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Immune status of patients with haemophilia A before exposure to factor VIII: first results from the HEMFIL study

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Summary

Previous cross-sectional studies showed that some patients with haemophilia A (HA) without inhibitor presented a pro-inflammatory profile during factor VIII (FVIII) replacement therapy. Furthermore, an anti-inflammatory/regulatory state was described in HA patients after inhibitor development. However, no study investigated the levels of these biomarkers before exposure to exogenous FVIII. This study investigated the immunological profile of previously untreated patients (PUPs) with HA in comparison with non-haemophiliac boys. A panel of chemokines and cytokines was evaluated in the plasma of 40 PUPs with HA and 47 healthy controls. The presence of microparticles was assessed in the plasma of 32 PUPs with HA and 47 healthy controls. PUPs with HA presented higher levels of CXCL8 (IL8), IL6, IL4, IL10, IL2, IL17A (IL17), and lower levels of CXCL10 (IP-10) and CCL2 (MCP-1) than the age-matched healthy controls ($P < 0.05$). We also observed higher levels of microparticles derived from endothelium, erythrocytes, platelets, leucocytes, neutrophils, and T lymphocytes in patients in comparison with controls ($P < 0.05$). Compared with controls, PUPs with HA presented a distinct immunological profile, characterized by a prominent pro-inflammatory status that appears to be regulated by IL4 and IL10.

Keywords: chemokines, cytokines, microparticle, haemophilia A, PUPs.

Haemophilia A (HA) is an inherited X-linked bleeding disorder caused by the deficiency of coagulation factor VIII (FVIII), affecting 1:5000–10 000 new-born males worldwide. Treatment of bleeding episodes requires administration of FVIII-containing products either on demand or on a prophylactic basis (Hoyer, 1994).

The main treatment-related complication in patients with HA is the development of neutralising antibodies (inhibitors), which occurs in 20–30% of patients (Lollar, 2004). During the administration of factor products, patients with HA develop a pro-inflammatory immunological profile, characterised by interleukin (IL) 2, tumour necrosis factor (TNF) and interferon-γ (IFNG), stimulating the synthesis of

immunoglobulin (IgG)1 anti-FVIII with no inhibitory activity (Hu et al, 2007; Chaves et al, 2010; Silveira et al, 2015). Otherwise, patients with HA and inhibitors present an anti-inflammatory/regulatory immunological profile mediated by neutrophils and monocytes with high production of IL5 and IL10 (Hu et al, 2007; Chaves et al, 2010; Silveira et al, 2015).

It has been shown that microparticles derived from leucocytes can trigger the release of inflammatory proteins, such as IL6, CXCL8 (also termed IL8) and CCL2 (also termed MCP-1), as an attempt to increase platelet activation and fibrin deposition during vascular injury (Mesri & Altieri, 1998; Distler et al, 2005). Although microparticles are involved in physiological haemostasis, few studies have evaluated their

plasma levels in patients with haemophilia (Proulle *et al*, 2004, 2005; Mobarrez *et al*, 2013). Moreover, the levels of cytokines, chemokines and microparticles in untreated patients with HA are still not known.

An evaluation of the immunological profile in previously untreated patients (PUPs) with HA may contribute to a better understanding of how these biomarkers behave before exposure to exogenous FVIII. This can be important in understanding why some patients develop inhibitors and others do not.

Material and methods

Study population

This case-control study is a subset of the HEMFIL Study, an ongoing cohort project that includes patients with HA who had never been exposed to FVIII attending haemophilia treatment centres in Brazil. After enrolment, patients are referred for clinical evaluation for the collection of socio-demographic, clinical and laboratory data by completing standardized forms. These forms were translated from the RODIN study (Gouw *et al*, 2013), with kind permission of the RODIN Study group. Patients are followed up until 75 exposure days (ED) and/or upon inhibitor development. However, the present work targeted at studying patients at inclusion (T0), before any exposure to FVIII.

Participants of this study were PUPs with HA diagnosed at five Brazilian haemophilia treatment centres – Fundação HEMOMINAS (Minas Gerais), Fundação HEMORIO (Rio de Janeiro), Fundação HEMEPAR (Paraná) Fundação HEMOSC (Santa Catarina) and Fundação HEMOES (Espírito Santo). They were enrolled consecutively from February 2013 to August 2016.

