The Effect of Desmopressin on Platelet Function:  
A Selective Enhancement of Procoagulant COAT-Platelets  
in Patients with Primary Platelet Function Defects

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Key points

1. DDAVP (increasing VWF/FVIII) is the drug of choice for mild hemophilia A and VWD, and (by unclear mechanisms) for platelet function disorders.

2. *In vivo* DDAVP selectively and markedly enhances the ability to form procoagulant platelets, by enhancing intracellular Na\(^+\) and Ca\(^{2+}\) fluxes.
Abstract

Desmopressin (DDAVP) is clinically efficacious in patients with mild platelet function disorders but it is not known which mechanisms mediate this effect. Our aim was to evaluate the impact of in vivo DDAVP administration in these patients. We assessed VWF, FVIII, PFA-100, platelet-aggregation, platelet-activation, thrombin-generation, and platelet intracellular Na⁺/Ca²⁺ fluxes in patients with mild platelet function disorders receiving a test-dose of DDAVP (0.3 µg/kg) before, 2, and 4 hours after drug administration. We found: 1) No significant changes for P-selectin-expression, PAC1-binding, delta-granule content and secretion, and platelet-aggregation induced by ADP, collagen, arachidonate, and ristocetin. 2) Significant decreases of secretion of alpha-granules and GPIIb-IIIa activation induced by ADP, convulxin, and thrombin. 3) Significant increases of procoagulant platelets induced by convulxin/thrombin, and platelet-dependent thrombin-generation (median about 1/3). 4) Significant increases of intracellular Na⁺/Ca²⁺ concentrations. We show that in vivo DDAVP selectively and markedly enhances the ability to form procoagulant platelets and increases platelet-dependent thrombin-generation, by enhancing Na⁺/Ca²⁺ mobilization. This report indicates that the beneficial haemostatic effect of DDAVP is not limited to an increase in large VWF multimers. An enhancement of platelet procoagulant activity appears to be an additional and—at least in platelet disorders—possibly clinically relevant mechanism of DDAVP’s action.

Key words

Desmopressin, DDAVP, platelet function, platelet procoagulant activity, thrombin generation, intracellular Na⁺ and Ca²⁺
Introduction

1-deamino-8-D-arginine vasopressin (DDAVP, desmopressin), a synthetic analogue of the natural antidiuretic hormone L-arginine vasopressin (AVP), was originally employed for treating diabetes insipidus and enuresis. The favourable pharmacodynamic profile of DDAVP compared to AVP and other mimetic synthetic compounds derives from its minimal effect on V1 vasopressin receptors (mediating vasoconstriction) and almost selective antidiuretic action, mediated by V2 receptors.

In a seminal publication 1977 Mannucci et al. showed that DDAVP can prevent bleeding during dental extraction and major surgery in patients with mild haemophilia A or von Willebrand disease (VWD). This haemostatic effect of DDAVP is mediated via activation of V2 vasopressin receptors on endothelial cells and intracellular cAMP-dependent signalling leading to exocytosis of von Willebrand factor (VWF) and tissue plasminogen activator (t-PA) from Weibel-Palade bodies. The rise in factor VIII activity (FVIII:C) is thought to be secondary to the rise of its carrier protein, VWF.

Subsequently, it has been observed that DDAVP is clinically efficacious in patients with various, generally mild disorders of the platelet function as well and, although data are still limited almost exclusively to case reports, its use is recommended for bleeding prophylaxis in patients at low personal bleeding risk. An enhanced hemostasis in patients with disorders of the platelet function might be mediated by the DDAVP-induced rise of circulating VWF high molecular weight multimers, leading to an increased platelet adhesion to the injured vessel wall; however, although this mechanism is biologically plausible it has not yet been proven. On the other hand, the documented efficacy of DDAVP in patients with type 3 VWD (lacking VWF in
endothelial stores\textsuperscript{12,13} and in patients with Bernard Soulier syndrome (lacking glycoprotein Ib, the platelet receptor for VWF)\textsuperscript{14,15} clearly indicates that additional mechanisms are responsible for the \textit{in vivo} haemostatic effects of DDAVP as well. In this regard, the lack of efficacy in patients with Glanzmann thrombasthenia\textsuperscript{16-18} indicates that DDAVP cannot substitute for a missing or severely dysfunctional fibrinogen receptor. Several hypothetical mechanisms have been proposed for explaining the clinical benefit of DDAVP in patients with platelet disorders, but their physiological role is uncertain.\textsuperscript{9,11}

The aim of the present work was to investigate the effect of \textit{in vivo} DDAVP administration on platelet adhesive and procoagulant functions. In particular, we have also investigated the impact of DDAVP on the generation of COAT platelets. This is a subpopulation of highly procoagulant platelets induced by combined activation with collagen and thrombin, and characterized by the expression of negatively charged aminophospholipids, surface retention of \(\alpha\)-granule proteins (such as factor V/Va and VWF) and preferential binding of factor Xa\textsuperscript{,19,20} whose potential clinical relevance is increasingly recognized.\textsuperscript{21}
Materials and Methods

Patients

We enrolled patients diagnosed with mild congenital platelet function defects at our institution receiving as a part of routine work-up an elective test dose of DDAVP. A diagnosis of primary platelet function disorder was made if the patient had not taken any drugs known to affect platelet function for at least 10 days before investigation, had normal platelet counts, an abnormal (but not absent) platelet aggregation to standard platelet agonists (ADP, arachidonic acid, collagen), an abnormal (but not absent) agglutination to ristocetin, and a positive bleeding history. Platelet aggregation abnormalities had to be confirmed in a blood sample drawn at a later time point.

