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As an extended half-life recombinant FVIII, Esperoct® offers a simple way to reach higher trough FVIII activity levels compared to standard half-life treatments.**1,4-9

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In adults and adolescents (12 years and over)[†] with severe haemophilia A, Esperoct® demonstrated:

A simple, fixed dose:^{††1,4}
50 IU/kg every 4 days

Higher trough FVIII activity levels
vs. SHL treatments:^{1,4-9}

Mean trough FVIII activity levels of 3%

Low ABR:^{1,4}
Median total ABR^{‡§} of 1.18

*40°C storage for up to 3 months before reconstitution¹ **Esperoct® is licenced for the treatment and prophylaxis of bleeding in patients 12 years and above with haemophilia A (congenital factor VIII deficiency).

The safety and efficacy of Esperoct in previously untreated patients have not yet been established.¹

This Novo Nordisk advertisement is intended for UK Healthcare Professionals

Prescribing Information

Esperoct® powder and solvent for solution for injection Turoctocog alfa pegol Esperoct 500 IU Esperoct 1000 IU Esperoct 1500 IU Esperoct 2000 IU Esperoct 3000 IU **Indication:** Treatment and prophylaxis of bleeding in patients 12 years and above with haemophilia A (congenital factor VIII deficiency) **Dosage and administration:** The dose, dosing interval and duration of the substitution therapy depend on the severity of the factor VIII deficiency, on the location and extent of the bleeding, on the targeted factor VIII activity level and the patient's clinical condition. **On demand treatment and treatment of bleeding episodes:** Required dose IU = body weight (kg) x desired factor VIII rise (%) (IU/dL) x 0.5 (IU/kg per IU/dL). **Mild haemorrhage:** early haemarthrosis, mild muscle bleeding or mild oral bleeding. Factor VIII level required (IU/dL or % of normal): 20-40. Frequency of doses: 12-24, until the bleeding is resolved. **Moderate haemorrhage:** More extensive haemarthrosis, muscle bleeding, haematoma. Factor VIII level required (IU/dL or % of normal): 30-60. Frequency of doses: 12-24, until the bleeding is resolved. **Severe or life-threatening haemorrhages:** Factor VIII level required (IU/dL or % of normal) - 60-100. Frequency of doses: 8-24, until the threat is resolved. **Perioperative management:** **Minor surgery including tooth extraction:** Factor VIII level required (IU/dL or % of normal): 30-60. Frequency of doses (hours): within one hour before surgery; repeat after 24 hours if necessary. Duration of therapy: single dose or repeat injection every 24 hours for at least 1 day until healing is achieved. **Major surgery:** Factor VIII level required (IU/dL or % of normal): 80-100 (pre- and post-operative). Frequency of doses (hours): Within one hour before surgery to achieve factor VIII activity within the target range. Repeat every 8 to 24 hours to maintain factor VIII activity within the target range. Repeat injection every 8 to 24 hours as necessary until adequate wound healing is achieved. Consider continuing therapy for another 7 days to maintain a factor VIII activity of 30% to 60% (IU/dL). **Prophylaxis:** The recommended dose is 50 IU of Esperoct per kg body weight every 4 days. Adjustments of doses and administration intervals may be considered based on achieved factor VIII levels and individual bleeding tendency. **Paediatric population:** The dose in adolescents (12 years and above) is the same as for adults. In children below 12 years long-term safety has not been established. **Method of administration:** Intravenous injection (over approximately 2 minutes) after reconstitution of the powder with 4 mL supplied solvent (sodium chloride 9 mg/mL (0.9%) solution for injection). **Contraindications:** Hypersensitivity to the active substance or to any of the excipients, or to hamster protein. **Special warnings and precautions for use:** Name and the batch number of the administered product should be clearly recorded to improve traceability. **Hypersensitivity:** Allergic-type hypersensitivity reactions are possible due to traces of hamster proteins, which in some patients may cause allergic reactions. If symptoms of hypersensitivity occur, patients should be advised to immediately discontinue the use of the medicinal product and contact their physician. Patients should be informed of the early signs of hypersensitivity reactions including hives, generalised urticaria, tightness of the chest, wheezing, hypotension, and anaphylaxis. In case of shock, standard medical treatment for shock should be implemented. **Inhibitors:** The formation of neutralising antibodies (inhibitors) to factor VIII is a known complication in the management of individuals with haemophilia A. These inhibitors are usually IgG immunoglobulins directed against the factor VIII pro-coagulant activity, which are quantified in Bethesda Units (BU) per mL of plasma using the modified assay. The risk of developing inhibitors is correlated to the severity of the disease as well as the exposure to factor VIII, this risk being highest within the first 50 exposure days but continues throughout life although the risk is uncommon. The clinical relevance of inhibitor development will depend on the titre of the inhibitor, with low titre posing less of a risk of insufficient clinical response than high titre inhibitors. Patients treated with coagulation factor VIII products should be monitored

