UNIVERSIDADE D
COIMBRA

Raquel Moreira de Pinho

PHARMACOKINETIC ANALYSIS OF CLOTTING FACTORS: IDENTIFYING INDIVIDUAL CHARACTERISTICS OF VARIABILITY

Dissertação no âmbito do Mestrado em Farmacologia Aplicada orientada pela Professora Doutora Ana Cristina Bairrada Fortuna e pela Professora Doutora Marília João Rocha, apresentada à Faculdade de Farmácia da Universidade de Coimbra

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"If you can't fly then run, if you can't run then walk, if you can't walk then crawl, but whatever you do you have to keep moving forward"

Martin Luther King Jr,

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Abstract

Hemophilia is a rare hypocoagulation disorder that, depending on the lacking coagulation cascade factor, have different denominations. This study will focus only on Hemophilia A (HA), which is characterized by reduced levels of FVIII. Its treatment is based on the replacement of the normal levels through FVIII concentrates, namely recombinant FVIII (either Standard half-life or Extended half-life). However, pharmacological response is heterogenic namely due to disease evolution and FVIII concentrates pharmacokinetic profiles, which seem to be influenced by individual characteristics. Therefore, this study aimed at identifying the factors underlying the variability observed in treatment response with recombinant FVIII.

The present observational retrospective study included patients with more than 17 years old and at least one FVIII concentrate prescribed between the period of 1st January of 2018 and 30th June of 2020 at the immune-hemotherapy service of University Hospital Centre of Coimbra (CHUC, EPE). Indeed, 46 patients were enrolled, 34 diagnosed with severe HA (73.9%) whereas 10 presented mild disease (21.7%). During the time of the study, patients had *switch* on treatment, mostly related to the FVIII concentrate which led to an increase in extended half-life concentrates. Overall, most of the patients (n=40) had their levels monitored, although 19 had one blood sample scheme, leaving a short sample for further pharmacokinetics analysis. This later analysis involved patients monitored on the same day (n=4) where the two, with the same concentrate and same dose, had different values in their maximum concentration (49% *versus* 79%) and the minimum concentrations (0.8% *versus* 7.70%). The other two patients, with different concentrate and distinct doses, had similar values for minimum concentration (1.35% *versus* 1.50%). Regarding the patients monitored in different days, two of them were administered with different doses of the same concentrate and revealed different maximum concentrations (81% *versus* 47%). Additionally, patients prescribed with extended half-life concentrates had lower half-life than the standard half-life ones. The inter-variability verified can be justified by the age of the patients since half-life decreases and clearance increases as the age enhances.

To conclude, the results herein found highlight the heterogenicity in treatment of HA patients as well as the importance of performing a well-defined protocol in monitoring clotting factors. This is essential to collected precisely FVIII levels and to identify inter-variability factors to improve clinical outcomes in this population.

Keywords: hemophilia A, FVIII concentrates, individual variability factors, therapeutic drug monitoring; pharmacokinetics.

Resumo

Hemofilia é uma doença rara hemorrágica. As diferentes formas da doença relacionam-se com as baixas concentrações de um determinado fator de coagulação. O presente estudo focou-se apenas nos doentes com hemofilia do tipo A (HA). Como a HA é explicada pelas baixas concentrações de FVIII, o seu tratamento consiste na reposição desses níveis para valores normalidade através da administração de concentrados de FVIII recombinantes (nomeadamente os Standard half-life e os Extended half-life). No entanto, a resposta farmacológica é muito heterogénea devido à evolução da própria doença e ao comportamento farmacocinético dos concentrados, o qual, por sua vez, depende das características individuais de cada doente. Assim, o objetivo principal do presente estudo consistiu na identificação de fatores individuais que contribuem para esta variabilidade no tratamento.

Tratou-se de um estudo retrospetivo, o qual incluiu doentes com pelo menos 18 anos de idade e com uma ou mais prescrições de concentrado de FVIII recolhidas entre 1 de janeiro de 2018 e 30 de junho de 2020, no serviço de imuno-hemoterapia do Centro Hospitalar e Universitário de Coimbra (CHUC, EPE). Este estudo envolveu 46 doentes, 34 diagnosticados com HA severa (73.9%) e 10 com HA ligeira (21.7%).

Durante o tempo do estudo, os doentes sofreram um *switch* no tratamento, maioritariamente, relacionado com o concentrado prescrito, o que levou a um aumento nos concentrados de extended half life. Em termos de regime instituído, a mudança de regime de profilaxia para *on-demand* foi menos comum.

A maior parte dos doentes foram submetidos a monitorização dos níveis de FVIII (n=40) apesar de 19 apresentarem apenas uma análise sanguínea, comprometendo o número de doentes que puderam ser submetidos a análise farmacocinética (n=9). A análise farmacocinética englobou doentes monitorizados no mesmo dia (n=4), dois dos quais, apesar de terem sido administrados com o mesmo concentrado e a mesma dose, apresentaram valores de concentração máxima (49% *versus* 79%) e concentração mínima diferentes (0.8% *versus* 7.70%); os outros dois doentes obtiveram valores similares de concentração mínima (1.35% *versus* 1.50%) apesar de serem administrados com concentrados e doses diferentes. Nos doentes monitorizados em dias distintos, dois deles foram tratados com diferentes doses do mesmo concentrado, apresentando, expectavelmente concentração máxima de 81% e 47%. Doentes prescritos com extended half-life FVII obtiveram valores de tempo de semivida menores que os concentrados standard half-life.

Esta variabilidade inter-individual pode ser justificada pela diferença de idades dos doentes pois os mais velhos atingiram menores valores de tempo de semivida e maiores valores de clearance ou, pela limitação do tempo pós-infusão das amostras sanguíneas (máximo de 24 horas).

Assim, os resultados obtidos no presente trabalho evidenciam a heterogeneidade no tratamento de doentes com HA, assim como realçam a importância da aplicação de um protocolo de monitorização bem definido para a colheita de amostras de forma a permitir correlacionar variabilidade inter-individual com os *outcomes* clínicos do tratamento de doentes com HA.

Palavras-chave: hemofilia A, concentrados FVIII, variabilidade individual, monitorização terapêutica de fármacos; farmacocinética.

List of Abbreviations

A

ABR Annual bleed rate
aPTT Activated partial thromboplastin time
AUC Area under the curve
AUMC Area under the first moment of the curve
APC Activated protein C

B

BDD B-domain deleted
BDT B-domain truncated
BHK Baby Hamster Kidney
BMI Body Mass Index
BW Bodyweight

C

CL Clearance
C_{max} Maximum/peak of plasma concentration
C_{trough} Minimum/trough plasma concentration
CHO Chinese hamster ovary cells
CFCs Clotting factor concentrates

E

EDs Exposure days
EHL Extended Half-life

F

FIIa Thrombin
FIX Factor IX
FM Fat mass
FV Factor V
FVa Factor V activated
FVIIa Factor VII activated
FX Factor X
FXa Factor X activated
FIX Factor IX
FIXa Factor IX activated
FVIII Factor VIII
FVIII:C Factor VIII activity
FVIIIa Factor VIII activated
FXI Factor XI
FXIII Fibrin-stabilizing factor
FXIIIa Fibrin-stabilizing factor activated

H

HAV Hepatitis A virus
HBV Hepatitis B virus
Hek293 Human embryonic kidney cell line
HA: Hemophilia A
HCV Hepatitis C virus
HIV Human immunodeficiency virus

I

IgG Immunoglobulin G
IVR Incremental *in vivo* recovery or *In vivo* recovery or recovery
IU International Units
ISTH International Society on Thrombosis and Haemostasis

L

LRP1 Low-density lipoprotein receptor-related protein

M

MRT Mean residence time

P

PK Pharmacokinetics
pdFVIII Plasma derived FVIII

R

rFVIII Recombinant concentrates

S

SHL Standard Half-life

T

t½ Terminal half-life
TAT1% Time spent above the threshold
TDM Therapeutic drug monitoring
TF Tissue factor

V

Vc Acute volume of distribution
Vss Volume at the steady-state
Vd Volume of distribution
vWF von Willebrand Factor
vWF:Ag von Willebrand Factor antigen

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Chapter 1- Introduction

1.1. Hemophilia

The dynamic physiological mechanism named hemostasis is important to maintain the normal blood flow after blood vessels trauma (Gale, 2011). It involves two coordinated systems: (a) the procoagulant, englobing the primary and secondary hemostasis to cease the blood loss through the thrombus formation and, (b) the anticoagulant system, englobing negative regulators towards the first system (Gale, 2011). Hemostasis disorders arise from the unbalance of these coordinated systems (Gale, 2011). For instance, the group of hypercoagulation diseases are caused by an anticoagulant deficiency whereas, in hypocoagulation diseases procoagulant activity is in deficit (Gale, 2011).

Hemophilia is acknowledged as the "royal family disease" after the discovery that, Queen of England Victoria (1837 to 1901) carried the hemophilia gene and her son expressed it, dying with 31 years old with a brain haemorrhage (Franchini and Mannucci, 2014).

Clinically, hemophilia leads patients to a continuous and uncontrollable bleeding (Srivastava *et al.*, 2020) due to the poor clotting activity (i.e. hypocoagulation). The faulty coagulation process is related to the deficiency regarding the clotting plasma factors (Lippi *et al.*, 2012). Depending on the missing clotting plasma factor, hemophilia may be classified as: Hemophilia A (HA), associated to the Factor VIII (FVIII); Hemophilia B, related to the Factor IX (FIX) or, Hemophilia C, as a defect on Factor XI (FXI) (Lippi *et al.*, 2012; Palta, Saroa and Palta, 2014). Regardless of the different types, the present work focuses on HA.

1.1.1. Epidemiology

Hemophilia A, like other types of hemophilia, is an inherited disorder (Carcao, Moorehead and Lillicrap, 2018). The expression of FVIII is coordinated by the FVIII gene, located in the long X chromosome arm, at Xq28 (Carcao, Moorehead and Lillicrap, 2018). This gene is special as it presents, at intron 22, two distinct extra coding elements, the FVIIIA and FVIIIB **(Figure 1)** (Carcao, Moorehead and Lillicrap, 2018). These extra copies are involved in the most common mutations associated to HA. These mutations are recurrent inversion mutations exerted by an intrachromosomal recombination (Carcao, Moorehead and Lillicrap, 2018).

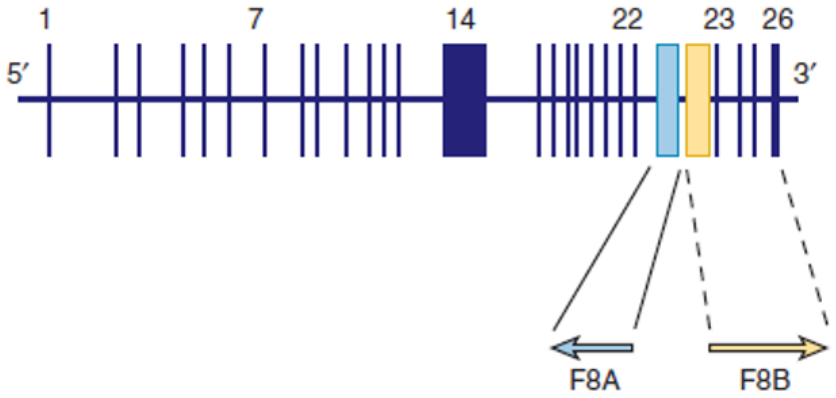

Figure 1- Representation of Factor VIII gene and the extra copies, F8A and F8B. *Copyrights from it (Carcao, Moorehead and Lillicrap, 2018).*

This recurrent mutation, is mostly verified in severe type of HA, which correspond to approximately 45% of cases (F Giannelli, 2001). Furthermore, it is only expressed at male germline, in sperm cells, reason for men being the most affected by HA whereas, women are mainly carriers of the gene (Carcao, Moorehead and Lillicrap, 2018; Srivastava *et al.,* 2020).

Generally, pathogenic mutations in FVIII gene will lead to a dysfunctional FVIII protein as they will interfere at the: (I) secretion, targeting the folding and intracellular processing; (II) activation, as it becomes slower; (III) stability, affecting the structure of the cofactor FVIIIa and/or (IV) function, with abnormal interaction with serine protease Factor IX (FIXa) as further abnormal tenase complex (Fang, Wang and Wang, 2007).

1.1.2. Incidence and Prevalence

The most common form of all hemophilia's is HA, englobing 80 to 85% of the cases (Srivastava *et al.*, 2020). Data from the 2019 annual report revealed that, the worldwide incidence of HA was 24.6 cases per 100,000 male birth patients while the prevalence, surrounded the 17.1 cases per 100,000 male patients (World Federation of Hemophilia, 2020). Since that the current worldwide number of males is estimated to be 3.8 billion, the number of patients with HA is expected to be 794,000 (World Federation of Hemophilia, 2020).

In Portugal, the number of HA patients is around 700 to 800 cases with the incidence, approximately, 22 cases per 100,000 males' births (Café *et al.*, 2019).

1.1.3. Factor VIII Protein Levels

Laboratory testing is essential to assess the clot rate formation upon activation of the coagulation cascade (Winter, Flax and S.Harris, 2017).

Accurate diagnosis for HA should be suspected when a patient, regardless of the age, presents a clinical medical history of easy bruising, spontaneous bleeding with no specific underlying reasons or, an excessive bleeding after any trauma or surgery (Srivastava *et al.*, 2020). In this context the prothrombin time (PT) and the activated partial thromboplastin time (aPTT) should be performed **(Figure 2)** (Winter, Flax and S.Harris, 2017).

In inherited deficiency disorders usually the PT will be normal whereas aPTT will be prolonged (Winter, Flax and S.Harris, 2017). Additionally, a prolonged aPTT may be related to different clotting factors defects or, to an immunologic response explained by the production of inhibitors as it will be discussed in **1.6. Immunogenicity** (Peyvandi *et al.*, 2020).

Therefore, mixing studies are the next step to accurately confirm the abnormal aPTT prolongation (Peyvandi *et al.*, 2020; Winter, Flax and S.Harris, 2017). These studies usually involve an equal volume (50:50) of the citrated patient plasma mixed with normal pooled plasma. Provided that aPTT will correct (not prolonged) this explains that, the correction of the time, was due to a factor that was previously missing on patient plasma (i.e. hemophilia) (Winter, Flax and S.Harris, 2017). In case of not properly corrected, it means that other cause may be involved namely, an immunologic response (Peyvandi *et al.*, 2020; Winter, Flax and S.Harris, 2017).

In a scenario where the deficiency is a result of a missing clotting factor, confirming tests are required to know which clotting protein is lacking. They will measure coagulation activity of the patient, usually represented by F:C, as "F" for factor and "C" for plasma concentration at that time (Björkman and Berntorp, 2001). In case of HA, representation is FVIII:C with the values express in international units (IU) or percentage (%), per dL or mL, which means that 1 IU is the plasma activity present in 1 dL (or mL) of normal pooled plasma (Fijnvandraat *et al.*, 2012). One-stage assays or chromogenic assays are an example of these test as they either be helpful to a definitive diagnosis of HA or, for monitoring treatment (Peyvandi *et al.*, 2020).

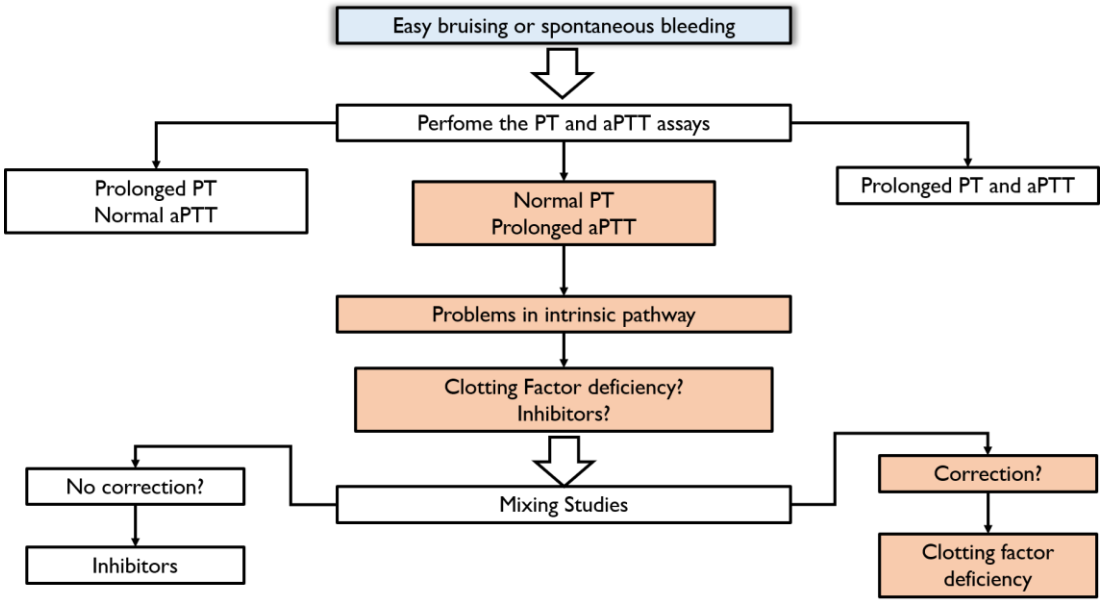

Figure 2 - Representation of the procedure to assess FVIII levels.

One-stage assays are often use in clinical practice (Srivastava *et al.*, 2020) where the patients' citrated plasma is mixed with plasma FVIII deficient (levels <1 IU/dL) and compared to a standard reference, with FVIII levels known (Winter, Flax and S.Harris, 2017). Results are represented with the clotting time in the y-axis and, the FVIII levels on the x-axis (Potgieter, Damgaard and Hillarp, 2015). As an example, if the results shows a FVIII:C of 7% comparing to the standard plasma and, if this standard reference had a FVIII:C about 85 IU/dL, this means that patients present a concentration FVIII 6 IU/dL (7% x 85) (World Federation of Hemophilia, 2010).

On the other hand, chromogenic tests use the patient plasma in mix with other coagulation cascade factors such as thrombin or prothrombin, factors IX and X, calcium and phospholipids in order to encourage the activation of FVIII and, subsequently will interact with factor X (FX) as reported in **1.3. Biological Mechanism of Factor VIII** (Teichman, Razzaq and Sholzberg, 2018). Here, the assay will measure the rate of FVIII to form the FX cofactor (FXa) by adding a chromogenic substrate (p-nitroanaline) that will reproduce a yellow colour (due to specific affinity towards FXa) (World Federation of Hemophilia, 2010). The emitted colour is proportional to the amount of FVIII present in patient plasma (Teichman, Razzaq and Sholzberg, 2018).

In addition, the two methods have discrepancies between them, in which FVIII:C levels tend to be 15-20% lower with one-stage assays. This has implications for interpretation of pharmacokinetic parameters, particularly with overestimation of the half-life (Delavenne and Dargaud, 2020). Chromogenic assays are more accurate to detect FVIII:C between 0.1-2 IU/dL

(Srivastava *et al.*, 2020) being hence, ascribed as the best method for monitoring clotting factor concentrates (CFCs) (Delavenne and Dargaud, 2020; EMA, 2017). However, these assays are not always available in clinical practice so, guidelines emphasize that for pharmacokinetic studies it is important to use always the same method to reduce data variability (Delavenne and Dargaud, 2020; Srivastava *et al.*, 2020).

1.1.4. Degrees of Severity

Healthy patients present a FVIII:C between 40 to 150 IU/dL whereas, HA ones will have values below this normal range (Fijnvandraat *et al.*, 2012; Srivastava *et al.*, 2020). Depending on the residual FVIII activity, HA severity may be classified into, mild, moderate and severe **(Table 1)** (Srivastava *et al.*, 2020).

Table 1- Factor VIII levels associated to severity and bleeding occurrences.

Residual FVIII Levels (IU/dL)	Severity	Bleeding Manifestations
40-150	Healthy	None
5-40	Mild	Bleeding with major trauma/surgeries
1-5	Moderate	Bleeding with minor trauma/surgeries Occasional cases of spontaneous bleeding
<1	Severe	Bleeding with minor trauma/surgeries Recurrent spontaneous bleeding in joints or muscles

Severe HA is described by recurrent and spontaneous bleeds with 90% starting at the joints (knees, elbows, ankles, shoulders and wrists) (Jayandharan and Srivastava, 2008), 10 to 25% at the muscles (i.e. lower legs or forearms) (Peyvandi, Garagiola and Young, 2016), and 5-10% for other sites (Srivastava *et al.*, 2020). As for newborns and children with severe phenotype, it is common for them to experience soft tissue and intramuscular bleeding, mucocutaneous bleeding, extracranial and intracranial hemorrhage (Peyvandi, Garagiola and Young, 2016; Srivastava *et al.*, 2020). The previous 2019 annual report also offered predictions regarding the incidence and prevalence for severe phenotypes. Namely, the incidence was estimated for 9.5 cases per 100,000 males, whilst prevalence was 6.0 cases per 100,000 males. Globally, on those 794,000 patients with HA, 270,000 are severe (World Federation of Hemophilia, 2020).

In contrast, mild hemophilia patients are often under-diagnosed (Benson *et al.*, 2018) as they only bleed when triggered by a trauma or major surgeries (Srivastava *et al.*, 2020). Thus, moderate patients, are more sensible to bleeding in minor surgeries and they may even start to experience spontaneous bleedings (Srivastava *et al.*, 2020).

1.1.5. Clinical Evidence of Uncontrolled Factor VIII Levels

1.1.5.1. Low Levels of FVIII

Extreme low FVIII levels (<1IU/dL) represent 60 to 70% of the hemophilia population (Jayandharan and Srivastava, 2008). These patients may experience approximately 15 to 35 spontaneous bleeding into the joints and muscles, per year, without a proper management (Jayandharan and Srivastava, 2008).

Prolonged spontaneous bleeding into the joints, also referred as hemarthrosis, will promote the release of iron from hemoglobin, stimulating the production of cytokines and pro-angiogenic factors (Melchiorre, Manetti and Matucci-cerinic, 2017). This will lead to an acute intra-articular inflammation named synovitis, and hypertrophy of synovium, called hemophilic synovitis (Jayandharan and Srivastava, 2008; Melchiorre, Manetti and Matucci-cerinic, 2017).

A continuous environment of inflammation and hypertrophy will induce a chronic and vicious cycle on the same joint named as target joint, that will progressively lead to bone damage, osteoporosis, degeneration of the articular cartilage and atrophy of the muscles (Melchiorre, Manetti and Matucci-cerinic, 2017). Hemophilic arthropathy is the denomination for this final stage of the worst clinical outcome of the disease as patients experience extreme disability in their lives (Melchiorre, Manetti and Matucci-cerinic, 2017).

Another consequence is the bleeding into the muscle (Kumar and Carcao, 2013). The mainly concern remains at the muscle located on the pelvis as it is difficult to control the loss of blood as well as prolonged hematomas that may lead to atrophy of the tendons, ossifications or hemophilic pseudotumor (Kumar and Carcao, 2013).

Moreover, even though central nervous bleeding is less frequent to happen (<5%) in hemophilia, it should not be disregarded, since it could be life-threatening (Jayandharan and Srivastava, 2008). The simplest headaches for a long period of time or, a simplest somnolence, in HA may be an early diagnosis for intracranial bleeding (Srivastava *et al.*, 2020). If not properly detected, there is a strong probability for permanent neurological damage (Jayandharan and Srivastava, 2008).

In conclusion, low levels of FVIII are dangerous for the patient life and so, a proper control of FVIII levels is crucial to not just prevent the irreversible damages in joints but also, to reduce the consequences of clinical futures in patients life (Davari *et al.*, 2019). Indeed, uncontrolled levels will increase patient related outcomes such chronic pain, disability, society deprivation associated with anxiety and depression, all subscribed for a negative impact in quality of life (Davari *et al.*, 2019).

1.1.5.2. High Levels of FVIII

In contrast to the poor levels, elevated levels of FVIII are related to the increased risk for thrombosis, particularly the venous thrombosis (Kamphuisen, Eikenboom and Bertina, 2001). Several studies concluded that high FVIII levels (≥150 IU/dL) were observed in 57% of the patients with recurrent venous thrombosis (Kamphuisen, Eikenboom and Bertina, 2001). This can be explained through the increase of thrombin and fibrin rate or, by the influence of high FVIII levels on activated protein C (essential to down-regulate coagulation cascade) in inducing resistance (Kamphuisen, Eikenboom and Bertina, 2001).

On other hand, arterial thrombosis can also happen with higher levels of FVIII (Kamphuisen, Eikenboom and Bertina, 2001). The explanation may rely on von Willebrand factor (vWF) which is increased due to forces in stenosis vessels, stimulating platelet adhesion/aggregation at the damage arterial wall or, the higher FVIII levels itself will increase thrombin formation and platelets activation (Kamphuisen, Eikenboom and Bertina, 2001).

1.1.6. Factors of Variability of Factor VIII Levels

Plasma FVIII levels can vary considerably between patients, affecting the pharmacokinetic profiles, consequently requiring drug adjustments (Turecek *et al.*, 2020). The major factors that determine FVIII variability include: age, bodyweight, gender/ethnicity, vWF antigen levels, ABO blood group, immunogenicity, pregnancy and liver diseases (Franchini, 2006; Miesbach *et al.*, 2009; Turecek *et al.*, 2020; Wang *et al.*, 2017). All this variability factors will be explained further at **1.7.5 Factors that Contribute to Inter-Individual Variability.**

1.2. Treatment Management

1.2.1. Evolution History

Over the years, HA treatment suffered strong changes. The first therapeutic option was available in 1840 with blood transfusions **(Figure 3)** (Peyvandi, Garagiola and Young, 2016). From 1950 to 1960, patients were treated with fresh frozen plasma but, unfortunately, the amount of FVIII was not enough hence, patients either died at early ages or, lived longer with low quality of life due to several comorbidities (Swiech, Picanço-Castro and Covas, 2017).

The year of 1964 was marketed by Judith Pool discovery as she attaining large amounts of FVIII in thawing plasma allowing better care in HA (Franchini and Mannucci, 2014). Years later **(Figure 3),** plasma-derived FVIII (pdFVIII) concentrates were the first home treatment, developed from patients pooled plasma that offered a better control in bleeding as well as an

improvement in patient's quality of life (Franchini, 2013). Seven years later, mild severity gained an effective, safer and cheaper treatment, the desmopressin (DDAVP) (Franchini, 2013).

However, treatment journey was not always a golden path as in 1980 a "dark era" was witnessed, after several patients prescribed with pdFVIII, were infected with human immunodeficiency virus (HIV) or hepatitis C virus (HCV) **(Figure 3)** (Franchini, 2013). Most of these patients either died or lived with severe sequels (Franchini, 2013).

In parallel with the development of new viral inactivation steps DNA technology was exponentially increasing contributing for the important cloning of the FVIII gene **(Figure 3)** (Franchini and Mannucci, 2014). As a result, it was finally possible to reproduce the FVIII protein in mammalian cells by recombinant DNA technology (Franchini and Mannucci, 2014). These new drugs, named recombinant concentrates (rFVIII), had their efficacy proven only in 1989 (Franchini and Mannucci, 2014) and the first launch in 1992 (Swiech, Picanço-Castro and Covas, 2017). Since then, rFVIII also had their improvements allowing for their categorization into generations **(1.4.3. Recombinant Concentrates)**.

Furthermore, innovations did not stop in rFVIII area **(Figure 3)** and, since 2010 there has been an interest for gene therapy as it is a more specific and accurate to stimulate the body to synthetize the missing protein (Consortium, 2017). As an opportunity to improve even more the quality of life of this population, this still being a field with a lot of research and clinical trials.

