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Risk factors for antibody formation in children with hemophilia: methodological aspects and clinical characteristics of the HEMFIL cohort study

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Up to 35% of patients with hemophilia A and 5% with hemophilia B develop neutralizing antibodies which can inhibit the therapeutic activity of factor replacement (inhibitors). Despite the clinical relevance of antifactor VIII and IX neutralizing antibodies, there is still a major gap on the knowledge of risk factors for their development. Furthermore, most of the studies on risk factors for inhibitor development come from Caucasian and Afro-American populations. The HEMFIL is a Brazilian prospective cohort study of previously untreated children with hemophilia, which primary aim is to identify new risk factors related to inhibitor development. This manuscript aims at describing the study design and its methodology. After the diagnosis, children are followed up to 75 exposure days or to inhibitor development. Standardized forms and blood samples are collected to describe clinical characteristics and to perform the measurement of immunological and genetic biomarkers at three time points; Inclusion time (T0), at inhibitor development or at 75 exposure days without inhibitors (T1) and after immune tolerance induction for patients in whom it is indicated and performed (T2). Currently, 120 children have been included, of whom, 95 have completed the follow-up. For severe/moderately severe hemophilia A, the cumulative incidence of inhibitors at 75 exposure days was 35% (95% confidence interval, 26–46%). The inclusion of additional patients and a longer follow-up will allow the analysis of risk factors for inhibitor development. *Blood Coagul Fibrinolysis* 32:443–450 **Copyright © 2021 Wolters Kluwer Health, Inc. All rights reserved.**

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Introduction

Hemophilia A and B are inherited X-linked bleeding disorders caused by deficiency of coagulation factor (F) VIII and IX, affecting 1 : 5000–10 000 and 1 : 40 000–50 000 new-born males worldwide, respectively. Treatment of bleeding episodes requires administration of FVIII or FIX-containing concentrates [1].

The main treatment-related complication in patients with hemophilia is the development of neutralizing antibodies, which occurs respectively in 20–35% and 1–5% of patients with hemophilia A and B within the first 75 exposure days [1,2]. These antibodies (inhibitors) block the protein epitopes, neutralizing the therapeutic activity of the infused factor [1,2]. Patients with inhibitors present hemorrhages of difficult control and lack of response to infusions of the deficient factor concentrates [1]. Currently, immune tolerance induction (ITI) is the unique treatment available for eradication of persistent anti-FVIII inhibitors, which occurs in 60–80% of cases [2]. For antifactor IX inhibitors treatment is more complex and less efficient.

Inhibitor development results from individual genetic predisposition associated with environmental and/or exogenous conditions. Studies targeting on biological mechanisms are scarce although crucial for discovering new treatments and to develop preventive strategies [3–6].

With the emergence of new, nonfactor replacement therapies, and specially Emicizumab (Roche, Switzerland), the therapeutic context for treating hemophilia patients with or without inhibitors has changed significantly in recent years. However, these therapies are costly and may not be accessible to patients living in less resourced countries. Moreover, bleeding episodes and surgical procedures in patients with hemophilia A using Emicizumab still demand use of FVIII concentrates, which will expose patients to inhibitor development. Thus, prospective cohort studies, nevertheless remain of interest in documenting the epidemiology of inhibitors, even in the era of nonfactor replacement therapies.

The HEMFIL study is an ongoing, prospective cohort study of patients with hemophilia, which the primary aim is to identify new risk factors related to inhibitor development. This manuscript aims at describing the study design and its methodology.

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Background

The development of neutralizing antibodies in patients with hemophilia is the result of individual genetic predisposition associated with environmental or exogenous conditions. Studies targeting a better understanding of the biological mechanisms behind inhibitor development are crucial for providing appropriate treatment and to develop preventive strategies.

Type and severity of hemophilia, family history of inhibitors, genetic mutation associated with the disease and the intensity of deficient factor infusions have been described as factors associated with increased risk for inhibitor development [4,7]. Patients who have been initially exposed to factor concentrate in an intensive mode, as in surgeries or severe bleeding, seem to present a higher risk of inhibitor development [7].