Overall, we investigated 93 patients (69 women) with a median age of 42 years (interquartile range 27 – 52 years, range 18 – 77 years). The observed platelet aggregation defects were as follows: 38 patients had an impaired response to a single agonist (ADP n=32, collagen n=4, arachidonic acid n=1, ristocetin n=1), 28 patients had an impaired response to two agonists (ADP and collagen n=25, collagen and arachidonic acid n=3), 24 patients had an impaired response to three agonists (ADP, collagen and arachidonic acid), and 3 patients had an impaired response to all four investigated agonists. Because of their rarity, we did not include patients with Glanzmann thrombasthenia and Bernard-Soulier syndrome. The study was approved by the deputy ethical board and patients gave informed written consent. The study was conducted in accordance with the Declaration of Helsinki.
DDAVP

DDAVP (Octostim®, Ferring Pharmaceutical, Saint-Prex Lausanne, Switzerland) was infused at a dosage of 0.3 μg/kg body weight over 30 minutes in 100 ml 0.9% NaCl.

Blood sampling and coagulation assays

Whole blood samples were obtained before, 2 and 4 hours after DDAVP-infusion with a 19-gauge needle from the antecubital vein into three 10 ml plastic tubes each containing 1 ml 0.13 M trisodium citrate pH 5.5 for platelet aggregation studies, two 3.8 ml plastic syringes (Monovette, Sarstedt, Nümbrecht, Germany) containing 0.38 ml 0.129 M buffered citrate pH 5.5 for platelet function analyser (PFA) studies, and two 10 ml plastic syringes (Monovette, Sarstedt, Nümbrecht, Germany) containing 1 ml of 0.106 M trisodium citrate for flow cytometric and coagulation studies.

Analysis of coagulation parameters

Coagulation assays and VWF analysis were performed as described.23

Platelet function analyzer (PFA)

The PFA (PFA-100® and INNOVANCE® PFA-200™ System, Siemens Healthcare Diagnostics, Germany) is a tool for investigating von Willebrand factor and platelet function.24

Light transmission platelet aggregation tests (LTA) in platelet rich plasma (PRP)

LTA testing was performed as already described.25 Specifically, venous blood samples were collected into buffered 0.13 M trisodium citrate (9:1 blood-to-citrate ratio), transported to the laboratory at room temperature (RT) and processed immediately. PRP was prepared by centrifugation at 150g for 15 minutes at RT. The
platelet count was adjusted to 250 $\times 10^6$/ml with platelet poor plasma (PPP). The aggregometer (APACT 4004®, LABiTec GmbH, Ahrensburg, Germany) was then calibrated using a cuvette containing PRP (0% light transmission) and a second cuvette containing PPP (100%). Platelet aggregation was induced by increasing concentrations of four agonists. ADP (Sigma-Aldrich, St. Louis, MO, USA) was used at final concentrations of 4, 6, and 10 $\mu$M for male patients and concentrations of 3, 4, and 6 $\mu$M for female patients. Collagen (Horm®, Nycomed, Linz, Austria) was used at final concentrations of 1.5, 3, and 4 $\mu$g/ml. Arachidonic acid (Bio Data/Medonic Servotec AG, Interlaken, Switzerland) was set at 2 mM. Ristocetin (Socochim SA, Lausanne, Switzerland) concentrations were 1.5 mg/ml and 0.5 mg/ml. Two-hundred $\mu$L of PRP pre-warmed at 37°C for 1 min were added to the aggregometer cuvette and run for an additional minute in order to exclude spontaneous aggregation. Thereafter, 20 $\mu$L of the agonist were added and the response recorded. If the response to one agonist was out of normal range, the test was repeated once more either to confirm the result or to rule out technical failures. LTA started 1 hour after blood collection and was completed within 2.5 hours.