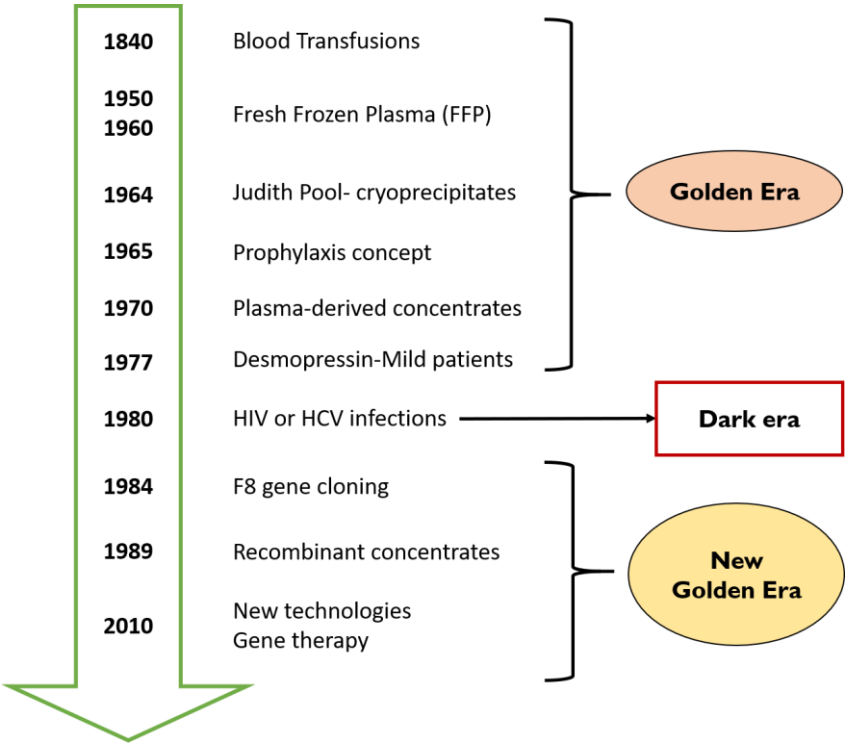

Figure 3 - Treatment evolution in hemophilia A.

1.2.2. Therapeutic Regimens

In HA, the available regimens are distinguished by the final purpose of the treatment. For instance, situations of episodic bleeding, patients are treated under the on-demand regimen whereas, prophylaxis regimen can help in preventing the worsening of clinical outcomes (Srivastava *et al.*, 2020).

The on-demand approach consists on the administration of the CFCs only when the bleeding episode starts to occur in order to reduce the pain and manage the impact of the bleeding at that time (Srivastava *et al.*, 2020; Steen Carlsson *et al.*, 2003). Since the use is recommended only at the start of an episode, the chances of a small bleeding turning into a larger hemorrhage and, subsequently, develop the worst clinical outcome are high, so the goal standard regimen remains on prophylaxis (Steen Carlsson *et al.*, 2003).

Prophylaxis concept emerged in 1965 by Ahlberg after his observation of less hemorrhages and less cases of hemophilic arthropathy, in moderate HA patients (Hazendonk *et al.*, 2018). Accordingly, it was mandatory to stablish prophylaxis as a regimen that maintained FVIII levels above 1 IU/dL to convert the severe stage into moderate/milder stage and, hence, experience less HA symptoms (Collins *et al.*, 2011). Therefore, an effective prophylaxis involves regular intravenous infusions of CFCs not only to prevent and preserve the musculoskeletal function but also, to allow a normal life-style and a better quality of life (Srivastava *et al.*, 2020).

Prophylaxis may be subdivided in three classes (primary, secondary, tertiary) based on when patient started the treatment management **(Table 2)** (Srivastava *et al.*, 2020). Usually, when the patients start the prophylaxis early in life (i.e. primary and secondary) the chances of getting better long-term outcomes are higher so, in clinical practice, this is seen as a goal standard choice (Blatn *et al.*, 2016; Srivastava *et al.*, 2020). In contrast, later prophylaxis will only reduce the pain and inflammation as well as slowing down the progression of HA (Blatn *et al.*, 2016; Srivastava *et al.*, 2020).

Table 2 - Type of prophylaxis by the age of initiation.

Class of prophylaxis	Age of initiation	Expected clinical history
Primary	<3 years old	✓ Physical exams and/or imaging test with no joint disease ✓ Before the second evident joint bleed
Secondary	≥3 years old	✓ Before the onset of a joint disease ✓ After two or more joint bleed
Tertiary	Any age; mostly adults	✓ After a documented joint disease

The intensity of prophylaxis depends on dose and frequency of administration **(Table 3)** (Srivastava *et al.*, 2020). For instance, the Swedish prophylaxis (or the Malmö protocol) is the highest intense regimen where the patient receives 25 to 40 IU/kg every two days for, at least, three times per week (e.g. Monday, Wednesday, Friday) (Blanchette, 2010). The Dutch regimen (or Utrecht protocol) is of intermedium intensity once patients are prescribed with 15 to 30 IU/kg two or three times per week to avoid spontaneous bleeding (Blanchette, 2010; Blatn *et al.*, 2016). The lowest intense regimen is considered when the dose varies between 10 to 15 IU/kg for two or three days per week (Srivastava *et al.*, 2020).

In alternative to these three main protocols, it is possible to start at low intensity (once weekly infusion) and escalate the frequency (Srivastava *et al.*, 2020). This is called the Canadian protocol (Blatn *et al.*, 2016), which is an important strategy in young children to enhance treatment compliance (Srivastava *et al.*, 2020).

Table 3 - The advantages and disadvantages of each prophylaxis intensity regimen in HA. *(Srivastava et al., 2020), (Carcao e lorio, 2015).*

Regimens	Intensity	Advantages	Disadvantages
Swedish (Malmö protocol)	Higher	➢ Guarantees minimum 1 IU/dL levels ➢ Lower annual joint bleeds ➢ Better long-term joint outcomes ➢ Great for active lifestyle patients	❖ Adherence (more infusions) ❖ Expensive (more doses) ❖ High overtreated mild phenotypes
Dutch (Utrecht protocol)	Moderate	➢ Less expensive ➢ More quality of life than low intensity ➢ Reduce chances of bleeding to 90% ➢ Low annual joint bleeds (1 per year) ➢ Good for adolescents and adults	❖ Undertreated patients ❖ Slightly worse long-term musculoskeletal outcomes
Low intensity		➢ Less expensive (many countries can afford) ➢ Reduce bleeding incidence *versus* on-demand by 80% ➢ Reduced annual joint bleeds to 3 per year	❖ Unknown long-term effect on musculoskeletal outcomes *(hypothesis to be worse than others)*

1.3. Biological Mechanism of Factor VIII

FVIII is a glycoprotein synthesized by the liver sinusoidal cells, Kupffer cells or even by the hepatocytes (Thompson, 2003). Briefly, in terms of structure, FVIII is a complex protein (Bolton-Maggs and Pasi, 2003) with an arrangement of : (NH2) A1-a1-A2-a2-B-A3-C1-C2 (COOH) **(Figure 4)** (Fay, 2004). In Golgi complex, this protein suffers proteolysis with two intracellular cleavages within the B-domain, resulting in a heavy chain with variable size (A1-A2-B domain) and light chain with constant size (A3-C1-C2 domain) **(Figure 5)** (Carcao, Moorehead and Lillicrap, 2018; Fay, 2004). FVIII is then secreted to the bloodstream as an inactive heterodimer, forming a noncovalent linking with the multimeric protein vWF (Carcao, Moorehead and Lillicrap, 2018).

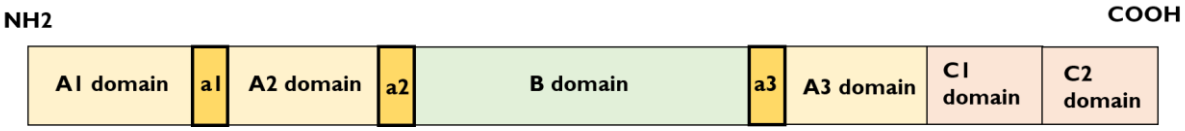

Figure 4 - Structure of Factor VIII before secretion.

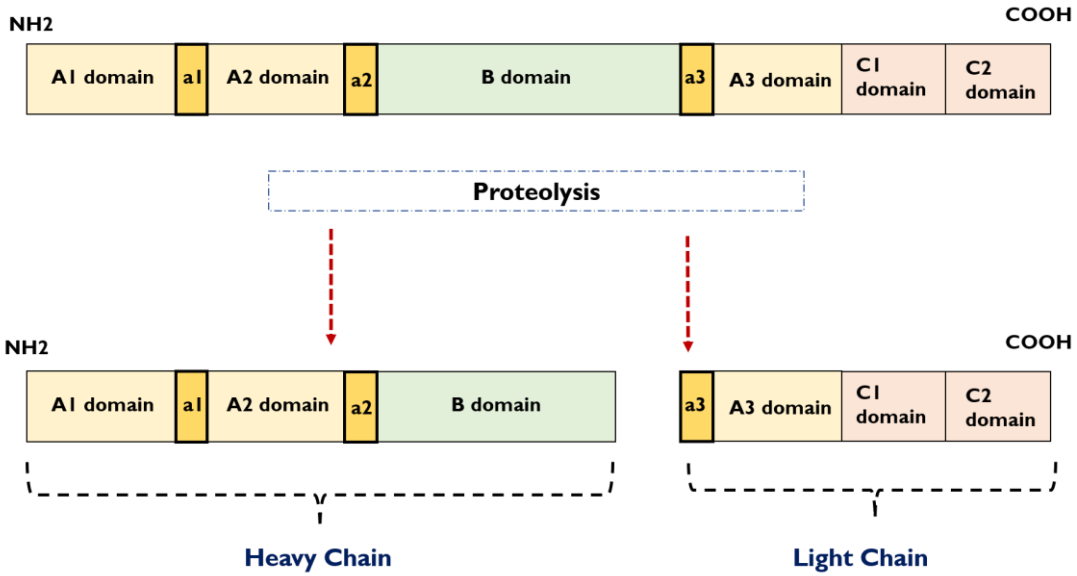

Figure 5 - Heterodimer structure of Factor VIII.

Coagulation cascade is activated upon a vascular damage (*initiation phase*) **(Figure 6).** This stimulation to the blood vessels leads to the release of tissue factor (TF) which, further, will bind to the activated factor VII (FVIIa) **(Figure 6)** (Palta, Saroa and Palta, 2014). The interaction between both factors is crucial to start the activation of the factor X (FX) and factor IX (FIX). The *initiation phase* ends with thrombin (FIIa) synthesized through the bound of activated FX (FXa) and activated factor V (FVa) (i.e. the prothrombinase complex) **(Figure 6)** (Kumar and Carcao, 2013). The amount of FIIa is very small at this stage (approximately 2% of the required concentration) therefore, the coagulation process is ineffective to arrange a proper platelet plug (Kumar and Carcao, 2013).

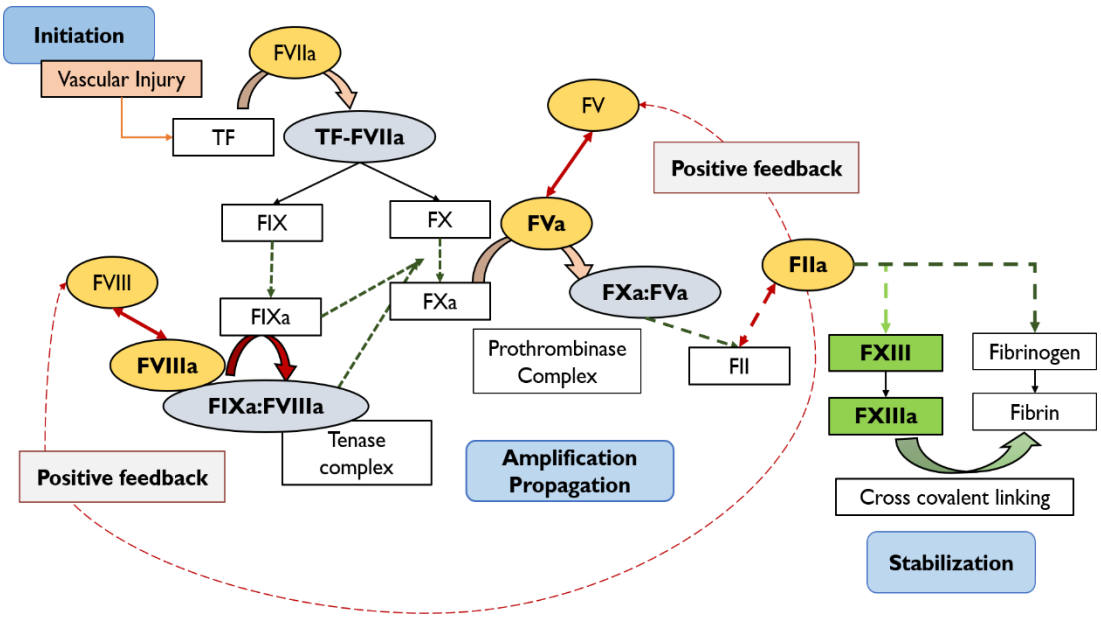

Figure 6 - Mechanism of coagulation cascade.

Abbreviations: ***TF:*** *tissue thromboplastin or tissue factor;* ***FVIIa:*** *factor VII activated;* ***FIX:*** *factor IX;* ***FIXa:*** *factor IX activated;* ***FX:*** *factor X;* ***FXa:*** *factor X activated;* ***FVIII:*** *factor VIII;* ***FVIIIa:*** *factor VIII activated; FV: factor V;* ***FVa:*** *factor V activated;* ***FII:*** *prothrombin;* ***FIIa:*** *Thrombin;* ***FXIII:*** *fibrin-stabilizing factor;* ***FXIIIa:*** *fibrin-stabilizing factor activated*

As a result FIIa will activate FVIII by interacting at heavy chain sites (at Arg372 in A1-A2 domain and Arg740 in A2-B domain) and at light chain as well (at Arg1689 in B- A3 domain) **(Figure 7),** to dissociate FVIII from vWF (Carcao, Moorehead and Lillicrap, 2018; Mazurkiewicz-Pisarek *et al.*, 2016). FVIII will then change his structure to an heterotrimer (A1, A2 and A3-C1-C2 domain) **(Figure 7)** (Carcao, Moorehead and Lillicrap, 2018).

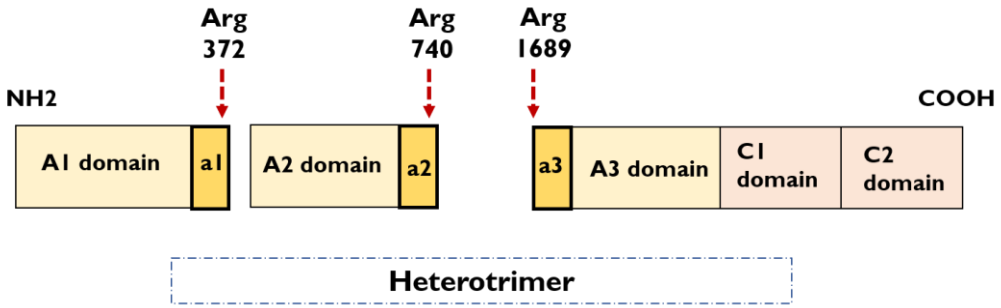

Figure 7 - Heterotrimer structure of Factor VIII.

Parallel to FVIII activation, FIIa will also target factor V (FV) to activate his cofactor (FVa) (Kumar and Carcao, 2013). Consequently, FVIIIa interacts with FIX and form the tenase complex (FVIIIa:FIXa), enhancing the activation of the FX (Kumar and Carcao, 2013) whereas, FVa binds to the FXa (prothrombinase complex) to create more prothrombin (FII), completing the *amplification phase* with this positive feedback response **(Figure 7)** (Kumar and Carcao, 2013). This mechanism guarantees a continuous production of FIIa at the surface of activated platelets (*propagation phase)* (Palta, Saroa and Palta, 2014). Furthermore, FIIa acts on fibrinogen to form the fibrin monomers (Hall, 2016), which are weakly connected. Therefore, FIIa activates the factor XIII (FXIIIa) to covalently link the monomers and held together a strong clot to cease the blood (*stabilization phase*) **(Figure 7)** (Kumar and Carcao, 2013).

To sum up, FVIIIa plays a critical role in the middle phase of coagulation (*amplification/propagation*) being an essential cofactor for the intrinsic tenase complex and prothrombinase complex in order to increase the levels of thrombin and cease the blood loss with thrombus formation (Carcao, Moorehead and Lillicrap, 2018; Fang, Wang and Wang, 2007).

1.4. Factor VIII Concentrates

1.4.1. Definition

Since hemophiliacs do not synthesize enough amount of FVIII, the treatment rational is the replacement of FVIII levels by CFCs administration (Srivastava *et al.*, 2020). The FVIII concentrates are a wide group that englobes plasma-derived products (pdFVIII) and the recombinant ones (rFVIII) **(Figure 8)** (Srivastava *et al.*, 2020). However, these drugs are expensive and may not be supported in poor the health system (Srivastava *et al.*, 2020). Instead, they opt between the cryoprecipitates or the fresh frozen plasma **(Figure 8)**, even though these are not submitted to a safety inactivation procedure like pdFVIII. Therefore patients have a high probability of developing infections (Srivastava *et al.*, 2020).

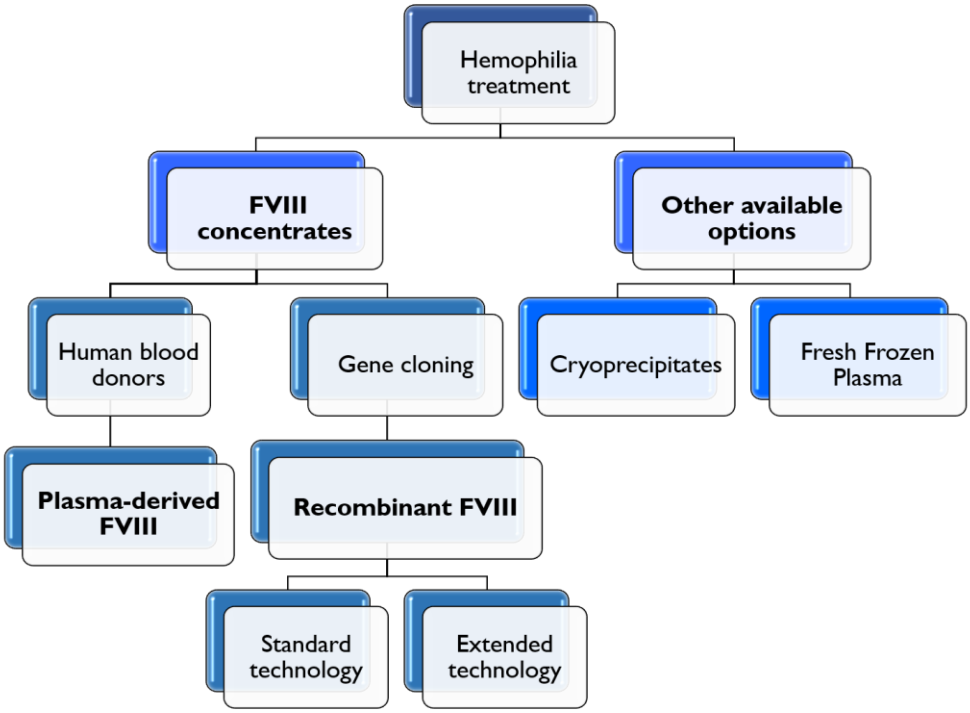

Figure 8 - Representation of the different drug groups for HA treatment.

1.4.2. Plasma-derived concentrates

Plasma FVIII concentrates had been available since 1970 (Franchini, 2013). They are obtained by cryoprecipitation which is the result of a precipitation process at cold temperatures (Burnouf, 2007).

As explained previously **(1.2.1 Evolution History)**, this first home replacement treatment had a terrible path started in the first part of 1980 with the HIV transmission, in 60-70% of the severe HA (Cafuir and Kempton, 2017), and almost 95% infected HCV in 1990 (Cafuir and Kempton, 2017; Raso and Hermans, 2018). Besides these viruses, there were more transmissible as descried in **Table 4** (World Health Organization, 2004).

Table 4 - Classification of the different type of viruses *(Klamroth, Gröner and Simon, 2014).*

Classification	Name
Enveloped	Human Immune deficiency Virus (HIV)
	Hepatitis C Virus (HCV)
	Hepatitis B Virus (HBV)
Non-enveloped	Hepatitis A virus (HAV)
	Parvovirus B19 (B19V)

As these concentrates derived from plasma donors, aforementioned contaminations only happened due to the lack of viral purification steps within the manufacturing process (Franchini, 2013). Since then, the scientific community and pharmaceutical industries improved the safeness of pdFVIII by introducing viral inactivation techniques such as: (a) dry heat; (b) pasteurization; (c) vapor heat and, (d) solvent/detergent **(Table 5)** (Klamroth, Gröner and Simon, 2014). In addition, screening tests started to be mandatory (i.e. nucleic acid amplification testing) (Franchini, 2013).

In the past, dry heat treatments were pdFVIII concentrates submitted to temperatures between 60-80°C, for 24 to 96 hours, only inactivating the HIV (Burnouf, 2018). Therefore, alternatives had emerged and products are now either submitted to higher temperatures like 80°C or 100°C, for 72 hours or 30 minutes respectively, targeting a wide variety of virus, including HIV, HCV and hepatitis HAV (Burnouf, 2018).

On the other hand, pasteurization is a highly effective method as the drug undergoes for heat treatment (60°C), for 10 hours, in the presence of the FVIII stabilizers (i.e. sugars, amino acids, or acetate) to prevent loss of activity (Klamroth, Gröner and Simon, 2014) (Burnouf, 2018). Similar to this procedure is vapour heat where, water vapour is added before heating the product to 60°C for 10 hours (Klamroth, Gröner and Simon, 2014).

Finally, the solvent/detergent technology is the most effective for lipid membranes of certain virus (Burnouf, 2018). It consists in a mix of organic solvents (e.g. tri-n-butyl-phosphate) and detergents (e.g. Tween-80 or Triton X-100) that will target the membrane of the virus and, consequently, inactivate them (Burnouf, 2018). It is effective against HIV, HBV, and HCV as well as the West Nile virus, Dengue virus and Zika virus (Burnouf, 2018).

Alongside with viral inactivation procedures, pdFVIII concentrates go through purification by chromatography methods that will separate the viruses from the protein (World Health Organization, 2004). There are several variants of chromatography assays but the most used on pdFVIII are the affinity chromatography and the ion exchange chromatography (Burnouf, 2007). The difference between them is on the molecules that are used to separate the components. For instance, the affinity chromatography is more specific and using ligands such heparin, metals or gelatine (Burnouf, 2007) whereas, the ion exchange uses electric charges molecules (Burnouf, 2007).

Prior to formulation, pdFVIII are filtered to remove smaller viruses that could be present in the product (Klamroth, Gröner and Simon, 2014). The method used in FVIII is nanofiltration with filters pores ranging from 35 to 15 nm (Klamroth, Gröner and Simon, 2014), allow the protein to go through whilst the virus is retained on the nanofilter (Burnouf, 2018) (Klamroth, Gröner and Simon, 2014).

A side but important note regards the possibility of the contamination and subsequent transmission of prions within pdFVIII (Klamroth, Gröner and Simon, 2014). Prions are associated with fatal neurodegenerative disorders like the Creutzfeldt-Jakob disease (Klamroth, Gröner and Simon, 2014). They are resistant to inactivation procedures (Burnouf, 2007) and therefore the only steps that have been proved to be efficient are precipitation, chromatography and filtration (Klamroth, Gröner and Simon, 2014).

Table 5 - Summary of plasma-derived concentrates characteristics available in Portugal.

Trade Product Name	Year of approval	Manufacture	Product Characteristics			Reference
			Active Substance	Viral Inactivation	Viral purification	
Emoclot	1999	Kedrion S.p.A.	FVIII	S/D Dry heat	Ion exchange chromatography	(INFARMED. IP., 1999)
Fanhdi	2001	Grifols	FVIII	S/D Dry heat	Heparin ligand chromatography	(INFARMED. IP., 2001)
Octanate	2015	Octapharma	FVIII	S/D	Ion exchange chromatography	(INFARMED. IP., 2015) (Octapharma , 2009)
HaemateP	2000	CSL Behring	FVIII +vWF	Pasteurization	Multiple precipitation	(INFARMED. IP., 2000)
Wilate	2012	Octapharma	FVIII +vWF	S/D	Ion exchange chromatography	(INFARMED. IP., 2012) (Stadler *et al.*, 2006)

Abbreviations: **FVIII:** *factor VIII;* **vWF:** *von Willebrand factor;* **S/D:** *solvent/detergent*

1.4.3. Recombinant Concentrates

Cloning was a big step for HA treatment. Recombinant FVIII concentrates consists on an heterologous transfection of the FVIII DNA plasmids into a cell line that is, then, cultured and stabilized by plasma proteins derived from humans or animals (Raso and Hermans, 2018). In contrast with the previous products rFVIII are safer, justified by the significant reduction of the transmission of viruses and/or prions (Raso and Hermans, 2018; Saenko *et al.*, 2003).

The concentrates evolved over the years in terms of the manufacturing methods and, on the incorporated technology to gain more efficacy in bleeding control. To simplify, several generations (four exactly) were created to distinguish each drug **(Figure 9)**. Furthermore, these generations are only validated to the first manufactured products, the standard half-life (SHL) while, the extended half-life (EHL) ones are the recently innovations formulated to prolong the rFVIII activity (Raso and Hermans, 2018).

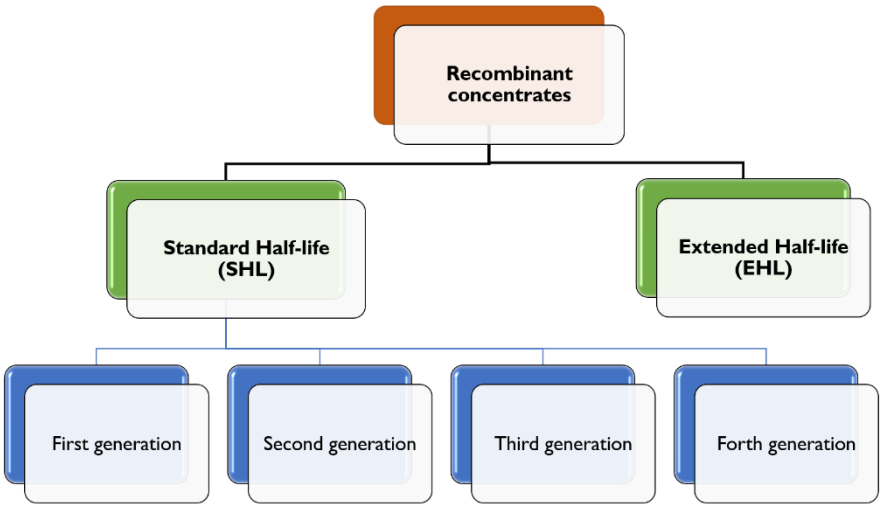

Figure 9 - Representation of the different recombinant FVIII drug generations.

1.4.3.1. Standard Half-life

1.4.3.1.1. First Generation

The first SHL product was launched in 1992 by FDA, named as Recombinate® or, also known as Antihemophilic Factor (Raso and Hermans, 2018). This product was cultured in the non-human cell line (Chinese Hamster Ovary [CHO] cells), using animal proteins in medium culture (e.g. bovine-insulin, -aprotinin and -albumin) and, human albumin as stabilizer (Franchini and Lippi, 2010). For viral safety, affinity chromatography by a monoclonal antibody (immunoaffinity) was introduced alongside with ion exchange chromatography (Franchini and Lippi, 2010). Unfortunately, this first generation had a reported risk of transmission of nonenveloped viruses and prions associated to the Creutzfeldt–Jakob disease (Swiech, Picanço-Castro and Covas, 2017) and, hence, the pharmaceutical industries started to improve their manufacturing process by developing new generations of rFVIII.

1.4.3.1.2. Second Generation

The second generation of SHL products is characterized by the use of human proteins in medium culture (e.g. human serum albumin) instead of the animal-derived and, by the replacement of albumin as stabilizer for sucrose (Swiech, Picanço-Castro and Covas, 2017).