for the development of inhibitors by appropriate clinical observations and laboratory tests. If the expected factor VIII activity plasma levels are not attained, or if bleeding is not controlled with an appropriate dose, testing for factor VIII inhibitor presence should be performed. In patients with high levels of inhibitor, factor VIII therapy may not be effective and other therapeutic options should be considered. **Cardiovascular events:** In patients with existing cardiovascular risk factors, substitution therapy with factor VIII may increase the cardiovascular risk. **Catheter-related complications:** If a central venous access device (CVAD) is required, the risk of CVAD-related complications including local infections, bacteraemia and catheter site thrombosis should be considered. **Paediatric population:** Listed warnings and precautions apply both to adults and adolescents (12-18 years). **Excipient-related considerations:** Product contains 30.5 mg sodium per reconstituted vial, equivalent to 1.5% of the WHO recommended maximum daily intake of 2.0 g sodium for an adult. **Fertility, pregnancy and lactation:** Animal reproduction studies have not been conducted with factor VIII. Based on the rare occurrence of haemophilia A in women, experience regarding the use of factor VIII during pregnancy and breast-feeding is not available. Therefore, factor VIII should be used during pregnancy and lactation only if clearly indicated. **Undesirable effects:** Adverse events in clinical trials which could be considered serious include: (≥1/10): Rash, erythema, pruritis, injection site reactions (<1/10,000): Factor VIII inhibition, hypersensitivity. The Summary of Product Characteristics should be consulted in relation to other adverse reactions. **MA numbers and Basic NHS Price:** Esperoct 500 IU EU/1/19/1374/001 £425 Esperoct 1000 IU EU/1/19/1374/002 £850 Esperoct 1500 IU EU/1/19/1374/003 £1,275 Esperoct 2000 IU EU/1/19/1374/004 £1,700 Esperoct 3000 IU EU/1/19/1374/005 £2,550 **Legal category:** POM. For full prescribing information please refer to the SmPC which can be obtained from the Marketing Authorisation Holder: Novo Nordisk Limited, 3 City Place, Beehive Ring Road, Gatwick, West Sussex, RH6 0PA. **Marketing Authorisation Holder:** Novo Nordisk A/S, Novo Allé, DK-2880 Bagsvaerd, Denmark. **Date last revised:** September 2020

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ABR, annualised bleed rate; EHL, extended half-life; FVIII, factor VIII; rFVIII, recombinant factor VIII; SHL, standard half-life

[†]Previously treated patients, 12 years and above. ^{††}Prophylaxis: The recommended dose is 50 IU of Esperoct per kg body weight every 4 days. Adjustments of doses and administration intervals may be considered based on achieved factor VIII levels and individual bleeding tendency. [‡]Total ABR includes all bleeds: spontaneous, traumatic and joint bleeds⁴

References: 1. Esperoct® Summary of Product Characteristics. 2. Adynovi® Summary of Product Characteristics. 3. Elocta® Summary of Product Characteristics. 4. Giangrande P et al. Thromb Haemost 2017; 117:252-261. 5. Tiede A et al. J Thromb Haemost 2013; 11:670-678. 6. Advate® Summary of Product Characteristics. 7. NovoEight® Summary of Product Characteristics. 8. Nuwiq® Summary of Product Characteristics. 9. Refacto AP® Summary of Product Characteristics.

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

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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 (IFN γ), stimulating the synthesis of

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.

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, CA, 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 ($1500 \times g$ for 15 min followed by $13\,000 \times g$ 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 $14\,000 \times g$ for 90 min at 15°C . The resultant microparticle pellet was resuspended in 100 μl of $1 \times$ 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

Table I. Characteristics of the study population.

