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CASE REPORT

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Platelet function and microvesicle generation in patients with hemophilia A

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Abstract

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

KEYWORDS

calcium, factor VIII, Hemophilia A, microvesicles, platelets, prophylaxis

1 | INTRODUCTION

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. Blood tests were performed before (72 hours 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 Ca^{2+} mobilization. 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. Our results do not support any effect of FVIII on platelet function in patients with severe HA treated under the regime of prophylaxis.

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 (ie, 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 was to analyze whether the administration of FVIII modifies this hemorrhagic phenotype, comparing a baseline sample (washing period/72 hours without factor VIII) with respect to a sample 15 minutes after administration of FVIII.

2 | METHODS

2.1 | Study design

We have performed a case/control study conducted between July 2015 and April 2016. 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.

2.2 | Human samples and reagents

Blood samples were obtained before (baseline sample, 72 hours 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 was used for measurements other than platelet function, that is, 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-leukocyte 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]

2.3 | 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 Ca 2 + 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.

3 | RESULTS

3.1 | General features of HA patients

Descriptive data are shown in Table S1. 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 had 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.

3.2 | Blood analysis data

The hemogram of controls and patients before and after treatment with FVIII is shown in Table S2. There were no differences between controls and patients in any variable, although leukocyte and lymphocyte 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 S3. As expected, we observed statistically significant differences in the amount of FVIII before and after FVIII infusion, as well as with control subjects ($P < .001$). In the rest of the parameters, VWF:Ag and VWF:Rco, no significant differences were observed between groups.

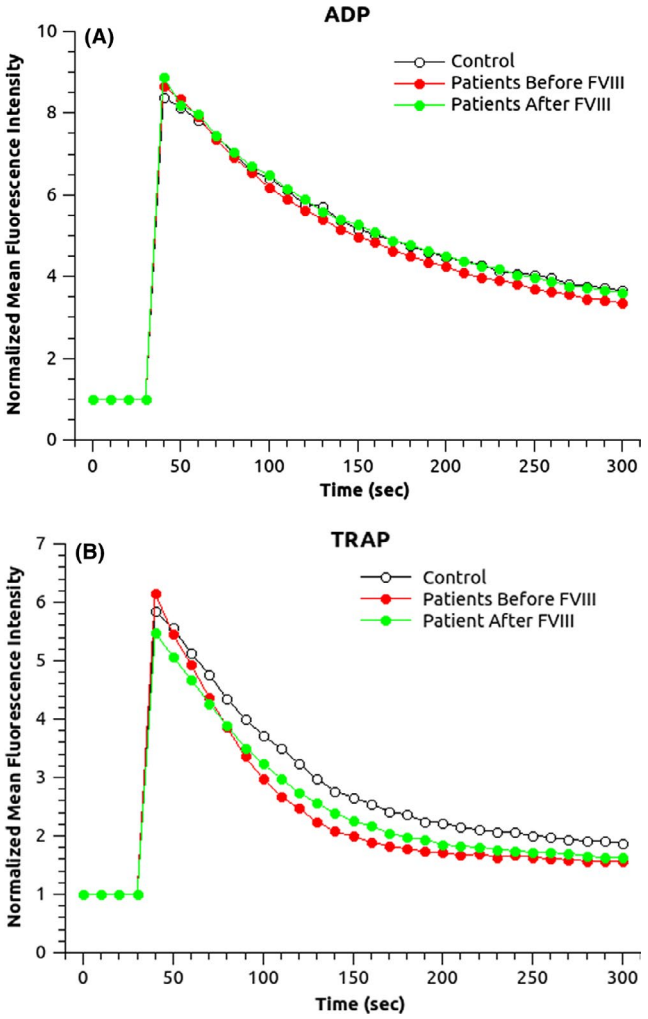

FIGURE 1 Calcium responses to ADP (A) or TRAP(B) of platelets as measured with Fluo-3 by flow cytometry

3.3 | Platelet function

The data obtained in the PFA-100 study are shown in Table S4. No statistically significant differences were observed between the groups. Similarly when platelet aggregation function was analyzed with Chrono-log, there were no