The type of factor concentrates used is the most debated nongenetic risk factor. Studies have shown that recombinant FVIII concentrates are more immunogenic [6–9] in comparison with plasma-derived ones [10]. Recently, the HEMFIL study reported a cumulative incidence of 36% [95% confidence interval (CI), 25.7–48.7%] for all antifactor VIII neutralizing antibodies and 27% (95% CI, 17.5–39.6%) for high-titer in a cohort of previously untreated patients (PUPs) with severe hemophilia A under exclusive use of ADVATE (Shire, Lexington, USA) [5]. These results are in agreement with the results from the SIPPET Study, which found a cumulative incidence of high-titer antifactor VIII neutralizing antibodies with recombinant FVIII concentrates of 28.4% (95% CI, 19.6–37.2) [6].

Although the biological mechanism of inhibitor development in congenital hemophilia has not yet been elucidated, it is well accepted that the infused protein is endocytosed by antigen-presenting cells, which cleaves the protein and present their peptides to CD4 T cells via complex of histocompatibility human (HLA) class II [11]. In addition, some fragments can also be presented to CD8 T cells through HLA class I. These interactions result in the clonal expansion of the active T lymphocytes, which secret cytokines and induce the production of FVIII inhibitory antibodies (IgG) by B lymphocytes [12,13]. Neutralizing antibodies against FVIII are predominantly of IgG4 subclass. However, the subclasses IgG1 and IgG2 are also observed [12]. In parallel, regulatory T cells produce regulatory cytokines and regulate the innate and adaptive immune response [14,15]. It is believed that these regulatory mechanisms may decrease the production of neutralizing antibodies against the infused FVIII.

It has been shown that during the initial infusion of FVIII concentrate some patients develop a pro-inflammatory response profile that involves IgG1 synthesis without FVIII inhibitory activity [13,16]. After the development of neutralizing antibodies against FVIII, this response shifts towards an anti-inflammatory/regulatory profile mediated by neutrophils and monocytes, with high expression of IL-5 and IL-10 and low levels of IL-2, IL-4, IFN-γ and TNF-α, which favors the synthesis of antifactor VIII IgG4 antibodies [13,16].

Anti-FVIII antibodies, mostly IgG1, IgM and IgA have been reported in healthy individuals [17] as well as in patients with either inherited or acquired hemophilia A [16,17–19]. Significantly, anti-FVIII IgG4 is predominantly found in patients with congenital hemophilia A who developed inhibitors, especially those with high-titer [17–19]. Except for one report [20], studies addressing anti-FVIII antibodies did not include the collection of blood samples before the first infusion of FVIII. Cannavò *et al.* [20] reported that among patients with severe hemophilia A who had never been exposed to FVIII concentrates, the presence of nonneutralizing antibodies was associated with inhibitor development. However, the specific subclass(es) of anti-FVIII immunoglobulin has not been examined.

An evaluation of immunological biomarkers in PUPs with hemophilia in a prospective cohort study design may contribute to a better understanding of the immune mechanisms involved in inhibitor development. This may shed light on the understanding why some patients develop inhibitors and others do not.

Our group reported that before the first FVIII infusion, patients with hemophilia A presented higher levels of microparticles, CXCL8/IL-8, IL-6, TNF, IL-4, IL-10 and IL-17 in comparison with controls without hemophilia [21]. Our hypothesis is that this inflammatory/regulatory cytokine and chemokine profile might be a result of subclinical bleeding, which could induce the activation of coagulation and inflammation.

The evaluation of cytokine and chemokine profile in a hemophiliac animal model with inhibitor suggests that low levels of TGF-β associated with high levels of pro-inflammatory cytokines may favor the development of an immune response against FVIII after gene therapy [22]. However, to the best of our knowledge, studies evaluating the immunological profile of patients with hemophilia before the first infusion of factor-containing products and after development of neutralizing antibodies or at 75 exposure days in patients without development are still lacking.

