Epidemiological aspects and complications of congenital hemophilia A in Brazil

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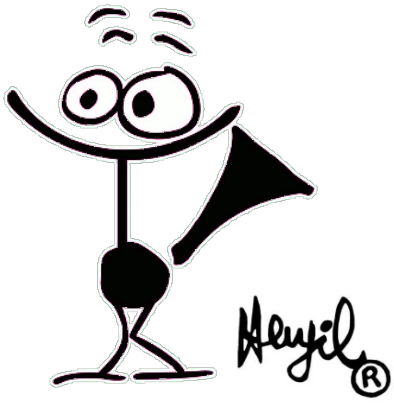
CHAPTER 1

INTRODUCTION



1.1 Hemophilia A

Hemophilia A (HA) is an inherited X-linked bleeding disorder caused by coagulation factor (F) VIII deficiency, affecting 1:5,000-10,000 newborn males worldwide. Treatment of bleeding episodes requires the administration of FVIII-containing concentrates (Iorio et al, 2010).

In its severe form (FVIII plasma levels below 1% of normal), HA is characterized mainly by joint and muscle bleeding (Pio et al, 2009). Bleeding episodes may occur spontaneously or after trauma, and their severity varies according to the residual coagulant activity of deficient FVIII. Severe HA may also present hemorrhages in internal organs, which may lead to death depending on the affected site and severity. Patients with moderate HA have FVIII activity levels between 1% and 5% of normal and present milder hemorrhagic episodes, which occur mainly after trauma. However, some patients can have a more severe bleeding phenotype and present spontaneous bleeding. Mild HA is characterized by FVIII plasma levels between 5% and 40% of normal and is associated with hemorrhages after trauma or surgical procedures (Antonarakis et al, 1995; White et al, 2001).

Patients with severe HA should receive preventive and regular infusions of FVIII concentrate (prophylaxis), to maintain sufficiently high levels of the protein (at least > 1%), avoiding bleeding episodes. Prophylaxis consists of regular FVIII replacement one to three times a week, which is generally called “primary prophylaxis” when it begins before the second joint bleeding and, therefore, before osteochondral changes in patients up to three years of age. Alternatively, secondary prophylaxis refers to prophylaxis starting after two or more joint bleedings, but before the osteochondral changes are evident. Tertiary prophylaxis applies to prophylaxis starting after evidence of osteochondral changes (Ministry of Health, 2015). Prophylaxis is indicated for patients with severe and moderately-severe HA (FVIII levels < 2%), but also for patients with bleeding phenotype regardless of higher FVIII levels (Rezende et al, 2024). Prophylaxis has a positive impact on preventing joint damage and improving the quality of life of people with hemophilia (Aledort et al, 2019).

In addition to FVIII replacement, bispecific antibodies have recently emerged as an innovative and promising strategy for prophylaxis in HA. An example of this approach is emicizumab (Hemlibra), used for prophylaxis of bleeding episodes in HA. Emicizumab binds to factor IXa and factor X, effectively circumventing the need for the deficient or impaired FVIII and enhancing FX activation to support clot formation (Kitazawa et al., 2017).

In addition to the bleeding episodes and their acute complications, patients with HA suffer especially from three potential types of chronic complications: (i) arthropathy, which occurs as a result of recurrent joint bleeding and can lead to permanent motor disability; (ii) infectious diseases transmitted by plasma-derived products, of which infections with human immunodeficiency virus (HIV) and hepatitis B and C are the main problems; and (iii) the development of neutralizing alloantibodies against FVIII (inhibitors). The occurrence of the first two complications has reduced drastically after the implementation of prophylaxis, the production of

recombinant clotting factor concentrates, greater blood product safety through viral purification/inactivation of plasma-derived products, and the implementation of serological screening and nucleic acid testing in blood banks. Thus, currently, the main treatment-related complication in patients with HA is the development of inhibitors, which occurs in 20%-35% of patients with severe HA within the first 75 exposure days (ED) (Ehrenforth et al, 1992; Antun et al, 2015).

Anti-FVIII inhibitors block the protein active sites, neutralizing the therapeutic activity of infused factor FVIII (Antun et al, 2015). Patients with inhibitors lack a sufficient response to replacement with FVIII concentrates, leading to impairment of hemostatic control. In such cases, bypassing agents may be needed, either for episodic or prophylactic treatment (Srivastava et al, 2020). These agents are activated prothrombin complex concentrate and recombinant activated FVII (rFVIIa), which are products capable of generating thrombin independently of the FVIII pathway. In Brazil, both products are provided by the Ministry of Health for inhibitor treatment of patients who do not respond to higher than conventional doses of FVIII (active inhibitors). However, bypassing agents are high-cost medications and are generally less effective than FVIII replacement (Messori, 2018). More recently, prophylaxis with bypassing agents for patients with HA and inhibitors has been replaced by emicizumab in many countries.

Inhibitor development results from genetic predisposition in interaction with environmental and/or exogenous conditions. Type and severity of hemophilia, family history of inhibitors, the type of genetic mutation associated with the disease and the intensity of FVIII replacement have been described as factors associated with increased risk for inhibitor development (Ter Avest et al, 2008; Gouw et al, 2013; Peyvandi et al, 2016; Jardim et al, 2018). Patients who have been initially exposed to FVIII concentrate in an intensive mode, as in surgeries or severe bleeding, seem to present a higher risk of inhibitor development (Gouw et al, 2013). The source of FVIII concentrate (whether plasma-derived or recombinant) used is the most debated non-genetic risk factor. Studies have shown that recombinant FVIII concentrates are more immunogenic in comparison with plasma-derived ones. Therefore, targeting a better understanding of the biological mechanisms behind inhibitor development may support better treatment and the development of preventive strategies.

When patients develop high-responding persistent inhibitors requiring the use of bypassing agents, immune tolerance induction (ITI) is indicated. Currently, ITI is the unique treatment for eradicating persistent anti-FVIII inhibitors (Antun et al, 2015). In Brazil, the ITI regimen adheres to the National Program for Inherited Bleeding Disorders established by the Ministry of Health. The initial ITI regimen for all patients with HA is a low dose of 50 IU/kg three times a week. After the first six months of ITI, if the inhibitor titer does not decrease by more than 20% after the peak, the regimen can be escalated to a high dose of 100 IU/kg daily. If there is no

response with high-dose ITI using recombinant FVIII, plasma-derived FVIII may be used as an alternative (Camelo et al, 2021). ITI is a time-consuming and costly treatment, which is successful in about 70% of patients.

1.2. Hemophilia A in Brazil

Brazil has the fourth largest population of people with hemophilia worldwide, after the United States, India and China, accounting for 11.141 patients with HA.

Since 1993, the Brazilian Ministry of Health has provided blood products for distribution in the Unified Health System (SUS) at state blood banks. Since 2001, the purchase of clotting factor concentrates has been centralized, with the Ministry of Health being the sole national purchaser of these products. In 2007, the Ministry of Health implemented the home-dose program, which consisted of delivering 3 doses of FVIII concentrate for home treatment to be used for immediate replacement in case of bleeding. In 2009, a national registry of inherited bleeding disorders - the Hemovida web coagulopathies Computerized System - was created. This system uses a web platform to collect sociodemographic, clinical, laboratory, and treatment data from patients with inherited bleeding disorders (Rezende et al, 2017). The registry also has a medication inventory control system that links information from the Federative Units (individual states or regions that make up the federal country) to the Ministry of Health. The system is fed by state hemophilia treatment centers.

As of 2011, the Ministry of Health began to promote a series of advances in the policy of care for patients with hemophilia. Treatments such as ITI and prophylaxis were implemented, which ensured visible improvements in the hemophilia outcomes and quality of life of patients. In 2012, Brazil began purchasing recombinant FVIII concentrates (Ministry of Health, 2015). This demand was justified by the need for replacement products with high safety, combined with a significant demand for the implementation of ITI and prophylaxis. Also in 2012, home treatment was fully implemented for moderate and mild hemophilia (Ministry of Health, 2015). In 2014, a public-private partnership rendered the acquisition of high quantities of a third-generation recombinant FVIII. Since then, this is the only recombinant FVIII option available in Brazil, used to treat 70-80% of patients with HA. The remaining patients are treated with plasma-derived FVIII, also made available by the Ministry of Health (Ministry of Health, 2015).

In 2019, Emicizumab was incorporated into the Brazilian healthcare system and approved for use in patients with HA and inhibitors who failed ITI. In 2023, access to this medication was expanded to all patients with moderate or severe HA and inhibitors above 2 BU/mL and with a documented need for the use of bypassing agents before.

1.3. Mortality and comorbidities in hemophilia

With the improvement in life expectancy of patients with hemophilia over the years, health complications normally associated with aging, such as cardiovascular disease and cancer, have begun to be observed in this population (Tuinenburg et al, 2008). With the emergence of new, non-factor replacement therapies, emicizumab, the therapeutic context for treating HA patients with or without inhibitors has changed significantly in recent years. Emicizumab prophylaxis is effective and can help to improve joint health outcomes and health-related quality of life, especially for patients with HA and inhibitors. 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 HA using Emicizumab still demand the use of FVIII concentrates, which will expose patients to the risk of inhibitor development. Thus, studies focused on documenting the epidemiology of inhibitors, even in the era of non-factor replacement therapies, are still needed. An evaluation of the immunological profile in previously untreated and/or minimally treated patients with HA may contribute to a better understanding of how immunological biomarkers behave before exposure and/or on first EDs to exogenous FVIII. This may be important to understand why some patients develop inhibitors and others do not. Indeed, the determination of risk factors involved in antibody formation may help in its prevention and thereby reduce the burden of the disease.

The analysis of the epidemiological data of a population is essential for the planning of public health interventions. In Brazil, since 2009, descriptive data on inherited bleeding disorders (including hemophilia) have been published annually by the Ministry of Health through the "Profile of Hereditary Coagulopathies in Brazil". However, only a few studies have explored quality of life and the mortality of hemophilia (and its causes) in Brazil. This knowledge is of fundamental relevance in understanding the impact of public policies related to hemophilia in Brazil and the main causes of death in these patients.

Aim of the Thesis

The work presented in this thesis aims to identify epidemiological aspects of HA in Brazil and how hemophilia complications impact mortality and life quality in severe patients with HA.

It also aims to explore potential associations between immunological, clinical, and genetic risk factors for developing inhibitory antibodies against exogenous FVIII in a Brazilian prospective cohort study. Furthermore, it aims to identify whether the occurrence of past inhibitors has a negative impact on health status, including joint health and health-related quality of life of patients with HA.

Outline of the Thesis

The first part of this thesis focuses on the mortality of hemophilia in Brazil to evaluate the mortality rate and its causes in patients with hemophilia in Brazil. This knowledge enables the public manager to focus on public policies aimed at reducing the main causes of mortality in this population. Furthermore, it contributes to evaluating the impact of prophylaxis, home treatment, and ITI actions, implemented in 2012 by the Brazilian government.

- **Chapter 2** aimed to analyze mortality and its causes in Brazilian patients with hemophilia from 2000 to 2014.

The second part of this thesis describes studies about risk factors of inhibitor development in a Brazilian cohort.

- **Chapter 3** presents an illustrated and brief review of the development of inhibitors in patients with congenital hemophilia A.

- **Chapter 4** describes the study design and methodology of the HEMFIL study which primary aim is to identify new risk factors for inhibitor development.

- The inhibitor incidence in HA in the HEMFIL Study is presented in **chapter 5.**

- **Chapters 6 and 7** focus on identifying the immunological biomarkers of patients with HA before and after exposure to factor VIII infusions.

- **Chapter 8** presents a prediction model for inhibitor development in HA, which was constructed using a network of clinical variables and biological biomarkers.

The third part of this thesis describes the life quality of patients with hemophilia in a Dutch cohort.

- **Chapter 9** shows the relation between the occurrence of past inhibitors on health status, including joint health and health-related quality of life of patients with HA.

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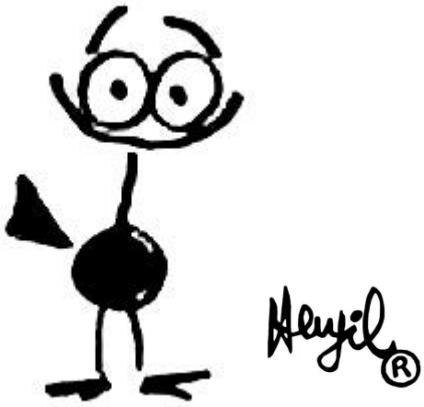
CHAPTER 2

Mortality of patients with haemophilia in Brazil: First report

Jardim LL, van der Bom JG, Caram-Deelder C, Gouw SC, Leal Cherchiglia M, Meireles Rezende S. Mortality of patients with haemophilia in Brazil: First report. Haemophilia. 2019 May;25(3):e146-e152. doi: 10.1111/hae.13730. Epub 2019 Mar 15. PMID: 30875453.



ABSTRACT

Introduction: Brazil has the fourth largest world population of patients with haemophilia. However, mortality rates in this population are unknown.

Aim: To analyse mortality and its causes in Brazilian patients with haemophilia from 2000 to 2014.

Methods: The number of deceased patients with haemophilia and causes of death were obtained from the Brazilian National Mortality Information System (SIM), according to the 10th International Classification of Diseases (ICD-10). Standardized mortality ratios (SMR) were calculated to estimate the rate of overall death of patients with haemophilia relative to that of the Brazilian general male population.

Results: A total of 784 deaths were identified in the period of 15 years. Mortality of patients with haemophilia was 13% higher when compared with the general male population (SMR 1.13, 95% CI: 1.01-1.16). Haemorrhage was the main cause of death (n = 254; 32.4%) of which 137 (54%) was intracranial haemorrhage. The total number of deaths due to HIV decreased over the years, and an increase in deaths due to cancer and cardiovascular disease was observed. A total of 129 deaths (16.5%) were related to hepatitis infection, of whom, 109 (86.5%) patients also presented with cirrhosis and hepatocellular carcinoma or other liver diseases.

Conclusion: Mortality rate of Brazilian patients with haemophilia decreased over the evaluated period. Intracranial haemorrhage is still an important cause of death in these patients, which requires major effort for prevention. Death due to age-related cardiovascular disease and cancer has increased over the years, following the same tendency observed in developed countries.

Keywords: Brazil, epidemiology, factor VIII, haemophilia, mortality, standardized mortality ratios

INTRODUCTION

Haemophilia is an inherited X-linked bleeding disorder, characterized by the deficiency of factor VIII (haemophilia A) or factor IX (haemophilia B). Haemophilia A and haemophilia B affect respectively 1:5000-10 000 and 1:35 000-50 000 newborn males worldwide [1,2]. The treatment of haemophilia is based on the episodic and/or prophylactic replacement of clotting factor concentrates [3]. Despite the technological progress, mortality of patients with haemophilia is still higher than that of the general male population in different countries as United States, The Netherlands and United Kingdom [4,5]. However, studies investigating the mortality of haemophilia patients in developing countries are scarce [6,7]. To our concern, mortality in patients with Haemophilia in Latin America has not been published.

Brazil has the fourth largest world population of haemophilia patients, after United States, India and China, accounting for 12,119 individuals registered in 2016 [8] of whom 10,123 and 1,996 patients are registered as having haemophilia A and haemophilia B, respectively. Haemophilia care in Brazil is guaranteed by the National Public Health System (*Sistema Único de Saúde)*. In 2009, the Brazilian Ministry of Health initiated a web-based registry of patients with inherited bleeding disorders, including haemophilia [9]. From 2011 onwards, haemophilia care improved as a result of the implementation of health policies involving increased purchase of factor concentrates leading to the implementation of immune tolerance induction (ITI), prophylaxis and home treatment. Since mortality studies can be useful as a tool to monitor progress of care in a population, it can be of value for policymakers and stakeholders to evaluate health policies. The aim of the study was to investigate the mortality of patients with haemophilia in Brazil and to evaluate the causes of death from 2000 to 2014.

ME THODS

Data source

This retrospective study was performed using the Brazilian National Mortality Information System (*Sistema de Informação sobre Mortalidade*—SIM) as data source. The SIM system is based on the notifications provided by the death certificate registration. The death certificate is unique for the entire country since 1975 and is mandatory for registration of death and for burial purposes. The death certificate is composed of two parts for primary cause of death and comorbidities. Part I has four lines, the first is for the ICD-10 code of the primary cause of death, characterized as the disease or injury that initiates the sequence of morbid states, or the circumstances of the accident or violence, which led directly to death. The other three lines are filled in with the complications of the underlying cause, such as intermediate clinical states that eventually reach the terminal or immediate cause. Part II is filled in with the contributing causes, which are comorbidities without a direct relation to the primary cause of death.

We had access to the mortality information of the entire Brazilian male population, primary and secondary causes of death, at patient level, from 2000 to 2014. Data on the general male population alive in Brazil at the same period were provided by the Brazilian Institute of Geography and Statistics (IBGE). The IBGE is a public institute responsible for providing information about the country population, economy and geosciences. The decennial national census provides the official population count all over the national territory. The data are also available by assessing the website http://ibge.gov.br This information is grouped by age and geographical region (north, northeast, southeast, south and midwest).

Outcome definitions

Deaths and causes of death of male patients with congenital haemophilia A and haemophilia B were identified according to the 10th International Classification of Diseases (ICD-10). Deceased individuals with haemophilia were selected from SIM when at least one of the fields in the death certificate reported ICD10-code "D66," for

haemophilia A (hereditary factor VIII deficiency), or "D67," for haemophilia B (hereditary factor IX deficiency). Haemophilia A and haemophilia B were analysed as one group.

In order to evaluate comorbidities of deceased patients with haemophilia, we surveyed all ICD-10 codes mentioned in any of the fields of death certificate. The most frequent codes were related to HIV, hepatitis, cancer, kidney diseases, haemorrhage and cardiovascular diseases such as diabetes, hypertension, angina, myocardial infarction and thrombosis. Then, a survey of all the codes related to these complications was performed followed by a new search on the SIM database. Among patients with hepatitis, we also looked for codes of liver diseases including cirrhosis, liver failure, fibrosis and liver cancer. Among patients with haemorrhage, codes for intracranial haemorrhage were specifically researched.

The following codes were researched in the fields of the death certificate: B20, B21, B22, B23, B24 and R75 for HIV; B16, B17 and B18 for hepatitis B and hepatitis C; K72, K73, K74, K75, K76, C22, C23 and C24 for liver disease; N17, N18, N19, N99.0 and P96.0 for kidney disease; R04, R58, D62, K92.0, K92.2, H11.3, H35.6, H43.1, H45.0, K25, K26, K27 and K28 for haemorrhage; I60, I61, I62, P10, P52 and S06 intracranial haemorrhage; E10, E11, E12, E13, E14, I10, I11, I12, I13, I15, I20, I21, I22, I23, I24, I25, I26, I27, I31, I32, I33, I46, I49, I50, I63, I64, I65, I66, I67 for cardiovascular disease; C00 until to D48, Z85 and Z86 for cancer.

Patients with acquired haemophilia or other coagulation defects were not included in the analysis. Therefore, the code D68.4, "Acquired coagulation factor deficiency," as well as all other codes derived from CID D68, ie, "Other coagulation defects," were not analysed.

Statistical analysis

The standardized mortality ratio (SMR) refers the number of observed deaths divided by the number that was expected if the mortality rate in the study group and its specific age distribution was the same as that in the general population. Thus, an estimation of the number of patients with haemophilia who were alive between 2000 and 2014 was needed to calculate the expected number of deaths. In the period from 2009 to 2014, the exact number of patients with haemophilia is available from annual publications by the Ministry of Health through the "Profile of inherited bleeding disorders in Brazil".10-12 For the period 2000-2009, the number of patients with haemophilia in Brazil is unknown and had to be estimated. We estimated the numbers of patients with haemophilia for each year from 2000 to 2014, by multiplying the mean prevalence of haemophilia of the period 2009-2014, per age category with the general male population of each corresponding age group.

To compare the overall mortality rate in the haemophilia population with the Brazilian male population, the SMRs were calculated for the total period 2000-2014. To evaluate time trends in mortality rates in the haemophilia population, age-adjusted mortality rates were calculated per three-year periods: (a) from 2000 to 2002; (b) from 2003 to 2005; (c) from 2006 to 2008; (d) from 2009 to 2011; and (e) 2012 to 2014, using the World Health Organization (WHO) population age distribution as weights13

The analyses were performed in strata of age (0-4, 5-9, 10-14, 15-19, 20-29, 30-39, 40-49, 50-59, 60-69, 70-79 and > 80 years old) and 3-year period. Statistical analyses were performed using Stata (STATA Corp LP, Texas, USA).

RESULTS

Mortality rates of patients with haemophilia in Brazil

From January 2000 to December 2014, 784 deaths of patients with haemophilia were identified. The overall mortality of patients with haemophilia was 13% higher when compared with the general male population (SMR, 1.13; 95% CI: 1.01-1.16). SMR was 1.51 (95% CI: 1.29-1.74) in 2000-2002 and 0.89 (95% CI: 0.74-1.04) in 2012-2014 (Table 1).

There was a major variation of SMR between years 2000 and 2008 and a tendency to a plateau which ranged between 0.83 and 0.94 from 2009 to 2014 (Figure 1).

Table 1. Standardized mortality ratios for males with haemophilia A and B

Period	Observed deaths, n	SMRs	95% CI
2000-2002	182	1.51	[1.29, 1.74]
2003-2005	166	1.32	[1.12, 1.53]
2006-2008	166	1.19	[1.01, 1.38]
2009-2011	129	0.86	[0.71, 1.02]
2012-2014	141	0.89	[0.74, 1.04]
2000-2014	784	1.13	[1.01, 1.16]

95% CI, 95% confidence interval; N, number; SMRs, standardized mortality ratios.

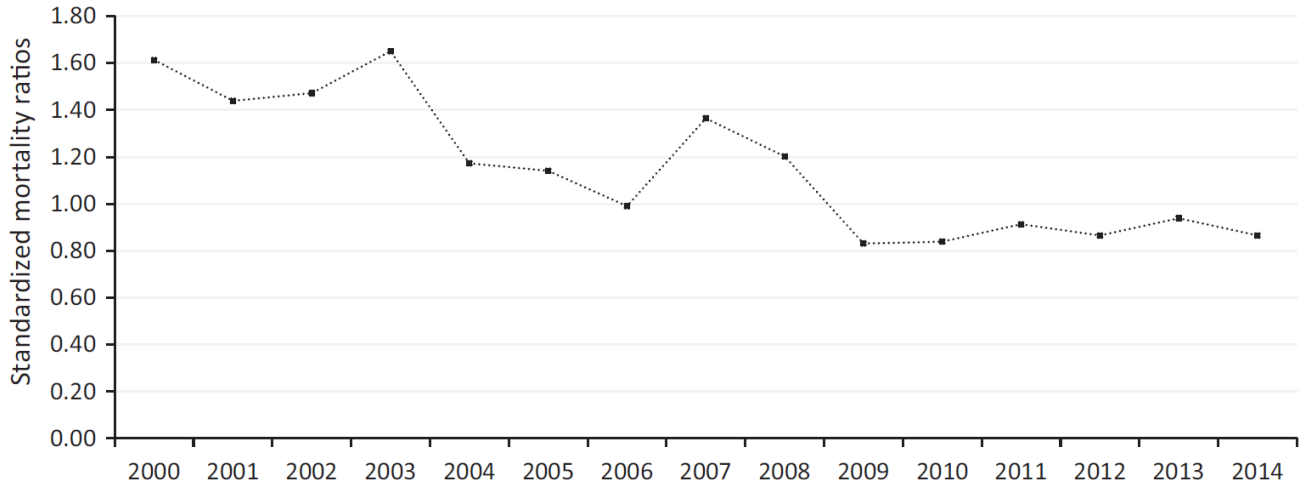


Figure 1. Standardized mortality ratios (SMRs) for males with haemophilia A and haemophilia B by year

Causes of death and other related disease

Table 2 presents the causes of death categorized according to the time periods. Haemorrhage was the most frequent cause of death, reported in 254/784 (32.4%) patients with haemophilia, and in most patients (n = 137; 54%), it was due to intracranial haemorrhage. HIV as a cause of death in patients with haemophilia decreased from 30.8% of all deaths in 2000-2002 to 11.3% in 2012-2014. The opposite was observed on cancer and cardiovascular disease, which increased from 7.7% to 12.1% and 13.7% to 25.5%, respectively, from 2000-2002 to 2012-2014. A total of 129 patients died due to hepatitis infection, of whom 109 (86.5%) also presented liver diseases (Table 2).

The frequency of HCV and HBV infection in male patients who died with haemophilia A and haemophilia B was the highest in the age group 40-49 years in all the periods Analysed (Table 3). As expected, there was no death related to hepatitis B or C infection in patients with haemophilia in the age range of 0-14 years and only one death in the age group 15-19 years in all the periods analysed (Table 3).

Table 2. Causes of death and other conditions in male patients with haemophilia A and B according to the ICD-10 classification[a]

	2000-2002 (n = 182)	2003-2005 (n = 166)	2006-2008 (n = 166)	2009-2011 (n = 129)	2012-2014 (n = 141)
Haemorrhage, n (%)	56 (30.8)	52 (31.3)	54 (32.5)	45 (34.9)	47 (33.3)
Intracranial haemorrhage, n (%)	29/56 (51.8)	28/52 (53.8)	28/54 (51.9)	21/45 (46.7)	31/47 (66.0)
HIV, n (%)	56 (30.8)	38 (22.9)	29 (17.5)	17 (13.2)	16 (11.3)
Cardiovascular disease, n (%)	25 (13.7)	33 (19.9)	29 (17.5)	29 (22.5)	36 (25.5)
HCV and HBV, n (%)	25 (13.7)	27 (16.3)	28 (16.9)	17 (13.2)	32 (22.7)
Liver disease, n (%)	24/25 (96)	23/27 (85.2)	23/28 (82.1)	14/17 (82.4)	25/32 (78.1)
Kidney disease, n (%)	9 (4.9)	12 (7.22)	19 (11.6)	15 (11.6)	9 (6.4)
Cancer, n (%)	14 (7.7)	12 (7.22)	9 (5.4)	3 (2.3)	17 (12.1)
Other, n (%)	24 (13.3)	22 (13.3)	22 (13.3)	16 (12.4)	16 (11.3)

n, number of patients.
[a]Some patients presented more than one related cause of death.

Table 3. Number of male patients (%) with HCV and HBV infection who died with haemophilia A and B by age groups and 3-year period

Age group, in years	2000-2002	2003-2005	2006-2008	2009-2011	2012-2014	Total
0-4, n (%)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
5-9, n (%)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
10-14, n (%)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
15-19, n (%)	1 (4)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0.8)
20-29, n (%)	1 (4)	5 (18.5)	3 (10.7)	2 (11.8)	2 (6.3)	13 (10)
30-39, n (%)	6 (24)	7 (25.9)	5 (18.9)	4 (23.5)	3 (9.4)	25 (19.4)
40-49, n (%)	14 (56)	9 (33.3)	12 (42.9)	4 (23.5)	8 (25)	47 (36.4)
50-59, n (%)	3 (12)	3 (11.1)	5 (18.9)	3 (17.6)	5 (15.6)	19 (14.7)
60-69, n (%)	0 (0)	3 (11.1)	2 (7.2)	3 (17.6)	9 (28.1)	17 (13.2)
70-79, n (%)	0 (0)	0 (0)	1 (3.6)	1 (5.9)	5 (15.6)	7 (5.4)
>80, n (%)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Total, n	25	27	28	17	32	129

n, number of patients.
[a]Some patients presented more than one related cause of death.

Death due to haemorrhage was the most common cause of mortality, especially in adult patients with haemophilia. The highest frequency was observed in the age range of 40-49 years in all periods evaluated. Although numbers are small, there was a trend towards a reduction of deaths due to haemorrhage in the age group 0-4 years from year 2000 to year 2014 (Table 4).

In 137 patients who died of intracranial haemorrhage, 33.6% and 12.4% were patients of the age groups 40-59 and 0-4 years, respectively. It was observed a decrease in the number of deaths due to intracranial haemorrhage in the age groups 0-4 and 5-9 years from year 2000 to year 2014, despite small numbers (Table 5).

Table 4. Number of male patients (%) with haemorrhage who died with haemophilia A and B by age groups and 3-year period

Age group, in years	2000-2002	2003-2005	2006-2008	2009-2011	2012-2014	Total
0-4, n (%)	6 (10.7)	7 (13.5)	5 (9.3)	3 (6.7)	1 (2.1)	22 (8.7)
5-9, n (%)	4 (7.1)	4 (7.7)	2 (3.7)	2 (4.4)	2 (4.3)	14 (5.5)
10-14, n (%)	1 (1.8)	2 (3.8)	2 (3.7)	0 (0)	0 (0)	5 (2)
15-19, n (%)	3 (1.8)	5 (9.6)	6 (11.1)	1 (2.2)	4 (8.5)	19 (7.5)
20-29, n (%)	8 (14.3)	6 (11.5)	6 (11.1)	4 (8.9)	6 (12.8)	30 (11.8)
30-39, n (%)	7 (12.5)	4 (7.7)	6 (11.1)	9 (20)	5 (10.6)	31 (12.2)
40-49, n (%)	15 (26.8)	11 (21.2)	12 (22.2)	9 (20)	9 (19.1)	56 (22)
50-59, n (%)	5 (8.9)	6 (11.5)	8 (14.8)	5 (11.1)	9 (19.1)	33 (13)
60-69, n (%)	6 (10.7)	4 (7.7)	5 (9.3)	8 (17.8)	7 (14.9)	30 (11.8)
70-79, n (%)	1 (1.8)	2 (3.8)	1 (1.9)	4 (8.9)	3 (6.4)	11 (4.3)
>80, n (%)	0 (0)	1 (1.9)	1 (1.9)	0 (0)	1 (2.1)	3 (1.2)
Total, n	56	52	54	45	47	254

n, number of patients.

Table 5. Number of male patients (%) with intracranial haemorrhage who died with haemophilia A and B by age groups and 3-year period

Age group, in years	2000-2002	2003-2005	2006-2008	2009-2011	2012-2014	Total
0-4, n (%)	5 (17.2)	6 (21.4)	4 (14.3)	1 (4.8)	1 (3.2)	17 (12.4)
5-9, n (%)	3 (10.3)	4 (14.3)	2 (7.1)	1 (4.8)	2 (6.5)	12 (8.8)
10-14, n (%)	0 (0)	1 (3.6)	2 (7.1)	0 (0)	0 (0)	3 (2.2)
15-19, n (%)	1 (3.4)	2 (7.1)	5 (17.9)	0 (0)	4 (12.9)	12 (8.8)
20-29, n (%)	6 (20.7)	2 (7.1)	3 (10.7)	1 (4.8)	3 (9.7)	15 (10.9)
30-39, n (%)	0 (0)	1 (3.6)	1 (3.6)	2 (9.5)	3 (9.7)	7 (5.1)
40-49, n (%)	7 (24.1)	5 (17.9)	1 (3.6)	5 (23.8)	5 (16.1)	23 (16.8)
50-59, n (%)	3 (10.3)	3 (10.7)	6 (21.4)	3 (14.3)	8 (26.4)	23 (16.8)
60-69, n (%)	4 (13.8)	2 (7.1)	2 (7.1)	4 (19)	3 (9.7)	15 (10.9)
70-79, n (%)	0 (0)	2 (7.1)	1 (3.6)	4 (19)	1 (3.2)	8 (5.8)
>80, n (%)	0 (0)	0 (0)	1 (3.6)	0 (0)	1 (3.2)	2 (1.5)
Total, n	29	28	28	21	31	137

n, number of patients.

DISCUSSION

We have studied, for the first time, mortality and its related causes in Brazilian patients with haemophilia, which is the fourth world largest population affected by the disease. We found that mortality relative to that of the general Brazilian population decreased from 2000 to 2014, with no difference in mortality of haemophilia patients and the general population in 2014 (SMR, 0.89; 95% CI: 0.74-1.04).

Haemorrhage remained the main cause of death, mostly due to intracranial bleeding. In the past 30 years, haemophilia care has improved significantly worldwide. This is reflected by nearly normal life expectancies of patients with haemophilia reported in developed countries,14 especially when patients infected with HIV are excluded from analysis [4,15]. However, studies in low- and middle-income countries are scarce [6,7], and therefore, mortality and its causes are virtually unknown in these populations.

In 2006, the mortality of patients with haemophilia in the Netherlands (1992-2001) was 2.3 higher in comparison with the general male population (SMR 2.3; 95% CI: 1.9-2.8).4 When patients with HIV were excluded, the analysis showed a lower SMR of 1.7 (95% CI: 1.3-2.7]. In the present study, the mortality in haemophilia patients evaluated between 2000 and 2014 was 13% higher when compared with the general male population in Brazil (SMR, 1.13; 95% CI: 1.01-1.16).

Our findings show a progressive decrease in the SMRs throughout the years 2000-2014. These findings likely reflect the improvement in haemophilia care, which took place in Brazil in the last 10 years. In 2007, the Ministry of Health implemented a short-home treatment programme, which comprised delivering three doses of factor concentrate for home treatment to be used for immediate replacement in case of bleeding. In 2009, a web-based, national registry on inherited bleeding disorders was created [9], In 2012, prophylaxis, full home treatment and ITI programmes were implemented.

Although this study was not designed to investigate this impact, it is likely that these treatment strategies influenced mortality of patients with haemophilia in Brazil. to intracranial haemorrhage according to age range, we found that it decreased in the very young patients (age range, 0-4 years), from 17.2% (2000-2002) to 3.2% (2012-2014). It is tempting to suggest that this might result from the implementation of early primary prophylaxis for severe haemophilia on this age group. Andersson et al recently reported that children on regular, frequent prophylaxis have a lower risk of intracranial haemorrhage compared with those using non-frequent or no prophylaxis [19]. However, since the present study was not designed to investigate the impact of health policies on the outcome measurements, further studies will be needed to confirm this.

We found an important reduction in mortality due to HIV infection from 30.8% (2000-2002) to 11.3% (2012-2014). In the 1980s and 1990s, mortality in patients with haemophilia was strongly influenced by infectious diseases, mainly HIV and hepatitis infection [4,17].

In the following years, due to better treatment and preventive strategies, the number deaths resulting from HIV infection has gradually decreased to almost zero [17,20,21]. In our study, although there has been a slight increase in the rate of death due to hepatitis throughout the years, most cases (56%) occurred in the first period (2000-2002) evaluated and in patients in the age range of 40-49 years. As expected, there was no death due to hepatitis B or C in the age groups 0-14 years. However, at the calendar period 2012-2014, the age group 60-69 years showed the highest frequency (28.1%). This data suggests that patients with haemophilia and hepatitis are probably living longer despite their infectious status. This improvement likely reflects the universal access that the Brazillian patients with haemophilia have to vaccination for hepatitis A and hepatitis B and also to the antiviral therapy for hepatitis C since 1993. Despite all improvements, chronic liver diseases, mainly resulting from hepatitis C infection, are still an important cause of mortality in patients with haemophilia [5,16,22]. In our study, liver disease in patients with hepatitis decreased from 2000-2002 to 2003-2005, but remained constant thereafter.

With the improvement in the life expectancy of patients with haemophilia over the years, comorbidities typically related to the normally ageing population, such as cardiovascular diseases and cancer, have risen in this population [16,23]. However, despite the increase in mortality from cardiovascular diseases from the 2000s, these rates remain clearly lower in patients with haemophilia when compared with that expected in the general male population. This has led some authors to hypothesize that haemophilia can be protective of cardiovascular disease [5,16,24]. In our study, mortality due to cardiovascular disease increased from 13.7% to 25.5%, respectively, from 2000-2002 to 2012-2014.

This study has some limitations. First, the prevalence of haemophilia from 2000 to 2014 was estimated. However, the estimated prevalence of the years 2010, 2012 and 2014 and the prevalence published by the Brazilian Ministry of Health [10-12] were very similar. Second, this study was dependent on death certificates, which are liable to incorrect filling, especially regarding the cause of death and the correct codes in the respective lines.

