

Platelet Function and Microvesicles Generation in Patients with Hemophilia A

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ABSTRACT

Aim: In the present work we have studied the role of platelets and microvesicles in patients with severe hemophilia A (HA) treated under the regimen of prophylaxis. We have analyzed whether the administration of coagulation factor FVIII modifies this hemorrhagic phenotype in a cohort of 16 patients with diagnosis of severe HA, who were on prophylactic treatment with recombinant FVIII.

Methods: Blood tests were performed before (72h without FVIII, baseline sample) and after 15 minutes of FVIII infusion. As a control group, 15 healthy subjects were studied. Platelet aggregation was determined by closure time, optical aggregation, impedance aggregation and flow cytometry. We also studied the expression of the platelet activation markers P-selectin, CD63, platelet-tissue factor, formation of platelet-leukocyte aggregates and tissue factor exposure. The total number of platelet and endothelial microvesicles were also analyzed by flow cytometry, as well as platelet cytosolic Ca²⁺ mobilization.

Results: We found no significant differences in platelet function in patients with severe HA in prophylactic treatment before and after FVIII infusion. After FVIII administration, patients presented fewer endothelial microvesicles, indicating that the treatment does not increase one of the possible thrombotic risk markers of these patients. The total amount of plasma microvesicles and the platelet microvesicles were decreased in patients with HA compared to the control group.

Conclusions: Our results do not support any effect of FVIII on platelet function in patients with severe HA treated under the regime of prophylaxis.

INTRODUCTION

Hemophilia A (HA) is a recessive hemorrhagic disease linked to the X chromosome, characterized by reduced levels of clotting factor VIII (FVIII). According to factor VIII levels, HA is classified as severe (less than 1%), moderate (1-5%) or mild (6-30%). In severe and moderate forms, the disease is characterized by hemorrhagic episodes on the joints (i.e., hemarthrosis), soft tissues and muscles after minor trauma or even spontaneously (1). Although the lack of FVIII is the main factor predisposing to the disease, the differences in bleeding phenotype could be related to several factors that may influence clinical presentation and response to treatment in patients with HA (2).

Among these factors, the role of platelets and their associated microvesicles expressing tissue factor on their membrane has been considered to be important (3). Moreover, several studies have also reported alterations in platelet function that challenged the classical view maintaining that platelet function is normal in hemophilic patients (4-5). However, the association between platelet function and HA is far from clear and a recent review has discussed the possible existence of a publication bias to favor only publication of studies with positive results (6).

The present work is directed to the investigation of some modulating factors of the hemorrhagic phenotype in patients with severe HA treated under the regimen of prophylaxis. Among the modulating factors that we are going to study are the platelets and microvesicles. The main objective of the study is to analyze if the administration of FVIII modifies this hemorrhagic phenotype, comparing a baseline sample (washing period / 72h without factor VIII) with respect to a sample 15 minutes after administration of FVIII.

METHODS

Study design.

We have performed a case/control study conducted between July 2015 and April 2016. The project was approved by the Ethics Committee of Clinical Research (CEIC) of the Hospital Universitario Virgen de la Arrixaca (Murcia, Spain) with the reference number 2013-10-1-HCUVA. The rules of Good Clinical Practices in Research and the ethical guidelines of the 1975 Declaration of Helsinki (1983 Revision) have been fully respected. Informed consent was obtained from all the patients, selected from the database of the Hemophilia Unit of the Region of Murcia with severe HA diagnosis and who were in treatment prophylactic with recombinant FVIII at the time of the study. The inclusion and exclusion criteria can be consulted in the supplementary document. Control subjects were obtained from healthy coworkers and doctors who voluntarily agreed to participate.

Sixteen patients with severe HA were included in the study. Although this may be considered as a low sample, these were all the patients available in our area at the time of the study. It is important to remember that HA is a rare disease.

Human samples and reagents.

Blood samples were obtained before (baseline sample, 72h without administering factor VIII) and after infusion of factor VIII (15 minutes after). This time was selected following a previous report of the National Hemophilia Foundation (7). They were always extracted by the same expert nurse, with a 21G needle without the use of compressor or tourniquet, to minimize platelet stimulation. A total of 18 mL were extracted in each subject. The first 2.5 ml were used for measurements other than platelet function, i.e. hemogram, following the recommendations of the International Society on Thrombosis and Haemostasis (8). The rest of blood was distributed as indicated in the supplementary document. Briefly, we determined platelet count, measurement of factor VIII, von Willebrand antigen and cofactor von Willebrand, platelet function by impedance using the Roche Multiplate analyzer system, platelet-related primary hemostasis using the PFA-100 System, platelet aggregation by means of light optical aggregometry, flow cytometry for aggregation, activation and platelet-leucocytes conjugates and platelet cytosolic calcium. Finally, microvesicles were also analyzed in a Gallios flow cytometer in the Laboratory of Flow Cytometry-Coulter Cytometry Center and Related Techniques in Valencia.

The conceptual basis for performing so many different techniques to measure platelet function is that although each one is different, all of them are different from each other, having their advantages and disadvantages, and analyzed differently, with different nuances, sometimes even from very different angles. Therefore, we decided to perform a comprehensive battery of platelet function tests, since it is not known how hemophilia or FVIII interferes with them. Thus, by using a wide spectrum of techniques we will have more possibilities of detecting any change that hemophilia or the administration of FVIII could produce on platelet function, as it is done to assess the efficacy of new antiplatelet agents (9) or to assess platelet function in certain pathologies or circumstances (10) The value, importance and relevance of each of these techniques are very well detailed elsewhere (10-11).

Statistics.

Statistical analysis was performed with SPSS 20.0 computer software (SPSS Inc., Chicago, IL, USA). To compare the quantitative variables, the Student's t-test was used. Cytosolic Ca²⁺ was analyzed using a one-way analysis of variance followed by multiple Tukey-Kramer comparisons. Data are expressed as the mean and the standard deviation. A p level lower than 0.05 was used to indicate a significant difference.

RESULTS.

General features of HA patients.