The HEMFIL study aim

To quantify the associations between potential immunological, clinical and genetic risk factors for the development of inhibitory antibodies against exogenous FVIII and FIX in patients with hemophilia before and during their first 75 infusions of the deficient factor concentrates.

The study name

The current HEMFIL study was named to honor Mr Henrique de Souza Filho (1944–1988), also known as

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Table 1 Collected variables

Variables of the inclusion
Name; date of birth; sex; ethnic origin; birth place; consanguinity; type of hemophilia; residual clotting factor activity at diagnosis; assessment of FVIII and FIX activity levels; blood group; *F8*/*F9* mutation; type of delivery; gestational age at delivery; use of blood products/blood components by the mother during delivery of the patient; use of blood products/blood components in the patient's first month of life; abnormal bleeding in the first week after birth; intracranial bleeding after the first week after birth; exclusive breastfeeding time; date of diagnosis of hemophilia, reason for diagnosis; associated bleeding disorder (if any); performance of circumcision; presence of other diseases. Information about family history of hemophilia, hemophilia carriers, prenatal diagnosis during pregnancy, diagnosis of inhibitors in other family members with hemophilia
Variables of follow-up (treatment-related)
Bleeding events during the analyzed period; number of bleeds, severity and location; use of coagulation factor concentrates during the evaluated period; type of replacement (prophylaxis or episodic); cumulative number of exposure days until the data of consultation; surgeries or invasive procedures during the evaluated period; total number of hospitalization days during the period evaluated; school status; total number of days out of school or day care due to hemophilia; need for school assistance due to hemophilia; inhibitor detection in the described period; inhibitor titer; maximum inhibitor titer in the evaluated period; modification of treatment in relation to the previous period; type of treatment (prophylaxis/on demand) until next appointment; indication of home treatment; weight at the time of consultation; patient participation in other studies
Variables of follow-up (related to the exposure of factor VIII and factor IX)
Date of treatment; the number of EDs; reason for treatment [episodic, prophylaxis, surgery (pre and post), head trauma and so on] and treatment follow-up; bleeding location; bleeding severity; total number of units used in the days of infusion; product name; extravasation of factor concentrate; type (recombinant or plasma-derived) and commercial name of factor concentrate
Variables of follow-up (related with the immunological profile)
Bacterial and/or viral infections during the evaluated period; vaccination with respective date of application in the period; reaction to vaccination; allergies, chronic diseases; family history of allergy; all medications used in the period

BU, Bethesda Unit; ED, exposure day.

Henfil. Henrique was one of the greatest Brazilian cartoonists, also journalist and writer, the creator of characters with great popularity in the country. Henfil had hemophilia and died from AIDS, after contamination with blood transfusion.

Methodology

Study design and study population

HEMFIL is a prospective cohort study of patients with severe and moderately severe (below 2% plasma FVIII or FIX) hemophilia. We enroll children with hemophilia A or B before the first infusion of any factor concentrate, although we considered inclusion of children who had up to five exposure days. One exposure day is one calendar day that the patient receives any infusion of factor concentrate. Children are included consecutively at the moment of diagnosis in five Brazilian Hemophilia Treatment Centers by one of the hemophilia doctors attending in each of the comprehensive centers in the states of Minas Gerais, Rio de Janeiro, Espírito Santo, Santa Catarina and Paraná. The standardized forms are filled at each Hemophilia Treatment Center. All data receive a code for privacy reasons (Table 1).

Implicated period and end point

It has been shown that the majority of patients with hemophilia will develop inhibitors within the first 20 exposure days [23]. However, van den Berg *et al.* [24] reported that 21% of inhibitors occurs after 20–50 exposure days and recommend that 75 exposure days shall be the cut off point for prospective cohort studies of PUPs with hemophilia.