Some of the doctors might not be skilled to complete the death certificate appropriately. Thus, we could not rule out unreported deaths and misclassification of haemophilia and causes of death. Furthermore, it was not always possible to ascertain which ICD-10 was related to the basic cause of death and which related to comorbidities. However, as described by Lima and Queiroz [25] the registration of deaths in Brazil had an increment of approximately 80% of all deaths in 1980-1991 to over 95% in 2000-2010, the latter being the period evaluated in our study. Furthermore, to avoid misclassification in the death certificate, we decided to perform the analyses of haemophilia A and haemophilia B as one group, since the clinical characteristics of both diseases are similar. Third, it was not possible to further investigate other determinants of mortality as we had no access to clinical data of the patients, such as severity, type of treatment, inhibitor development and others.

In conclusion, we studied, for the first time, mortality data of the fourth largest world population of patients with haemophilia and found that mortality of patients with haemophilia decreased over the years. Death due to HIV infection showed an important reduction, and death due to cardiovascular disease and cancer have increased over the years, following the same tendency observed in the developed countries. Haemorrhage and intracranial haemorrhage are the main causes of death, requiring further attention and major efforts for prevention.

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Disclosure

JVDB has been a teacher of educational activities of Bayer. The other authors state that they have no conflict of interest.

Authorcontribution

LLJ performed the research, analysed the data and wrote the paper; JVDB, SCG, CCD and MLC contributed with study design and data analysis; SMR designed the research, contributed to data analysis and wrote the paper. All authors revised and approved the final version of the manuscript. The authors stated that they had no interests which might be perceived as posing a conflict or bias.

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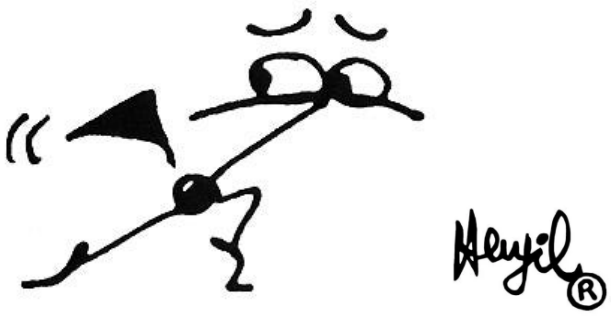
CHAPTER 3

Development of inhibitors in hemophilia A: An illustrated review

Jardim LL, Chaves DG, Rezende SM. Development of inhibitors in hemophilia A: An illustrated review. Res Pract Thromb Haemost. 2020 May 26;4(5):752-760. doi: 10.1002/rth2.12335. PMID: 32685884; PMCID: PMC7354390.



ABSTRACT

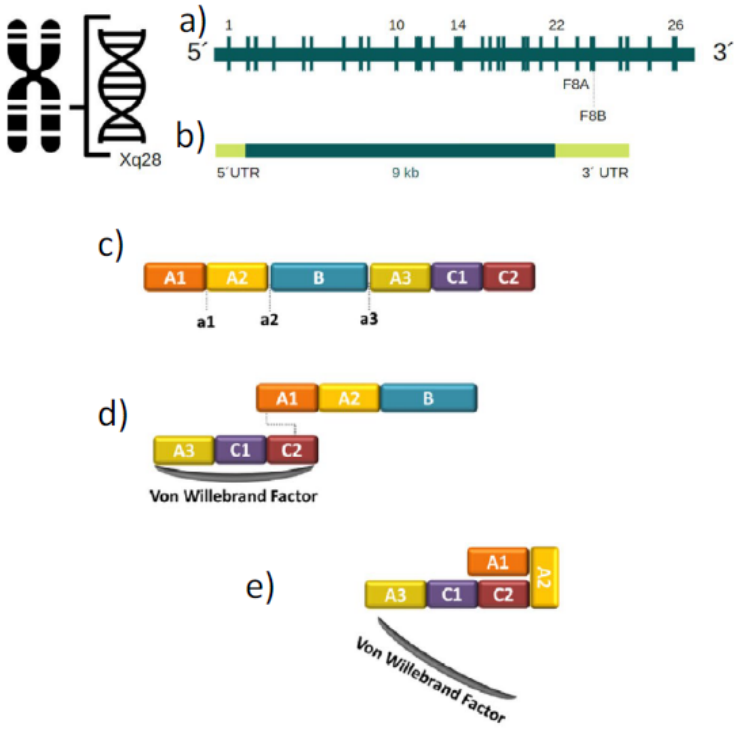
This illustrated review focuses on the development of inhibitors in patients with congenital hemophilia, which is the most serious treatment-related complication in these patients. Hemophilia A (HA) is an inherited X-linked bleeding disorder affecting 1:5000-10 000 newborn males worldwide. It results from the deficiency of coagulation factor VIII (FVIII), due to mutation(s) in its coding gene (F8). Treatment requires administration of FVIII-containing products either on demand or as prophylaxis, which can induce inhibitor development in 20%-35% of patients. Inhibitors are alloantibodies that neutralize the procoagulant activity of exogenous FVIII. During the initial administration of FVIII-containing products, patients with HA can develop a proinflammatory immune response with synthesis of anti-FVIII IgG1, which has no FVIII inhibitory activity. However, in patients with inhibitors, immune response shifts toward an anti-inflammatory/regulatory pattern favoring the synthesis of anti- FVIII IgG4 antibodies. Patients with inhibitors present with bleeding episodes that are difficult to control, and they have reduced response to FVIII replacement. Currently, immune tolerance induction is the available treatment for eradication of persistent high-titer inhibitors. Despite the clinical relevance, the immunological mechanisms for inhibitor development in patients with HA remains unexplained.

Keywords: antibody, factor VIII, hemophilia, immune response, inhibitor

ESSENTIALS

- Hemophilia A (HA) is a bleeding disorder caused by the deficiency of coagulation factor VIII (FVIII).
- The main treatment-related complication in patients with HA is the development of inhibitor.
- Inhibitors are alloantibodies that neutralize the procoagulant activity of infused FVIII.
- The reasons why only 20%-30% of the patients with HA develop inhibitors remain a challenge.

Factor VIII - Gene and protein[1-2]



a) Structure of factor VIII gene (*F8*);

b) Transcription of messenger RNA, with 2 noncoding regions (5´UTR and 3´UTR);

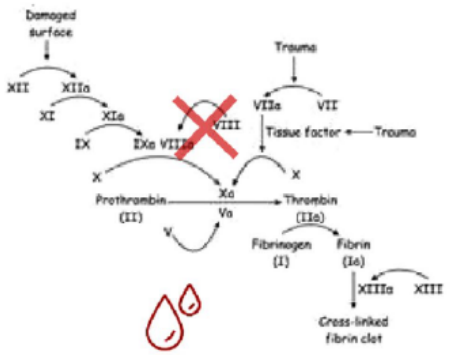
c) Primary structure of FVIII, representing the domains and the breakpoints in acidic regions a1, a2, and a3;

d) Inactive FVIII associated with von Willebrand factor. Acidic regions a1, a2, and a3 contains interaction sites recognized by FX and thrombin;

e) Protein activation after thrombin cleavage and dissociation of von Willebrand factor.

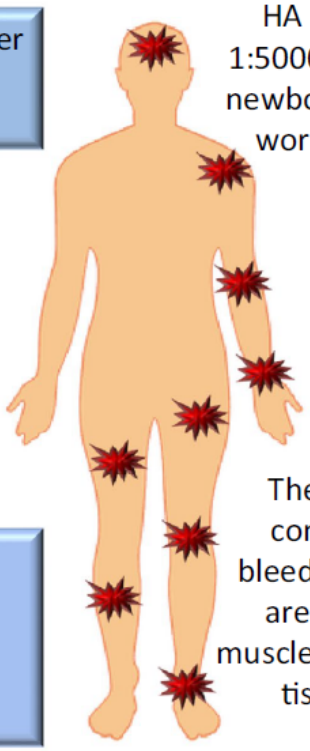
Hemophilia A[1,3-4]

Hemophilia A (HA) is an inherited X-linked bleeding disorder caused by the deficiency of coagulation FVIII due to mutations in *F8*.



The main determinant of the bleeding phenotype is the residual level of FVIII:

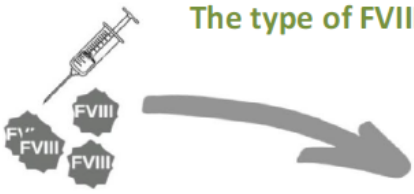
FVIII <1%:	FVIII 1%-5%:	FVIII >5%-40%:
SEVERE	MODERATE	MILD



HA affects 1:5000-10 000 newborn males worldwide.

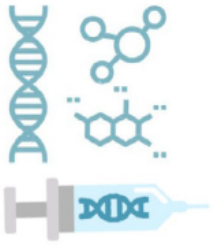
The most common bleeding sites are: joint, muscles and soft tissues.

Treatment of hemophilia A



The type of FVIII concentrate can be:

OR



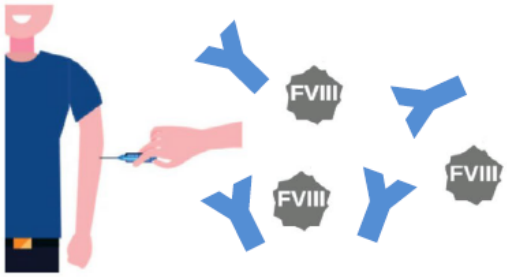
PLASMA-DERIVED (pdFVIII)

RECOMBINANT (rFVIII)

Treatment of bleeding episodes requires intravenous infusion of FVIII products either on demand or on a prophylactic basis.[3]

Inhibitors in hemophilia A

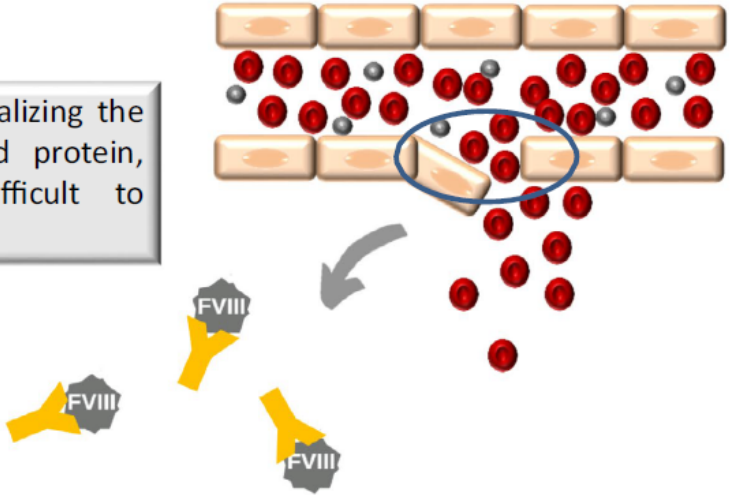
Neutralizing alloantibodies (inhibitors) are the main treatment-related complication in patients with severe HA.[5-6]



During initial administration of FVIII-containing products, the immune system develop a pro-inflammatory response which involves synthesis of antibodies against FVIII.[7-10]

Inhibitors bind FVIII epitopes, neutralizing the therapeutic activity of the infused protein, leading to bleeding that is difficult to control.[7,11]

Cumulative incidence of inhibitor development in patients with HA is 20%-35%.



In about 95% of patients with HA who develop inhibitors, it occurs within the first 75 exposure days to FVIII replacement.[12]

Inhibitors in hemophilia A[11]

An inhibitor is confirmed when there is an antibody titer above 0.6 Bethesda Unit (BU)/mL in two consecutive plasma measurements. Then, inhibitor is classified as:

High-titer inhibitors:
Titer ≥5 BU/mL

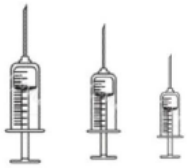
Low-titer inhibitors:
Titer <5 BU/mL

Risk factors for inhibitor development[4,13-14]

Mutations in genes related to immune response

Family history of inhibitors

Type of *F8* mutation



Intensity of FVIII infusions

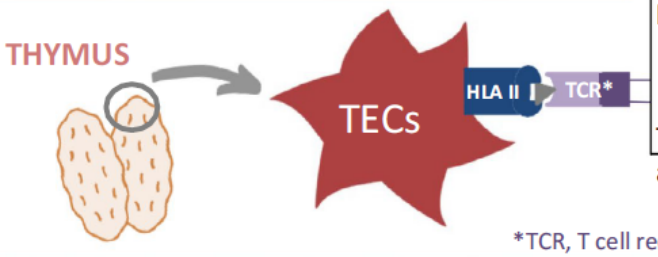
Younger age at first exposure to FVIII

Type of FVIII concentrate used (pdFVIII or rFVIII)

Immunology of inhibitors in hemophilia A

Part I: Central tolerance for the deficient protein[6,15]

Immature T cells enter the thymus and interact with thymic epithelial cells (TECs)



T cells that do not recognize "self antigens" presented by class II human leukocyte antigen (HLA)

Signaling for survival

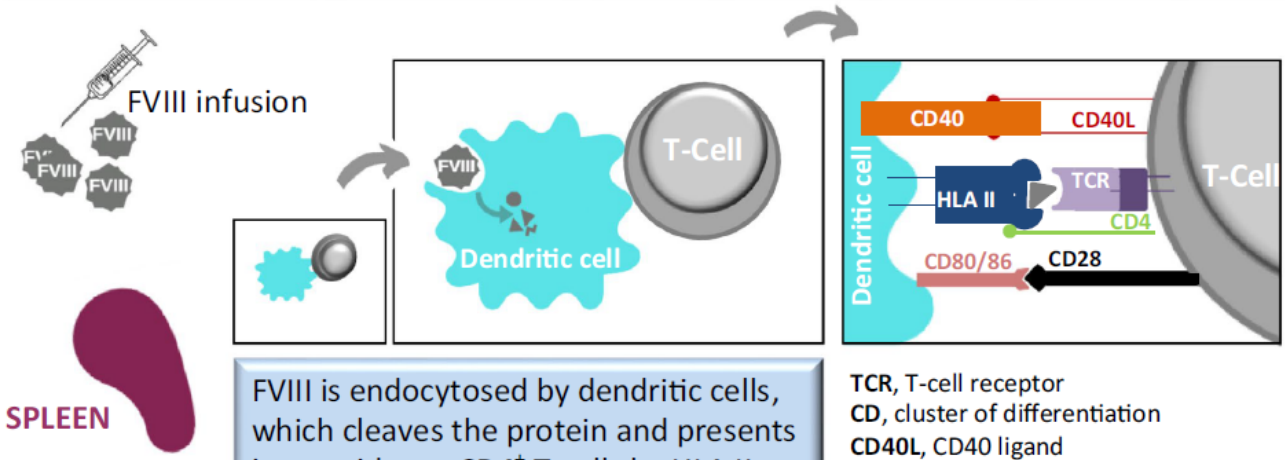
T cells that recognize "self-antigens" presented by class II HLA

Signaling for apoptotic death

*TCR, T cell receptor

As a result of their genetic mutation, some patients with HA are unable to express large portions or any amount of FVIII. These patients are not able to eliminate high-affinity FVIII-reactive T cells. These T cells will then populate peripheral tissues and may induce the development of inhibitors when patients are treated with FVIII.

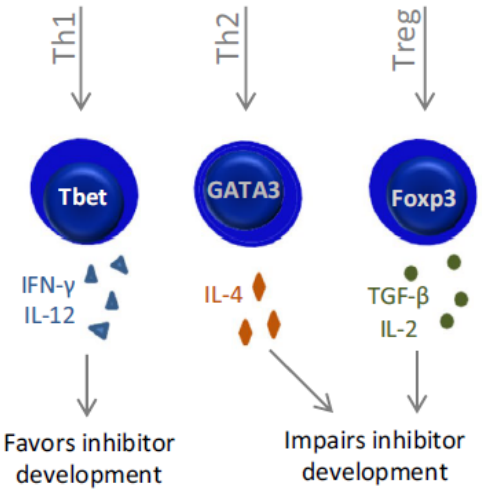
Part II: Primary response[6-9,15-17].



SPLEEN

FVIII is endocytosed by dendritic cells, which cleaves the protein and presents its peptides to $CD4^+$ T cells by HLA II.

TCR, T-cell receptor
CD, cluster of differentiation
CD40L, CD40 ligand

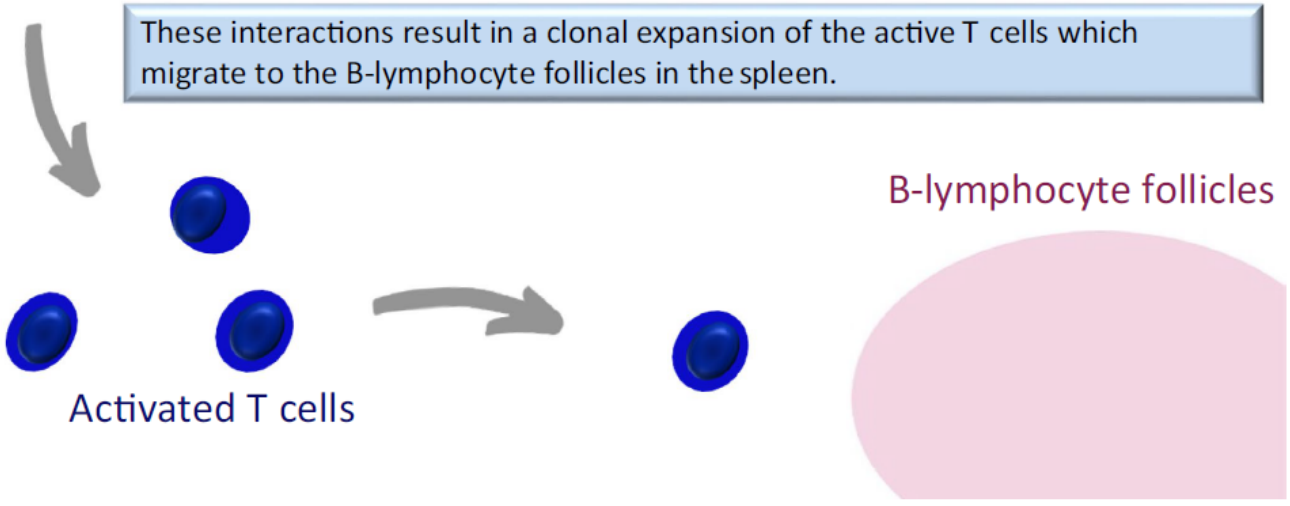


Differentiation of T cells after their activation depends on the stimuli of specific cytokines.

Transcription factors: Tbet, cell-specific transcription factor / GATA3, zinc finger transcription factor 3 / Foxp3, Forkhead box p3

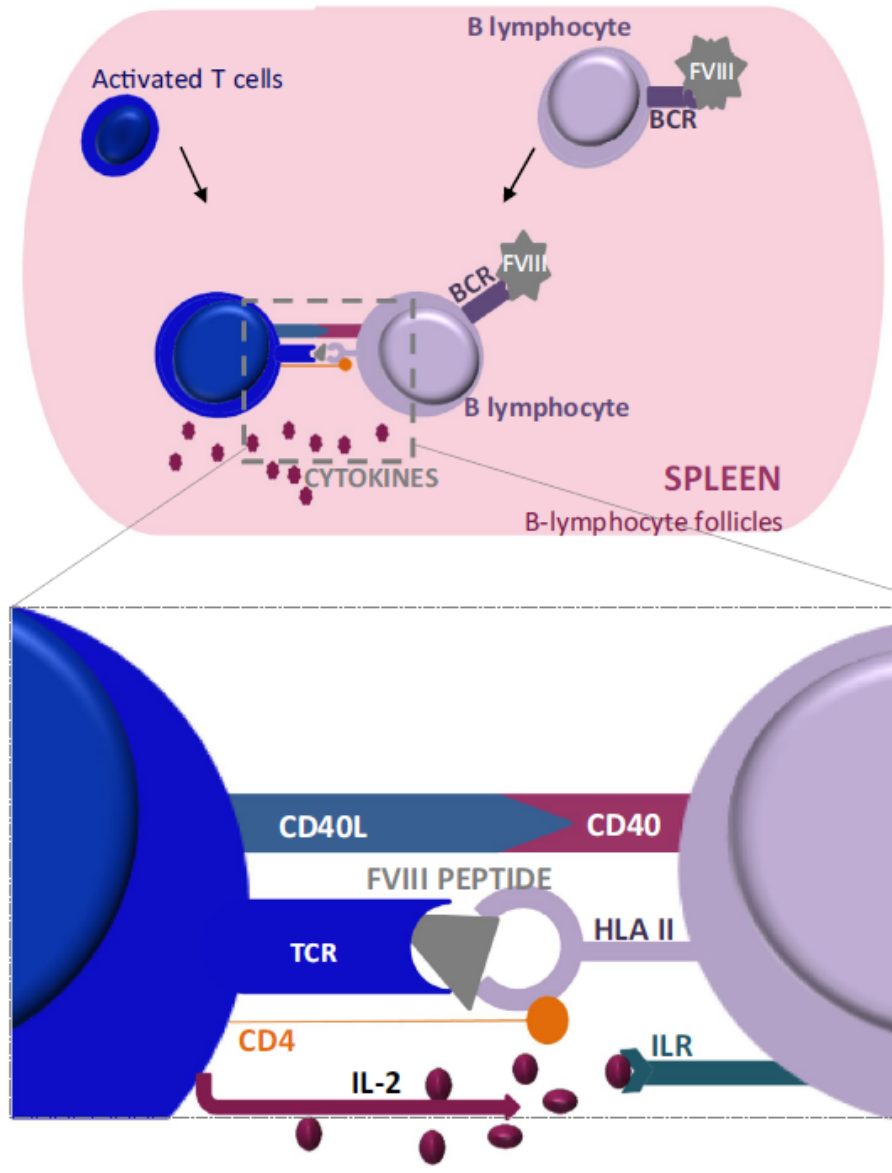
Transcription factors: Tbet, cell-specific transcription factor / GATA3, zinc finger transcription factor 3 / Foxp3, Forkhead box p3

These interactions result in a clonal expansion of the active T cells which migrate to the B-lymphocyte follicles in the spleen.



Immunology of inhibitors in hemophilia A

Part III: Primary response[6,15-17].

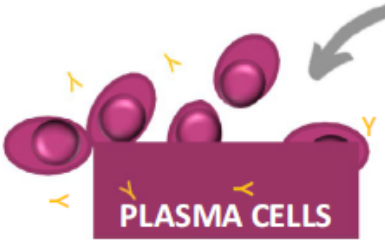


Follicular B lymphocytes that have been primed by infused FVIII and have internalized FVIII through the BCR present FVIII peptides via class II HLA on the membrane surface in the B lymphocyte follicle.

Interaction between activated T cells and B lymphocytes through TCR-HLA, co-stimulatory molecules, and cytokine stimulation triggers the differentiation of follicular B lymphocytes.

Figure legend:

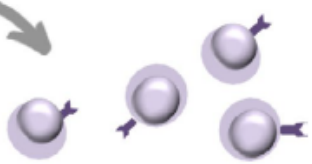
BCR, B-cell receptor
TCR, T-cell receptor
HLA II, class II human leukocyte antigen
IL, Interleukin
ILR, Interleukin receptor



Involved in long-lasting antibody production

Follicular B lymphocyte can differentiate into:

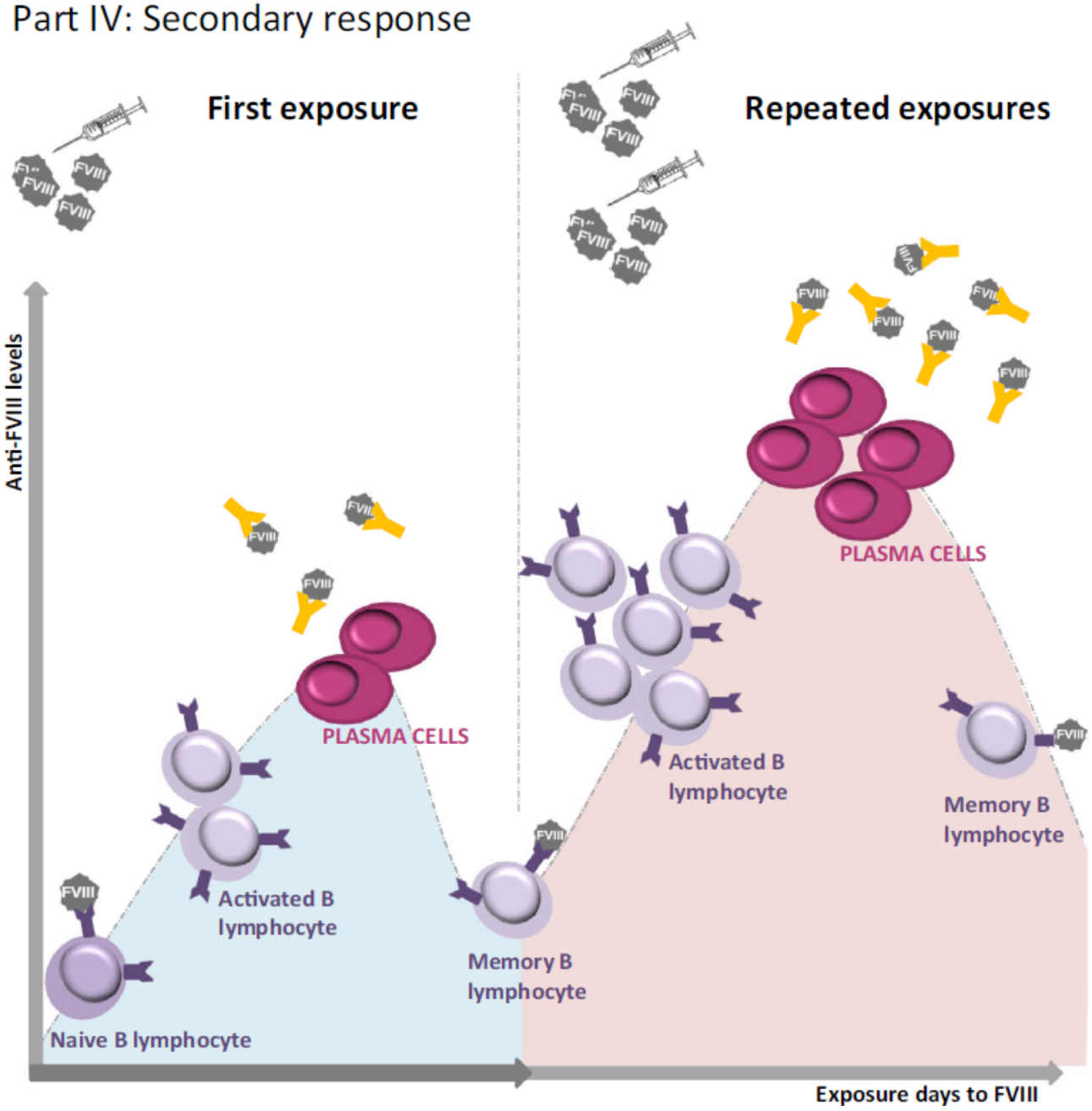
OR



MEMORY B LYMPHOCYTES

Immunology of inhibitors in hemophilia A

Part IV: Secondary response



Memory B lymphocytes can develop a faster and stronger immune response against FVIII than naive B lymphocytes, with production of high-affinity neutralizing antibodies.[15]

Anti-FVIII antibodies, mostly IgG1, IgM, and IgA, have been reported in healthy individuals. Anti-FVIII IgG4 is predominantly found in patients with HA who developed inhibitors, especially those with high titer.[17-19]

Inhibitors in hemophilia A

Treatment[5]

Bypassing agents such as activated prothrombin complex concentrate and activated recombinant factor VII are needed to treat/prevent bleeding episodes in patients with HA and high-titer inhibitors.

Emicizumab is a nonfactor therapy recently approved for the prevention of bleeding in patients with HA and inhibitors.

Immune tolerance induction (ITI)[20]

Immune tolerance induction (ITI) is the unique available treatment for eradication of persistent high-titer inhibitors in HA. ITI is effective in 60%-80% of treated patients with HA.

However, ITI is a demanding and high-cost treatment and requires frequent infusions of FVIII for months to years

Final Remarks

Inhibitor development is the main complication of HA, affecting about 30% of patients. ITI can eradicate inhibitors but is costly and not successful for all patients. Furthermore, inhibitors can recur. Therefore, a better understanding of biological mechanisms, epidemiology, and risk factors for inhibitor development is needed.

Acknowledgments

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Relationship disclosure

The authors state that they have no conflict of interest.

Author contributions

LLJ, DGC, and SMR created the capsules and the conceptual design and wrote the paper. All authors critically revised the manuscript and approved the final version.

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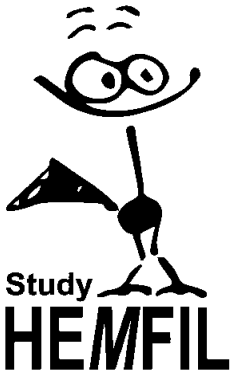
CHAPTER 4

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

Jardim LL, Santana MP, Chaves DG, van der Bom J, Rezende SM. Risk factors for antibody formation in children with hemophilia: methodological aspects and clinical characteristics of the HEMFIL cohort study. Blood Coagul Fibrinolysis. 2021 Oct 1;32(7):443-450.



ABSTRACT

Up to 35% of patients with hemophilia A (HA) and 5% with hemophilia (HB) develop neutralizing antibodies which can inhibit the therapeutic activity of factor replacement (inhibitors). Despite the clinical relevance of anti-factor 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.

Keywords: Antibody, Factor VIII, Hemophilia, Immune tolerance, Immunology, Inhibitor

INTRODUCTION

Hemophilia A (HA) and B (HB) are inherited X-linked bleeding disorders caused by deficiency of coagulation factor (F) VIII and IX, affecting 1:5,000-10,000 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 HA and HB within the first 75 exposure days (ED). [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 anti-factor 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, non-factor 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 HA 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 non-factor 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.

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 non-genetic 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 anti-factor VIII neutralizing antibodies and 27% (95% CI, 17.5%-39.6%) for high-titer in a cohort of previously untreated patients with severe HA 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 anti-factor 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. [16,17] 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-gamma and TNF-alpha, which favors the synthesis of anti-factor VIII IgG4 antibodies. [16,17]

Anti-FVIII antibodies, mostly IgG1, IgM and IgA have been reported in healthy individuals [18] as well as in patients with either inherited or acquired HA. [16,18-20] Interestingly, anti-FVIII IgG4 is predominantly found in patients with congenital HA who developed inhibitors, especially those with high-titer [18-20]. Except for one report [21], studies addressing anti-FVIII antibodies did not include the collection of blood samples before the first infusion of FVIII. Cannavò et al reported that among patients with severe HA who had never been exposed to FVIII concentrates, the presence of non-neutralizing antibodies was associated with inhibitor development [21]. However, the specific subclass(es) of anti-FVIII Ig has not been examined.

An evaluation of immunological biomarkers in previously untreated patients (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 HA presented higher levels of microparticles, CXCL8 / IL-8, IL-6, TNF, IL-4, IL-10, and IL-17 in comparison with controls without hemophilia. [22] 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-beta associated with high levels of pro-inflammatory cytokines may favor the development of an immune response against FVIII after gene therapy. [23] 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 ED 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

This HEMFIL study was named to honor Mr. Henrique de Souza Filho (1944 - 1988), also known as 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 factor VIII or IX) hemophilia. We enroll children with HA or HB before the first infusion of any factor concentrate, although we considered inclusion of children who had up to five ED. One ED 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 HTC. All data receive a code for privacy reasons (Table 1).

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.

Implicated period and end point

It has been shown that the majority of patients with hemophilia will develop inhibitors within the first 20 ED [24]. However, van den Berg et al (2019) reported that 21% of inhibitors occurs after 20-50 ED and recommend that 75 ED shall be the cut off point for prospective cohort studies of PUPs with hemophilia. [25]

In the HEMFIL Study, children are followed up to 75 ED and/or upon inhibitor development. Immunological biomarkers are tested before the first ED, i.e., at inclusion time (T0); at inhibitor development time (T1-INH^+) or at 75 ED 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) (Figure 1).

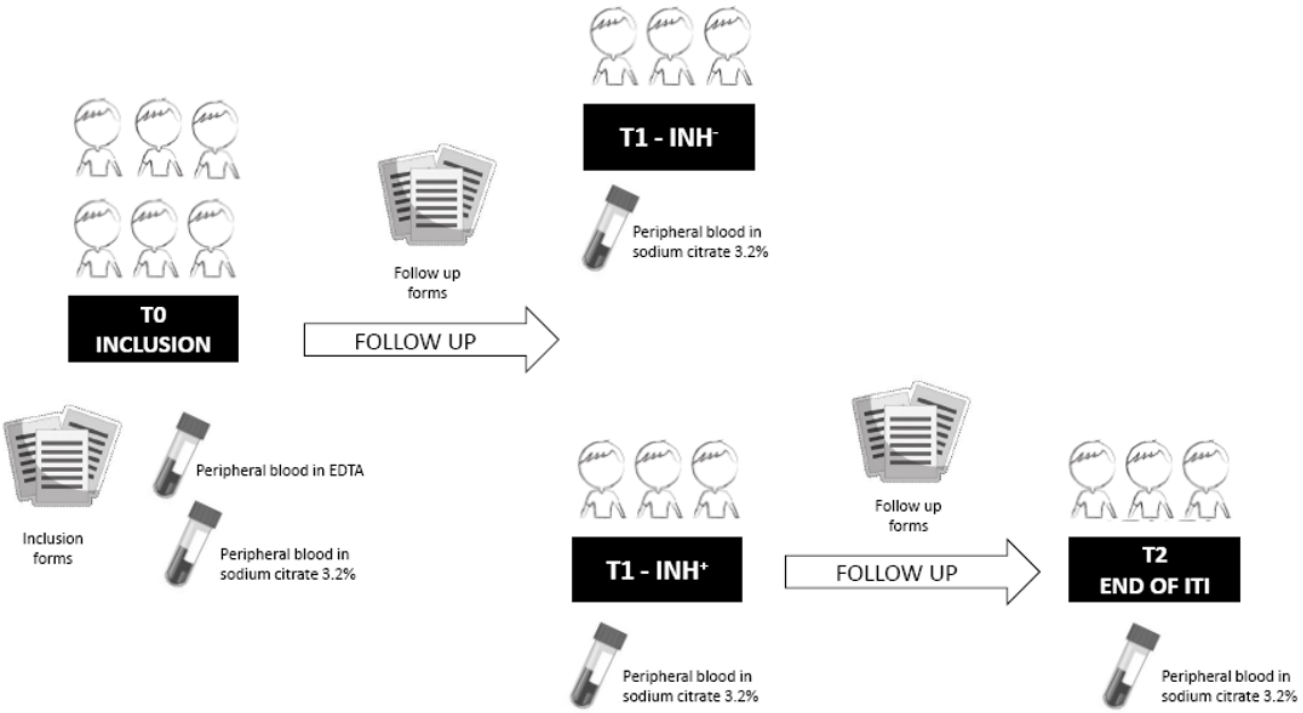


Figure 1. Time line of biological samples collection in the HEMFIL study. T0, inclusion of the patient in the study; T1, development of anti-factor neutralizing antibody (T1-INH+) or no antibody at 75 exposure days (T1-INH-); T2, end of immune tolerance induction. ITI, Immune tolerance induction.

The primary outcome for T1-INH$^+$ is the development of any inhibitor defined as a positive antibody titer above 0.6 Bethesda Unit (BU)/mL in two consecutive measurements 2-4 weeks apart. [26] The secondary outcome is the development of high-titer inhibitor (≥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 [17,27] 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 acquirement

At the enrolment time and every two 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 ED.

Type of treatment

In Brazil, patients with HA are either treated with plasma-derived (different brands) or recombinant FVIII concentrate (ADVATE®, Shire, Massachusetts, EUA). Hemophilia B patients are treated with plasma-derived FIX concentrate (different brands). Prophylactic treatment is available for all patients with severe/moderately-severe (below 2% factor levels) hemophilia A and B.

All patients with HA 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 HA 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 (Figure 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 1,500 rpm for 15 minutes 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 twenty healthy adults and twenty 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 [22].