Kogenate Bayer® (also marked as Kogenate FS® outside of Europe Union) is a drug of this generation, using octocog alfa as the active substance and Baby Hamster Kidney (BHK) as the cell line used to express the FVIII (Franchini and Lippi, 2010). Moreover, in viral purification, S/D and filtration were coupled to chromatography to guarantee the inactivation of the viruses (Raso and Hermans, 2018).

Helixate NexGen® (marketed as Helixate FS® outside of Europe Union) was also part of this generation, but it was recently withdrawn from the European Union as requested by marketing authorization holder, Bayer AG (European Medicines Agency, 2019).

1.4.3.1.3. Third Generation

The third generation emerged aiming to recreate rFVIII drugs with reduced chances of viruses transmission through the loss of the animal or human-derived proteins throughout all the manufacturing process (Franchini and Lippi, 2010). This is the most extensive generation so far introduced in the market and the most prescribed in clinical practice.

The first drug fitting into this category was ADVATE® using the same active substance as the previous generation (octocog alfa) but a different cell line, CHO cells (Franchini and Lippi, 2010). It is the reference drug used in comparative and bioequivalence studies for new drugs. Its pharmacokinetics was studied in 195 subjects with severe HA through at all age window **(Table 6)** (European Medicines Agency, 2009). The drug proved efficacy in controlling and preventing bleeds as prophylaxis, with 88.5% of successful rate, by infusing only one or two doses (Shapiro, 2007). The annual bleed rate (ABR) for standard prophylaxis (25–40 IU/kg, 3–4 times a week) was 6.0 whereas, on-demand was 18.5 suggesting that ADVATE® is more efficient in patients who adhere to prophylaxis (Shapiro, 2007). Finally, ADVATE® is safe with no inhibitors detected (Shapiro, 2007).

Table 6 - Pharmacokinetic parameters of ADVATE® regarding patients age *(European Medicines Agency, 2009).*

Pharmacokinetic Parameters					
Age group	Cmax (IU/dL)	AUC_t (IU*h/dL)	$t_{1/2}$ (h)	CL (mL/h/kg)	Incremental recovery (IU/dL per IU/kg)
Adults ≥18 years	111.3 ± 27.1	1538.5 ± 519.1	12.9 ± 4.3	3.6 ± 1.2	2.2 ± 0.6
Adolescents 12 to <18 years	107.6 ± 27.6	1317.1 ± 438.6	12.1 ± 3.2	4.1 ± 1.0	2.1 ± 0.6
Children 5 to <12 years	100.5 ± 25.6	1506.6 ± 530.0	11.8 ± 3.8	3.8 ± 1.5	2.0 ± 0.5
2 to 5 years	90.8 ± 19.1	1180.0 ± 432.7	9.6 ± 1.7	4.8 ± 1.5	1.8 ± 0.4

Abbreviations: ***AUC_t:*** *area under the plasma FVIII activity curve from 0 to the last measurable point;* ***Cmax:*** *maximum concentration;* ***CL:*** *clearance;* ***$t_{1/2}$:*** *terminal half-life.*

Refacto AF® (moroctocog alfa) emerged as the first product to have the B-domain deleted (BDD) from the FVIII structure (Franchini and Lippi, 2010). The rational of removing one domain of the mature protein could lead to think that this product would be not effective as the standard molecule. However, upon its delivery into the bloodstream, the FVIII molecule is activated by thrombin (FIIa) and the conformational structure changes for an heterotrimer **(Figure 7)** with no B-domain involved (Carcao, Moorehead and Lillicrap, 2018). Therefore, the removal of BDD does not interfere with FVIII function. Indeed, it enhances the secretion even more, as studies concluded higher levels (i.e.17-fold higher) of FVIII mRNA (Orlova *et al.*, 2013).

In terms of efficacy, moroctocog alfa prevents spontaneous bleeding in a defined prophylaxis routine, especially in patients with history of target joints (Recht *et al.*, 2009). Direct comparisons of moroctcog and ADVATE® **(Table 7)** were performed in 30 patients (>12 years old) to prove bioequivalence. The results of pharmacokinetics parameters (AUC_{0-t} ; $AUC_{0-\infty}$; incremental recovery) were all within the 80-125% interval (i.e. 90% CI) (Recht *et al.*, 2009). The baseline PK parameters are represented in **Table 8**.

Table 7 - Pharmacokinetic parameters and bioequivalence results between ReFacto AF® and ADVATE® *(Recht et al., 2009).*

Pharmacokinetic Parameters				
Concentrate	AUC_{∞} (IU*h/mL)	AUC_t (IU*h/mL)	In vivo Recovery (%)	Incremental recovery (IU/dL per IU/kg)
ReFacto AF®	14.7 ± 6.1	13.8 ± 5.7	112 ± 22	2.35 ± 0.47
ADVATE®	16.5 ± 6.3	15.0 ± 5.4	114 ± 30	2.39 ± 0.65
90% log-transformed CI	81.6–94.8%	83.3–96.9%	ND	92.5–108%

Abbreviations: ***AUC_t:*** *area under the plasma FVIII activity curve from 0 to the last measurable point,* ***AUC_{∞}:*** *area under the plasma FVIII activity curve extrapolated to infinity;* ***CI:*** *confidence interval;* ***ND****: not defined.*

Table 8 - Pharmacokinetic parameters of moroctocog alfa for adolescents/adults *(European Medicines Agency, 2009).*

Pharmacokinetic Parameters					
Age group	AUC_t (IU*h/mL)	$t_{1/2}$ (h)	CL (mL/h/kg)	MRT (h)	Incremental recovery (IU/dL per IU/kg)
Adolescents/Adults ≥12 years	19.9 ± 4.9	14.8 ± 5.6	2.4 ± 0.75	20.2 ± 7.4	2.4 ± 0.38

Abbreviations: ***AUC_t:*** *area under the plasma FVIII activity curve from 0 to the last measurable point;* ***CL:*** *clearance;* ***MRT:*** *mean residence time;* ***$t_{1/2}$:*** *terminal half-life.*

Furthermore, moroctocog alfa revealed to be effective in prophylaxis regimen (30 IU/kg; three times per week) in 94 patients as 60.6% of them did not experience spontaneous bleedings, contributing for a low annual bleeding rate (ABR=3.9) (Recht et al., 2009). Safety of moroctocog alfa was proved in clinical trials with no significant immunogenicity response (Recht et al., 2009).

NovoEight® (turoctocog alfa) is other product of the third generation with improvements of the B-domain (Ezban, Vad and Kjalke, 2014). This drug uses the B-domain truncated (BDT) and, even though the B-domain is not essential for the FVIII activity, it is usually highly glycosylated as a result of post-translational changes (N-linked glycosylation and O-linked glycosylation) **(Figure 10)** to a proper intracellular transport and subsequent processing of the FVIII protein (Orlova *et al.*, 2013).

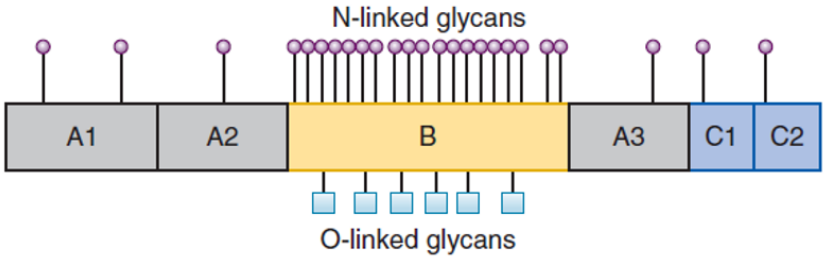

Figure 10 - Illustration of post-translational modifications within FVIII *(Carcao, Moorehead and Lillicrap, 2018).*

Moreover, the full-length structure drugs have, usually, nineteen N-linked glycosylation, making them more difficult to express the FVIII (Ahmadian *et al.*, 2016). Therefore, turoctocog alfa has the advantage of being express more easily due to the truncation of the respective domain as it has only four N-linked glycosylation (two in A1 domain, one in A3 domain and one at C1 domain) (Ezban, Vad and Kjalke, 2014) and one O-linked glycosylation at the B-domain (Ser750) **(Figure 11)** (Raso and Hermans, 2018).

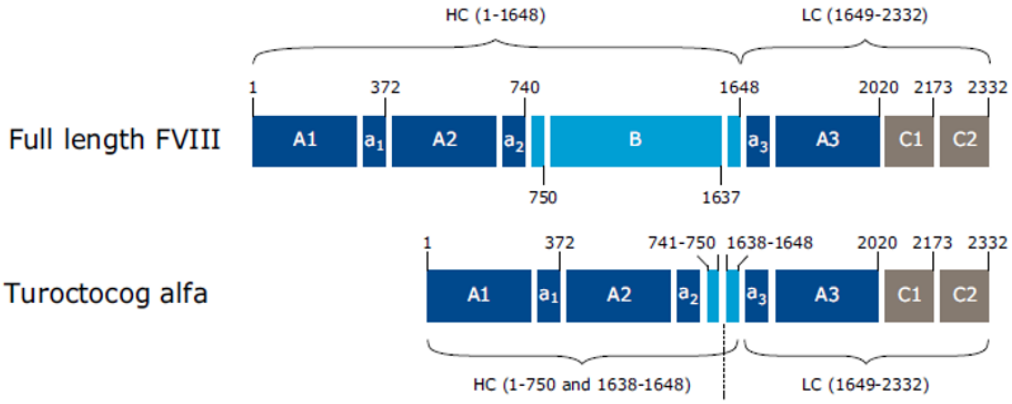

Figure 11- Design representation of turoctocog alfa *(Ezban, Vad and Kjalke, 2014).*

Turoctocog alfa pharmacokinetics parameters **(Table 9)** were accessed after a single infusion of 50 IU/kg in already previous treated patients (European Medicines Agency, 2013). In addition, the bioequivalence was tested with ADVATE®, involved 23 male patients with severe HA receiving the 50 IU/kg of each drug with four days of washout (Martinowitz *et al.*, 2011). The results of pharmacokinetics endpoints were within the 90% of confidence interval (80-125%), proving their bioequivalence **(Table 10)** (Martinowitz *et al.*, 2011).

The efficacy and safety were investigated in adults and adolescents, in the GUARDIAN™ I trial (Lentz *et al.*, 2013). Accordingly, in 150 patients turoctocog alfa showed to be effective in control bleedings successively with one to two infusions (Lentz *et al.*, 2013). Patients had an ABR of 3.7 bleeds per patient per year under prophylaxis regimen (Lentz *et al.*, 2013). In terms of safety, it was hypothesized that it would have a bigger immunologic response due to the engineered B-domain truncated (BDT) (Ezban, Vad and Kjalke, 2014). Even so, the clinical safety data showed no concerns of this matter as none of the patients enrolled in the study developed inhibitors (Lentz *et al.*, 2013).

Table 9 - Pharmacokinetic parameters of turoctocog alfa regarding the age *(European Medicines Agency, 2013).*

Age group	**Pharmacokinetic Parameters**						
	Cmax (IU/dL)	AUC_t (IU*h/dL)	$t_{1/2}$ (h)	CL (mL/h/kg)	MRT (h)	Vss (mL/kg)	Incremental recovery (IU/dL per IU/kg)
Adolescents/ Adults ≥12 years	163 ± 50	1963 ± 773	11.22 ± 6.86	2.86 ± 0.94	14.54 ± 5.77	38.18 ± 10.24	2.9 ± 0.6
Children 6 to <12 years	125 ± 27	1437 ± 348	9.42 ± 1.52	3.70 ± 1.00	11.61 ± 2.32	41.23 ± 6.00	2.5 ± 0.6
0 to 6 years	112 ± 31	1223 ± 436	9.99 ± 1.71	4.59 ± 1.73	12.06 ± 1.90	55.46 ± 23.53	2.2 ± 0.6

*Abbreviations: **AUC_t:** area under the plasma FVIII activity curve from 0 to the last measurable point; **CL:** clearance; **Cmax:** maximum concentration; **MRT:** mean residence time; **$t_{1/2}$:** terminal half-life; **Vss:** volume of distribution at steady state conditions.*

Table 10 - Pharmacokinetic parameters and bioequivalence results between NovoEight® and ADVATE® *(Martinowitz et al., 2011).*

Pharmacokinetic Parameters						
Concentrate	AUC (IU*h/mL)	AUC_t (IU*h/mL)	Incremental recovery (IU/dL per IU/kg)	CL (mL/h/kg)	$t_{1/2}$ (h)	Cmax (IU/mL)
NovoEight®	11.9942	11.3044	0.01839	4.1687	9.8586	0.9723
ADVATE®	11.8128	11.1397	0.01816	4.2327	10.5524	0.9858
90% CI	[0.9227; 1.0513]	[0.9231; 1.0519]	[0.9234; 1.0565]	[0.9512; 1.0838]	[0.9808; 1.1681]	[0.9498; 1.0823]

Abbreviations: ***AUC:*** *area under the plasma FVIII activity curve;* ***AUC_t:*** *area under the plasma FVIII activity curve from 0 to the last measurable point;* ***CI:*** *confidence interval;* ***Cmax:*** *maximum concentration;* ***CL:*** *clearance* ***$t_{1/2}$:*** *terminal half-life*

Kovaltry® (octocog alfa) emerged based on Kogenate Bayer® but adding new features on its technology (Mahlangu *et al.,* 2018). The remain characteristics are the amino acid sequence, the full-length structure and the cell line chosen to express FVIII (i.e. BHK) (Mahlangu *et al.*, 2018). The innovations started with the addition of the human heat shock protein 70 (HSP70) gene, an intracellular chaperone that will ensure the proper folding of FVIII and, consequently, increase the protein expression (Mahlangu *et al.*, 2018). Moreover, the N-terminal glycans present 96% of sialic acid (i.e. sialylation) (Mahlangu *et al.*, 2018) which seems to be responsible for the 10% prolonged half-life (12.2h *versus* 13.4h) and slower clearance (0.043 *versus* 0.036 dl/h/kg) of Kovaltry® comparing to Kogenate Bayer® (Raso and Hermans, 2018).

Clinical data was evaluated in an extensive LEOPOLD clinical trial (Mahlangu *et al.*, 2018). The pharmacokinetics was evaluated at LEOPOLD 1, on 26 previous treated patients after a single infusion of 50 IU/kg **(Table 11)**. Additionally, the LEOPOLD 1 assessed the efficacy in 62 patients aged from 12 to 65 years old (Mahlangu *et al.*, 2018). Among them, 44 patients (71%) were treated prophylactic three times per week and 18 patients (29%) treated prophylactic twice per week (Mahlangu *et al.*, 2018). No major differences were verified in ABR for each patient group as the median of total bleeds were low (1.0 for twice times/week regimen and 2.0 three times/week) (Mahlangu *et al.*, 2018). As for safety, no immunologic response was observed in previous treated patients and no serious adverse events were observed (Mahlangu *et al.*, 2018).

Table 11 - Pharmacokinetic parameters of octocog alfa regarding the age *(Mahlangu et al., 2018) and (European Medicines Agency, 2016).*

Pharmacokinetic Parameters				
Age group	AUC (IU*h/dL)	$t_{1/2}$ (h)	CL (mL/h/kg)	Vss (mL/kg)
Adults ≥18 years	1858 ± 38	14.8 ± 34	0.03 ± 38	0.56 ± 14
Adolescents 12 to <18 years	1523 ± 27	13.3 ± 24	0.03 ± 27	0.61 ± 14
Children 6 to <12 years	1242 ± 35	14.1 ± 31	0.04 ± 35	0.77 ± 15
0 to 6 years	970 ± 25	13.3 ± 24	0.05 ± 25	0.92 ± 11

Abbreviations: ***AUC:*** *area under the plasma FVIII activity curve;* ***CL:*** *clearance;* $\boldsymbol{t_{1/2}}$***:*** *terminal half-life;* ***Vss:*** *Volume of distribution at steady state conditions.*

The last third generation drug that was introduced in the market was Afstyla® (lonoctocog alfa), which has the most unique technology. It is a single chain with the truncated B-domain that serves as a linkage between the heavy chain and the light chain **(Figure 12)** (Al-Salama and Scott, 2017). The rational is that, endogenous FVIII have both chains connected by a noncovalent divalent metal ion (Ca^{2+} or Mn^{2+}) (Fang, Wang and Wang, 2007), which is easily to dissociate and becoming inactive (Zollner *et al.*, 2014). Therefore, lonoctocog alfa is covalently linked by a BDT enhancing the stability and increasing the chances to interact with vWF and, subsequently, prolong the half-life comparatively with the full-length molecules (Schmidbauer *et al.*, 2015) (Al-Salama and Scott, 2017).

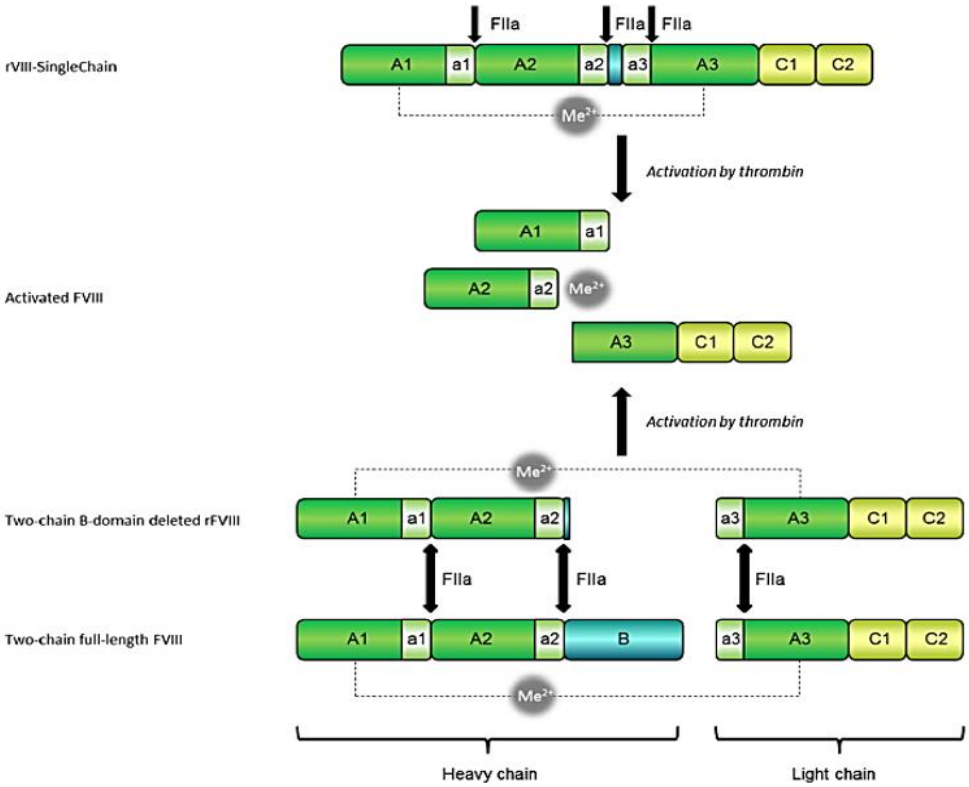

Figure 12 - Illustration of the design lonoctocog alfa in comparison with other structures *(Schmidbauer et al., 2015)*.

Clinical data of lonoctocog alfa was assessed in the AFFINITY program (Schiavoni *et al.*, 2019). The first part of the study was carried out the pharmacokinetics and short-term safety after a single dose (50 IU/kg) of lonoctocog alfa, in previous treated patients with severe HA **(Table 12)** (Raso and Hermans, 2018). In contrast with ADVATE®, lonoctocog alfa has a slightly better half-life (14.5 ± 3.8h vs. 13.3 ± 4.4h) expected by the technology proposed and, a reduction in clearance by 28–31% (Klamroth *et al.*, 2016; Raso and Hermans, 2018).

Table 12 - Pharmacokinetic parameters of lonoctocog alfa regarding the age *(European Medicines Agency, 2017).*

Pharmacokinetic Parameters							
Age group	AUC_{∞} (IU*h/dL) Mean (CV %)	Cmax (IU/dL) Mean (CV %)	MRT (h) Mean (CV %)	$t_{1/2}$ (h) Mean (CV %)	CL (mL/h/kg) Mean (CV %)	Vss (mL/kg) Mean (CV %)	Incremental recovery (IU/dL per IU/kg) Mean (CV %)
Adults ≥18 years	1960 (33.1)	106 (18.1)	20.4 (25.8)	14.2 (26.0)	2.90 (34.4)	55.2 (20.8)	2.00 (20.8)
Adolescents 12 to <18 years	1540 (36.5)	89.7 (24.8)	20.0 (32.2)	14.3 (33.3)	3.80 (46.9)	68.5 (29.9)	1.69 (24.8)
Children 6 to <12 years	1170 (26.3)	83.5 (19.5)	12.3 (16.8)	10.2 (19.4)	4.63 (29.5)	67.1 (22.3)	1.66 (19.7)
0 to 6 years	0.80 (31.0)	80.2 (20.6)	12.4 (25.0)	10.4 (28.7)	5.07 (29.6)	71.0 (11.8)	1.60 (21.1)

Abbreviations: ***AUC_{∞}:*** *area under the plasma FVIII activity curve extrapolated to infinity;* ***Cmax:*** *maximum concentration;* ***CL:*** *clearance;* ***MRT****: mean residence time;* ***$t_{1/2}$:*** *terminal half-life;* ***Vss:*** *volume of distribution at steady state.*

In terms of efficacy, which was evaluated on the second and third part of the program, lonoctocog alfa showed to be as efficient as the others in control the bleeds (93.8%) with either one or two doses (median dose of 31.7 IU/kg) recording low registry of ABR (1.14) (Raso and Hermans, 2018). As for the safety, no adverse events were reported and no inhibitors were detected (Raso and Hermans, 2018).

1.4.3.1.4. Fourth Generation

This generation is only described by Nuwiq® (simoctocog alfa) (Lissitchkov *et al.*, 2019). The difference between the previous generations refers to the use of human embryonic kidney cell line (Hek293) to express the FVIII (Swiech, Picanço-Castro and Covas, 2017). The previous cell lines, CHO and BHK, have been described as a potential source for an immunologic response due to the presence of glycans epitope *N*-glycolylneuraminic acid (Neu5Gc) and Galα1→3Gal groups that are not present in the human form of the protein hence, being a source for the activation of the immune system (Raso and Hermans, 2018). Furthermore, using

the human cell line has the advantage to mimic the endogenous FVIII, particularly, in post-translational modifications such sulfation (Lissitchkov *et al.*, 2019). Sulfation occurs in Golgi apparatus and it is important for FVIII function (Orlova *et al.*, 2013). This modification targets the tyrosine residues (Tyr) located near to the acidic domains of the structure **(Figure 13)** and all of them (six in total) are crucial for the activity of FVIII (Orlova *et al.*, 2013).

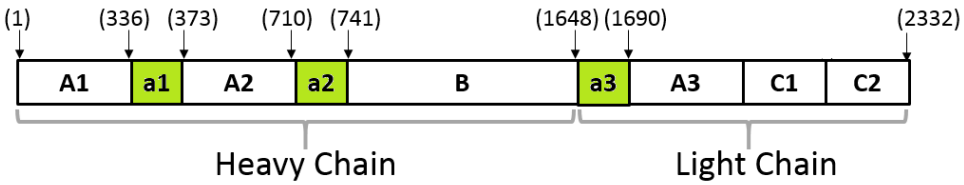

Figure 13- Representation of the acidic sites and potential sulfation sites *(Mazurkiewicz-Pisarek et al., 2016).*

Moreover, it has been demonstrated that the sulfation of Tyr 1680 is the key for the binding of vWF to FVIII, conferring more stability and protection against early degradation/elimination of the bloodstream (Cafuir and Kempton, 2017; Raso and Hermans, 2018). The other drugs, from second to third generations, have the Tyr 1680 sulfated as well but in less proportion (1% to 6.5% in second-generation and 15% in third-generation) (Raso and Hermans, 2018) whereas, simoctocog alfa has every tyrosine fully sulfated including the Tyr 1680 (Lissitchkov *et al.*, 2019). All these characteristics were created to decrease immunogenicity of the patients improving drug residence time in bloodstream (Lissitchkov *et al.*, 2019).

The pharmacokinetics was evaluated in 22 adolescents/adults in GENA-01 program whilst, the estimations from children were done in GENA-03, which enrolled 26 patients. All of them received 50 IU/kg infusion of simoctocog alfa **(Table 13)** (Lissitchkov *et al.*, 2019). Overall, the drug proved to be effective in treating bleeds with standard prophylaxis (30–40 IU FVIII/kg) both in adults and children, as well as safer in terms of adverse effects and immunologic response (Lissitchkov *et al.*, 2019).

Table 13 - Pharmacokinetic parameters of simoctocog alfa regarding the age *(European Medicines Agency, 2014).*

Pharmacokinetic Parameters				
Age group	AUC $_t$ (IU*h/mL)	$t_{1/2}$ (h)	CL (mL/h/kg)	Incremental *in vivo* recovery (% per IU/kg)
Adolescents/ Adults ≥12 years	22.6 ± 8.0	14.7±10.4	3.0±1.2	2.5±0.4
Children 6 to <12 years	13.2±3.4	10.0±1.9	4.3±1.2	1.9±0.4
0 to 6 years	11.7±5.3	9.5±3.3	5.4±2.4	1.9±0.3

Abbreviations: ***AUC$_t$:*** *area under the plasma FVIII activity curve from 0 to the last measurable point;* ***CL:*** *clearance;* ***$t_{1/2}$:*** *terminal half-life.*

1.4.3.2. Extended half-life

Over the years, the SHL were the best option to manage HA patients. However, these drugs deliver the active substance for a short period of time (8h to 12h) (Cafuir and Kempton, 2017). This characteristic obligates a more frequent infusions (3 to 4 times weekly) to maintain the minimum levels of FVIII activity (Cafuir and Kempton, 2017). This is a burden for some patients and a reason that can justify the poor compliance in some cases (Mannucci, 2020). Therefore, since 2010, pharmaceutical industries had been investing resources to manufacture new drugs with a prolonged half-life time, requiring lower frequency of infusions (Mannucci, 2020). This is the field where the extended half-life (EHL) started.

For some time, researchers tried to find the best definition that could significantly serve for a drug to fit into EHL category. Today, an EHL drug must conquer three points: (1) innovative engineering technology to clearly extend the half-life time; (2) use bioequivalence cut off limits (80%-125%) to compare the exposure (AUC) between SHL and EHL. If the ratio between the two products is above those limits then, there is reassurance that the EHL product will have better AUC ratios on the population; (3) the half-life ratio extension needs to be of at least 1.3 higher (Mahlangu *et al.*, 2018). Based on these criteria, lonoctocog alfa was excluded as the AUC ratio was below the 125% (not "biodifferent") and its half-life was only extended 1.09 h, remaining comparable to ADVATE® (Ar, Balkan and Kavaklı, 2019).

In terms of the technology, EHL may use chemical modifications (PEGylation) or being fused with Fc domains of serum proteins with long half-life times (Fc fusion) as it will be explained in next sections.

1.4.3.2.1. PEGylation

PEGylation is a chemical modification that consists on a covalent bound between polyethylene glycol (PEG) molecule to FVIII (Ar, Balkan and Kavaklı, 2019). The advantage of this conjugation is that, PEG serves as a "shield" from the clearance receptors (prolonging the half-life) and the immunogenic epitopes (reducing the immunogenicity) (Fogarty, 2011).

The first product was Adynovi® (Adynovate® outside the EU) using rurioctocog alfa pegol as the active substance and CHO cell line to express activity (Cafuir and Kempton, 2017). The design involved the full-length molecule ADVATE®, shield with a weighted 20 kDa PEG molecule (Cafuir and Kempton, 2017). After the assessment of pharmacokinetics parameters **(Table 14)** it was observed that the half-life time was extended to 14-19.6 hours, which is 40% longer than octocog alfa (ADVATE®) (Cafuir and Kempton, 2017). Furthermore, it also demonstrated to be efficient in controlling bleeding episodes with prophylaxis (40-50 IU/kg) since the ABR value was lower (median 1.9) (Cafuir and Kempton, 2017).