Descriptive data are shown in Table 1 (suppl). The most frequent genetic mutation was the reversal of intron 22, which was detected in 9 patients (56.25%). The inversion of intron 1 was detected in one patient, mutations of type missense in two patients (one with involvement of exon 23 of the F8 gene (p.Pro2153Leu), affectation of exon 7 (p.Phe276Leu) in another one and finally a nonsense mutation in exon 18 (p.Arg1966X) in another patient. In 3 patients (18.75%), the study had not been carried out yet. Five patients were positive for hepatitis C virus (HCV) and 3 of these were also positive for HIV. The weekly

consumption of FVIII was of 6.78 ± 3.26 IU, with 9 patients receiving it 3 times a week and 6, every two days. Only one patient received it twice a week. Regarding inhibitors, 11 patients had never presented them and 5 patients have had inhibitors at some point in their lives. No inhibitor was detected in the last year prior to the study in any patient. Although 10 patients only had HA, 3 patients with HIV were in treatment with triple antiretroviral therapy, with good analytical controls and different stages of the disease, one patient had autism and one patient, epilepsy (both in treatment with risperidone, aripiprazole and/or sodium valproate) and another one had asthma with occasional treatment with antihistamines. The HCV positive patients had all been treated for years with interferon; at the moment of the study, none of them was under treatment for different reasons: undetectable copies of viral RNA (in two patients), poor tolerance to the drug and no liver fibrosis greater than 2. Two patients were smokers. A single 9-year-old patient with mental retardation was a carrier of Porth-A-Cath. As negative controls, we selected 15 healthy subjects of a range age between 26 and 48 years with a median of 36 years. Three of them were smokers.

Blood analysis data.

The hemogram of controls and patients before and after treatment with FVIII is shown in Table 2 (suppl). There were no differences between controls and patients in any variable, although leucocytes and lymphocytes number was significantly lower after administering FVIII to HA patients. Similarly, mean platelet volume was also significantly decreased in FVIII-treated HA patients. The data obtained in the special coagulation study are shown in Table 3 (suppl). As expected, we observed statistically significant differences in the amount of FVIII before and after FVIII infusion, as well as with control subjects ($p < 0.001$). In the rest of the parameters, VWF:Ag and VWF:Rco, no significant differences were observed between groups.

Platelet function.

The data obtained in the PFA-100 study are shown in Table 4 (suppl). No statistically significant differences were observed between the groups. Similarly, when platelet aggregation function was analyzed with Chronolog, there were no significant differences between groups (suppl, Table 5). Equally, after analysing platelet function in complete blood with the Multiplate technique, no significant differences were found among groups (suppl, Table 6), except for a lower aggregation percentage in patients after the infusion with FVIII and the controls, when TRAP was used. Finally, the aggregation study and platelet activation markers expression, platelet-leukocyte aggregates and tissue factor exposure performed by flow cytometric analysis did not reveal any significant differences between groups (suppl, Table 7). Regarding calcium levels, both agonists, TRAP and ADP, elevated cytoplasmic calcium in all three groups but there were no significant differences between them (Figure 1). Although calcium release was lower in patients when TRAP was used, the analysis of the area under the curve of the calcium responses gave no statistical significance (Figure 2).

Study of plasma microvesicles (MVs).

There was a lower number of total MVs in HA patients as compared to the controls and the administration of FVIII eliminated these differences (suppl, Table 8). The number of CD62+ MVs was greater in the controls than in both

samples of patients. Among patients, the number of MVs of endothelial origin (CD144+) decreased significantly after the infusion of FVIII. In relation to age in patients (suppl, Table 9), there were no significant differences in the total number of MVs or in MVs of platelet origin. However, a decrease in MVs of endothelial origin (CD144+) was observed after infusion of FVIII in the younger (<18 years) patients (suppl, Table 9).

DISCUSSION

In our study, performed in patients with severe HA in prophylactic treatment, platelet function was essentially normal and did not change after the infusion of FVIII. We will discuss these data sequentially.

Hemogram.

Although most hematological parameters were completely normal, we found that administration of FVIII resulted in a lower mean platelet volume in the HA patients and the reason is at present unknown. Since MPV was not elevated basally in these patients, we can rule out the possibility of a possible elevation of platelet volume to compensate for the deficit of FVIII. Although the reduction of MPV after FVIII administration was very modest, we are not certain about the clinical significance of this acute effect. More studies would be necessary to elucidate this aspect.

As expected, our patients showed a very low plasma FVIII levels which were normalized after its infusion. However, neither VWF:Ag nor VWF:Rco exhibited differences with the control values, which is in agreement with data reported previously (12).

Platelet function.

Platelet function was essentially normal, as suggested by the results obtained with different methodologies. With the use of PFA-100, a tendency to the increase in the obturation time (OT) was observed in patients receiving FVIII, but no significant differences were obtained. There are mixed results in the literature, both with an increase in the baseline OT (13-15) or no differences in patients with HA (16-19). However, we believe our data are the first to show that the administration of FVIII to HA patients does not change OT.

Regarding the Multiplate system, we believe that our study is the first one to use it in HA patients. A significant reduced aggregation was observed between patients after administration of FVIII and the controls in response to TRAP. A data that may be of interest since this decrease was observed clearly in 5 patients, 4 of which were VHC+ and 2 of them HIV+. A reduced platelet function has been also described in the hepatitis C virus infection (20-21), and this possibility clearly merits further study.

The turbidimetric platelet aggregometry remains the gold standard for the diagnosis of platelet function disorders, and our data are also the first to explore it in HA patients. Again, no significant changes were observed after the administration of FVIII in comparison to the baseline or control values.

The expression of P-selectin (CD62P) and CD63, and an increase in the number of platelet-leukocyte conjugates or in the exposure of tissue factor are related to a variety of pathologies with elevated thromboembolic risk. Moreover, P-selectin is considered the gold standard for platelet activation. Our study of flow cytometry, however, could not find a significant difference between groups.

There are conflicting results in the literature, even from the same laboratory (22-24), but it seems that our results agree with most of these studies, both in human patients (23, 25) and mice (24), thus suggesting that there is no platelet activation in patients with severe HA.