	Patients (<i>n</i> = 40)	Controls (<i>n</i> = 47)
Age in months, median (IQR)	10 (5.0–13.5)	12.2 (7.7–16.7)
Race, <i>n</i> (%)		
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, <i>n</i> (%)		
Severe	38 (95.0)	NA
Moderate*	2 (5.0)	NA
Reason for diagnosis, <i>n</i> (%)		
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

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 $P < 0.05$.

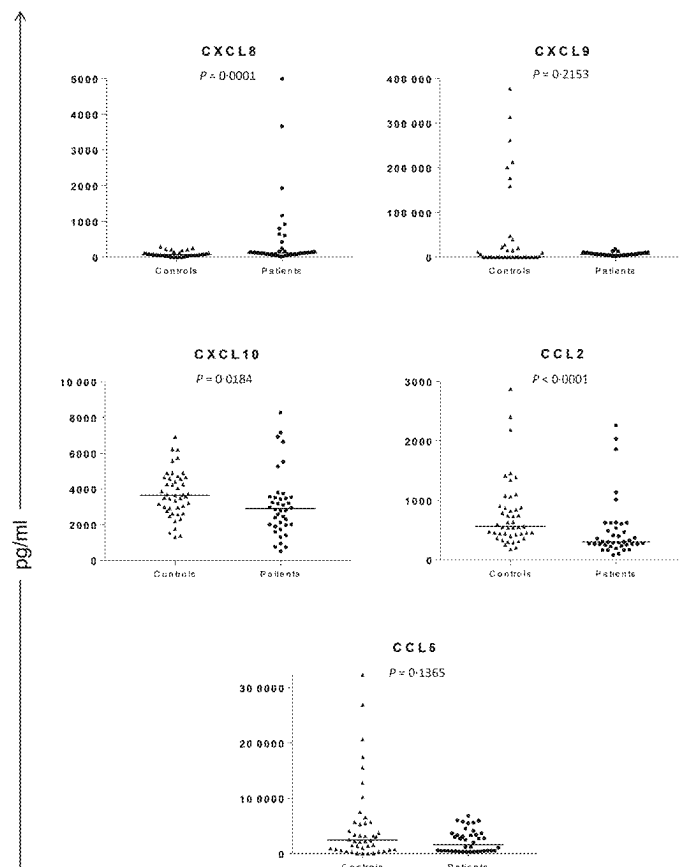


Fig 1. Representation of the chemokines analysed. Each point in the graph represents one measurement of one individual. The continuous line represents the median of the results in the group of individuals analysed for each chemokine. Comparison between groups was performed by Mann–Whitney test. The differences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