The control group consisted of healthy boys recruited during routine consultation at Paediatric Primary Care Centre from the University Hospital, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil. Their health status was determined by a comprehensive medical history and examination to rule out bleeding symptoms, use of medications, recent vaccination and evidence of chronic/acute illness. Patients and control subjects with conditions that might influence the levels of chemokines and cytokines at blood collection, such as allergies, vaccination, infection and inflammation, as well as use of medications for the treatment of any morbid condition, were excluded or postponed.

The parents/guardians of all patients with HA and controls signed a written informed consent to participate in the study. The research was approved by institutional ethical committees.

Sample collection

The peripheral blood of controls and PUPs with HA was collected in a 4 ml tube containing sodium citrate 3·2% as anticoagulant. Blood was immediately centrifuged at 1500 rpm for 15 min to obtain plasma. Plasma samples were stored at −80°C until analysis of chemokines, cytokines and microparticles. Patients' samples were collected before the first infusion of FVIII-containing products. In controls, samples were obtained at the time blood was drawn for routine visits to the Paediatric Primary Care Centre.

Determination of FVIII activity levels

FVIII activity was measured in patients at diagnosis, to determine the type and severity of haemophilia. Tests were performed in the haemophilia treatment centre where the patients were recruited.

Analysis of chemokines and cytokines

Measurements of chemokines [CXCL8 (IL8), CCL5 (RANTES), CXCL9 (MIG), CCL2 (MCP-1), CXCL10 (IP-10)] and cytokines [IFNG, TNF, IL2, IL4, IL6, IL10 and IL17A (IL17)] were performed in duplicate and recorded in pg/ml, according to the human Cytometric Bead Array kit (BD Biosciences, San Jose, CS, USA). Acquisition was performed on BD Accuri™ C6 Flow Cytometer (BD Biosciences).

The panel of chemokines was chosen to comprise a wide spectrum of molecules responsible for the attraction of leucocytes to inflammation sites. Additionally, cytokines were designed to be a representative panel of anti-inflammatory/regulatory (IL4 and IL10) and pro-inflammatory molecules (IFNG, TNF, IL2, IL6 and IL17A). The data were analysed using the FCAP software v1.0.1 (BD Biosciences, Franklin Lakes, NJ, USA).

The detection limits for chemokines were: CXCL8 (0·2 pg/ml), CCL5 (1·0 pg/ml), CXCL9 (2·5 pg/ml), CCL2 (2·7 pg/ml), CXCL10 (2·8 pg/ml). And for cytokines: IFN-γ (3·7 pg/ml), TNF (1·7 pg/ml), IL2 (2·6 pg/ml), IL4 (0·4 pg/ml), IL6 (1·9 pg/ml), IL10 (1·5 pg/ml) and IL17A (8·3 pg/ml). Levels below the detection limit of each cytokine/chemokine were defined as 0 pg/ml.

Microparticle analysis

Microparticles were prepared as described elsewhere (Campos *et al*, 2010). In brief, platelet-free plasma was obtained by double centrifugation (1500 × *g* for 15 min followed by 13 000 × *g* for 5 min at room temperature). The latter was diluted in 300 µl of sodium citrate (0·124 mol/l) (BD Biosciences) with 5000 units of heparin (Roche, Rio Janeiro, Brazil) in the ratio 1:3 and centrifuged at 14 000 × *g* for 90 min at 15°C. The resultant microparticle pellet was resuspended in 100 µl of 1× annexin V binding buffer (BD Biosciences). Microparticles isolated from plasma were gated based on their forward (FSC) and side (SSC) scatter distribution as compared to the distribution of synthetic 0·7–0·9 µm SPHERO™ Amino Fluorescent Particles (Spherotech Inc., Libertyville, IL, USA). Considering the presence of phosphatidylserine residues on

the microparticles surface, events for positive staining were assessed for annexin V (BD Biosciences). Phenotypic characterization of the microparticles to determine their cellular origin was performed using monoclonal antibodies (BD Biosciences) specific for endothelial cells (ITGAV; CD51/61-PE), erythrocytes (GYPA; CD235a-PECy5), platelets (ITGA2B; CD41a-PERCP-Cy5.5), leucocytes (PTPRC; CD45-APC), neutrophils (CEACAM1; CD66-PE), monocytes (CD14-PERCP) and T lymphocytes (CD3-PE). The samples were analysed in a Flow Cytometry FACSCalibur (BD Biosciences). Over 100 000 events were acquired for each sample, to reach at least 2000 events within the microparticles gate.