Calcium study.

Intraplatelet free Ca^{2+} has been used in the functional study of platelets and in the monitoring of therapies with platelet antagonists (26). In our study, we observed, in all groups, a rapid increase in intracellular calcium and a subsequent decrease in the signal after 30 seconds, although without returning to the previous basal situation, before addition of the agonist. In our study, we used thrombin (more potent physiological platelet activator) and ADP (weaker physiological platelet agonist), which are the most frequently used agonists (27-27), without observing significant differences among the groups studied. However, in the case of ADP, the level of calcium was similar between the control group and the hemophilic patients after infusion of FVIII, although, it was lower in the baseline sample of patients. When TRAP was used as an agonist. it was found that the controls presented a higher response to calcium release than patients (both before and after the administration of factor VIII). Perhaps these results indicate that patients have a lower level of extracellular Ca^{2+} available to be used when the agonist acts at not very high concentrations, although, in our study, these differences were not statistically significant. To the best of our knowledge, this is the first study in which intra-platelet calcium is analyzed in patients with hemophilia. In conclusion, we have not observed significant differences in the response of intraplaquetary Ca^{2+} to ADP or TRAP stimulation in relationship with the infusion of FVIII compared to a cohort of healthy subjects.

Microvesicles.

Microvesicles (MVs) are small membrane particles of 0.1–1.0 μm shed by either activated or apoptotic cells (28) in response to a variety of stimulatory factors, and they can originate from platelets, among other cells (29), playing a important role on hemostasis and thrombosis (28). The data obtained in our study showed significant differences between HA patients and controls, with untreated patients having a lower amount of total MVs than the controls. We also observed a lower number of CD62+ MVs in patients, both in the baseline sample and after the infusion of FVIII in comparison to the controls. Our patients showed a lower amount of CD144+ microvesicles after the infusion of FVIII, a decrease which was also observed in the study of Mobarrez et al. (30). It is likely that this decrease may be related to the inclusion of these MVs in the platelet thrombus.

Differences in the number of MVs with respect to age have also been described (31), but we did not observe statistically significant differences in the number of MVs according to the age of patients, except for a lower number of endothelial MVs in the younger patients before FVIII administration.

In our study we did not observe statistically significant differences in the number of total MVs and those of platelet origin between patients (before and after FVIII administration). The group of patients in our study was a homogeneous group with respect to their underlying disease, that is, they all have severe HA. Our results are in agreement with preliminary data that could

not find differences in the number of platelet MVs in patients with severe HA (32-33). However, Artoni et al. (34) observed more platelet-derived MVs in patients with severe HA. In these three papers, the measurement of the MVs or the cytometer used was not specified, thus it is difficult comparing it with our results.

Conclusions

In our study, performed in patients with severe Hemophilia A in prophylactic treatment, platelet function was essentially normal and did not change after the infusion of FVIII. The amount of total microvesicles and those of platelet origin are significantly decreased in patients with Hemophilia A compared to healthy subjects. The administration of FVIII was accompanied with a lower amount of microvesicles of endothelial origin, suggesting that FVIII treatment does not increase the thrombotic risk in these patients. Our results do not support the use of platelet aggregation studies in patients with severe Hemophilia A.

Acknowledgments

We would like to thank the financial support from the Pfizer company to carry out this project.

Author's contributions

Dr. Melero, Dr. Romecín and Dr. Iyú performed all the experiments except those done at the Laboratory of Flow Cytometry-Coulter Cytometry Center and Related Techniques in Valencia, Dr. García-Bernal performed the calcium experiments, Ms. García-Navarro was the technician lab responsible for the optimal maintenance of all the equipment and reagents, Dr. Moraleda and Dr. García-Candel were the clinicians responsible for the management of all patients, Dr. Atucha and Dr. García-Candel were the main designer of all protocols and study, and Dr. García-Estañ was the main writer of the manuscript.

Conflict of interest.

We gratefully acknowledge the financial support provided by Pfizer. However, Pfizer has not any role in the design, realization, discussion and writing of the project and the present manuscript.

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Figures

Figure 1. Calcium responses of platelets as measured with Fluo-3 by flow cytometry.