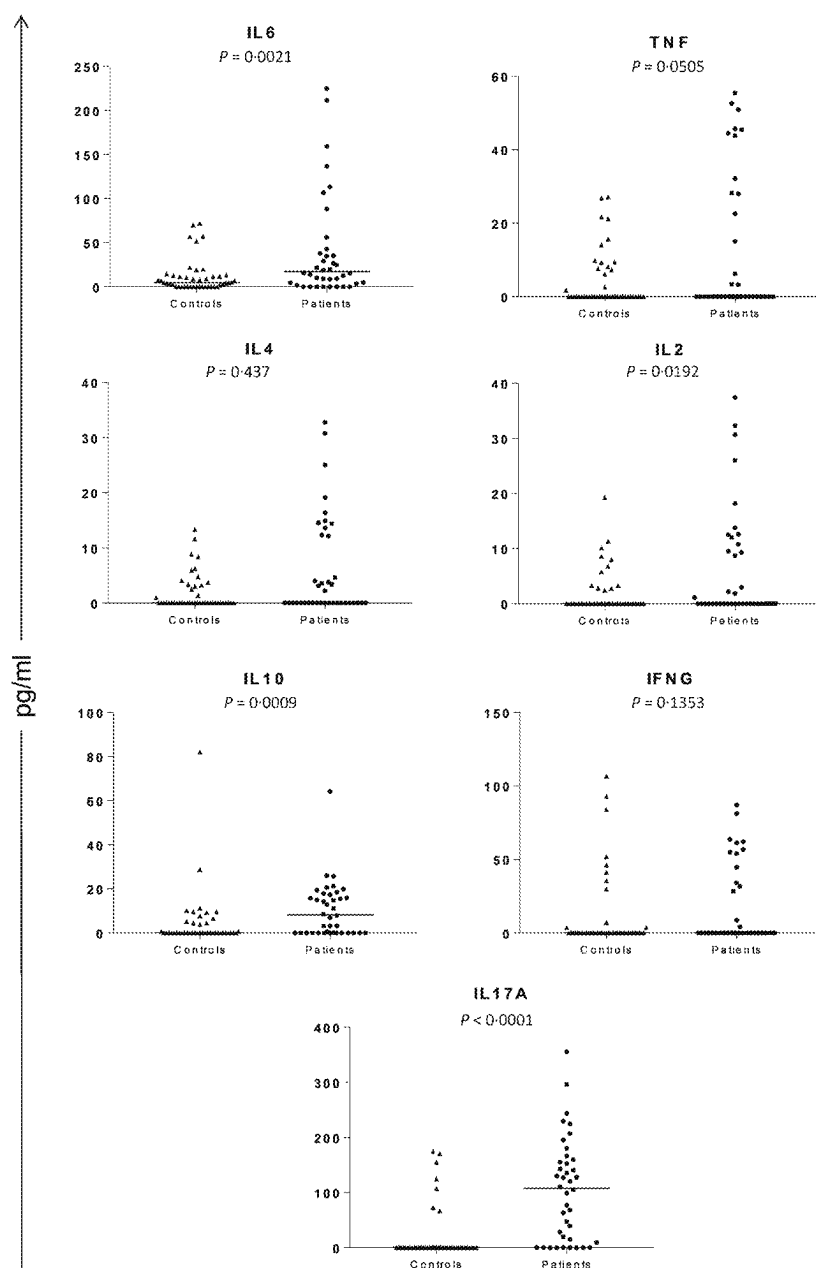


Fig 2. Representation of the cytokines analysed. Each point in the graph represents one measurement of one individual. The continuous line represents the median of the results in the group of individuals analysed for each cytokine. Comparison between groups was performed by Mann-Whitney test. The differences 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 $\leq 1\%$) whereas 2 (5%) had moderately-severe HA (FVIII:C 1–2%) (Table 1).

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;