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 recombinant FVIII (ADVATE®, Shire, Lexington) diluted in 1X phosphate-buffered saline (PBS) overnight at 4°C. Plates are washed and blocked with PBS 1X-bovine serum albumin (BSA) 3% for 1 hour at 37°C. Plasma samples are diluted in PBS 1X-BSA 0.1% and incubated for 1 hour at 37°C. The test is revealed after plate incubation for 1 hour at 37°C with biotin-conjugated anti-human Immunoglobulin antibody IgM, IgG3 and IgG4 (SIGMA-Aldrich, St. Louis, EUA) or horseradish peroxidase (HRP) IgG1 (Sanquin, Amsterdam, Netherlands) in proper dilutions. The resulting absorbance is measured in ELISA plate reader at 492 nm wavelength after the addition of 50µL of sulfuric acid 1M 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 (CV), 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 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 which were performed in different days. The inter-assay CV for the 1:40 dilution was 12%.

Analysis of chemokines and cytokines

Measurements of cytokines (IFN-gamma, 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, CS, USA). Acquisition is performed on BD Accuri™ C6 Flow Cytometer (BD Biosciences) as previously described. [22]

Immunophenotyping analysis

Immunophenotyping is performed within 24 hours 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 phosphate-buffered saline wash (PBS-W) [PBS 0.5% (w/v) bovine serum albumin and 0.1% (w/v) sodium azide] and fixed with fluorescence activated cell sorter MaxFacs Fix fixative solution (MFF) [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 Biosciensce, San Jose, United States).

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 HA phenotypes, respectively. [28-30] Analysis of introns 1 and 22 are performed in all patients with HA with FVIII:C < 2%, with the inverse PCR technique, according to protocols already established. (29,29) Samples of non-severe patients with HA and for the ones who are not carriers of introns 1 and 22 inversion and samples of all patients with HB 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, 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 200X. Analyses of the genomic data obtained are performed using Illumina BaseSpace Suite (Illumina).

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, TriColour.

Data analysis plan

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

The levels of cytokines, chemokines and anti-factor subclasses will be analyzed by comparing the levels of patients who developed inhibitors *versus* patients who reached 75 ED 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 < 0.05. Statistical analyses will be performed using GraphPad Prism 7 software (GraphPad Software, San Diego, 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.

Table 3. Baseline characteristics of children with HA.

		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)

[a]No clinical bleeding at/before hemophilia diagnosis. [b]At first infusion. #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; n, number of patients; ED, Exposure days; IQR, Interquartile range; HA, hemophilia A; HB, haemophilia B.

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 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 HA and 16 with HB. A total of 10 patients with HB have completed 75ED and no patient developed inhibitor. Of all children with HA, 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-titre and 53/85 (62%) patients reached 75ED 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 HA under exclusive use of a third-generation recombinant factor VIII concentrate at 75 ED 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: (i) the study aim which target at discovering new risk factors for inhibitor development, focusing on genetic and phenotypic biomarkers of the immune system; (ii) 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 time-points; (iii) 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. Firstly, 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. Secondly, some patients might not be included before the first ED 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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Author contributions

LLJ perform the research and wrote the paper; *JGVB* contributed with study design and revised the paper; DGC and SMR designed the research, and wrote the paper. All authors critically revised the manuscript and approved the final version.

Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

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CHAPTER 5

Inhibitor incidence in haemophilia A under exclusive use of a third-generation recombinant factor VIII concentrate: results of the HEMFIL Cohort Study

Jardim LL, van der Bom J, Brommonschenkel CC, Gouw SC, Rezende SM; on the behalf of the HEMFIL Study Group. Inhibitor incidence in haemophilia A under exclusive use of a third-generation recombinant factor VIII concentrate: results of the HEMFIL Cohort Study. Br J Haematol. 2019 Jul;186(1):152-155.

Dear Editor,

The development of an inhibitor is the most serious complication in patients with haemophilia A (HA). Of all risk factors associated with inhibitor development, type of factor VIII (FVIII) concentrate is the most debated. Several studies have shown that recombinant (r) FVIII concentrates are associated with a higher risk for inhibitor development in comparison with plasma-derived (pd) ones (Iorio et al, 2010; Gouw et al, 2013; Calvez et al, 2014, 2018; Collins et al, 2014; Peyvandi et al, 2016). The SIPPET (Survey of Inhibitors in Plasma Product-Exposed Toddlers) Study was the first randomized clinical trial to compare FVIII concentrates according to their source (Peyvandi et al, 2016). This study showed that the cumulative incidence was 44_5% [95% confidence interval (CI), 34.7–54.3] vs. 26.8% (95% CI, 18.3-35.2) for all inhibitors and 28.4% (95% CI, 19.6–37.2) vs.18.6% (95% CI, 11.1–26.9) for high-titre inhibitors in users of rFVIII and pdFVIII concentrates, respectively. This resulted in 87% higher incidence of inhibitors in patients using rFVIII in comparison with pdFVIII [hazard ratio (HR), 1.87; 95% CI, 1.17–2.96] (Peyvandi et al, 2016).

The RODIN (Research of Determinants of Inhibitor Development) Study found that a second-generation fulllength rFVIII was associated with an increased risk of inhibitor development as compared with other rFVIII products (HR, 1.60; 95% CI, 1.08–2.37) (Gouw et al, 2013). Subsequent retrospective studies reported similar results, 75% (HR, 1.75; 95% CI, 1.11–2.76) Calvez et al, 2014) and 55% (HR, 1.55; 95% CI, 0.97–2.49) (Collins et al, 2014) among patients using the same brand of rFVIII. In the latter, 45 out of 128 (35.2%; 95% CI, 27.4–43.8) patients treated with a second-generation rFVIII (Kogenate Bayer/Helixate NexGen, both from Bayer HealthCare, Barmen, Germany) developed inhibitors compared with 42 out of 172 (24.4%; 95% CI, 18.6–31.4) with ADVATE_ (P = 0.04). Recently, a French cohort study of previously untreated patients (PUPs) with severe HA (Calvez et al, 2018) found that the cumulative incidence of high-titre inhibitors at 75 exposure days (ED)was 12.7% (95% CI, 7.7–20.6) with a pdFVIII (Factane; LFB, Paris, France), 20.4% (95% CI, 14.0–29.1) and 31.6% (95% CI, 23.5–41.7) with two rFVIII, ADVATE (Shire, Lexington, USA) and Kogenate FS (Bayer HealthCare), respectively (Calvez et al, 2018). These findings suggest that the immunogenicity may not be the same for different rFVIII concentrates. In the literature, estimates of immunogenicity per product have limited precision, due to the relatively low number of patients treated with each source. In order to improve future precision regarding the estimated immunogenicity of the rFVIII product used in Brazil, this study aimed to evaluate the incidence of inhibitors in a cohort of Brazilian PUPs with HA under the exclusive use of a thirdgeneration rFVIII concentrate (ADVATE) in the HEMFIL Study.

HEMFIL is an ongoing, prospective cohort study. Patients were enrolled consecutively at the time of diagnosis in five Brazilian haemophilia treatment centres (HTC) from 2013 to 2018, and were followed for up to 75 ED. After enrolment, patients were clinically evaluated (clinical and laboratory data) and completed standardized forms for collection of socio-demographic data (Table I). FVIII activity (FVIII:C) was measured at diagnosis and inhibitor measurements were performed every 5–10 ED. The primary outcome was the development of inhibitor, defined as a positive antibody titre above 0.6 Bethesda Units (BU)/ml in two consecutive measurements 2–4 weeks apart (White et al, 2001). The secondary outcome was the development of high titre inhibitor (≥5 BU/ml). Time to inhibitor development was calculated by Kaplan-Meier cumulative incidence with cumulative ED as the time variable. All parents/guardians signed a written informed consent. The study was approved by institutional ethical committees. A total of 70 PUPs with severe HA (baseline FVIII: C < 1%) were included, 61 (85%) of whom completed the follow-up. Inhibitor was detected in 24/63 patients of which 17 were high-titre. Among patients who developed inhibitors, 17 (68%) were subsequently treated with immune tolerance. A total of 37/61 patients reached 75 ED without developing an inhibitor. There are 9 patients under follow-up, 6 (67%) of whom reached at least 30 ED. Inhibitor development occurred before 20 ED in 19/24 (79%) PUPs. The median time for inhibitor development was 14 ED (95% CI, 7.5–18) with a median age of 15.0 months [interquartile range (IQR), 12.0–19.5]. Indeed, this corroborates the study by Calvez et al (2018) who detected inhibitor at a median of 14 ED (IQR, 8.0–20.0) and a median age of 16.0 months (IQR, 12.0–24.0).

In our study, the cumulative incidence at 75 ED was 36% (95% CI, 25.7–48.7%) for all inhibitors, 27% (95% CI, 17.5–39.6%) for high-titre inhibitors and 13% (95% CI, 6.0–24.3%) for low-titre inhibitors (Fig 1).

Our results are similar to the results of the SIPPET Study, which found a 28.4% cumulative incidence of high-titre inhibitors with rFVIII concentrates (Peyvandi et al, 2016). However, in the SIPPET study, only 13 of 126 (10 3%) patients were treated with ADVATE.

Table 1. Characteristics of patients included in the study.

		Completed follow-up (n = 61)	
	All patients (n = 70)	No inhibitor* (n = 37)	Inhibitor (n = 24)
Age at baseline, months; median (IQR)	12·6 (7·0–14·0)	10·0 (5·3–14·0)	12·0 (8·0–14·0)
Age at inhibitor development, in months; median (IQR)	NA	NA	15·5 (12·0–20·8)
Weight at baseline, kg; median (IQR)	9·3 (8·0–11·0)	9·0 (8·0–11·8)	9·5 (9·0–11·0)
Ethnicity; n (%)			
White	45 (64)	25 (67)	15 (62)
Black	13 (19)	5 (14)	4 (17)
Mixed	11 (16)	6 (16)	5 (21)
Indian native	1 (1)	1 (3)	0 (0)
Reason for diagnosis; n (%)			
Bleeding	58 (83)	30 (81)	20 (83)
Family history	12 (17)	7 (19)	4 (17)
Type of treatment; n (%)			
Prophylaxis†	58 (83)	37 (100)	15 (62)
On demand	12 (17)	0 (0)	9 (38)
ED at inhibitor development; median (IQR)	NA	NA	14·0 (7·5–18·5)
Inhibitor titre; n (%)			
Low (<5 BU/ml)	7 (10)	NA	7 (29)
High (≥5 BU/ml)	17 (24)	NA	17 (71)
More than 5 consecutive ED to FVIII‡; n (%)			
Yes	13 (19)	10 (27)	3 (12)
No	57 (81)	27 (73)	21 (88)
Allergy; n (%)	12 (17)	6 (16)	3 (12)
Family history of haemophilia; n (%)	35 (50)	19 (51)	12 (50)
Family history of inhibitor; n (%)	2 (3)	1 (3)	1 (4)

BU, bethesda units; ED, exposure days; FVIII, factor VIII; IQR, interquartile range; n, number of patients; NA, not applicable.
*Patients who completed 75 exposure days without inhibitor development.
†Prophylaxis was defined as ADVATE_ infusions at 1–3 times a week, with the aim of preventing bleeds.
‡On at least one occasion during the treatment.

The main strength of our study is related to its methodological design as a cohort study of PUPs under exclusive use of a third generation rFVIII who were consecutively enrolled at diagnosis and followed-up prospectively with inhibitor development as outcome. Indeed, except for SIPPET (Peyvandi et al, 2016), all previous studies evaluating inhibitor incidences have been performed in Caucasian populations whereas the Brazilian population is widely mixed. Studying inhibitor incidence in different populations is important because it probably varies according to ethnicity (Gunasekera et al, 2015). The results of our study suggest that immunogenicity of ADVATE does not seem to be influenced by different ethnic background. Unfortunately, we were unable to compare inhibitor development with pdFVIII or other brands of rFVIII concentrates, as currently, in Brazil, ADVATE is the only type of rFVIII concentrate purchased by the Ministry of Health and, therefore, available for treatment of HA. Another limitation worth mentioning is that the measurement of inhibitors was not performed in a central laboratory. However, it was performed according to national recommendation in all participating HTCs.

In conclusion, the cumulative incidence of inhibitors with the exclusive use of ADVATE in PUPs with severe HA was 36% for all inhibitors and 27% for high-titre inhibitors

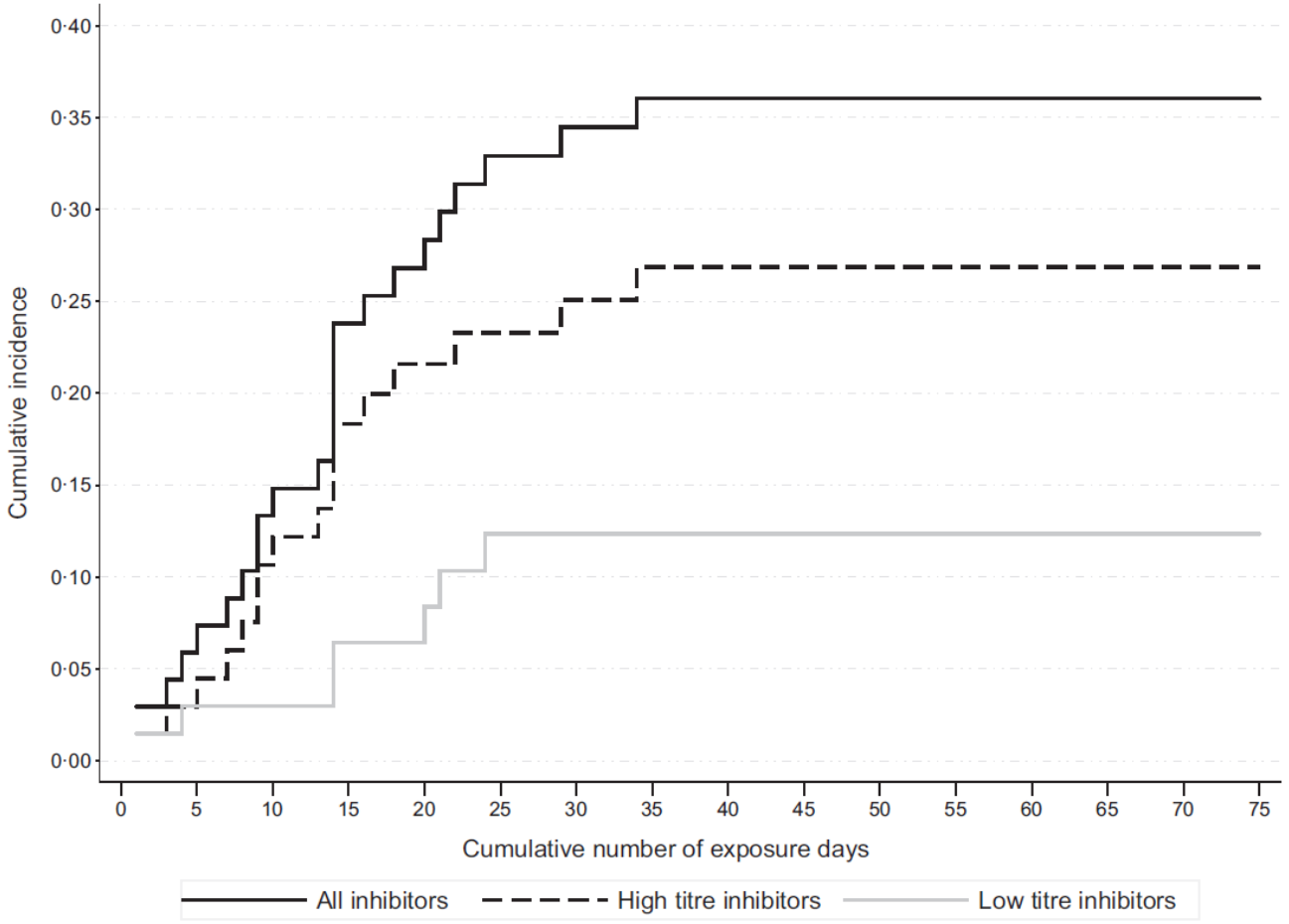

Figure 1. Cumulative incidence of inhibitor development according to cumulative number of factor VIII exposure days for all inhibitors, high titre and low titre inhibitors.

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Author contributions

L. L. Jardim performed the research, analyzed the data and wrote the paper; J. van Der Bom contributed with study design and data analysis; C. C. Brommonschenkel collected clinical data; S. C. Gouw contributed with data analysis; S. M. Rezende 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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CHAPTER 6

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

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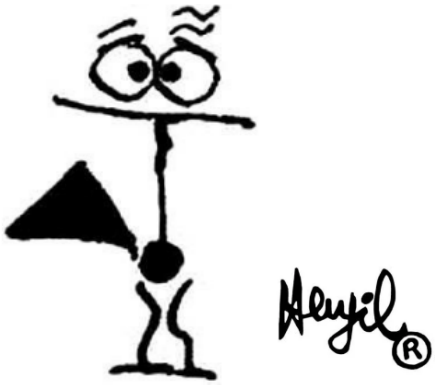


ABSTRACT

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.

INTRODUCTION

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, characterized by interleukin (IL) 2, tumour necrosis factor (TNF) and interferon-c (IFNG), stimulating the synthesis of immunoglobulin (IgG)1 anti-FVIII with no inhibitory activity (Hu et al, 2007; Chaves et al, 2010; Silveira et al, 2015).

Otherwise, patients with HA and inhibitors present an antiinflammatory/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 sociodemographic, clinical and laboratory data by completing standardized forms. These forms were translated from the RODIN study (Gouw et al, 2013), with kind permission of the RODIN Study group. Patients are followed up until 75 exposure days (ED) and/or upon inhibitor development. However, the present work targeted at studying patients at inclusion (T0), before any exposure to FVIII.

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

The control group consisted of healthy boys recruited during routine consultation at Paediatric Primary Care Centre from the University Hospital, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil. Their health status was determined by a comprehensive medical history and examination to rule out bleeding symptoms, use of medications, recent vaccination and evidence of chronic/acute illness.

Patients and control subjects with conditions that might influence the levels of chemokines and cytokines at blood collection, such as allergies, vaccination, infection and inflammation, as well as use of medications for the treatment of any morbid condition, were excluded or postponed. The parents/guardians of all patients with HA and controls signed a written informed consent to participate in the study. The research was approved by institutional ethical committees.

Sample collection

The peripheral blood of controls and PUPs with HA was collected in a 4 ml tube containing sodium citrate 3.2% as anticoagulant. Blood was immediately centrifuged at 1500 rpm for 15 min to obtain plasma. Plasma samples were stored at -80°C until analysis of chemokines, cytokines and microparticles. Patients' samples were collected before the first infusion of FVIII-containing products. In controls, samples were obtained at the time blood was drawn for routine visits to the Paediatric Primary Care Centre.

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

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

The panel of chemokines was chosen to comprise a wide spectrum of molecules responsible for the attraction of leucocytes to inflammation sites. Additionally, cytokines were designed to be a representative panel of anti-inflammatory/regulatory (IL4 and IL10) and pro-inflammatory molecules (IFN-Gama, 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: IFNG (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 (1,500 x g for 15 min followed by 13,000 x g for 5 min at room temperature). The latter was diluted in 300 mL of sodium citrate (0.124 mol/l) (BD Biosciences) with 5,000 units of heparin (Roche, Rio Janeiro, Brazil) in the ratio 1:3 and centrifuged at 14,000 x g for 90 min at 15°C. The resultant microparticle pellet was resuspended in 100 µL of 1 x 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 lm SPHERO™ Amino Fluorescent Particles (Spherotech Inc., LibertyviµLe, IL, USA).

Considering the presence of phosphatidylserine residues on the microparticles surface, events for positive staining were assessed for annexin V (BD Biosciences). Phenotypic characterization of the microparticles to determine their cellular origin was performed using monoclonal antibodies (BD Biosciences) specific for endothelial cells (ITGAV; CD51/61-PE), erythrocytes (GYPA; CD235a-PECy5), platelets (ITGA2B; CD41a-PERCP-Cy5.5), leucocytes (PTPRC; CD45-APC), neutrophils (CEACAM1; CD66-PE), monocytes (CD14-PERCP) and T lymphocytes (CD3-PE). The samples were analysed in a Flow Cytometry FACSCalibur (BD Biosciences). Over 100,000 events were acquired for each sample, to reach at least 2,000 events within the microparticles gate.

For the determination of microparticles/µL the formula microparticles/µL = (N x 400)/(100 x 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 $P < 0.05$.

In an exploratory analysis, individuals were considered high or low responders for each cytokine evaluated. The cutoff 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 v2 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 [interquartile range (IQR), 5.0–3.5 months]. The control group consisted of 47 healthy non-haemophiliac Brazilian boys, with a median age of 12.2 months (IQR, 7.7–16.7).

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

Table 1. Characteristics of the study population.

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

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

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; 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.

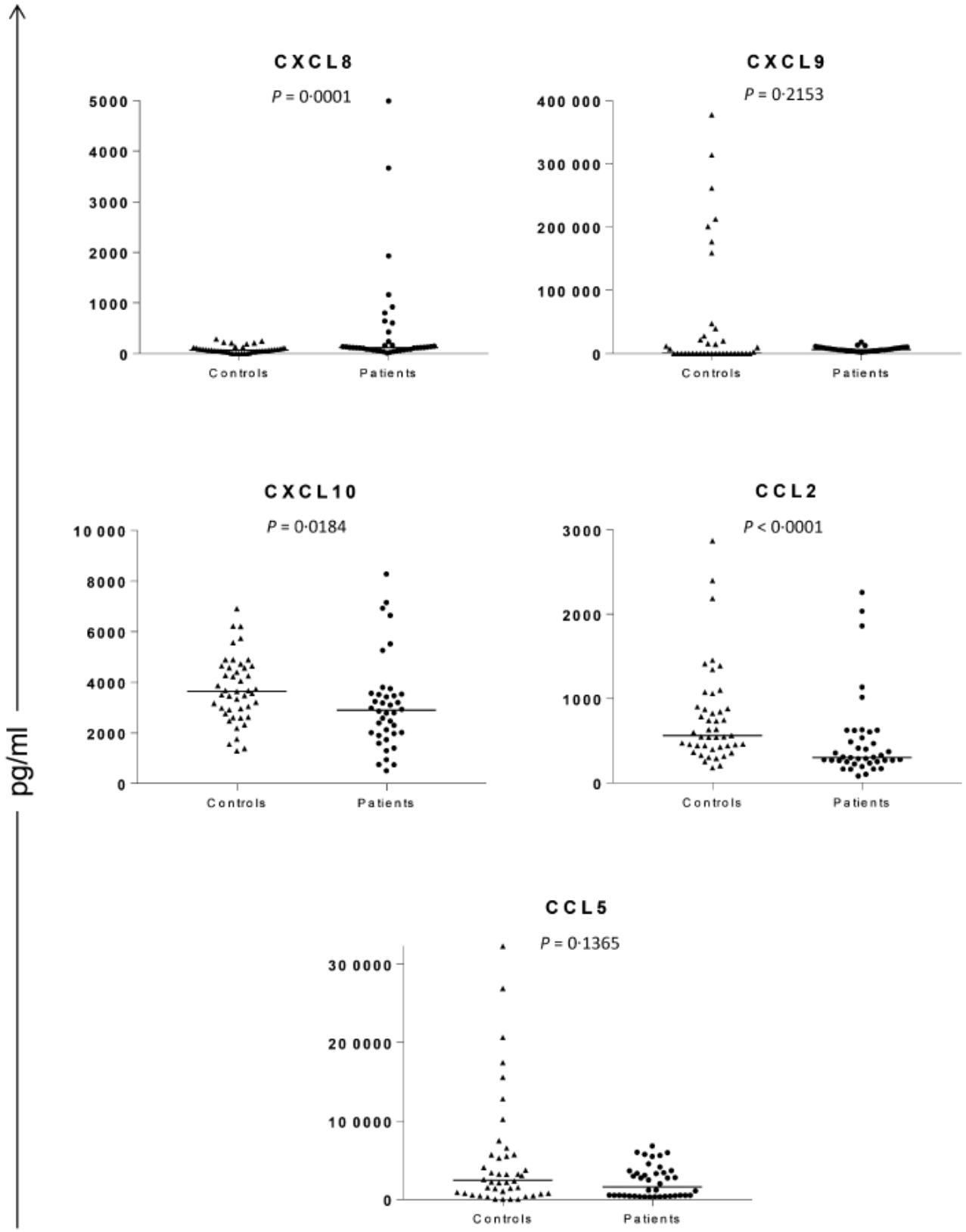


Figure 1. Representation of the chemokines analysed. Each point in the graph represents one measurement of one individual. The continuous line represents the median of the results in the group of individuals analysed for each chemokine. Comparison between groups was performed by Mann–Whitney test. The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

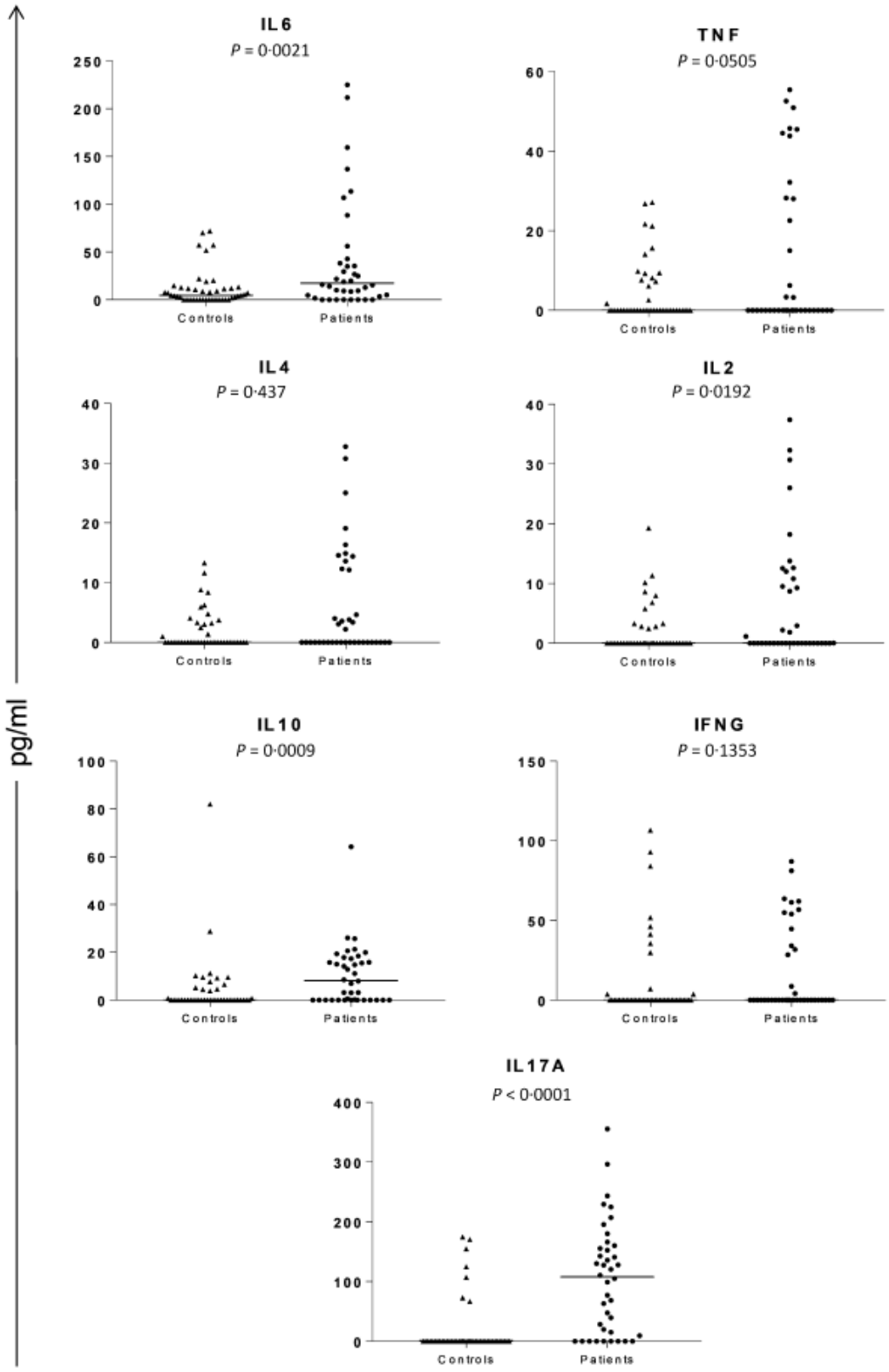
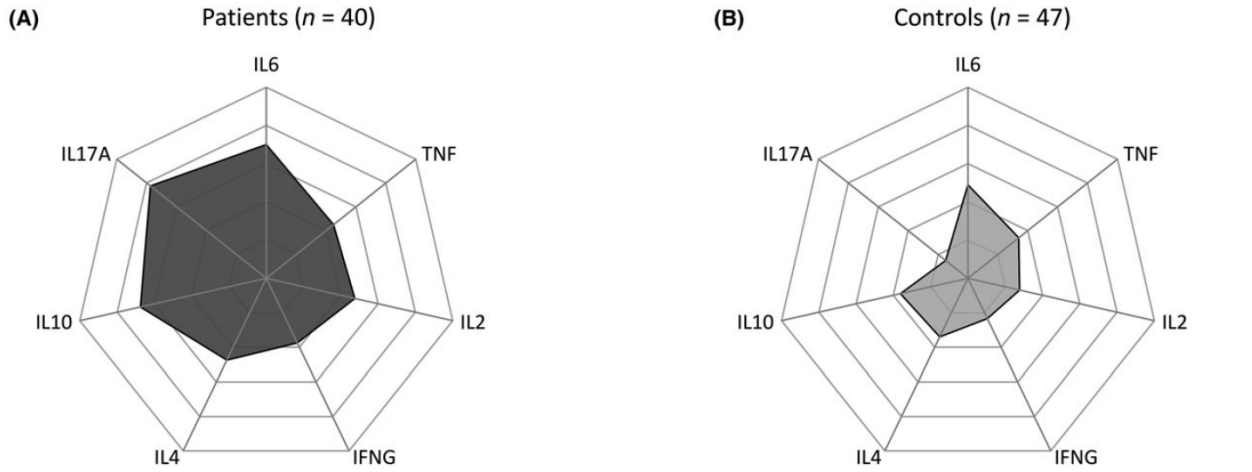


Figure 2. Representation of the cytokines analysed. Each point in the graph represents one measurement of one individual. The continuous line represents the median of the results in the group of individuals analysed for each cytokine. Comparison between groups was performed by Mann–Whitney test. The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



Group	IL6, *n* (%)	TNF, *n* (%)	IL2, *n* (%)	IFNG, *n* (%)	IL4, *n* (%)	IL10, *n* (%)	IL17A, *n* (%)
Patients	28 (70·0%)	18 (45·0%)	19 (47·5%)	15 (37·5%)	19 (47·5%)	27 (67·5%)	31 (77·5%)
Controls	23 (48·9%)	16 (34·0%)	13 (27·7%)	11 (23·4%)	16 (34·0%)	17 (36·2%)	7 (14·9%)
P-value	0·05	0·21	0·02	0·07	0·13	< 0·01	< 0·01

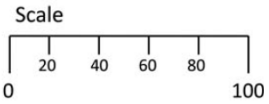


Figure 3. Radar graphical representation of cytokine patterns in patients and controls. This chart summarizes the percentage of high cytokine balance in previously untreated patients (dark grey area) and controls (light grey area). The cut-off point between low and high levels of each cytokine was defined 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 v2 test. The differences were considered statistically significant when $P < 0.05$.

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 in angiogenesis-dependent disorders, such as wound repair and conditions characterized by persistent neovascularization.

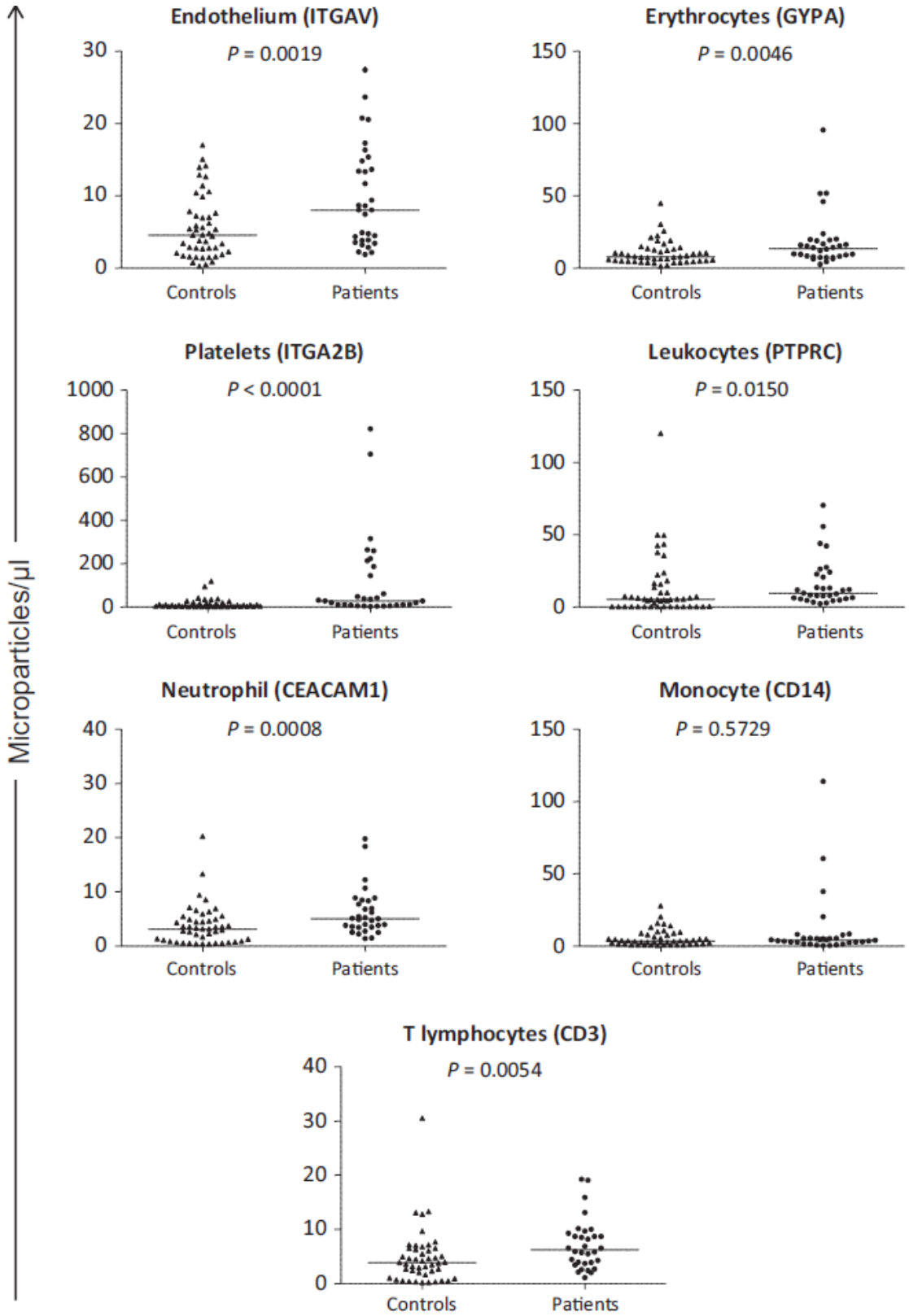


Figure 4. Representation of the microparticles analysed. Each point in the graph represents one measurement of one individual. The continuous line represents the median of the results in the group of individuals analysed for each microparticle. Comparison between groups was performed by Mann–Whitney test. The differences were considered statistically significant when P < 0.05.

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 hich 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 (Hrachovinov.a 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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CHAPTER 7

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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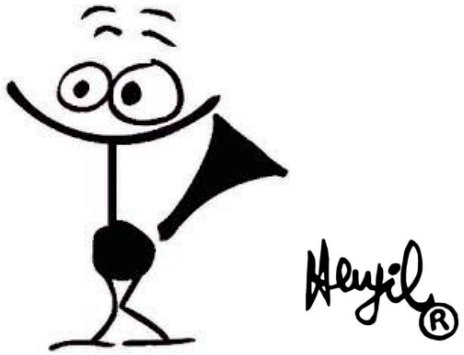


ABSTRACT

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

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.