For the determination of microparticles/µl the formula microparticles/µl $= (N \times 400)/(100 \times 60)$ was used, in which N corresponds to the number of events; 400 µl to the total volume of sample before analysis; 60 µl to the sample volume analysed, and 100 µl relates to the original volume of microparticle suspension. Data were analysed using the FlowJo software v10.1r5 (FlowJo LLC, Ashland, OR, USA).

Statistical analysis

The analyses of chemokines and cytokines were performed using the mean of the duplicate measurements. The number of events and respective percentages were calculated for the categorical variables and the median with interquartile range (IQR) for the continuous variables.

Comparison between groups was performed by a double-sided Mann–Whitney test. The differences were considered statistically significant when $P < 0.05$.

Table I. Characteristics of the study population.

	Patients ($n = 40$)	Controls ($n = 47$)
Age in months, median (IQR)	10 (5·0–13·5)	12·2 (7·7–16·7)
Race, n (%)		
White	27 (67·5)	35 (74·5)
Black	6 (15·0)	9 (19·1)
Mixed	6 (15·0)	–
Indian native	1 (2·5)	–
Unknown	–	3 (6·4)
Severity of HA, n (%)		
Severe	38 (95·0)	NA
Moderate*	2 (5·0)	NA
Reason for diagnosis, n (%)		
Bleeding	31 (77·5)	NA
Family history	9 (22·5)	NA

*Two patients presented 1%–2% of factor VIII:C.
IQR, interquartile range; n, number of patients; HA, Haemophilia A; NA, not applicable

Fig 1. Representation of the chemokines analysed. Each point in the graph represents one measurement of one individual. The continuous line represents the median of the results in the group of individuals analysed for each chemokine. Comparison between groups was performed by Mann–Whitney test. The differences were considered statistically significant when $P < 0.05$.

In an exploratory analysis, individuals were considered high or low responders for each cytokine evaluated. The cut-off point between low and high levels of each cytokine was defined by calculating the median from the values obtained for the control group. Individuals with cytokine levels above the median were considered high responders. Based on the percentage of high responder individuals, the radar chart was built to characterize the balance of distinct inflammatory and anti-inflammatory/regulatory cytokines in PUPs with HA and controls. Each axis was connected to the central polygon area, which represents the magnitude of cytokine profiles. The increasing or decreasing central polygon area reflects major or minor contribution of a given cytokine profile in

untreated patients with HA and control groups. The frequency of high and low cytokine producers was compared by contingency table analysis and χ^2 test.

Statistical analyses were performed using GraphPad Prism 5 software (GraphPad Software, San Diego, CA, USA).

Results

Patients

A total of 40 PUPs with HA [FVIII coagulant activity (FVIII: C) < 2%] were included in the study from February 2013 to August 2016. Patients had a median age of 10·0 months

