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Effect of the First Factor VIII Infusions on Immunological Biomarkers in Previously Untreated Patients with Hemophilia A from the HEMFIL Study

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Abstract

Hemophilia A (HA) is an inherited bleeding disorder which requires continuous replacement with factor (F) VIII concentrate. The main complication of HA is the development of neutralizing alloantibodies which inhibit FVIII activity (inhibitors). The objective of this study was to investigate the effect of the first FVIII infusions on immunological biomarkers in previously untreated patients with HA. Plasma samples were collected at enrollment before any FVIII infusion (T0) and at inhibitor development (INB +/T1) or up to 35 exposure days without inhibitors (INB −/T1). Anti-FVIII antibodies (immunoglobulin M, immunoglobulin G [IgG] 1, IgG3, and IgG4), chemokines (CCL2, CCL5, CXCL8, CXCL9, and CXCL10), and cytokines (interleukin [IL]-2, IL-4, IL-6, IL-10, interferon-γ, tumor necrosis factor, and IL-17) were assessed. A total of 71 children with severe HA were included, of whom 28 (39.4%) developed inhibitors. Plasma levels of anti-FVIII IgG4, IL-6, and CXCL8 were higher at INB +/T1 when compared with INB −/T1. This group presented a mixed cytokine profile and higher plasma levels of CXCL9 and CXL10 when compared with INB +/T1. We conclude that exposure to FVIII triggers a proinflammatory response mediated by IL-6 and CXCL8 in patients with HA who developed inhibitors. Regardless of inhibitor status, the immune system of all HA patients is stimulated after infusions of FVIII.

Keywords
- hemophilia A
- factor VIII
- inhibitors
- immune response
- immunological biomarkers

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Introduction

Hemophilia A (HA) is an inherited bleeding disorder caused by factor (F) VIII deficiency due to mutations in the FVIII gene (*F8*).[1] Treatment of HA requires replacement with FVIII concentrates or nonfactor-based therapies.[2,3] One of the major complications in HA is the development of neutralizing alloantibodies which inhibit the activity of FVIII (inhibitors). The dosage of inhibitors is quantified by the Bethesda assay. Inhibitors above and below 5 Bethesda units [BU]/mL are considered as high and low titers, respectively.[4] Immunological mechanisms leading to inhibitor development are not yet completely understood.

Earlier studies suggested that repeated infusions of FVIII alter the immune response, which contributes to inhibitor development.[4–6] These studies reported the presence of an immune anti-inflammatory/regulatory profile in patients with inhibitors in comparison with patients without them. Otherwise, patients without inhibitors exhibit an immune proinflammatory profile.[5,6] However, these studies had a cross-sectional design and enrolled patients with long-standing inhibitors. Furthermore, they did not assess immune biomarkers before FVIII replacement, on the course of replacement, nor at the time of inhibitor development. In this study, we evaluated a panel of biomarkers of the immune system before the first FVIII infusion (T0) and at inhibitor development (INB+/T1) or up to 35 exposure days (EDs) without inhibitor development (INB−/T1) in previously untreated patients (PUPs) with HA.

Methods

Study Population

We enrolled male PUPs with severe HA (FVIII activity [FVIII:C] <1%) who were participants of the HEMFIL Cohort Study. PUPs were attended at four hemophilia treatment centers (HTCs) in Brazil (Minas Gerais, Paraná, Rio de Janeiro, and Santa Catarina).[7] For this study, PUPs were included before any exposure to FVIII and were treated either on demand or prophylactically with recombinant FVIII (ADVATE Alfa octocog; Takeda, Lexington, United States). Patients' data were collected through standardized forms. Since all PUPs in the HEMFIL Study developed inhibitors within the first 35 EDs to FVIII, for this report, we included patients who were followed up until 35 EDs or up to inhibitor development. All parents/guardians signed a written informed consent form. The study was approved by the institutional ethics committees.

Sample Collection and Processing

Blood samples were collected in tubes containing sodium citrate 3.2% as anticoagulant for the patients at the time of diagnosis (T0), during the first 35 EDs in INB− (T1/INB−), and at inhibitor development in INB+ (T1/INB+). Samples were centrifuged and immediately frozen at −80°C. Plasma samples were thawed at 37°C before assays.

Determination of the Coagulant Activity of FVIII and Inhibitor Assessment

At diagnosis, FVIII:C was measured in each HTC. Plasma samples were diluted in imidazole buffer and supplemented with FVIII-deficient plasma and cephalin. Calcium chloride was used as activator and the time of clot formation was recorded. The FVIII:C was calculated using a calibration curve obtained with plasma pool of normal controls.

Inhibitors were measured by the Bethesda assay with Nijmegen modification[8] in each HTC. Once positive (≥0.6 BU/mL), the inhibitor status was confirmed if the second test, assessed 2 to 4 weeks later, yielded a positive result (≥0.6 BU/mL).