Table 14 - Pharmacokinetic parameters of rurioctocog alfa pegol, after a single infusion of 45 ±5 IU/Kg to adults and adolescents and 50±10 IU/Kg to children *(European Medicines Agency, 2018).*

Pharmacokinetic Parameters							
Age group	AUC_{∞} (IU*h/dL)	Cmax (IU/dL)	$t_{1/2}$ (h)	CL (mL/h/kg)	MRT (h)	Vss (dL/kg)	Incremental recovery (IU/dL per IU/kg)
Adults ≥18 years	2589 ± 848	145 ± 29	15.01 ± 3.89	2.16 ± 0.75	19.70 ± 5.05	0.40 ± 0.09	2.87 ± 0.61
Adolescentes 12 to <18 years	1900 ± 841	117 ± 28	13.80 ± 4.01	2.58 ± 0.84	17.73 ± 5.44	0.54 ± 0.22	2.34 ± 0.62
Children 6 to 12 years	2259 ± 514	-	11.93 ± 2.58	2.80 ± 0.67	17.24 ± 3.73	0.46 ± 0.04	-
<6 years	2190 ± 1593	-	12.99 ± 8.75	3.49 ± 1.21	18.74 ± 12.60	0.54 ± 0.03	-

Abbreviations: ***AUC_{∞}:*** *area under the plasma FVIII activity curve extrapolated to infinity;* ***Cmax:*** *maximum concentration;* ***CL:*** *clearance;* ***MRT****: mean residence time;* ***$t_{1/2}$:*** *terminal half-life;* ***Vss:*** *volume of distribution at steady state.*

Moreover, Jivi® (damoctocog alfa pegol) is expressed in BHK cells and uses the B-domain deleted structure linked to a single 60 kDa PEG molecule through an amino acid substitution by cystine (Cafuir and Kempton, 2017). The comparisons made so far were with Kogenate Bayer® in previous treated patients with HA. Once again, the half-life **(Table 15)** proved to be higher (19 hours *versus* 13 hours) with a median ABR between 1.9 and 3.9 depending on the prophylactic days of infusion (five and seven respectively) (Cafuir and Kempton, 2017).

Table 15 - Pharmacokinetic parameters of damoctocog alfa pegol after a single infusion of 60 IU/Kg in adolescents/adults *(European Medicines Agency, 2019).*

Pharmacokinetic Parameters						
Age group	AUC (IU*h/dL) Mean (CV %)	Cmax (IU/dL) Mean (CV %)	$t_{1/2}$ (h) Mean (CV %)	CL (dL/h/kg) Mean (CV %)	MRT (h) Mean (CV %)	Vss (mL/kg) Mean (CV %)
Adolescents/ Adults ≥12 years	3710 (33.8)	163 (14.7)	17.1 (27.1)	0.0160 (33.7)	24.4 (27.5)	0.391 (16.3)

Abbreviations: ***AUC:*** *area under the plasma FVIII activity;* ***Cmax:*** *maximum concentration;* ***CL:*** *clearance;* ***MRT****: mean residence time;* ***$t_{1/2}$:*** *terminal half-life;* ***Vss:*** *volume of distribution at steady state.*

Esperoct® (Turoctocog alfa pegol) is the last and the most recent EHL introduced in the market. It englobes the B-domain truncated molecule (turoctocog alfa) glycoconjugated to a PEG substance of 40 kDa (Raso and Hermans, 2018), expressed in CHO cells (Cafuir and Kempton, 2017). Glycoconjugation means that, the PEG molecule is place, via enzymatic, in one of the o-linked glycans at B-domain (Raso and Hermans, 2018). Furthermore, its pharmacokinetics profile **(Table 16)** evidence an extensive half-life time, ranging from 11.6h to 27.3h. This characteristic seems to contribute to the good control of bleeding by prophylaxis (median ABR of 1.3) in previous treated patients (Raso and Hermans, 2018).

Table 16 - Pharmacokinetic parameters of turoctocog alfa pegol after a single infusion of 60 IU/Kg regarding the age *(European Medicines Agency, 2019).*

Pharmacokinetic Parameters							
Age group	AUC_{∞} (IU*h/dL) Mean (CV %)	Cmax (IU/dL) Mean (CV %)	$t_{1/2}$ (h) Mean (CV %)	CL (mL/h/kg) Mean (CV %)	MRT (h) Mean (CV %)	Vss (mL/kg) Mean (CV %)	Incremental recovery (IU/dL per IU/kg) Mean (CV %)
Adults ≥18 years	3686 (35)	134.4 (23)	19.9 (34)	1.4 (32)	25.2 (29)	37.7 (27)	2.63 (22)
Adolescents 12 to <18 years	3100 (44)	133.2 (9)	15.8 (43)	1.5 (43)	21.7 (45)	33.4 (10)	2.79 (12)
Children 6 to 12 years	2503 (42)	119.6 (25)	14.2 (26)	2.4 (40)	17.3 (31)	41.2 (25)	1.99 (25)
<6 years	2147 (47)	101.2 (28)	13.6 (20)	2.6 (45)	17.0 (22)	44.2 (34)	1.80 (29)

Abbreviations: ***AUC:*** *area under the plasma FVIII activity extrapolated to infinity;* ***Cmax:*** *maximum concentration;* ***CL:*** *clearance;* ***MRT****: mean residence time;* ***$t_{1/2}$:*** *terminal half-life;* ***Vss:*** *volume of distribution at steady state.*

1.4.3.2.2. Fc Fusion

The Fc domain of immunoglobulins establish fusions with other molecules of the body such as, cytokines or growth factors (Mancuso and Mannucci, 2014). Endothelial cells express the neonatal Fc receptor (FcRn), at the same site where IgG coexist to protect the vasculature (Mancuso and Mannucci, 2014). The fusion between both components (i.e. FcRn and Fc domain of IgG) is documented at epithelial cells of certain organs (e.g. lungs, kidneys, intestine) (Mancuso and Mannucci, 2014). In addition, studies proved that, FcRn protects IgG from lysosomal degradation on the vascular endothelium, recycling it back to the bloodstream (Mancuso and Mannucci, 2014; Roopenian and Akilesh, 2007) prolonging ,then, the half-life time up to 21 days **(Figure 14)** (Cafuir and Kempton, 2017).

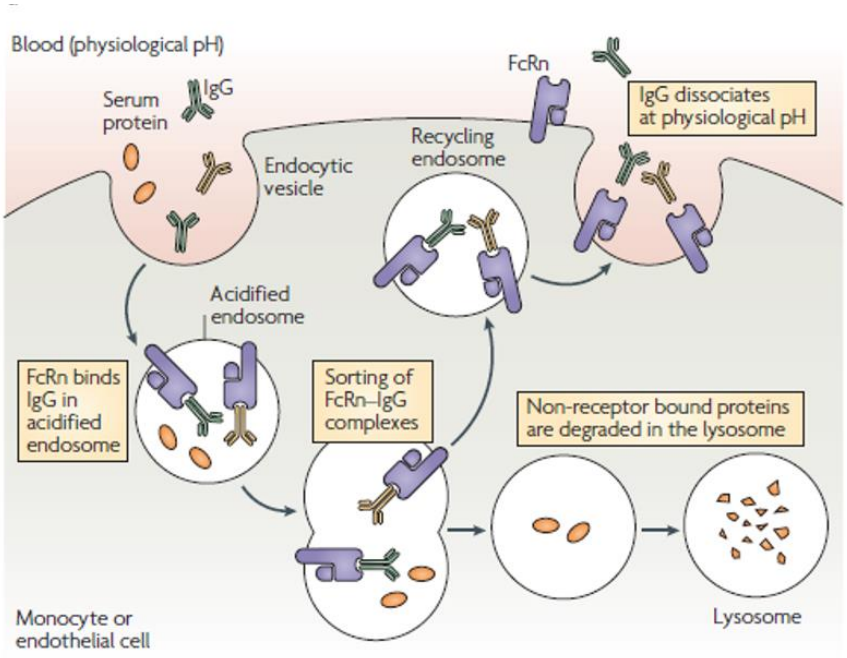

Figure 14 - The neonatal Fc receptor (FcRn) and IgG mechanism in vascular endothelium *(Roopenian and Akilesh, 2007).*

Therefore, this mechanism was replicated to HA treatment as a new opportunity to prolong the FVIII life on the bloodstream and enhancing his activity. Elocta® (Efmoroctocog alfa) is the only product currently available which links covalently the Fc portion of IgG1 to the molecule (Mancuso and Mannucci, 2014). It has the BDD and is expressed in Hek293 cells (Mancuso and Mannucci, 2014).

The pharmacokinetics parameters of Efmoroctocog alfa were studied in A-LONG clinical trial in three different age groups (n=27 with ≥15 years, n=27 with 6-11 years, n=24 with <6 years) after a single infusion of 50 IU/kg **(Table 17)**. Accordingly, the half-life time enhanced as the age rise (European Medicines Agency, 2015; Frampton, 2016).

Table 17 - Pharmacokinetic parameters of efmoroctocog alfa after a single dose of 50 IU/kg regarding the age *(European Medicines Agency, 2015).*

Pharmacokinetic Parameters							
Age group	Cmax (IU/dL)	AUC/Dose (IU*h/dL per IU/kg))	$t_{1/2}$ (h)	CL (mL/h/kg)	MRT (h)	Vss (mL/kg)	Incremental recovery (IU/dL per IU/kg)
Adolescents/ Adults ≥15 years	131 (104-165)	47.5 (41.6-54.2)	20.9 (18.2-23.9)	2.11 (1.85-2.41)	25.0 (22.4-27.8)	52.6 (47.4-58.3)	2.49 (2.28-2.73)
Adolescents 12 to <18 years	-	40.8 (29.3-56.7)	17.5 (12.7-24.0)	2.45 (1.76-3.41)	23.5 (17.0-32.4)	57.6 (50.2-65.9)	1.91 (1.61-2.27)
Children 6 to 11 years	-	32.8 (28.2-38.2)	15.9 (13.8-18.2)	3.05 (2.62-3.55)	20.7 (18.0-23.8)	63.1 (56.3-70.9)	2.08 (1.91-2.25)
<6 years	-	25.9 (23.4-28.7)	14.3 (12.6-16.2)	3.86 (3.48-4.28)	17.2 (15.4-19.3)	66.5 (59.8-73.9)	1.88 (1.73-2.05)

Abbreviations: ***AUC:*** *area under the plasma FVIII activity;* ***Cmax:*** *maximum concentration;* ***CL:*** *clearance;* ***MRT****: mean residence time;* ***$t_{1/2}$:*** *terminal half-life;* ***Vss:*** *volume of distribution at steady state.*

In comparison with other rFVIII (ADVATE®), the same doses (25, 50 and 65 IU/kg) were infused in adolescents/adults (≥12 years). Results showed a longer half-life for efmoroctocog alfa (1.5 to 1.7-fold longer) contributing for a geometric mean of half-life time approximately 19h with single infusions *versus* 11-12h of ADVATE® (Frampton, 2016). In addition, efmoroctocog alfa can prolong the time with levels above 1% (Frampton, 2016). In children, the same conclusions were observed in half-life with 1.4-fold (6-11 years) and 1.7-fold (<6 years) longer in previous treated patients with ADVATE® (n=16) (Frampton, 2016). As a prophylaxis regimen, following 25–65 IU/kg every 3–5 days, the average ABR was 1.6 which was maintain on ASPIRE trial (Frampton, 2016). Besides this, the bleeding episodes (757 in total) were well manageable with 1-2 doses with a rate of success of 91.8% (Cafuir and Kempton, 2017; Frampton, 2016).

1.5. Switching

Switching is a clinical decision made by health care professional in which is suggested a swap from one concentrate to another (Coppola *et al.*, 2016). This exchange may be between different category of concentrates (i.e. pdFVIII to rFVIII) or, within the same type but different technologies (SHL to EHL), which is more common (Yu *et al.*, 2019). This is a multifactorial decision **(Table 18)** that reunites several clinical concerns and expectations emerged from

the patient or from the health professionals. Furthermore, switch is an individual assessment where the benefits and potential risks should be balanced (Escobar *et al.*, 2019).

Regardless of the type of concentrate chosen, the switch should always be considered when the efficacy is impaired (i.e. patients experience frequent bleeds and/or have target joints) (Escobar *et al.*, 2019). When this happens is more frequent to swap from a SHL concentrate to EHL since, this latter technology, present a lot of advantages in compliance and quality of life of the patients **(1.4.3.2 Extended half-life).** Additionally, EHL is recommended in patients that are not capable to undertake prophylaxis with SHL or, for patients who are currently struggling with adherence to the treatment (Escobar *et al.*, 2019).

On the other side, patients should not be submitted to a switch if, they are not experiencing breakthrough bleeds (or they are minimal) or, the regimen (on-demand or prophylaxis) management has no issues related and /or, they are less severe patients with minimal bleeding associated treatment (Escobar *et al.,* 2019).

Table 18 - Summary of the possible clinical reasons behind the switch *(Coppola et al., 2016)* and *(Escobar et al., 2019).*

Subject	Major Concern	Clinical Expectations
Patients	Safety	Avoid side effects: Hypersensitivity/allergy
	Quality of life	Possibility to increase physical activity. Participation in society Good control and protection with bleedings Less infusions /Less venipunctures
	Economics	Cost-savings
Health Care Professionals	Type of concentrate	Safety Efficacy Patient future compliance Assessment which new regimen is the best
	Pharmacokinetics	Initial parameters assessment Monitoring behavior Tailoring new regimen
	Immunogenicity	Medical history Monitoring long-term

Guidelines provide some recommendations for this clinical decision, advising professionals that, this swap should be in patients with more than 150 exposure days (EDs) along with no prior inhibitors history (Rayment *et al.*, 2020). The choice behind the number of EDs is explained by the vulnerability of inhibitor development in patients with less than 50 EDs, which puts them at a higher risk **(1.6. Immunogenicity)** (Escobar *et al.,* 2019).

After patient evaluation and the reasons for a switch, the next step is to know which the ideal regimen is and which the best drug that should be chosen is. Trial-and-error is a common approach where two scenarios can happen: either the dose is kept the same and only the frequency is adjusted, or the dose and frequency from pivotal data studies are used (Yu *et al.*, 2019). However, it is a mistake forgetting that switch is a process where the previous PK is known and the change will be for a PK unknown behaviour (Yu *et al.*, 2019). Also, CFCs have high inter-patient variability which means that, each patient will respond differently so, this approach is a risk to underdosing and subsequently, for more bleeding (Escobar *et al.*, 2019; Yu *et al.*, 2019).

Moreover, after the switch, PK should be evaluated and tailored for a better clinical response (Yu *et al.*, 2019). Some reviews describe the possibility to do a PK assessment after single-dose infusion or, after several doses for more accurate results due to steady-state conditions (Escobar *et al.*, 2019). Trace PK profile may be based on classic studies but, it is better to estimate the profile by pharmacometrics (modelling techniques) because there is no need for an unnecessary "wash-out" period that leaves patients prone to bleedings and, also less number of samples required (less burden) (Escobar *et al.*, 2019).

Generally, trace a personalized regimen involves a definition of trough optimal levels to overcome the bleeds and target joints (Escobar *et al.*, 2019). If the aim is to maintain the previous dosing regimen, high trough levels will be the objective for the switch whereas, if the dosing interval is better to be prolonged for better adherence, for instances, then trough levels are maintained and time spent at a lower factor level is considered (Escobar *et al.*, 2019).

The PK parameters will be adjusted to each situation as explained in **1.7.4.2.1 Pharmacokinetic parameters for prophylaxis** that sums up the importance of AUC, peak levels and trough levels as the main PK parameters to choose the right dosing regimen (Morfini and Farrugia, 2019).

After achieving the right regimen for each patient, it is recommended a monitoring process of these patients upon 10 EDs, 4 weeks and 3 months (Escobar *et al.*, 2019). Clinical evaluation should be focus on microbleeds, evaluate the joint progression (bone density and structure), diagnosis of possible inhibitors formation and testing in a long-term neurological impairment if patients were switch to a PEGylated drug (Escobar *et al.*, 2019).

1.6. Immunogenicity

Antibodies associated to CFCs were first reported in 1940 by Lawrence, described them as neutralizing alloantibodies (Nakar and Shapiro, 2019). They are an immunologic response to

the treatment with CFCs (Witmer and Young, 2013). Consequently, the treatment becomes ineffective, promoting a higher susceptibility for bleeding episodes (Witmer and Young, 2013).

Neutralizing alloantibodies are currently designed as inhibitors of FVIII (Carcao and Goudemand, 2018). They have a high affinity to certain epitopes present on A2, C1, and C2 FVIII domains (Miller, 2018), interfering with the FVIII either by blocking the mechanism (restrict the binding sites for FIX, phospholipids, and vWF) or removing it from circulation (i.e. enhanced clearance) (Miller, 2018; Witmer and Young, 2013).

Moreover, antibodies are not only "neutralizing" towards FVIII. Indeed, patients can synthesize "non-neutralizing" or "non-inhibitory" antibodies. Although they do not express a function directly to FVIII (Miller, 2018), it has been reported their impact on the catabolism of CFCs (Lebreton *et al.*, 2011) and pharmacokinetics (Turecek *et al.*, 2020). These types of antibodies may be important as biomarkers for the neutralizing antibodies, after one study discovered positive inhibitors 1.5 years later in patients previously with non-neutralizing inhibitors (Abdi *et al.*, 2020).

Besides these two categories, the immune system can also develop auto-antibodies in non-hemophilia patients, as a condition named acquired hemophilia (Carcao and Goudemand, 2018). It is a very rare condition with an incidence of about 1 case per million people *per* year (Cugno *et al.*, 2014). Half of patients diagnosis with acquired hemophilia usually are related to clinical conditions such autoimmune disorders, tumours (Cugno *et al.*, 2014) or to the postpartum as a rare adverse event (risk between 7% to 21%) (Franchini, 2006). In addition, age could explain the idiopathic cases, particularly in elderly as they are more vulnerable (Cugno *et al.*, 2014).

1.6.1. Screening

Inhibitors detection is possible using either the Bethesda assay or the modified version, the Nijmegen Bethesda assay, which is more sensitive and specific (Srivastava *et al.*, 2020). Both assays measure the concentration (also named as titer) of inhibitors (Witmer and Young, 2013). Results are expressed in Bethesda Units (BU) meaning that, 1 BU is the equivalent inhibitor amount in 1 millilitre (mL) of human plasma that neutralizes FVIII by 50% (Miller, 2018). To consider a positive result, the titer has to be higher than 6.0 BU (Srivastava *et al.*, 2020).

Furthermore, inhibitors should be screened before they interfere with the efficacy of the treatment and, preferably, when there is a high risk for their development, which is within the first 20 EDs (Carcao and Goudemand, 2018). For children, they should be tested from 5 until

20 EDs, and then every 10 EDs until the 50 EDs. Afterwards, screening inhibitors should be performed twice per year until 150 EDs since the risk is much lower (Carcao and Goudemand, 2018). In adults, normally the risk is lower and should be considered when: (a) after intensive treatments; (b) before undergoing a major surgery; (c) clinical response to the treatment is suboptimal (Carcao and Goudemand, 2018).

As for non-inhibitors, they cannot be detected with the previous gold standard assays (Srivastava *et al.*, 2020). Instead, enzyme-linked immunosorbent assay (ELISA) or fluorescence-linked immunoassay are recommended (Srivastava *et al.*, 2020).

1.6.2. Characterization

In terms of the structure, inhibitors are polyclonal immunoglobulin G (IgG), often from the IgG4 or IgG1 subclasses (Cugno *et al.*, 2014). After positive results, inhibitors can be classified in terms of peak activity as low-titer inhibitors (<5.0 BU) or, as higher-titer inhibitor (>5.0 BU), both requiring different managements (Srivastava *et al.*, 2020).

Starting with low-titer inhibitors (LTI), they are frequently IgG1 subclass and tend to disappear spontaneously after 6 months without need management, which is why they are often described as transient inhibitors. Nonetheless, patients with this type of inhibitors should be monitoring closely, every 2-4 weeks, because LTI can easily convert into higher-titer inhibitor (HTI) (Carcao and Goudemand, 2018).

In contrast, HTI are usually IgG4 subclass and persistent inhibitors (Carcao and Goudemand, 2018) meaning that, after a long period without a CFCs exposure but after 3-5 days of re-introducing CFCs, their response may increase (i.e. anamnestic response) (Srivastava *et al.*, 2020). These inhibitors are undetectable creating resistance to the CFCs (Carcao and Goudemand, 2018).

In addition to their activity response, inhibitors may express different kinetics behaviour. Inhibitors can be type I, when they act as a second-order kinetics (i.e. dose-dependent inhibition) inactivating fully FVIII activity; or type II which have a more complex kinetics with only a partial inactivation of FVIII activity (Cugno *et al.*, 2014; Witmer and Young, 2013). The prevalence of type I is seen in severe HA patients whereas, type II is more common in mild hemophilia or in acquire hemophilia (Cugno *et al.*, 2014; Witmer and Young, 2013).

1.6.3. Antibodies Prevalence and Incidence

Incidence is related to the number of the new inhibitors in HA cases over a period of time (Carcao and Goudemand, 2018; Tieu, Chan and Matino, 2020). Usually, in severe HA the incidence rounds the 30% whereas in moderate/mild HA it is approximately 3-13% (Tieu, Chan

and Matino, 2020). As for prevalence, it represents the total number of people with inhibitors in HA population at a specific time (Carcao and Goudemand, 2018). In severe HA, prevalence is 5-10% which means that, at any time, approximately 5-10% of the patients with severe type will present inhibitors (Carcao and Goudemand, 2018). However, this is influenced by the incidence rate, the type of inhibitors found (HTI and LTI), eradication with immune tolerance induction programs and the deaths related to inhibitors (Carcao and Goudemand, 2018).

For non-inhibitors, studies have been reported a prevalence of 2-3% in healthy individuals but, higher values for hemophilia patients and a wide range related to the severity of the disease (12% to 54%) (Cannav *et al.*, 2017).

1.6.4. Risk Factors for Development of Inhibitors

Since the focus of this project is not on HA inhibitors, the background of risk factors for inhibitors occurrence is represented at **Figure 15** (Blatn *et al.*, 2016). They are classified in two categories: the ones that are genetic (unmodified ones) or not related to genetics (environmental/modified) **(Figure 15)** (Garagiola, Palla and Peyvandi, 2018).

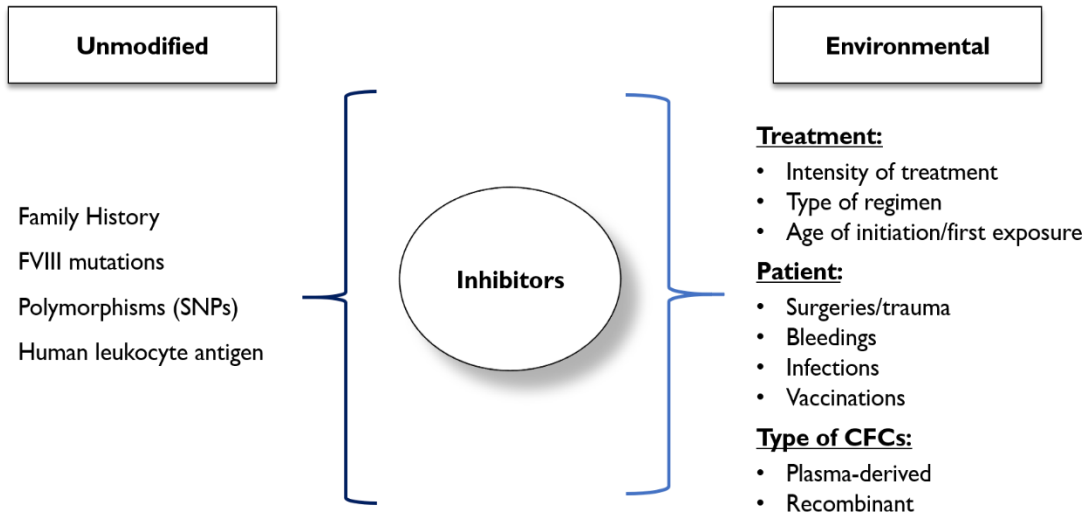

Figure 15- Representation risk factors for inhibitors development in HA treatment.

1.6.5. Management of inhibitors

Upon detection of inhibitors and their classification as LTI or HTI, it is crucial to trace a proper management plan. Generally, if the inhibitor is LTI, it is possible to use porcine recombinant FVIII (prFVIII), usually prescribed for patients with acquired HA (Carcao and Goudemand, 2018). In 2015, the European Medicines Agency authorized the Obizur® (susoctocog alfa), a prFVIII, which is a high-purity B-domain deleted structure manufactured by recombinant technology in BHK cells (Mannucci and Franchini, 2017). Desmopressin

(DDAVP) is a synthetic vasopressin analogue with proved efficacy towards mild HA patients (Loomans *et al.*, 2018) as well as an option for LTIs when displaying a type II kinetics (Carcao and Goudemand, 2018).

As previously referred, LTI can easily turn into HTI which promotes the use of bypassing agents such as plasma-derived activated prothrombin complex concentrates (aPCC) and recombinant factor VII activated (rFVIIa). Both have an efficacy of about 80-90% for manage bleedings with inhibitor patients (Tjønnfjord and Holme, 2007). Feiba® (Factor Eight Inhibitor Bypass Activity) is an aPCC with viral inactivation process containing zymogens, factor II (FII), factor VII (FVII), factor IX (FIX), factor X(FX) as well as their activated forms (FIIa, FVIIa, FIXa and FXa) which will help to restore hemostasis and stop bleeds (Tjønnfjord and Holme, 2007). The recommended dose is 50-100 IU/kg with 200 IU/kg the maximum dose per day (Carcao and Goudemand, 2018).

As for NovoSeven®(eptacog alfa), it is a rFVIIa (Carcao and Goudemand, 2018). Pharmacologically, activated factor VII (FVIIa) is not enzymatically capable of activating itself which means that FVIIa needs a partner, the tissue factor (TF), to form a stable complex (Hedner, 2006). This complex can easily activates factor (FXa) and generates a sufficient amount of thrombin, crucial to activate the cofactors FVIII and FV (Hedner, 2006). Another advantage of rFVIIa in terms of the mechanism of action is that, FVIIa is not easily inactivated by antithrombin so it is possible to establish TF:FVIIa complex without neutralizers (Hedner, 2006). The dose will depend on the severity of the bleeding episode but generally, it is recommended bolus infusion of 90 μg/kg and interval of 2 to 3 hours between multiple infusions (European Medicines Agency).

Bypass agents are effective but prophylaxis with them, in a long term, is not cost-effective as the efficacy decreases as the morbidity risk increases (Blair, 2019).

Hemlibra®(Emicizumab) is a humanized bispecific monoclonal antibody (IgG1) that binds to FIXa and FX mimicking the function of FVIII (Blair, 2019; Sankar, Weyand and Pipe, 2019). It is the first non-factor replacement therapy administered subcutaneously prescribed for inhibitors management (Blair, 2019). Contrary to the previous drugs, this one has a long half-life (approximately 27 days) and it has no structural similarities to FVIII which may be the reason to work in these patients since it does not induce or enhance their development (Blair, 2019).

1.7. Pharmacokinetic of FVIII concentrates

1.7.1. Absorption

The route of CFCs administration is *via* intravenous so, contrary to other routes (e.g. oral), the bioavailability on the bloodstream is 100% (Hermans and Dolan, 2020) hence, absorption does not occur (Hermans and Dolan, 2020).

1.7.2. Distribution

The distribution process is influenced by the content of human body fluids and plasma proteins (e.g. albumin), since some of them can have mechanism towards the active substance influencing the available plasma concentration (Rosenbaum, 2017).