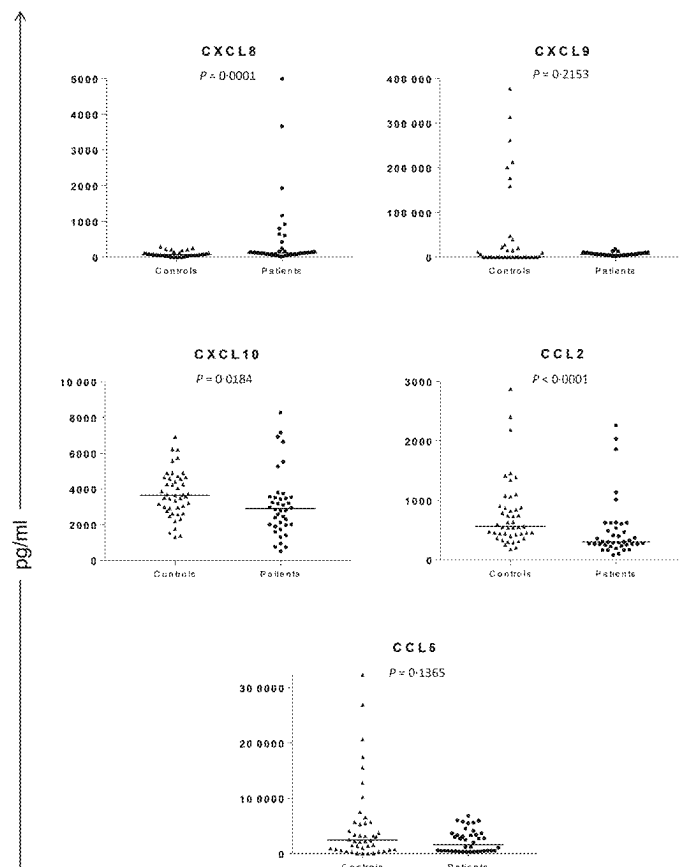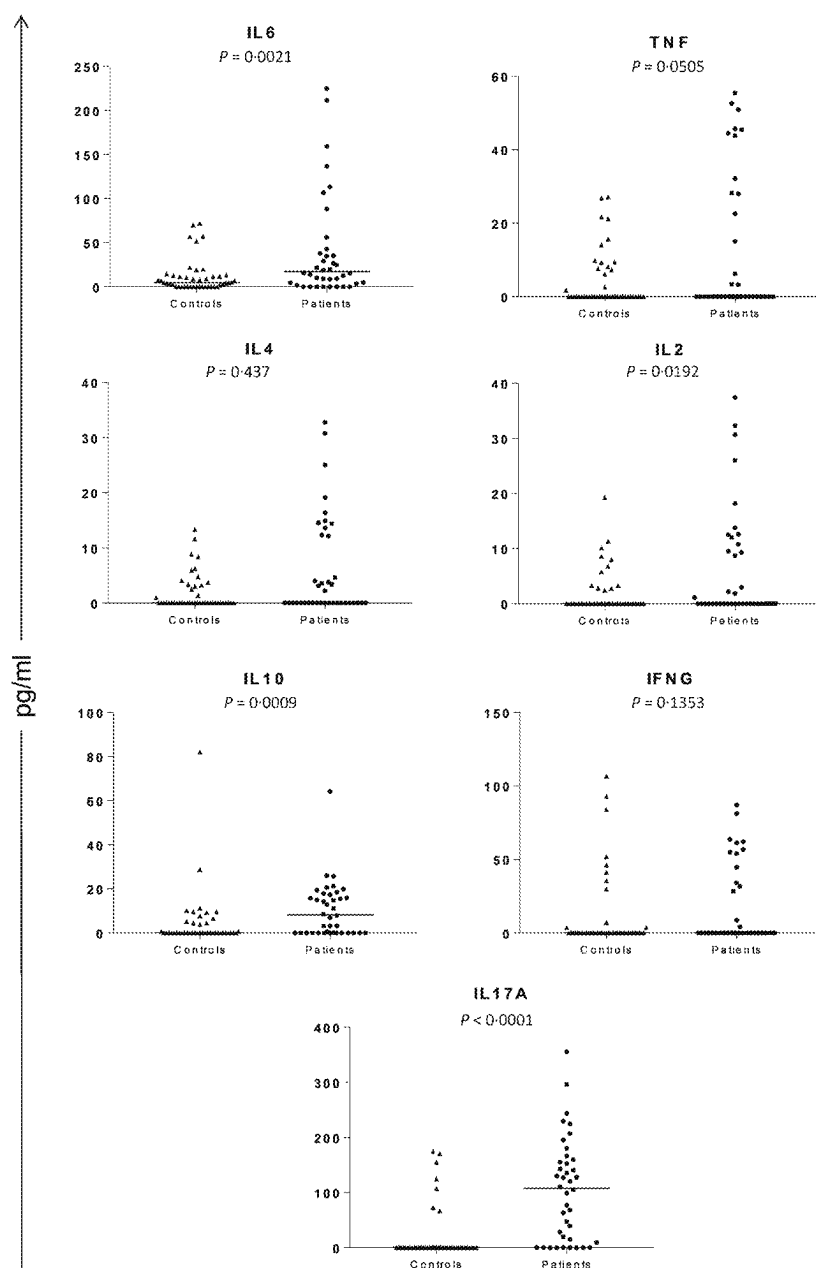Fig 2. Representation of the cytokines analysed. Each point in the graph represents one measurement of one individual. The continuous line represents the median of the results in the group of individuals analysed for each cytokine. Comparison between groups was performed by Mann–Whitney test. The differences were considered statistically significant when $P < 0.05$.