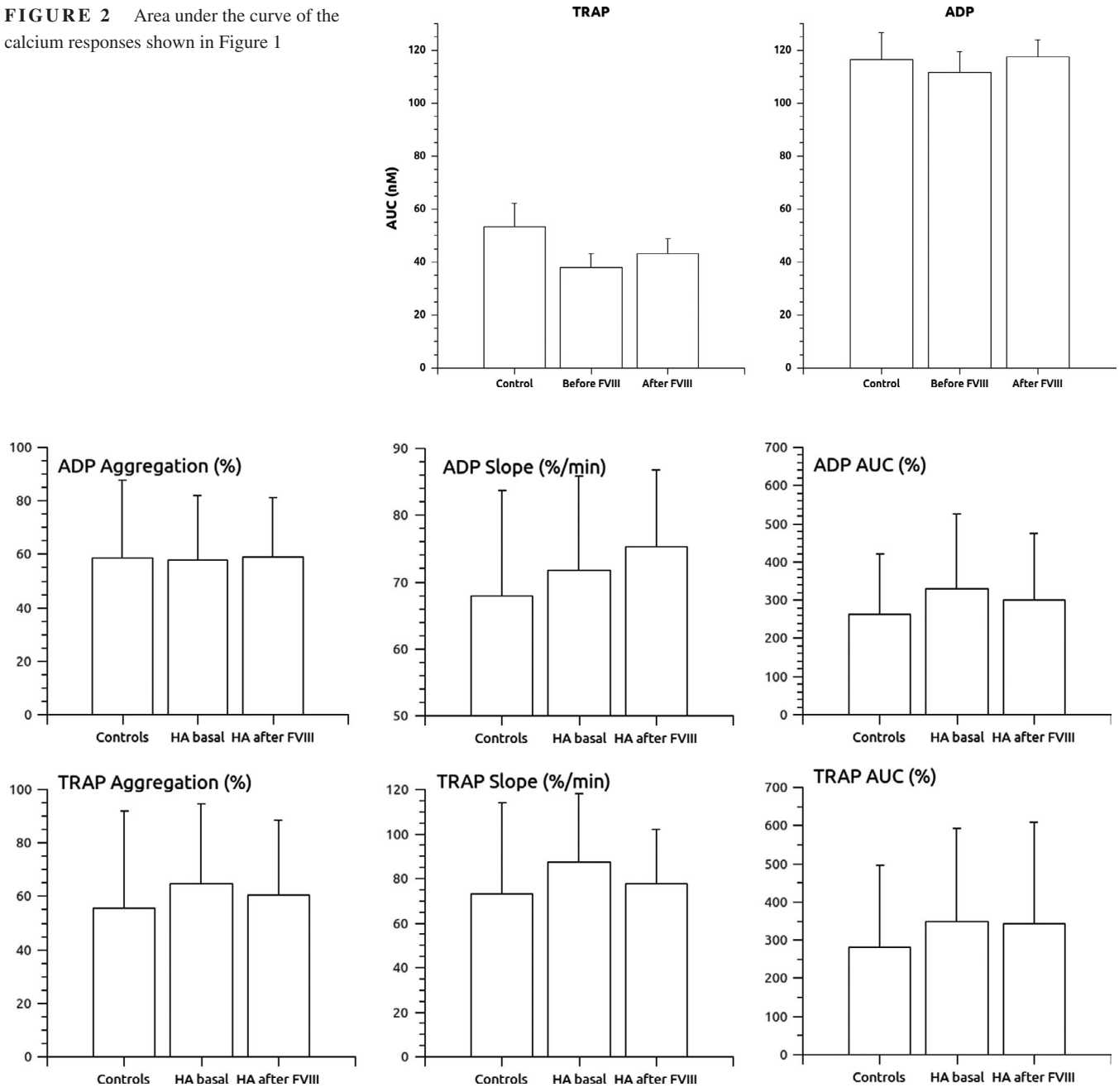

FIGURE 2 Area under the curve of the calcium responses shown in Figure 1

FIGURE 3 Platelet function by optical aggregation with PRP (with Chrono-log). AUC: area under the curve

significant differences between groups (Table S5). Equally, after analyzing platelet function in complete blood with the Multiplate technique, no significant differences were found among groups (Table S6), 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 marker expression, platelet-leukocyte aggregates, and tissue factor exposure performed by flow cytometric analysis did not reveal any significant differences between groups (Table S7). 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 (Figures 2-5).