**Flow cytometric analysis of platelet function**

PRP from the patient and a normal donor was diluted to 10 $\times 10^6$ platelets/ml with Tyrode’s buffer. Analysis of surface glycoproteins (GP) Ibα (by a monoclonal anti-human CD42b antibody coupled with PE, Dako), GPIIb-IIIa (anti-hCD41-FITC and anti-hCD61-FITC, Becton Dickinson), baseline P-selectin expression (anti-CD62P-PE, Becton Dickinson) and PAC-1 binding (PAC1-FITC, Becton Dickinson) were performed in a 100 $\mu$l volume containing platelets at a final concentration of 5 $\times 10^6$/ml and combinations of relevant antibodies at saturating concentrations. After incubation in the dark for 15 min at RT, 1'000 $\mu$l of Tyrode’s buffer were added and platelets
were immediately analysed by flow cytometry (FACSCanto). Platelet reactivity, assessed by secretion of α-granules and activation of GPIIb-IIIa, was investigated with increasing concentrations of ADP (baseline and final ADP concentrations of 0.5, 5.0, and 50 μM), convulxin (5, 50, and 500 ng/ml), and thrombin (0.05, 0.5, and 5 nM) in a 100 μl volume containing platelets at a concentration of 5 x10⁶/ml, anti-CD62P-PE and PAC1-FITC. After incubation in the dark for 10 min at 37°C, 1’000 μl of Tyrode’s buffer were added to the platelets and the sample was analysed immediately. COAT platelets, expressing negatively charged aminophospholipids and retaining α-granule proteins on their surface, were induced by simultaneous activation with convulxin (500 ng/ml) and thrombin (5 nM) and detected by Annexin V-binding and in some experiments by co-staining with anti-FV/Va. Surface expression of negatively charged phospholipids was investigated with Annexin V-FITC (Roche) at baseline and after activation for 8 min at 37°C in the dark with either 2 μM Ionophore A 23187 (Sigma) or the combination of 500 ng/ml convulxin and 5 nM thrombin. Immediately prior to analysis platelets were diluted with 500 μl calcium-containing buffer. Finally, in order to evaluate content and secretion of dense granules, platelets were diluted to a final concentration of 5 x10⁶/ml with Hank’s buffer and loaded with mepacrine (final 0.17 and 1.7 μM) into a 100 μl volume for 30 min at 37°C in the dark. Secretion of dense granule was assessed after an additional 10 min incubation at 37°C with buffer versus 5 nM thrombin. Immediately prior to analysis platelets were diluted with 1’000 μl Hank’s buffer.

**Thrombin generation.**

Thrombin generation (TG) in platelet-rich plasma (PRP) was measured by calibrated automated thrombography (CAT) with commercial reagents as published by Hemker et al. In order to exclude the effect of plasmatic factors and therefore exclusively...
assess platelet-dependent TG we developed following modifications of the CAT method: gel-filtered platelets (GFP) were normalized to a concentration of 200 x10⁶/ml with 10 mM Hepes, 140 mM NaCl, pH 7.5 and re-suspended in factor V (FV) deficient human plasma. Specifically, 60 μl of GFP were added to 20 μl of working buffer containing (final concentrations) CaCl₂ (2 mM), MgCl₂ (1 mM), CVX (500 ng/ml) and thrombin (5 nM) in transparent round-bottom 96-well plates and incubated 2 min at 37°C in a Fluoroskan (Thermo Fisher Scientific AG, Reinach, Switzerland). Twenty μl of FV-deficient human plasma were added and the measurement was started after addition of 20 μl of FluCa (Fluca reagent, Thrombinoscope BV, Maastricht, The Netherlands). Each measurement was calibrated against the fluorescence curve obtained with a fixed amount of thrombin-α₂-macroglobulin complex (Thrombin calibrator, Thrombinoscope). A TG curve was obtained for 60 minutes using a dedicated software program (Thrombinoscope, Synapse BV, Maastricht, The Netherlands).²⁷

Visualization of intracellular free Ca²⁺ and Na⁺ changes by flow cytometry

We employed an in-house modification of the method described by Monteiro et al.²⁸ Eighty μl of PRP adjusted to 300 x10⁶ platelets/ml with PPP were pipetted into 720 μl Tyrode’s buffer in a 1.5 ml polypropylene microtube. After gentle mixing they were incubated with 1.6 μl 1 mM fluo-3 AM (invitrogen, Eugene, OR, USA) at a final concentration of 2 μM for 15 min at RT in the dark. Fifty μl of fluo-3 AM loaded platelets were then pipetted into 214 μl Tyrode’s buffer and 6 μl GPRP (Gly-Pro-Arg-Pro peptide, final concentration 2 μM) in a 5 ml round bottom polystyrene tube and acquired on a FACSCanto cytometer (Becton Dickinson) for 30 seconds at room temperature (baseline). Afterwards, platelets were activated by the addition of 30 μl agonist (final concentrations: convulxin 500 ng/ml and thrombin 5 nM) and acquisition
was resumed up to 6 minutes. In order to investigate cytoplasmic Na\(^+\) the same staining procedure was performed with CoroNa green (Sigma-Aldrich, St. Louis, MO, USA) at a final concentration of 5 \(\mu\)M in calcium-containing Tyrode’s buffer and platelet activated with convulxin and thrombin as described above. Results were analyzed by DIVA software (Becton Dickinson).

**Statistical analysis.**

Quantitative data are expressed as median, range and interquartile range. The 95% confidence interval (95%CI) for the proportions (p) was calculated by following equation: “p ± 1.96 x standard error (S.E.)”, where the S.E. was estimated by the quadratic square of \(p(1-p)/n\) (n = sample size). Quantitative data were analysed by non-parametric tests as required employing SigmaStat software (version 3.5; Systat Software Inc., San Jose, CA, USA). Significance was set at the 5% level.
Results

The effect of in vivo DDAVP on VWF, FVIII:C, and in vitro bleeding time

The efficacy of DDAVP (0.3 μg/kg body weight) was documented in all 93 patients by its ability to induce an increase in VWF and FVIII:C, and a shortening of the in vitro bleeding times assessed by PFA-system (Figure 1).