[interquartile range (IQR), 5·0–3·5 months]. The control group consisted of 47 healthy non-haemophiliac Brazilian boys, with a median age of 12·2 months (IQR, 7·7–16·7).

A total of 38 (95%) PUPs had severe HA (FVIII:C ≤ 1%) whereas 2 (5%) had moderately-severe HA (FVIII:C 1–2%) (Table I).

At the time of inclusion/blood collection, 31 (77·5%) patients had suffered bleeding episodes of variable severity (bruises, gum bleeding, puncture site bleeding after blood collection, cephalohaematoma and haemarthrosis). The remaining 9 patients (22·5%) were diagnosed before the onset of any clinical bleeding. These patients had no reported bleeding at the time of blood collection. There was no family history of inhibitors in never treated patients enrolled in this study and 26 (65%) had family history of HA.

Analysis of chemokines and cytokines

Levels (in pg/ml) of CXCL8 [median, 118·7; interquartile range (IQR), 76·2–221·1; $P = 0.0001$], IL6 (median, 17·4; IQR, 3·7–52·7; $P = 0.0021$), IL4 (median, 0; IQR, 0–13·3; $P = 0.0437$), IL10 (median, 8·2; IQR, 0–17·7; $P = 0.0009$), IL2 (median, 0; IQR, 0–11·7; $P = 0.0192$) and IL17A (median, 107·8; IQR, 10·9–159; $P < 0.0001$) were increased in

PUPs compared with controls (Figs 1 and 2). Otherwise, patients presented lower levels of CXCL10 (median, 2902; IQR, 1989–3565; $P = 0.02$) and CCL2 (median, 303; IQR, 251·3–588·2; $P < 0.0001$) in comparison with controls (Figs 1 and 2).

There was no difference in the plasma levels of TNF (median, 0; IQR, 0–40·9; $P = 0.0505$), IFNG (median, 0; IQR, 0–42·6; $P = 0.1353$), CXCL9 (median, 6454; IQR, 4469–9064; $P = 0.2153$) and CCL5 (median, 16024; IQR, 4819–35802; $P = 0.1365$) between PUPs and controls (Figs 1 and 2).

The radar chart demonstrates that a higher proportion of patients, i.e., 67·5%, 70·0% and 77·5%, respectively, presented with IL10, IL6 and IL17A levels above the median (Fig 3). On the other hand, the control group presented a lower proportion of all cytokines tested and a more expressive area towards IL6 (Fig 3).

Microparticle analysis

When compared with controls, PUPs with HA presented higher levels of microparticles derived from endothelium (median, 8·3; IQR, 3·8–15·2; $P = 0.0019$) erythrocytes (median, 13·5; IQR, 7·8–19·1; $P = 0.0046$), platelets (CD41a;