In the HEMFIL study, children are followed up to 75 exposure days and/or upon inhibitor development. Immunological biomarkers are tested before the first exposure day, that is, at inclusion time (T0); at inhibitor development time (T1-INH^+) or at 75 exposure days without inhibitor (T1-INH^-) and at the end of ITI in those patients in whom this treatment is performed, independently of success or failure (T2) (Fig. 1).

The primary outcome for T1-INH^+ is the development of any inhibitor defined as a positive antibody titer above 0.6 BU/ml in two consecutive measurements 2–4 weeks apart [25]. The secondary outcome is the development of high-titer inhibitor ($\geq$5 BU/ml). The third outcome is the response to ITI, independent of success or failure.

The enrollment of patients is ongoing and started on January 2015. A sample size calculation has not been performed. Difficulties for sample size calculation related to the scarcity of previous studies addressing the performance of immunological biomarkers in such patients. Considering that the very few studies evaluating those biomarkers in patients with hemophilia [13,26] included less than 30 patients, a minimum of 30 patients in T1-INH^+ and 30 patients in T1-INH^- has been established as a starting point to analyze the comparison between the two groups.

Data acquisition

At the enrolment time and every 2 months during the follow-up, patients are referred for clinical evaluation for the collection of socio-demographic, clinical and inhibitor tests by completing standardized forms. These forms were translated from the RODIN study [3], with kind permission of the RODIN study group.

Determination of factor levels and inhibitor testing

Factor activity levels are measured twice times at the moment of the diagnosis of hemophilia to determine the type and severity. Tests are performed in Hemophilia Treatment Centers where the patients are recruited. Inhibitor tests are also performed in Hemophilia Treatment Centers at the moment of the diagnosis of all patients and every 5–10 exposure days until 75 exposure days.

Type of treatment

In Brazil, patients with hemophilia A are either treated with plasma-derived (different brands) or recombinant FVIII concentrate (ADVATE, Shire, Massachusetts, USA). Hemophilia B patients are treated with plasma-derived FIX concentrate (different brands). Prophylactic

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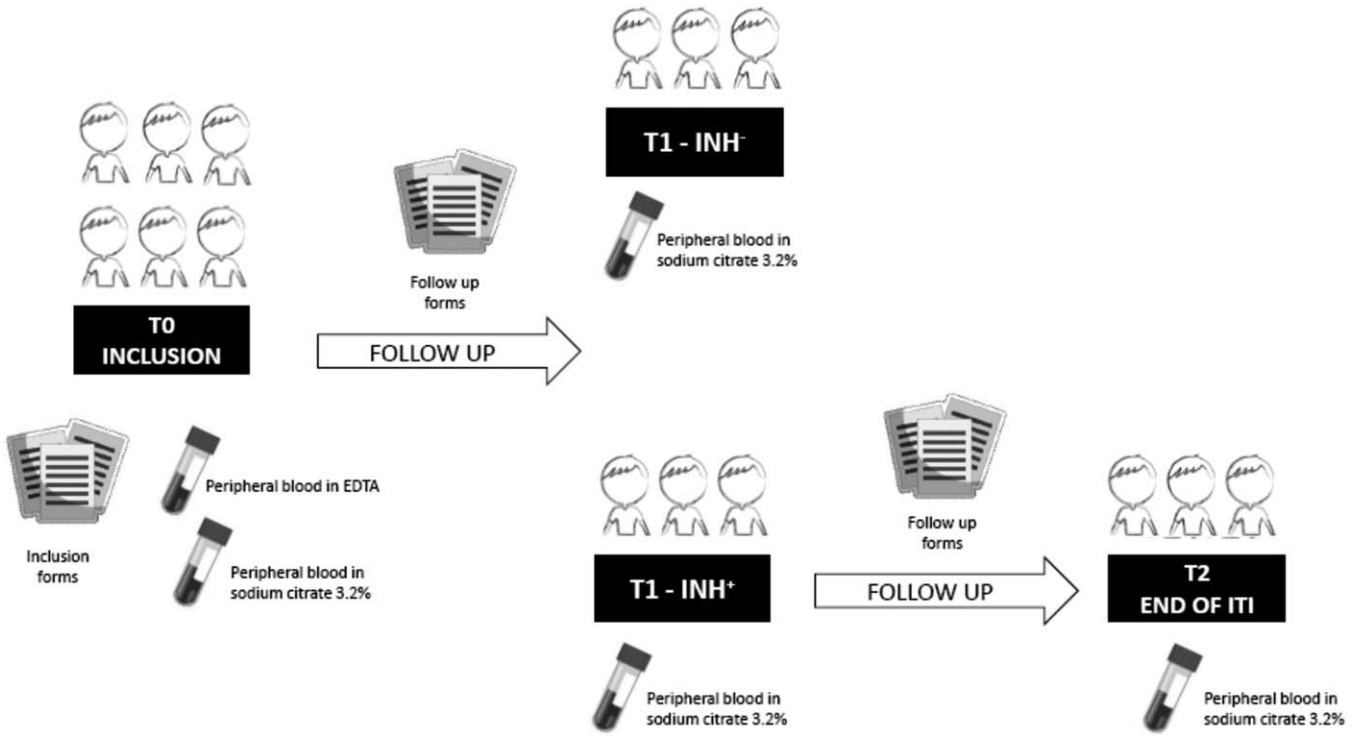
Fig. 1



