Epidemiological aspects and complications of congenital hemophilia A in Brazil

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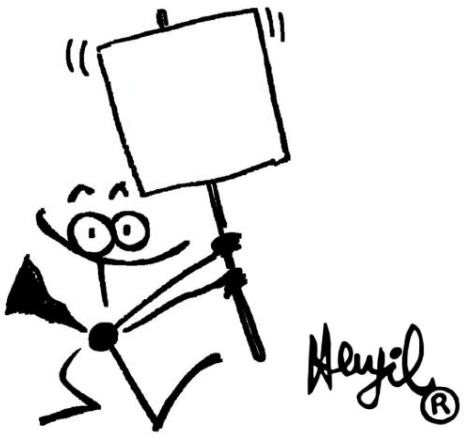
CHAPTER 10

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.

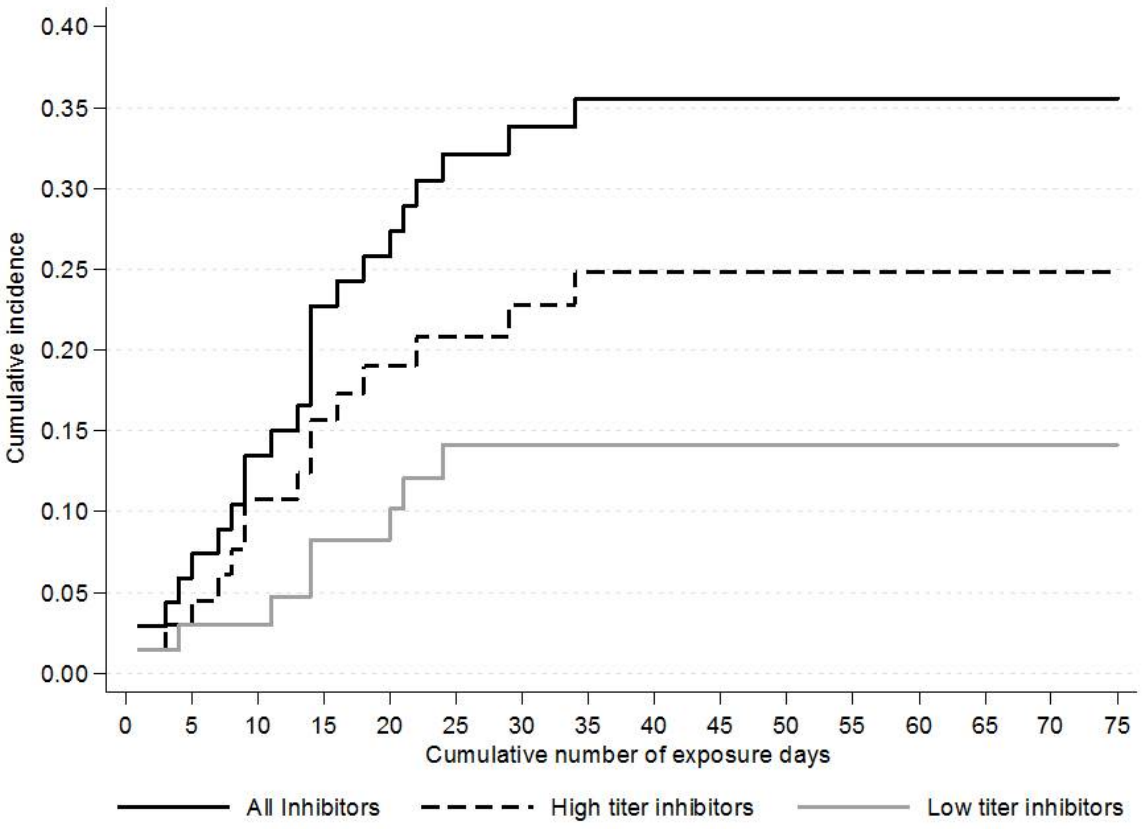


Figure 1. Cumulative incidence of inhibitor development according to the cumulative number of factor VIII exposure days for all inhibitors and high/low titer inhibitors

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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