The effect of in vivo DDAVP on platelet aggregation

In order to evaluate whether infusion of DDAVP would also have a direct impact on platelet function we first tested platelet aggregation in platelet-rich-plasma (PRP) from 10 patients (6 women, median age 44 years, range 16 – 60 years) 4 hours after DDAVP. We assessed platelet aggregation 3 minutes after addition of increasing concentrations of ADP, collagen, arachidonic acid, and ristocetin. In addition we also recorded the time delay between agonist's addition and onset of the aggregation curve (lag phase) for collagen and arachidonic acid. At baseline, 4 patients had a blunted aggregation in response to a single agonist (ADP n=3, collagen n=1), one had a blunted aggregation in response to ADP and collagen, and 5 had a blunted aggregation in response to ADP, collagen and arachidonic acid. Table 1 and Figure 2 demonstrate that we did not observe any significant change between baseline and platelets drawn 4 hours after start of the DDAVP infusion.

The effect of in vivo DDAVP on surface expression of GPIb and GPIIb-IIIa

Subsequently, we investigated platelets by flow cytometry, comparing several phenotypic and functional characteristics before, 2 and 4 hours after DDAVP (n=16). Since we did not observe any significant difference at 2 versus 4 hours we pooled the data for these two time points. We observed a slight, statistically significant decrease of the fibrinogen receptor (Fig. 3A): GPIIb decreased by 10.6% (95% C.I.: -12.3 – -
1.3; p=0.006, Wilcoxon signed rank test; n=16) and GPIIIa decreased by 4.3% (95% C.I.: -9.9 – -0.5; p=0.044). We did not find a statistically significant change in GPIb surface expression, which showed a median decrease of -3.5% (95% C.I.: -8.2 – 3.3; p=0.323).

The effect of in vivo DDAVP on platelet dense granule
Figure 3B illustrates that both dense granule content (median change -0.01%; 95% C.I.: -5.0 – 13.7; p=0.322; n=16) and their thrombin-induced secretion (median change -0.03; 95% C.I.: -1.4 – 0.7; p=0.462) were not significantly affected by DDAVP.

The effect of in vivo DDAVP on α-granule secretion and GPIIb-IIIa activation
Secretion of platelet α-granules was assessed by P-selectin surface expression and agonist-induced activation of GPIIb-IIIa by PAC-1 binding. ADP-induced secretion of α-granules was significantly reduced hours after DDAVP (Fig. 3C), with a median decline of 35.7% (95% C.I.: -41.7 – -22.5; p<0.001; n=16) and ADP-induced activation of the fibrinogen-receptor (Fig. 3D) decreased by 15.4% (95% C.I.: -24.3 – -12.0; p<0.001). Similarly, thrombin-induced secretion of α-granules (Fig. 3C) decreased by 9.9% (95% C.I.: -15.5 – -4.0; p<0.001) and GPIIb-IIIa activation (Fig. 3D) declined by 8.2% (95% C.I.: -19.2 – -0.5; p=0.004). Finally, while convulxin-induced secretion of α-granules (Fig. 3C) significantly declined by 11.9% (95% C.I.: -20.6 – -2.7; p=0.019), the decrease in convulxin-induced GPIIb-IIIa activation (Fig. 3D) just did not reach statistical significance (median change -4.0%; 95% C.I.: -12.0 – -0.4; p=0.080). Of note, the levels of circulating platelets expressing P-selectin (median 0.9% before versus 0.8% after DDAVP; p=0.083, Wilcoxon Signed Rank
Test) or binding PAC-1 (median 1.3% before versus 0.8% after DDAVP; \( p=0.057 \), Wilcoxon Signed Rank Test) did not increase after DDAVP.

**The effect of in vivo DDAVP on COAT platelet generation**

COAT platelets were induced by simultaneous activation with convulxin (500 ng/ml) and thrombin (5 nM).\(^{19}\) After DDAVP, a relative increase of at least 5% COAT platelets was observed in 13 of the 16 patients (81%) initially investigated by flow cytometry. Among these patients median COAT platelets relative increase was 18.1% (95% C.I.: 11.1 – 45.2) 2 hours after DDAVP and 42.3% (95% C.I.: 19.5 – 74.8) 4 hours after DDAVP. Figure 4A illustrates a typical patient, in whom COAT platelets progressively increased from 15.9% to 30.6% 4 hours after DDAVP. We therefore assessed COAT platelet generation in 52 other patients. Overall, COAT platelets at baseline were 24.8% (IQR: 17.0 – 33.3) and significantly increased to 27.8% (IQR: 19.5 – 34.5) and 33.9% (IQR: 26.4 – 41.9) 2 hours and 4 hours after DDAVP, respectively (\( p<0.001 \), \( n=52 \)). Again, a relative increase in COAT platelet generation of at least 5% was not observed in all individual patients. Thirty-seven out of 52 patients (71%) showed an increase in COAT platelets of median 18.1 (95% C.I.: 13.8 – 27.3; \( p<0.001 \)) 2 hours after DDAVP (Fig. 4B). The proportion of responding patients increased to 45/52 (86.5%) at 4 hours, with a median COAT platelets relative increase of 32.9% (95% C.I.: 23.8 – 38.9; \( p<0.001 \)). Of note, the levels of circulating platelets binding Annexin-V did not increase after DDAVP (1.9% at baseline; 1.2% 2 hours and 1.4% 4 hours after DDAVP; \( p=0.052 \)).