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 longstanding 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 the addition of 50 µL/well of 1 M H2SO4 (Sigma-Aldrich), the optical density (OD) was measured using 492nm 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 10minutes 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 analyses were performed using the Spearman correlation test. Data included in the 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 each 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 those who did not (p<0.01). Detailed data of the 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). 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).

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 CXCL8was 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).

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.

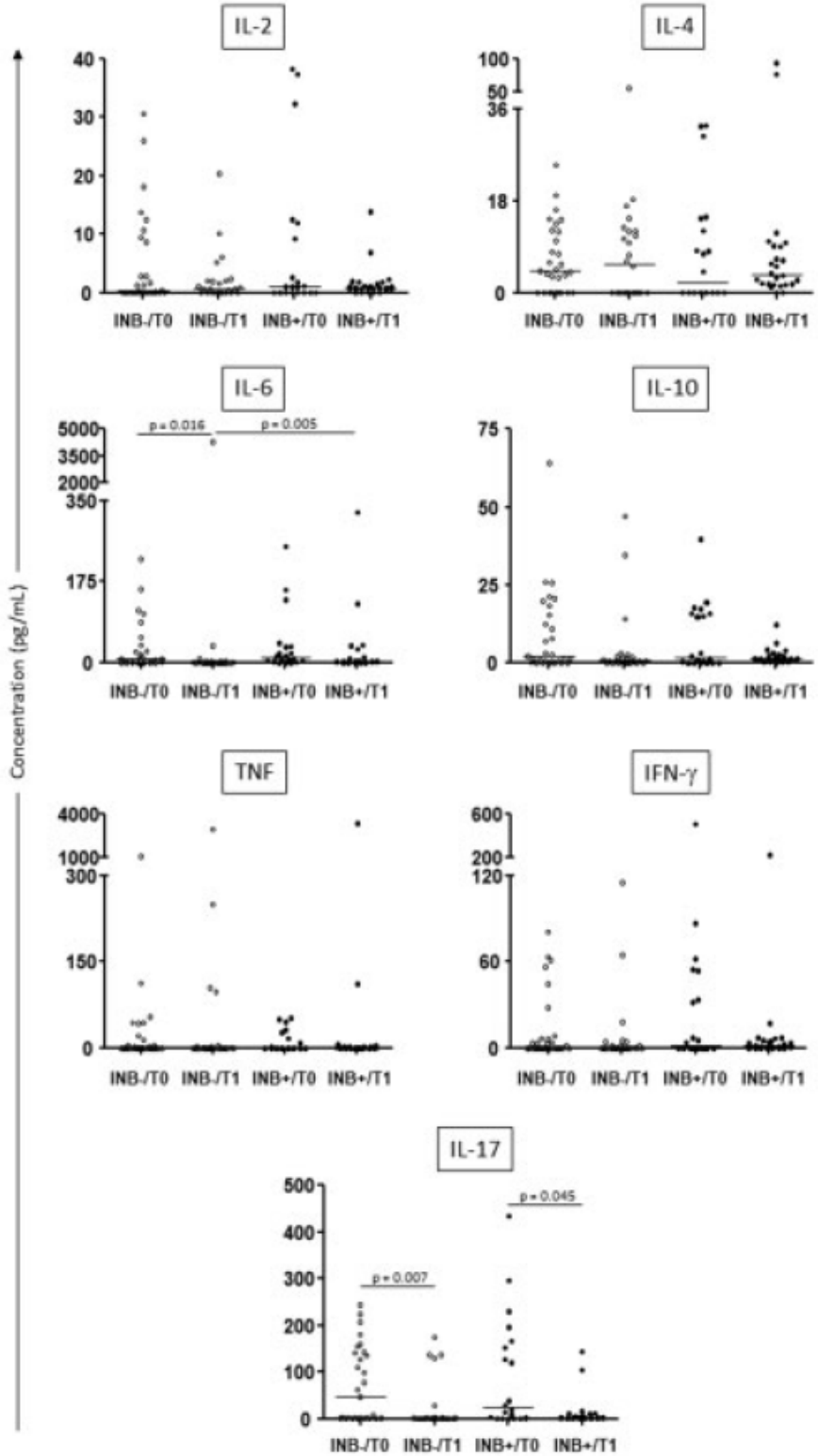


Figure 1. Representation of plasma cytokine concentration (in pg/mL) in all patients by group and time point. Each circle represents the mean concentration of two measurements of each cytokine. Empty circles represent patients without inhibitor (INB-) and black filled circles represent patients with 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.

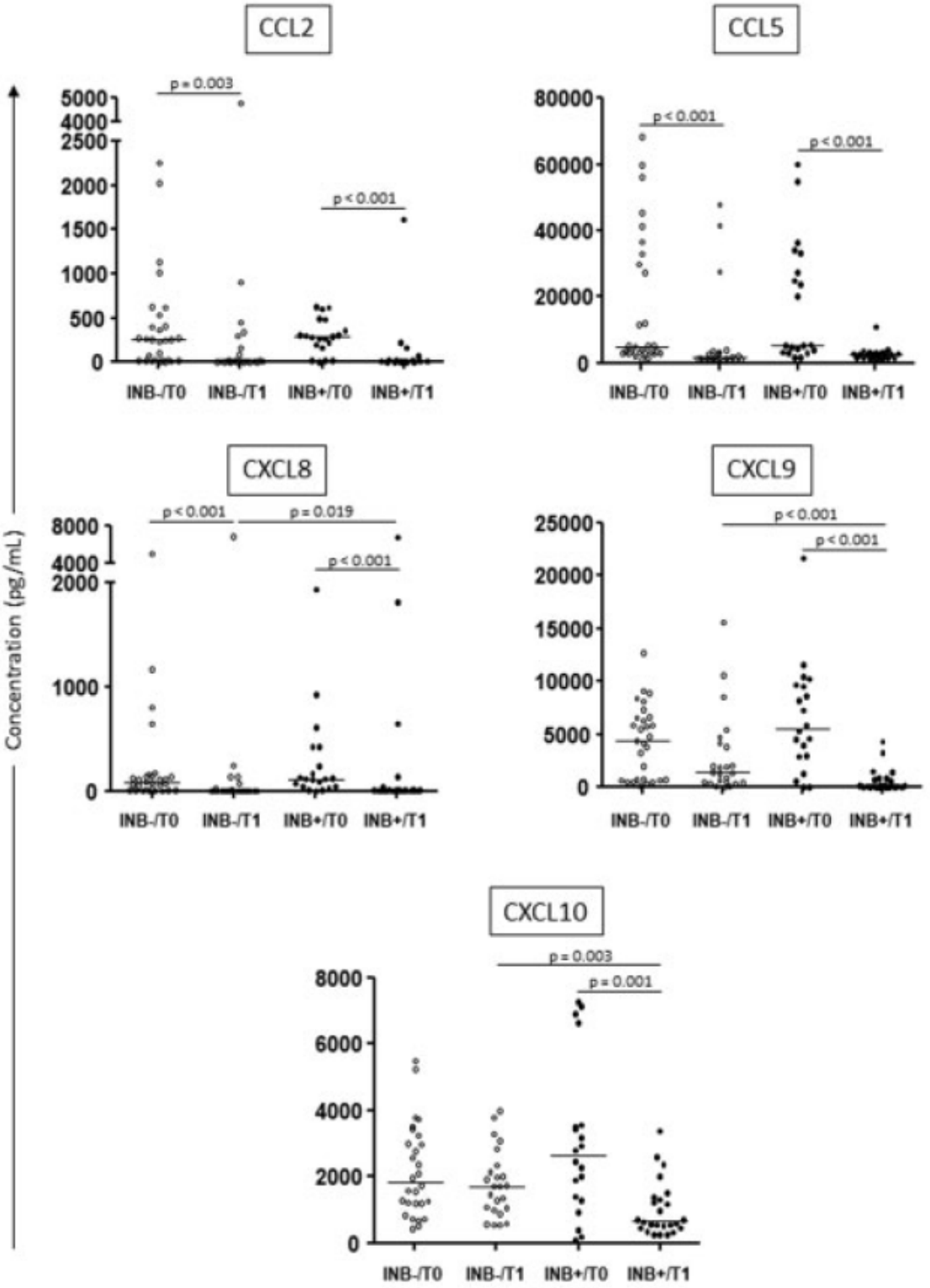


Figure 2. Representation of plasma chemokine concentration (in pg/mL) in all patients by group. Each circle represents the mean concentration of two measurements of each chemokine. Empty circles represent patients without inhibitor (INB-) and black filled circles represent patients with inhibitor (INB+). 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.

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 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.

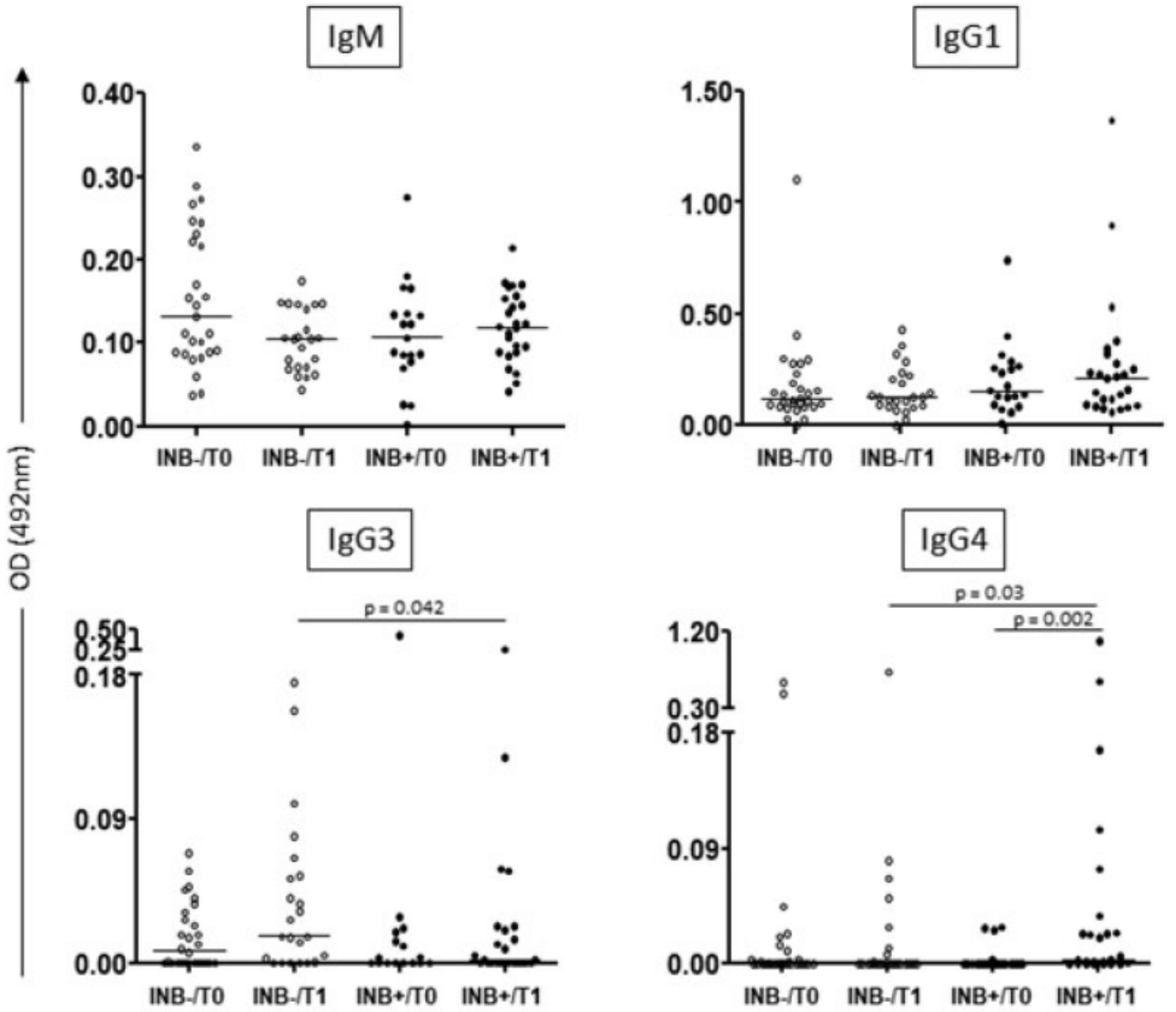
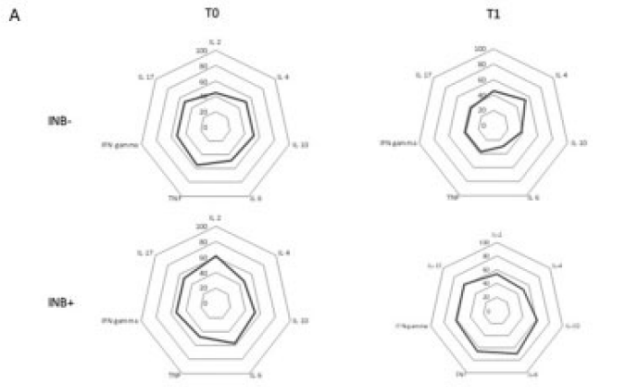


Figure 3. Levels of anti-FVIII IgM, IgG1, IgG3, and IgG4 antibodies in all included patients by group. Each empty circle represents the mean OD of two measurements of immunoglobulin assessed in each patient without inhibitor (INB-). Each black circle represents the mean OD of two measurements of immunoglobulin 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.

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.

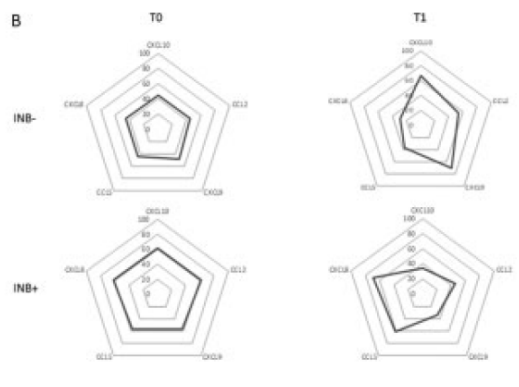
A proinflammatory Immune profile was found in INB+/T1 The analysis of radar charts of the cytokine profile in INB+/ T1 revealed a significantly higher proportion of patients with increased levels of IL-6 (69.2 vs. 29.2%; p=0.005) when compared with the INB-/T1 (Fig. 4A). Analyses of the chemokine profile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



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

Figure 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.

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 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).

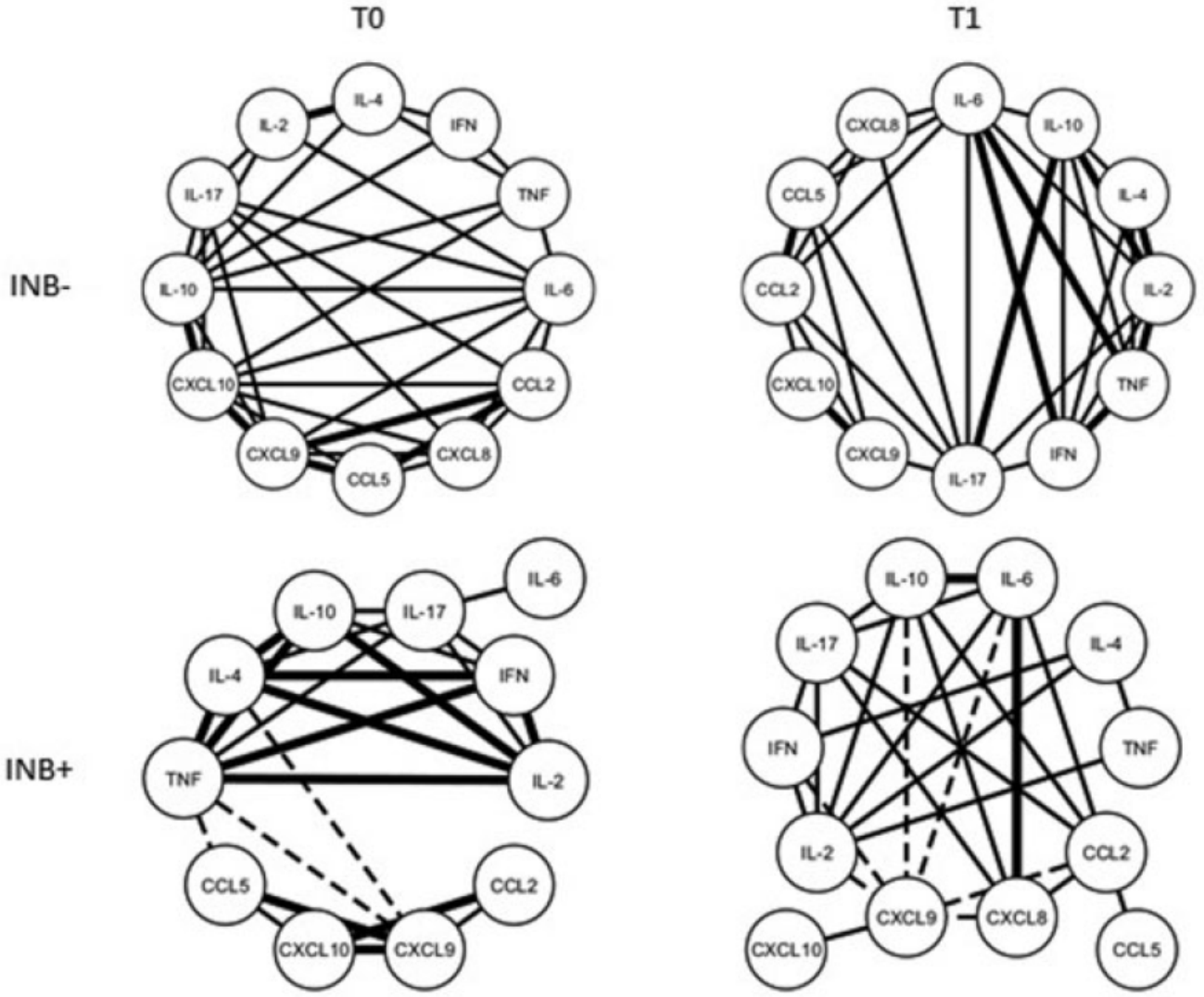


Figure 5. Representation of correlation network of immunological biomarkers. Solid lines correspond to positive correlation between biomarkers. Dotted lines correspond to negative correlation between biomarkers. Thicker lines represent strong correlations (r >0.68).

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 those who did not. We found an impaired network between cytokines and chemokines before 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 the 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 that 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 with 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 exposure 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.

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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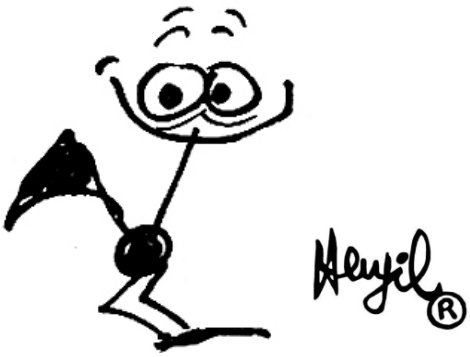
CHAPTER 8

Prediction of inhibitor development in previously untreated and minimally treated children with severe and moderately severe hemophilia A using a machine-learning network

Jardim LL, Schieber TA, Santana MP, Cerqueira MH, Lorenzato CS, Franco VKB, Zuccherato LW, da Silva Santos BA, Chaves DG, Ravetti MG, Rezende SM. Prediction of inhibitor development in previously untreated and minimally treated children with severe and moderately severe hemophilia A using a machine-learning network. J Thromb Haemost. 2024 Sep;22(9):2426-2437.



ABSTRACT

Background: Prediction of inhibitor development in patients with hemophilia A (HA) remains a challenge.

Aim: To construct a predictive model for inhibitor development in HA by utilizing a network of clinical variables and biomarkers.

Methods: Previously untreated and minimally treated children with severe/moderately-severe HA, participants of the HEMFIL Cohort Study, were followed-up until reaching 75 exposure days (ED) without inhibitor (INH-) or upon inhibitor development (INH+). Clinical data and biological samples were collected before the start of factor VIII (FVIII) replacement (T0). A predictive model (HemfilNET) was built to compare the networks and potential global topological differences between INH- and INH+ at T0, considering the network robustness. For validation, the "leave-one-out" cross-validation technique was employed. Accuracy, precision, recall, and F1-score were used as evaluation metrics for the machine-learning model.

Results: We included 95 children with HA (CHA), all treated with recombinant FVIII. Inhibitors were detected in 31 (33%) CHA. The algorithm, featuring 37 variables, identified distinct patterns of networks at T0 for INH+ and INH-. The accuracy of the model was 74.2% for CHA INH+ and 98.4% for INH-. By focusing the analysis on CHA with high-risk *F8* mutations for inhibitor development, the accuracy in identifying CHA INH+ increased to 82.1%.

Conclusion: Our machine-learning algorithm demonstrated an overall accuracy of 90.5% for predicting inhibitor development in CHA, which further improved when restricting the analysis on CHA with a high-risk *F8* genotype. However, our cohort consists of an admixed population and the model need to be validated in other cohorts. Yet, missing data for some variables hindered more precise predictions.

Keywords: Hemophilia A, factor VIII, machine-learning, inhibitor, previously untreated children

Essentials:

- The algorithm presented an overall accuracy of 90.5% to predict inhibitor development in children with hemophilia A.
- The algorithm accuracy for inhibitor prediction increased when the analysis was restricted to patients with *F8* high-risk mutations.

INTRODUCTION

Hemophilia A (HA) is an inherited bleeding disorder resulting from mutation in the factor VIII (FVIII) gene (*F8*). Patients with HA have low FVIII activity and, therefore, require frequent administration of FVIII-containing products or emicizumab [1]. However, up to 35% of patients with HA receiving FVIII replacement can develop neutralizing alloantibodies (inhibitors) within the first 75 exposure days (ED) [1]. The presence of inhibitors remains a challenging complication of hemophilia treatment, increasing the risk of morbidities, such as excessive bleeding, chronic pain, and functional disability [1]. Therefore, early prediction of inhibitor development may allow the implementation of preventive interventions and personalized treatments, which can improve patients' clinical outcomes.

Because of the potential influence of FVIII in non-genetic biomarkers, the ideal acquisition of data for inhibitor prediction analyses is before the first infusions of FVIII-containing products. However, as several patients demand urgent treatment for bleeding episodes upon diagnosis, the collection of biological samples before FVIII replacement is challenging. Furthermore, hemophilia is a rare disease and such analysis require prospective, large cohort studies, which are scarce in the field. Therefore, predictive studies on inhibitor development are still lacking.

In recent years, artificial intelligence (AI) has emerged as a foundational scientific discipline, finding widespread application across various fields of knowledge, including medicine, biology, physical phenomena, chemistry, and others [2-9]. In this context, machine learning methods have emerged as promising tools for investigating associations between clinical and laboratory information in diverse diseases [10-25] including hemophilia [26-28]. These algorithms capture intricate hierarchical interactions among multiple risk factors, unveiling patterns that traditional statistical approaches might fail to detect.

Complex networks, represented by graphs with non-trivial topological features, have proven to be potent instruments for studying systems across various domains. These systems are based on relationships between entities, such as those present in social, biological, and communication [29-36]. The utilization of network-based approaches offers insights into the structural patterns of these systems and their dynamic behavior [37-39]. However, studies elucidating the interactions of diverse biomarkers as a network and exploring other risk factors related to inhibitor development in HA are still lacking.

In this study, we employed the Network Node Dispersion (NND) method [40] as a potential tool for investigating the prediction of inhibitor development in previously untreated and minimally treated children with HA (CHA), who are participants in the HEMFIL study.

METHODS

Patients

The HEMFIL is a prospective Brazilian cohort study that aims to identify new risk factors related to inhibitor development in CHA [41]. The present study included previously untreated (0 ED) and minimally treated (up to 5 ED) children with severe/moderately-severe HA (FVIII<2%) from The HEMFIL Study. All CHA included in this report have completed the follow up until 75 ED or until inhibitor development and were treated exclusively with recombinant FVIII concentrate (ADVATE®; Takeda Pharmaceuticals, United States).

Immune biomarkers

At inclusion time (T0), peripheral blood was collected in tubes containing sodium citrate to perform immunological phenotyping of monocytes (CD14+HLA-DR+) neutrophils (CD16+HLA-DR+), T and B-cells (CD4+HLA-DR+; CD8+HLA-DR+; CD19+CD5+; CD19+CD80+; CD19+CD86+ -). Samples were centrifuged for plasma obtention and the following biomarkers were evaluated: specific anti-FVIII antibodies (IgM, IgG1, IgG3, IgG4), chemokines (CCL2, CCL5, CXCL8, CXCL9, CXCL10) and cytokines (IL-2, IL-4, IL-6, IL-10, IFN-gamma, TNF,

IL-17). Phenotypic characterization of microparticles (MPs) was also performed to determine their cellular origin by using monoclonal antibodies specific for endothelial cells (CD51/61), erythrocytes (CD235a), platelets (CD41a-PERCP), leukocytes (CD45), neutrophils (CD66), monocytes (CD14) and T lymphocytes (CD3). Details of blood collection and the methodology for assessing immunological biomarkers were previously described [41,42].

Factor VIII assessment and genotyping of factor VIII gene

Factor VIII activity levels were measured twice at diagnosis to determine the type and severity of HA. Patients were classified as severe HA when plasma FVIII was < 1 U/dL and moderately-severe HA when FVIII was 1-2 U/dL [43]. Inhibitor assessment was performed at diagnosis and at every 5-10 ED until 75 ED [43]. These tests were performed in the Hemophilia Treatment Centers where the patients were recruited. All laboratories have internal and external quality control in place.

DNA was extracted from peripheral blood using QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). FVIII gene (*F8*) genotyping was performed for assessment of introns 1 and 22 inversions [44,45] and all the DNA samples ware sequenced using a next generation sequence custom panel - AmpliSeq Custom DNA Panel (Illumina; San Diego, California). According to the type of *F8* variant identified, CHA were classified in two groups: "High-risk" and "Low-risk' for inhibitor development. Large insertion/deletion, nonsense mutations and introns 1 and 22 inversions were considered as high-risk mutations and the remaining were considered as low-risk mutations [46,47].

Outcome

The primary outcome was the development of any inhibitor defined as a positive antibody titer above 0.6 Bethesda Unit (BU)/mL in two consecutive measurements, 2-4 weeks apart [23]. High-titer inhibitor was considered if ≥5 BU/mL at any time [42].

Statistical analysis

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 analyses were performed by Mann-Whitney test. The differences were considered significant when P-values were < 0.05. Statistical analyses were performed using GraphPad Prism 7 software (GraphPad Software, San Diego, United States). A predictive model (The HemfilNET) was used to compare the network and potential global topological differences of the patients' profiles at T0.

The HemfilNET

Several methods have been proposed for representing information as network structures [29-37]. The most straightforward and intuitive approach is to use categorical variables that contain a finite number of distinct categories or groups. In this study, each vertex in the resulting network represents an individual (CHA). Two vertices are linked if they share the same category. Figure 1-A illustrates the relationship between the genetic variants in the dataset under consideration.

For continuous variables $(x_1, x_2, \ldots, x_N)$, we create a complete network with N vertices whose links between CHA i and j have weights $w_{ij} = |x_i - x_j|$, the absolute difference between two variable values. Thus, $w_{ij} = 0$ if, and only if, the values coincide. The more significant the difference, the greater the dissimilarity between the values in each pair of CHA. Here, we propose using the maximization of the Network Node Dispersion (NND)[20]as weight thresholding [48-50], reducing the number of edges, and increasing the vertex's

heterogeneity in terms of the node distance distributions. Starting from a complete network in which all vertices are interconnected, the highest weights are progressively removed and the NND is computed until its maximum value is reached. Then, the resulting network is considered unweighted. Figure 1-B shows the distribution of CD4+HLA-DR+, while Figure 1-C represents the network obtained through the maximization of the NND. Readers are encouraged to consult the Supplementary Information (SI) section for a more comprehensive explanation of the methodology.

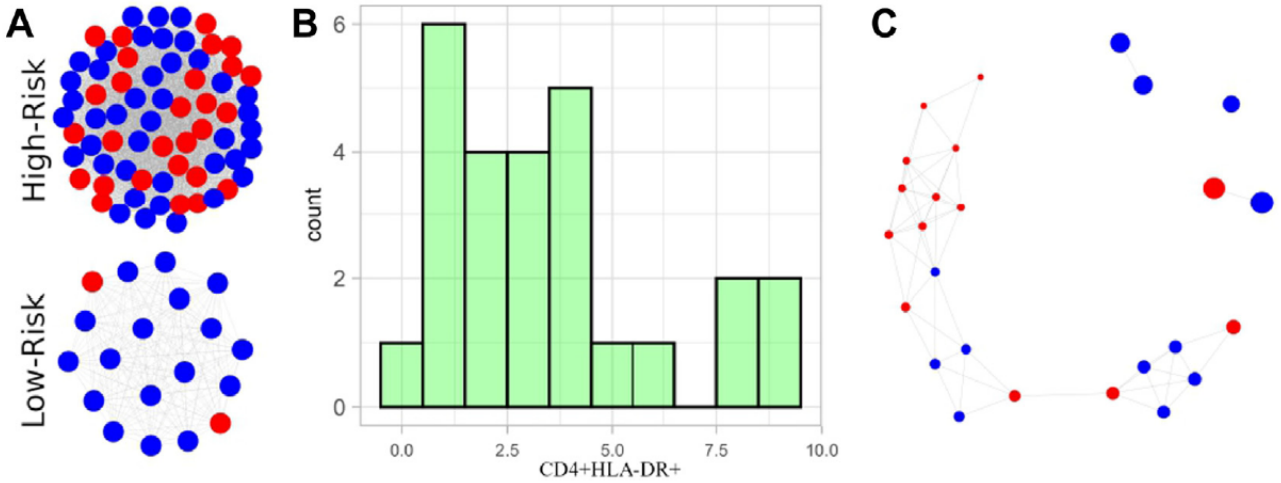


Figure 1. Structural relationships among children with hemophilia A for *F8* variant and CD4+HLA-DR+ variable. Each vertex represents one CHA at inclusion time (T0). (A) Genetic variant network according to high- or low-risk mutations. (B) Distribution of CD4+HLA-DR+ counts. (C) Structural relationship between all CHA (vertices) for the CD4+HLA-DR+ variable. The size of the vertices is directly proportional to the values of the CD4+HLA-DR+. Therefore, larger vertices represent higher CD4+HLA-DR+ values. In A and C, red vertices correspond to CHA who developed inhibitors, and blue vertices correspond to CHA who reached 75 exposure days without inhibitors. / CHA, children with hemophilia.

It is important to emphasize that each variable undergoes an individual transformation into a structural relationship between pairs of vertices (CHA) containing measurements of that variable. This approach eliminates the need for missing data imputation methods. The resulting structure is a multilayered network, where each layer represents the network of a specific variable. These layers are interconnected through vertices shared between different layers, forming an intricate structure known as the HemfilNet. This method unveils patterns and areas of high interconnectivity, offering profound insights into the relationships between variables.

To understand the structure of the HemfilNet, we propose to characterize the networks based on their organizational structure into distinct groups or communities, a well-established approach in network analysis [51-54]. These communities consist of tightly interconnected nodes that fulfill critical functional roles within the network. Identifying such communities is crucial for comprehending the underlying similarities found in various real-world systems. In the present study, we employed the Louvain method [55] to classify the communities within each network as either inhibitor (C_i) or non-inhibitor clusters (C_n). To determine the cluster type of a CHA in a given layer, we calculated the fraction of CHA INH+ in that layer, denoted by F. This fraction represents the probability that a random CHA in this cluster will develop an inhibitor. If the fraction f of vertices in the whole network that develops inhibitors is greater than F, we classify the cluster as C_n; otherwise, we classify it as C_i.

The model aims to assess the variation in clusters across different features through the construction of a network that interrelates distinct CHA clustered in multiple layers. The underlying premise is that if a CHA

exhibits similar clustering patterns (C_i or C_n) for two different features, the predictive signal provided by these features for the outcome remains consistent. Conversely, when two features contain CHA with dissimilar clusters, the information diversity between them is maximized. To formalize this notion, let $N_{\alpha\beta}$ denote the number of CHA sharing the same cluster class for features α and β. The *layer similarity network* is represented as a directed and weighted network, where vertices correspond to variables and edges correspond to the relationships between these clusters by a weight $W_{\alpha\beta} = N_{\alpha\beta}/N_{\alpha\alpha}$. A $W_{\alpha\beta}$ value of one indicates that all CHA observed in layers α and β belong to the same cluster class in both layers. Conversely, a zero $W_{\alpha\beta}$ suggests that all CHA observed in layers α and β exhibit distinct cluster classifications patterns in these respective layers.

Figure 2 illustrates the layer similarity network, where at least 70% of the vertices ($W_{\alpha\beta} \geq 0.7$) belong to either cluster class (C_i or C_n). Figure 2A presents the network analysis encompassing all CHA. Figures 2B and 2C depict individuals categorized based on their inhibitor development status. It is important to note the discernible topological differences in the networks that characterize the two groups. Classification patterns in INH+ are distinct in comparison with INH-.

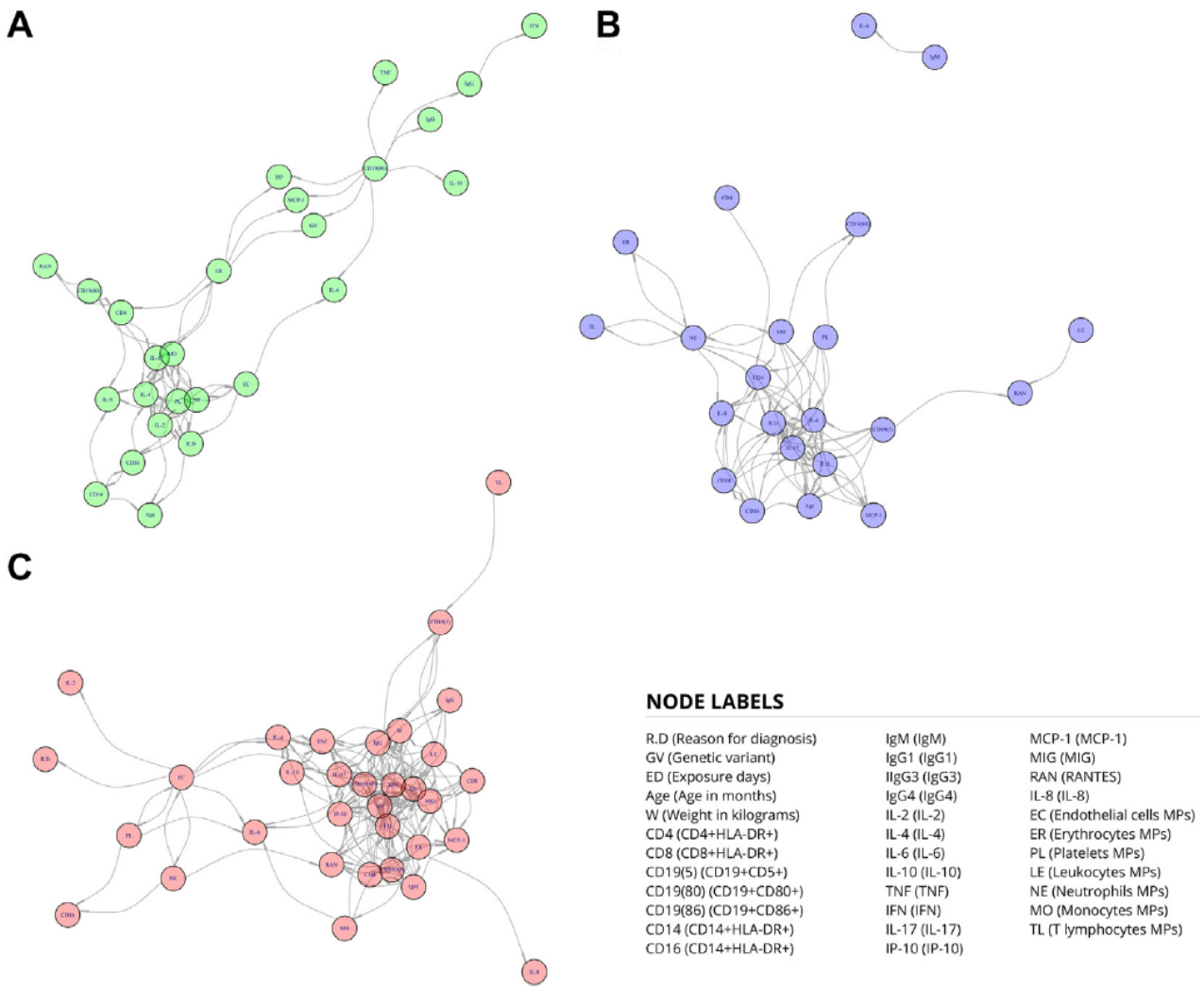


Figure 2. The Layer Similarity Network. A directed link between two features exists if at least 70% of the individuals who have measures on both variables belong to the same class (INH- or INH+). In panel A, the network considers all children with hemophilia (CHA). In contrast, panel B shows CHA who did not develop inhibitor (INH-) and panel C, CHA who developed inhibitor (INH+).