Assessment of Anti-FVIII Antibodies

Enzyme-linked immunosorbent assay (ELISA) was performed for the detection of anti-FVIII antibodies. For this, 96-well plates (Invitrogen, Nunc MaxiSorp, Thermo Scientific, Massachusetts, United States) were coated overnight at 4°C with 100 µL of recombinant FVIII (ADVATE) diluted in phosphate buffer saline (PBS) 1X (0.1 IU/well). The plates were washed three times with 100 µL/well of washing solution (PBS 1X [Sigma-Aldrich, St. Louis, United States], 1% Tween 20 [Sigma-Aldrich]) between steps. The plates were incubated for 1 hour at 37°C with 200 µL/well of blocking solution (PBS 1X [Sigma-Aldrich], 1% bovine serum albumin [VWR Life Science, Radnor, United States]). Plasma samples (100 µL/well) diluted 1:40 in blocking solution were added and incubated for 1 hour at 37°C. Plates were incubated for 1 hour with 100 µL/well with the following antibodies diluted in blocking solution: goat polyclonal anti-human IgM-Biotin (B1265, Sigma-Aldrich; 1:40,000); mouse monoclonal anti-human IgG1-HRP (M1328; Sanquin; 1:10,000); mouse monoclonal anti-human IgG3-Biotin (B3523, Sigma-Aldrich; 1:1,000); and mouse monoclonal anti-human IgG4-Biotin (B3648, Sigma-Aldrich; 1:3,000). Peroxidase-labeled streptavidin (Sigma-Aldrich; 100 µL/well) diluted in blocking solution (1:5,000) was added and plates were incubated for 30 minutes at room temperature, excepted for immunoglobulin G (IgG) 1. O-Phenylenediamine (Sigma-Aldrich; 100 µL/well) was added and plates were incubated at room temperature for 30 minutes. After addition of 50 µL/well of 1M H_2SO_4 (Sigma-Aldrich), the optical density (OD) was measured using 492 nm filter in the ELISA reader. Experiments were performed in duplicates.

For each plate of ELISA assay tested, we included (1) an adult normal control pool, composed of plasma of 20 healthy adults; (2) a children normal control pool, composed of plasma of 20 healthy children, (3) a positive sample for each antibody; and (4) a blank. Plasma samples of the control groups were tested individually before the pool was made. To evaluate intra-assay coefficient of variation (CV), the pool of the control group was titrated and each dilution was replicated 10 times in the same assay. The intra-assay CV for the 1:40 dilution was 20%. The inter-assay CV was calculated based on the results of six different measurements of the positive control titrated from 1:10 to 1:640 in separate assays performed on different days. The inter-assay CV for the 1:40 dilution was 12%.

Assessment of Cytokines and Chemokines

Plasma samples were centrifuged for 10 minutes at 32,000 × g for platelet-poor plasma separation. Cytokines (interleukin [IL]-2, IL-4, IL-6, IL-10, interferon-γ [IFN-γ], tumor necrosis factor [TNF], and IL-17) and chemokines (CCL2, CCL5, CXCL8, CXCL9 and CXCL10) were measured using commercial kits (Cytometric Bead Array; BD Biosciences, San Jose, United States) as previously described.[7]

Molecular Tests

Inversions of intron 1 (Inv1) and 22 (Inv22) were detected using a polymerase chain reaction-based method.[9,10] For samples negative for inversions, *F8* exons and intron–exon boundaries were sequenced using a customized panel of next-generation sequencing (Illumina; California, United States). Data analyses were performed using Illumina's BaseSpace Suite. Patients carrying null (introns 1 and 22 inversions, nonsense, frameshift, and large deletions) and nonnull (missense and splice site) F8 pathogenic variants were classified as high and low risk of inhibitor development, respectively.[11] The frequency of patients carrying high-risk variants was compared between the groups with (INB +) and without inhibitors (INB −).

Statistical Analysis

The number of events and percentages for categorical variables were calculated. Median with interquartile range (IQR) for the continuous variables was calculated. Fisher's exact test was used to compare frequencies. Comparison between groups was performed using the double-sided Mann–Whitney test. Correlation analyzes were performed using the Spearman correlation test. Data included in figures presented correlation coefficients above 0.5. Correlations were considered strong when $r > 0.68$.[12] Radar charts were constructed using the frequency of patients with levels of biomarkers above the median of all patients in time point. The differences were considered statistically significant when $p < 0.05$. Graphpad Prism 5.0 software was used for data analysis and Cytoscape (version 3.7.1) was used for network design.

Results

Study Population

We enrolled 71 PUPs with severe HA, median age 10.0 months (IQR: 6.5–14.0 months), of whom 39.4% developed inhibitors during the study. Inhibitor development occurred with a median of 13 EDs (IQR: 9–17) (T1/INB +). Five patients (17.9%) developed inhibitors after 20 EDs. The median ED in T1/INB− was 8 (IQR: 4–22 ED). The median inhibitor titer was 8.4 BU/mL (IQR: 3.1–36.1). Patients with high-titer ($n = 18$; 64.3%) and low-titer inhibitors ($n = 10$; 35.7%) presented a median titer of 22.4 BU/mL (IQR: 9.0–71.5) and 2.1 BU/mL (IQR: 0.9–3.1), respectively. Inversion of intron 22 was more prevalent in patients who developed inhibitors when compared with the ones who did not ($p < 0.01$). Detailed data of included patients are summarized in ►**Table 1**. The frequency of patients with null mutations was significantly higher in the group INB+ when compared with the group INB− (92.8 vs. 58.1%, respectively; $p = 0.01$).