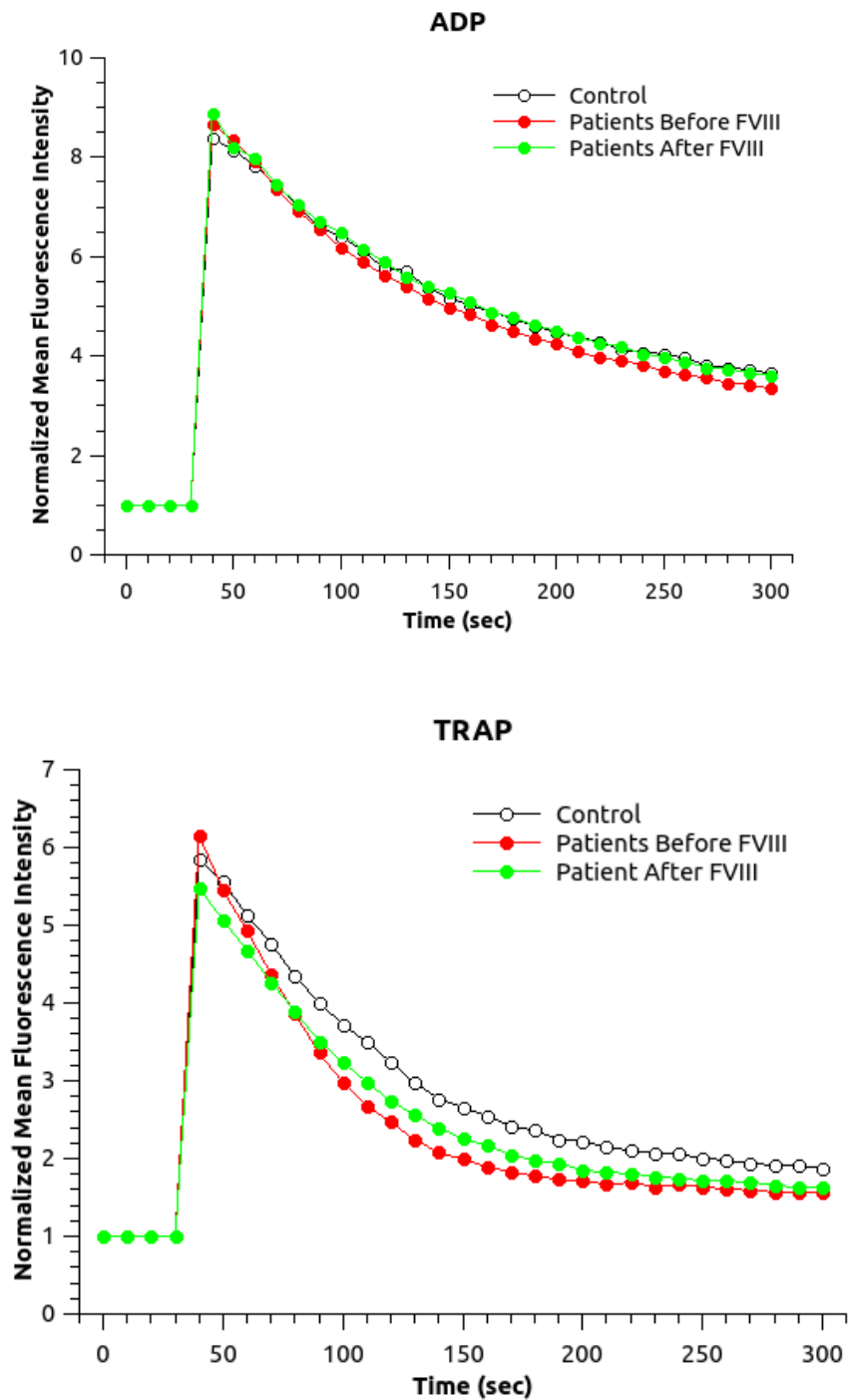


Figure 2. Area under the curve of the calcium responses shown in figure 1.

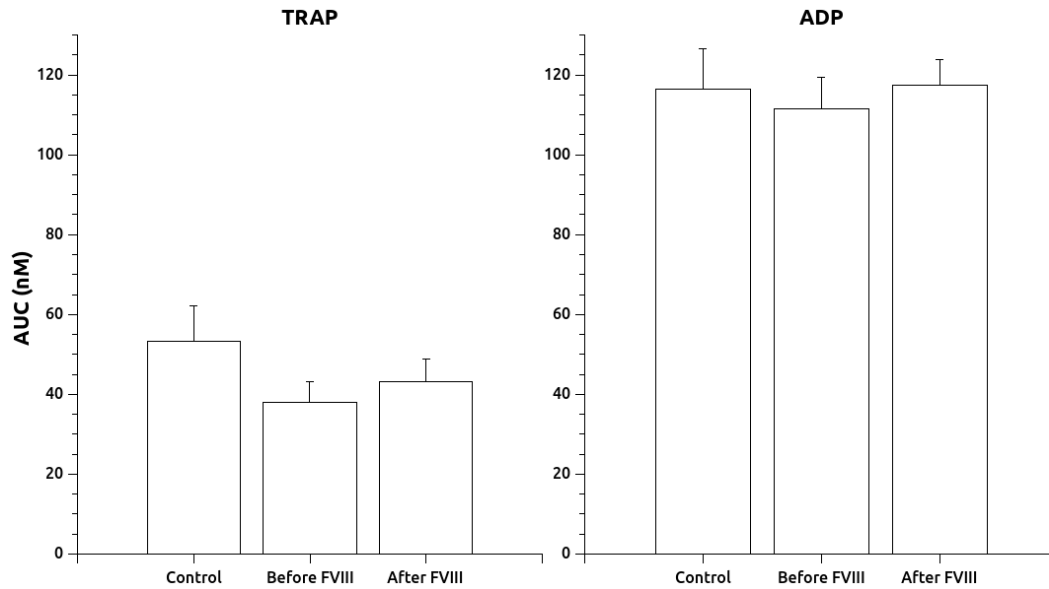


Figure 3. Platelet function by optical aggregation with PRP (with Chronolog). AUC: area under the curve.

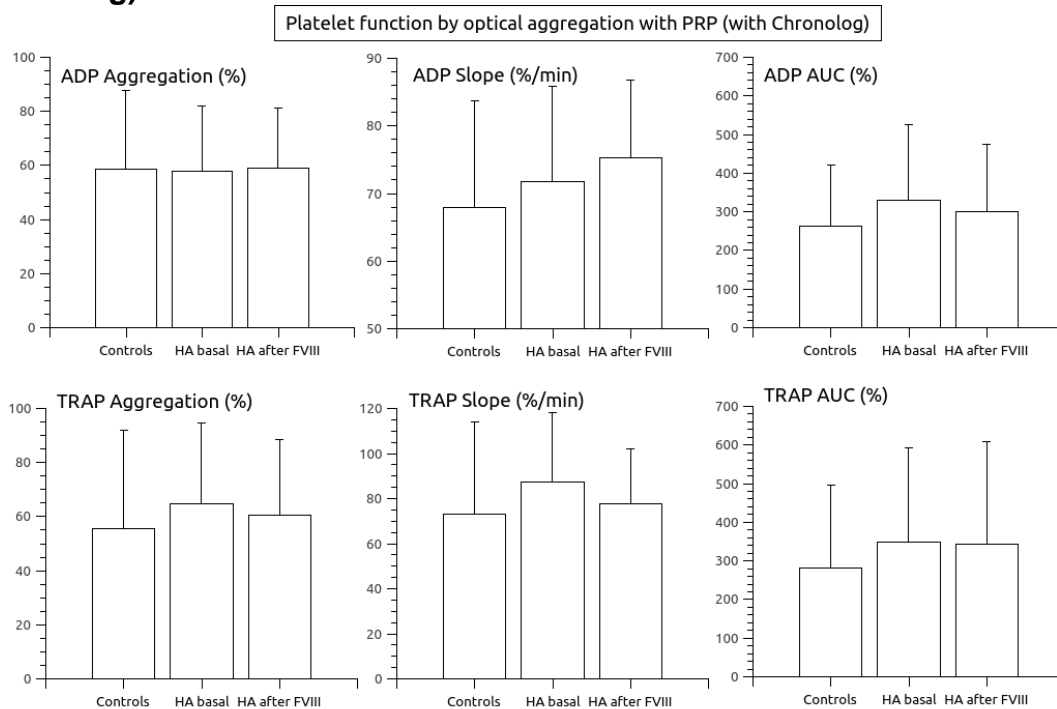


Figure 4. Platelet function by whole blood impedance (with Multiplate). AUC: area under the curve; U: units; UA; arbitrary units.