In general, after the administration, drugs are distributed to the site(s) of the body to complete their main mechanism of action (Curry and Whelpton, 2010). In the case of FVIII, the action is exerted mainly on the bloodstream (Orlova *et al.*, 2013). Most of the FVIII distributes on the extracellular space (specifically at the intravascular compartment) due to its large molecular weight (Hermans and Dolan, 2020) (Björkman and Berntorp, 2001). Moreover, FVIII has high affinity to bind noncovalently and reversibly to vWF **(section 1.3)**, creating a well balance complex that properly regulates the amount of the free form (i.e. in circulation) and, the one bound to vWF (i.e. as a complex) (A.Noe, 1996).

The literature describes FVIII plasma concentration around the 0.8 nmol/L whereas, for vWF is about 35 nmol/L **(Figure 16)** (Turecek *et al.*, 2020). As vWF concentrations are higher, it is expected at steady-state conditions, an excess of 50 molars (Turecek *et al.*, 2020), representing 1:50 as FVIII molar ratio per vWF monomer (Thompson, 2003). In addition, the dissociation constant (Kd) is estimated to be between 0.2 and 0.5 nanometres (nm) which means that the affinity between the two molecules is high **(Figure 16)** (Terraube, O'Donnell and Jenkins, 2010).

Furthermore, most of FVIII is linked to vWF (approximately 95 to 97%) (Lillicrap, 2008; Turecek *et al.*, 2020). Thus, since the vWF serves as a shield for FVIII elimination, when FVIII is in free form it is expected to have a faster elimination (half-life estimated 2 hours) while, when complexed, the elimination is much longer (half-life estimated 12 hours) (Turecek *et al.*, 2020).

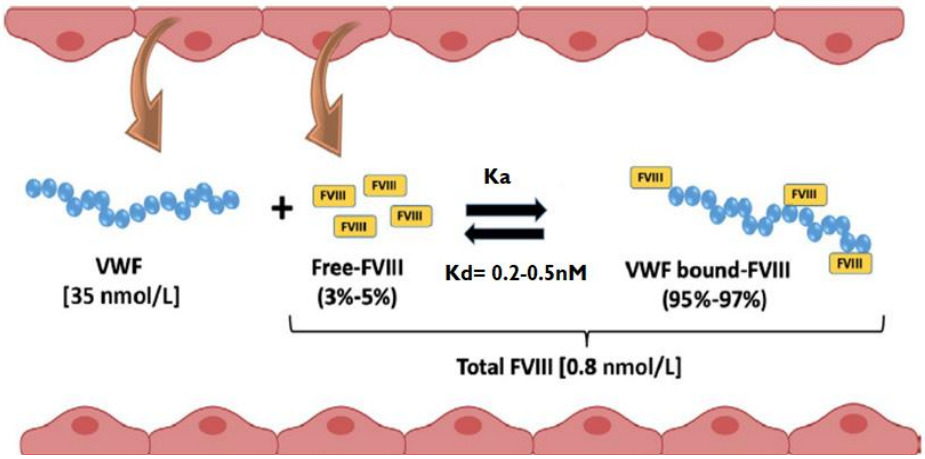

Figure 16 - Representation of the *in vivo* distribution equilibrium between FVIII and VWF. *Adapted from (Turecek et al., 2020).*

Abbreviations: ***FVIII:*** *Factor VIII****; Kd:*** *dissociation constant****; Ka:*** *association constant****; vWF:*** *von Willebrand Factor*

1.7.3. Elimination

Drug elimination involves metabolism and/or excretion (Rosenbaum, 2017). The metabolism is a gathering of several chemical reactions, consisting on two phases carried out either by the liver or secondary organs/fluids (e.g. plasma, intestinal flora, lungs, brain) (Curry and Whelpton, 2010). These chemical reactions, subsequently, will facilitate the excretion of the drug, either by the liver or kidneys (Curry and Whelpton, 2010).

Elimination of CFCs is quite different from generic drugs. Firstly, the erasing of the drug from the bloodstream can happen either in a free form or FVIII-vWF form (Swystun *et al.*, 2018). If the drug is removed as a free form, it needs to be firstly inactivated by a process called catabolism (Orlova *et al.*, 2013). This reaction aims to target the A2 domain to destabilize the protein. It may be through a spontaneous dissociation, since the A2 domain has a weakly interaction with A1/A3-C1-C2 structure (kd ≈ 0.2µmol/L) (Lenting, Mourik, van and Mertens, 1998), or by proteolytic cleavage played by the activated protein C (APC) or by FXa (Orlova *et al.*, 2013). Secondly, another reason for the elimination being different is because, the excretion process is assumed by the liver cells and not by the kidneys (Pipe, 2010).

Reviews described FVIII clearance mainly by the low-density lipoprotein receptor-related protein (LRP1), an endocytic receptor expressed commonly on the hepatocytes membrane and Kupffer cells and also in vasculature structures such the surface of smooth muscle cells, fibroblasts and macrophages (Lenting, Schooten, Van and Denis, 2007) (Sarafanov *et al.*, 2001; Turecek *et al.*, 2020).

The LRP1 receptor has activity towards the A3 domain (at 1804-1834), A2 domain (at 484-509), and C-terminal of the C2 domain (Orlova *et al.*, 2013). The first two domains present a high affinity towards LRP1 (Lenting, Schooten, Van and Denis, 2007) whereas, the latter

domain shares the same site as vWF in binding to FVIII (Orlova *et al.*, 2013). This overlap is the reason for vWF to reduce FVIII clearance, mediated by LRP1, at an extent of almost 90% (Orlova *et al.*, 2013). In contrast, when both are not linked together, C2 domain may act as another site for LRP1 to exert the clearance process (Sarafanov *et al.*, 2001). Nonetheless, LRP1 has been associated with polymorphisms, in particular, the LDLR c.1773C/T genotype, which influences the PK and may also play a part in the inter-individual responses to treatment (Lunghi *et al.*, 2019).

Cell surface heparin sulfate proteoglycans (HSPGs) are components from the extracellular matrix, acting as co-receptors for LRP1 or as independent receptors (catabolic receptors) (Sarafanov *et al.*, 2001). Furthermore, *in vivo* HSPGs can interact within the A2 domain (at 558-565) (Orlova *et al.*, 2013) and facilitate the presentation to the fragments of LRP1 (Lenting, Schooten, Van and Denis, 2007). Hence, it is possible to prolonged the half-life of FVIII if, simultaneously, the HSPGs and LRP1 receptors are blocked, like previously tested in mice (Sarafanov *et al.*, 2001).

Another receptor involved is the asialoglycoprotein or Ashwell receptor (ASGPR), which is expressed by hepatocytes and structurally composed of two transmembrane protein subunits, asialoglycoprotein receptor-1 (ASGR-1) and asialoglycoprotein receptor-2 (ASGR-2) (Terraube, O'Donnell and Jenkins, 2010). Its activity is either towards the FVIII unbounded, through the B-domain (Mei *et al.*, 2010), or to the complex itself (Terraube, O'Donnell and Jenkins, 2010). In mice, when ASGR-1 was blocked, levels of FVIII and vWF raised *versus* ASGR-2 (Terraube, O'Donnell and Jenkins, 2010). Hence, targeting ASGR-1 seems to be a good strategy to reduce the elimination process of FVIII.

More receptors **(Table 19)** have been recently described as having endocytosis activity for FVIII and vWF, in hepatic macrophages and liver sinusoidal endothelial cells (LSECs). They are currently considered the new major *in vivo* regulators (Turecek *et al.*, 2020). Additionally, LSECs are the cells with the highest endocytosis capacity as they offer high ability for lysosomal activities important to the clearance of several blood components (Poisson *et al.*, 2017). However, it is still not totally clear their specific role in regulating the clearance of both forms of FVIII (i.e. unbound and bound) (Turecek *et al.*, 2020).

Table 19 - Summary of the potential clearance receptors involved in FVIII elimination.

Cell Location	Receptor	Reference
Kupffer cells	LRP1	(Lenting, Schooten, Van e Denis, 2007)
Hepatocyte's membrane	LRP1	(Lenting, Schooten, Van e Denis, 2007)
	ASGPR* • ASGR-1 • ASGR-2	(Terraube, O'Donnell e Jenkins, 2010)
	HSPGs*	(Lenting, Schooten, Van e Denis, 2007)
	LDL-R*	(Lenting, Schooten, Van e Denis, 2007)
Hepatic macrophages	SR-A1	(Turecek *et al.*, 2020)
	LRP1	(Turecek *et al.*, 2020)
	MGL	(Turecek *et al.*, 2020)
	Siglec-5*	(Turecek *et al.*, 2020)
Liver sinusoidal endothelial cells	STAB2	(Turecek *et al.*, 2020)
	CLEC4M	(Turecek *et al.*, 2020)

Abbreviations: ***ASGPR:*** *Ashwell receptor;* ***ASGR-1****: asialoglycoprotein receptor-1;* ***ASGR-2:*** *asialoglycoprotein receptor-2;* ***CLEC4M****: C-type lectin domain family 4 member M;* ***HSPGs:*** *heparin sulfate proteoglycans;* ***LRP1:*** *low-density lipoprotein receptor-related protein;* ***LDL-R:*** *low-density lipoprotein receptor;* ***MGL****: macrophage galactose-type lectin;* ***SR-A****: scavenger receptor class A member;* ***Siglec-5****: sialic acid binding immunoglobulin-like lectin 5;* ***STAB2****: stabilin-2*

***in vitro binding FVIII results**

CFCs are excreted by the liver, either as FVIII-free or FVIII-vWF, through the cellular mechanisms involving several receptors **(Figure 17).** Hence, kidneys do not represent a big role in elimination of CFCs (Pipe, 2010).

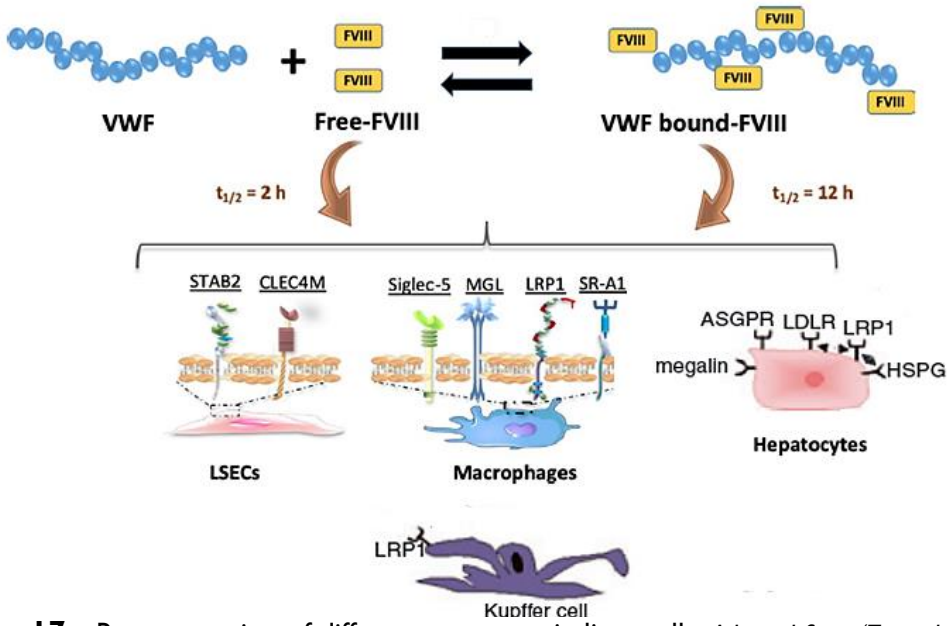

Figure 17 - Representation of different receptors in liver cells. *Adapted from (Turecek et al., 2020) and (Lenting, Schooten, Van e Denis, 2007).*

Abbreviations: ***ASGPR:*** *Ashwell receptor;* ***CLEC4M****: C-type lectin domain family 4 member M; FVIII: factor VIII;* ***HSPGs:*** *heparin sulfate proteoglycans;* ***LRP1:*** *low-density lipoprotein receptor-related protein;* ***LDL-R:*** *low-density lipoprotein receptor; LSECs:* liver sinusoidal endothelial cells; ***MGL****: macrophage galactose-type lectin;* ***SR-A****: scavenger receptor class A member;* ***Siglec-5****: sialic acid binding immunoglobulin-like lectin 5;* ***STAB2****: stabilin-2;* $\mathbf{t_{1/2}}$***:*** *terminal half-life;* ***vWF:*** *von Willebrand Factor*

1.7.4. Pharmacokinetic parameters

Usually, pharmacokinetic behavior is determined by repeated measures of drug concentrations in plasma over time. However, for clotting factors, "drug concentrations" differ from those of general drugs (Shapiro, Korth-Bradley and Poon, 2005). As well-known, clotting factors are endogenous zymogens thus, their activity is measured by bioassays such the one-stage or chromogenic assays **(section 1.1.3**) (Shapiro, Korth-Bradley and Poon, 2005). Hence, the obtained results are often understood as "plasma concentrations" which semantically is not well accepted (Björkman and Berntorp, 2001). Instead, the terms are "activity" or "level" of FVIII in plasma (Björkman and Berntorp, 2001).

For simplicity, pharmacokinetic parameters will be divided as standard and as non-standard/specific parameters.

1.7.4.1. Standard Parameters

Standard parameters is a term used to group up the "basic" pharmacokinetic parameters that characterize the general pharmacokinetic processes (Björkman and Berntorp, 2001; Delavenne and Dargaud, 2020). For instance, the distribution is evaluated through the volume of distribution (Vd), which corresponds to the apparent volume , in which, the drug distributes to achieve the same activity levels as observed in plasma (Iorio *et al.*, 2017). Therefore, after the infusion, Vd is achievable following the **(Equation1):**

$$\text{Vd} = \frac{\text{Dose (IU/kg)}}{\text{Plasma level (IU/mL or dL)}} \qquad \text{(Equation1)}$$

Literature value for Vd is approximately 48 mL/kg (i.e. 0.048 L/kg) (McEneny-King *et al.*, 2017) which is close to the plasma volume in the body (i.e. 3L) (McEneny-King *et al.*, 2017).

Nonetheless, when the FVIII concentrates infusion equals the elimination, steady-state conditions are attainable (Rosenbaum, 2017). In this moment, Vd is not the right term, instead volume at the steady-state (Vss) is the most suitable, as it will represent the equilibrium between compartments (plasma and surrounding tissues) (Rosenbaum, 2017). The value of Vss will always exceed the Vd meaning that, even the large complexes like FVIII are not totally confined to the plasma space (Björkman and Carlsson, 1997). The formula for Vss is presented in **Table 20** (Shapiro, Korth-Bradley and Poon, 2005)**.**

After administration and distribution, molecules can remain on the body so, mean residence time (MRT) is another essential PK parameter that describes this phenomenon (Shapiro, Korth-Bradley and Poon, 2005). It is influenced by distribution (i.e. Vss) and elimination (Björkman and Carlsson, 1997). In addition, MRT estimation **(Table 20)** will depend on the area under of FVIII concentration vs. time curve (AUC) and the area under the first moment of the curve (AUMC) (Morfini, 2002). Both AUC and AUMC are extrapolated to infinity and assess by trapezoid method following **Equations (2)** and **(3),** respectively as C_1 and $t_1$being the first plasma level and the respective time and, C_2 and t_2 the second measures of plasma and time (Shapiro, Korth-Bradley and Poon, 2005).

$$\text{AUC} = \frac{(C_1 + C_2) \times (t_2 - t_1)}{2} \qquad \text{(Equation 2)}$$

$$\mathrm{AUMC} = \frac{(C_1 \times t_1 + C_2 \times t_2) \times (t_2 - t_1)}{2} \qquad \text{(Equation 3)}$$

Furthermore, elimination is determined by clearance (CL) as the value serves to understand the efficacy of the organs such kidneys and/or liver in removing the drug from plasma (Rosenbaum, 2017). The results should be interpreted as the volume of plasma cleared of FVIII concentrates per time unit (Björkman and Carlsson, 1997). This parameter has the particularity to be the constant of proportionality between the rate of elimination and plasma levels **Equation 4** (Rosenbaum, 2017) which means that, when values of CL are high, the rate of elimination will be higher as well (Rosenbaum, 2017). Literature mean CL values in healthy adults with 70 kg surround the 200 mL/h (Björkman and Berntorp, 2001) or 3mL/h/kg (Björkman, Folkesson and Jönsson, 2009), however both are not static values and, yet it may vary from 1.8 to 6 mL/h/kg (Björkman, 2003).

$$\text{Rate of elimination (IU/h)} = \text{CL (mL/h)} \times \text{FVIII:C (IU/mL)} \qquad \text{(Equation 4)}$$

Hence, the amount of FVIII excreted will remain constant per unit of time (Morfini, 2002). Since the elimination is analysed at the slope of the curve **Equation 5** to estimate the constant of elimination (Ke), it should follow equation **5**, as $C1$ and $C2$ two plasma FVIII levels within the terminal section of the curve whilst t1 and t2 are the matching time points (Shapiro, Korth-Bradley and Poon, 2005).

$$\mathrm{Ke} = \frac{(\ln C1 - \ln C2)}{(t2 - t1)} \qquad \text{(Equation 5)}$$

Moreover, the terminal half-life (t½) is helpful to express the rate of the overall elimination during the terminal phase (Toutain and Bousquet-Mélou, 2004). Focus on the equation of t½ **Equation 6** it is quick to understand that will depend on CL and Vd thus, if the drug presents a higher value of terminal half-life (longer elimination), it will be associated with more availability of the drug in the bloodstream (higher Vd) and less ability for the liver to clear the drug (lower CL) (Rosenbaum, 2017; Toutain and Bousquet-Mélou, 2004). Hence, as it depends on other kinetics parameters, t½ is an hybrid PK parameter (Toutain and Bousquet-Mélou, 2004).

$$t_{1/2} = \frac{0.632 \times \text{Volume of distribution}}{\text{Plasma clearance}} \qquad \text{(Equation 6)}$$

As an hybrid parameter, it is difficult to associate values of terminal half-life to clinical features of the patients such as age, body weight or, liver diseases (Toutain and Bousquet-Mélou, 2004). Despite this, terminal half-life helps prescribing dose regimens, particularly for prophylaxis **(section 1.7.4.2.1).** Moreover, average values of plasma half-life vary between 12-14 hours (Björkman, Folkesson and Jönsson, 2009), although recent data have been reported inter-patient variations (Nogami and Shima, 2015). Indeed, among 42 individuals with severe HA, half-life has a wide interval of values between 7.4 to 20.4 hours (Nogami and Shima, 2015). Some other recent studies have also reported intervals from 6 to 25 hours (Hazendonk *et al.*, 2018) or 5.3 hours to 28.8 hours for others (Turecek *et al.*, 2020). Several factors determine the pharmacokinetics as it will be discussed in **section 1.7.5.**

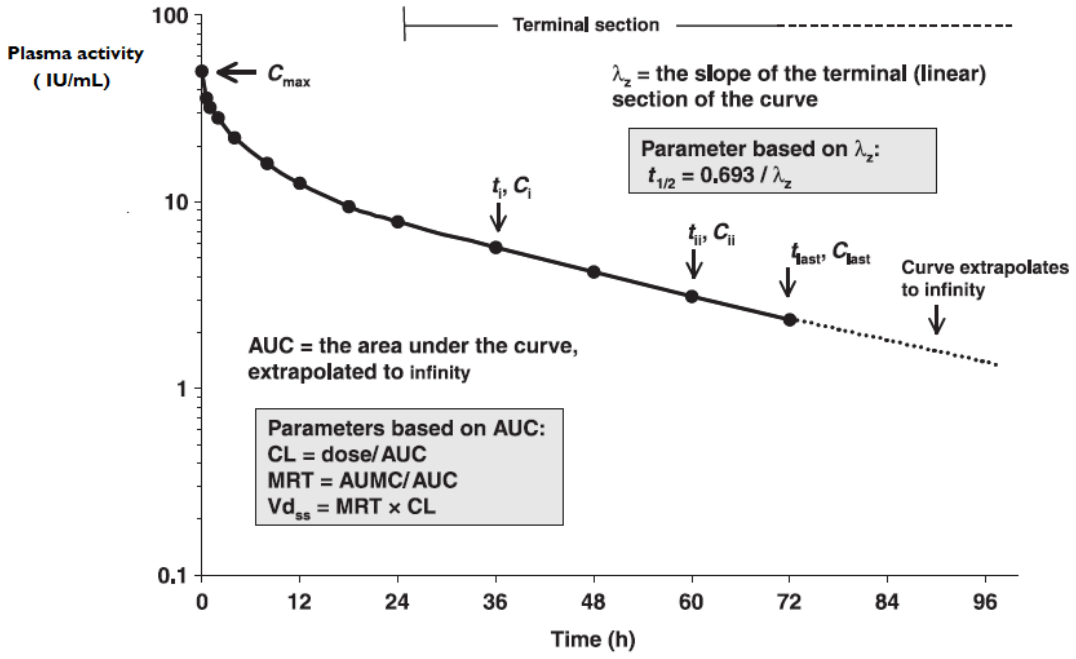

Figure 18 - Representation of the slope curve and several equations that can be applied to assess the patient's pharmacokinetic profile. *Based on (Shapiro, Korth-Bradley and Poon, 2005).*

Table 20 - Summary of the basic/fundamental pharmacokinetic parameters of FVIII concentrates. *Based on (Morfini, 2002) and (Hermans and Dolan, 2020).*

Parameters	Units	Equation	Definition
Volume of Distribution at the steady state (Vss)	mL/kg	$MRT \times CL$	Theorical volume necessary for a certain amount of drug achieve the same activity level as observed in plasma, upon equilibrium between plasma and surrounding tissues.
Clearance (CL)	mL/h/kg	$\frac{Dose}{AUC}$	Volume of plasma cleared of drug per time unit.
Mean Residence Time (MRT)	h	$\frac{AUMC}{AUC}$	The average amount of time that a single molecule unit of the drug remains in plasma or body.
Half-life (t½)	h	$\frac{MRT}{1.443}$	Time to plasma activity level decrease by ½ after equilibrium has reached.
		$\frac{\ln_2}{ke}$	Terminal half-life is a linear regression of logarithmic points in the last portion of activity portion, as elimination becomes constant.

Abbreviations: ***AUC:*** *area under the curve;* ***AUMC:*** *area under the first moment of the curve;* ***ke:*** *constant of elimination*

1.7.4.2. Non-standard

In contrast, non-standard or specific parameters are a better choice to evaluate the effectiveness of regimens and to improve their safeness (Delavenne and Dargaud, 2020). They involve patient clinical events in estimation and interpretation of the results which are useful for therapeutic monitoring (Delavenne and Dargaud, 2020).

Based on **Figure 19**, it is possible to see when the patient reaches the maximum or peak of plasma activity (C_{max}) and the minimum level, also referred as trough level (C_{trough}) (Delavenne and Dargaud, 2020). Furthermore, measure of AUC and the time spent above the threshold (TAT1%) is essential to prevent the risk of bleeding as it will be exploited in section **1.7.4.2.1 Pharmacokinetic parameters for prophylaxis** (Delavenne and Dargaud, 2020).

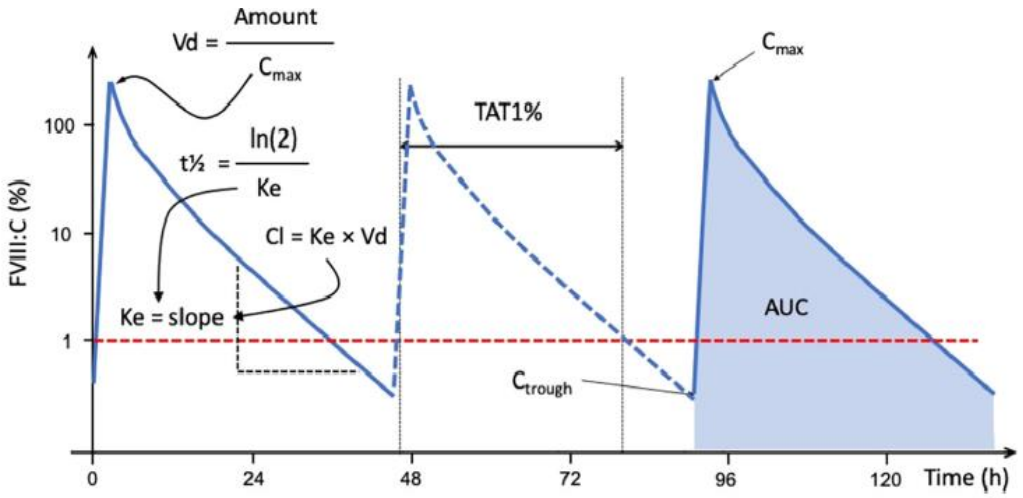

Figure 19 - Representation the FVIII pharmacokinetics profile with several equations that can be applied to the standard and non-standard parameters. *Copyrights to (Delavenne and Dargaud, 2020).*

Abbreviations: ***AUC****- area under the curve;* ***C_{max}****- maximum concentration;* ***C_{trough}****- minimum concentration;* ***CL-*** *clearance;* ***Ke****- constant of elimination;* ***TAT1%-****time above the threshold of 1%;* ***t½-*** *half-life;* ***Vd****- volume of distribution.*

In general, TAT1% is estimated by gathering the administered dose and, the t½ of the concentrate infused on the patient (Delavenne and Dargaud, 2020). This is where the complexity comes in TAT1% , as the terminal half-life is sensitive to the minimum limit of quantification of the quantitative assay (Iorio *et al.*, 2017). For this reason, it is required for an extensive pharmacokinetic analysis that involves 5 to 6 samples for two or three days after infusion of dose (Delavenne and Dargaud, 2020).

Incremental *in vivo* recovery (IVR) or, *in vivo* recovery or, simply recovery, is another non-standard parameter. It directly gives the rise (recovery) of the plasma FVIII activity after administration of a dose (Delavenne and Dargaud, 2020). IVR corresponds to the ratio between peak levels **Equation 7** (Shapiro, Korth-Bradley and Poon, 2005) as the observed peak is directly measured while the expected peak can be assessed either through body weight (BW) or plasma volume (Shapiro, Korth-Bradley and Poon, 2005). The average value, in adults, may vary between 0.020-0.025 IU/dL *per* IU/kg (Barnes, 2013).

$$\text{IVR (IU/dL per IU/kg)} = \frac{\text{Observed peak (IU/dL)}}{\text{Expected peak (IU/dL per IU/kg)}} \qquad \text{(Equation 7)}$$

From the previous equation, in order to assess the expected peak, calculations may change if it is according with patients' plasma level **Equation 8** or with BW (**Equation 9; Figure 20**) (Shapiro, Korth-Bradley and Poon, 2005).

$$\text{IVR (\%)} = \frac{100 \times \text{Maximum rise from baseline} \times \text{plasma volume}}{\text{Dose}} \qquad \text{(Equation 8)}$$

Therefore, the estimated peak by BW is better (Shapiro, Korth-Bradley and Poon, 2005). This formula does not take into account the individual pharmacokinetics such as CL, Vd, and t½ (Hazendonk *et al.*, 2018) as it only measures one value of FVIII:C or the highest of all after a short-infusion (Collins *et al.*, 2011).

$$\text{IVR (IU/dL per IU/kg)} = \frac{\text{Maximum rise from baseline} \times \text{BW}}{\text{Dose}} \qquad \text{(Equation 9)}$$

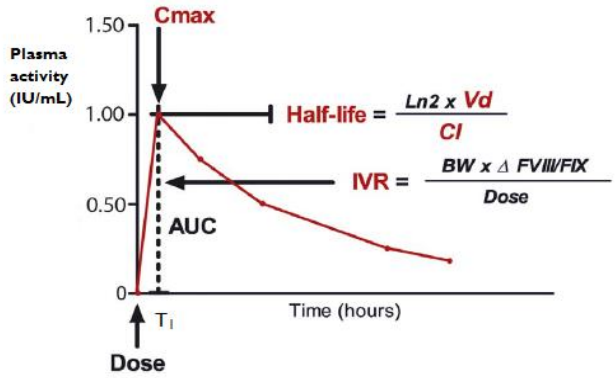

Figure 20- Representation of the FVIII plasma levels *versus* time curve and several equations that can be applied in pharmacokinetics analysis. *Based on (Hazendonk et al., 2018).*

*Abbreviations: **AUC**- area under the curve; **C_{max}**- maximum concentration; **CL-** clearance; **IVR:** in vivo Recovery; Δ- increment of FVIII plasma levels; **t½-** half-life; **Vd**- volume of distribution.*

No matter the formula chosen, the use of IVR in prophylactic regimens is not reliable since IVR has a poor correlation with C_{trough} (Björkman, 2003).