Table 1 Characteristics of the patients included

	INB− group	INB+ group	*p*-Value
Number of patients (%)	43 (60.6)	28 (39.4)	–
Age in months, median (IQR)	10.0 (6.0–14.0)	9.5 (7.0–12.5)	0.17
Race, *n* (%)			
White	30 (69.8)	17 (60.7)	0.44
Black	8 (18.6)	4 (14.3)	0.66
Mixed	4 (9.3)	6 (21.4)	0.18
Native	1 (2.3)	ND	–
Asian	ND	1 (3.6)	–
Reason for diagnosis, *n* (%)			
Bleeding	24 (55.8)	15 (53.6)	0.86
Family history	5 (11.6)	7 (25.0)	0.16
Bleeding + family history	14 (32.6)	6 (21.4)	0.33
F8 mutation, *n* (%)			
Inversion of intron 22	11 (25.6)	21 (75.0)	<0.01[a]
Missense	8 (18.6)	1 (3.6)	0.12
Nonsense	6 (14.0)	1 (3.6)	0.18
Small deletions/ insertions	4 (9.3)	3 (10.7)	0.60
Large deletion	3 (7.0)	ND	–
Splice site mutation	3 (7.0)	ND	–
Inversion of intron 1	1 (2.3)	ND	–
Initiation codon mutation	ND	1 (3.6)	–
Inhibitor titer in BU/mL, median (IQR)	ND	8.4 (3.1–36.1)	–
High titer (≥5 BU/mL), *n* (%)	ND	18 (64.3)	–
Low titer (<5 BU/mL), *n* (%)	ND	10 (35.7)	–

Abbreviations: BU, Bethesda unit; INB, inhibitor; IQR, interquartile range; ND, not defined.
[a]Statistically significant.

Plasma Concentration of IL-6 Is Significantly Higher in INB +/T1 in Comparison with INB −/T1

The median plasma concentration of IL-6 and IL-17 in INB −/T0 was higher ([7.9 pg/mL; IQR: 0.9–35.5] and [47.4 pg/mL; IQR: 1.6–141.8], respectively) when compared with INB −/T1 ([1.0 pg/mL; IQR: 0.0–3.4; $p = 0.016$] and [1.0 pg/mL; IQR: 0.0–2.8; $p = 0.007$], respectively). In INB +/T0, median plasma IL-17 (24.2 pg/mL; IQR: 0.4–162.9) was higher when compared with INB +/T1 (2.5 pg/mL; IQR: 1.2–7.6; $p = 0.045$). INB +/T1 presented a higher concentration of IL-6 (median, 3.9 pg/mL; IQR: 1.7–12.0) when compared with INB −/T1 (median, 1.0 pg/mL; IQR: 0.0–3.4; $p = 0.005$). No significant differences were found in plasma concentrations of IL-2, IL-4, IL-10, IFN-γ, and TNF (►**Fig. 1**).

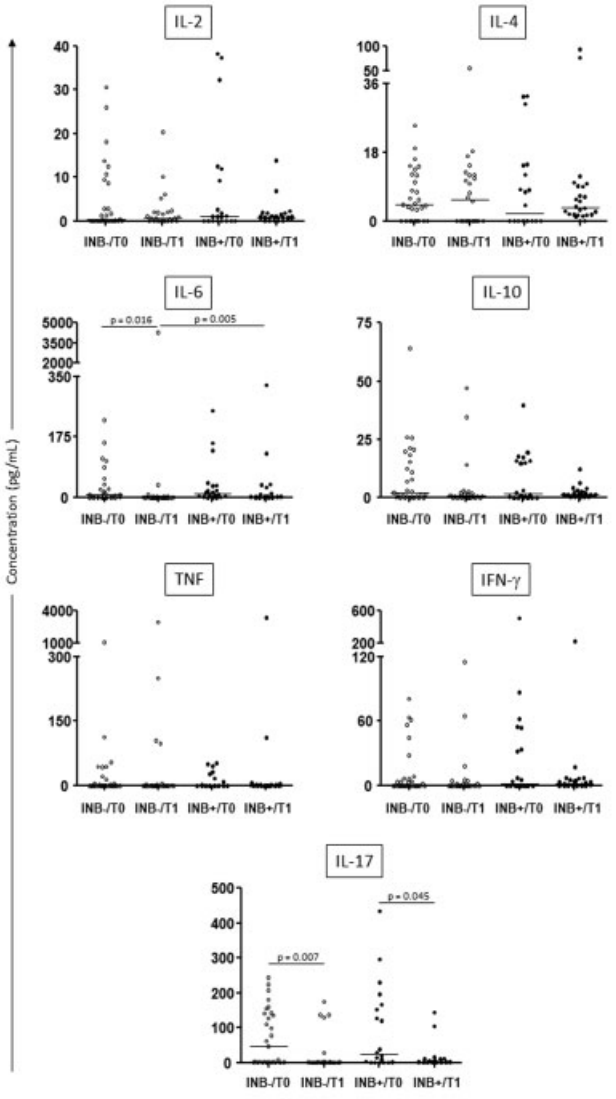


Fig. 1 Representation of plasma cytokine concentration (in pg/mL) in all patients by group and time point. Each circle represents the mean concentration of two measurements of each cytokine. *Empty circles* represent patients without inhibitor (INB−) and *black filled circles* represent patients with inhibitor (INB+). *Horizontal lines* represent the median concentration of each measured cytokine in the respective group. IFN, interferon; IL, interleukin; T0, time at enrollment, before any FVIII infusion; T1, time point after FVIII infusion; TNF, tumor necrosis factor.