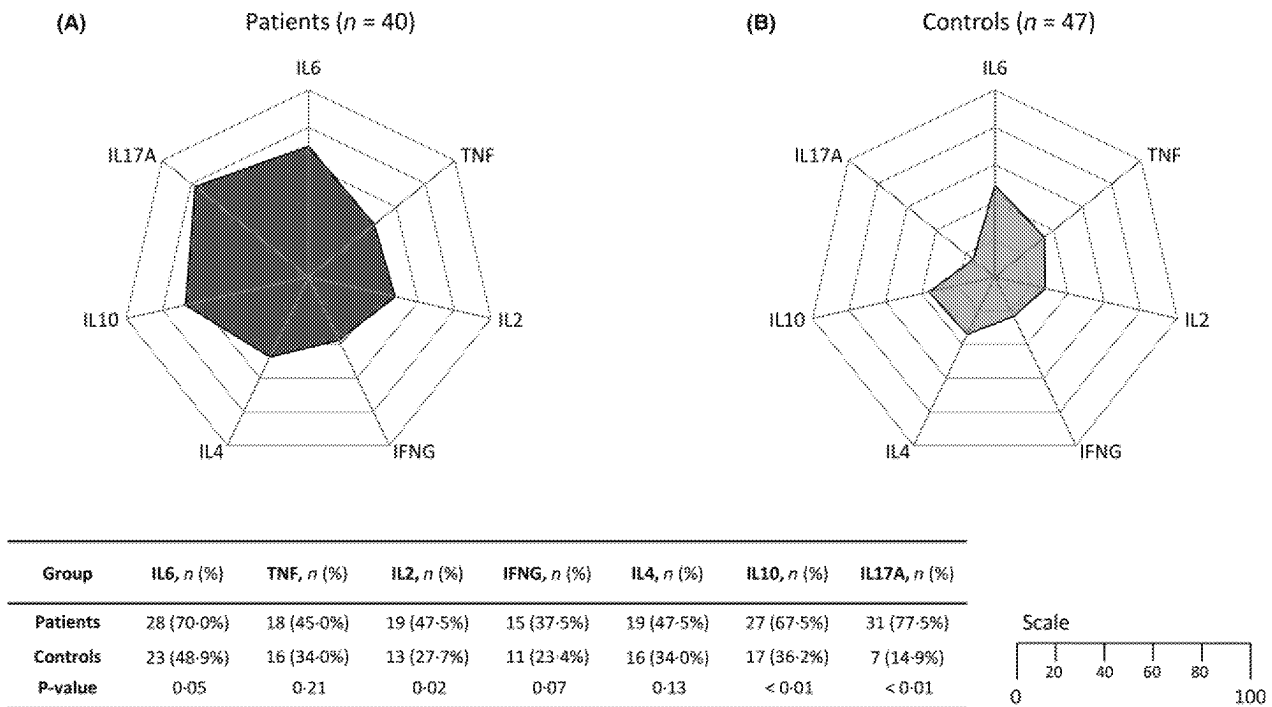


Fig 3. Radar graphical representation of cytokine patterns in patients and controls. This chart summarizes the percentage of high cytokine balance in previously untreated patients (dark grey area) and controls (light grey area). The cut-off point between low and high levels of each cytokine was defined 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 $P < 0.05$.

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

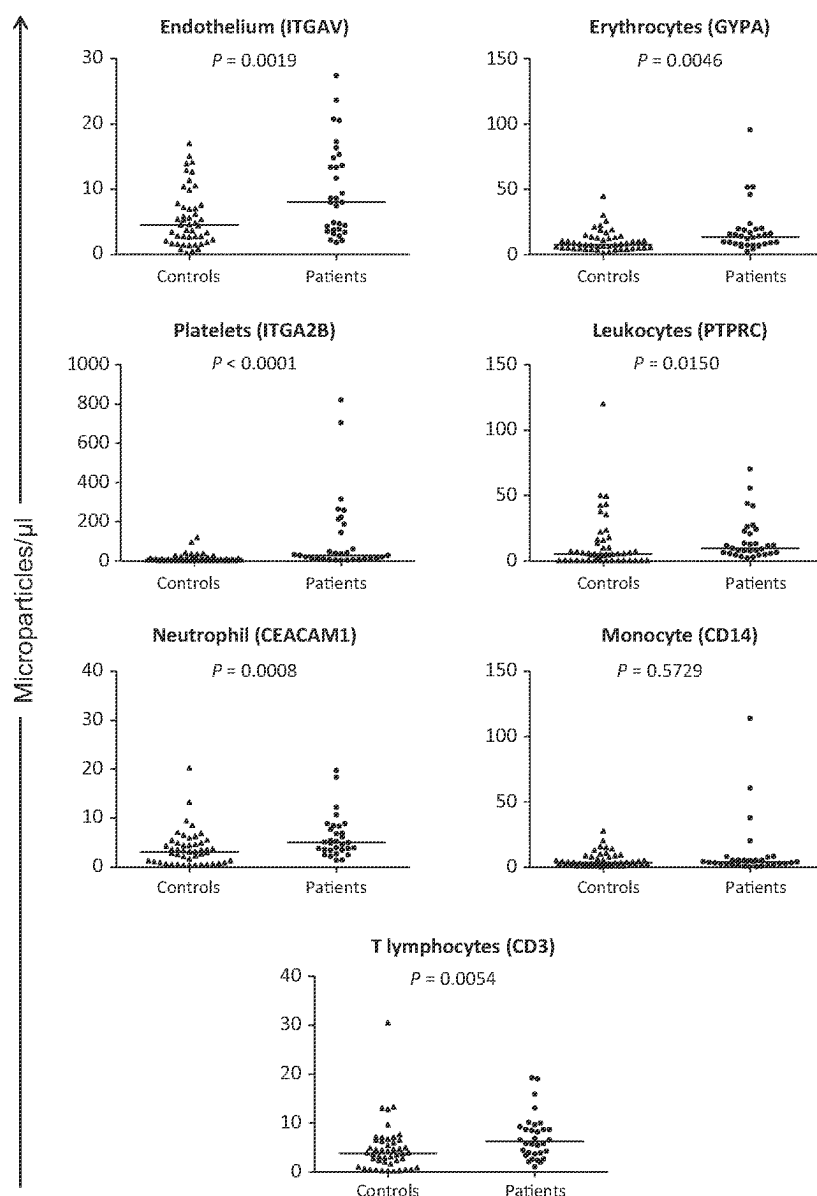


Fig 4. Representation of the microparticles analysed. Each point in the graph represents one measurement of one individual. The continuous line represents the median of the results in the group of individuals analyzed for each microparticle. Comparison between groups was performed by Mann–Whitney test. 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.

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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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