3.4 | Study of plasma microvesicles (MVs)

There were a lower number of total MVs in HA patients as compared to the controls and the administration of FVIII

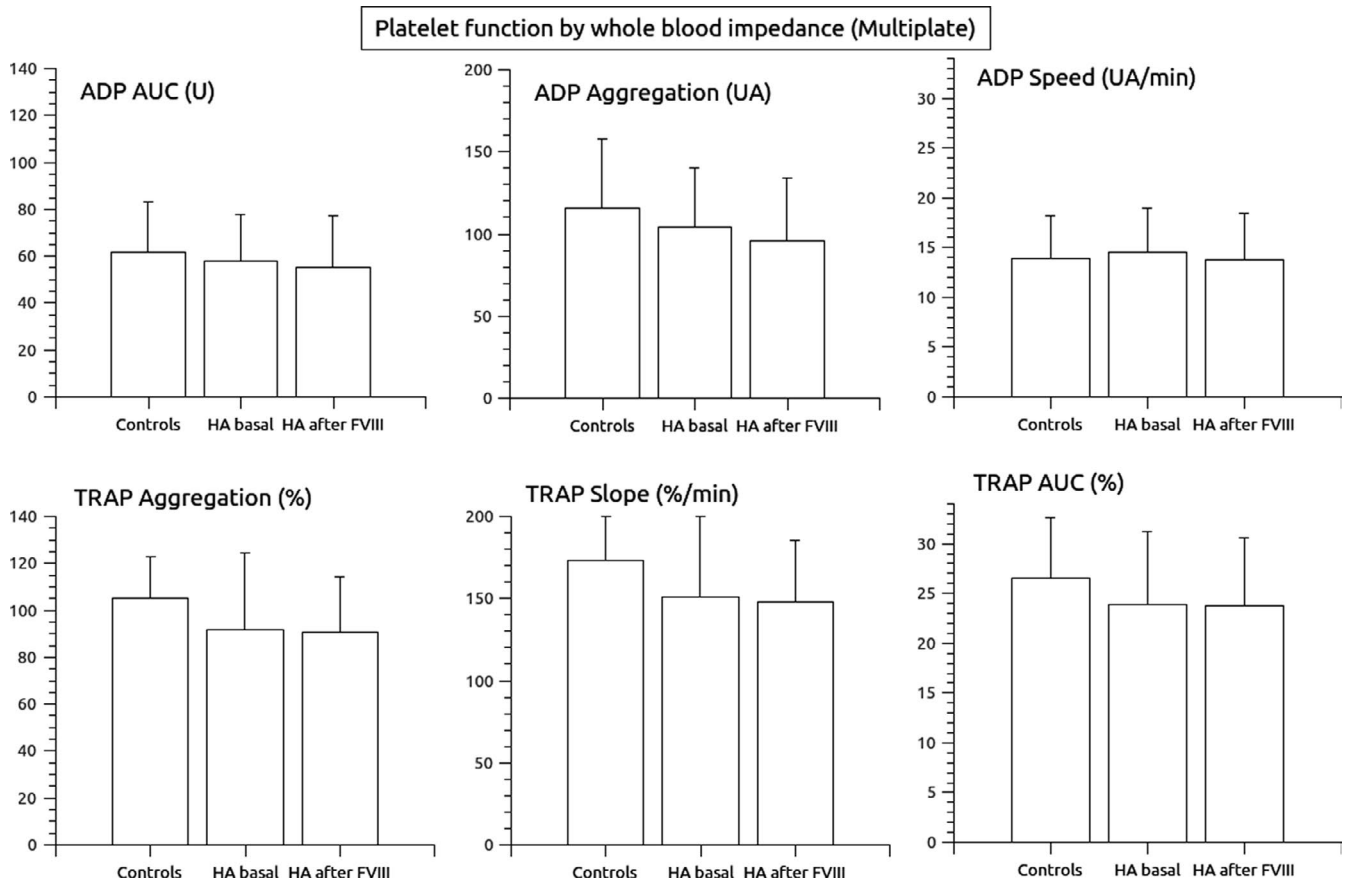

FIGURE 4 Platelet function by whole blood impedance (with Multiplate). AUC: area under the curve; U: units; UA; arbitrary units

eliminated these differences (Table S8). 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 (Table S9), 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 (Table S9).

4 | 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.

4.1 | 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 in 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]

4.2 | 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] and with 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.