High Plasma Concentration of CXCL8 Is a Hallmark of INB +/T1

Median plasma concentrations of CCL2 (254.4 pg/mL; IQR: 26.7–474.7), CCL5 (4,937.0 pg/mL; IQR: 3,107.0–31,340.0), and CXCL8 (80.2 pg/mL; IQR: 7.8–143.3) in INB −/T0 were higher than those in INB −/T1 ([15.5 pg/mL; IQR: 6.8–149.4; $p = 0.003$], [1,551.0 pg/mL; IQR: 1,210.0–3,177.0; $p < 0.001$], and [0.0 pg/mL; IQR: 0.0–22.7; $p < 0.001$], respectively).

Median plasma concentrations of CCL2 (289.9 pg/mL, IQR: 174.5–455.3), CCL5 (5,390.0 pg/mL; IQR: 3,462.0–31,725.0), CXCL8 (116.1 pg/mL; IQR: 41.6–379.3), CXCL9 (5,602.0 pg/mL; IQR: 2,956.0–9,655.0), and CXCL10 (2,632.0 pg/mL; IQR: 1,326.0–3,562.0) in INB +/T0 were higher than those in INB +/T1 ([14.0 pg/mL; IQR: 10.0–24.7; $p < 0.001$], ([2,694.0 pg/mL; IQR: 1,659.0–3,145.0; $p < 0.001$], [4.7 pg/mL; IQR: 2.4–13.5; $p < 0.001$], [151.5 pg/mL; IQR: 78.2–822.4; $p < 0.001$], and [650.4 pg/mL; IQR: 469.7–1,357.0; $p = 0.001$], respectively) (►**Fig. 2**).

The comparison of INB −/T1 and INB +/T1 revealed that the median plasma concentration of CXCL8 was higher in INB +/T1 (4.7 pg/mL; IQR: 2.4–13.5) than that in INB −/T1 (0.0 pg/mL; IQR: 0.0–22.7; $p = 0.019$). In contrast, median plasma concentrations of CXCL10 (1,726.0 pg/mL; IQR: 1,021.0–2,310.0) and CXCL9 (1,421.0 pg/mL; IQR: 518.2–4,119.0) were higher in INB −/T1 than those in INB +/T1 ([650.4 pg/mL; IQR: 469.7–1,357.0; $p = 0.003$] and [151.5 pg/mL; IQR: 78.2–822.4; $p < 0.001$], respectively) (►**Fig. 2**).

Exposure to FVIII Was Associated with Increased Levels of Specific Anti-FVIII IgG4 in INB +/T1

The median OD of anti-FVIII IgG3 was significantly higher in INB −/T1 (0.017; IQR 0.001–0.055) than that in INB +/T1 (0.002; IQR 0.000–0.022; $p = 0.042$) (►**Fig. 3**).

The median OD of anti-FVIII IgG4 was significantly higher in INB +/T1 (0.004; IQR: 0.000–0.031) than that in INB +/T0 (0.000; IQR: 0.000–0.000; $p = 0.002$). Additionally, the median

