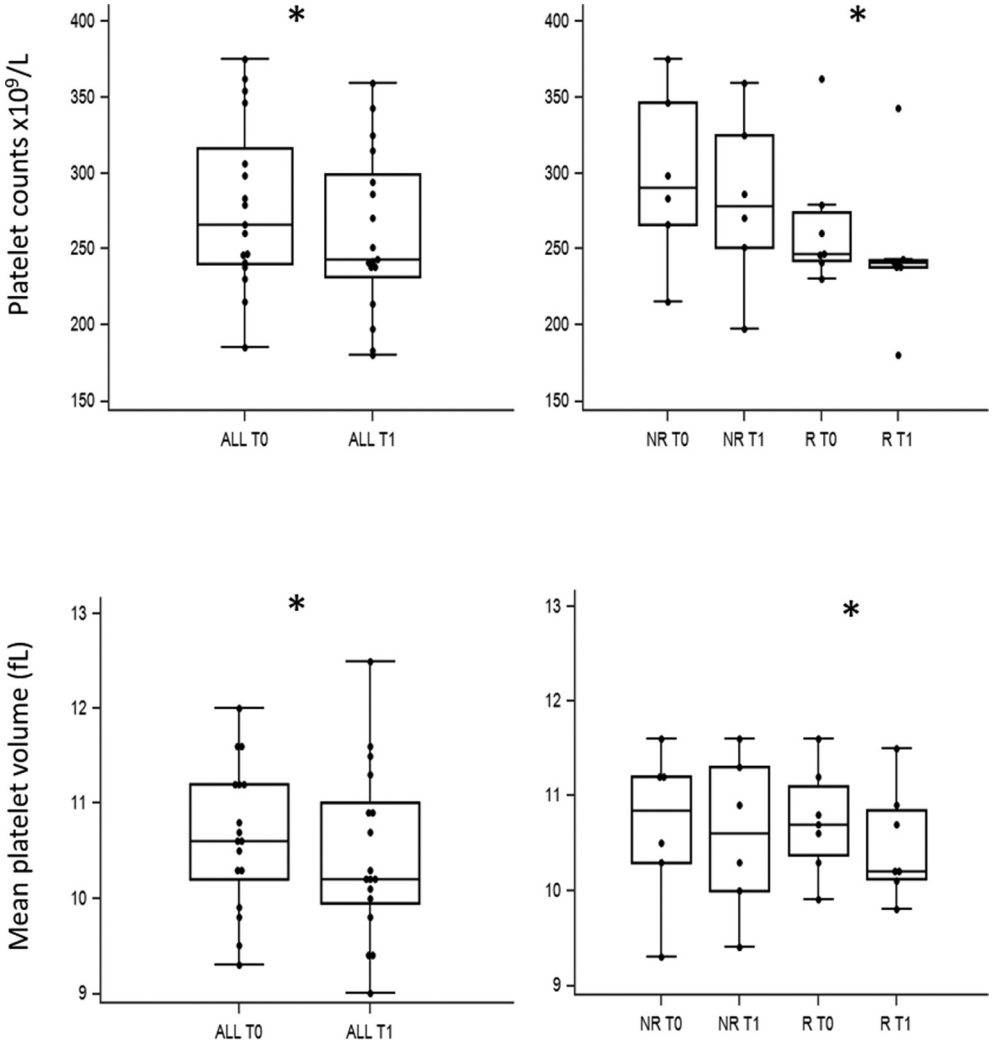

Fig. 3 Effect of 1-desamino-8-D-arginine vasopressin (DDAVP) on platelet counts and on mean platelet volume. Data are presented for all patients (17) at T0 and T1 on the left, then discriminating nonresponders (NR, $n = 6$) and responders (R, $n = 7$).