The Network-Based Classification Model

In this study, we employed a straightforward model to assess the robustness of a similarity network in predicting inhibitor development within a dataset. Given the limited size of the dataset and the presence of missing values for some variables, we opted for the leave-one-out cross-validation technique [56-59]. Using this technique, the model was trained on all data points, except for one observation, which was set aside for testing. This iterative process was performed for each observation in the dataset, ensuring that each data point was exclusively used for testing.

Initially, a layer similarity network was constructed for the training set, consisting of distinct groups of CHA: CHA INH- denoted as G^{NI} and CHA INH+ represented as G^{I}. Subsequently, the cluster class for each CHA in the test set was determined based on similarity for each feature.

For each CHA in the test set, two networks were generated by selectively removing links from G^{NI} and G^{I}. The connection between two features was retained only if the test individual belonged to the same cluster class in both features; otherwise, it was deleted. The primary objective was to evaluate which of the resulting systems exhibited greater robustness to link removal. If the integrity of the G^{NI} network was better preserved compared to that of the G^{I} network, the test CHA was classified as INH-; conversely, it was classified as INH+.

This approach allowed us to discriminate the predictive power of the constructed networks and ascertain the potential of the layer similarity network in classifying CHA with respect to inhibitor development. By testing the resilience of the networks to link removal, we gained insights into their reliability as tools for distinguishing between INH- and INH+ individuals within the test set.

Several methods exist to assess the impact of link removal on the G^{I} and G^{NI} networks. Network robustness is generally defined as its ability to withstand failures while maintaining structural integrity under targeted or untargeted attacks [60-67]. In this study, our primary focus is on the robustness of the network G under link failures, leading to the formation of a new network G_f, denoted by $R(G|G_f)$ given by the sum of all link weights in G_f divided by the sum of all link weights in G. Consequently, $R(G|G_f)$ takes a value of one only if the network G_f is identical to G, and zero if G_f contains no links.

Let G_f^I and G_f^{NI} be the networks generated by the deletion of links of G^{I} and G^{NI} induced by the test CHA, the number $\Gamma = R(G^I|G_f^I) - R(G^{NI}|G_f^{NI})$ measures which of the two systems (inhibitor or non-inhibitor) is more affected by the link removal process. If $\Gamma > 0$, the G^{NI} network is more affected by the test individual, and the test CHA is classified as a potential inhibitor developer. On the other hand, if $\Gamma < 0$, the inhibitor system is more affected by the test CHA and, therefore classified as a potential non-inhibitor. In consideration of the imbalanced dataset encompassing patients with and without inhibitors, it is noteworthy that the magnitudes of $R(G^I|G_f^I)$ and $R(G^{NI}|G_f^{NI})$ exhibit similarity. This observation underscores a compelling outcome arising from the proportional relationship between the weights within the network $G_f^{I\ or\ NI}$ and the overall weights within the full network $G^{I\ or\ NI}$.

During each CHA test, the model provides a Γ value, along with the highest robustness obtained between G^{I} and G^{NI}. The first value is associated with the test outcome, while the robustness value reflects the confidence level in correctly selecting the outcome.

Data sharing statement

The data that support the findings of this study are available on request from the corresponding author, SMR. The data are not publicly available due to ethical restrictions and privacy of research participants.

RESULTS

Patients

A total of 95 CHA who have completed the follow-up were included in the present analysis. At inclusion the median age was 10.0 months [Interquartile range (IQR), 6.0 - 15.0], with a median weight of 9.7 kg (IQR, 8 - 11). A total of 19 (20%) were minimally treated [median of 2 ED (IQR, 1 - 4 ED)] (Table 1). There was no statistically difference in the main clinical characteristics and levels of biomarkers between CHA who were previously treated or minimally treated (Table S1).

A total of 64/95 (67.4%) CHA reached 75 ED without inhibitor development (INH-). Inhibitor was detected in 31/95 (32.6%) patients (INH+) of whom, 22 (68.9%) were high-titer. The median time for inhibitor development was 14 ED (IQR, 7 - 21) with a median age of 13 months (IQR, 10 - 17) (Table 1).

Immunological biomarkers

Figure 3 shows that, at T0, a higher proportion of CHA INH- presented a more expressive area towards IL-6, TNF, IgG4, IgG3 and MPs derived from erythrocytes. On the other hand, a higher proportion of CHA INH+ presented levels of lymphocytes TCD4, TCD8, B lymphocytes below the median and a more expressive area towards monocytes-derived microparticles, IgG3 and most cytokines (IL-4, IL-2, IL-17, IFN-gamma and IL-10). These results shows that the immunological profile at baseline, i.e., before the first/minimal exposure to FVIII differs in CHA INH+ when compared with CHA INH-.

The Network-Based Classification Model Results

The construction of our forecasting model was conducted with a strategic focus on cross-validation, specifically employing the Leave-One-Out Cross-Validation (LOOCV) method. In LOOCV, each data point in the dataset is sequentially selected as a test point, while the model is trained using all the other data points. This iterative process is repeated for each observation in the dataset, enabling a comprehensive evaluation of the model's performance under various test conditions. The choice of LOOCV proved to be crucial, especially considering the limited size of our dataset. This approach maximizes the utilization of each available data point, providing a robust estimate of the model's capability to make accurate and generalizable predictions.

Figure 4-A displays the values obtained for the robustness and the Γ parameter for each individual subjected to the test. Notably, as Figure 4-B shows, greater robustness is associated with an increased ability to discriminate between individuals who develop inhibitors and those who do not. All individuals tested with a robustness exceeding 23.5% demonstrated correct classifications, implying that a higher degree of information contained within the study variables may be correlated with a higher model accuracy.

Figure 4-C displays the confusion matrix from the experiment, encompassing all CHA in the dataset, in contrast to the model restricted to individuals with high-risk *F8* mutations associated with HA. The positive and negative predictive values of the model were, respectively, 74.2% and 98.4%. The overall accuracy is 90.53%. The recall was 95.8% and the F1-score was 83.7%. When the analysis was restricted to patients with *F8* high-risk mutations, the accuracy to identify CHA INH+ increased from 74.2% to 82%, but decreased from 98.4% to 87.8% to identify CHA INH-. In the Supplementary Materials, we analyzed the ROC curve for this experiment and found that the area under the curve (AUC) was 0.9435.

Interestingly, all nine CHA patients who were not correctly classified at T0 (eight CHA INH+ classified as INH- and one CHA INH- classified as INH+) had some missing data points, accounting for a low robustness value in the classification. These missing data points included MPs counts and immunophenotyping, suggesting that the misclassification could be attributed to misinformation rather than to the features' capacity to predict the outcome.

Table 1. Baseline characteristics of the children included.

		Completed outcome (*n* = 95)	
	All *n* = 95	Children with HA with no inhibitor *n* = 64	Children with HA with inhibitor *n* = 31
Age at baseline in mo, median (IQR)	10 (6-5)	11 (7-17)	8 (4-12)
Age at first infusion in mo, median (IQR)	11 (6-16)	11 (7-17)	9 (6-13)
Age at inhibitor development in mo, median (IQR)	NA	NA	13 (10-17)
Minimally treated patients, *n* (%)	19 (20)	15 (23)	4 (13)
ED at inclusion time, median (IQR)	2 (1-4)	2 (1-3.5)	3 (2-5)
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 (< 1.0%)	92 (97)	63 (98)	29 (94)
Moderately severe (1.0%-1.9%)	3 (3)	1 (2)	2 (6)
Skin color, *n* (%)			
White	57 (60)	45 (70)	16 (53)
Black	22 (23)	11 (17)	7 (22)
Mixed	15 (16)	7 (11)	8 (25)
Indian native	1 (1)	1 (2)	0 (0)
Reason for diagnosis, *n* (%)			
Bleeding	76 (80)	55 (86)	22 (71)
Family history[a]	18 (20)	9 (14)	9 (29)
ED at inhibitor development, median (IQR)	NA	NA	14 (7-21)
Inhibitor titer, *n* (%)			
Low	NA	NA	9 (29)
High	NA	NA	22 (71)
More than 5 consecutive ED to FVIII[b], *n* (%)			
Yes	9 (9)	6 (9)	3 (9)
Classification according with the *F8* variant[c], *n* (%)			
High-risk mutations	60 (63)	35 (55)	25 (81)
Intron 22 inversion	*40 (67)*	*19 (54)*	*21 (84)*
Intron 1 inversion	*4 (6)*	*4 (12)*	*0 (0)*
Large insertion/deletion	*6 (10)*	*5 (14)*	*1 (4)*
Nonsense mutation	*9 (15)*	*7 (20)*	*2 (8)*
Start Lost	*1 (2)*	*0 (0)*	*1 (4)*
Low-risk mutations	28 (29)	23 (36)	5 (17)
Missense	*16 (57)*	*14 (61)*	*2 (6)*
Small deletion/insertion	*9 (32)*	*6 (26)*	*3 (10)*
Splice site[d]	*3 (11)*	*3 (13)*	*0 (0)*
Deleterious variant not detected	7 (8)	6 (9)	1 (3)

ED, Exposure days; F8, factor VIII gene; HA, hemophilia A; IQR, interquartile range.
[a] No clinical bleeding at/before hemophilia diagnosis; [b] At first infusion; [c] Large insertion/deletion, nonsense mutations mutation, Introns 1 and 22 inversions were considered as high-risk mutation; the remaining were considered as low-risk mutations. [d] Splice site includes splice acceptor, splice donor, splice region and start lost mutations.

In consideration of the significance ascribed to each variable within the framework of our classification model, we conducted a sensitivity analysis to identify the variables that, when included, most effectively characterize patients as INH- or INH+. To achieve this objective, we assessed the model's accuracy by examining the presence or absence of each variable in the dataset. The heightened importance associated with specific variables is intricately linked to the model's capacity to generate more precise predictions when the respective variable is considered. Figure 5 shows a visual representation of the normalized values that clarify the relative importance of each variable in facilitating accurate outcome classifications. The three most influential variables include CD19+CD5+ (B1 cells), CD4+HLA-DR+ (activated TCD4 lymphocytes) and genetic variant.

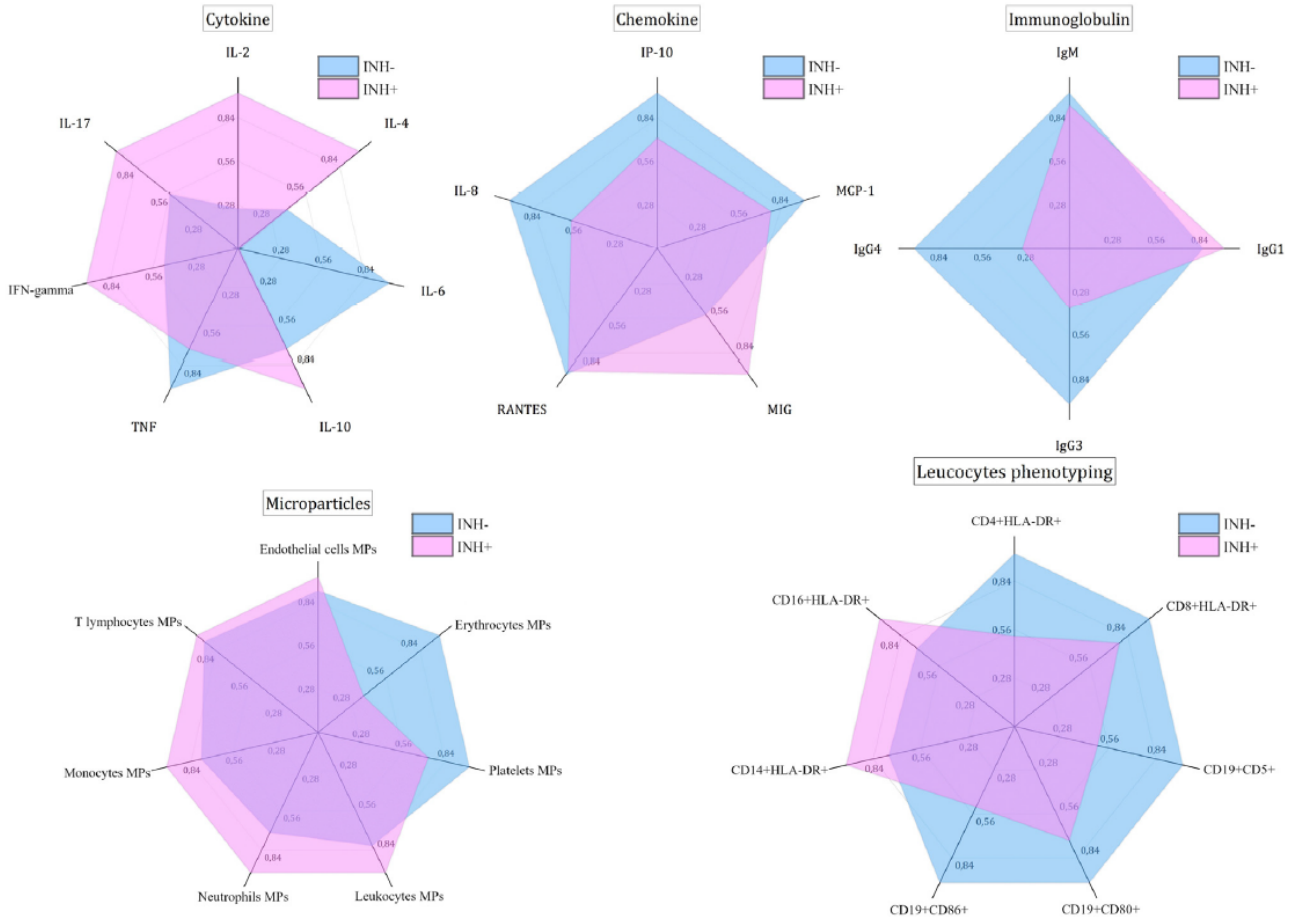


Figure 3. Radar charts of plasma cytokines, chemokines, Immunoglobulins, microparticles and leucocyte phenotyping. Each axis represents the proportion of CHA with level of biomarkers above the median. The increase or decrease of the areas of the central polygon respectively reflects the more or less proportion of children with high or low variable levels, respectively. Children who completed the follow-up without inhibitor development (INH-) are represented in blue and children with inhibitors (INH+) are represented in pink. Measurements represent the mean of duplicates and were performed at inclusion time (T0). MPs, microparticles; IFN, interferon; IL, interleukin; TNF, tumor necrosis factor; Ig, Immunoglobulin.

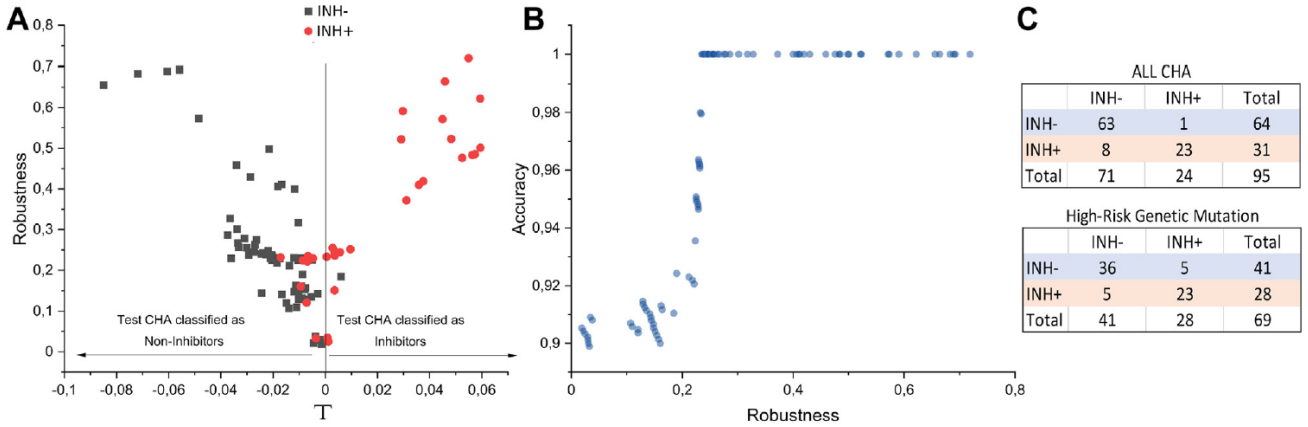


Figure 4. **Robustness and accuracy of the model**. (A) Each point represents a CHA who developed (INH+; in red) or did not develop (INH-; in black) inhibitors. The x-axis represents the gamma value used in the decision-making process, and the y-axis shows the robustness. (B) Evolution of accuracy when considering higher robustness as a decision-making criterion. (C) Confusion Matrix for the classification experiment when considering all CHA and when the analysis was restricted to those with a high-risk *F8* variant. CHA, children with hemophilia. INH+, CHA who developed inhibitor; INH-, CHA who completed the follow-up without inhibitor; *F8*, factor VIII gene.

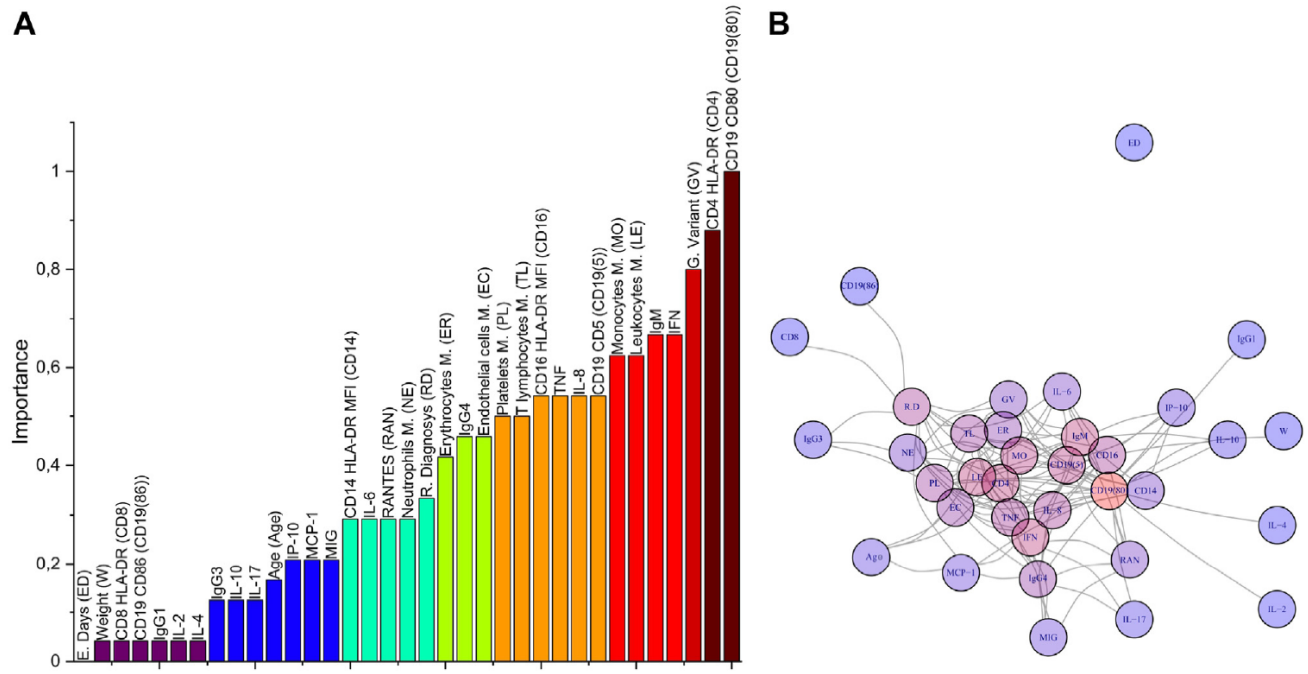


Figure 5. Normalized importance values of each variable. (A) Normalized importance values of each variable, classified based on their weight on inhibitor development. An optimization process was conducted to examine groups with similar variables using the D-metric, which measures the impact of the retrieval of each variable on the correct classification of the patients (B) The HemfilNET network links variables based on their positive contribution to the prediction of inhibitor development and only includes variables that have a positive impact on the prediction of inhibitor development.

DISCUSSION

By using 37 variables, including clinical variables and biomarkers, we developed a network-based machine learning algorithm to predict inhibitor development in HA This technique reduces the number of edges in weighted networks by identifying the maximum node distance distribution heterogeneity in the resulting network [36-38]. Each transformed network serves as a layer in a multilayered structure, offering valuable insights into the patterns and interactions among various factors [39,40]. The integration of the structural framework derived from the networks, accomplished through clustering and similarity methodologies, offers a unique perspective that elucidates the varying degrees of similarity or distinction among different CHA. This suggests the potential to validate the significance of these biomarkers in other cohorts, thereby underscoring their potential application in clinical practice.

Machine learning methodologies have already been used in hemophilia with a focus on disease severity [26-28], genetic variants [26], FVIII protein structure [27], thrombin generation and gene therapy [28]. These methodologies have become promising tools in understanding molecular interactions and in identifying more assertive treatment strategies.

Here, we developed a prediction model for inhibitor development in HA using a machine-learning network. The algorithm presented an overall accuracy of 90.5% to predict inhibitor development in CHA. The positive and negative predictive values of the model were, respectively, 74.2% and 98.4%. When the analysis was restricted to CHA with *F8* high-risk mutations, the accuracy to identify CHA INH+ increased from 74.2% to 82%, but decreased from 98.4% to 87.8% to identify CHA INH-. These results align with previous studies reporting that patients with *F8* high-risk mutations are at an increased risk of developing inhibitors, regardless of other influencing variables [46,47,68]. Indeed, the SIPPET study showed that specific mutations may influence clinical outcomes [69]. However, the accuracy of predicting HIN- decreased from 98.4% to 87.8% when the analysis was restricted to *F8* high-risk mutations, suggesting that *F8* genotype has a lower impact on patients who will not develop inhibitors than on patients who will do so. The main variables associated with inhibitor development were CD19+CD5+ (B1 cells), CD4+HLA-DR+ (activated TCD4 lymphocytes) and genetic variant.

The main advantage of our methodology is that there is no need to input values for missing data. When addressing missing data, it is crucial to recognize that imputing values can significantly influence the importance of certain attributes, particularly when the imputed values fail to accurately reflect the true distribution of the data. Such discrepancies can lead to errors in the model's ability to identify the most relevant features for classification. In fact, when performing missing value imputation on small datasets, there is a greater tendency to introduce higher levels of uncertainty, given that these datasets inherently contain less information compared to larger counterparts. This heightened uncertainty may result in less accurate imputations, negatively impacting the model's ability of generalization [70,71]. We conducted a comparison between HemfilNet and two widely used classifiers in the literature, namely Random Forest (RF) and Logistic Regression (LR), to gain insights into this effect. It is noteworthy that both RF and LR necessitate complete data. However, our classifier outperformed them, achieving an overall accuracy of 62.10% for RF and 60.00% for LR.

The biological basis and physiopathology of inhibitor development is not fully understood. It is well accepted that the neutralizing antibodies formation results from individual genetic predisposition associated with environmental and/or exogenous conditions [72-74]. Cross-sectional studies have described the immune profile of CHA [48,49], but only few studies have evaluated this profile prospectively [69,74,75]. Our group previously reported that, regardless of inhibitor status, repeated infusions of FVIII can modulate the immune system of patients with HA [51]. As such, before the first FVIII infusion, patients with HA present higher levels of MPs, CXCL8/IL-8, IL-6, TNF, IL-4, IL-10, and IL-17 in comparison with controls without hemophilia [42]. Furthermore, the administration of FVIII-containing products seems to trigger a pro-inflammatory response mediated by IL-6 and CXCL8/IL-8 in patients with HA [75] However, these studies only evaluated the individual contribution of each biomarker, without considering the connection between them.

Our study has major strengths. The HEMFIL Study is a well characterized prospective cohort of CHA with a long follow-up. Blood samples were collected at the time of diagnosis of HA, predominantly before (80% of patients) or shortly after few (less than 5) exposures to FVIII products. Rigorous precautions were implemented at the time of blood collection to ensure that CHA were free from any conditions that could potentially influence immune biomarkers, such as allergies, recent vaccinations, ongoing infections, inflammation, or the use of medications to treat any underlying medical conditions [41]. Notably, all CHA included in this analysis received treatment with a unique type of recombinant Factor VIII (ADVATE®; Takeda Pharmaceuticals, United States), minimizing the possibility of bias caused by the immunogenicity of different types of factors [41].

Our study has some limitations worth discussing. Firstly, 19 out of 95 (20%) CHA received minimal treatment (< 5 ED) with FVIII at T0 due to bleeding at enrollment, requiring immediate FVIII replacement. Nevertheless, comparison between the general characteristics of previously untreated children and minimally treated children, as well as the levels of immunological biomarkers, exhibited no significant differences between the two groups. Yet, the "exposure days at inclusion" variable (0 to 5 days) was not relevant for the model (Figure 5(B). Notably, our model accurately predicted the outcomes of all 19 minimally treated children. Secondly, since immunophenotyping requires fresh blood samples, it was not performed in all included CHA. Thirdly, due to the rarity of HA and the need to assess blood samples prior to FVIII replacement, we were unable to validate the model in other cohorts. However, we will make the model and guidance available online for public access and test. It worth to mention that that all patients included in this study received the same recombinant factor VIII and this may limit the replication of the HemfilNET in cohorts treated with other products. Fourthly, missing data for some variables hindered the model's accuracy, preventing more precise predictions. Lastly, our cohort consists of an admixed population, which may limit the generalizability of the results to other populations of CHA.

In conclusion, our machine-learning algorithm had a high overall accuracy to predict inhibitor development, which improved upon restricting the analysis to CHA with high-risk *F8* mutation. The insights gained from this analysis hold promise for the development of predictive models concerning inhibitor susceptibility, potentially laying the foundation for future clinical strategies and personalized treatment approaches tailored to patients with HA.

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Author contributions

LLJ perform the research and wrote the paper; TAS and MGR contributed with study design and with the machine-learning evaluation; DGC and SMR designed the research and wrote the paper; MPS, MHC, CSL, VKBF selected the patients and collected the clinical data; LWZ contributed with genetic analyses; BASS contributed with immunological measurements. All authors critically revised the manuscript and approved the final version.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

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Supplementary Information

Prediction of inhibitor development in previously untreated children with hemophilia A using a machine-learning network

The D-measure and the maximum NND method

The identification and quantification of dissimilarities between networks constitute a fundamental and important challenge. In the year 2017, Schieber et al [40]. introduced a dissimilarity measure known as the D-measure, designed for application in networks encompassing both directed and undirected links, each linked to a distinct structural aspect. This metric relies on a series of probability distribution functions (PDFs) that comprehensively depict the connectivity distances among the network nodes. The comparison of networks is achieved through the utilization of standard information-theoretic metrics. The D-metrics presents an efficient and highly precise approach for network comparison within the realm of scientific inquiry.

The distribution of distances at each node i, denoted as $P_i = \{p_i(j)\}$, where $p_i(j)$ represents the fraction of nodes connected to node i at a distance j, encapsulates detailed information regarding the network topology. The D-measure combines disparities in distance distributions between networks and intra-network heterogeneity through the Network Node Dispersion (NND), which serves as a characterization of the mean diversity in network topology, as represented by the distance distributions of nodes, among network components. More specifically, considering a network G with N vertices, and the set of distance distributions $\{P_1, \ldots, P_N\}$ the NND is given by

$$NND(G) = \frac{J(P_1,P_2,\ldots,P_N)}{\log(d+1)},$$

being $J(P_1, P_2, \ldots, P_N) = \frac{1}{N}\sum_{i,j}^{N} p_i(j)\log\left(\frac{p_i(j)}{\mu_j}\right)$, the Jensen-Shannon divergence between the N probability distributions, d its diameter and $\mu_j = \sum_{i=1}^{N} p_i(j)/N$ the average probability among all node distance distributions.

Here, we propose using the maximization of the Network Node Dispersion (NND) [40] as weight thresholding [48-50] reducing the number of edges, and increasing the vertex's heterogeneity in terms of the node distance distributions. The removal of high-weight links to maximize the NND of the resulting network aims to remove uninformative links while maintaining greater topological diversity. For instance, consider a system of N vertices that can connect randomly with a uniform probability p, where the value $p = 0$ represents a disconnected network, and $p = 1$ represents a complete network.

An interesting aspect of this analysis is the phenomenon of bond percolation. In this system of random connections, there is a phase transition at $p = 1/N$, below which the network consists of small isolated groups, and above which a large connected component appears. The subcritical phase carries little information about the relationships between vertices, whereas supercritical values capture more connections, on average. However, connection probability values close to one imply a low diversity of information among different groups of vertices because the network tends to form a single giant cluster. Schieber et al [40]. demonstrated that this system has a maximum NND at twice the critical percolation point, i.e., $p = 2/N$. This implies that the network has the highest heterogeneity of topological connections in the supercritical phase, at a probability twice that of the percolation threshold.

To enhance our comprehension of the methodology and its transformation of discrete variables $Y = \{y_i\}$ into networks containing topological insights about the examined system, we explored specific scenarios. In order to simplify the analysis while maintaining generality, we considered 100 measurements of a variable $Y =$

$\{y_1, y_2, \ldots, y_{100}\}$. In the first scenario, we selected $y_i = i$, resulting in a linear progression of variable values based on measurement identifiers. In the second scenario, we adopted logarithmic growth with $y_i = log(i)$, leading to closer values among measurements with higher identifiers. In the third scenario, we utilized $y_i = i^2$, causing measurements with higher identifiers to be more distantly separated. For this variable, we constructed a comprehensive network wherein each vertex i signifies a measurement y_i, and the links (with associated weights) connecting these vertices are determined by the absolute difference between their values. Consequently, the link weight increases as the disparity between two values of the variable Y grows.

Supplementary Figure 1 illustrates the functional relationships used to derive the Y variable values and showcases the resulting networks achieved through NND maximization. In the case of linear growth in Y, the resulting network exhibits greater density compared to other scenarios, leading to the emergence of only two distinct groups using the Louvain method. In contrast, a logarithmic increase in Y values results in a network primarily characterized by a substantial cluster composed of nodes with higher Y values. Conversely, a square power increase prompts nodes with higher values to form isolated clusters, driven by the increasing dissimilarity between these nodes and others within the network as Y values increase.

Comparison to other classifiers

Here, we compare our methodology with two well-established approaches in the scientific literature: Random Forest (RF) and Logistic Regression (LR). Both methodologies require complete datasets of patients in both the training and test sets. To fulfill these data processing needs, we employed median imputation for continuous variables and mode imputation for categorical variables. We train our model in a balanced dataset via synthetic balanced samples are generated according to ROSE [32].

The RF method correctly predicted 46 INH- but only 13 INH+. The overall accuracy is 62.10%, sensitivity 42.94%, specificity 71.88%, precision 41.94% and F1-score 41.94%. The LR method, on the other hand, correctly predicted 43 INH- and 15 INH+. The overall accuracy is 60.05%, sensitivity 48.39%, specificity67.18%, precision 41.67% and F1-score 44.78%.

Figure 3 illustrates the ROC curves for the two models under consideration, as well as for HemfilNet. For the latter, a linear transformation was applied to the Γ parameter, which varied between [0, 1]. A value of zero represents the impossibility of developing an inhibitor, while a value of 1 represents almost absolute certainty of developing an inhibitor.

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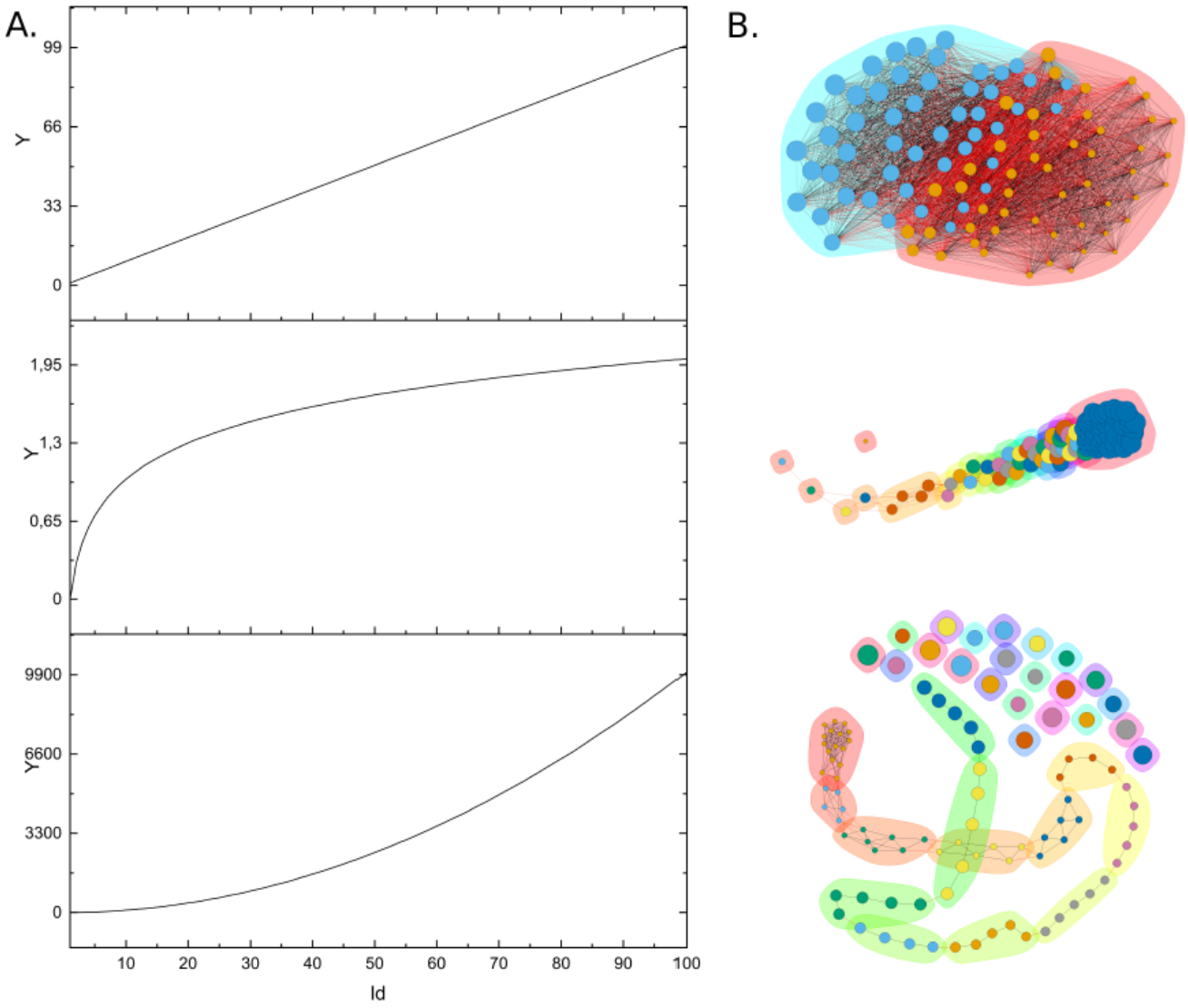
HemfilNET on the constrained dataset with immunophenotyped patients

In the main text, it is stated that model misclassification may be attributed to the absence of variable information. To address this, a sensitivity analysis of the classification performance on the constrained dataset, which includes only immunophenotyped patients was performed. In a Leave-One-Out Cross Validation experiment, the HemfilNet model demonstrated an overall accuracy of 86.36%, sensitivity of 88.89%, specificity of 84.62%, precision of 80.00% and F1-score of 84.21%.

