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) 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 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 µL) such as ADP (2 µM), arachidonic acid (ASPI, 0.5 mM), thrombin receptor activating peptide (TRAP, 10 µM), collagen (COL, 2 µg/mL), ristocetin (RISTO, 1.2 mg/mL) and epinephrine (EPI, 5 µM) were added to 300 µL 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 µL 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 21ºC. Agonists (25 uL) used were: epinephrine (5 µM), arachidonic acid (1 mM), thrombin (10 µM), ristocetine (1.2 mg/ml), ADP (2 µM) and collagen (2 µg/ml).
- 3 mL of blood collected in a citrate tube (3.2%), for flow cytometry (FACSCanto TM system, BD Biosciences). The agonist TRAP (20 µL 10 M) was used for all tests and 480 µL 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 µL 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 µL of the following antibodies: CD61 for platelets, CD62P and CD63 for platelet activation, CD45 and FT for leukocytes. Cytosolic calcium was also meaured 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 µL of the MVs were diluted with 35 µL 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 ± S.D.	60 ± 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 ($x10^6$/µL)	5.29±0.27	5.13±0.49	5.07±0.45
Hemoglobin (g/dL)	15.94±0.67	14.98±2.01	14.83±2.01
Hematocrit (%)	46.65±2.19	44.23±5.37	43.64±5.43
MCV (fL)	88.23±2.93	85.93±5.87	85.72±6.03
MCH (pg/cell)	30.18±1.2	29.08±2.5	29.14±2.5
MCHC (g/dL)	34.23±1.38	33.82±1.04	33.97±1.26
Platelets ($x10^3$/µL)	233.93±40.8	231.37±48.45	230.37±42.76
MPV (fL)	8.31±1.36	9.02±1.52	8.77±1.59 +
Reticulated platelets (%)	2.9±1.52	2.43±1.07	3.16±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 $\pm$ 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; 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 ± 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 ± 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 µL. 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.