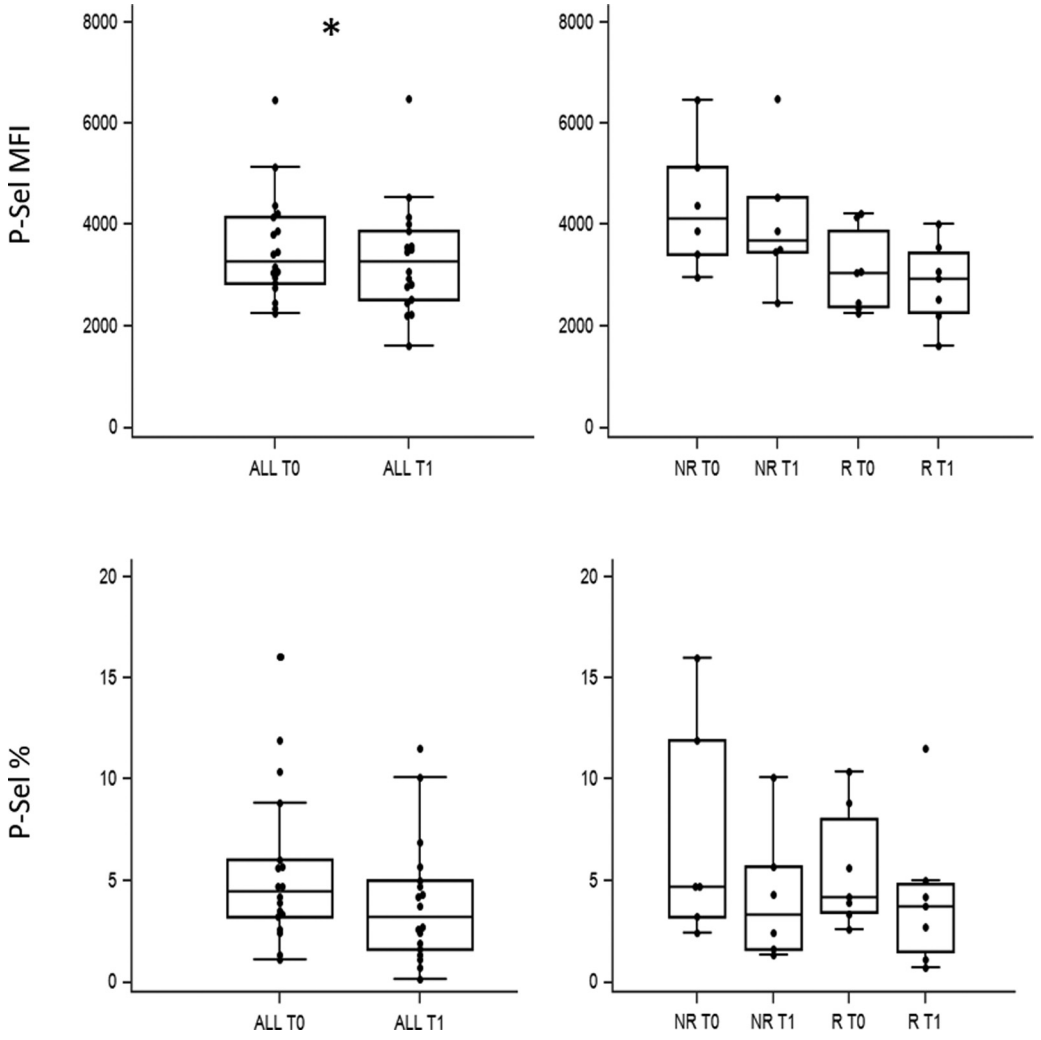

Fig. 4 Effect of 1-desamino-8-D-arginine vasopressin (DDAVP) on P-selectin expression. Data are presented for all patients (18) at T0 and T1 on the left, then discriminating nonresponders (NR, $n = 6$) and responders (R, $n = 7$). P-selectin was measured in a whole blood no-wash flow cytometry method.

labeled by PAC-1 decreased significantly to 33% ($p = 0.02$) after DDAVP infusion, while there was no variation in MFI ($p = 0.77$; ►Fig. 5).

Analyses of subgroups (responders and nonresponders) disclosed a significant decrease of labeled platelets with PAC-1 in the nonresponders group ($p = 0.02$).

Discussion

The main goal of this study was to investigate the induction of PMPs formation after in vivo infusion of DDAVP. For the 13 tested patients, a 37% average increase in PMPs' release was indeed observed 1 hour after DDAVP infusion. Although this increase is not statistically significant, due to individual variations and the relatively small size of the series, the two groups (responders or nonresponders) could clearly be distinguished by showing or not an increase above 2 CV of PMPs numbers. The proposition that two response patterns to DDAVP exist is reinforced by the fact that other different responses to DDAVP were observed between the two groups. Indeed, specific responses were also identified considering platelet counts, MPV, and PAC-1 binding.