Group	IL6, n (%)	TNF, n (%)	IL2, n (%)	IFNG, n (%)	IL4, n (%)	IL10, n (%)	IL17A, n (%)
Patients	28 (70·0%)	18 (45·0%)	19 (47·5%)	15 (37·5%)	19 (47·5%)	27 (67·5%)	31 (77·5%)
Controls	23 (48·9%)	16 (34·0%)	13 (27·7%)	11 (23·4%)	16 (34·0%)	17 (36·2%)	7 (14·9%)
P-value	0·05	0·21	0·02	0·07	0·13	<0·01	<0·01
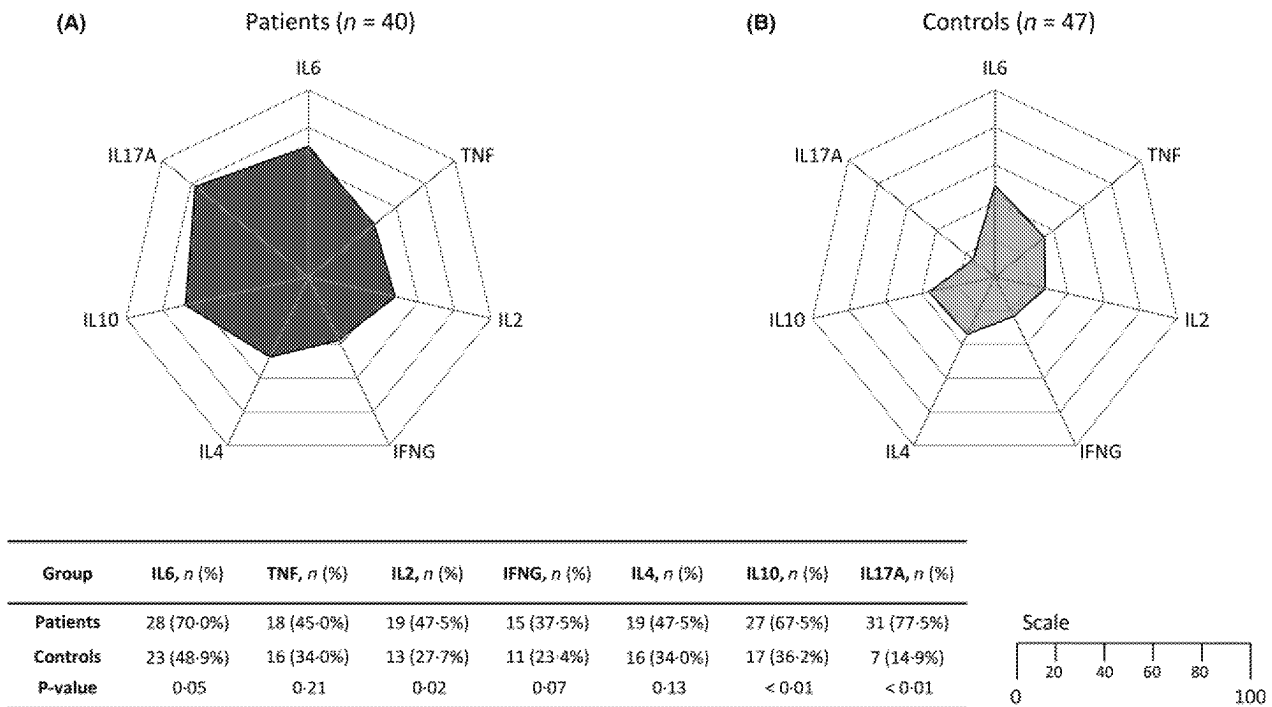
Fig 3. Radar graphical representation of cytokine patterns in patients and controls. This chart summarizes the percentage of high cytokine balance in previously untreated patients (dark grey area) and controls (light grey area). The cut-off point between low and high levels of each cytokine was defined by calculating the median from the values obtained for the control group. Each axis represents the proportion of individuals with the levels of cytokine above the median. The increase or decrease of central polygon areas reflect higher or lower contribution of inflammatory or regulatory cytokine balance in patients and controls. The contingency table presents the frequency of individuals with higher levels of each cytokine in patients and controls. Comparison between the groups was performed by χ^2 test. The differences were considered statistically significant when $P < 0.05$.

WITN3428037_0006

median, 29·1; IQR, 8·1–206·2; $P < 0.001$), leucocytes (median, 9·5; IQR, 5·6–22·7; $P = 0.0150$), neutrophils (median, 5; IQR, 3·5–8·4; $P = 0.0008$), and T lymphocytes (median, 6; IQR, 3·7–9·1; $P = 0.0054$) (Fig 4). On the contrary, the plasma levels of monocytes (median, 4; IQR, 2·2–5·3; $P = 0.5729$) did not differ between the groups.

Discussion

This study aimed at investigating the immunological profile in PUPs with HA in comparison with healthy age-matched boys. PUPs presented significantly higher levels of CXCL8, IL6, IL2, IL4, IL10, IL17A and lower levels of CXCL10 and CCL2 in comparison with controls. Furthermore, patients presented increased levels of microparticles derived from endothelial cells, erythrocytes, platelet, leucocytes, neutrophils and T lymphocytes in comparison with controls. Therefore, PUPs with HA presented a distinct immunological profile characterized by a prominent pro-inflammatory status regulated by IL4 and IL10.