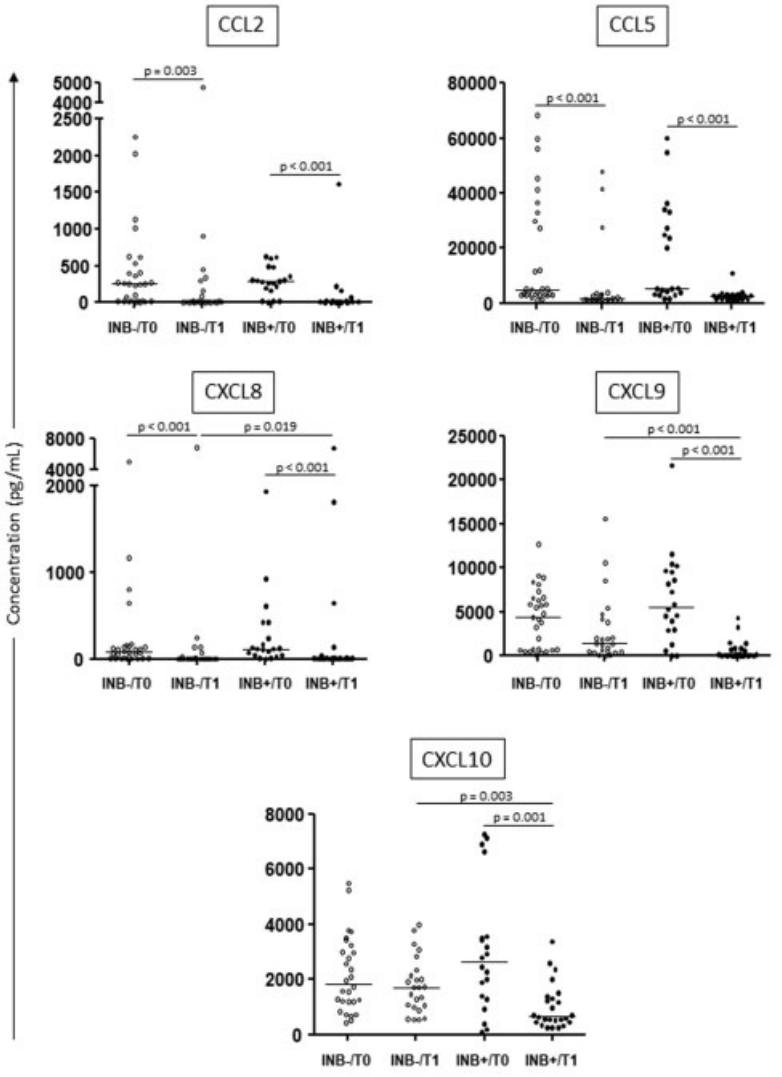


Fig. 2 Representation of plasma chemokine concentration (in pg/mL) in all patients by group. Each circle represents the mean concentration of two measurements of each chemokine. *Empty circles* represent patients without inhibitor (INB−) and *black filled circles* represent patients with inhibitor (INB+). *Horizontal lines* represent the median concentration of each measured chemokine in the respective group. T0, time at enrollment, before any FVIII infusion; T1, time point after FVIII infusion.

OD of anti-FVIII IgG4 in INB +/T1 was significantly higher than the median OD in INB −/T1 [0.000; IQR: 0.000–0.012; $p = 0.028$] (►**Fig. 3**). Levels of anti-FVIII immunoglobulin M (IgM) did not change after FVIII exposure in the groups.

Correlation analysis between Bethesda titers and OD values of anti-FVIII specific antibodies of all patients revealed a Spearman correlation coefficient (ρ) of 0.271 ($p = 0.057$) for anti-FVIII IgG1 and ρ = 0.41 ($p = 0.003$) for anti-FVIII IgG4 at stratum 1–35 ED.

A Proinflammatory Immune Profile Was Found in INB +/T1

The analysis of radar charts of the cytokine profile in INB +/T1 revealed a significantly higher proportion of patients with increased levels of IL-6 (69.2 vs. 29.2%; $p = 0.005$) when compared with the INB −/T1 (►**Fig. 4A**).

Analyses of the chemokine profile in INB −/T1 revealed a significantly higher proportion of patients with increased levels of CXCL10 (66.7 vs. 34.6%; $p = 0.028$) and CXCL9 (70.8 vs. 34.6%; $p = 0.012$) when compared with INB +/T1, respectively. INB +/T1 had significantly higher proportion of patients with increased levels of CXCL8 (69.2% vs. 29.2%; $p = 0.005$) when compared with INB −/T1 (►**Fig. 4B**).

An Impaired Network between Cytokines and Chemokines Was Observed in PUPs Who Developed Inhibitors

The chemokine/cytokine networks assembled according to the status of inhibitor development and stratum are presented in ►**Fig. 5**. INB +/T0 shows a substantially lower number of neighborhood connections when compared with INB −/T0. INB −/T0 presents strong edges of high correlation indexes between almost all cytokines, except IL-17 and IL-6. An overall analysis shows that the cross-talk between cytokines and chemokines is impaired in INB +/T0.