**The effect of in vivo DDAVP on thrombin generation**

Thrombin generation (TG) induced by simultaneous platelet activation with convulxin and thrombin was measured by CAT\(^{27}\) in platelet-rich plasma (PRP) and in gel-
filtered platelets (GFP) reconstituted with factor V-deficient plasma (FV-DP) in 38 patients (Figure 5 and Online Supplemental Material). In PRP initial TG rate before DDAVP was median 10.2 nM/min (IQR: 7.0 – 13.5) and significantly increased by 49.2% (95% C.I.: 34.8 – 79.0) 4 hours after DDAVP to 14.1 nM/min (IQR: 9.6 – 18.3; p<0.001; n=38). This was expected as a consequence of DDAVP-induced increased in VWF and FVIII:C. In order to specifically assess platelet-dependent procoagulant activity, GFP at baseline and 4 hours after DDAVP were reconstituted with FV-DP and their ability to sustain TG was assessed. Figure 5 (GFP) demonstrates that platelet dependent initial TG rate significantly increased by 36.3% (95% C.I.: 27.5 – 49.0), from median 8.2 nM/min (IQR: 5.2 – 9.8) at baseline to 11.4 nM/min (IQR: 6.5 – 14.0; p<0.001; n=38) 4 hours after DDAVP. Of note, the level of circulating thrombin-antithrombin complexes did not increase after DDAVP (2.6 μg/L at baseline; 2.2 μg/L 2 hours and 2.1 μg/L 4 hours after DDAVP; p=0.065).

Intracellular free Ca$^{2+}$ changes and COAT platelet generation after DDAVP

In order to explore the mechanisms involved in the formation of procoagulant COAT platelets we studied intracellular free Ca$^{2+}$ flux. Figures 6A and 6B show the rapid and sustained increase of intracellular Ca$^{2+}$ observed in native platelets up to 6 minutes after simultaneous activation with convulxin and thrombin. Among patients in whom DDAVP induced a significant increase of COAT platelet generation we observed an enhanced ability to mobilize Ca$^{2+}$ after DDAVP compared to baseline (Figures 6C and 6D). On the other hand, in patients among whom COAT platelets remained unchanged, Ca$^{2+}$ mobilization after DDAVP was lower than at baseline (Figure 6D).
Intracellular Na\(^+\) changes and COAT platelet generation after DDAVP

Similar experiments performed with CoroNa green showed a slower and less marked increase of cytosolic Na\(^+\), peaking at 2 to 4 minutes after activation with convulxin and thrombin (Figure 7A and 7B). This transient intracellular Na\(^+\) increase was enhanced by DDAVP among individuals reacting with an increased COAT platelet generation, while remained unchanged in those patients whose COAT platelet generation was unaffected by DDAVP (Figure 7C).
Discussion

We investigated the effect of an *in vivo* DDAVP infusion, at a standard dose of 0.3 μg/kg body weight, on different aspects of platelet function in patients with congenital mild platelet function defects.

Our data are consistent with previous reports indicating that DDAVP administration does not induce by itself platelet activation.\textsuperscript{18,30} In fact, surface expression of P-selectin and negatively charged phospholipids, and binding of PAC-1 (a monoclonal antibody recognising an activation-dependent epitope on the fibrinogen receptor, GPIIb-IIIa) did not change after DDAVP. Additionally, we did not observe signs of *in vivo* increased thrombin generation, as circulating TAT-complexes did not increase after DDAVP-infusion.

Second, our results summarized in Table 1 and Figure 2 are in line with several reports failing to observe an enhanced agonist-induced platelet aggregation after DDAVP infusion.\textsuperscript{5,31-33} However, our data appear to be in contrast with one study published by Balduini et al. reporting an enhancement of collagen- and ADP-induced platelet aggregation.\textsuperscript{18} Beside different experimental conditions (whole blood anticoagulated with sodium heparin versus buffered citrate), the main reason for the discrepant finding may rely in the time course of the effect. As depicted in Figure 2 of their publication, Balduini et al. observed a rapid increase in agonist-induced platelet aggregation which subsequently declined and lost statistical significance 3 hours after DDAVP start.\textsuperscript{18} We investigated platelet aggregation 4 hours after DDAVP infusion and from this perspective the data from both publications are congruent. Noteworthy, the effect of DDAVP on VWF appears to have a similar time-dependent course (Figure 1) and it may be possible that an early, transient enhancement in
GPIIb-IIIa dependent platelet aggregation may be mediated by the higher levels of circulating VWF in the patient plasma. Three observations are in line with this hypothesis. First, De Marco et al. showed that in vivo DDAVP administration improved ADP-induced platelet aggregation in two afibrinogenemic patients but that this effect was lost at 4 hours. Second, several publications demonstrate that VWF interaction with GPIIb-IIIa can mediate platelet-platelet contacts. Third, Kasirer-Friede et al. demonstrated that clustering of GPIb-IX complexes by VWF up-regulates the adhesive functions of GPIIb-IIIa.

Moreover, our data do not support a direct pro-aggregant effect of DDAVP on platelet function. On the contrary, investigating with flow cytometry platelet activation induced by graded concentrations of ADP, thrombin and convulxin we observed a decreased α-granule secretion (Figure 3C) and PAC-1 binding (Figure 3D) after DDAVP. It is known that DDAVP promotes formation of cAMP in endothelial cells and in platelets as well. Therefore the above mentioned observations are likely to be the consequence of the well-known inhibitory effect of increased cAMP levels in platelets on their function.