Chemokines are proteins involved in the recruitment of inflammatory cells to the site of injury or immune response (Mantovani, 1999). CXCL8 is mainly produced by neutrophils and macrophages and is involved in inflammation and angiogenesis (Koch *et al*, 1992; Curfs *et al*, 1997). It acts as an important chemokine in the setting of infection and vascular injury (Koch *et al*, 1992; Curfs *et al*, 1997). In this study, higher levels of CXCL8 and lower levels of CXCL10 and CCL2 were characteristic of the PUPs with HA group. Koch *et al* (1992) suggested that CXCL8 might be involved

Fig 4. Representation of the microparticles analysed. Each point in the graph represents one measurement of one individual. The continuous line represents the median of the results in the group of individuals analyzed for each microparticle. Comparison between groups was performed by Mann–Whitney test. The differences were considered statistically significant when $P < 0.05$.
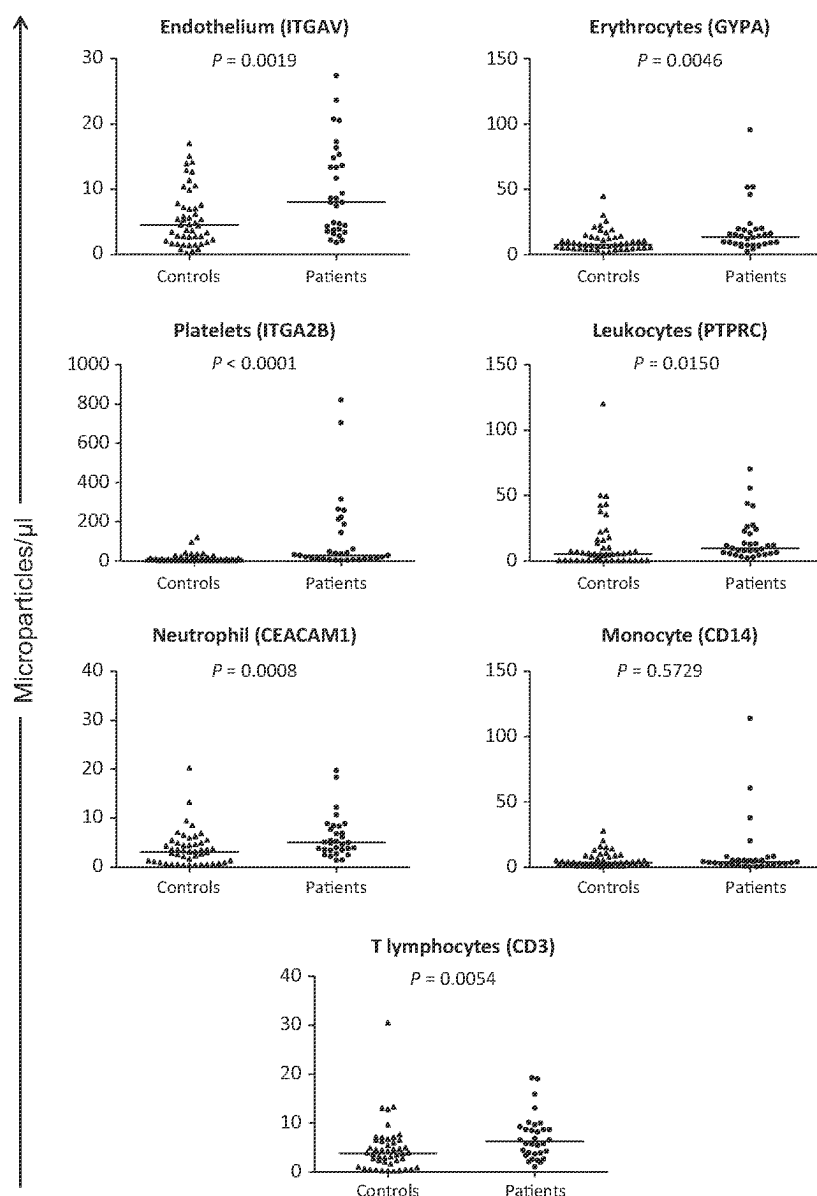
in angiogenesis-dependent disorders, such as wound repair and conditions characterized by persistent neovascularization. The increased levels of CXCL8 in PUPs might reflect vascular injury as consequence of bleeding and as an attempt to heal wound in affected individuals with severe HA.