1.7.4.2.1. Pharmacokinetic parameters for prophylaxis

Prophylaxis rationale emerged from the observation of fewer bleeding events on mild/moderate patients (FVIII:C 1-5 IU/dL). It was straight away hypothesized that a C_{trough} above 1 IU/dL was the ideal to reverse the severe state into a milder state (Collins *et al.*, 2010). Additionally, as the time spent below this C_{trough} increases, higher is the risk for bleeding episodes and breakthrough bleeds (Collins *et al.*, 2010). Additionally, measure TAT1% along with C_{trough} is essential to understand the pharmacokinetic response and adjust prophylaxis regimens to the patient lifestyle (Collins *et al.*, 2010).

Another parameter to make prophylactic regimens more effective is the t½ (Carcao and Iorio, 2015; Collins *et al.*, 2010). As studied by Collins *et al.,* among the children and adults that were administered with (30 IU/kg), the ones with shorter t½ reached the C_{trough} of 1% more quickly (44 hours and 46.4 hours, respectively) than those with longer t½ (78 hours and 103.3 hours, respectively) (Collins *et al.*, 2010).

Moreover, other studies demonstrated that peak levels and AUC also describe the efficacy of prophylaxis (Valentino *et al.*, 2016). A *post hoc* comparison between pharmacokinetic-guided prophylaxis with standard weight adjustments prescribing ADVATE® concluded that, peak levels and AUC may be associated with the risk of joint and non-joint bleeding (Valentino *et al.*, 2016). Specifically, higher values of both parameters were positively correlated with lower bleeds. However, it is important to establish that these findings are for prophylaxis given every third day in severe patients and it cannot be extrapolated to other regimens (Valentino *et al.*, 2016).

In clinical practice, it is common the doubt on what parameter should be used for prophylaxis. To clear these questions, each parameter should be tailored to the circumstances of the patient lifestyle (Collins et al., 2011). For instance, an individual with HA that frequently practices sports is more prone to the risk of bleeding and injuries than sedentary patients (Morfini and Farrugia, 2019). Therefore, assessing the time to attain peak levels is more relevant to define the exact time of the next infusion (Escobar et al., 2019; Morfini and Farrugia, 2019). In opposition, if the patient is more sedentary, it is more important to maintain minimal FVIII levels to protect against bleedings (Escobar et al., 2019; Morfini and Farrugia, 2019). In case of patient is not adhering to the treatment properly, the best scenario would be reducing dosing frequency along with the trough level and TAT1% (Delavenne and Dargaud, 2020). The utility of AUC will remain on the evaluation of the total exposure to understand the overall protection and preventing, then, subclinical bleeds and target joints (Delavenne and Dargaud, 2020; Escobar et al., 2019).

1.7.4.2.2. Pharmacokinetic parameters for surgeries or on-demand

In surgeries or acute bleedings, the goal is to achieve a certain level above the C_{trough} but not too high above the C_{max} (Hazendonk *et al.*, 2018). To manage these clinical situations, peak levels and IVR are the most important parameters to take into account (Hazendonk *et al.*, 2018). Peak levels will depend on the location of the bleeding and the severity of the surgery **(Table 21)** (Hazendonk *et al.*, 2018). IVR equation usually used is the one proposed by Prowse **(Equation 10)** which is simplified by the ratio of only the post-infusion peak level (IU/dL) and the dose infused (IU/kg) dose **(Equation 10)** (Björkman and Berntorp, 2001; Morfini, 2017).

$$IVR = \frac{\text{Post-infusion peak (IU/dL)}}{\text{Dose (IU/kg)}} \quad \text{(Equation 10)}$$

However, the peak of the activity itself is not found directly after the end of infusion (Björkman and Berntorp, 2001). Reports have found peaks of FVIII at 10 to 15 minutes or more delayed, at 1 to 2 hours, which makes IVR dependent on rigorous sampling process (Björkman and Berntorp, 2001). These discrepancies evidence the inter-patient variability that induces pharmacokinetic protocols to recommend extrapolation of plasma activity based on three samples (15, 30, and 60 minutes) (Delavenne and Dargaud, 2020). Others also use IVR-extrapolated at time 0 which is equivalent to the C_{max} of FVIII at that time, being the ratio of C_{max} and dose the right equation (Morfini, 2017). Regardless, sit has been also reported that IVR is important to determine the loading dose of a new CFC as it only requires two samples, one at the baseline and the other after post-infusion, following **Equation 11** (Shapiro, Korth-Bradley and Poon, 2005):

$$\text{Number of IU required} = \text{BW (kg)} \times \text{desired rise (IU/dL)} \times \text{Reciprocal IVR (IU/kg per IU/dL)} \quad \text{(Equation 11)}$$

Table 21 - Guidance in peak levels and duration of administration of FVIII concentrates for treatment in acute bleedings and/or surgeries. *Based on (Srivastava et al., 2020).*

	Lower-dose regimen		**High-dose regimen**	
Hemorrhage	Peak factor level (IU/dL)	Duration (days)	Peak factor level (IU/dL)	Duration (days)
Joint	10-20	1-2 [a]	40-60	1-2 [a]
Superficial muscle No neurovascular compromise (except lipossomas)	10-20	2-3 [a]	40-60	2-3 [a]
Iliopsoas or deep muscle with neurovascular injury OR substantial blood loss				
Initial	20-40	1-2	80-100	1-2
Maintenance	10-20	3-5 [b]	30-60	3-5 [b]
Intracranial Bleeding				
Initial	50-80	1-3	80-100	1-7
Maintenance	20-40	8-14	50	8-21
	30-50	4-7	----	-----
Throat and Neck				
Initial	30-50	1-3	80-100	1-7
Maintenance	10-20	4-7	50	8-14
Gastrointestinal				
Initial	30-50	1-3	80-100	7-14
Maintenance	10-20	4-7	50	
Renal	20-40	3-5	50	3-5
Deep laceration	20-40	5-7	50	5-7
Major surgery				
Pre-operative	60-80	--------	80-100	----------
Post-operative [c]	30-40	1-3	60-80	1-3
	20-30	4-6	40-60	4-6
	10-20	7-14	30-50	7-14
Minor surgery				
Pre-operative	40-80	----------	50-80	----------
Post-operative [d]	20-50	1-5	30-80	1-5

Notes: ***a-*** *May be longer if necessary;* ***b-*** *Sometimes longer as secondary prophylaxis during physical therapy;* ***c-*** *Duration referring to sequential days post-surgery (depending on treatment and the patient response);* ***d-****Depending on procedure; doses will depend on half-life of treatment used.*

1.7.5. Factors that Contribute to Inter-Individual Variability

1.7.5.1. von Willebrand Factor

vWF is a plasma glycoprotein that interacts with certain domains of FVIII, resulting in the vWF-FVIII complex. This complex is beneficial, in particular for FVIII because it can: (a) stabilize the FVIII structure as a heterodimer in the bloodstream facilitating the activation for thrombin; (b) protects FVIII from proteolysis cleavage by FXa or APC which will inactivate the protein; (c) modulates interaction with serine protease FIXa and, (d) regulates the cellular uptake within clearance circulation removal (Lenting, Schooten, Van and Denis, 2007; Terraube, O'Donnell and Jenkins, 2010).

Furthermore, studies have shown that vWF levels (vWF:Ag) are positively correlated with t½ of FVIII. For each rise of 0.1 IU/dL in vWF:Ag an increase of 16.6 minutes in FVIII t½ is observed (Turecek *et al.*, 2020). Also, plasma FVIII and vWF levels can vary between 0.5 to 2 IU/mL in healthy individuals counting for 15% of inter-individuality (Turecek *et al.*, 2020).

1.7.5.2. ABO Blood Type

ABO blood group is a system of antigens consisting on three determinant structures such A, B, and H (Turecek *et al.*, 2020). A study made in twins demonstrated that approximately 30% of plasma levels of vWF were influenced by the ABO blood group (Wang *et al.*, 2017). Moreover, as FVIII presents affinity towards vWF, indirectly the ABO blood group will also contribute to variability in FVIII (Turecek *et al.*, 2020). In fact, patients from O blood group have 20-30% lower vWF:Ag levels versus the non-O blood (type A, B or AB) (Turecek *et al.*, 2020; Wang *et al.*, 2017). As explained before, vWF protects FVIII from early elimination so, if the blood O type group has less of vWF then, a shorter t½ value is expected whereas, CL would be higher values. Indeed, studies comparing the different types of blood groups proved lower t½ in O-blood patients (15.3 hours) comparing, non-O blood patients (19.7 hours) (Kamphuisen, Eikenboom and Bertina, 2001; Turecek *et al.*, 2020).

Although the mechanism it is not yet elucidated, some hypotheses have been purposed. Before secretion, vWF is submitted to glycosylation with some ABO(H) structure similarities present in glycan structures from the proteins (Turecek *et al.*, 2020). One is a possible effect of the ABO group on the N-linked oligosaccharide of vWF chains as they share carbohydrate structure similarities (Wang *et al.*, 2017). Alternatively, it is also questionable the H antigen expression ABO group, on regulating vWF levels or, in ADAMTS13 which is an important protease for vWF proteolysis (Wang *et al.*, 2017).

It was also studied the chance of Rhesus blood group (RhD) phenotype, defined by the protein present in erythrocytes membrane, target the FVIII PK but no influence on pharmacokinetics was observed (Turecek *et al.*, 2020).

1.7.5.3. Gender and Race

Mean levels of vWF and FVIII were demonstrated to be significantly higher for females than males (Wang *et al.*, 2017).

As for ethnicities, levels of FVIII and vWF are 20% higher in African Americans than Caucasians (Terraube, O'Donnell and Jenkins, 2010; Wang *et al.*, 2017). Typically, the prevalence of the ABO system tends to vary within racial groups. However, the influence in FVIII clearance remains consistent in studies that involved different ethnicities (Turecek *et al.*, 2020; Wang *et al.*, 2017).

1.7.5.4. Age

Age is associated with changes in several organs starting with maturation throughout the pediatric phase (Anker, van den *et al.*, 2018) and a decline of the functions from adults to elderly ages (Thürmann, 2019). These changes are also observed in the coagulation system as aging rises several clotting factors, particularly FVIII, in healthy individuals (Miesbach *et al.*, 2009).

Several studies reveal an inverse relationship between age and clearance which, in turn, will influence the half-life (Björkman, 2013). Clearance of FVIII showed to be greater in children than adults (Björkman, 2013) while, the half-life is described as shorter in children aged between 1-6 years (9.4 hours) than in patients within 10 and 65 years old (11.1 hours) (Hermans and Dolan, 2020; Turecek *et al.*, 2020).

Younger ages have a liver ratio mass of 30-35% (Curry and Whelpton, 2010) as this tends to decrease with aging as well as their respective functions (Thürmann, 2019). For CFCs, this means that elimination by the liver will be at a lower rate, prolonging FVIII half-life (Thürmann, 2019). Another possible explanation could be the lower expression of LRP1 verified in aging rats which will also decrease the CL and longer t½ (Sagare, Deane and Zlokovic, 2012).

There have been studies that describe a positive correlation between aging and vWF levels (Favaloro, Franchini and Lippi, 2014) as an influence for secretion and clearance (Turecek *et al.*, 2020). Once again, indirectly this will affect the FVIII with older patients presenting more vWF complexed to FVIII, more time in circulation, and more protected from CL (Turecek *et al.*, 2020).

Moreover, since vWF is also influenced by the ABO blood group, one study was able to analysed the same influence but associated with aging (Albánez *et al.*, 2016). In that study, about 207 individuals were divided into three age categories (young, middle, or older) and it was measured vWF:Ag levels as well as the ABO antigen (Albánez *et al.*, 2016). Conclusions were that the ABO blood system is submitted to changes throughout ages and, A- and B- antigens were the main factor for high values of vWF (Albánez *et al.*, 2016). This supports findings of an increase in vWF levels of 0.16 IU/dL for every 10-years, being more significant in non-O individuals than O blood group ones (Turecek *et al.*, 2020).

1.7.5.5. Bodyweight

The increase of BW leads to higher body fat and therefore there is less plasma volume available per kg of BW (Tiede *et al.*, 2020).

Usually, patients are classified, in terms of weight, through the body mass index (BMI) determined through **equation 12** (Curry and Whelpton, 2010):

$$BMI = \frac{BW}{(H)^2} \qquad \text{(Equation 12)}$$

A underweight patient is someone with BMI <20 kg/m^2 whereas, overweight has BMI >25 kg/m^2(Hunt, 2018). Additionally, obesity is diagnosed when patients present a BMI >30 kg/m^2 and subdivided as moderate (BMI: 30-35 kg/m^2), severe (BMI: 35-40 kg/m^2), morbid (BMI >40 kg/m^2) and super-morbid (BMI >50 kg/m^2) (Curry and Whelpton, 2010; Hunt, 2018). Therefore, overweight/obese patients will have lower plasma volume than underweight patients, as they will present more plasma volume per kg of BW (Tiede *et al.*, 2020).

For FVIII, these situations may impact its levels if the body fat and BW compositions are underestimated in clinical practice (Tiede *et al.*, 2020). As explained before, FVIII is confined to intravascular space. The vasculature represents a small fraction of body fat tissue volume (0.005 to 0.010%) so, more or less percentage of fat in this space will not have an impact on the distribution or elimination (McEneny-King *et al.*, 2016). Therefore, it has been recommended the use of ideal body weight (IBW) **(Equation 13)** for dose estimations instead of the actual BW(Tiede *et al.*, 2020).

$$IBW(kg) = \text{Height (cm)} - 100 - \left[\frac{\text{Height (cm)}-150}{4}\right] \qquad \text{(Equation 13)}$$

Moreover, for dose adjustments according to BW, IVR is usually used, following **Equation 14:**

$$\text{Dose} = \frac{\text{Total body weight (kg) x desired increase in FVIII level (\%)}}{\text{IVR}} \quad \text{(Equation 14)}$$

Body metrics can be a tool to predict pharmacokinetic parameters and subsequently helping in dosing tailoring (Henrard, Speybroeck and Hermans, 2013). Typically, IVR average value is 2 IU/dL *per* IU/kg (McEneny-King *et al.*, 2017) or, also common, the value of 0.5 dl/kg which is the plasma volume considered to achieve *this recovery* (Henrard, Speybroeck and Hermans, 2011). In previous equation **(Equation 9)**, it is possible to conclude a proportional correlation between IVR and BW and, thus, as weight increases IVR will also rises (Collins *et al.*, 2011). A pioneering study was able to analysed the influence of several morphological variables on FVIII *recovery* in 201 patients (Henrard, Speybroeck and Hermans, 2013). Four groups were created according to the BMI: the underweight (BMI <18.5 kg/m^2), normal weight (BMI between 18.5 and 24.9 kg/m^2), overweight (BMI between 25.0 and 29.9 kg/m^2) and, obese patients (BMI ≥30.0 kg/m^2) (Henrard, Speybroeck and Hermans, 2013). BMI was found to be the best predictor for IVR, as values of 1.60 were related to patients with BMI <20 kg/m^2, 2.14 for BMI between 20 and 30 kg/m^2 and 2.70 for BMI ≥30 kg/m^2 (Henrard, Speybroeck and Hermans, 2013). Consequently, assuming the same IVR value (2 IU/dL *per* IU/kg) for every patient, does not take into consideration the physiological characteristics of that patient (Henrard, Speybroeck and Hermans, 2013), leading to doses errors.

Furthermore, the same researchers also found a high IVR dependency on fat mass index (FMI) (Henrard, Speybroeck and Hermans, 2011). FMI is another metric that englobes information on BW, height, and the fat mass (FM), calculated by the formula **15** (Alpízar *et al.*, 2020):

$$\text{FMI} = \frac{\text{FM (kg)}}{\text{height (m}^2\text{)}} \quad \text{(Equation 15)}$$

Whereas the fat mass (FM) calculated by the formula **16** (Alpízar *et al.*, 2020):

$$\text{FM} = \frac{\text{FM(\%)} \times \text{BW(kg)}}{100} \quad \text{(Equation 16)}$$

Moreover, at the study they recruited 46 patients with different severity of HA as well as different BMI categories (Henrard, Speybroeck and Hermans, 2011). Patients were then divided in three groups according to their FMI where 9 had FMI <15.0%, 11 were between 15.0% and 19.9%, and 26 with FMI ≥20.0% (Henrard, Speybroeck and Hermans, 2011). Mean

IVR values increased from 1.74 to 1.89 and 2.35, respectively, suggesting that higher percentage of FMI are associated to higher FVIII levels and *vice versa* (Henrard, Speybroeck and Hermans, 2011). Also, patients with FMI ≥ 20.0% were overtreated while patients with FMI <15% were undertreated when assumed IVR of 2, supporting the idea of the importance for variability and the need for individualization (Henrard, Speybroeck and Hermans, 2011). At the end, they defended the use of the value of 2 for *recovery* only when the patient presented a normal BW as well as an FMI between 15-20% (Henrard, Speybroeck and Hermans, 2011).

Hence, FVIII dosing should be defined according to the weight of each patient (underweight and overweight patients) and BMI and FMI are good to predict IVR in different body compositions (Henrard, Speybroeck and Hermans, 2013).

A side note, BMI was also found to be a good predictor for pharmacokinetic parameters comparing to other metrics such as IBW, Lean Bodyweight, Adjusted Bodyweight, or Body Surface Area. However, this was described in a population with no inclusion of underweight patients, no extremely body muscle mass patients, and neither anaemic ones so, BMI results were not tested yet in these physiological characteristics (Tiede *et al.*, 2020). Plus, BMI is not good for t½ so, for prophylaxis, this metrics is not helpful since half-life has an impact to target trough levels. Therefore it is possible to conclude that BMI is and may be useful to manage patients in surgeries or acute bleedings (Tiede *et al.*, 2020). Also, for adjustments of dose in children, the total of BW gives a poor description of PK so either LBW or BSA can be applied (Björkman, Folkesson and Jönsson, 2009). BSA has a relationship between the surface area (S), body weight (BW), and height (H) (Curry and Whelpton, 2010):

$$S=BW^{0.425} \times H^{0.725} \times 71.84 \qquad \text{(Equation 17)}$$

1.7.5.6. Immunogenicity

Inhibitors, as they express capacity to enable FVIII protein, will unavoidably impact the pharmacokinetics. It is reported that inhibitors enhance the Vss and CL whereas half-life remains unchanged and IVR is lower (Björkman and Berntorp, 2001; Shapiro, Korth-Bradley and Poon, 2005).

As for "non-inhibitory" antibodies, although they do not express a function directly to FVIII (Miller, 2018), in 42 adults with severe/moderate HA with high-titer there was a decrease in half-life compared to patients without antibodies (Abdi *et al.*, 2020; Turecek *et al.*, 2020). In contrast with the previous class, they do not affect IVR or distribution itself (Björkman, 2003). The mechanism is still not understood (Abdi *et al.*, 2020) but these antibodies account for 17%

of inter-individual variability in FVIII half-life (Turecek *et al.*, 2020) as an increased clearance and shorter t½ (Abdi *et al.*, 2020; Turecek *et al.*, 2020).

1.7.5.7. Liver Diseases

The liver, as explained before, is the main organ for the synthesis of clotting factors hence, diseases such as chronic- and acute liver failure or even cirrhosis, may have an impact on PK (Miesbach *et al.*, 2009). FVIII is the most affected by these conditions as their levels will be elevated (Miesbach *et al.*, 2009) as well as for vWF that, increases in acute failure and higher in liver cirrhosis (Holestelle *et al.*, 2004).

The mechanism is still poorly understood but it might be related to either vWF or LRP1 (Holestelle et al., 2004). Since vWF co-exists with FVIII, the higher the levels the better protection of FVIII from elimination thus, prolonged time with FVIII levels elevated (Holestelle et al., 2004). If on the one hand the liver is also the main organ to synthesize clotting factors, on the other it clear CFCs so, in liver disease conditions, the predominant receptor LRP1 might be less expressed, resulting in a lower CL and an increase in half-life (Holestelle *et al.*, 2004).

Such conditions should not be ignored in HA population since that, a lot of patients were or may be infected with HCV (Miesbach *et al.*, 2009), which contributes for higher risk to develop chronic liver disease or hepatocellular carcinoma (Pradhan-Sundd *et al.*, 2021). Plus, this might impact more mild patients since, they still have more residual activity than severe patients (Miesbach *et al.*, 2009).

An important reminder is that FVIII is also produced by other organs tissues such as lungs, kidneys, spleen, lungs, and brain that might also impact the plasma levels but, no studies related yet this possible influence on PK (Holestelle *et al.*, 2004).

1.7.5.8. Pregnancy

Overall, healthy pregnant women experience changes in their cardiovascular profile with an increased risk of stroke (Feghali, Venkataramanan and Caritis, 2015). This is directly related to hemostatic changes with the rise of most clotting factors whilst anticoagulants factors decrease as well as the fibrinolytic activity (Franchini, 2006).

Women with HA are rare so it is more common to report them as carriers (Leebeek, Duvekot and Kruip, 2020). Carriers may be classified as obligatory (certain to have the X-chromosome) or as possible (chance to have the affected gene) (Leebeek, Duvekot and Kruip, 2020). The fact that they have only one chromosome affected, it is expected to present 50%

of the normal FVIII levels (Chi *et al.*, 2008) so, pregnant women still present a high risk for bleeding during and after pregnancy (Leebeek, Duvekot and Kruip, 2020).

In terms of FVIII levels, the tendency is to rise during the first half of the pregnancy (Franchini, 2006), however not all carriers showed the same plasma activity as wide range of values from 5 IU/dL to 219 IU/dL (Chi *et al.*, 2008) have been reported. This wide range shows inter-individual variations and an important fact that justify the importance of a properly manage in carriers HA women with some of them being at higher risk of bleeding (if show lower levels) than others (Chi *et al.*, 2008).

Since carriers can reach 50% of normal levels, usually there is no need for therapeutic intervention even though the use of DDAVP proved to be effective in carriers with FVIII levels below 40 IU/dL (Franchini, 2006). It is a fact that pregnancy always comes with adjustments to PK parameters due to several changes within the body but, for this disease, women suffering if HA are rare and analysis of PK in this population is not reproducible.

1.7.6. Individualized Treatment

Individualized regimen could be *a priori* if the dose regimens are adjusted to patient clinical information (i.e. weight, age, genetics) or*, a posteriori* after the administration of the concentrate to understand the behaviour of the drug in a specific patient (Abrantes, 2019). The need of monitoring and tailoring treatment in FVIII concentrates is grounded on (1) inter-individual variability pharmacokinetic response; (2) higher concentrations or low concentrations may be dangerous for the patient; (3) treatment is expensive for the patient (Carcao and Iorio, 2015).

(1) <u>Same treatment inter-individual variability pharmacokinetic response</u>

Hemophilia population is considered heterogenic not only for the differences in response to the treatment but also, for the variability related to the bleedings (Sørensen *et al.*, 2015). As explained in **section 1.1.4**, hemophilia severity is established and accepted by the current guidelines as an association between bleeding characteristics and residual FVIII:C (Nogami and Shima, 2015)**.** However, recent studies are showing that patients with the same residual FVIII:C can present different clinical bleedings (Jayandharan and Srivastava, 2008). For example, in 5 to 10% of severe patients with levels FVIII <1IU/dL, mild phenotype were observed whereas 15% of moderate patients presented a frequent bleeding registry (Nogami and Shima, 2015)**.**

In terms of frequency of bleedings, it is expected from severe patients to present, on average, 15 to 35 spontaneous joint and muscle bleeds (Jayandharan and Srivastava, 2008). But, similarly to the example before, within mild and moderate patients, clinical irreversible

arthropathy were observed (Sørensen *et al.*, 2015). Furthermore, studies made in this population revealed that arthropathy can happen either in patients that bleed frequently or in absence of it as a subclinical characteristic (Sørensen *et al.*, 2015).

Besides the different clinical phenotypes, as a goal of prophylaxis is to maintain levels above 1IU/dL to prevent clinical futures, it is predictable that assuming this limit for all HA patients may not be the ideal in clinical practice (Sørensen *et al.*, 2015). In fact, some patients with levels above 1% may not bleed whereas others may bleed with 3 IU/dL trough levels (Sørensen *et al.*, 2015). As for TAT1% the same must be applied as this measure should not be interpreted as 1IU/dL is a critical value to be above and prevent risk for every patient (Collins *et al.*, 2010).

All these arguments prove the existence of heterogenicity between HA patients (Nogami and Shima, 2015)**.** As this is explained by multifactorial factors (Nogami and Shima, 2015)**,** monitoring every patient is important, regardless of the severity classification (Sørensen *et al.*, 2015). Furthermore, individual trough levels and TAT1% should not be considered 1 IU/dL for all of them, instead, it should be remember the dynamics of parameters that may vary from 2 IU/dL to 15-20 IU/dL to the upper limit which is around 60-160 IU/dL (Morfini, 2017).

(2) Higher concentrations or low concentrations may be dangerous for the patient;

FVIII concentrates have an extensive therapeutic range when compared to general drugs that are submitted to monitoring/tailoring. In this case, it is dangerous for patients life to not present the minimum levels for therapeutic effect (subtherapeutic levels) as patient will suffer from hemorrhages or arthropathy (Carcao and Iorio, 2015). On other hand, FVIII levels above the maximum desirable (supratherapeutic levels) can also be a risk for thrombosis even though this is less harmful and can easily be manageable, as explained in **section 1.1.3** (Carcao and Iorio, 2015).

(3) Treatment is expensive for the patient

Despite the rareness of HA, replacement care is overall very expensive (Café *et al.*, 2019). In Portugal, is prophylactic regimens is estimated an annual mean cost of 26,333€ per HA patient being one of the regimens that contributes to 89% of the costs (i.e. 50,712€) (Café *et al.*, 2019).

Tailoring prophylaxis will help to choose the best dose and frequency according to the patients' characteristics and thus, preventing the waste of money in ineffective treatments (Carcao and Iorio, 2015). One study pharmacokinetics guided-dosing showed a reduction in annual consumption could decrease by 31% with pharmacokinetics dosing (Carlsson *et al*., 1997) as well as the reduction in annual joint bleeding rate (Iannazzo *et al.,* 2017).

Since in Portugal is estimated a cost of 40.4 million euros are spent per year by the National Health System (Café *et al*., 2019), monitoring CFCs and tailor regimens based on the needs of each patient could bring a cost-effective system in hemophilia care. Also, it could improve adherence in patients that cannot afford short dosing often (Carcao and Iorio, 2015). Moreover, as the study by Café *et al.,* patients with antibodies will have more costs (7.6 times higher) than the patients without these complications, due to the external treatments required for the proper management (Café *et al*., 2019)**.** For a better perception of the costs involved per patient, Café *et al.* estimated costs of 134,032€ for patients with antibodies, whereas 40,318€ was the cost associated with the patient without the adverse event (Café *et al*., 2019)**.**

Based on these arguments for individualization, is the case to conclude and apply the slogan "one size does not fit all" (Morfini, 2017). Plus, tailoring PK will not be just effective and safe but also well economically balanced (Björkman and Berntorp, 2001).

Chapter 2- Aims and objectives

2.1. Aim

As hemophilic patients exhibit heterogenic clinical status and the treatment is very diversified, this investigation aimed at identifying the factors that contribute for the variability observed in treatment response of HA patients admitted at the University Hospital Centre of Coimbra (CHUC, EPE).