In summary, our data are in line with the concept that DDAVP does not exert a direct pro-aggregant effect on platelet function but that, by means of the transient increase of VWF, it may enhance agonist induced platelet aggregation. This effect appears to be mediated by direct interaction of VWF with GPIIb-IIIa and not with GPIb-α.

The most interesting observation of our study is the ability of in vivo DDAVP infusion to enhance the generation of procoagulant COAT platelets (Figures 4). COAT
platelets are a subpopulation of platelets generated by simultaneous activation with collagen and thrombin. Only these activated platelets express negatively charged phospholipids and high levels of $\alpha$-granule factor V/Va on their surface, are able to bind exogenous factor Xa, and are most efficient in sustaining thrombin generation. We named this subset of Collagen And Thrombin activated platelets as “COAT platelets” in order to convey the concept of their surface being covered by a layer, a coat of adhesive and procoagulant proteins.

In the present work we show a progressive increase in COAT platelets 2 and 4 hours after DDAVP infusion (Figure 4B). Since COAT platelets are procoagulant we also investigated their functional relevance by assessing the ability to sustain thrombin generation. As expected and already published by Keularts et al. DDAVP infusion improves, by means of increased VWF-levels, thrombin generation in PRP (Figure 5 and Online Supplemental Material). We therefore modified the CAT assay in order to specifically assess platelet-dependent thrombin-generation (see Methods Section). As depicted in Figure 5, after DDAVP we observe a significant rise in platelet ability to sustain thrombin generation, paralleling the increased percentage of COAT platelets and documenting their functional relevance.

These data are in line with a report by Horstman et al. indicating increased platelet microparticle formation and procoagulant activity after DDAVP. Our work expands this previous observation on two aspects. First, while Horstman et al. investigated blood samples of six patients before and one hour after DDAVP, we analysed a total of 68 patients before, 2 and 4 hours after DDAVP. Our data provide solid evidence for an enhanced procoagulant activity and show that this effect increases up to 4 hours after DDAVP (Figure 4), a time point at which the VWF-increase and
related effects are already declining (Figure 1). Second, since Horstman et al. evaluated procoagulant activity by a Russell’s viper venom assay in plasma samples (platelet-rich, platelet-poor, and particle-filtered plasma)\textsuperscript{42} their results may be partly explained by the DDAVP-induced increase in VWF and FVIII:C.\textsuperscript{29} Our work provides clear evidence that DDAVP promotes a specific enhancement of platelet-dependent thrombin-generation (Figure 5).

Which mechanisms may be involved in the procoagulant DDAVP effect? The observation that DDAVP-induced enhancement of COAT platelet generation still increases when plasmatic VWF and FVIII-levels are already declining suggests that the procoagulant effect may be mediated by intracellular signaling events distinct from the rapid rise in cAMP production leading to VWF exocytosis from endothelial Weibel-Palade bodies.\textsuperscript{3,43} Studies performed in cortical and medullary renal tubules indicate that stimulation of V2 receptors induces several intracellular signaling events which take longer than cAMP production and are responsible for the sustained (up to 12 hours) antidiuretic effect following DDAVP administration.\textsuperscript{44} Several proteins become phosphorylated upon DDAVP action\textsuperscript{45,46} and some of them are involved in Na\textsuperscript{+} retention.\textsuperscript{47,48} Of note, an increase in intracellular Na\textsuperscript{+} has been shown to promote an increase of intracellular Ca\textsuperscript{2+} \textsuperscript{49,50} and could thus facilitate an agonist-induced, high and prolonged rise in intracellular Ca\textsuperscript{2+} required for the exposure of negatively charged aminophospholipids on the platelet surface.\textsuperscript{51} Our experiments visualizing intracellular Ca\textsuperscript{2+} and Na\textsuperscript{+} mobilization (Figures 6 and 7) suggest that DDAVP may modulate the ability to generate COAT platelets by enhancing both Na\textsuperscript{+} and Ca\textsuperscript{2+} intracellular fluxes.
Although pathways underlying the sustained cytosolic Ca\textsuperscript{2+} increase required for phosphatidylserine expression on the platelet surface involve extracellular Ca\textsuperscript{2+} influx through Ca\textsuperscript{2+} channels, the mechanisms are still not fully elucidated. Recent work points to a peculiar role of Na\textsuperscript{+} influx, in particular when platelets are simultaneously activated by collagen and thrombin\textsuperscript{52,53}. Indirect evidence for this comes also from the observation that a reduction of extracellular Na\textsuperscript{+} inhibits exposure of phosphatidylserine after platelet activation with convulxin and thrombin\textsuperscript{54}.

DDAVP may facilitate an increased intracellular Na\textsuperscript{+} concentration by interacting with Na\textsuperscript{+} pumps, such as the Na\textsuperscript{+}/H\textsuperscript{+} exchanger, the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger, and/or the Na\textsuperscript{+}/Cl\textsuperscript{-} cotransporter. The involvement of the Na\textsuperscript{+}/H\textsuperscript{+} exchanger has been proposed by Tomasiak et al., who working with \textit{in vitro} DDAVP concentrations ranging from 100 to 500 nM demonstrated a Na\textsuperscript{+}/H\textsuperscript{+} exchanger-induced increase in mean platelet volume (MPV) and platelet procoagulant activity\textsuperscript{55}. Among our patients we observed a slight, statistically significant decrease of the MPV 4 hours after DDAVP (7.9 fl at baseline, 7.9 fl 2 hours, and 7.7 fl 4 hours after DDAVP; p=0.045, n=93). This could be explained by the fact that after an \textit{in vivo} infusion (0.3 µg/kg b.w.) the expected pharmacological DDAVP concentration is around 1-5 nM\textsuperscript{18}, much lower than the concentrations tested by Tomasiak et al\textsuperscript{55}. We are not aware of publications evaluating the impact of DDAVP on other platelet Na\textsuperscript{+} channels. However, the transient nature of the observed Na\textsuperscript{+} increase (Figures 7 A-B) suggests a role for the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger\textsuperscript{52,53}.