CCL2 is mainly produced by monocytes and is significantly increased in presence of thrombin (Gu et al, 2000; Sato et al, 2016). As result of FVIII deficiency, we expect PUPs to have lower levels of thrombin in comparison with healthy children (Young et al, 2013). Therefore, this could explain the lower levels of CCL2 observed in patients when compared with controls.

Cytokines are involved in the activation and inhibition of cell functions, such as immunological response, angiogenesis and vascular injury (Pober & Cotran, 1990; Abbas et al, 1996). Since they regulate cell differentiation, cell repair, normal turnover and migration of cells into injury sites, defective regulation of the cytokine network may play a role in the pathogenesis of diseases and clinical settings (Pober & Cotran, 1990; Abbas et al, 1996).

In our study, PUPs with HA presented higher plasma levels of cytokines IL6, IL2, IL4, IL10 and IL17A, when compared with controls. IL6 is a pro-inflammatory cytokine which activates the immune system and stimulates the initial neutrophil infiltration during acute inflammation (Scheller et al, 2008). IL4 and IL10 are complex multifunctional Th2 cytokines presenting anti-inflammatory and anti-inflammatory/regulatory characteristics, respectively. (Saraiva & O'Garra, 2010; Sachin et al, 2012). During early inflammatory response, cytokines such as IL4 and IL10 can be released as an attempt to balance the microenvironment and control inflammation, producing a mixed cytokine profile (Hu et al, 2007). In this study, the higher levels of IL2 and IL17A found in PUPs when compared with controls also suggest the occurrence of a pro-inflammatory response mediated by T cells (Oliveira et al, 2013; Silveira et al, 2015). Our hypothesis is that IL6 and CXCL8 are secreted in response to an initial inflammatory process occurring in PUPs with HA. The question here is why these patients present an inflammatory response before starting replacement with FVIII? We hypothesize that this is likely to occur due to bleeding, which stimulates inflammation. Indeed, nearly 80% of the patients included in our study reported bleeding episodes of variable severity at inclusion/blood collection.

Microparticles are a heterogeneous population of small fragments (0·1–1·0 µm) released from apoptotic or activated cells (Wolf, 1967). The recruitment of microparticles that express phosphatidylserine and tissue factor on their surface is associated with coagulation activity, which is amplified in response to tissue injury (McEver, 2001; Morrissey et al, 2011). Vascular dysfunction, pro-inflammatory response and transport of cytokines are also related to microparticle levels (Jy et al, 2010). Additionally, leucocyte-derived microparticles can stimulate the release of chemokines and cytokines in attempt to increase a procoagulant and pro-inflammatory

activity (Mesri & Altieri, 1998; Distler et al, 2005). Therefore, microparticles could serve as a useful tool to explore coagulation activity in response to tissue injury.

Circulation microparticles have been related to a shorter tail-vein bleeding time in HA mice, after injection of soluble P-selectin immunoglobulin (Hrachovinová et al, 2003). Increased levels of procoagulant microparticles were observed in patients with HA and inhibitors after infusion of recombinant activated factor VII (Proulle et al, 2004). Furthermore, microparticle levels increased during a bleeding episode in young patients with HA (Proulle et al, 2005). In our study, PUPs presented higher levels of microparticles derived from endothelial cells, erythrocytes, platelet, leucocytes, neutrophils and T lymphocytes when compared with controls. It will be interesting to compare the immunological profile of PUPs with HA with the profile found after the onset of FVIII replacement and upon inhibitor development. The ongoing HEMFIL study will address these issues.

This study has some limitations. (i) Fourteen patients were not included in the cohort because they presented clinical bleeding at the time of enrolment, which required immediate therapeutic intervention. These patients had and median age of 11 months (IQR, 7·5–15·0) and all 14 had severe HA. (ii) PUPs were a little younger than controls, although this difference was not significant.

We conclude that PUPs with HA, in comparison with healthy controls, present a prominent pro-inflammatory status characterized by high levels of CXCL8, IL6, IL2 and IL17A, balanced with higher levels of IL10.

Author contributions

LLJ performed the research, analysed the data and wrote the paper; ACOSC contributed to the performance of the immunological techniques; ACSS selected the controls, collected clinical data; MPS, MHC, AP, VF and CL selected the patients and collected clinical data; JGVB contributed with study design and data analysis; DGC and SMR designed the research, contributed to data analysis and wrote the paper. All authors revised and approved the final version of the manuscript.

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