2.2. Specific Objectives

To attain the aforementioned aim, the following specific objectives were established:

- Literature review of the typical kinetics behavior of infused FVIII concentrates in HA patients;
- Descriptive analysis of the demographic, clinical, pharmacological data from HA patients prescribed with FVIII concentrates between 1st January of 2018 and 30th June of 2020;
- Collection of plasma FVIII levels and the administered dose of each infused concentrate on HA patients;
- Assessment of the influence of inter-individual variability factors on the FVIII kinetics and efficacy;
- Estimation of the pharmacokinetic parameters as well as the administered dose for each patient;
- Identify the published pharmacokinetic model more suitable to define the first dose to administered;
- Suggest a monitoring protocol to be used at the clinical practice at CHUC, EPE.

Chapter 3- Research methodology

3.1. Study Design

This research was an observational and retrospective study accepted by the Health Ethics Committee of CHUC, EPE. Clinical patient process file was assessed through the hospital database *SClinico* as well as the prescribed treatment through the integrated system of drug management (SGICM4).

All patient information was codified in numbers to guarantee the anonymity, respecting the rights of the patients involved.

3.2. Screening of patients

As the project focused only HA patients, other coagulopathies such as Hemophilia B, Hemophilia C or von Willebrand disease were excluded. Inclusion criteria were as follow: patients with more than 17 years old with at least one FVIII concentrate prescription between the period of 1st January of 2018 and 30th June of 2020 at the immune-hemotherapy service of CHUC, EPE. This study excluded pregnant women as well as those with no clinical information available.

3.3. Data collection

SClinico database was used to compile demographic and clinical data whereas SGICM4 was required for collection of the treatment regimen designed for each patient. To identify the factors of variability underlying FVIII pharmacokinetics, the FVIII levels were collected as well as the blood type and vWF antigen (vWF:Ag), resorting to the SGICM4 platform.

Data was organized in an *Excel* document, building a database with:

- Demographic data: Age, weight (kg), gender, height (m);
- Clinical data: Severity, inhibitors, complications of the disease, viral infections, blood type (A, B, AB, O);
- Pharmacological data: concentrate category/generation, applied regimen (prophylaxis/on-demand), time for infusion, date of start and finish the treatment, *switch* in treatment;
- Biochemical data: measurements of FVIII levels (time, data, value), vWF:Ag.

After the database was completed, it was possible to describe and analyze HA patients considering the previously collected demographic, clinical and pharmacological data. Additionally, the monitoring of FVIII levels were also described regarding the number of monitored patients, the number of collected samples, posology regimen and disease severity.

The assessment of plasma FVIII levels and the administered dose of each infused concentrate was only considered in patients under prophylaxis regimen that had a minimum of two blood samples and enough data related to the infusions (defined days as well as the hour prescribed to the infusion). To standardize the analysis, patients were divided based on the samples monitoring (*"same day"* or *"different days"*) and on the frequency of prophylaxis ("three times *per* week" or "two times *per* week")

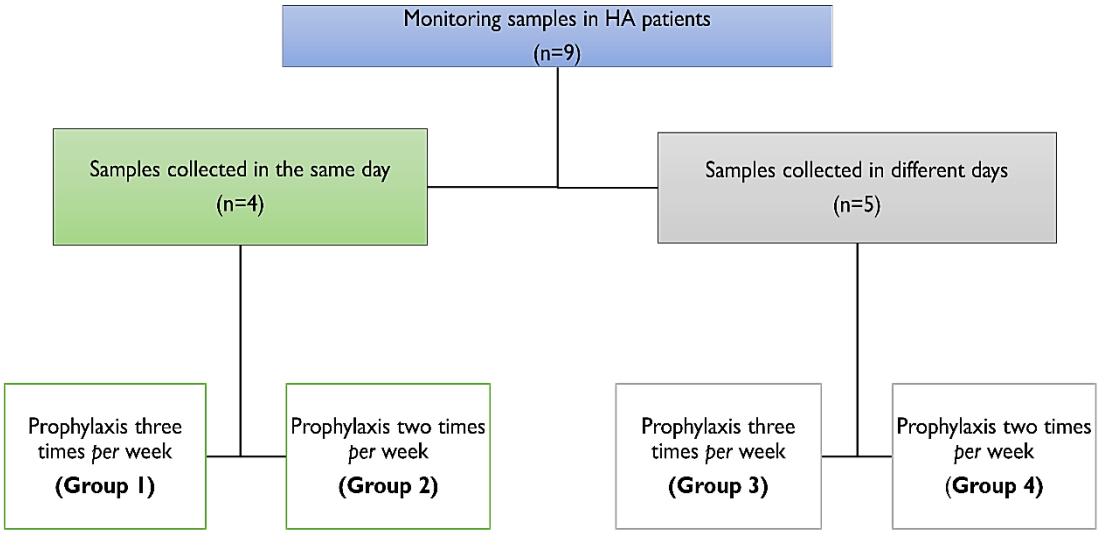

Figure 21- Distribution of HA patient's groups in FVIII levels analysis regarding their monitoring samples process.

For pharmacokinetic estimations, HA patients were enrolled only when presenting at least three FVIII levels and complete information regarding dose, frequency, accurate time of sample collection and the history about the last infusion. The pharmacokinetics parameters assessed included C_{max}, C_{trough}, AUC and AUMC, Vss, CL, t½ and MRT according with equations provided through **1.7.4 Pharmacokinetic parameters.**

The assessment of the influence of inter-individual characteristics on FVIII kinetics and efficacy of the treatment was not possible to attained due to the lack of clinical information in these considering patient

3.4. Data analysis

Microsoft Excel Office (2016 version) and *Statistical Package for the Social Sciences* (IBM SPSS 23 version) were used for statistical analysis including descriptive statistics, which enrolled mean, median, mode, maximum and minimum values as well as standard variations. In addition, Microsoft Excel® was also required to estimate the pharmacokinetic parameters applying bibliographic models.

Chapter 4- Results

4.1. Description of the study population

Among the 65 HA patients followed at CHUC, EPE, only 46 matched to the inclusion criteria of the study.

- Demographic data

All patients were masculine (100%), all of them prescribed with at least one FVIII concentrate between 1st January of 2018 and 30th June of 2020 at the immune-hemotherapy service. The mean of age for these patients was 43.7 years old varying between 20.0 years (minimum) and 78.0 years old (maximum) **(Figure 22)**.

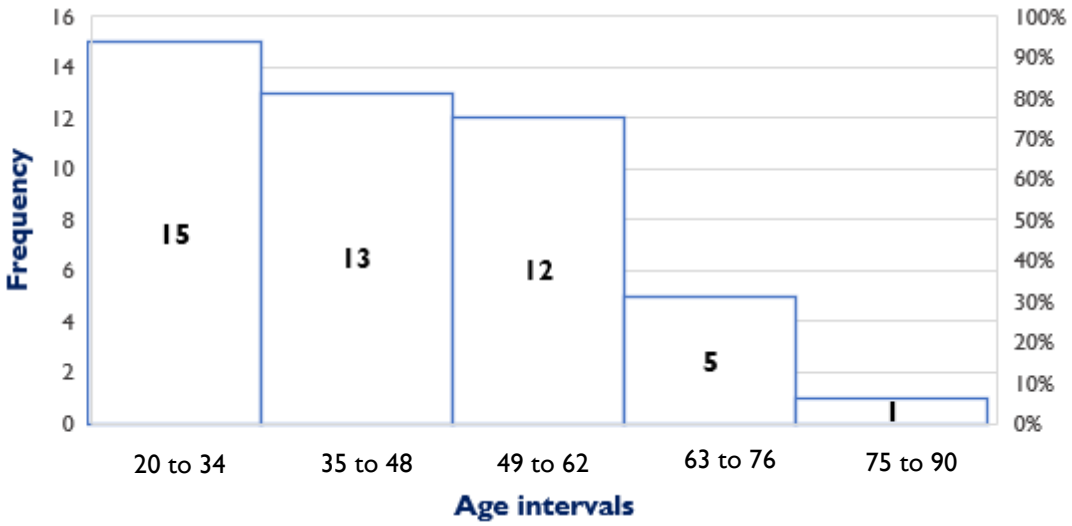

Figure 22 - Distribution of HA patients regarding their age. Results expressed in absolute (left y axis) and relative frequency (right y axis).

Regarding patient weight, it was only reported for 41 patients. Accordingly, the mean weight of test population was 78.5 kg varying between 58 kg (minimum) and 148 (maximum) **(Figure 23).** Note that IMC was not possible to be herein determined since, the height was not reported in any of the patients at the time of the study.

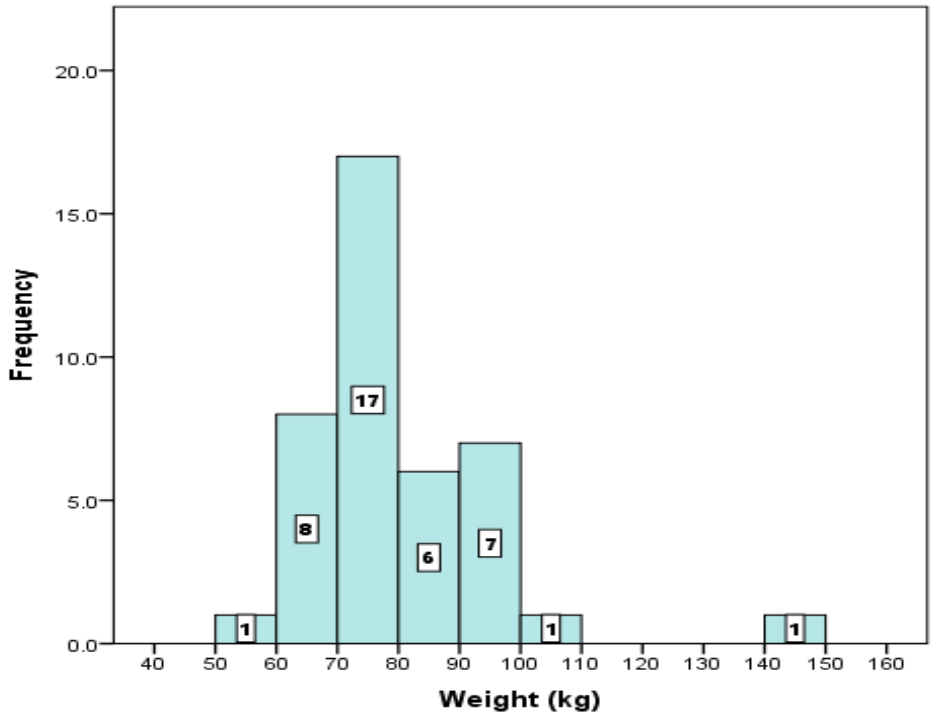

Figure 23 - Distribution of HA patients regarding their weight. Results expressed in absolute frequency (left y axis) and weight (x axis).

- Clinical data

Clinically, 34 patients were diagnosed with severe HA (73.9%) whereas 10 were mild (21.7%) and 2 had no definition in terms of their severity (4.3%) **(Figure 24).** There were no moderate patients in the tested population. The mean age of severe group was 39.9 years old for the while 54 years old was the mean for mild HA patients **(Figure 25).**

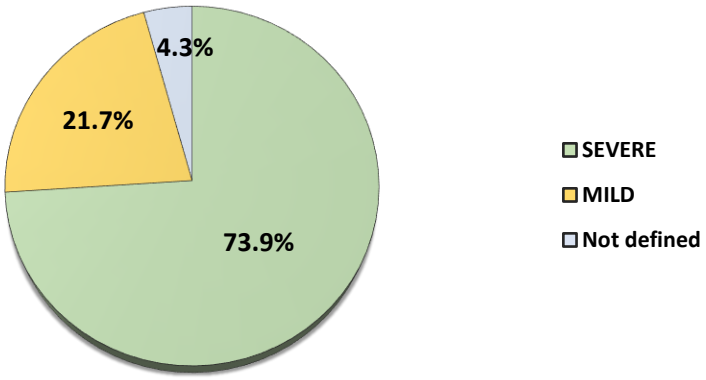

Figure 24 - Pie chart of severity distribution among the HA patients herein tested (results expressed in relative frequency, %).

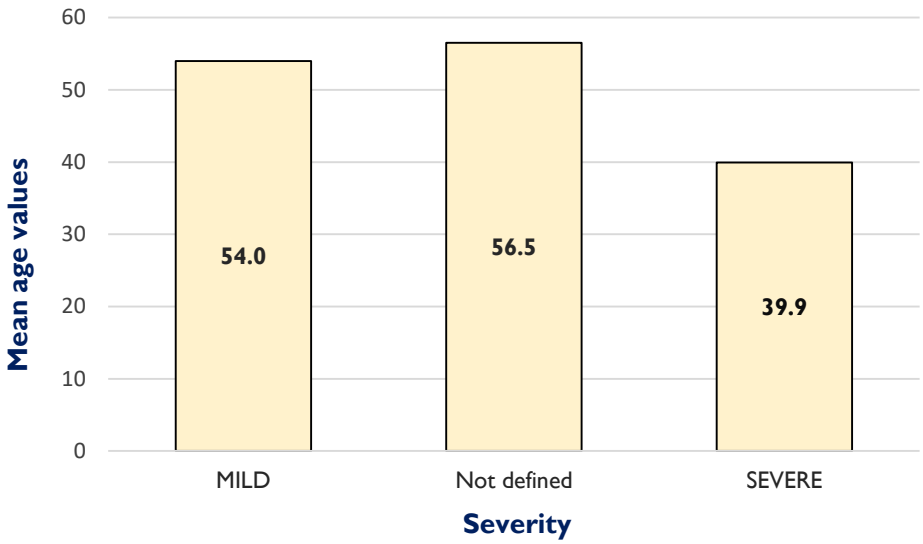

Figure 25 - Distribution of severity grade of the disease by mean of age in HA patients.

In terms of the presence or absence of inhibitors **(Figure 26)**, most of the patients in the study (93.5%) presented negative results, meaning that only 3 patients (6.5%) presented inhibitors. Additionally, all the positive inhibitors were classified as low titer inhibitors (<0.5 BU) and all of them were diagnosed with severe HA.

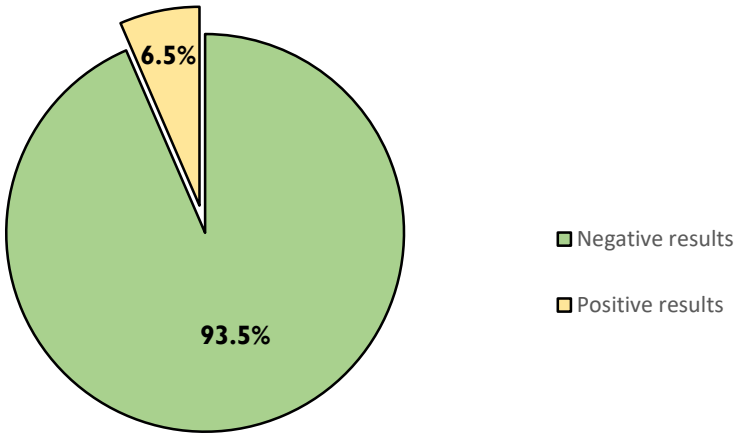

Figure 26 - Distribution of HA patients by inhibitors (results expressed as relative frequency in percentage, %)

Furthermore, **Table 22** sums up the overall clinical history in terms of the viral infections and immunizations of patients. As these patients often develop complications such hemorrhages, arthropathy or the urgency for replacement surgery, **Table 23** illustrates the number of patients with complications of this matter (n=11).

Table 22 - Overall relative frequency of the virus infections reported in HA patients.

Infection / Patients	HAV	HBV	HCV	HIV
Infected	2.2%	2.2%	6.5%	2.2%
Immunized	8.7%	23.9%	17.4%	10.9%
Not infected/immunized	89.1%	73.9%	76.1%	87%
Total	100%	100%	100%	100%

Abbreviations: ***HAV****-hepatitis A virus;* ***HBV****- hepatitis B virus;* ***HCV****- hepatitis C virus;* ***HIV****- human immunodeficiency virus*

Table 23 - Distribution of HA complications in patients (n=11). Percentages are in relation to all the analysed population.

Clinical HA complications	Frequency	Relative Frequency (%)
Arthropathy	3	6.5%
Located hemorrhages	5	10.9%
• Stomach	1	
• Elbows	3	
• Ankles	1	
Surgery Replacement	3	6.5%
• Knees	2	
• Hip	1	
Total	11	23.9%

It is important to highlight that blood type and the levels of vWF (vWF:Ag) were not requested throughout the period of time (from1st January of 2018 to 30th June of 2020), precluding the results to be analyzed.

- Pharmacological data

As the period of this retrospective study was extensive, most of the patients suffered *switch* in their treatment. Therefore, the description will be always made comparing "before *switch*" and "*after switch*". Hence, between 1st January of 2018 and 30th June of 2020, 20 patients *switched* their treatment, 18 had no changes, and 8 had no specific reports regarding *switch* or not **(Figure 27).** The number of patients that had the *switch* is sum up in **Table 27** alongside with the modality of the *switch***.**

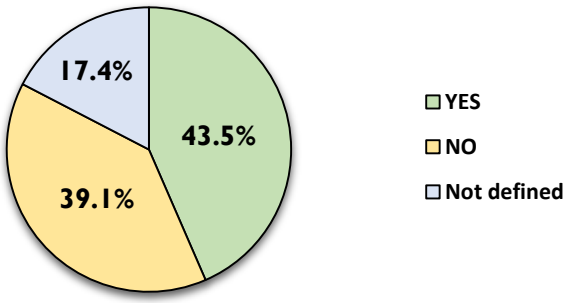

Figure 27 - Distribution of the HA patient's *switch* treatment. Results presented in relative frequency (%).

Only 4 patients suffered changes regarding their pharmacological regimen **(Table 24)**. In fact, focusing on **Table 24**, before the *switch* there was 25 patients under prophylaxis (23 severe, 1 mild and 1 not defined) while *after switch* 27 patients were under prophylaxis (25 severe, 1 mild and 1 not defined).

Onto the other regimen, before *switch* there was 17 patients on-demand (10 severe, 6 mild and 1 not defined) whereas the number decreased to 15 patients (after *switch* 8 severe, 6 mild and 1 not defined) **(Table 24)**.

Overall, the percentage of the test population, after *switch* and on prophylaxis was 54.7% while they were only 32.6% on demand. The patients that previously did not have their severity and regimen defined remained the same (8.7%).

Table 24 - Distribution of the regimen of HA patients by regimen before and after the switch.

Before Switch								
Regimen	**Severity**						**Total**	
	Severe		**Mild**		**Not defined**			
	N	%	N	%	N	%	N	%
Prophylaxis	23	49.9	1	2.2	1	2.2	25	54.3
On-demand	10	30.3	6	13.0	1	2.2	17	37
Not defined	1	2.2	3	6.5	0	0	4	8.7
After Switch								
Regimen	**Severity**						**Total**	
	Severe		**Mild**		**Not defined**			
	N	%	N	%	N	%	N	%
Prophylaxis	25	54.3	1	2.2	1	2.2	27	58.7
On-demand	8	17.4	6	13.0	1	2.2	15	32.6
Not defined	1	2.2	3	6.5	0	0	4	8.7

Not defined- means not described

In terms of the dose frequency, patients who were under prophylaxis had prescriptions three days *per* week (Monday,Wednesday,Friday) or two days *per* week (Tuesday, Friday), before the *switch* **(Table 25)**. After *switching*, the common frequency of infusion was two times *per* week (either Monday/Thursday or Tuesday/Friday) **(Table 25)**. However, for some patients (n=9) with a reported switch, it was not possible to assure that the frequency of infusion remained the same. Due to this uncertainty, these patients were classified as "not defined" **(Table 25)**.

Table 25 - Distribution of FVIII concentrates by the days of infusion per regimen prescribed to the patients.

Before Switch						
	Regimen					
	Prophylaxis		On-demand		Not defined	
Frequency of dosing	N	%	N	%	N	%
Three days *per* week	8	17.4	0	0	0	0
Two days *per* week	12	26.2	0	0	0	0
Not defined	5	10.9	0	0	4	8.7
Not applicable	0	0	17	37	0	0
After Switch						
	Regimen					
	Prophylaxis		On-demand		Not defined	
Frequency of dosing	N	%	N	%	N	%
Three days *per* week	1	2.2	0	0	0	0
Two days *per* week	12	26.1	0	0	0	0
Not defined	14	30.4	0	0	4	8.7
Not applicable	0	0	15	32.6	0	0

Not defined: means not described; *Not applicable*: is related to the on-demand regimen

Furthermore, the prescription of concentrates **(Table 26)** had variations before and after the switch. Although ADVATE® was the most prescribed concentrate before any *switch (*n=23), after *switch*, ADVATE® was equal to Adynovi® (n=12). This means that before any *switch,* the third generation was the most prescribed (n=34) as SHL the class of FVIII concentrates most prevalent (n=40) **(Figure 28)**.

After *switching*, the prevalence of PEGlyation (n=12) and Fc-Fusion (n=4) increased. Even though SHL class was still predominant (n=27), there was a significant increase in EHL (n=16) **(Figure 29).**

At the end, most of the switches made in the tested population were more related to FVIII concentrates prescribed (n=16), alongside to the days/frequency of dosing (n=12) **(Table 27).** Changes related to the regimen (from prophylaxis to on-demand) were less common **(Table 27).**

Table 26 - Distribution of the FVIII concentrates in HA patients, before and after the switch.

Concentrates	**Classification**	Before the switch	After the switch
		N	N
Kogenate®	SHL, 2nd Generation	4	2
ADVATE®	SHL, 3rd Generation	23	12
ReFacto AF®	SHL, 3rd Generation	5	3
Kovaltry®	SHL, 3rd Generation	6	6
Elocta®	EHL, Fc-Fusion	2	4
Nuwiq®	SHL, 4th Generation	2	4
Emicizumab®	Monoclonal Antibody	1	1
NovoSeven®	rFVIIa	1	1
Adynovi®	EHL, PEGylated	1	12
DDAVP®	Not applicable	1	1

Abbreviations: ***SHL****-standard half-life;* ***EHL****-extended half-life;* ***rFVIIa****-Recombinant activated Factor VII*

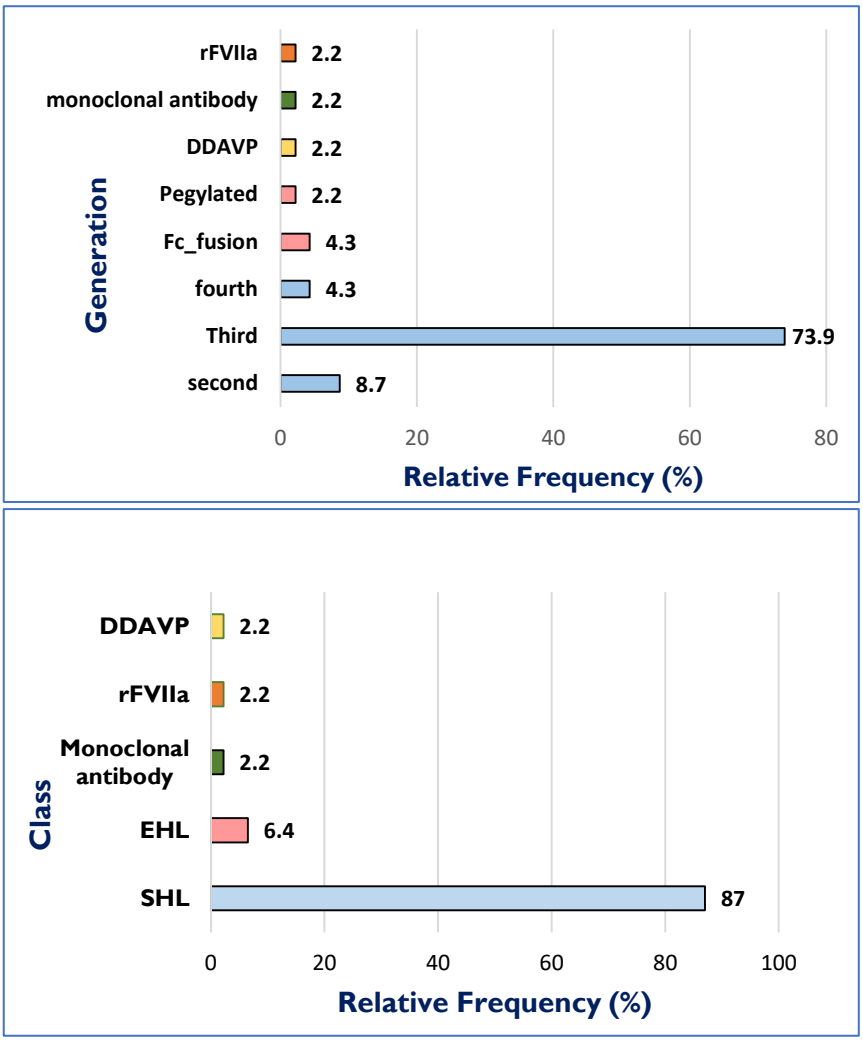

Figure 28 - Distribution of FVIII concentrates prescribed before the switch, by generations and classes.

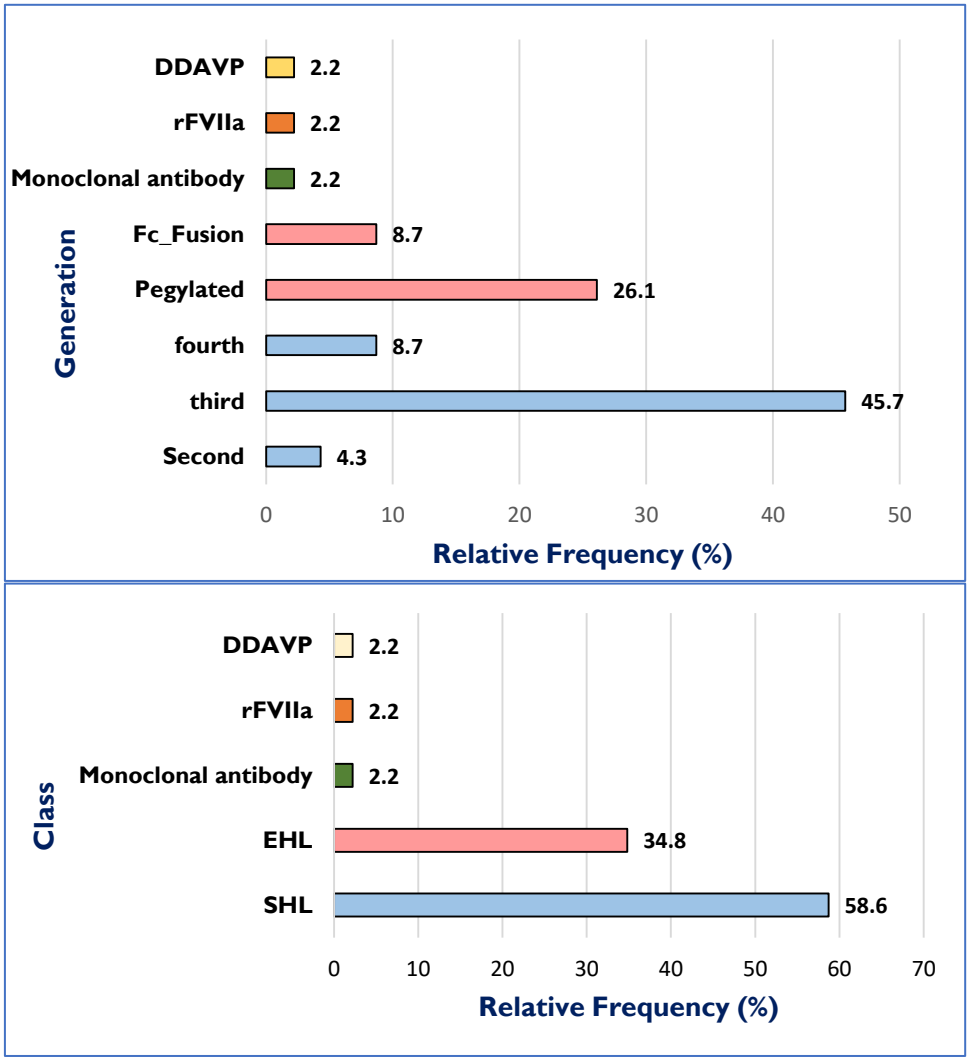

Figure 29 - Distribution of FVIII concentrates prescribed after switch, by generations and classes.