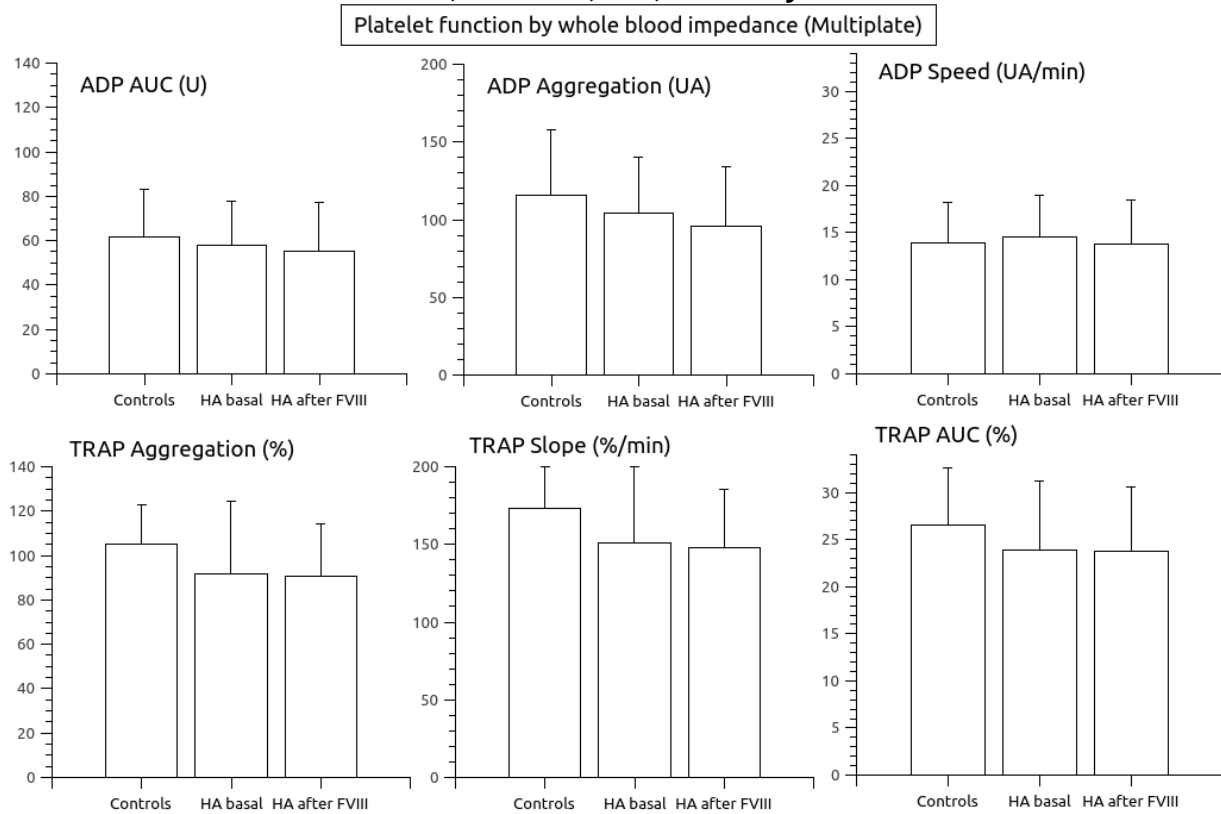
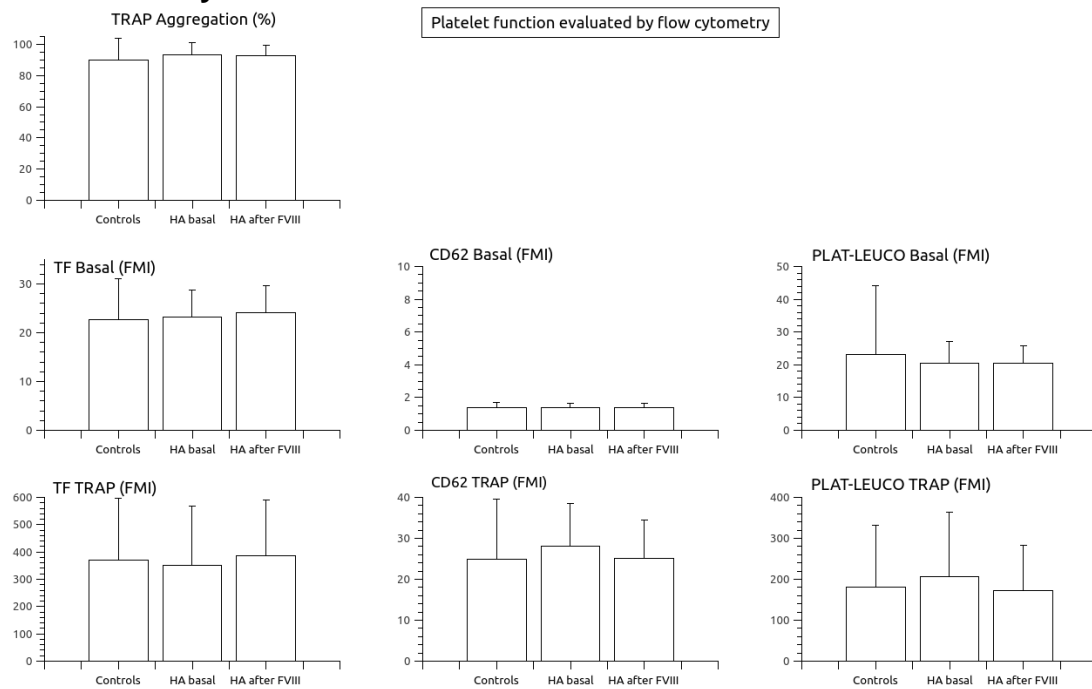


Figure 5. Platelet function evaluated by flow cytometry. FMI: fluorescence mean intensity.