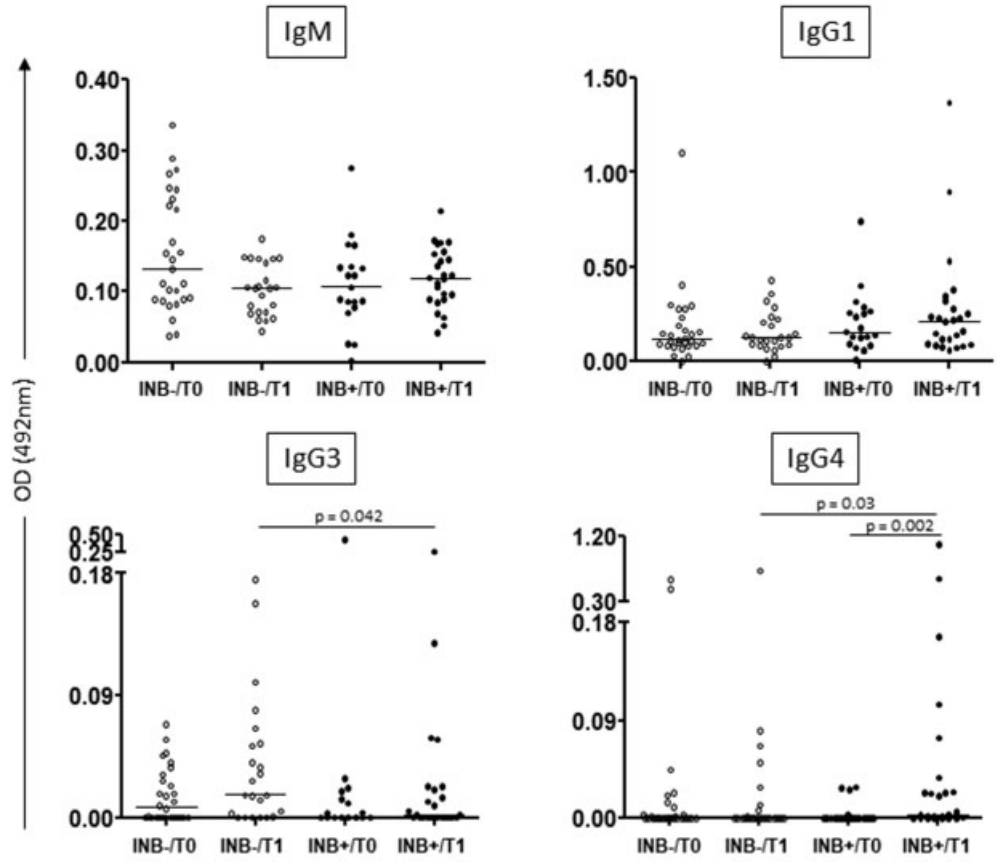
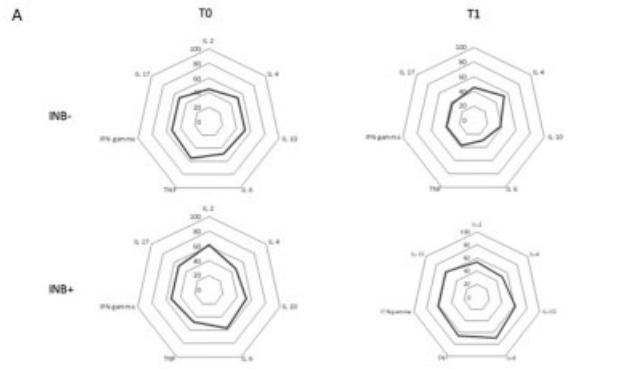
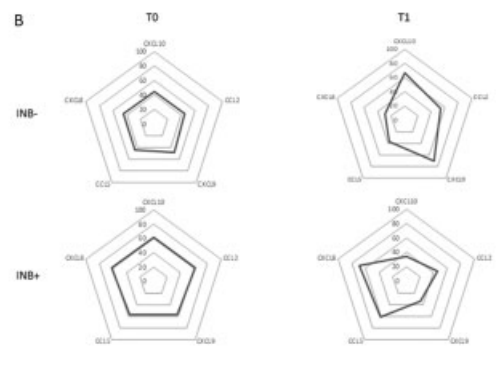


Fig. 3 Levels of anti-FVIII IgM, IgG1, IgG3, and IgG4 antibodies in all included patients by group. Each *empty circle* represents the mean OD of two measurements of immunoglobulin assessed in each patient without inhibitor (INB−). *Each black circle* represents the mean OD of two measurements of immunoglobulin assessed in each patient with high and low titer inhibitor (INB+). The *horizontal lines* represent the median OD of the respective immunoglobulin in each stratum. IgG, immunoglobulin G; IgM, immunoglobulin M; OD, optical density; T0, time at enrollment, before any FVIII infusion; T1, time point after FVIII infusion.



Group/Stratum	IL-2, n (%)	IL-4, n (%)	IL-10, n (%)	IL-6, n (%)	TNF, n (%)	IFN-γ, n (%)	IL-17, n (%)
INB-/T0	13 (44.8)	15 (51.7)	15 (51.7.3)	14 (48.3)	16 (55.2)	15 (51.7)	15 (51.7)
INB+/T0	13 (61.9)	10 (47.6)	11 (52.4)	12 (57.1)	10 (47.6)	11 (52.4)	11 (52.4)
INB-/T1	11 (45.8)	13 (54.2)	9 (37.5)	7 (29.2)*	9 (37.5)	9 (37.5)	9 (37.5)
INB+/T1	14 (53.8)	13 (50.0)	16 (61.5)	18 (69.2)*	17 (65.4)	16 (65.5)	16 (61.5)

*p<0.05



Group/Stratum	CCL2, n (%)	CCL5, n (%)	CXCL8, n (%)	CXCL9, n (%)	CXCL10, n (%)
INB-/T0	13 (44.8)	13 (44.8)	13 (44.8)	14 (48.3)	13 (44.8)
INB+/T0	13 (61.9)	12 (57.1)	13 (61.9)	12 (57.1)	13 (61.9)
INB-/T1	13 (54.2)	9 (37.5)	7 (29.2)*	17 (70.8)*	16 (66.7)*
INB+/T1	12 (34.6)	16 (61.5)	18 (69.2)*	9 (34.6)*	9 (34.6)*

*p<0.05

Fig. 4 Radar charts containing the proportion of patients with high concentration of plasma cytokines (**A**) and chemokines (**B**) at T0 and T1. Each axis represents the proportion of individuals with cytokine and chemokine levels above the median. The increase or decrease of the areas of the central polygon respectively reflects the more or less contribution of the inflammatory or regulatory balance of cytokines and chemokines in INB+ and INB−. Comparison between groups was performed using Fisher's exact test. The differences were considered statistically significant when $p < 0.05$ and are highlighted with "*". IFN, interferon; IL, interleukin; T0, time at enrollment, before any FVIII infusion; T1, time point after FVIII infusion; TNF, tumor necrosis factor.