In conclusion, this is the first report showing that \textit{in vivo} administration of DDAVP selectively and markedly enhances the ability to form procoagulant COAT platelets and increases platelet-dependent thrombin-generation up to 4 hours after infusion.
Our work indicates that the beneficial haemostatic effect of DDAVP is not limited to an increase in large VWF multimers and consecutive FVIII:C increment. An enhancement of platelet procoagulant activity is an additional and – at least in platelet disorders – possibly clinically relevant mechanism of DDAVP action. DDAVP appears to increase COAT platelet formation by facilitating intracellular Na\(^+\) flux and the subsequent sustained increase of cytosolic free Ca\(^{2+}\).
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**Author contributions**

GC performed research, analysed the data, and wrote the manuscript

MS performed research

SR performed research

TC performed research

MP performed research

MR performed research

EG performed research

AH performed research

CT performed research and wrote the manuscript

PK performed research and wrote the manuscript

LA designed research, analysed the data, and wrote the manuscript

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We like to thank professor Bernhard Lämmle for his enthusiastic support and intellectual contributions to this research project, Gabi Barizzi for her thoughtful and competent supervision of platelet function investigations, and Therese Jost for her outstanding administrative assistance.

**Conflicts of interests**

The authors do not have any conflict of interest.
Table 1. Impact of DDAVP on platelet aggregation (n=10).

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<th>Agonist</th>
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<th>Parameter</th>
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<th>4 hrs after DDAVP</th>
<th>P value *</th>
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<td></td>
<td></td>
<td>median</td>
<td>IQR</td>
<td>median</td>
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<td>ADP</td>
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<td>16-48</td>
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<td>10-41</td>
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<tr>
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<td>52</td>
<td>39-71</td>
<td>61</td>
<td>45-77</td>
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<tr>
<td></td>
<td>High</td>
<td>73</td>
<td>57-81</td>
<td>75</td>
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<td>Collagen</td>
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<td>4.0 µg/ml</td>
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<tr>
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<td>96</td>
<td>90-98</td>
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</table>

* Statistics: Wilcoxon signed rank test. IQR: interquartile range
Figure Legends

Figure 1. Effect of *in vivo* DDAVP on VWF, FVIII, and PFA-100

Whole blood was drawn before, 2 and 4 hours after start of the DDAVP infusion in 93 patients with congenital platelet function defects. **Panel A:** Median VWF:RCo increased from 77% (IQR: 56 – 104) to 236% (IQR: 186 – 292) 2 hours after DDAVP and declined to 187% (IQR: 154 – 248) at 4 hours (p<0.001, Friedman RM ANOVA); **Panel B:** Median VWF:Ag increased from 80% (IQR: 63 – 112) to 210% (IQR: 163 – 251) and 175% (IQR: 142 – 228) 2 and 4 hours after DDAVP, respectively (p<0.001); **Panel C:** FVIII:C increased from 89% (IQR: 70 – 115) to 204% (IQR:169 – 242) and 167% (IQR:138 – 205), respectively (p<0.001); **Panel D:** PFA-system, epinephrine/collagen. The closure time shortened from 159 sec (IQR: 125 – 211) to 91 sec (IQR: 77 – 105) 2 hours after DDAVP and slightly lengthened to 109 sec (IQR: 90 – 129) at 4 hours (p<0.001); **Panel E:** PFA-system, ADP/collagen. The closure time with the ADP/collagen cartridge shortened from 101 sec (IQR: 86 – 123) to 61 sec (IQR: 54 – 70) and 66 sec (IQR: 60 – 82), respectively (p<0.001). Box plots visualize the median value (horizontal line within the box), the 25th and 75th percentiles (lower and upper borders of the box), the 10th and 90th percentiles (lower and upper whiskers), and each outlier outside the 10th and 90th percentiles (open circles).

Figure 2. Effect of *in vivo* DDAVP on platelet aggregation in platelet-rich plasma

Whole blood was drawn before and 4 hours after start of the DDAVP infusion in 10 patients with congenital platelet function defects. **Panel A:** ADP-induced platelet aggregation at 3 minutes; **Panel B:** Lag phase of collagen-induced platelet aggregation; **Panel C:** Collagen-induced platelet aggregation at 3 minutes; **Panel D:**
Lag phase of arachidonate-induced platelet aggregation; **Panel E**: Arachidonate-induced platelet aggregation at 3 minutes; **Panel F**: Ristocetin-induced platelet agglutination at 3 minutes. See Methods Section for details on agonists concentrations. Changes were not statistically significant (see Table 1 for p values).