Model for public access and testing available at: https://github.com/t-schieber/Prediction-of-inhibitor-development-HA



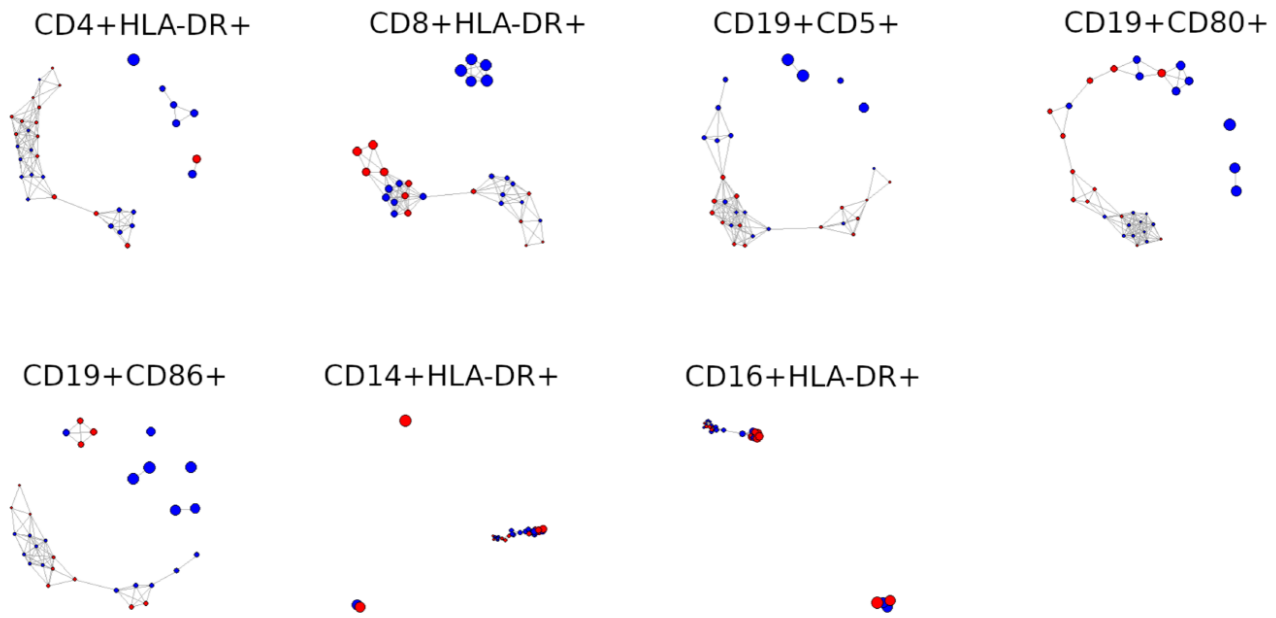
Supplementary Figure 1: (A) illustrates three artificially generated variable values, while (B) showcases the three networks generated using the maximum Network Node Dispersion method, along with the clusters identified through the Louvain method.

Supplementary Table

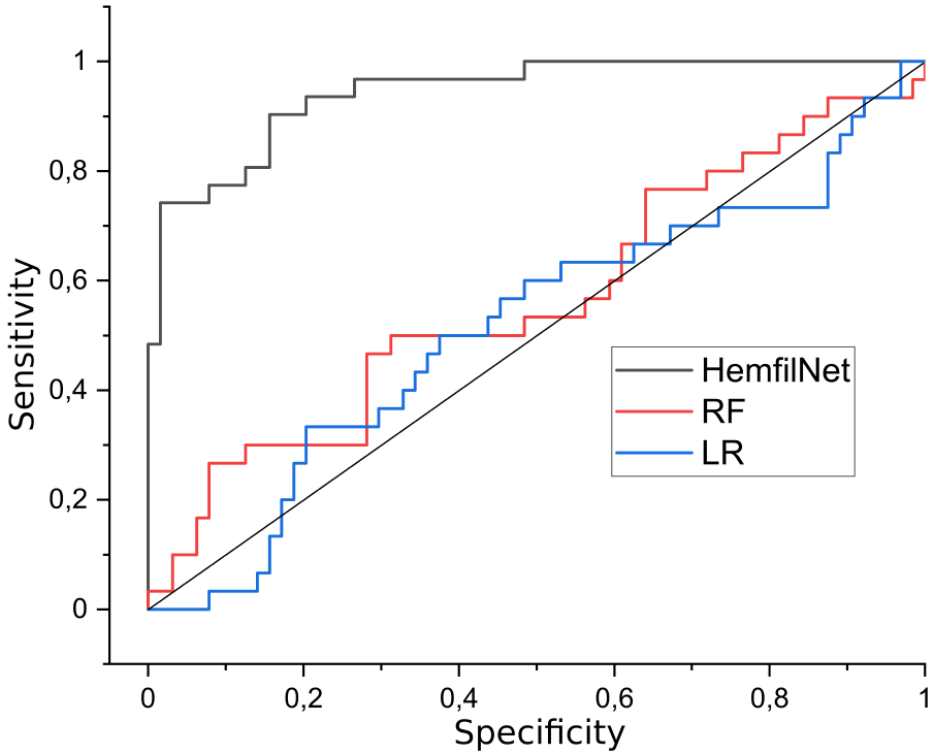
Table S1. Comparison of immunological markers in previously untreated and minimally treated children

	Previously Untreated Children (0ED)	Minimally treated children (1 to 5 ED)	p value
	n = 76	n = 19	
Age at baseline in months, median (IQR)	10 (5-14)	12 (8-23)	0,07
Weight at baseline in kg, median (IQR)	10 (9.1-11.8)	9.4 (7.7-11)	0,24
Inhibitor development, n (%)	27 (35.5)	4 (21)	0.22
High-risk mutations for inhibitor development*, n (%)	48 (63%)	12 (63%)	
Immunological phenotyping			
CD4+HLA-DR+, median (IQR)	2.8 (2.1-4.1)	2.4 (1.3-4.5)	0,22
CD8+HLA-DR+, median (IQR)	5.2 (3.1-8.6)	5.3 (2.2-6.8)	>0,99
CD19+CD5+, median (IQR)	5.3 (3.9-6.4)	4.2 (2.4-5.0)	0,09
CD19+CD80+, median (IQR)	1.3 (0.8-8.7)	3.1 (1.4-7.8)	0,85
CD19+CD86+, median (IQR)	1.6 (0.8-3.7)	3.2 (1.7-3.8)	0,14
CD14+HLA-DR+, median (IQR)	8330 (1688-15258)	13358 (4530-20556)	0,31
CD16+HLA-DR+, median (IQR)	4740 (1803-15926)	17873 (5509-23952)	0,92
Specific anti-FVIII antibodies			
IgM, median (IQR)	0.08 (0.4-0.1)	0.1 (0.07-0.2)	0,11
IgG1, median (IQR)	0.15 (0.1-0.2)	0.14 (0.1-0.2)	0,81
IgG3, median (IQR)	0.02 (0.01-0.06)	0.03 (0.01-0.04)	0,09
IgG4, median (IQR)	0.02 (0.01-0.08)	0.01 (0.01-0.05)	0,08
Cytokines			
IL2, median (IQR)	1.0 (0.01-2.4)	0.4 (0.01-2.2)	0,56
IL4, median (IQR)	11.1 (0.01-23)	14 (0.01-25)	0,82
IL6, median (IQR)	44 (0.7-128)	36 (1.0-130)	0,97
IL10, median (IQR)	6.6 (0.01-37)	3.5 (0.01-10.6)	0,24
TNF, median (IQR)	14.4 (1.4-178)	3 (0.8-109)	0,07
IFN-gamma, median (IQR)	24 (0.01-77)	29 (0.01-62)	0,06
IL17, median (IQR)	68 (1-181)	57 (0.01-93)	0,26
IP10, median (IQR)	2423 (716-3717)	1275 (636-2787)	0,26
Chemokines			
MCP-1, median (IQR)	192 (22-634)	31 (13-388)	0,59
MIG, median (IQR)	4921 (246-9431)	3411 (180-5007)	0,35
RANTES, median (IQR)	3454 (2640-8527)	3519 (2950-4433)	0,11
CXCL8, median (IQR)	158 (5.8-767)	165 (5.0-734)	0,16
Phenotypic characterization of microparticles			
Endothelial cells MPs (CD51/61), median (IQR)	4.1 (3.3-8.9)	4.0 (3.1-5.5)	0,73
Erythrocytes (CD235a), median (IQR)	13.8 (8.9-16.6)	10.7 (7.6-26.2)	0,66
Platelets MPs (CD41a), median (IQR)	6.2 (3.4-10.67)	5.3 (5.0-21.5)	0,19
Leukocytes MPs (CD45), median (IQR)	6.4 (4.2-8.4)	11.9 (4.9-13)	0,11
Neutrophils MPs (CD66), median (IQR)	3.6 (2.6-6.0)	5.7 (2.5-9.7)	0,36
Monocytes MPs (CD14), median (IQR)	4.4 (1.8-4.7)	8 (4.3-14)	0,09
T lymphocytes MPs (CD3), median (IQR)	5.4 (2.8-8.7)	6.6 (4-9.2)	0,42

ED, Exposure days; n, number of patients; IQR, interquartile range; MPs, microparticles; IFN, interferon; IL, interleukin; TNF, tumor necrosis factor; Ig, Immunoglobulin. *According to the type of F8 variant identified. Large insertion/deletion, nonsense mutations mutation, Introns 1 and 22 inversions were considered as high-risk mutation



Supplementary Figure 2 [The HemfilNet]: Networks obtained via the maximization of the Network Node Dispersion method for the continuous variables in the study. Red nodes represent children with hemophilia A (CHA) who developed inhibitors, and blue nodes represent CHA who did not develop inhibitors. MPs, microparticles; IFN, interferon; IL, interleukin; TNF, tumor necrosis factor; Ig, Immunoglobulin.



Supplementary Figure 3: ROC (Receiver Operating Characteristic) curves comparing the performance of three employed classification methods show the following AUC values: HemfilNet (AUC – 0.9435), Random Forest (AUC – 0.5651), and Logistic Regression (AUC – 0.5141).

CHAPTER 9

Inhibitor history and health status in patients with haemophilia A in the Netherlands

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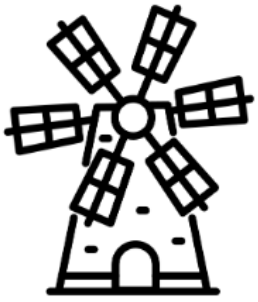

ABSTRACT

Background: Factor VIII inhibitors in persons with haemophilia A (PWH) can lead to ineffective factor VIII treatment replacement. Inhibitors have been reported to be associated with excessive bleeding, joint damage, and reduced health outcomes.

Aim: To describe the consequences of current and past inhibitors on joint health and activity of adult and paediatric persons with severe and non-severe haemophilia A in the Netherlands.

Methods: This cross-sectional study analysed data from the sixth Haemophilia in the Netherlands (HiN6), conducted during 2018–2019. We evaluated questionnaires and the medical records of Dutch males with haemophilia. Primary outcomes were joint health status, activity level assessed by the Haemophilia Activities List (pedHAL/HAL), self-reported pain level and Health status. Outcomes were compared between persons with and without an inhibitor history.

Results: A total of 494 PWH were included with a median age of 50 years [Interquartile range (IQR)30-61)]. A history of inhibitor development was reported by 73 (14.7%) participants, 33 severe PWH and 40 non-severe HA, of whom 24 (4.9%) repored current inhibitors. The annual joint bleeding rate, chronic joint impairment, pain intensity and the frequency of hospitalization for orthopaedic surgery did not differ between persons with and without an inhibitor history regardless of severity. Limitations in lifting, climbing stairs, kneeling, walking and self-care did not differ in persons < 61 years with and without an inhibitor history, even not when stratified by inhibitor titre. In contrast, in older PWH >61 years, the HAL Scores were worse in persons with an inhibitor history than in those without ($p<0.05$).

Conclusion: A history of inhibitors was not clearly associated with worse joint health or activity/participation level in persons with haemophilia in The Netherlands. However, improvement in care is still needed especially for older persons with haemophilia and an inhibitor history.

Keywords: Haemophilia, Inhibitor, Joint, quality of life.

INTRODUCTION

The main treatment-related complication in haemophilia is the development of neutralizing alloantibodies (inhibitors), which occurs in 20%-35% of persons with haemophilia A (PWH), within the first 75 exposure days (ED) to Factor VIII (Ehrenforth et al, 1992; Jardim et al, 2021). In persons with non-severe HA, the risk for inhibitor development also persists after 75 ED with a reported cumulative incidence of 13% at 100 ED (van Velzen et al, 2017). Persons with inhibitors present haemorrhages that are difficult to control due to a lack of response to FVIII replacement of the deficient factor. Bypassing agents such as activated prothrombin complex concentrates or recombinant activated Factor VII are used to treat bleeding episodes (Oldenburg et al, 2018; Srivastava et al, 2020). Yet, these are less effective than Factor VIII replacement. Currently, immune tolerance induction (ITI) is the unique treatment available for the eradication of persistent anti-FVIII inhibitors. Inhibitor eradication is achieved in 60%-80% of cases (Ter Avest et al, 2010).

Until recently, bypassing agents were the only option for prophylactic therapy to prevent bleeds and joint damage. With the emergence of new, non-factor replacement therapies, such as Emicizumab the therapeutic context for prophylactic therapy in PWH with inhibitors has changed significantly (Carcao et al, 2019). Emicizumab prophylaxis is highly effective in preventing bleeds in PWH with and without inhibitors (Oldenburg et al, 2017; Mahlangu et al, 2018). In this context, we expect that joint health outcomes and health-related quality of life will improve dramatically within the next years for PWH with inhibitors. However, emicizumab prophylaxis is costly and may not be accessible to persons living in less-resourced countries (Carcao et al, 2019). Knowledge on the disease burden in PWH with an inhibitor history treated in the pre-emicizumab era can provide the basis for future follow-up studies in PWH treated with novel treatment options. Therefore, this study aimed to assess the consequences of inhibitors on joint health and health related quality of life of PWH in the Netherlands in the period before the widespread use of emicizumab.

METHODS

Study design and study population

The Haemophilia in the Netherlands 6 (HIN-6) Study is a cross-sectional study among PWH conducted in the Netherlands from 01 June 2018 to 01 July 2019 (Hassan et al, 2021).

We evaluated data from male adult and pediatric persons with severe (FVIII/FIX <0.01 IU/mL), moderate (FVIII/FIX 0.01–0.05 IU/mL), and mild (FVIII/FIX >0.05–0.40 IU/mL) HA who participated in the HIN-6 study and had complete data about inhibitor development. Non-severe haemophilia was defined as persons with moderate or mild haemophilia. The study was approved by the Institutional Review Board of the Leiden University Medical Centre (NL59114.058.17) (Hassan et al, 2021).

Data acquisition

Data were obtained from the HIN-6 Study questionnaires and medical records. The questionnaires contained question on self-reported treatment characteristics, joint bleeding rate, level of joint impairment, orthopaedic interventions, hospital admissions, extent of chronic joint pain, and pain intensity. In addition, data from the Haemophilia Activities List (HAL), the Paediatric Haemophilia Activities List (PedHAL) and self-reported pain level and Health status accessed by the RAND-36 were also assessed.

Inhibitor

In the questionnaire, persons were asked whether they ever had an inhibitor. To confirm the data, inhibitor history, inhibitor treatment and inhibitor characteristics were cross-checked from electronic health records from all persons who gave informed consent for data collection.

Inhibitor status was based on the Bethesda unit (BU) assay, using each center's own cut-off level, which varied from >0.6 BU to >1.0 BU. A current inhibitor was defined as being currently inhibitor positive. A past inhibitor was defined as having been inhibitor-positive in the past but currently inhibitor-negative (Hassan et al JTH 2020). High-titer inhibitor was considered if ≥5 BU/mL at any time (White et al, 2001). A current inhibitor was defined when the inhibitor was positive (above the cut-off) at the time of completion of the questionnaire. A past inhibitor was defined if the patient reported a positive inhibitor in the past but currently was inhibitor-negative.

Outcomes

The outcomes of interest were joint health status, activity level assessed by the (pediatric) Haemophilia

Activities List (pedHAL/HAL), self-reported pain level, and Health status accessed by the RAND-36.

Joint health

Joint health status was self-reported and assessed by the presence of bleeding in the last 12 months, the annualized joint bleeding rate, level of joint impairment, and orthopedic interventions, pain. Annualized joint bleeding rate (AJBR) was defined as the number of self-reported bleeds in joint (knee, elbow, ankle and wrist) bleeding rate, defined as the total of reported joint bleeds in the preceding 12 months The annual joint bleeding rate was defined as the number of self-reported joint bleeds (knee, elbow, ankle and wrist) for which they received at least two days of factor VIII or DDAVP in the preceding 12 months (Hassan et al, 2021).

Activity level

The questionnaire evaluating activity level includes the Haemophilia Activities List version 2.0 (HAL for adults/PedHAL for children aged 4 to 18 years) which is a self-perceived functional ability of persons with

haemophilia (van Genderen et al, 2006). The HAL (49 questions) and PedHAL (53 questions) assess limitations in activities in the previous month across 7 domains: lying down/sitting/kneeling/standing, functions of the legs, functions of the arms, use of transportation, self-care, household tasks, leisure activities/sports. Items were classified as impossible, always, usually, sometimes, rarely, never, or not applicable. Domain scores were normalized to a 100-point scale, where higher scores represent a better functional status. Domain scores were only calculated if a minimum of 50% of items of a domain were scored.

Pain

The presence of pain was self-reported assessed by. The persons answered questions about the presence of pain and/or chronic pain, intensity, the location of the chronic pain, activities associated with the pain and interference of pain in the normal daily activities. The time frames related to pain occurring in the previous four weeks and/or in the last 12 months.

Health status-related quality of life

In adults, the health status was evaluated by the RAND 36-Item Health Survey version 1.0 (RAND-36) (VanderZee et al, 1996; Solovieva et al, 2004) which contains 36 items that measure perceived health status. Thus, eight domains are evaluated: physical functioning, social functioning, role limitations due to physical problems, limitations due to emotional problems, mental health, vitality, bodily pain and general health perception. Domain scores were converted to a 100-point scale. A higher score indicates a better health status.

Data analysis

The number of events and respective percentages were calculated for the categorical variables and the median with interquartile range (IQR) for the continuous variables. The joint scores, (Ped)HAL scores, as well as prevalence of reported joint impairment, were stratified by age (less than 21 years old; 21-40; 41-60; more than 61 years), severity of haemophilia and presence of current and/or past inhibitor.

Median differences between joint scores, (Ped)HAL scores, and RAND 36 domain scores were compared between groups of persons with and without inhibitors, stratified by age, and severity of haemophilia.

Statistical analyses were performed using RStudio software version 1.3.1073 (company, Boston, United States).

Results

In total, 494 PWH were included, with median age was 50 years (IQR, 30-61), of whom 206 (41.7%) had severe HA, 72 (14.4%) moderate HA and 216 (43.7%) mild HA (Table 1).

Table 1. Characteristics of included persons

	All n = 494
Age, median (IQR)	50 (30, 61)
Age group	
< 21 years old, n (%)	17 (3.4)
21-40 years old, n (%)	131 (26.6)
41-60 years old, n (%)	188 (38)
>61 years old, n (%)	158 (32)
Height, median (IQR)	182 (177, 187)
Weight, median (IQR)	83 (74, 92)
Type of hemophilia	
Severe, n (%)	206 (41.7)
Moderate, n (%)	72 (14.6)
Mild, n (%)	216 (43.7)
Inhibitor	
Total, n (%)	73 (14.7)
Past, n (%)	49 (9.9)
Current, n (%)	24 (4.9)
High titer, n (%)	24/73 (32.9)

n, number of persons; IQR, Interquartile range.

Overall, 73 persons with a positive inhibitor history, 33/73 (45%) with severe HA and 40/73 (55%) non-severe. Of the 73 PWH with a positive inhibitor history, 24 (33%) had a current inhibitor and 49 (67%) had an inhibitor in the past. A total of 55/73 (75%) underwent immune tolerance induction (ITI). Of persons with past inhibitors, 24/73 (33%) had high titer inhibitors and of the 24 PWH with current inhibitors 7 (29%) had high titer inhibitors. Considering only persons with severe HA, 6/33 (18.2%) had current and 27/33 (81.8%) had past inhibitors. A total of 18/40 (45%) of persons with non-severe HA had current inhibitors and 22 (55%) past inhibitors.

Joint bleeding and impairment

Overall, 74 (35.9%) PWH with severe HA reported at least one bleeding event in the past 12 months, for which they received at least two days of factor VIII or DDAVP (Table 2). PWH with severe haemophilia with current inhibitors presented a higher annual joint bleed rate of 10 (IQR, 6-15) in comparison with PWH with past inhibitors 4.5 (IQR, 2-10) and without inhibitor history 3 (IQR, 2-5).

Table 2. Characteristics of included persons stratified by severity of haemophilia.

	Severe HA			Moderate HA			Mild HA		
	n = 173	n = 6	n = 27	n = 61	n = 3	n = 8	n = 187	n = 15	n = 14
	Without inhibitor	Current inhibitor	Past inhibitor	Without inhibitor	Current inhibitor	Past inhibitor	Without inhibitor	Current inhibitor	Past inhibitor
Age, median (IQR)	49 (31, 61)	48 (32, 60)	47 (33, 56)	48 (31, 61)	70 (66, 74)	57 (33, 66)	56 (39, 66)	50 (42, 62)	58 (50, 70)
Prophylaxis, n (%)									
Now	150 (86)	4 (67)	25 (100)	11 (18)	1 (33)	0 (0)	6 (2.7)	1 (6.7)	1 (7.1)
Treated bleeding in the past 12 months treated for at least two consecutive days, n (%)									
No bleeding	36 (20)	1 (17)	6 (22)	19 (31)	0 (0)	6 (75)	142 (62)	2 (13)	9 (64)
Joint	102 (58)	3 (50)	18 (67)	23 (38)	0 (0)	1 (13)	18 (7.8)	4 (27)	2 (14)
Mucous membrane	6 (3.4)	0 (0)	1 (3.7)	6 (9.8)	1 (33)	0 (0)	17 (7.4)	1 (6.7)	0 (0)
Muscle	51 (29)	2 (33)	6 (22)	20 (33)	0 (0)	1 (13)	29 (13)	2 (13)	2 (14)
Bleeding from a wound	20 (11)	1 (17)	3 (11)	12 (20)	1 (33)	0 (0)	27 (12)	7 (47)	2 (14)
Other	16 (9.1)	1 (17)	2 (7.4)	5 (8.2)	1 (33)	0 (0)	17 (7.4)	1 (6.7)	1 (7.1)
I don't know	6 (3.4)	1 (17)	0 (0)	0 (0)	0 (0)	0 (0)	6 (2.6)	1 (6.7)	0 (0)
Annual joint bleed rate, treated for at least two consecutive days, n (%)									
	3 (2, 5)	10 (6, 15)	4.5 (2, 10)	2 (1, 3)	0	0	2 (1, 4)	0	0
Have you ever had joint bleeding?									
yes, n (%)	66 (38.2)	2 (33.3)	6 (67)	30 (79)	2 (67)	6 (75)	98 (46)	6 (60)	7 (58)
Pain in the past 4 weeks, n (%)									
None/very mild	55 (31.8)	2 (33.3)	6 (22.2)	32 (52.5)	2 (66.7)	3 (37.5)	136 (72.7)	7 (46.7)	6 (42.9)
Mild/moderate	94 (54.3)	4 (66.7)	16 (59.3)	24 (39.3)	0	5 (62.5)	60 (32.1)	5 (33.3)	5 (35.7)
Severe/very severe	13 (7.5)	0	3 (11.1)	1 (1.6)	1 (33.3)	0	6 (3.2)	1 (6.7)	1 (7.1)
Pain interference with normal activities during the past 4 weeks? , n (%)									
Not at all	66 (41)	1 (17)	7 (28)	30 (53)	2 (67)	5 (63)	130 (64)	5 (38)	7 (54)
A little/ somewhat	87 (50.3)	5 (83.3)	14 (51.9)	24 (39.3)	1 (33.3)	3 (37.5)	62 (33.2)	7 (46.7)	6
A lot/very much	9 (5.2)	0	4 (14.8)	3 (4.9)	0	0	10 (5.3)	1 (6.7)	0

n, number of persons; IQR, Interquartile range; HA, haemophilia A.

In PWH with moderate HA the median number of joint bleeds in the last 12 months was 2 (IQR, 1-3) and 2 (IQR, 1-4) in mild HA., 23/61 (38%) of non-inhibitor PWH with moderate HA reported joint bleeds. A total of 6/8 (75%) of persons with an inhibitor history had bleeding in the past 12 months.

The majority of persons with mild HA (153/216; 71%) did not report any bleeding that needed at least two days of treatment. However, 7/15 (47%) of persons with a current inhibitor in this group reported bleeding from a wound.

The frequency of reported joint impairments, pain intensity and the frequency of hospitalization for orthopaedic surgery did not substantially differ between persons with and without a history of inhibitors independent of haemophilia severity (Table 3).

Table 3. Information about joint impairment and pain

	SEVERE HA				NON SEVERE HA			
	Patients without inhibitor n = 173		Patients with inhibitor n = 33		Patients without inhibitor n = 288		Patients with inhibitor n = 40	
	n	%	n	%	n	%	n	%
Joint limitation								
Shoulder								
Light/Moderate	60	34,7	15	45,5	43	14,9	5	12,5
Severe	11	6,4	3	9,1	6	2,1	0	0
Elbow								
Light/Moderate	128	74,0	31	93,9	32	11,1	2	5
Severe	45	26,0	7	21,2	3	1,0	0	0
Wrist								
Light/Moderate	59	34,1	14	42,4	21	7,3	4	10
Severe	2	1,2	0	0,0	0	0,0	0	0
Hip								
Light/Moderate	47	27,2	5	15,2	30	10,4	4	10
Severe	12	6,9	1	3,0	1	0,3	0	0
Knee								
Light/Moderate	85	49,1	20	60,6	56	19,4	13	32,5
Severe	62	35,8	11	33,3	1	0,3	0	0
Ankle								
Light/Moderate	129	74,6	32	97,0	63	21,9	8	20
Severe	105	60,7	15	45,5	23	8,0	2	5
Other								
Light/Moderate	13	7,5	4	12,1	8	2,8	2	5
Severe	1	0,6	0	0,0	1	0,3	0	0
Do you ever have pain?								
Yes	157	90,8	30	90,9	174	60,4	29	72,5
Pain intensity, n (%)								
None/very mild	55	31,8	8	24,2	140	48,6	19	47,5
Mild/moderate	94	54,3	20	60,6	84	29,2	15	37,5
Severe/very severe	13	7,5	3	9,1	7	2,4	3	7,5
Chronic pain due to hemophilia in the past 12 months?								
Dont know	4	2,3	2	6,1	19	6,6	3	7,5
No	42	24,3	4	12,1	107	37,2	16	40
Yes	110	63,6	24	72,7	47	16,3	10	25

When do you have chronic pain?								
When walking	71	41,0	15	45,5	27	9,4	5	12,5
When climbing stairs	45	26,0	8	24,2	20	6,9	2	5
At night	42	24,3	6	18,2	11	3,8	2	5
At rest	53	30,6	8	24,2	17	5,9	2	5
When bearing weight	75	43,4	16	48,5	28	9,7	7	17,5
Other	6	3,5	3	9,1	4	1,4	0	0
Chronic pain								
Shoulder	35	20,2	10	30,3	14	4,9	4	10
Elbow	81	46,8	14	42,4	9	3,1	0	0
Wrist	10	5,8	1	3,0	4	1,4	2	5
Hip	20	11,6	3	9,1	11	3,8	1	2,5
Knee	69	39,9	16	48,5	21	7,3	5	12,5
Ankle	127	73,4	24	72,7	30	10,4	6	15
Other joint	3	1,7	2	6,1	6	2,1	2	5
Have you ever been admitted to hospital for orthopedic surgery?								
Dont know	3	1,7	0	0,0	2	0,7	2	5
No	92	53,2	12	36,4	228	79,2	29	72,5
Yes	78	45,1	20	60,6	53	18,4	8	20
In which joints have you had orthopedic surgery?								
Shoulder	4	2,3	0	0	3	1,0	2	5
Elbow	27	15,6	4	12,1	1	0,3	1	2,5
Wrist	0	0,0	1	3,0	0	0,0	0	0
Hip	20	11,6	6	18,2	10	3,5	2	5
Knee	78	45,1	22	66,7	30	10,4	4	10
Ankle	64	37,0	14	42,4	30	10,4	3	7,5
What type of orthopedic surgeries have you had?								
Joint replacement	56	32,4	14	42,4	16	5,6	3	7,5
Arthrodesis	35	20,2	8	24,2	16	5,6	1	2,5
Synovectomy	13	7,5	4	12,1	4	1,4	2	5
Radiosynovectomy	5	2.9	2	6,1	0	0,0	0	0
Other	16	9,2	2	6,1	18	6,3	3	7,5

n, number of patients; IQR, Interquartile range; HA, haemophilia A.

Considering the joint scores, we did not find differences between persons with and without inhibitors. However, severe PWH with more than 41 years presented higher scores than persons younger than 40 years ($p<0.05$). In the non-severe group, the differences were significant only in PWH without inhibitors, where older persons (>61 years old) presented higher scores than persons below this age.

Orthopedic interventions

A total of 78 (45%) of the PWH with severe HA without inhibitors had ever had orthopedic surgery, while fewer of those with an inhibitor history (20, 60.6%) had orthopedic surgery (Table 3).

The same was observed in the non-severe PWH, similar proportions of 53 (18.4%) of the persons without inhibitors and 8 (20%) with inhibitors had orthopedic surgery, being knee and ankle joints that were most frequently targets for surgery in persons with and without inhibitors.

Activity Level

In PWH younger than 60 years of age, limitations for lifting, climbing stairs, kneeling, walking and self-care did not differ by inhibitor history, neither when stratified by inhibitor titre (Figure 1). Older persons (>61 years old) with severe HA, with inhibitor history had more limitations in physical activity than those without an inhibitor history.

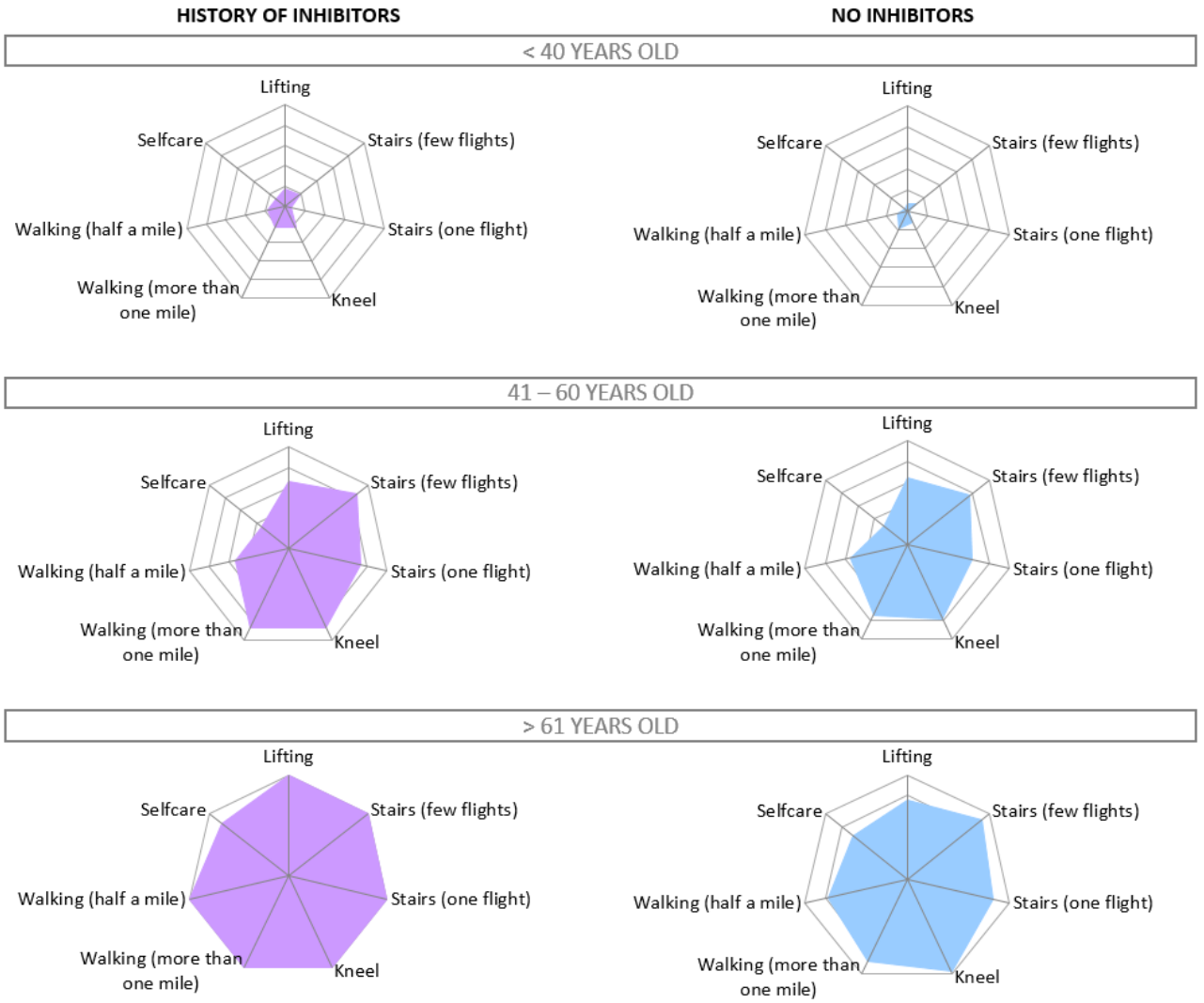

Figure 1. Proportion of PWH with severe hemophilia who report limitations in the domains of the (ped)HAL questionnaire. The chart summarizes the percentage of patients with (in purple) and without (in blue) history of inhibitor who report limitations (bit or severe) for lifting, climbing stairs, kneeling, walking and self-care. Each axis displays the proportion by age group from 0 to 100%. Only severe haemophilia A patients were included in this figure.

Overall, HAL scores for PWH with non-severe HA were higher in comparison with severe HA, but the score did not differ between PWH with and without inhibitors, regardless of HA severity (p>0.05) (Table 4). Severe PWH with 40 years or older presented lower HAL scores (Table 4; Figure 1) and the scores were even lower

above 61 years old. In PWH with non-severe HA older than 61 years, those with inhibitors (currently or past) presented lower scores for all domains in comparison with younger persons, except for self-care.

The joint scores were similar between persons with and without inhibitor history, across haemophilia severity and ages groups (Table 4). However, severe PWH with more than 41 years presented higher scores than persons younger than 40 years ($p<0.05$). In the non-severe group, the differences were significant only in PWH without inhibitors, where older persons (>61 years old) presented higher scores than persons below this age (Table 4).

Table 4. Haemophilia Activities List (HAL) Scores of patients with and without a history of inhibitors, by severity and age group.