Time line of biological samples collection in the HEMFIL study. T0, inclusion of the patient in the study; T1, development of antifactor neutralizing antibody (T1-INH$^+$) or no antibody at 75 exposure days (T1-INH$^-$); T2, end of immune tolerance induction. ITI, immune tolerance induction.

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treatment is available for all patients with severe/moderately-severe (below 2% factor levels) hemophilia A and B.

All patients with hemophilia A included in the HEMFIL study are treated with the same recombinant FVIII (ADVATE).

Blood research and sampling

At T0, about 4 ml of blood from patients with hemophilia A is collected from peripheral vein in a tube containing sodium citrate 3.2% as anticoagulant, to performance of immunological tests. Another 4 ml of blood is also collected in a tube containing EDTA, for genetic tests.

At T1-INH$^+$ or T1-INH$^-$ time and at T2 time, another sample of blood (4 ml) is collected in a tube containing sodium citrate 3.2% as anticoagulant to perform immunological tests (Fig. 1).

Peripheral blood collected in citrate is also used for performing immunological phenotyping of monocytes, neutrophils, T and B cells. Then, the sample is immediately centrifuged at 1500 rpm for 15 min for plasma obtention and stored at −80 °C until assessment.

At the time of blood collection of patients and controls, participants must be free of conditions that might have influenced the immunological biomarkers such as allergies, vaccination, infection and inflammation, as well as use of medications for the treatment of any morbid condition.

Control group

Plasma samples from 20 healthy adults and 20 healthy children are used as two different negative control groups for immunological assays validation purpose. Healthy adults are male blood donors with median age of 28 years [interquartile range (IQR), 22.5–30.0]. Children are non-hemophiliac boys with median age of 11.5 months (IQR, 8.5–13.0 months), recruited during routine consultation at Pediatric Primary Care Centre from the University Hospital, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil. The 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 [21].

Analysis of immunoglobulins

Measurements of anti-FVIII subclasses are performed by an in-house ELISA and the results provided in optical density. Samples are diluted 1 : 40. Immunosorbent 96-well plates (Nunc MaxiSorp flat-bottom; Thermo Fisher, Massachusetts, USA) are coated with 100 µl of 1 IU/ml

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recombinant FVIII (ADVATE, Shire, Lexington) diluted in 1× PBS overnight at 4 °C. Plates are washed and blocked with PBS 1X-bovine serum albumin (BSA) 3% for 1 h at 37 °C. Plasma samples are diluted in PBS 1X-BSA 0.1% and incubated for 1 h at 37 °C. The test is revealed after plate incubation for 1 h at 37 °C with biotin-conjugated antihuman Immunoglobulin antibody IgM, IgG3 and IgG4 (SIGMA-Aldrich, St. Louis, Missouri, USA) or horseradish peroxidase IgG1 (Sanquin, Amsterdam, the Netherlands) in proper dilutions. The resulting absorbance is measured in ELISA plate reader at 492 nm wavelength after the addition of 50 µl of sulfuric acid 1 mol/l to stop the reaction. Samples of patients and controls are assayed in duplicates.