**Figure 3. Effect of DDAVP on platelet surface glycoproteins, dense-granule content, agonist-induced α-granule secretion and GPIIb-IIIa activation**

Whole blood was drawn before, 2 and 4 hours after start of the DDAVP infusion in 16 patients with congenital platelet function defects (results at 2 and 4 hours after DDAVP were similar and were therefore pooled). **Panel A**: surface density of GP IIb (CD41; p=0.006, Wilcoxon signed rank test; n=16), GP IIIa (CD61; p=0.044), and GPIb (CD42b; p=0.323); **Panel B**: Dense-granule content (p=0.322) and their thrombin-induced secretion (p=0.462); **Panel C**: Secretion of α-granules induced by ADP (p<0.001), thrombin (p<0.001), and convulxin (p=0.019); **Panel D**: Activation of GPIIb-IIIa induced by ADP (p<0.001), thrombin (p=0.004), and convulxin (p=0.080).

**Figure 4. Effect of DDAVP on COAT platelet formation**

**Panel A**: COAT platelets at baseline (15.9%), 2 hours (24.5%), and 4 hours (30.6%) after DDVP in a typical patient. For comparison COAT platelets of the normal control (22.3%). **Panel B**: Relative COAT platelet increase in responding patients (n=37/52 at 2h; n=45/52 at 4h; p<0.001 for both comparisons).

**Figure 5. Effect of DDAVP on thrombin generation**

Panel A: Initial thrombin generation rate in platelet-rich plasma (PRP) and gel-filtered platelets (GFP) reconstituted with factor V-deficient plasma (FV-DP) at baseline and 4 hours after DDAVP (p<0.001 for both comparisons, Wilcoxon signed rank test;
n=38). Panel B: Relative increase of the initial thrombin generation rate (iTGR) in PRP and GFP/FV-DP at 4 hours compared to baseline (p<0.001 for both comparisons, Wilcoxon signed rank test; n=38).

Figure 6. Effect of DDAVP on intracellular free Ca$^{2+}$ concentration after simultaneous platelet activation with convulxin and thrombin

Panel A: Intracellular Ca$^{2+}$ course in a representative patient before DDAVP administration (P1: baseline; P2: <30 sec; P3: 1 min; P4: 2 min; P5: 4 min; P6: 6 min after simultaneous platelet activation with convulxin and thrombin). Panel B: The figure summarizes intracellular Ca$^{2+}$ course in 15 patients at baseline (median fold-increase, 95% C.I.). Panel C: Intracellular Ca$^{2+}$ after simultaneous platelet activation with convulxin and thrombin in a representative patient after DDAVP administration. Panel D: The figure depicts the relative intracellular Ca$^{2+}$ course after DDAVP compared to baseline (median fold-change, 95% C.I.) in patients with enhanced COAT platelet generation (median increase 29%, n=11) and those without (n=4).

Figure 7. Effect of DDAVP on intracellular Na$^+$ concentration after simultaneous platelet activation with convulxin and thrombin

Panel A: Intracellular Na$^+$ course in a representative patient before DDAVP administration (P1: baseline; P2: <30 sec; P3: 1 min; P4: 2 min; P5: 4 min; P6: 6 min after simultaneous platelet activation with convulxin and thrombin). Panel B: The figure summarizes the intracellular Na$^+$ course in 5 patients at baseline (median fold-increase, 95% C.I.). Panel C: The figure depicts the relative intracellular Na$^+$ course after DDAVP compared to baseline (median fold-change, 95% C.I.) in patients with enhanced COAT platelet generation (median increase 39%, n=3) and those without (n=2).
Figure 1D

![Box plot showing FPA-100 EPICollagen (Closure time, sec) before, 2h after, and 4h after DDAVP treatment.]

Figure 1E

![Box plot showing FPA-100 ADPCollagen (Closure time, sec) before, 2h after, and 4h after DDAVP treatment.]

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Figure 2A

ADP-induced platelet-aggregation (%)

before  after

DDAVP

Figure 2B

Collagen - Lag phase (sec)

before  after

DDAVP
Figure 2E

Figure 2F

Arachidonate - Lag phase (sec)

before
after

DDAVP

Ristocetin-induced platelet-aggregation (%)

before
after

DDAVP
Figure 3D
Figure 4A

**Patient, Baseline (15.9%)**

**Normal control (22.3%)**

**Patient, 2h after DDAVP (24.5%)**

**Patient, 4h after DDAVP (30.6%)**

Figure 4B

**DDAVP-reversed clotting, COAT data (% increase)**

**Time after DDAVP**

0  2h  4h
Figure 6D

[Graph showing data points and error bars over time with labels for baseline, <30 sec, 1 min, 2 min, 4 min, 6 min after CVX/Thr.

- Black dots with error bars: COAT platelets increased after DDAVP.
- Gray dots with error bars: COAT platelets unchanged after DDAVP.

Y-axis: COAT (Relative fold change compared to pre-DDAVP).
X-axis: Time after CVX/Thr (baseline, <30 sec, 1 min, 2 min, 4 min, 6 min).]
Figure 7A

Figure 7B

Figure 7C
The effect of desmopressin on platelet function: a selective enhancement of procoagulant COAT-platelets in patients with primary platelet function defects

Giuseppe Colucci, Monika Stutz, Sophie Rochat, Tiziana Conte, Marko Pavicic, Marianne Reusser, Evelyne Giabbani, Anh Huynh, Charles Thürlemann, Peter Keller and Lorenzo Alberio

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