			Age groups			
			≤ 20	**21 to 40**	**41 to 60**	**>= 60**
		HAL Score, median (IQR)	**n = 9**	**n = 38**	**n = 66**	**n = 40**
a)	**Severe HA without inhibitor (n = 173)**	Lying / sitting / kneeling / standing	100 (95, 100)	93 (75, 100)	53 (35, 68)	30 (20, 43)
		Functions of the legs	100 (99, 100)	87 (62, 100)	38 (24, 56)	20 (9, 40)
		Functions of the arms	100 (100, 100)	100 (85, 100)	75 (45, 90)	45 (25, 76)
		Use of transportation	100 (100, 100)	100 (93, 100)	80 (53, 95)	33 (20, 60)
		Self care	100 (100, 100)	100 (100, 100)	90 (68, 100)	60 (40, 93)
		Household tasks	100 (100, 100)	100 (97, 100)	76 (53, 93)	53 (23, 76)
		Leisure activities and sports	100 (100, 100)	94 (79, 100)	60 (40, 80)	40 (22, 57)

b)		**HAL Score, median (IQR)**	**n = 0**	**n = 12**	**n = 15**	**n = 6**
	Severe HA with inhibitor (n = 33)	Lying / sitting / kneeling / standing	NA	95 (68, 100)	43 (33, 50)	21 (18, 32)
		Functions of the legs	NA	78 (67, 93)	36 (27, 46)	3 (0, 15)
		Functions of the arms	NA	100 (88, 100)	60 (53, 78)	32 (30, 42)
		Use of transportation	NA	100 (95, 100)	60 (40, 77)	23 (15, 27)
		Self care	NA	100 (97, 100)	80 (72, 100)	54 (48, 75)
		Household tasks	NA	100 (88, 100)	67 (58, 77)	15 (4, 48)
		Leisure activities and sports	NA	80 (71, 97)	58 (44, 69)	39 (33, 42)

c)		**HAL Score, median (IQR)**	**n = 8**	**n = 72**	**n = 92**	**n = 96**
	Non-Severe HA without inhibitor (n = 288)	Lying / sitting / kneeling / standing	100 (96, 100)	100 (95, 100)	100 (88, 100)	93 (70, 100)
		Functions of the legs	100 (100, 100)	100 (100, 100)	100 (86, 100)	96 (64, 100)
		Functions of the arms	100 (100, 100)	100 (100, 100)	100 (99, 100)	100 (80, 100)
		Use of transportation	100 (100, 100)	100 (100, 100)	100 (100, 100)	100 (93, 100)
		Self care	100 (100, 100)	100 (100, 100)	100 (100, 100)	100 (96, 100)
		Household tasks	100 (100, 100)	100 (100, 100)	100 (100, 100)	100 (93, 100)
		Leisure activities and sports	100 (99, 100)	100 (97, 100)	100 (91, 100)	100 (81, 100)

d)

	HAL Score, median (IQR)	n = 0	n = 9	n = 15	n = 16
Non-Severe HA with inhibitor (n = 40)	Lying / sitting / kneeling / standing	NA	100 (99, 100)	100 (86, 100)	81 (36, 91)
	Functions of the legs	NA	100 (100, 100)	100 (89, 100)	73 (37, 92)
	Functions of the arms	NA	100 (100, 100)	100 (95, 100)	90 (60, 100)
	Use of transportation	NA	100 (100, 100)	100 (100, 100)	88 (67, 100)
	Self care	NA	100 (100, 100)	100 (100, 100)	100 (94, 100)
	Household tasks	NA	100 (100, 100)	100 (100, 100)	93 (80, 100)
	Leisure activities and sports	NA	100 (97, 100)	84 (73, 96)	95 (85, 100)

Legend	HAL Score
High	> 89
Low	< 89 and > 51
Very low	<50

n, number of patients; IQR, Interquartile range; HA, haemophilia A; HAL, Haemophilia Activities List.

Pain

Overall, 111/494 (22.4%) PWH reported serious or very serious pain. Indeed, a smaller proportion (27/494; 5.4%) of the participants stated that pain interfered a lot or very much with their normal activities during the past month (Table 3).

A total of 390/494 (78.9%) of PWH reported that ever had pain (Table 3). Considering only the severe PWH, 134/206 (65%) reported chronic pain due to haemophilia in the previous 12 months, while in the group with non-severe HA, only 57/288 (18%) experienced the same condition.

Focus on the comparison between those with and without inhibitor history.

Table 5. RAND 36 scores of persons with and without a history of inhibitors, by severity and age group.

	Severe HA			Non-Severe HA		
Score, median (IQR)	Persons without inhibitor	Persons with current inhibitor	Persons with past inhibitor	Persons without inhibitor	Persons with current inhibitor	Persons with past inhibitor
	n = 173	n = 6	n = 27	n = 288	n =18	n = 22
Physical functioning	65 (40, 90)	55 (36, 55)	55 (33, 85)	95 (85, 100)	95 (47, 100)	87 (65, 100)
Role functioning/emotional	76 (63, 80)	76 (63, 80)	80 (64, 84)	80 (72, 88)	84 (58, 92)	76 (68, 88)
Energy/fatigue	65 (50, 75)	65 (56, 70)	60 (45, 75)	100 (100, 100)	100 (37, 100)	100 (62, 100)
Emotional well-being	91 (50, 100)	76 (55, 82)	100 (66, 100)	100 (100, 100)	100 (33, 100)	100 (100, 100)
Social functioning	75 (48,100)	75 (56, 90)	75 (56, 100)	100 (75, 100)	81 (65, 100)	100 (56, 100)
Pain	67 (57, 90)	62 (48, 75)	67 (45, 80)	89 (67, 100)	77 (58, 100)	79 (61, 100)
General health	60 (45, 75)	38 (26, 56)	60 (45, 75)	75 (55, 85)	57 (45, 80)	65 (50, 85)
Health change	50 (50, 50)	62 (43, 75)	50 (25, 50)	50 (50, 50)	50 (43, 56)	50 (50, 50)

n, number of persons; IQR, Interquartile range; HA, haemophilia A.

Health status-related quality of life

Considering PWH, we did not observe differences between persons with an inhibitor (current or past) and those without an inhibitor considering the pain domain. Severe PWH with a current inhibitor had worse scores in the "general health" domain [38 (IQR, 26-56)] than persons without an inhibitor [60 (IQR, 45-75)] and persons who had inhibitors in the past [60 (IQR 45-75)] (Table 5). Overall, non-severe PWH without inhibitors presented better Rand 36 scores than non-severe PWH with current or past inhibitors.

Discussion

In this study we evaluated and compared health outcomes of PWH in the Netherlands, before the introduction of emicizumab prophylaxis, such as bleeding rates, joint limitations, orthopaedic interventions, joint score, chronic pain. In general, for physical activity, we found differences between persons with and without inhibitor history in the assessment of limitations for lifting, climbing stairs, kneeling, walking and self-care. However, these differences were significant ($p<0.05$) in the older age group (>61 years) in comparison with younger persons (< 60). Persons between 41 and 60 years of age had a higher frequency of general limitations than persons under 40 years of age, but limitations were similar between persons with and without inhibitors in these age groups. This may indicate that inhibitor persons have received better care since the 1960s, which may have contributed to a positive impact on their quality of life. Probably, in the Netherlands, most persons with inhibitor started ITI as soon as inhibitor was detected. Therefore, they did not suffer the consequences of increased bleeding. Unfortunately, we did not have access to time between inhibitor detection and ITI in this cohort.

Despite improvements in inhibitor treatment, which includes ITI and prophylaxis with bypassing agents, there is still a large proportion of severe PWH with inhibitors who experience pain and chronic pain when walking, climbing stairs, at night, at rest and when carrying weight. We found that there is still a significant frequency of joint bleeding and orthopaedic surgeries due to complications of HA in our study. In recent years, haemophilia treatment has improved significantly around the world. It was shown in a Dutch cohort that the average life expectancy of persons with severe haemophilia was 73 years (Hassan et al, 2021b), which is approaches the life expectancy of people without haemophilia. In our study, older persons in general and especially those with an inhibitor history reported a higher proportion of joint limitation and lower HAL scores (and lower HRQOL?). Despite improved access to treatment, persons still demand care and major efforts to prevent bleeding, especially elderly persons.

This study has some limitations. We assessed the impact of having an inhibitor history on self-reported health outcomes at any time in the past. Thus, our results could have been influenced by memory bias and misclassification This factor was minimized with the conference of the medical report of all PWH included in the HIN-6 population.

Limited data on inhibitors in the past, duration of the inhibitor presence and ITI treatment, and data on long-term prophylaxis were lacking.

In conclusion, Dutch PWH aged > 60 years, with a positive inhibitor history had worse joint health and reported more limitations in activities than those without an inhibitor history. Although joint problems continue to be frequently reported in elderly PWH, a history of inhibitors was not clearly associated with worse joint health or activity/participation level in persons with haemophilia in The Netherlands. It will be interesting to compare these data after the introduction of Emicizumab.

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Author contributions

LLJ and SMR performed the research and wrote the paper; JVDB, FRR and SCG developed the protocol, designed the research and wrote the paper; LFDVV, JE, EAMB, LH, MC, SEMS and FWGL were involved in the development of the protocol, provided data from participating haemophilia centres and reviewed the manuscript. All authors critically revised the manuscript and approved the final version.

Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

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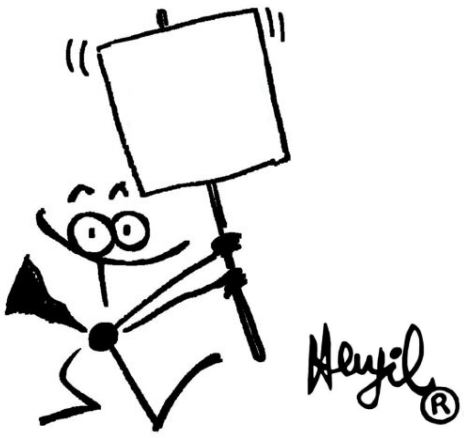
CHAPTER 10

SUMMARY AND GENERAL DISCUSSION



SUMMARY

Chapter 2

In Chapter 2, standardized mortality ratios (SMR) were calculated to estimate the rate of overall death of patients with hemophilia (A and B) relative to that of the Brazilian general male population. Data were adjusted for age and calendar period.

Between January 2000 and December 2014, there were 784 recorded deaths among patients with hemophilia in Brazil. The overall mortality of patients with hemophilia was 13% higher when compared with the general male population (SMR, 1.13; 95% CI: 1.01-1.16). However, there was a decline from 2000 to 2014, with no significant difference observed in 2014 (SMR, 0.89; 95% CI: 0.74-1.04). Hemorrhage remained the main cause of death, affecting 254 of the 784 patients (32.4%), mostly due to intracranial bleeding (137/254; 54%). The percentage of deaths due to HIV among hemophilia patients decreased from 30.8% in 2000-2002 to 11.3% in 2012-2014. The incidence of deaths attributed to cancer and cardiovascular diseases increased, rising from 7.7% to 12.1% and from 13.7% to 25.5%, respectively, during the same periods. Additionally, 129 patients died from hepatitis infection, with 109 (86.5%) also suffering from liver disease.

Chapter 3

This illustrated review focuses on the development of inhibitors in patients with congenital HA, which is the most serious treatment-related complication in these patients. Inhibitors are alloantibodies that neutralize the procoagulant activity of FVIII. Initially, patients with HA can develop a pro-inflammatory immune response with the synthesis of anti-FVIII immunoglobulin (Ig) G1, which has no FVIII inhibitory activity. However, in patients with inhibitors, the immune response shifts towards an anti-inflammatory/regulatory pattern favoring the synthesis of anti-FVIII IgG4 antibodies. Patients with inhibitors present bleeding episodes that are difficult to control and they have reduced response to FVIII replacement. Tolerance induction is currently the primary treatment for eradication of high-titer inhibitors, but the underlying immunological mechanisms remain largely unexplained. The review highlights in an illustrated and didactic way the main risk factors for the development of inhibitors and the immunological interactions involved, including the cells and receptors that play a role in the production of these antibodies.

Chapter 4

Congenital hemophilia is a hemorrhagic disease resulting from a deficiency of factor VIII (FVIII) in hemophilia A (HA) or IX (FIX) in individuals with hemophilia B (HB). Hemophilia requires frequent infusions of FVIII or FIX concentrates for prophylaxis and/or treatment of hemorrhage. However, during treatment,

approximately 30% and 5% of patients with HA and HB, respectively, develop antibodies (inhibitors) that inhibit the coagulant activity of the infused factor promoting ineffective factor replacement. The pathophysiology related to the development of inhibitors is poorly understood. Previous studies have evaluated the immune profile of patients with hemophilia, but no study followed this profile at different stages of the disease. The HEMFIL Study aimed to evaluate the clinical, genetic and immunological risk factors related to inhibitor development. This is a prospective cohort of previously untreated/minimally treated (< 5 ED) patients with hemophilia, in which patients were monitored for up to 75 ED to factor or until inhibitor development. Blood samples were collected in three moments of the study: inclusion (T0), with 75 DE or the development of inhibitor (T1) and after ITI (T2), in those cases where it is accomplished. In addition to the immunological factors, clinical and genetic risk factors are evaluated. Chapter 4 describes the methodologic aspects of the cohort study.

Chapter 5

Chapter 5 reported, for the first time, the cumulative incidence of inhibitors in a cohort of Brazilian previously untreated patients (PUPs) with HA under the exclusive use of a third-generation recombinant factor VIII concentrate (ADVATE®, Takeda, USA). These results are part of the HEMFIL prospective study. Patients were included consecutively and followed for up to 75 ED and/or upon inhibitor development.

A total of 70 PUPs with severe HA (baseline FVIII:C < 1%) were included, 61 (85%) of whom completed the follow-up. Inhibitor was detected in 24/63 patients of which 17 were high-titre. Among patients who developed inhibitors, 17 (68%) were subsequently treated with ITI. The cumulative incidence was 36% [95%CI,26%-49%] for all inhibitors, 27% (95%CI,18%-40%) for high-titer and 13% (95%CI,6-24%) for low-titre inhibitors. This was an important step in understanding the immunogenicity of a unique brand of a third-generation recombinant factor VIII concentrate.

Chapter 6

This study aimed to investigate the immunological profile of previously untreated patients (PUPs) HA at the study baseline in comparison with healthy, non-hemophiliac boys. Compared with healthy controls, children with HA presented increased Microparticles (MPs) derived from T lymphocytes, platelets, neutrophils, leukocytes, monocytes and erythrocytes; high levels of the cytokines TNF, IL-10, IL-6, IL-4, and IL-2; elevated level of the chemokine IL-8 and reduced MIG. These results suggest that, even before FVIII exposure, the immunological profile of patients with HA differs from that of healthy controls, probably stimulated by microhemorrhages or subclinical bleeding.

After the beginning of treatment with factor VIII concentrate, MPs levels decreased, which may be associated with more efficient hemostasis in HA patients treated with FVIII. Regarding cytokines and chemokines, we observed an increase in the levels not only of IL-4, IL-6, IL-8 and IL-10 but also of TNF, IFN and IL-2, characterizing a pro-inflammatory profile.

Thus, in previously untreated patients with hemophilia (T0 phase of the study), the occurrence of bleeding, even if subclinical, could generate an inflammatory response, mediated by cytokines and chemokines, in addition to activating coagulation through the production of MPs. The production of MPs, in turn, can activate the synthesis of cytokines and IL-8, generating the pro-inflammatory profile identified in these patients. To our knowledge, this is the first study that evaluated the immunological profile in untreated patients with hemophilia A in comparison with normal controls. The study provides valuable insights into the immunological landscape of HA patients before FVIII replacement. An understanding of the immune status of HA patients before FVIII exposure could support the prediction of inhibitor development. Thus, this knowledge may guide personalized treatment strategies.

Chapter 7

The article resulting from this study reports the impact of initial FVIII infusions on immunological biomarkers in patients with HA from The Hemfil Study, who had not been minimally treated. Key findings include changes in immune responses following the first FVIII administration, with variations in specific biomarkers that may be associated with the development of inhibitors. The study suggests that monitoring these immunological changes could help predict the risk of inhibitor formation in newly treated patients. Overall, the results emphasize the need for careful observation of immune responses during early treatment stages.

Chapter 8

In this manuscript, we present a prediction model to compare the network and potential global topological differences of the patients' profiles at inclusion time at the Hemfil Study (T0) and their association with inhibitor development. The method transforms related variables into structural patterns via complex networks by maximizing the Network Node Dispersion. FVIII genotype was classified as high-risk (inversion, nonsense, frameshift mutations, large deletions and insertions) or low-risk mutations (the remaining).

A total of 95 children from the HEMFIL study who have completed the follow-up were included. Inhibitor was detected in 31 (33%) of whom 22(71%) were high-titer (>5UB). Our machine-learning algorithm demonstrated an overall accuracy of 90.5% for predicting inhibitor development in children with HA, which further improved when restricting the analysis to children with a high-risk F8 genotype.

Chapter 9

This cross-sectional study analyzed data from the sixth Hemophilia in the Netherlands (HiN6), conducted during 2018–2019. We evaluated the questionnaires including the Hemophilia Activities List (HAL) and the medical records of Dutch males with HA. Patients were stratified by age, HA severity, history of inhibitor and inhibitor titer. A total of 685 HA patients were included. The frequency of reported chronic joint problems due to hemophilia, pain intensity and the frequency of hospitalization for orthopedic surgery did not differ between patients with and without a history of inhibitors independent of the severity. Limitations for lifting, climbing stairs, kneeling, walking and self-care increased with age, but did not differ with a history of inhibitors in patients <60 years, even when stratified by titer. Yet, the differences between those with/without inhibitors were significant ($p<0.05$) in severe patients >60 years. Overall, HAL scores for non-severe HA patients were higher in comparison with severe HA, but the score did not differ by the history of past inhibitors, independent of the severity. In conclusion, a history of inhibitor was not associated with worse joint health or activity/participation level in patients with severe HA. Older patients reported several limitations, independent of inhibitor status. Although joint problems continue to be frequently reported in elderly people with HA, we did not observe differences between persons with or without inhibitors. It will be interesting to compare this data after the introduction of emicizumab.

DISCUSSION

1. Mortality

In chapter 2, we evaluated the mortality in patients with hemophilia in Brazil. In Brazil, since 2009, descriptive data on inherited bleeding disorders, including hemophilia prevalence, have been published annually by the Ministry of Health through the "Profile of Hereditary Coagulopathies in Brazil". However, data before 2009 have not been completely described. Our strategy was to calculate the mean prevalence of hemophilia between 2009 and 2014 and apply the result to the corresponding age of the general male population from 2000 to 2015. From 2000 to 2014, the overall mortality related to hemophilia A and B was 13% higher than that which was observed in the Brazilian male population. The study demonstrated, for the first time, that mortality among patients with hemophilia decreased over the years in Brazil and that, with the increase in life expectancy of these patients, we began to observe the development of diseases commonly related to aging, such as cardiovascular diseases and cancer, following the same trend reported in articles from developed countries. However, intracranial hemorrhage still presented itself as an important cause of death in patients with hemophilia A and B.

The course of mortality in hemophilia worldwide has been strongly influenced by the evolution of available treatments. With the emergence of plasma-derived products in the 1960s, there was a reduction in severe bleeding episodes in patients and a consequent increase in life expectancy, which until then was less than 30 years (Larsson SA, 1985). However, from 1980 onwards, infectious diseases transmitted by these products, which had not yet undergone the viral inactivation process, began to affect patients (Walker and Julian, 1998). The human immunodeficiency virus (HIV) became the leading cause of death among patients with hemophilia by the end of the 1990s (Chorba et al, 2001; Reitter et al, 2009) and infection by hepatitis C virus (HCV) affected more than half of the patients treated with plasma-derived factor concentrates before 1992 (Plug et al, 2006). In Brazil, 6.5% and 34.9% of the population of patients with hemophilia between 2005 and 2007 tested positive for HIV and HCV, respectively (Rezende et al, 2009).

With the improvement of techniques for detecting infectious agents, rigorous selection of blood donors and the advent of viral inactivation techniques in the late 1980s, deaths resulting from HIV infection were gradually reduced until they were no longer directly associated with the disease (Arnold et al, 2006; Reitter et al, 2009; Schramm et al, 2013). Nowadays, HCV infection in patients with hemophilia is a consequence of the contamination that occurred in the past. Although the advent of new antiviral drugs such as Sofosbuvir, Simeprevir and Daclatasvir has eradicated approximately 95% of cases of HCV infection in the last five years, liver diseases related to this infection remain an important cause of morbidity and mortality in patients with haemophilia to this day (Darby et al, 2007; Donald et al, 2006; Tuinenburg et al, 2009). Furthermore, patients who are co-infected with HIV and HCV have a worse prognosis, with a faster progression to hepatic complications (Donald et al, 2006). It is important to note that since 1995, there have been no reports of HIV infection or hepatitis resulting from the use of plasma-derived factor concentrates, which supports the high transfusion safety of these products (Reitter et al, 2009; Schramm et al, 2013). In Brazil acting antiviral drugs have been available since the 1990s.

Despite the great advances in the treatment of hemophilia in the last 50 years, the mortality of patients with hemophilia in developed countries is still higher than that of the general male population (Koumbarelis, 1994). In the Netherlands, standardized mortality ratios decreased over the years, but are still higher than that of the general male population, i.e., 1.4 (95%CI 1.2-1.7), considering all-cause mortality (Plug et al, 2006; Hassan et al, 2020). The analysis of individuals not infected with HIV and HCV shows that, if we exclude the effects related to infectious diseases, complications resulting from bleeding are still the main cause of death and reduced life expectancy of patients with hemophilia (Darby et al, 2007; Tuinenburg et al, 2008; Reitter et al, 2009). Furthermore, the mortality rate in individuals with the severe form of the disease was reported to be 5.1 times higher when compared to patients with moderate or mild hemophilia in the Netherlands (Plug et al, 2006) and approximately twice as high in the United Kingdom (1.81; 95% CI 1.54 - 2.16) (Darby et al, 2007). Hassan et al (2021) showed that in comparison with the general Dutch male

population, the mortality of patients with hemophilia still increased between 2001 and 2018 (SMR 1.4, 95% CI 1.2–1.7) and Intracranial bleeding is still an important related cause of death. Life expectancy for hemophilia patients has improved, approaching the average for the general population, especially among those who receive adequate treatment (Hassan et al, 2021).

A systematic review and meta-analysis of mortality and causes of death in people with hemophilia highlight those improvements in hemophilia treatments, such as the use of recombinant clotting factors and antiviral therapies. These have contributed to the reduction in mortality rates, particularly related to bleeding complications and HCV infections (Alam et al, 2021). However, mortality rates and causes of death showed significant regional variations, reflecting differences in access to and quality of health care (Alam et al, 2021).

In general, although life expectancy has increased, mortality is still mainly influenced by bleeding complications and other conditions associated with hemophilia. The meta-analysis conducted by Zwagemaker et al. (2021) focused on the incidence and mortality rates related to intracranial hemorrhages in patients with hemophilia. Despite treatment advances, intracranial hemorrhages remain a significant cause of mortality in these patients. Therefore, continued monitoring and the implementation of effective therapeutic approaches remain necessary to mitigate these risks (Zwagemaker et al., 2021). Further investigation into the factors contributing to mortality in individuals with hemophilia is essential to enhance understanding, inform targeted interventions, and ultimately improve both patient care and health-related quality of life.

Furthermore, the development of inhibitors, the most significant complication of hemophilia, is associated with increased morbidity, including hemophilic arthropathy and intracranial hemorrhage, both of which can also contribute to higher mortality rates (Lim et al, 2020). Therefore, the identification of predictive factors for inhibitor development is essential, as it could enable more targeted treatment for patients at high risk, preventing difficult-to-control bleeding and reducing the negative impact on their quality of life.

2. Inhibitor development in a Brazilian cohort

Chapters 3 to 8 describe the development of inhibitors in a prospective cohort, the HEMFIL Study. The cumulative incidence for inhibitor development was 36.0% (95%CI, 25.2-48.5), of which 25.0% (95%CI, 15.6-37.9) developed high-titer inhibitors. Our study reported that, regardless of inhibitor status, repeated infusions of FVIII can modulate the immune system of patients with HA. As such, before the first FVIII infusion, patients with HA present higher levels of MPs, CXCL8/IL-8, IL-6, TNF, IL-4, IL-10, and IL-17 in comparison with controls without hemophilia. Furthermore, the administration of FVIII-containing products seems to trigger a pro-inflammatory response mediated by IL-6 and CXCL8/IL-8 in patients with HA. However, these results only evaluated the individual contribution of each biomarker, without considering the connection between them. Thus, we proposed a predictive model for inhibitor development in HA by utilizing a network of clinical

variables and biomarkers. The model considered 37 variables, including clinical variables and biomarkers. We developed a network-based machine-learning algorithm to predict inhibitor development in children with HA from the HEMFIL Study.

Among the non-genetic predictive factors for inhibitor development, the type of factor VIII (FVIII) product used in hemophilia treatment is one of the most debated. Several studies have suggested that recombinant FVIII concentrates are associated with a higher risk of inhibitor development compared to plasma-derived products (Calvez et al, 2014; Collins et al, 2014; Peyvandi et al, 2016; Calvez et al, 2018). However, the RODIN study, an international cohort involving 576 previously untreated patients (PUPs) followed until inhibitor development or up to 75 exposure days without inhibitor occurrence, did not confirm this association (Gouw et al, 2013). The study reported no significant difference in inhibitor incidence between patients treated with plasma-derived or recombinant FVIII (Gouw et al, 2013). Nevertheless, when analyzing different recombinant concentrates, a specific second-generation FVIII product (Kogenate®FS, manufactured by Bayer HealthCare, Barmen, Germany) was associated with a 60% increased risk of inhibitor development compared to other recombinant products (Hazard ratio [HR]: 1.60; 95% CI, 1.08–2.37) (Gouw et al, 2013). Two subsequent retrospective cohort studies involving 395 French and 407 English patients reported a 75% (HR: 1.75; 95% CI, 1.11–2.76) (Calvez et al, 2014) and 55% (HR: 1.55; 95% CI, 0.97–2.49) (Collins et al, 2014) increased risk of inhibitor development, respectively, among patients treated with the same second-generation recombinant FVIII concentrate, Kogenate®FS (Bayer HealthCare, Barmen, Germany), compared to those receiving plasma-derived products. More recently, a French cohort study evaluated the incidence of inhibitors in PUPs with severe HA who received either plasma-derived FVIII concentrate (Factane®; LFB, Paris, France; n = 131) or two different recombinant FVIII products: Kogenate®FS (n = 127) and ADVATE® (Shire, Lexington; n = 137) (Calvez et al, 2018). The cumulative incidence of high-titer inhibitors was 12.7% (95% CI, 7.7–20.6) among those treated with Factane®, 20.4% (95% CI, 14.0–29.1) with ADVATE®, and 31.6% (95% CI, 23.5–41.7) with Kogenate®FS (Calvez et al, 2018). These findings suggest that immunogenicity may vary between different FVIII concentrates. However, in the literature, estimates of product-specific immunogenicity remain imprecise due to the relatively small number of patients treated exclusively with each product.

The SIPPET study was the first randomized clinical trial to compare the risk of inhibitor development between FVIII concentrates according to their source (Peyvandi et al, 2016). After adjustment for several confounders, this study reported a cumulative incidence of 44.5% (95% CI, 34.7–54.3) versus 26.8% (95% CI, 18.3–35.2) for all inhibitors and 28.4% (95% CI, 19.6–37.2) versus 18.6% (95% CI, 11.1–26.9) for high-titer inhibitors in patients using recombinant and plasma factor concentrates, respectively. This study concluded that the risk of inhibitor development was 87% higher in patients treated with recombinant FVIII (HR 1.87; 95% CI, 1.17 - 2.96) (Peyvandi et al, 2016). The SIPPET Study also analyzed genetic alterations in 235 patients

included in the study (Rosendaal et al, 2017). Rosendaal and collaborators (2017) examined the relationship between the type of FVIII concentrate used (plasma-derived or recombinant) and inhibitor development in patients stratified by mutation risk: high-risk mutations (including intron 1 and 22 inversions, nonsense mutations, frameshifts, and large deletions) and low-risk mutations (such as polymorphisms, missense mutations, and splice site variants). The study reported higher rates of inhibitor development among patients with low-risk mutations who were treated with recombinant FVIII compared to those receiving plasma-derived FVIII concentrates (Rosendaal et al, 2017). The reasons underlying the differences in immunogenicity between plasma-derived and recombinant FVIII concentrates, as well as among different recombinant products, remain unclear. It is hypothesized that variations in protein purification processes, post-translational modifications, and viral inactivation methods may alter the physicochemical properties of the FVIII molecule, thereby enhancing its immunogenic potential (Lai et al, 2017). Furthermore, as von Willebrand factor (vWF) is known to influence the half-life of FVIII by binding to its C2 domain, it is also believed that vWF may modulate FVIII immunogenicity. In 2012, Delignat et al. evaluated two groups of hemophilic mice: a control group receiving pure recombinant FVIII concentrate and a test group treated with recombinant FVIII in combination with vWF. The study demonstrated a significantly lower production of anti-FVIII IgG antibodies in the group receiving the FVIII/vWF complex compared to the control group ($p = 0.03$) (Delignat et al, 2012). Similarly, another study characterized FVIII peptides presented via MHC class II from cell cultures stimulated with either FVIII alone or the FVIII/vWF complex, showing that the presence of vWF can alter both the repertoire and presentation of FVIII antigens (Sorvilo et al, 2016). One hypothesis to explain these findings is that vWF binding reduces FVIII endocytosis by antigen-presenting cells (Dasgupta et al, 2007). Therefore, the absence or low concentration of vWF in FVIII concentrates may increase their immunogenicity (Behrmann et al, 2002)

In this thesis, we investigated the cumulative incidence of inhibitor development in the HEMFIL cohort study. The cumulative incidence of inhibitor development with exclusive use of a third-generation recombinant factor VIII (ADVATE, Takeda, USA) was 36% for all inhibitors and 27% for high-titer inhibitors. In 2021, we replicated the Kaplan-Meier cumulative incidence analyses, using exposure days (ED) as the time variable, on an expanded cohort of children with hemophilia A from the HEMFIL Study (n = 104). At that time, 85/104 (81%) children had completed the follow-up. Inhibitors were detected in 33/85 (38%) patients, of which 22 (69%) were high-titre and 53/85 (62%) patients reached 75ED without inhibitors. The cumulative incidence of inhibitors in HA patients at 75ED was 35% (95%CI,26%-46%) for all inhibitors, 25% (95%CI,17%-36%) for high-titer and 13% (95%CI,8%-23) for low-titer inhibitors (Figure 1). The median time for inhibitor development remains 14ED (IQR,7-21) with a median age of 13 months (IQR,10-17). The cumulative incidence of inhibitor development under the exclusive use of a third-generation recombinant FVIII concentrate remained constant 3 years after the first report reinforces the robustness of these data. Unfortunately, we did not have an arm with plasma-derived in the HEMFIL study for comparison of risk.

Although it is the major complication affecting patients with hemophilia today, the mechanisms underlying its development are still not fully understood. Findings from the HEMFIL study confirm that the initial days of exposure to FVIII concentrate are critical in modulating the immune response in HA patients predisposed to inhibitor development.

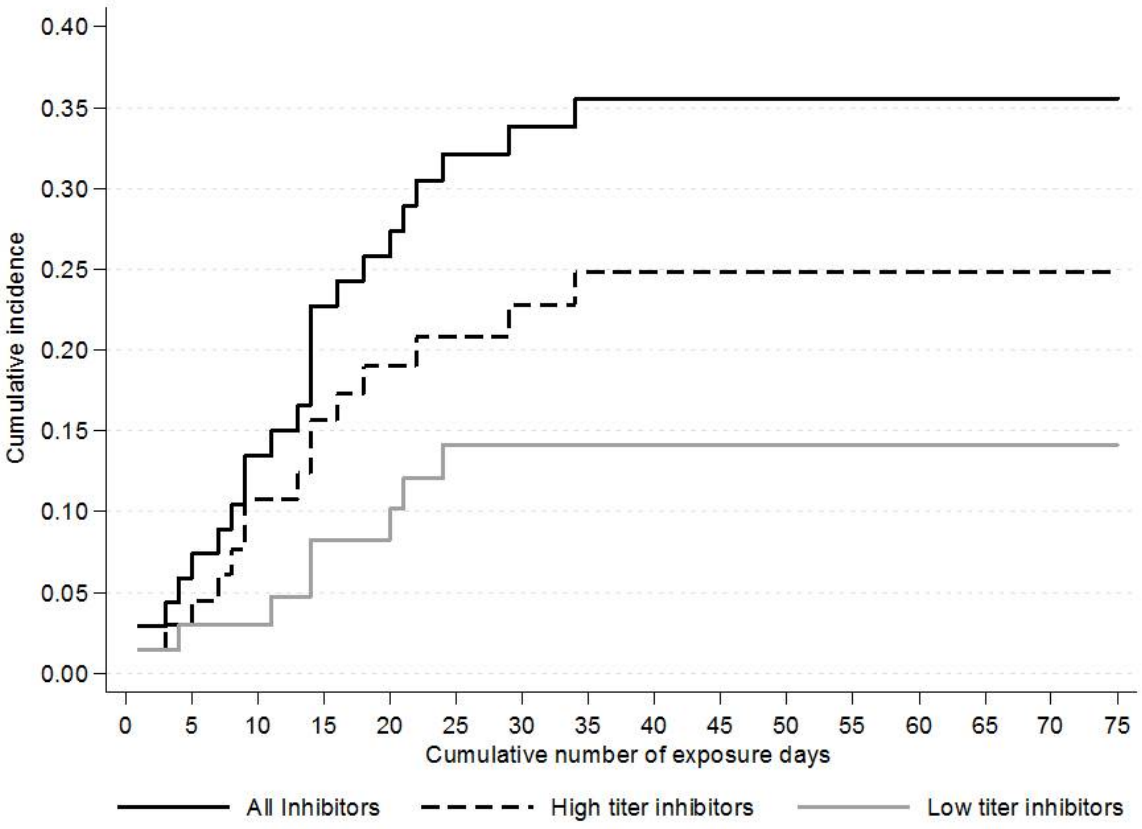


Figure 1. Cumulative incidence of inhibitor development according to the cumulative number of factor VIII exposure days for all inhibitors and high/low titer inhibitors

Chapters 3 to 8 suggest that FVIII acts as an immunomodulator of the immune system. Chemokines seem to be one of the elements that distinguish HA patients who develop inhibitors from those who do not during the initial exposures to FVIII.

Following intravenous administration of FVIII, antigen-presenting cells (APCs) process the protein and present its peptides to CD4+ T lymphocytes. Upon activation through interaction with APCs, these T cells migrate to the B cell follicles within the spleen (Niessen et al, 2008). In this environment, B cells—previously primed by FVIII via their B cell receptors—express FVIII-derived peptides bound to HLA class II molecules (Lollar, 2004). Activated CD4+ T cells recognize these HLA-II-peptide complexes on follicular B cells, promoting B cell activation. Subsequently, B cells differentiate into plasma cells and memory B cells, the latter capable of rapidly producing plasma cells upon re-exposure to FVIII (LeBien & Tedder, 2008). Plasma cells, residing

primarily in the bone marrow and spleen, may be short- or long-lived and secrete substantial amounts of anti-FVIII antibodies, neutralizing FVIII's procoagulant function. The persistence of inhibitors over many years, even in the absence of FVIII exposure, is likely due to the longevity of these plasma cells in certain patients (Hausl et al, 2002). Upon subsequent FVIII exposure following the primary immune response, inhibitor titers rise rapidly, driven by clonal expansion of memory CD4+ T cells and anti-FVIII memory B cells

Anti-FVIII antibodies with low affinity, mostly IgG1, IgM and IgA, have been reported in healthy individuals (Whelan et al, 2012) as well as in patients with either inherited or acquired HA (Hu et al, 2007; Chaves et al, 2010; Whelan et al, 2012, Montalvão et al, 2015, Hofbauer et al, 2015). It is believed that the production of low-affinity antibodies results from responses with extrafollicular onset. Interestingly, high-affinity anti-FVIII IgG4 is predominantly found in patients with HA who developed inhibitors, especially those of high-titer, implies differentiation of follicular T and B cells specific for FVIII, after higher levels of somatic hypermutation of the variable region of the immunoglobulins (Whelan et al, 2012, Montalvão et al, 2015, Hofbauer et al, 2015).