Supplementary document to Melero et al paper.**MATERIAL AND METHODS***Study design.*

We have performed an observational, prospective and longitudinal cohort study conducted between July 2015 and April 2016. The project was approved by the Ethics Committee of Clinical Research (CEIC) of the Hospital Universitario Virgen de la Arrixaca (Murcia, Spain). The rules of Good Clinical Practices in Research and the ethical guidelines of the 1975 Declaration of Helsinki (1983 Revision) have been fully respected. Informed consent was obtained from all the patients, selected from the database of the Hemophilia Unit of the Region of Murcia with severe HA diagnosis and who were in treatment prophylactic with recombinant FVIII at the time of the study. The inclusion criteria were:

- Patients aged between 6 and 65 years with HA in prophylactic treatment with recombinant factor VIII
- Patients without the presence of an inhibitor against factor VIII.
- Patients who are not being treated with non-steroidal anti-inflammatory drugs (NSAID) in the 15 days prior to the donation.
- Patients without episodes of infection and / or thrombosis and / or hemarthrosis in the last 3 months.
- Patients with platelet count > 100.000 / μL .

The exclusion criteria were refusal to participate in the study and patients who could not come to the hospital (due to clinical problems/comorbidities). Control subjects were obtained from healthy coworkers and doctors who voluntarily agreed to participate.

Sixteen patients with severe HA were included in the study. Although this may be considered as a low sample, these were all the patients available in our zone at the time of the study. It is important to remember that HA is a rare disease.

Human samples and reagents.

Blood samples were obtained before (baseline sample, 72h without administering factor VIII) and after infusion of factor VIII (15 minutes after). This time was selected following a previous report of the National Hemophilia Foundation (McDaniel M. RN (2013). Treatment of Hemophilia A and B. Nursing Working Group – Nurses' Guide to Bleeding Disorders (chapter 6, pp 1-9). New York. National Hemophilia Foundation). They were always extracted by the same expert nurse, with a 21G needle without the use of compressor or tourniquet, to minimize platelet stimulation. A total of 18 mL were extracted in each subject. The first 2.5 ml were used for measurements other than platelet function, i.e. hemogram, following the recommendations of the International Society on Thrombosis and Haemostasis (ISTH). Each blood sample was distributed as follows:

- 3 mL of blood collected in a tube with EDTA for completion of hemogram (platelet count) using the Cell-dyn system Sapphire System (Abbott).

- 3 mL of blood collected in a sodium citrate tube (3.2%) for measurement of factor VIII, von Willebrand antigen and cofactor von Willebrand (chromogenic special coagulation study) using the system Sysmex CA-1500 system (Siemens Spain). The von Willebrand antigenic factor (VWF:Ag) and the factor cofactor of ristocetin von Willebrand (FvW: Rco) were determined by automated immunoassay using the ACL TOP 700 system using the kits HemosIL and HemosIL VWF:Rco, respectively (Instrumentation Laboratory, Werfen Company).
- 3 mL in a tube with hirudin for platelet study by impedance using the Roche Multiplate analyzer system (Dynabyte Medical, Roche Diagnostics, Mannheim, Germany). Agonists (20 μ L) such as ADP (2 μ M), arachidonic acid (ASPI, 0.5 mM), thrombin receptor activating peptide (TRAP, 10 μ M), collagen (COL, 2 μ g/mL), ristocetin (RISTO, 1.2 mg/mL) and epinephrine (EPI, 5 μ M) were added to 300 μ L of blood patient.
- 3 mL of blood collected in a citrate tube (3.2%), for platelet-related primary hemostasis using the PFA-100 System (Siemens). The closure time is measured after adding 800 μ L of patients blood in wells containing the agonists (COL/EPI and COL/ADP).
- 3 mL of blood collected in a tube with citrate (3.2%), for the study of platelet function aggregation by means of light optical aggregometry (Chrono-log, Chrono-log Corp. Havertown, USA). In this assay, platelet rich plasma (PRP) was prepared by centrifuging blood at 1500g for 15 minutes at 21°C. Agonists (25 μ L) used were: epinephrine (5 μ M), arachidonic acid (1 mM), thrombin (10 μ M), ristocetine (1.2 mg/ml), ADP (2 μ M) and collagen (2 μ g/ml).
- 3 mL of blood collected in a citrate tube (3.2%), for flow cytometry (FACSCanto TM system, BD Biosciences). The agonist TRAP (20 μ L 10 M) was used for all tests and 480 μ L of whole blood for aggregation, activation and Platelet-Leuco conjugates in different tubes with their respective basal controls (without agonist). The samples for aggregation and conjugates platelet-leuco were stirred (1000 rpm) and incubated at 37°C in a Multi-Sample Agitator (manufactured at the University of Nottingham); after 5 minutes of reaction, the whole blood was fixed by addition of 83 μ L of AGGFix solution (Platelet Services) (1). For the activation test, the whole blood was incubated with EDTA (4 mM) and agonist, but without mixing, for 5 min and fixed by addition of PAMFix solution (Platelet Services). After fixation, samples were labeled with 10 μ L of the following antibodies: CD61 for platelets, CD62P and CD63 for platelet activation, CD45 and FT for leukocytes. Cytosolic calcium was also measured by flow cytometry using Fluo-3 in CD61+ cells. The cytometry data were processed using the Kaluza program (Beckman Coulter) in the Laboratory of Flow Cytometry-Coulter Cytometry Center and Related Techniques of the University of Valencia.
- The microvesicles were analyzed in a Gallios flow cytometer (Beckman Coulter) in the Laboratory of Flow Cytometry-Coulter Cytometry Center and Related Techniques in Valencia. In brief, plasmas were collected from aggregation tests by flow cytometry and frozen at -80°C. Isolation and identification of MVs was performed as described by Rank, et al (2). After several centrifugations, 5 μ L of the MVs were diluted with 35 μ L CaCl₂ (2.5 mM) and labeled with annexin V and cell-specific monoclonal antibody or isotype control: CD41 and CD62P for detection of MVs of platelet origin; CD144 for detection of MVs of endothelial

origin. The microvesicles were identified based on size and density using a commercial fluorescent beads pattern of the varied size range (0.1 to 1 μm) for microvesicles (Megamix-Plus SSC).