The study of Colucci et al[22] could provide a possible explanation, although it examined the generation of procoagulant COAT platelets induced by Convulxin (which is an agonist of the collagen receptor glycoprotein VI) together with thrombin in vitro 2 and 4 hours after administration of DDAVP. Indeed, these authors observed that the action of DDAVP is not uniform in all patients and on all platelets. DDAVP seems to enhance the formation of COAT platelets, a subtype of platelets activated by the combined action of collagen and thrombin, which are covered by procoagulant proteins and adhesion molecules. Such platelets can participate to thrombin generation[23] and have been shown to be an important source of PMPs besides classical platelets and megakaryocytes.[24] COAT platelets represent approximately 30% of all platelets[23,25] and contrary to other platelet subtypes they express phosphatidylserine on their surface.[23,26] Two pathways, calcium dependent or independent, can lead to the exposure of phosphatidylserine. The former is closely

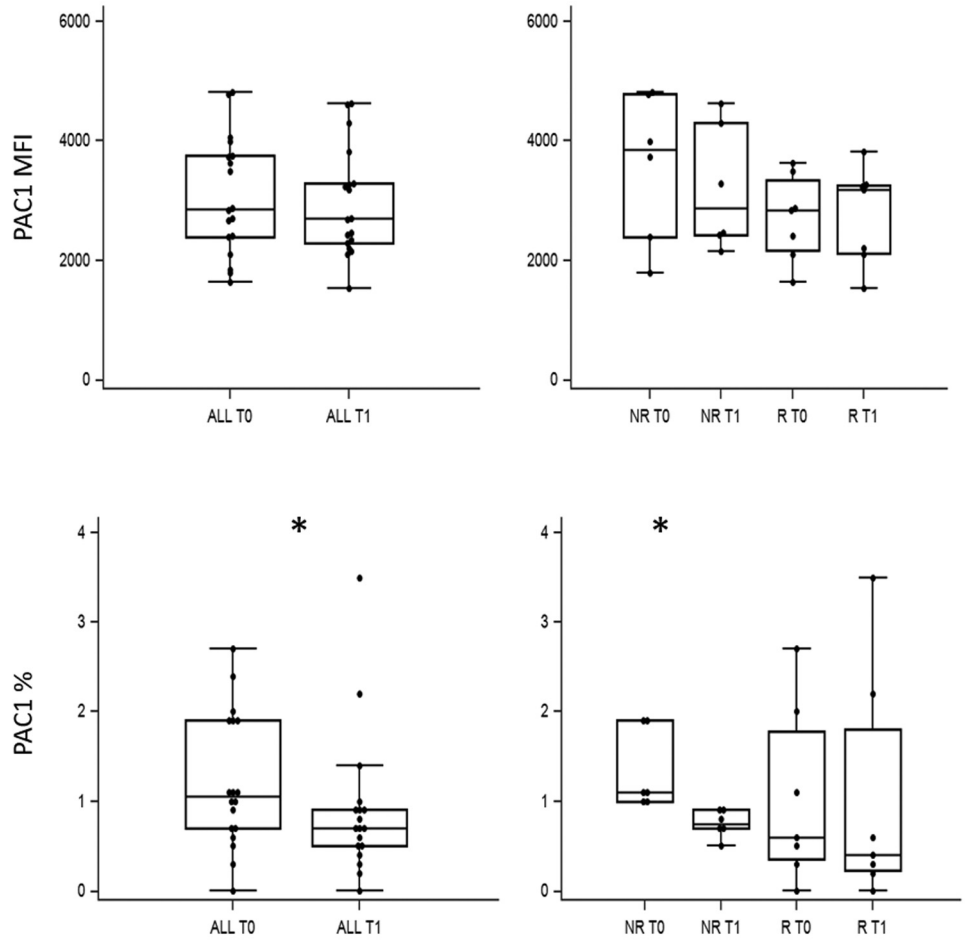

Fig. 5 Effect of 1-desamino-8-D-arginine vasopressin (DDAVP) on PAC-1 binding. Data are presented for all patients (18) at T0 and T1 on the left, then discriminating nonresponders (NR, $n = 6$) and responders (R, $n = 7$). PAC-1 was measured in a whole blood no-wash flow cytometry method.

linked to sodium metabolism,[27–29] itself modulated by DDAVP. Colucci et al[22] have demonstrated the presence of sodium and calcium flux modifications only in platelets from patients displaying an increase in COAT platelets generation after administration of DDAVP.[24]