The assay was validated by assessing specificity and precision of the test. Each ELISA plate assay is performed by running, in parallel, two controls composed of a pool of 20 adult without hemophilia and a pool of 20 healthy children. In addition, a positive sample for each IgG (1, 3 and 4) and a blank are included. Each plasma sample of the control group was tested individually before the pool was made.

To evaluate the intra-assay coefficient of variation, the pool of the control group was titrated as dilutions 1 : 10, 1 : 20, 1 : 40, 1 : 80, 1 : 160, 1 : 320 and 1 : 640 and each dilution was replicated ten times in the same assay. The intra-assay coefficient of variation for the 1 : 40 dilution was 20%. The inter-assay coefficient of variation was calculated based on the results of six different measurements of the positive control titrated from 1 : 10 to 1 : 640 in separate assays which were performed in different days. The inter-assay coefficient of variation for the 1 : 40 dilution was 12%.

Analysis of chemokines and cytokines

Measurements of cytokines (IFN-γ, TNF, IL-2, IL-4, IL-6, IL-10 and IL-17A) and chemokines [CXCL8 (IL8), CCL5 (RANTES), CXCL9 (MIG), CCL2 (MCP-1), CXCL10 (IP-10)] are performed in duplicates and recorded as mean in pg/ml, according to the human Cytometric Bead Array kit (BD Biosciences; San Jose, California, USA). Acquisition is performed on BD Accuri C6 Flow Cytometer (BD Biosciences) as previously described [21].

Immunophenotyping analysis

Immunophenotyping is performed within 24 h after blood collection in a containing sodium citrate 3.2% tube to evaluation of leukocyte cell activation status. Peripheral whole blood is immunostained in the dark for 30 min at room temperature with a combination of antibodies in 5-ml polystyrene tubes, according to the specifications on Table 2. After lysing/fixation procedure, the leucocytes are washed two times with PBS wash [PBS 0.5% (w/v) BSA and 0.1% (w/v) sodium azide] and fixed with fluorescence activated cell sorter MaxFacs Fix fixative solution [1% of paraformaldehyde, 1.02% of sodium cacodilate and 0.66% of NaCl, pH 7.2] and stored at 4 °C for 10 min before data acquisition in the flow cytometer. The reading is performed on a BD Accuri C6 flow cytometer (BD Biosciences).

Table 2 Panel used to perform the leucocytes phenotyping

	Marker/Reading channel			
Tube	FITC	PE	FITC	APC
1	CD4	HLA-DR	CD8	–
2	CD3	CD56	CD16	–
3	CD5	HLA-DR	CD19	–
4	CD16	HLA-DR	CD14	–
5	CD32	–	CD14	CD19
6	CD19	CD27	–	CD20
7	–	CD80	CD86	CD19
8	CD64	–	CD14	–
9	CD4	CD25	–	–

APC, allophycocyanin; CD, cluster of differentiation; FITC, fluoredceinisothiocyanate; FL, fluorescence channel; HLA, human leukocyte antigen; PE, phycoerythrin; TC, tricolor.

Genetic analysis

Peripheral blood collected in EDTA is used for DNA genomic extraction, for molecular analysis of FVIII (F8) and FIX (F9) gene and other genetic variants that may influence inhibitor development.