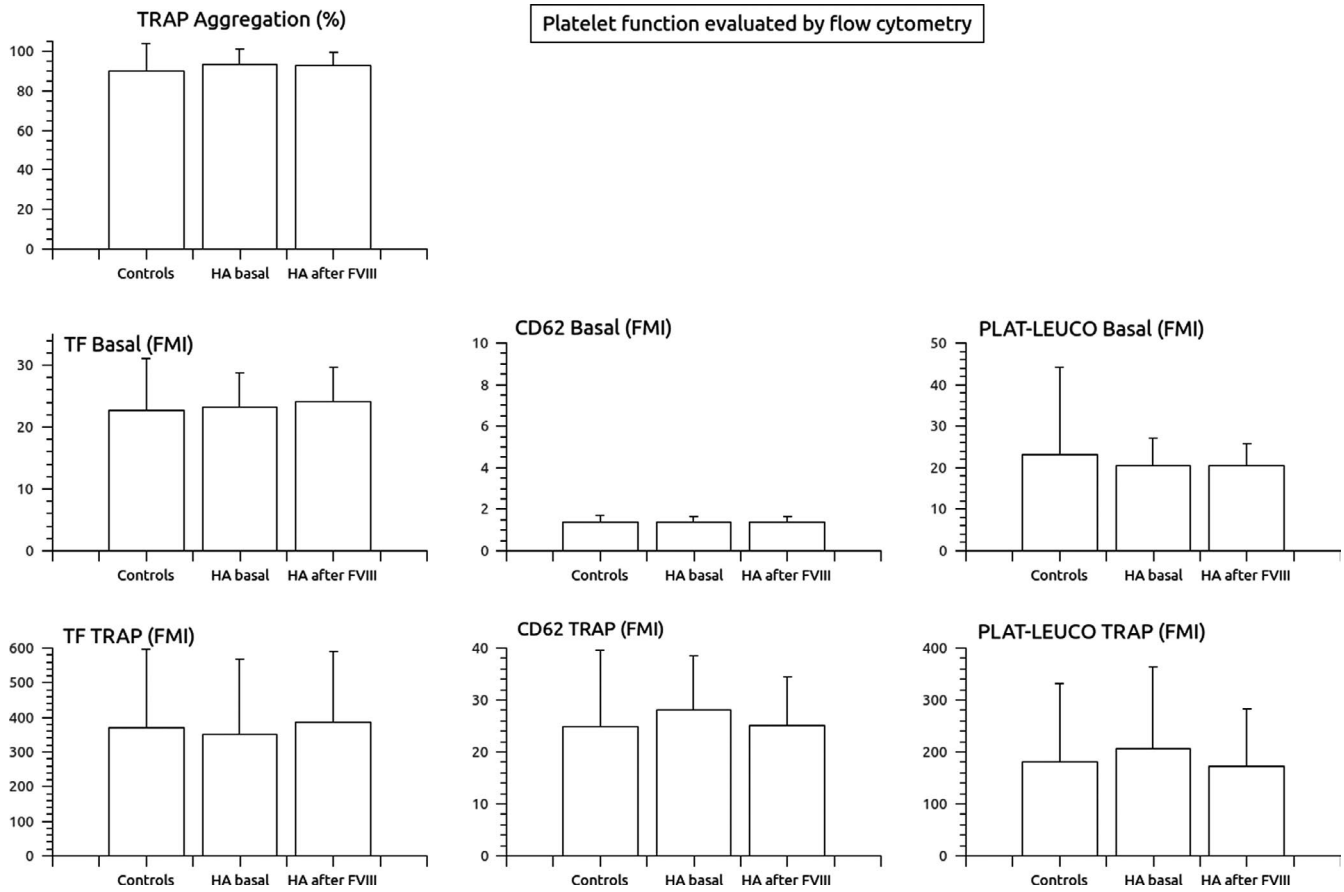

FIGURE 5 Platelet function evaluated by flow cytometry. FMI: fluorescence mean intensity

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 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 with 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,[16,22,23] but it seems that our results agree with most of these studies, both in human patients[16,24] and in mice,[23] thus suggesting that there is no platelet activation in patients with severe HA.

4.3 | Calcium study

Intraplatelet-free Ca^{2+} has been used in the functional study of platelets and in the monitoring of therapies with platelet antagonists.[25] 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,[26] 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 intraplatelet 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.

4.4 | Microvesicles

Microvesicles (MVs) are small membrane particles of 0.1-1.0 μm shed by either activated or apoptotic cells[27] in response to a variety of stimulatory factors, and they can originate from platelets, among other cells,[28] playing a important role on hemostasis and thrombosis.[27] 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 with the controls. Our patients showed a lower amount of CD144+ microvesicles after the infusion of FVIIII, a decrease which was also observed in the study of Mobarrez et al.[29] 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,[30] 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.[31,32] However, Artoni et al[33] 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.

4.5 | 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.

ACKNOWLEDGMENT

Published with written consent of the patient.

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.

AUTHOR'S CONTRIBUTIONS

Dr Melero-Amor, 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 laboratory technician 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.

ETHICAL APPROVAL

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.

DATA AVAILABILITY STATEMENT

Data are available at reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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