The study of Colucci et al[22] indeed describes two types of patients, that is, responders with an increase in COAT platelets generation 2 hours after DDAVP administration (71%) and nonresponders. We can thus hypothesize that responders in our study could correspond to patients who showed an increase in COAT platelets in the study of Colucci et al, although this has not been formally demonstrated, since we did not look at annexin binding on platelets. The lower proportion of responders here could be related to the fact that we collected the second sample 1 hour after the end of DDAVP infusion, while Colucci et al[22] performed samplings at later times. These authors also report a higher proportion of responders at 4 hours (86.5%) and it thus makes sense that only 54% of the patients were responders at 1 hour in our study.

Additionally, these results confirm those of the study from Horstman et al on PMPs,[11] where an increase in PMPs' release was observed after 1 hour for 27% of the patients. These results are also consistent with those of Trummer et al,[30] who reported an increase in PMPs in patients with either type 1 VWD or "unspecified bleeding diathesis" after DDAVP administration. Moreover, these authors reported that VWF binds these PMPs.[30] A hypothesis would be that DDAVP leads to platelet activation through the release of VWF multimers.

Yet, our results are not completely consistent with previous studies regarding platelet antigen expression. Indeed, it has been reported[22] that there was no impact of DDAVP on the expression of P-selectin nor PAC-1 binding on circulating platelets, while we observed a significant decrease in P-selectin expression and a significant decrease in PAC-1-positive platelets. Nevertheless, Colucci at al[21] found a decrease in both P-selectin and expression and PAC-1 binding after platelet activation by ADP or thrombin. A possible explanation for this discrepancy is again the time course of DDAVP's effects. We investigated for the expression of P-selectin and PAC-1 binding 1 hour after DDAVP infusion compared with 4 hours in the aforementioned study.[22] The decrease in P-selectin expression and PAC-1 labeling could be explained by the fact that DDAVP enhances the synthesis of cyclic adenosine monophosphate (cAMP), which has an inhibitory effect on platelet functions.[30–33] Since cAMP has

a short half-life, the effect we observed could be limited in time and thus not be detectable any longer at 4 hours.[22]

However, an impact of DDAVP on platelet counts and MPV was also observed. This is consistent with data reported by Tomasiak et al, suggesting again an activity on platelet sodium metabolism.[29] We have no clear explanation for the slight decrease in platelet counts after DDAVP infusion. We can make the hypothesis that part of activated platelets was cleared from the bloodstream. The difference between responders and nonresponders seems to reinforce this hypothesis. The decrease in MPV is not easier to understand. Indeed, there is a clear difference between responders and nonresponders and it seems to make sense to hypothesize that the release of microparticles from a platelet would be responsible for the decrease in platelet's volume.

These results, together with evidence from the literature, suggest that one of the beneficial effects of DDAVP infusion, besides the plasma levels of FVIII and VWF, could be related, at least in some patients, to the formation of COAT platelets and release of PMPs. This cannot be investigated by global coagulation tests, which are inevitably improved after DDAVP infusion. However, Horstman et al[11] have shown that infusion of DDAVP was responsible not only for an increase in PMPs but also for an improvement of procoagulant activity with shorter times of Russell's viper venom assay, which indirectly sustains this hypothesis.

Altogether, even if there still is no clear explanation about the mode of action of DDAVP, data from this study confirm that this compound has an action on platelets, resulting in the release of PMPs,[26] at least in some individuals.

What is known about this topic?

- DDAVP is efficient in patients with platelet function disorders, but its mechanism of action remains unknown.
- There is some evidence that DDAVP may promote generation of platelet-derived microparticles.

What does this paper add?

- DDAVP promotes the generation of platelet-derived microparticles in a part of the patients (responders).
- This suggests that DDAVP improvement of primary hemostasis could in part result from platelet activation.

Authors' Contributions

All authors had full access to the data. M.P. and N.A. performed the experiments and abstracted all the clinical and laboratory data and contributed equally to the work. M.T., M.S., and C.T. provided patients and analyzed the data. M.C.B. analyzed the data and wrote the manuscript. M.F. designed the study, analyzed the data, and wrote de manuscript. All authors read, gave comments on, and approved the final version of the manuscript.

Conflicts of Interests

The authors declare that they have no conflict of interest.

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