References:

- 1. Fox SC, Sasae R, Janson S, May JA, Heptinstall S. Quantitation of platelet aggregation and microaggregate formation in whole blood by flow cytometry. *Platelets* 2004; 15: 85–93.
- 2. Rank A, Nieuwland R, Liebhardt S, et al. Apheresis platelet concentrates contain platelet-derived and endothelial cell-derived microvesicles. *Vox Sanguinis* 2011; 100: 179–186.

Table 1. Characteristics of the patients.

Age (years). Median (range)	24.5 (9-39)
Body weight (Kg). Mean \pm S.D.	60 \pm 24.6
Age 1st hemorrhage (years). Median (range)	1 (1-1.75)
Target joint (% number of cases)	
No target joint	3 (18.75%)
1 joint	4 (25%)
2 joints	6 (37.5%)
≥ 3 joints	3 (18.75%)

Table 2. Hemogram parameters.

	CONTROLS	HA Basal	HA after FVIII
RBC ($\times 10^6/\mu\text{L}$)	5.29 \pm 0.27	5.13 \pm 0.49	5.07 \pm 0.45
Hemoglobin (g/dL)	15.94 \pm 0.67	14.98 \pm 2.01	14.83 \pm 2.01
Hematocrit (%)	46.65 \pm 2.19	44.23 \pm 5.37	43.64 \pm 5.43
MCV (fL)	88.23 \pm 2.93	85.93 \pm 5.87	85.72 \pm 6.03
MCH (pg/cell)	30.18 \pm 1.2	29.08 \pm 2.5	29.14 \pm 2.5
MCHC (g/dL)	34.23 \pm 1.38	33.82 \pm 1.04	33.97 \pm 1.26
Platelets ($\times 10^3/\mu\text{L}$)	233.93 \pm 40.8	231.37 \pm 48.45	230.37 \pm 42.76
MPV (fL)	8.31 \pm 1.36	9.02 \pm 1.52	8.77 \pm 1.59 +
Reticulated platelets (%)	2.9 \pm 1.52	2.43 \pm 1.07	3.16 \pm 2.27

Reticulocytes (%)	1.54±0.4	1.52±0.37	1.52±0.37
Leucocytes (x10 ³ /μL)	6.12±1.48	6.06±1.1	5.77±1.03 +
Neutrophils (x10 ³ /μL)	3.33±1	2.97±0.85	2.88±0.85
Lymphocytes (x10 ³ /μL)	2.09±0.53	2.24±0.48	2.05±0.36 +
Monocytes (x10 ³ /μL)	0.52±0.13	0.52±0.18	0.51±0.18
Eosinophils (x10 ³ /μL)	0.14±0.08	0.28±0.26	0.27±0.26
Basophils (x10 ³ /μL)	0.06±0.112	0.036±0.018	0.039±0.017

Data are mean ± S.D. +. p<0.05 between HA basal y HA after infusion of FVIII. RBC: red blood cells; MCV: Mean Corpuscular Volume); MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; MPV: mean platelet volume.

Table 3. Special coagulation parameters.

	CONTROLS	HA Basal	HA after FVIII
FVIII (U/dL)	102.5±24.94	3.23±4.18 *	89.05±34.82 +
VWF: Ag (%)	104.26±30.37	113.15±42.52	114.64±44.04
VWF: Rco (%)	93.54±21.32	97.85±25.34	97.16±27.15

Data are mean ± S.D. *.p<0.05 between Controls and HA basal; +. p<0.05 between HA basal y HA after infusion of FVIII. FVIII: Factor VIII; VWF: Ag. von Willebrand Factor Antigen; VWF: RCo. von Willebrand Factor Ristocetin Cofactor.

Table 4. Platelet function with PFA-100.

	CONTROLS	HA Basal	HA after FVIII
COL/EPI (sec)	118.6±22.14	119.12±30.0	124.33±27.04
COL/ADP (sec)	83.93±17.61	85.68±14.39	89.2±16.67

Data are mean ± S.D. COL/EPI: agonists collagen and epinephrine; COL/ADP: agonists collagen and adenosine diphosphate.

Table 5. Platelet function by optical aggregation with PRP (with Chronolog).

	CONTROLS	HA Basal	HA after FVIII
Aggregation ADP(%)	58.66±29.15	57.75±24.28	59.06±22.04
Slope ADP (%/min)	67.93±15.8	71.68±14.17	75.33±11.5
AUC ADP (%)	262.52±159.33	329.77±196.6	299.13±175.68
Aggregation COL(%)	75.66±17.79	81.12±9.45	85.62±7.95
Slope COL(%/min)	83.6±20.15	93.68±18.7	98.26±13.9 +
AUC COL(%)	344.52±109.82	441.35±166.78	442.56±128.66 +
Aggregation EPI (%)	69.64±29.08	65.87±25.86	67.66±25.98
Slope EPI(%/min)	42.14±16.19	43.56±15.87	40.8±8.26
AUC EPI(%)	316.17±151.38	318.08±159.01	669.25±1261.70
Aggregation TRAP(%)	55.4±36.46	64.93±29.94	60.6±27.95
Slope TRAP (%/min)	73±40.89	87.33±30.95	77.53±24.52
AUC TRAP(%)	281.26±215	349.09±244.58	342.95±267.61
Aggregation ASPI(%)	83.73±8.43	85±6.49	84.33±5.43
Slope ASPI(%/min)	92.06±24.64	91.68±31.07	89.06±29.09
AUC ASPI(%)	407.11±77.18	438.19±150.34	457.99±193
Aggregation RISTO(%)	89±4.67	90.06±6.87	88.86±7.3
Slope RISTO(%/min)	90.6±25.13	88.06±16.95	78.13±19.96 +
AUC RISTO(%)	473.78±84.39	480.24±103.98	503.98±128.8

Data are mean ± S.D. +. p<0.05 between HA basal y HA after infusion of FVIII. #. p<0.05 between Controls and HA after FVIII. AUC: area under the curve; ADP: adenosine diphosphate; COL: collagen; EPI: epinephrine; TRAP: thrombin; ASPI: arachidonic acid; RISTO: ristocetin.