The presence of inversions of introns 1 and 22 have been reported as responsible for approximately 5 and 45% of severe hemophilia A phenotypes, respectively [27–29]. Analysis of introns 1 and 22 are performed in all patients with hemophilia A with FVIII:C less than 2%, with the inverse PCR technique, according to protocols already established [27,28]. Samples of nonsevere patients with hemophilia A and for the ones who are not carriers of introns 1 and 22 inversion and samples of all patients with hemophilia B are sequenced. For this, we created a next generation sequence custom panel that included exomic regions of FVIII (*F8*) and FIX (*F9*) genes, von Willebrand factor gene and other genes of the immune system that might be involved with inhibitor development. The Illumina Design Studio tool (Illumina, San Diego, California, USA) was used to customize the regions of interest and select the enrichment panel for the construction of the AmpliSeq Custom DNA Panel for Illumina libraries, according to the manufacturer's instructions. The Miseq sequencer has been used to generate sequences with a mean coverage of 200×. Analyses of the genomic data obtained are performed using Illumina BaseSpace Suite (Illumina).

Data analysis plan

The number of events and respective percentages will be calculated for the categorical variables and the median with IQR for the continuous variables.

The levels of cytokines, chemokines and antifactor subclasses will be analyzed by comparing the levels of

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patients who developed inhibitors versus patients who reached 75 exposure days without any inhibitor development at T0 and T1 times. Patients who performed ITI will be analyzed in two different groups: patients who finished ITI with success versus patients who failed. Immunological profile of these two groups will be compared in T0, T1 and T2 times. Comparison between groups will be performed by Mann–Whitney test. Frequency and percentage values will be compared by the chi-square test or the Fischer exact test. The differences will be considered statistically significant when P values were less than 0.05. Statistical analyses will be performed using GraphPad Prism 7 software (GraphPad Software, San Diego, CA, United States).

To examine whether potential risk factors are independently associated with development of neutralizing antibodies, we intend to use Cox proportional hazard regression models with inhibitor development as the event and the cumulative number of exposure days as the time variable. The hazard ratio will be controlled for potential confounding factors such as genetic mutation, intensive use of factor concentrates products and family history of inhibitors.

Ethical considerations

The study has a multicenter design. Patients/guardians have to fill/respond to questionnaires during the follow-up and need additional blood sampling.

The study has been approved by the institutional ethical committees of the Faculty of Medicine, Universidade Federal de Minas Gerais, Brazil, and by the ethical committees of the participating comprehensive hemophilia treatment centers. The parents/guardians of all patients receive detailed information about the purpose

Table 3 Baseline characteristics of children with hemophilia A

		Completed outcome, $n=85$	
	Children with HA, $n=104$	Children with HA with no inhibitor, $n=53$	Children with HA with inhibitor, $n=32$
Age at baseline in months, median (IQR)	10 (6–17)	11 (7–17)	8 (4–12)
Age at first infusion in months, median (IQR)	11 (6–16)	11 (7–17)	9 (6–13)
Age at age at the beginning of prophylaxis in months, median (IQR)	15 (10–19)	16 (12–21)	12 (10–14)
Age at inhibitor development in months, median (IQR)	NA	NA	13 (10–17)
Family history of hemophilia, *n* (%)	56 (54)	27 (51)	18 (56)
Family history of Inhibitor, *n* (%)	3 (3)	1 (2)	1 (3)
Severity, *n* (%)			
Severe (<1%)	89 (86)	50 (94)	30 (94)
Moderate/severe (1–1.9%)	4 (4)	2 (4)	2 (6)
Moderate (2–4.9%)	2 (2)	1 (2)	0 (0)
Mild (>5%)	9 (9)	0 (0)	0 (0)
Skin color, *n* (%)			
White	62 (60)	37 (70)	17 (53)
Black	24 (23)	9 (17)	7 (22)
Mixed	17 (16)	6 (11)	8 (25)
Indian native	1 (1)	1 (2)	0 (0)
Reason for diagnosis, *n* (%)			
Bleeding	83 (80)	46 (87)	21 (66)
Family history[a]	21 (20)	7 (13)	11 (34)
Type of treatment at T1, *n* (%)			
Prophylaxis	82 (79)	53 (100)	20 (63)
Episodic	22 (21)	0 (0)	12 (23)
ED at inhibitor development, median (IQR)	NA	NA	14 (7–21)
Inhibitor titer, *n* (%)			
Low	NA	NA	10 (31)
High	NA	NA	22 (69)
More than 5 consecutive ED to FVIII[b], *n* (%)			
Yes	9 (9)	6 (11)	3 (9)
Allergy, *n* (%)	9 (9)	6 (11)	3 (9)
Genetic mutation, *n* (%)			
High-risk mutation	53 (51)	20 (38)	25 (78)
Low-risk mutation	45 (43)	28 (53)	6 (19)
Not tested yet	6 (6)	5 (9)	1 (3)