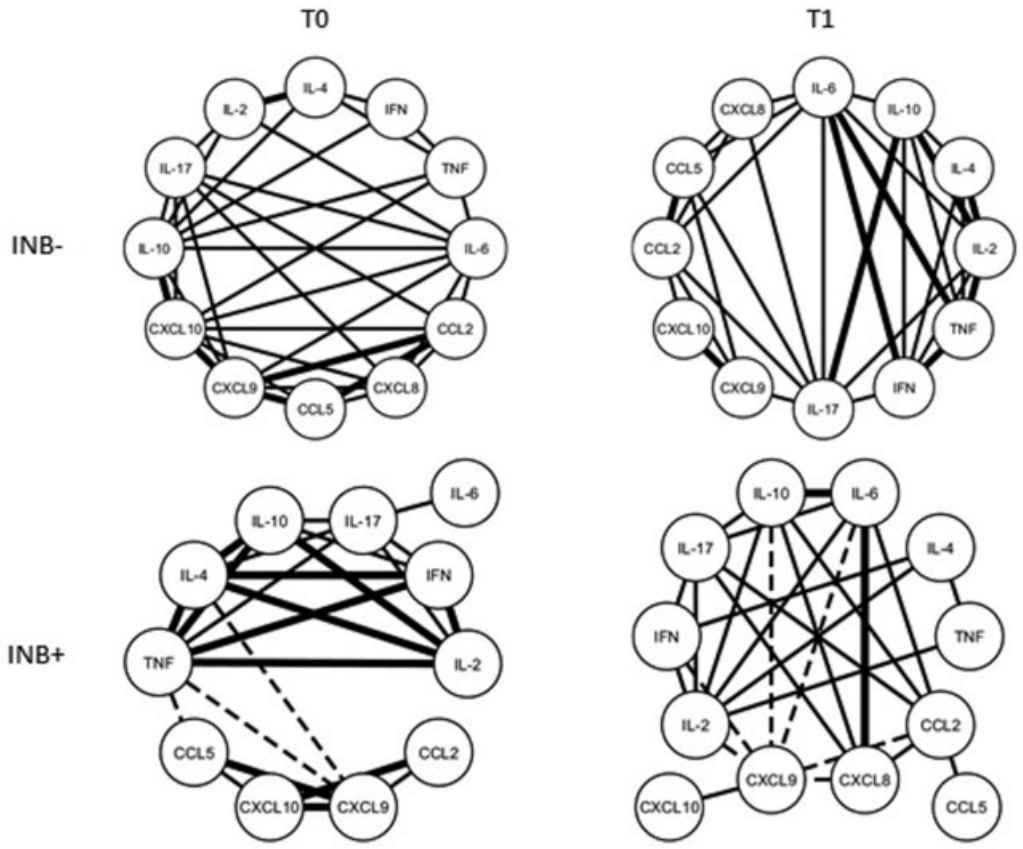


Fig. 5 Representation of correlation network of immunological biomarkers. *Solid lines* correspond to positive correlation between biomarkers. *Dotted lines* correspond to negative correlation between biomarkers. *Thicker lines* represent strong correlations ($r > 0.68$).

After the first exposures to FVIII, the network of INB −/T1 remains with a high number of connections. Correlations in INB −/T1 were stronger among cytokines when compared with INB −/T0, specially IL-6 and IL-10. A rearrangement of connections in INB +/T1 resulted in an intense cross-talking between cytokines and chemokines, particularly IL-6 and CXCL8 (►**Fig. 5**).

Discussion

We studied the effect of the first FVIII infusions on immunological biomarkers in PUPs with severe HA. We found that PUPs who developed inhibitors presented increased plasma levels of specific anti-FVIII IgG4, IL-6, and CXCL8 concentrations in comparison with the ones who did not. We found an impaired network between cytokines and chemokines prior to any exposure to FVIII in PUPs who developed inhibitors. Our results suggest that the development of inhibitors occurs in a proinflammatory microenvironment. Regardless of the inhibitor status, the immune system of all patients with HA is stimulated after repeated infusions of FVIII.