When evaluating the immunological profile in two groups of patients with hemophilia in a cross-sectional study, Chaves et al. (2010) found that patients without inhibitors presented a pro-inflammatory response profile mediated by T cells, probably inducing the synthesis of IgG1 anti-FVIII antibodies without inhibitory activity (Chaves et al, 2010). In the group of patients with inhibitors, an anti-inflammatory/regulatory profile was observed, mediated by neutrophils and monocytes, with high levels of IL-5 and IL-10 and low levels of IL-2, IL-4, IFN-gamma and TNF-alpha, probably inducing B lymphocytes to produce anti-FVIII antibodies of the IgG4 subclass (Chaves et al, 2010). The cytokine and chemokine profile in hemophilic mice that developed inhibitors after gene therapy was evaluated by Sun et al (2018). The results suggest that low levels of TGF-beta, associated with high levels of pro-inflammatory cytokines such as IL-1, IL-6, IL-12 and TNF-alpha, may favor the emergence of an immune response against FVIII (Sun et al, 2018).

Our work described that before the start of FVIII replacement, patients with HA have elevated levels of platelet-derived microparticles, total leukocytes, T lymphocytes, neutrophils, monocytes and erythrocytes, chemokine IL-8 and cytokines IL-6, TNF, IL-4, IL-10 and IL-17 when compared to the group of controls without hemophilia. We hypothesize that the presence of this profile characterized by increased pro-inflammatory and regulatory cytokines in patients with HA may result from microhemorrhages and/or subclinical bleeding, which could induce activation of coagulation and inflammation.

We also identified that before the exposure to FVIII, children with HA who developed inhibitors had increased levels of anti-FVIII IgG4, and 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 before 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. In addition, the analysis of the first FVIII infusions on immunological biomarkers in previously untreated patients with HA (de Oliveira et al, 2021) suggests that FVIII replacement triggers a pro-inflammatory 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 seems to be stimulated after repeated infusions of FVIII.

An evaluation of the immune profile of PUPs with HA may contribute to a better understanding of how immune biomarkers behave before exposure to exogenous FVIII. This may be important to understand why some patients develop inhibitors and others do not. However, analyzing the immune profile is challenging due to the dynamic nature of immune markers. Rather than measuring absolute levels of globulins, cytokines and chemokines, it is important to assess the complex interplay between these mediators. The levels of these biomarkers can vary considerably over time and in response to different stimuli, making data interpretation more complicated. Cross-sectional studies often capture a specific moment of the immune system dynamics, without considering how these molecules interact and modulate immune responses in real time and through time. Therefore, a more comprehensive analysis should include an investigation of different immunological biomarkers as a network and other risk factors related to inhibitor development. The use of network-based approaches has provided insights into the structural patterns of these systems and their dynamic behavior (Carpi et al., 2019; Schieber et al., 2023).

By using a machine-learning approach, we proposed a weight thresholding method that exploits the concept of maximizing the Network Node Dispersion (NND) (Schieber et al., 2017) as a potential tool for investigating predictive factors of inhibitor development in patients diagnosed with HA. A predictive model for inhibitor development in HA was developed by utilizing a network of clinical variables and biomarkers.

The algorithm presented an overall accuracy of 90.5% to predict inhibitor development in children with HA. The positive and negative predictive values of the model were, respectively, 74.2% and 98.4%. When the analysis was restricted to patients with *F*8 high-risk mutations, the accuracy of identifying children with HA and inhibitors increased from 74.2% to 82%, but decreased from 98.4% to 87.8% to identify children without inhibitors. These results align with previous studies reporting that patients with *F8* high-risk mutations are at an increased risk of developing inhibitors, regardless of other influencing variables (Rosendaal et al, 2017; Garagiola et al, 2018; Spena et al, 2018). Indeed, the SIPPET study showed that specific mutations may influence clinical outcomes (Paul et al, 2023). Our machine-learning algorithm had a high overall accuracy in predicting inhibitor development, which improved upon restricting the analysis to children with HA with a high-risk *F8* mutation.

3. Health status of persons with hemophilia A with and without inhibitors in the Netherlands

With the emergence of new, non-factor replacement therapies, more specifically emicizumab (Roche, Switzerland), the therapeutic context for treating hemophilia patients with inhibitors has changed significantly. Emicizumab prophylaxis is very effective and it is expected that joint health outcomes and health-related quality of life will improve dramatically, especially for patients with inhibitors. However, it is currently insufficiently known what the burden of disease is in patients with a history of inhibitors in the Netherlands. This knowledge can provide targets for future follow-up studies in cohorts of patients treated with novel treatment options.

The HIN6 Study is a cross-sectional study that included patients who participated in a nationwide postal survey conducted in the Netherlands from 01 June 2018 to 01 July 2019.

In Chapter 9, we evaluated data about all male persons with severe and non-severe HA who participated in the HIN 6 study and compared it with the health outcomes of persons with HA (PHA) in the Netherlands before the introduction of emicizumab prophylaxis. A total of 494 PHA patients were included with a median age of 45 years [Interquartile range (IQR)22-60]. A history of inhibitor development was reported by 73 (14.7%) patients, 33 severe PHA and 40 non-severe HA. The frequency of reported chronic joint problems, pain intensity and the frequency of hospitalization for orthopedic surgery did not differ between patients with and without a history of inhibitors, regardless of severity. Limitations in lifting, climbing stairs, kneeling, walking and self-care did not differ in patients < 61 years with a history of inhibitors in comparison with those without, even when stratified by inhibitor titer. In contrast, the HAL Scores differed between the two groups in patients >61 years with severe PHA ($p<0.05$). Overall, for all ages, HAL scores were higher for non-severe HA in comparison with severe HA, but the score did not differ by history of past inhibitors for non-severe PHA. A history of inhibitors was not associated with worse joint health or activity/participation level in patients with hemophilia in the Netherlands. This may indicate that inhibitor patients have received better care since the 1960s, which may have contributed to a positive impact on their quality of life. Probably, in the Netherlands, most patients with inhibitor started ITI as soon as an inhibitor was detected. Therefore, they did not suffer the consequences of increased bleeding. Unfortunately, we did not have access to time between inhibitor detection and ITI in this cohort.

Despite improvements in inhibitor treatment, which includes ITI and prophylaxis with bypassing agents, there is still a large proportion of severe PHA who experience pain and chronic pain when walking, climbing stairs, at night, at rest and when carrying weight. We found that there is still a significant frequency of joint bleeding and orthopedic surgeries due to complications of HA in our study. In recent years, hemophilia treatment has improved significantly around the world. It was shown in a Dutch cohort that the average life expectancy of patients with severe hemophilia was 73 years (Hassan et al, 2021), which is almost the life

expectancy of people without hemophilia. In our study, older patients reported a higher proportion of joint limitation and lower HAL scores. Despite improved access to treatment, patients still demand care and major efforts to prevent bleeding, especially among elderly patients. In conclusion, a history of inhibitor was not associated with worse joint health or activity/participation level in PHA in the Netherlands. Although joint problems continue to be frequently reported in elderly PHA, we did not observe differences between persons with or without inhibitors. It will be interesting to compare these data after the introduction of emicizumab.

CONCLUSION

Among individuals treated exclusively with third-generation recombinant FVIII, the overall incidence of inhibitor formation reached 36%, with 27% developing high-titer inhibitors. Despite its critical role in hemophilia management, the underlying mechanisms driving inhibitor emergence remain poorly understood. Our findings indicate that early exposure to factor VIII concentrates may trigger immunological pathways conducive to antibody production. Furthermore, this thesis demonstrated that mortality among patients with hemophilia has decreased over the years in Brazil and that, with the increase in life expectancy of these patients, we began to observe the development of diseases commonly related to aging, such as cardiovascular disease and cancer, following the same trend reported in articles from developed countries. Nevertheless, intracranial hemorrhage persists as a significant cause of death among individuals with hemophilia A and B in Brazil. Despite advancements in therapeutic access, there remains a substantial need for targeted interventions to minimize bleeding episodes, particularly in aging populations.

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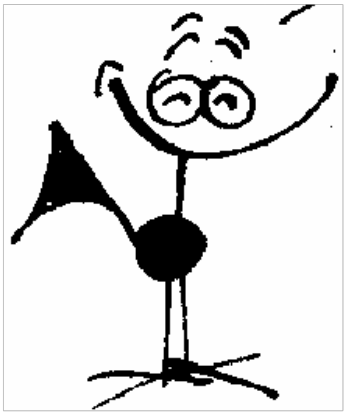
APPENDICES



NEDERLANDSE SAMENVATTING

Congenitale hemofilie is een X-gebonden stollingsstoornisstoornis die wordt gekenmerkt door een tekort aan factor VIII (FVIII) bij hemofilie A (HA) of IX (FIX) bij hemofilie B (HB). De behandeling bestaat uit infusie van FVIII- of FIX-concentraat of non-factor therapie ter preventie (profylaxe) en/of behandeling van bloedingen. De belangrijkste complicatie van hemofilie A is de ontwikkeling van remmende alloantistoffen (remmers) tegen de geïnjecteerde factor VIII, wat leidt tot ineffectieve behandeling. Deze remmers ontwikkelen zich bij ongeveer 10%-35% van de patiënten met HA. Er is weinig bekend over de risicofactoren die bijdragen aande ontwikkeling van remmers bij HA. Debelangrijkste zijn het type en de ernst van de hemofilie, de genmutatie die de hemofilie veroorzaakt en de intensiteit van de infusie met factorconcentraten. Er zijn weinig studies die de rol van immunologische en genetische markers hebben onderzocht (met uitzondering van die gerelateerd aan het gen dat codeert voor factor VIII). In Brazilië zijn er meer dan 13.000 geregistreerde patiënten met hemofilie, waarmee het de vierde grootste populatie van patiënten met hemofilie ter wereld is, na de Verenigde Staten, India en China. Het doel van het werk in dit proefschrift is daarom het onderzoeken van epidemiologische aspecten van HA in Brazilië en het analyseren hoe hemofiliecomplicaties de mortaliteit en kwaliteit van leven beïnvloeden bij patiënten met de ernstige vorm van HA. Daarnaast beoogt dit proefschrift ook mogelijke associaties te identificeren tussen immunologische, klinische en genetische risicofactoren voor de ontwikkeling van remmers in een Braziliaanse prospectieve cohortstudie. Verder beoogt het te identificeren of het voorkomen van eerdere remmers een negatieve impact heeft op de gezondheidstoestand, inclusief de gezondheid van de gewrichten en de gezondheidsgerelateerde kwaliteit van leven van patiënten met HA.

Het eerste deel van dit proefschrift richt zich op de hemofilie-gerelateerde mortaliteit in Brazilië en beschrijft het sterftecijfer en de belangrijkste doodsoorzaken bij hemofiliepatiënten. Deze kennis geeft aanknopingspunten voor overheidsbeleid ter vermindering van deze sterfte. Bovendien draagt het bij aan de evaluatie van de effecten van profylaxe, thuisbehandeling en ITI-behandeling, die in 2012 door de Braziliaanse overheid zijn geïmplementeerd (hoofdstuk 2).

Het tweede deel van dit proefschrift beschrijft studies naar risicofactoren voor de ontwikkeling van remmers in een Braziliaans cohort (hoofdstukken 3 tot en met 8).

Het derde deel van dit proefschrift beschrijft de levenskwaliteit van hemofiliepatiënten met remmers in een Nederlands cohort (hoofdstuk 9).

In hoofdstuk 2 werden gestandaardiseerde mortaliteitsratio's (SMR) berekend om het totale sterftecijfer van patiënten met hemofilie (A en B) te vergelijken met dat van de Braziliaanse algemene mannelijke bevolking. De gegevens werden gecorrigeerd voor leeftijd en kalenderperiode. Tussen januari 2000 en december 2014 werden er 784 sterfgevallen geregistreerd onder patiënten met hemofilie in Brazilië. De totale sterfte onder patiënten met hemofilie was 13% hoger vergeleken met de algemene mannelijke

bevolking (SMR, 1,13; 95% BI: 1,01-1,16). Er was een daling in SMR van 2000 tot 2014, zonder dat er in 2014 een significant verschil werd waargenomen (SMR, 0,89; 95% BI: 0,74-1,04). Bloedingen bleven de belangrijkste doodsoorzaak in 254 van de 784 patiënten (32,4%), voornamelijk intracraniële bloedingen (137 van de 254; 54%). Het percentage sterfgevallen als gevolg van hiv onder hemofiliepatiënten daalde van 30,8% in 2000-2002 tot 11,3% in 2012-2014. De incidentie van sterfgevallen als gevolg van maligniteiten en hart- en vaatziekten nam toe, respectievelijk van 7,7% naar 12,1% en van 13,7% naar 25,5% in dezelfde periodes. Daarnaast overleden 129 patiënten aan hepatitis.

In review in hoofdstuk 3 richt zich op de ontwikkeling van remmers bij patiënten met congenitale HA, de meest ernstige complicatie van behandeling bij deze patiënten. Remmers zijn alloantistoffen die de procoagulante activiteit van FVIII neutraliseren. Patiënten met HA kunnen een pro-inflammatoire immuunrespons ontwikkelen met initieel de ontwikkeling van van anti-FVIII immunoglobuline (Ig) G1, zonder FVIII-neutralizerend effect. Bij patiënten met remmers verschuift de immuunrespons naar een anti-inflammatoir/regulerend patroon dat de ontwikkeling van anti-FVIII IgG4-antilichamen bevordert. Bloedingen bij patiënten met remmers zijn moeilijker te behandelen door een verminderde of afwezige respons op FVIII behandeling. Immuuntolerantie-inductie therapie (ITI) is momenteel de primaire behandeling voor de eradicatie van remmers met een hoge titer, maar de onderliggende immunologische mechanismen blijven nog grotendeels onverklaard. Het geïllustreerde review bespreekt de belangrijkste risicofactoren voor de ontwikkeling van remmers en de immunologische interacties, inclusief de immuuncellen en receptoren die hierin een rol spelen.

Hoofdstuk 4 beschrijft de methodologische aspecten van de Hemfil-studie, een prospectief cohort van niet eerder behandelde/minimaal behandelde (< 5 behandeldagen met FVIII) patiënten met hemofilie, waarin patiënten werden gemonitord tot 75 behandelingsdagen of tot de ontwikkeling van een remmer. Bloedmonsters werden afgenomen op drie momenten in de studie: inclusie (T0), na bereiken van 75 behandelingsdagen of de ontwikkeling van een remmer (T1) en na bereiken van immuuntolerantie na ITI (T2). Naast de immunologische factoren werden ook klinische en genetische risicofactoren geëvalueerd.

Hoofdstuk 5 rapporteerde voor het eerst de cumulatieve incidentie van remmers in een cohort van Braziliaanse niet-eerder behandelde patiënten (PUP's) met ernstige HA (baseline FVIII:C < 1%) die behandeld werden met een derde-generatie recombinant factor VIII-concentraat (ADVATE®, Takeda, VS). Deze resultaten maken deel uit van de prospectieve HEMFIL-studie. Patiënten werden achtereenvolgens geïncludeerd en gevolgd tot 75 behandelingsdagen of tot aan de ontwikkeling van een remmer. In totaal werden 70 PUP's met ernstige HA geïncludeerd, van wie 61 (85%) de studie follow-up voltooiden. Remmers werden gedetecteerd bij 24 van de 61 patiënten, waarvan 17 een hoge titer remmer hadden. Van de patiënten die remmers ontwikkelden, werden 17 (68%) vervolgens behandeld met ITI. De cumulatieve incidentie was 36% [95%BI, 26%-49%] voor alle remmers, 27% (95%BI, 18%-40%) voor hoge titer remmers en 13% (95%BI, 6-24%) voor

lage titer remmers. Dit was een belangrijke stap in het begrijpen van de immunogeniciteit van een derde-generatie recombinant factor VIII-concentraat.

Hoofdstuk 6 richtte zich op het onderzoeken van het immunologische profiel van niet eerder behandelde patiënten (PUP's) met HA bij de start van het onderzoek in vergelijking met gezonde personen zonder hemofilie. Vergeleken met gezonde controles vertoonden kinderen met HA verhoogde concentraties micropartikels (MP's) afkomstig van T-lymfocyten, bloedplaatjes, neutrofielen, leukocyten, monocyten en erytrocyten; hogere concentraties van de cytokinen TNF, IL-10, IL-6, IL-4 en IL-2; verhoogde niveaus van de chemokine IL-8 en verlaagde MIG. Deze resultaten suggereren dat, zelfs vóór blootstelling aan factor VIII, het immunologische profiel van patiënten met HA verschilt van dat van gezonde controles, waarschijnlijk gestimuleerd door microbloedingen of subklinische bloedingen.

Na start van FVIII behandeling daalden de MP-niveaus, wat mogelijk verband houdt met een effectievere hemostase bij HA-patiënten die met factor VIII werden behandeld. Wat betreft cytokinen en chemokinen, zagen we niet alleen een toename in de IL-4, IL-6, IL-8 en IL-10 concentraties, maar ook van TNF, IFN en IL-2, wat een pro-inflammatoir profiel kenmerkt. Zo zou bij eerder onbehandelde patiënten met hemofilie (T0-fase van de studie) het optreden van bloedingen, zelfs subklinisch, een ontstekingsreactie kunnen opwekken, gemedieerd door cytokines en chemokines, naast de activatie van de stolling door de productie van MP's. De productie van MP's kan op zijn beurt de synthese van cytokines en IL-8 activeren, wat het pro-inflammatoire profiel genereert dat bij deze patiënten is geïdentificeerd. Voor zover wij weten is dit de eerste studie die het immunologische profiel bij onbehandelde patiënten met hemofilie A evalueerde in vergelijking met normale controles. De studie biedt waardevolle inzichten in het immunologische landschap van HA-patiënten vóór FVIII behandeling. Inzicht in de immuunstatus van HA-patiënten vóór blootstelling aan FVIII zou de voorspelling van de ontwikkeling van remmers kunnen ondersteunen. Deze kennis kan dus richtinggevend zijn voor gepersonaliseerde behandelstrategieën.

Hoofdstuk 7 beschrijft de impact van initiële FVIII-infusies op immunologische biomarkers bij patiënten met HA uit de Hemfil-studie, die nog niet met FVIII behandeld waren. De belangrijkste bevinding van deze studie zijn veranderingen in de immuunrespons na de eerste toediening van FVIII, met veranderingen in specifieke biomarkers die mogelijk gerelateerd zijn aan de ontwikkeling van remmers. De studie suggereert dat het monitoren van deze immunologische veranderingen kan helpen bij het voorspellen van het risico op remmervorming. Deze resultaten benadrukken het belang van zorgvuldige observatie van immuunreacties tijdens de vroege behandelingsperiode.

In Hoofdstuk 8 presenteren we een predictiemodel om de netwerk- en potentiële globale topologische verschillen van de patiëntprofielen op het moment van inclusie in de Hemfil-studie (T0) en hun associatie met de ontwikkeling van remmers te vergelijken. De methode transformeert gerelateerde variabelen in structurele patronen via complexe netwerken door de Network Node Dispersion te maximaliseren. Het FVIII-genotype

werd geclassificeerd als hoogrisico (inversies, nonsense mutaties, frameshift mutaties, grote deleties en inserties) of laagrisicomutaties (overige mutaties).

In totaal werden 95 kinderen uit de HEMFIL-studie die de follow-up hadden voltooid, geïncludeerd. In totaal ontwikkelden 31 (33%) patiënten remmers, van wie 22 (71%) patiënten een hoge titer hadden (> 5 BU). Ons machine-learning algoritme toonde een algehele nauwkeurigheid van 90,5% voor het voorspellen van de ontwikkeling van remmers bij kinderen met HA, wat verder verbeterde toen de analyse werd beperkt tot kinderen met een hoogrisico F8-genotype.

In hoofdstuk 9 beschrijft de levenskwaliteit van hemofiliepatiënten met remmers, die deelnamen aan de zesde Hemofilie in Nederland studie (HiN6), een cross-sectionele studie uitgevoerd in 2018-2019. We evalueerden de vragenlijsten, waaronder de Hemofilie Activiteiten Lijst (HAL), en data uit medische dossiers van Nederlandse mannen met HA. Patiënten werden gestratificeerd op leeftijd, ernst van HA, voorgeschiedenis van remmer en remmertiter. In totaal werden 685 HA-patiënten geïncludeerd. De frequentie van gerapporteerde chronische gewrichtsproblemen als gevolg van hemofilie, pijnintensiteit en de frequentie van ziekenhuisopname voor orthopedische chirurgie verschilden niet tussen patiënten met en zonder een voorgeschiedenis van remmers, onafhankelijk van de ernst van de hemofilie. Beperkingen bij tillen, traplopen, knielen, lopen en zelfzorg namen toe met de leeftijd, maar verschilden niet tussen patiënten <60 jaar met en zonder met een voorgeschiedenis van remmers, ook niet na stratificatie op remmertiter. Wel waren de verschillen tussen bij patiënten met ernstige hemofilie >60 jaar met en zonder remmers statistisch significant ($p<0,05$). Over het algemeen waren de HAL-scores voor patiënten met niet-ernstige HA hoger in vergelijking met patiënten met ernstige HA, maar de score verschilde niet tussen patiënten met en zonder remmer voorgeschiedenis, gecorrigeerd voor ernst van de hemofilie. Concluderend was een voorgeschiedenis van remmers niet geassocieerd met een de musculoskeletale gezondheid of dagelijkse activiteit/participatie bij patiënten met ernstige HA. Oudere patiënten rapporteerden verschillende beperkingen, onafhankelijk van de remmerstatus. Hoewel gewrichtsproblemen nog steeds frequent worden gemeld bij ouderen met HA, zagen we geen verschillen tussen personen met of zonder remmers. Het zou interessant zijn om deze gegevens te vergelijken met de kwaliteit van leven na de introductie van emicizumab.

Concluderend, was de totale incidentie van remmervorming onder personen die uitsluitend werden behandeld met een derde-generatie recombinant factor VIII 36%, waarbij 27% van de patiënten hoge titer remmers ontwikkelde. Ondanks de cruciale impact van remmers op de behandeling van hemofilie, blijven de onderliggende mechanismen van remmerontwikkeling nog weinig begrepen. Onze bevindingen wijzen erop dat blootstelling aan factor VIII-concentraten immunologische processen kan activeren die de productie van antilichamen stimuleren. Bovendien toonde dit proefschrift aan dat de sterfte onder hemofiliepatiënten in Brazilië in de loop der jaren is afgenomen en dat we, met de stijgende levensverwachting van deze patiënten, de ontwikkeling van ziekten die vaak verband houden met veroudering, zoals hart- en vaatziekten en

maligniteiten, zijn gaan waarnemen, in lijn met dezelfde trend die wordt beschreven in artikelen uit ontwikkelde landen. Desondanks blijven intracraniële bloedingen een belangrijke doodsoorzaak bij mensen met hemofilie A en B in Brazilië. Ondanks de vooruitgang in therapeutische opties blijft er een aanzienlijke behoefte aan gerichte interventies om bloedingsepisodes te minimaliseren, met name bij ouderen.

Acknowledgment

This thesis represents not only the culmination of a long academic journey but also the result of a year filled with enriching experiences in the Netherlands — a country so different from my own, yet one that welcomed me with generosity and offered a period of learning and personal growth.

I cherish every moment I spent in Leiden and would like to express my heartfelt thanks to all those who, in one way or another, contributed to making this chapter possible and who helped turn this experience into an unforgettable one.

In particular, I deeply thank my supervisors, Anske, Samantha, and Suely, for their invaluable guidance, clarity and encouragement. You taught me the power of collaboration and how much we can gain by sharing knowledge. You were true facilitators of my work, and I am sincerely thankful.

To Yvonne, thank you for your unwavering support, kindness, and patience throughout this journey.
I would also like to thank the families of the participants included in the research for this thesis for believing in the seriousness of our work. Our greatest motivation is to find evidence and better solutions to improve the quality of life for our patients.

To all the friends at the Department of Clinical Epidemiology in Leiden, my sincere and warmest thanks.

Curriculum Vitae

Leticia Lemos Jardim was born on June 25, 1987, in Belo Horizonte, Brazil. She obtained her bachelor's degree in Biomedical Sciences in 2010, followed by a master's degree and her first PhD degree in Sciences Applied to Adult Health from the Federal University of Minas Gerais (Brazil) in 2015 and 2019, respectively. In 2016, she was awarded a scholarship grant for international doctoral internships at the Clinical Epidemiology in Leiden University Medical Center, where she started her second PhD research, supervised by Prof. Dr. Anske van der Bom. During her PhD, she presented her research findings at various national and international conferences. She is currently working as a professor in the Department of Public Health of the Faculty of Medical Sciences of Minas Gerais (Brazil), and she is an epidemiological researcher at the Research Group on Health Policies and Social Protection at the René Rachou Institute (Brazil).

PORTFOLIO

	Year	Hours
Courses		
Leiden University Onboarding Programme Inform & Connect (2 activities) (done)	2025	5
Epidemiology an Introduction	2016	12
Writing course	2016	24
Advanced statistical methods in epidemiology	2019	75
Attended lectures, LUMC presentations, participation in meetings		
Journal Club (thrombosis and haemostasis)	2017	2
Scientific Committee	2017	2
Traineeship abroad		
Immunology laboratory (Amsterdam)	2016	28
Thrombin generation (Maastricht)	2017	30
Other activities		
Organization of the book *"Epidemiology and Biostatistics - Transforming data into information for health"*	2024	40
Teaching activities		
Epidemiology and biostatistics	2021-2025	40

LIST OF PUBLICATIONS

Publications resulting from this thesis

1. **Jardim LL**, van der Bom JG, Caram-Deelder C, Gouw SC, Leal Cherchiglia M, Meireles Rezende S. Mortality of patients with haemophilia in Brazil: First report. Haemophilia. 2019 May;25(3):e146-e152. doi: 10.1111/hae.13730. Epub 2019 Mar 15. PMID: 30875453.
2. **Jardim LL**, Chaves DG, Rezende SM. Development of inhibitors in hemophilia A: An illustrated review. Res Pract Thromb Haemost. 2020 May 26;4(5):752-760. doi: 10.1002/rth2.12335. PMID: 32685884; PMCID: PMC7354390.
3. **Jardim LL**, Santana MP, Chaves DG,van der Bom JG, Meireles Rezende S. Risk factors for antibody formation in children with hemophilia. Blood Coagulation & Fibrinolysis. 32(7):p 443-450, October 2021.
4. **Jardim LL**, van der Bom J, Brommonschenkel CC, Gouw SC, Rezende SM; on the behalf of the HEMFIL Study Group. Inhibitor incidence in haemophilia A under exclusive use of a third-generation recombinant factor VIII concentrate: results of the HEMFIL Cohort Study. Br J Haematol. 2019 Jul;186(1):152-155.
5. **Jardim LL**, Chaves DG, Silveira-Cassette ACO, Simões E Silva AC, Santana MP, Cerqueira MH, Prezotti A, Lorenzato C, Franco V, van der Bom JG, Rezende SM. Immune status of patients with haemophilia A before exposure to factor VIII: first results from the HEMFIL study. Br J Haematol.
6. de Oliveira LMM, **Jardim LL**, Santana MAP, Cerqueira MH, Lorenzato CS, Franco VKB, Zuccherato LW, Rezende SM, Chaves DG. Effect of the First Factor VIII Infusions on Immunological Biomarkers in Previously Untreated Patients with Hemophilia A from the HEMFIL Study. Thromb Haemost. 2021 Jul;121(7):891-899.
7. **Jardim LL**, Schieber TA, Santana MP, Cerqueira MH, Lorenzato CS, Franco VKB, Zuccherato LW, da Silva Santos BA, Chaves DG, Ravetti MG, Rezende SM. Prediction of inhibitor development in previously untreated and minimally treated children with severe and moderately severe hemophilia A using a machine-learning network. J Thromb Haemost. 2024 Sep;22(9):2426-2437.

Other publications

1. Santana MSP, Chaves DG, Souza RP, **Jardim LL**, Zuccherato LW, Santos BAS, Cerqueira MH, Lorenzato CS, Franco VKB, Rezende SM. Longitudinal Evaluation of Immunological Biomarkers in Previously Untreated/Minimally Treated Patients With Severe and Moderately Severe Haemophilia A During Exposure to Factor VIII: Results From the HEMFIL Study. Haemophilia. 2025 May 25. doi: 10.1111/hae.70048.
2. Zuccherato LW, Souza RP, Camelo RM, Dias MM, **Jardim LL**, Santana MAP, Oliveira AG, Lorenzato CS, Cerqueira MH, Franco VKB, Ribeiro RA, Etto LY, Roberti MDRF, Callado FMA, de Cerqueira MAF, Pinto ISS, Garcia AA, Anegawa TH, Neves DCF, Tan DM, Tou RP, Chaves DG, van der Bom J, Rezende SM; HEMFIL Study and the Brazilian Immune Tolerance (BrazIT) Study. Large deletions and small insertions and deletions in the factor VIII gene predict unfavorable immune tolerance induction outcome in people with severe hemophilia A and high-responding inhibitors. Thromb Res. 2024 Oct;242:109115. doi: 10.1016/j.thromres.2024.109115.
3. **Jardim LL**, Franco MB, de Oliveira NR, de Carvalho BN, Basques F, Ribeiro DD, Lisman T, Pereira LS, Rezende SM. Hypocoagulability in severe yellow fever infection is associated with bleeding: results

from a cohort study. Res Pract Thromb Haemost. 2024 Apr 26;8(4):102427. doi: 10.1016/j.rpth.2024.102427. eCollection 2024 May.

4. Machado AV, Ferreira WE, Vitória MAÁ, Magalhães Júnior HM, **Jardim LL**, Menezes MAC, Santos RPO, Vargas FL, Pereira EJ. COVID-19 and health systems in Brazil and around the world: effects on the working conditions and health of health workers. Cien Saude Colet. 2023 Oct;28(10):2965-2978. doi: 10.1590/1413-812320232810.10102023. Epub 2023 Jun 28.
5. Franco MB, **Jardim LL**, de Carvalho BN, Basques F, Ribeiro DD, Pereira LS, Rezende SM. Deficiency of coagulation factors is associated with the bleeding diathesis of severe yellow fever. Ann Hematol. 2023 Jul;102(7):1939-1949. doi: 10.1007/s00277-023-05262-x. Epub 2023 May 24.
6. Silva GDMD, Souza AA, Castro MSM, Miranda WD, **Jardim LL**, Sousa RP. Influence of socioeconomic inequality on the distribution of COVID-19 hospitalizations and deaths in Brazilian municipalities, 2020: an ecological study. Epidemiol Serv Saude. 2023 Feb 10;32(1):e2022303. doi: 10.1590/S2237-96222023000100021. eCollection 2023.
7. Santana MAP, Chaves DG, Camelo RM, Zuccherato LW, **Jardim LL**, Rezende SM; in behalf of the HEMFIL and BrazIT Study Groups. Prevalence of sporadic haemophilia A. Haemophilia. 2023 Mar;29(2):668-670. doi: 10.1111/hae.14742. Epub 2023 Jan 27.
8. Camelo RM, Dias MM, Caram-Deelder C, Gouw S, de Magalhães LP, Zuccherato LW, **Jardim LL**, de Oliveira AG, de Albuquerque Ribeiro R, Franco VKB, do Rosário Ferraz Roberti M, de Araújo Callado FMR, Etto LY, de Cerqueira MAF, Cerqueira MH, Lorenzato CS, de Souza IS, Serafim ÉSS, Garcia AA, Anegawa TH, Neves DCF, Tan DM, van der Bom J, Rezende SM; Brazilian Immune Tolerance (BrazIT) Study group.J Thromb Haemost. Time between inhibitor detection and start of immune tolerance induction: Association with outcome in the BrazIT study. 2022 Nov;20(11):2526-2537. doi: 10.1111/jth.15878. Epub 2022 Sep 26.
9. Martins NNN, **Jardim LL**, Pereira LS, Rezende SM, Gonçalves MLP. Blue-green cytoplasmic inclusions in neutrophils/monocytes of patients with yellow fever. Int J Lab Hematol. 2022 Aug;44(4):e168-e171. doi: 10.1111/ijlh.13827.
10. Rezende SM, **Jardim LL**, Lúcia Magalhães V, Teixeira Melo H, Araujo JPB, Genovez G. Building the National Program of Inherited Bleeding Disorders in Brazil. Blood Adv. 2019 Dec 6;3(Suppl 1):48-50. doi: 10.1182/bloodadvances.2019GS121559.
11. Duarte RCF, Rios DRA, Rezende SM, **Jardim LL**, Ferreira CN, Carvalho MDG. Standardization and evaluation of the performance of the thrombin generation test under hypo- and hypercoagulability conditions. Hematol Transfus Cell Ther. 2019 Jul-Sep;41(3):244-252. doi: 10.1016/j.htct.2018.08.007. Epub 2018 Dec 31.
12. Zuccherato LW, Elói-Santos SM, **Jardim LL**, Camelo RM, Chaves DG, Souza RP, Hollox EJ, Rezende SM. Variation of rs3754689 at lactase gene and inhibitors in admixed Brazilian patients with hemophilia A. Haematologica. 2019 Nov;104(11):e527-e529. doi: 10.3324/haematol.2019.220608. Epub 2019 Mar 14.
13. Zuccherato LW, Roberti MRF, **Jardim LL**, Rezende SM. Successful immune tolerance in a young female with inhibitor and severe haemophilia A due to a complex genetic rearrangement. Haemophilia. 2018 Jul;24(4):e283-e285. doi: 10.1111/hae.13560. Epub 2018 Jul 13.

AWARDS

1. “Best Hemostasis Work” Award - Brazilian Congress of Hematology, Hemotherapy and Cell Therapy - HEMO 2023 with the study ´Prediction of inhibitor development in children with severe hemophilia A: artificial intelligence analysis of the Hemfil study´. HEMO Congress in São Paulo.
2. 'Reach the World Travel Grant' by the International Society on Thrombosis and Haemostasis (ISTH) to present the study 'Prediction of Inhibitor Development in Previously Untreated Children with Hemophilia A using a Machine-Learning Network and Information Theory Quantifiers: Results from the HEMFIL Cohort Study.' at the ISTH 2023 Congress in Montreal.
3. 'Reach the World Travel Grant' by the International Society on Thrombosis and Haemostasis (ISTH) to present the study ´Prevalence of sporadic haemophilia A: An updated analysis of two cohort studies. In: International Society on Thrombosis and Haemostasis ' at the ISTH 2022 Congress in London.
4. 'Reach the World Travel Grant' by the International Society on Thrombosis and Haemostasis (ISTH) to present the study ´Clinical Characteristics and Incidence of Inhibitors in Previously Untreated Children with Haemophilia: An Update of the Hemfil Cohort Study.' at the ISTH 2021 Congress in Philadelphia.
5. 'Reach the World Travel Grant' by the International Society on Thrombosis and Haemostasis (ISTH) to present the study ´Clinical Characteristics and Incidence of Inhibitors in Patients with Haemophilia: Results of the HEMIL Cohort Study' at the ISTH 2019 Congress in Melbourn.
6. 'Reach the World Travel Grant' by the International Society on Thrombosis and Haemostasis (ISTH) to present the study 'Incidence in Hemophilia A under Exclusive Use of a Third-generation Recombinant Factor VIII Concentrate: Results of the HEMFIL Cohort Study.' at the ISTH 2018 Congress in Dublin.
7. Milwaukee SSC Award (The highest scored Reach the World abstracts), The Scientific and Standardization Committee (SSC) ISTH – Montpellier and the study: ´Immunological Profile of Previously Untreated Patients with Haemophilia A: Results from the HEMFIL Study.' at the ISTH 2016 Congress in Montpellier.

Images of the Thesis

The tree on the cover of this book is called the Yellow Ipê. It is native to Brazil and is one of the country's most iconic trees. It blooms even in times of drought, symbolizing strength, resilience, and the ability to overcome challenges, as well as the beauty that can emerge even in the face of adversity.

The “Graúna”, presented in all the chapters of this book, is a Portuguese denomination for a black bird. It is considered the most striking and popular drawing by cartoonist Henfil.

The HEMFIL is a Brazilian cohort study named to honor Mr. Henrique de Souza Filho (1944 - 1988), also known as Henfil. Henrique was one of the greatest Brazilian cartoonists, also a journalist and writer, and the creator of characters with great popularity in the country. Henfil had severe hemophilia A and died from AIDS, after contamination with clotting factor concentrates.