Number of high-risk mutations refer to null mutations, such as large insertion, large deletion, inversions and nonsense; low-risk mutations correspond to missense and splice site mutations. ED, exposure days; FVIII, factor VIII; HA, hemophilia A; IQR, Interquartile range; *n*, number of patients. [a] No clinical bleeding at/before hemophilia diagnosis. [b] At first infusion.

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of the study and signed a written informed consent. Each hemophilia treatment center has a local investigator, usually the assisting hematologist, responsible for the inclusion of the patients and ensuring that the study has been conducted in accordance with the protocol and ethical principles.

The parents/guardians of all patients receive detailed information about the purpose of the study and need to sign a written informed consent to participate.

The standardized forms are collected at each hemophilia treatment center. All data receive a code for privacy reasons.

Clinical characteristics

Currently, 120 children have been included, of whom 104 with hemophilia A and 16 with hemophilia B. A total of 10 patients with hemophilia B have completed 75 exposure days and no patient developed inhibitor. Of all children with hemophilia A, 93 (89%) are severe/moderately severe (FVIII:C $< 2\%$), and 85/93 (91%) have completed the follow-up. Inhibitor was detected in 32/85 (38%) patients of which 22 (69%) were high-titer and 53/85 (62%) patients reached 75 exposure days without inhibitors. A total of 25 patients (21%) presents a mild phenotype or were included recently and, therefore, are still under follow-up. The main characteristics of the patients are reported on Table 3. The cumulative incidence of inhibitors in patients with severe/moderately severe hemophilia A under exclusive use of a third-generation recombinant FVIII concentrate at 75 exposure days was 35% (95% CI, 25.9%-45.8%) for all inhibitors, 25% (95% CI, 16.8–35.4%) for high-titer inhibitors and 10% (95% CI, 7.9–23.2%) for low-titer inhibitors.

The inclusion of additional patients and a longer follow-up will allow a multivariate analysis of risk factors for inhibitor development with the role of confounding factors evaluation and, also, considering the immunological biomarkers contribution in different time points.

Strengths and limitations

The main strengths of the HEMFIL study are: first, the study aim which target at discovering new risk factors for inhibitor development, focusing on genetic and phenotypic biomarkers of the immune system; second, its methodological design as a cohort study of PUPs with hemophilia who are consecutively enrolled at the moment of diagnosis and followed-up prospectively, with biological samples collected before the first infusion of any factor concentrate and at further relevant timepoints; third, the inclusion of a Brazilian population which is widely admixed and considering that it has been reported that incidence of hemophilia inhibitors likely varies according to ethnicity. Some limitations are worth mentioning. First, assessment of factor levels and inhibitor testing are not performed in a centralized laboratory. However, all laboratories have internal and external quality control in place. Second, some patients might not be included before the first exposure day because they presented relevant clinical bleeding at the time of enrolment, which required immediate therapeutic intervention. However, these patients will be analyzed separately.

In conclusion, here we presented the study design and methodology of the HEMFIL cohort study, which is, to our knowledge, the first prospective cohort of patients with hemophilia conducted in Latin America.

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Conflicts of interest

The authors state that they have no conflicts of interest.

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