Inhibitor development in HA involves a classical T-cell-dependent immune response orchestrated by cytokines and chemokines influencing the attraction, activity, differentiation, proliferation, and survival of immune cells.[4,13–15] Studies have reported that cytokines play an important role in inhibitor development in HA patients,[5,6] but chemokines have been less explored.[7,16] Our study suggests that chemokines have a considerable role in inhibitor development in PUPs with HA. Although INB− and INB+ have similar plasma levels of cytokines and chemokines before FVIII exposure, analyses revealed significantly higher levels of IL-6 and CXCL-8 and significantly lower levels of CXCL9 and CXCL10 at inhibitor development in INB+. CXCL8 is mainly produced by macrophages and acts as a chemoattractant for granulocytes.[17] IL-6 is a pleiotropic cytokine that stimulates effector T-cell development and antibody production.[17] CXCL9 and CXCL10 are involved in the recruitment of effector T-cells to inflammation sites.[18] The radar charts in this study showed that after exposure to FVIII the proportion of patients who are high producers of IL-6 and CXCL8 is significantly increased in INB+. As a counterpart, the INB− group had a significantly greater proportion of patients who are high producers of CXCL9 and CXCL10. These results seem to indicate that INB+ presents a proinflammatory response which favors antigen presentation and activation of T and B lymphocytes.

Studies in hemophilia mice demonstrated that elevated levels of anti-inflammatory cytokines contribute to extended tolerance to FVIII.[19,20] Other studies associated the presence of higher levels of anti-inflammatory/regulatory cytokines to inhibitors.[4–6] Corroborating our findings, a recent study showed that a proinflammatory profile was predominant in HA mice that developed inhibitors.[21] This proinflammatory response might create a microenvironment that induces antigen presentation and activation of T-cells and antibody production.

The assessment of anti-FVIII-specific immunoglobulin revealed similar levels of anti-FVIII IgM in INB− and INB+ in our study. In contrast, a study in hemophilia mice detected a higher titer of anti-FVIII IgM after the first exposures to FVIII.[22] Analyses of anti-FVIII IgG3 levels revealed a significant increase of this immunoglobulin in INB −/T1. These results reinforce that these immunoglobulins are not relevant biomarkers for inhibitor development.[23–25]

The major subclasses of anti-FVIII antibodies found in patients with HA who develop inhibitors are IgG1 and IgG4.[5,24,26] A previous study using hemophilia mice demonstrated that inhibitor development is characterized by a prominent anti-FVIII IgG1 synthesis after four ED to FVIII.[22] In our study, anti-FVIII IgG4 in INB +/T1 was significantly higher when compared with INB +/T0. Despite significant results, anti-FVIII IgG1 and IgG4 showed low signals in ELISA and low correlation with BU. We hypothesize that this can be explained by the high inhibitory activity of low amounts of anti-FVIII antibodies and by the formation of immune complexes, which require low antibody levels to be formed.[27,28]

A correlation network study was performed to evaluate the interactions between cytokines and chemokines for INB− and INB +. Interestingly, we show that even before FVIII exposure, the network profiles of INB− and INB+ were different. INB −/T1 exhibited a complex cytokine–chemokine network. On the contrary, INB +/T0 revealed a compartmentalized network even before exposure to any FVIII. Therefore, our data show that the first ED to FVIII seems to be crucial for the activation of the immune system against FVIII. The network at INB +/T1 revealed a rearrangement of interactions with more cytokine–chemokine crosstalk. In INB −/T1 there is also a rearrangement of interactions between the biomarkers after FVIII infusions. However, in this group, the strongest correlations are observed between cytokines and no longer between chemokines as in T0. This suggests that FVIII seems to be recognized by the immune system of all PUPs with HA regardless of inhibitor development. However, in some patients the immune response is directed toward tolerance while in others FVIII promotes inhibitor development.[29] Our study suggests that there is an environment which predisposes to inhibitor development once, even before FVIII exposure, the network and immune profiles of INB− and INB+ are different.

This study has some limitations. FVIII and inhibitor tests were not performed centrally. However, external quality assessment programs were available for all HTCs. We did not evaluate FVIII kinetics nor inhibitor interaction to explain the low correlation between ELISA and the Bethesda test.

We conclude that PUPs with HA who developed inhibitors had increased levels of anti-FVIII IgG4, plasma concentration of IL-6, and CXCL8 in comparison with the ones who did not. They also presented an impaired network between cytokines and chemokines prior to any exposure to FVIII, suggesting that there might be a predisposing environment to inhibitor development even before FVIII replacement. Patients who did not develop inhibitors presented a mixed cytokine response and higher levels of CXCL9 and CXCL10. Nevertheless, the immune system of all patients with HA is stimulated by FVIII exposure regardless of inhibitor status.

What Is known about this topic?

- Development of neutralizing alloantibodies which inhibit factor VIII activity is one of the major complications in hemophilia A.
- Immunological mechanisms leading to inhibitor development is not yet completely understood.

What does this paper add?

- Patients who did not develop inhibitors presented a mixed cytokine profile and higher plasma levels of chemokines CXCL9 and CXL10.
- Exposure to FVIII triggers a proinflammatory response mediated by IL-6 and CXCL8 in patients with hemophilia A who developed inhibitors.

Authors' Contributions

L.M.M.O. and L.L.J. performed the research, analyzed the data, and wrote the manuscript; M.P.S., M.H.C., C.S.L., and V.K.B.F. selected the patients and collected the clinical data; L.W.Z. performed the molecular tests; D.G.C. and S. M.R. designed the research, contributed to data analysis, and wrote the manuscript. All authors revised and approved the final version of the manuscript.

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Conflict of Interest

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