Table 6. Platelet function by whole blood impedance (Multiplate).

	CONTROLS	HA Basal	HA after FVIII
AUC ADP (U)	61.66±21.47	58.14±19.57	55.06±21.87
Aggregation ADP (UA)	115.78±41.76	104.34±35.73	96.02±38.22
Speed ADP (UA/min)	13.96±4.19	14.48±4.36	13.82±4.58
AUC COL (U)	72.73±13.22	64.2±18.59	68.12±16.15
Aggregation COL (UA)	157.06±26.87	137.48±26.45	138.75±27.21
Speed COL (UA/min)	18.91±3.91	18.39±5.54	18.12±4.88
AUC TRAP (U)	105.26±17.58	91.53±33	90.75±23.74
Aggregation TRAP (UA)	173.16±26.7	150.66±49.41	147.62±38.09 #
Speed TRAP (UA/min)	26.56±5.97	23.96±7.29	23.73±6.85
AUC ASPI (U)	84.14±13.67	82.21±21.8	80.12±24.92
Aggregation ASPI (UA)	141.3±20.87	134.98±33.09	130.14±38.23
Speed ASPI (UA/min)	21.24±3.36	21.99±7.01	21.64±7.7
AUC EPI (U)	14.63±6.62	14.44±6.69	10.92±6.46
Aggregation EPI (UA)	32.83±17	30.51±11.96	24.15±12.66
Speed EPI (UA/min)	4.83±1.42	4.86±1.29	4.21±1.06
AUC RISTO H (U)	101±29.33	108.78±37.5	97.8±41.12
Aggregation RISTO H (UA)	210.59±51.9	215.9±66.4	195.91±65.91
Speed RISTO H (UA/min)	30.15±7.8	31.96±11.3	29.46±12.75
AUC RISTO L (U)	5.25±3.41	6.57±6.53	9.28±5.7
Aggregation RISTO L (UA)	13±4.77	15.87±10.93	20.5±10.67
Speed RISTO L (UA/min)	3.62±0.83	3.07±1.02	3.67±0.97

Data are mean ± S.D. #. $p < 0.05$ between Controls and HA after FVIII. AUC: area under the curve; U: units; UA: units arbitrary; ADP: adenosine diphosphate; COL: collagen; EPI: epinephrine; TRAP: thrombin; ASPI: arachidonic acid; RISTO: ristocetin.

Table 7. Platelet function evaluated by flow cytometry.

	CONTROL	HA Basal	HA after FVIII
Aggregation (%)	89.87±14.22	93.04±8.05	92.55±6.61
CD62 BASAL (FMI)	1.39±0.31	1.37±0.28	1.37±0.27
CD62 TRAP (FMI)	24.9±14.55	28.05±10.43	25.22±9.16
CD63 BASAL (FMI)	1±0.016	1±0.017	1.01±0.047
CD63 TRAP (FMI)	3.9±2.69	3.93±3.08	3.67±2.95
PLAT-LEUCO BASAL(FMI)	23.22±21	20.35±6.61	20.42±5.36
PLAT-LEUCO TRAP (FMI)	181.12±151.55	206.06±157.36	172.08±110.98
TF BASAL (FMI)	22.74±8.4	23.22±5.59	24.11±5.6
TF TRAP (FMI)	370.33±227	351.77±215.49	387.42±203.47

Data are mean \pm S.D. FMI: fluorescence mean intensity; PLAT: platelet; LEUCO: leucocytes; TF: tissue factor; TRAP: thrombin.

Table 8. Number of microvesicles in the study subjects.

	CONTROLS	HA Basal	HA after FVIII
Total MVs	633.42±258.28	434.06±245.13 *	479.71±148.46
CD41+ MVs	399.71±185.70	277.66±44.98	325.13±35.83
CD62+ MVs	483.35±200.63	323.8±46.57 *	336.86±34.48 #
CD41+CD62+ MVs	337.71±159.82	227.46±35.23	267.73±29.55
CD144+ MVs	4.43±7.28	2.13±0.54	1±0.32 +

Data are mean \pm S.D. *.p<0.05 between Controls and HA basal; +. p<0.05 between HA basal y HA after infusion of FVIII. #. p<0.05 between Controls and HA after FVIII. Values are number of MVs per μ L. MVs: microvesicles.

Table 9. Number of microvesicles according to the age of patients.

		<18 years (n=6)	>18 years (n=9)
Basal	Total MVs	352±167.28	488.22 ± 281.81
	CD41+ MVs	261.5±134.47	288.44±203.68
	CD62+ MVs	271.83±123.1	358.44±209.95
	CD41+CD62+ MVs	218.5±104.73	233.44±160.09
	CD144+ MVs	0.33±0.516 *	3.33±1.87
After FVIII	Total MVs	398.5±108.448	540.625±150.46
	CD41+ MVs	303.66±100.04	339.44±163.93
	CD62+ MVs	312.33±86.13	353.22±160.71
	CD41+CD62+ MVs	259.33±87.01	237.33±134.55
	CD144+ MVs	0.50±0.55	1.33±1.5

Data are mean ± S.D. *.p<0.05 between columns. Values are number of MVs per µL. MVs: microvesicles.