Table 27- Representation of the overall switch modality applied to the HA patients.

Modality of the switch	Number of patients (Frequency)	Number of patients (Relative Frequency, %)
FVIII Concentrate	16	34.8%
Dosing Frequency	8	17.4%
Regimen	4	8.7%
Days of infusion	12	26.1%
Dose	7	15.6%

4.2. Description of FVIII levels monitoring

Among the 46 patients, 40 (87.7%) had FVIII levels monitored whereas 6 (13.0%) were not subjected to any blood sampling monitoring. Of the 40 monitored patients, 31 had severe HA (67.4%) and 7 mild HA (15.2%). In addition, the total number of blood samples varied between them **(Figure 30)**. Nonetheless, most of them collected one blood sample (n=19) followed by two blood samples *per* patient (n=8).

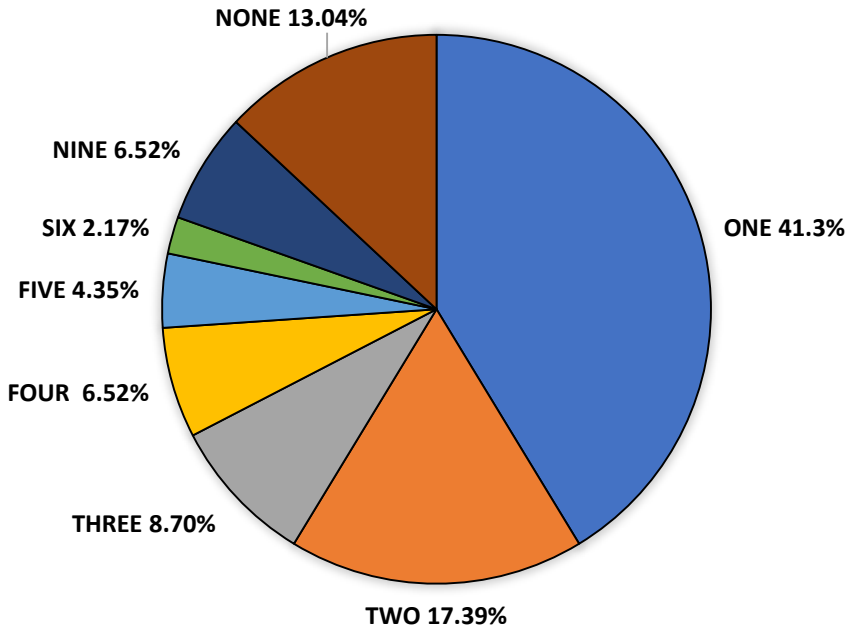

Figure 30 - Distribution of the blood sample FVIII levels scheme per patient.

Regarding the monitoring levels of FVIII, patients were organized by their posology regimen and disease severity **(Table 28)**. Most of the severe patients had only one blood sample either under prophylaxis regimen (n=9) or on-demand (n=7).

Table 28- Distribution of the blood sampling scheme applied to the HA patients.

Prophylaxis HA patients						
Number of blood samples *per* patient	**Severity**					
	Severe		Mild		Not defined	
	N	%	N	%	N	%
One	9	19.6	-	-	-	-
Two	1	2.2	1	2.2	1	2.2
Three	3	6.5	-	-	-	-
Four	3	6.5	-	-	-	-
Five	2	4.3	-	-	-	-
Six	1	2.2	-	-	-	-
Nine	3	6.5	-	-	-	-
None	1	2.2	-	-	-	-
On-demand HA patients						
Number of blood samples *per* patient	**Severity**					
	Severe		Mild		Not defined	
	N	%	N	%	N	%
One	7	15.2	2	4.3	1	2.2
Two	2	4.3	3	6.5	-	-
None	2	4.3	1	2.2	-	-
Not defined regimen HA patients						
Number of blood samples *per* patient	**Severity**					
	Severe		Mild		Not defined	
	N	%	N	%	N	%
Three	-	-	1	2.2	-	-
None	-	-	2	4.3	-	-

4.3. Analysis of the FVIII levels

To perform an accurate analysis of the FVIII levels, patients who had at least two blood samples were herein enrolled. Moreover, since on-demand patients only infused in acute situations, the evaluation was directed towards prophylaxis HA patients with a defined days of infusion as well as the hour prescribed to the infusion.

Therefore, the monitoring values were organized after separation of patients that had prophylaxis three times *per* week **(Group 1 and Group 3)** from those treated two times *per* week **(Group 2 and Group 4)**. The monitoring applied will be described as "levels on the same day" versus "levels in different days".

a) Monitoring samples collected in the same day

In Group 1, FVIII levels monitored on the same day are presented **Figure 31** and **Table 29**. Each patient was prescribed with ADVATE® (third generation, SHL class) with the same days of infusion (Monday, Wednesday, Friday). Patient A and C had a 2000 IU.

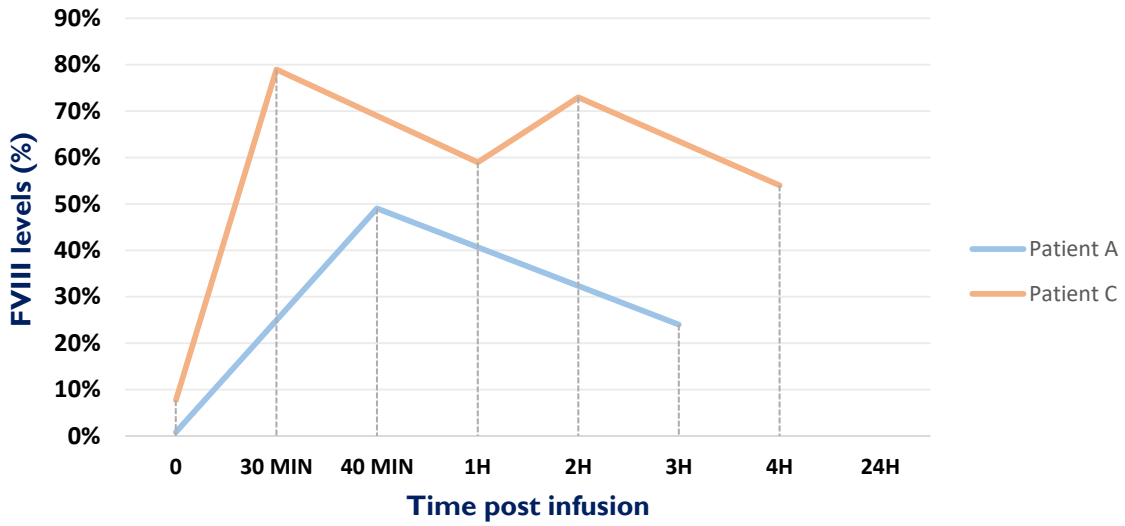

Figure 31 - Chart distribution of FVIII levels in **Group 1.** Results of FVIII expressed in percentage (%).

Table 29 - Distribution of FVIII levels of **Group 1.**

		Time post infusion							
	Dose (IU)	**Time Zero**	**30 min**	**40 min**	**1 hour**	**2 hours**	**3 hours**	**4 hours**	**24 hours**
Patient A	2000	0.80%	-	49%	-	-	-	24%	-
Patient C	2000	7.70%	79%	-	59%	73%	-	54%	-

At the pre-infusion time (time zero), the highest value was achieved in patient C (FVIII =7.70%) followed by patient A (FVIII=0.80%). Since there were no specific times for measures, it was not possible to establish correlations with each post-infusion time.

Even so, variations of the FVIII levels over the time were observed. For instance, after 40 minutes post-infusion, patient A reached 49% in FVIII levels, decreasing to 20%, 4 hours after administration. In patient C, time zero was significantly different, demonstrating a higher FVIII activity at 30 minutes and 2 hours after infusion. It is possible to observe that, at 4 hours post-administration, patient C had higher FVIII levels than patient A, both prescribed with ADVATE® (SHL, 3rd generation) with the same dose.

In **Group 2**, FVIII levels monitored on the same day are presented at **Figure 32** and **Table 30.** Patient B had Adynovi® prescription (PEGylated concentrate, EHL class) with 2500 IU dosed on Mondays and Thursdays. Patient D had ADVATE® prescription (same as **Group 1**) with 2000 IU for dose on Tuesdays and Fridays.

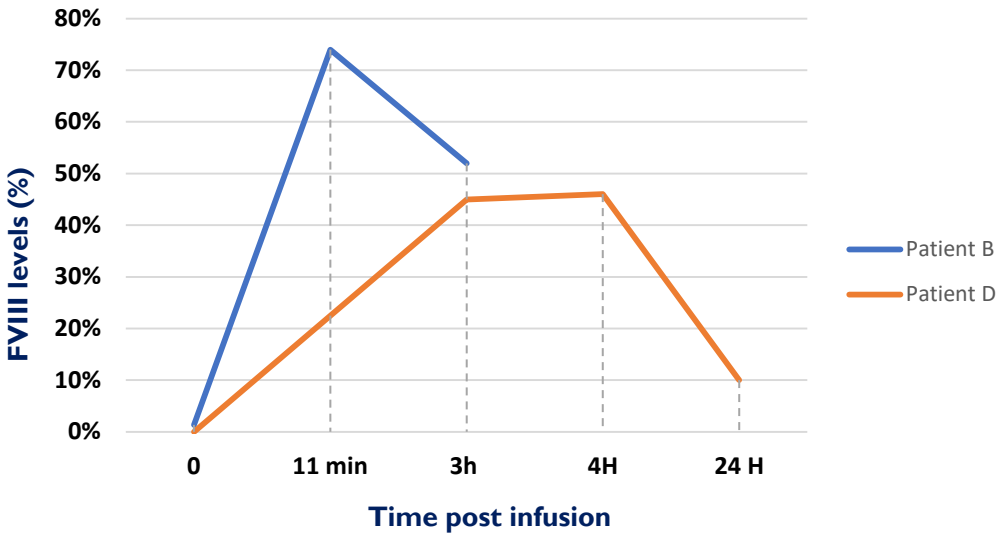

Figure 32 - Chart distribution of FVIII values of Patient B and D in **Group 2.** Results of FVIII expressed in percentage (%).

Table 30 - Distribution of the FVIII levels of the **Group 2.**

		Time post infusion				
	Dose (IU)	**Time Zero**	**11 min**	**3 hours**	**4 hours**	**24 hours**
Patient B	2000	1.35%	74%	52%	-	-
Patient D	1500	1.50%	-	45%	46%	10%

At the pre-infusion time (time zero), patient B and patient D had closer values (1.35% and 1.50%, respectively) with different doses and concentrates. Based on their blood sampling, C_{max} was attained 11 minutes post-dosing (FVIII=74%) for patient B and, 4 hours (FVIII=46%) for patient D.

At 24 hours post-infusion, patient D only presented 10% of FVIII levels which is considerably low considering that, the next infusion, was only 2 days after. This clearly evidence the importance of monitoring FVII for each patient.

b) Monitoring on different days

In this category, only 7 patients presented two or more samples although collected in different days. The monitoring values were also organized, separating the ones that had prophylaxis three times *per* week **(Group 3)** from those that received two times *per* week **(Group 4)**.

In **Group 3**, patient E had three different days for monitoring levels (monitoring 1, monitoring 2, monitoring 3) **(Figure 33)**. In the first monitoring, patient E was prescribed with Kovaltry® (Third generation, SHL) at the dose of 1750 IU whereas, in monitoring 2 and 3, patient *switched* the dose to 2000 IU, keeping the same concentrate and regimen. Additionally, monitoring 1 and 3 were measured only at 4 hours post-infusion, and, therefore, only monitoring 2 allowed the description of the patient levels profile over time **(Figure 33)**.

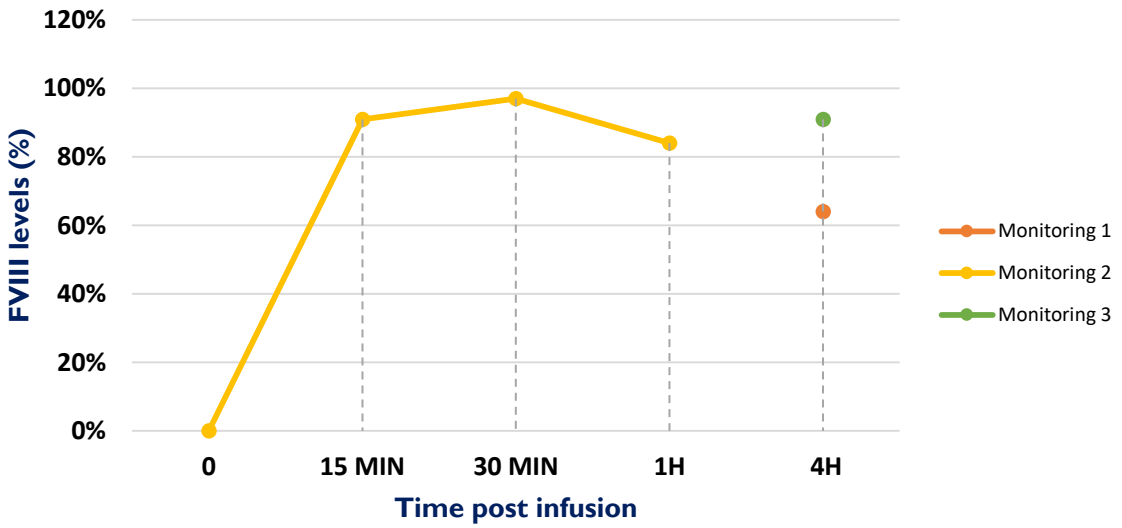

Figure 33 - Monitoring FVIII levels representation of patient E. Results of FVIII expressed in percentage (%).

Patient F had an extended monitoring level and belonged to the **Group 4**. As the previous one, FVIII levels were measured in three different days (monitoring 1, monitoring 2, monitoring 3) **(Table 31)**. In all measures, the regimen remained the same (prophylaxis on Mondays and Thursdays) as well as the dose (1500 IU) and the concentrate (Adynovi®). The first two monitoring were crucial to trace a profile of the variation levels until 72 hours **(Figure 34)**.

As patient G was administered with the same dose (1500 IU), **Figure 34** also represents the variations levels over time. This latter patient differs from F in days of dosing (Tuesdays and Fridays) and in the concentrate prescribed (ADVATE®). Each monitoring was collected in three separate days as Patient F **(Table 31)**.

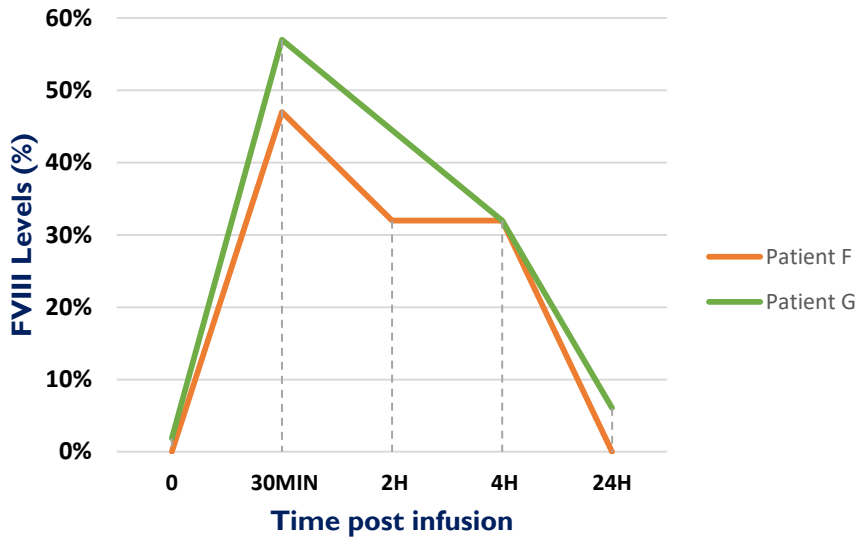

Figure 34 - Representation of the FVIII levels of patient F and patient G. Results of FVIII expressed in percentage (%).

Patient H had a higher dose (2000 IU), two different concentrates such ReFacto AF® (third generation, SHL class) and Elocta® (EHL class, Fc-fusion) and each monitoring corresponded to each concentrate. Although he maintained the dose, he *switched* not only the concentrate but, the days of prophylaxis infusion (Monday/Friday to Tuesday/Friday). The variation of levels is in **Figure 35** and **Table 31.**

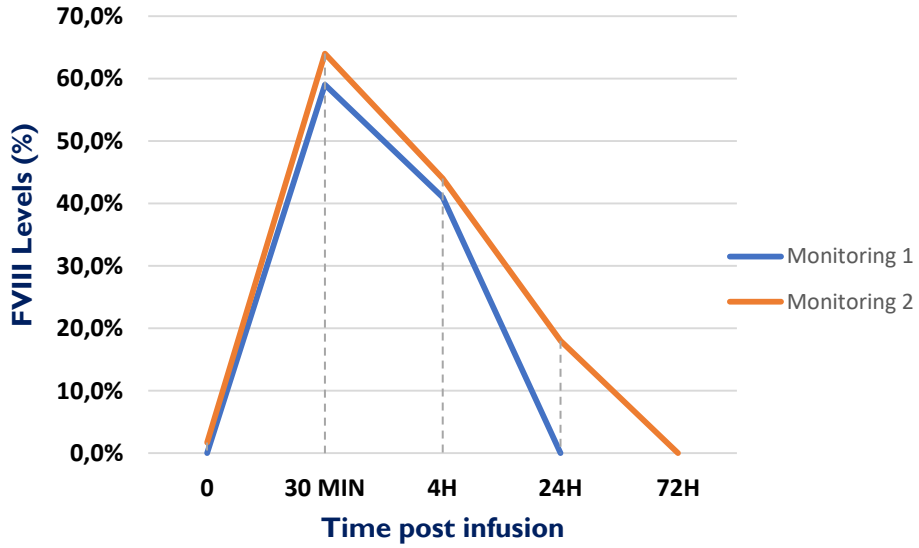

Figure 35 - Representation of patient H FVIII levels. Results of FVIII expressed in percentage (%).

Finally, patient I had the highest doses (3000 IU) for Adynovi® prescription on Tuesdays and Fridays. During the monitoring, this patient did not suffer from *a switch* of any kind. The profile was extended until the 75 hours post-infusion even though the measurements were into three separate days **(Figure 36)**.

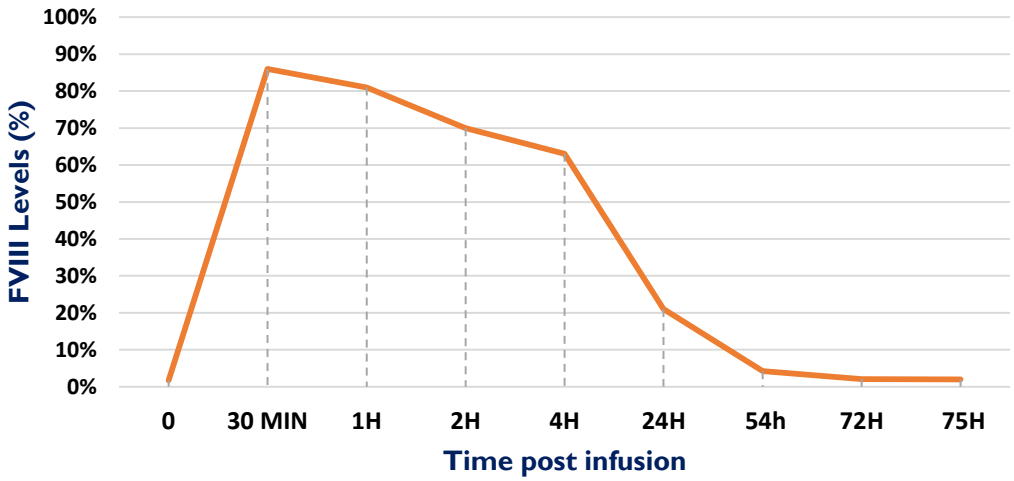

Figure 36 - Representation of patient I FVIII levels. Results of FVIII expressed in percentage (%).

Table 31- Summary of the FVIII levels obtained in HA patients monitored in different days. Results of FVIII levels expressed in percentage (%).

Prophylaxis three times *per* week											
			Time post infusion								
Patient code	Monitoring	Dose (IU)	0	15 MIN	30 MIN	1H	4H				
E	1	**1750**	-	-	-	-	64				
	2	**2000**	13.8	91	97	84	-				
	3	**2000**	-	-	-	-	91				
Prophylaxis two times per week											
			Time post infusion								
Patient code	Monitoring	Dose (IU)	0	30 MIN	1H	2H	4H	24H	54H	72H	75H
F	1	**1500**	<0.4	47	-	-	32	-	-	-	-
	2	**1500**	-	-	-	-	-	-	-	<0.7	-
	3	**1500**	-	-	-	32	-	-	-	-	-
G	1	**1500**	1.90	57	-	-	-	-	-	-	-
	2	**1500**	-	-	-	-	32	-	-	-	-
	3	**1500**	-	-	-	-	-	6.10	-	-	-
H	1	**2000**	6.3	59	-	-	41	10.7	-	-	-
	2	**2000**	1.70	64	-	-	44	18	-	3.4	-
I	1	**3000**	1.70	86	81	-	63				
	2	**3000**	-	-	-	-	-	21	-	2.10	-
	3	**3000**	-	-	-	70	-	-	4.20	-	2

4.4. Pharmacokinetic estimations for HA patients

Pharmacokinetic estimations were only considered on HA patients who had at least three FVIII levels and complete information regarding dose, frequency of dosing, accurate time of sample collection and the history about the last infusion. Therefore, only seven patients were considered to determine the pharmacokinetic parameters, among which four were monitored during the same day **(Table 32)** and three in different days **(Table 33)**.

Table 32 - Estimations of pharmacokinetic parameters in HA patients monitored on the same day.

Monitoring the same day	**Patient A**	**Patient B**	**Patient C**	**Patient D**
Collected Blood Samples (number)	3	3	5	4
Age (years)	53	47	35	43
Concentrate	ADVATE®	Adynovi®	ADVATE®	ADVATE®
Frequency	three days *per* week	two days *per* week	three days *per* week	two days *per* week
Dose (IU)	2000	2000	2000	1500
Maximum concentration **(C_{max})**	49%	74%	79%	46%
Trough level **(C_{trough})**	0.8%	1.35%	7.70%	1.50%
Area under the curve **(AUC)** (IU*h/dL)	694.4	1520.5	1727.6	207.0
Area under the first moment of curve **(AUMC)** (IU*h/dL)	1392.0	2386.5	13044.0	1740.0
Volume of distribution **(Vss)** (dL)	5.8	2.1	8.7	60.9
Clearance **(CL)** (dL/h)	2.9	1.3	1.2	7.2
Half-time **(t½)** (h)	11.413	10.821	19.929	13.153
Mean residence time **(MRT)** (h)	2.00	1.57	7.55	8.41

The results presented in **Table 32** include the pharmacokinetic estimations for the patients monitored on the same day. It is noteworthy the difference on the number of the taken blood samples. Patients on this set have also different ranges from 53 years old to the minimum of 35 years old. Most of them were prescribed with ADVATE® (patient A, C and D) while only one patient had a prescription of Adynovi® (Patient B).

In general, C_{max} varied from 46 to 79% whereas, C_{trough} varied from 0.8% to 7.70%. Comparing the patients taking prophylaxis three times *per* week, patient A achieved the lowest values in terms of the C_{max} (49% at 40 minutes post- infusion) and C_{trough} (0.8%) with the same dose and rFVIII as patient C (C_{max} of 79% at 30 minutes post-infusion and C_{trough} of 7.70%). Furthermore, patient A is older than his comparator (53 versus 35 years old) which was expected to present higher t½ and lower CL**.** However, patient A presented with a longer t½ than patient C (11.413h *vs.* 19.929h) and CL higher (2.9 dL/h *vs.* 1.2 dL/h).

Patient B and D had prophylaxis two times *per* week, although with different doses (2000 IU and 1500 IU, respectively) and different concentrates (Adynovi® and ADVATE®, respectively). Both presented similar C_{trough} levels (1.35% and 1.50%) but varied C_{max} (74% at 11 minutes post-infusion and 46% at 4h post-infusion, respectively). Patient B is slightly older than patient D (47 years old to 43 years old) and yet, the t½ shared 3 hours of difference (10.821 for B h and 13.153h for D) and CL as 6.1 dl/h higher (1.3 dL/h for B and 7.2 dL/h for D).

Table 33 presents the pharmacokinetic estimations for patients monitored in different days. All the patients were under prophylaxis two days *per* week, variating only the dose (from 1500 to 3000 IU) and the rFVIII (from Adynovi®, ADVATE®; ReFacto AF® to Elocta®).

Table 33 - Estimations of pharmacokinetic parameters in HA patients monitored in different days.

Monitoring different days	**Patient F**	**Patient G**	**Patient H**	**Patient H'**	**Patient I**
Blood Samples	5	4	4	5	9
Age (years)	26	24	68		34
Concentrate	Adynovi®	ADVATE®	ReFacto AF®	Elocta®	Adynovi®
Frequency	two days *per* week	two days *per* week	two days *per* week		two days *per* week
Dose (IU)	1500	1500	2000		3000
Maximum concentration **(Cmax)**	47%	57%	59%	64%	81%
Trough level **(C_{trough})**	0.4%	1.9%	6.3%	1.7%	1.7 %
Area under the curve **(AUC)** (IU*h/dL)	907.2	949.2	1324.4	1279.6	408.6
Area under the first moment of curve **(AUMC)** (IU*h/dL)	736.0	3256.0	10666.0	2944.0	2088.0
Volume of distribution **(Vss)** (dL)	1.3	5.4	12.2	3.6	37.5
Clearance **(CL)** (dL/h)	1.7	1.6	1.5	1.6	7.3
Half-time **(t½)** (h)	8.858	13.746	20.724	11.930	9.926
Mean residence time **(MRT)** (h)	0.81	3.43	8.05	2.30	5.11

Regarding patients monitored in different days, the number of collected blood samples varied from 4 (patient G and H) to 9 (Patient I). The age ranged from 24 and 68 years old. Here patients had different rFVIII starting with Adynovi® (Patient F and Patient I), ADVATE® (Patient G) to ReFacto AF® and Elocta® (patient H).

All the C_{max} presented correspond to 30 minutes post-infusion of the concentrate. The highest value was 81% (patient I) while the lowest was 47% (patient F), both with the same rFVIII (Adynovi®) but different doses (3000 IU and 1500 IU, respectively to patient I) which justifies the different values. As patient H had two monitoring's for each concentrate prescribed so it is possible to acknowledge that, it conquered better C_{max} with Elocta® (64%) than with ReFacto AF® (59%) for the same dose (2000 IU) and frequency (two days *per* week).

Considering the C_{trough} levels, they varied from 0.4% (patient F) to 6.3% (patient H with ReFacto AF®). Patient F had lower C_{trough} level (0.4%) comparing to patient I (1.7%). Patient H on the other hand, had lower C_{trough} with Elocta (1.7%) than ReFacto AF® (6.3%).

Patient H has switched concentrates, making it possible to compare his results with those from other patients administered with the same rFVIII class. For instance, when administered with ReFacto AF® patient H results were comparable to those observed in patient G administered with ADVATE® (both SHL). On the other hand, results with Elocta® were comparable with Adynovi® from patient F and patient I (both EHL). Furthermore, patient H which is older than patient G (68 years old), had higher t½ (20.724h *VS* 13.746h) and lower CL (1.5 dL/h *VS* 1.6 dL/h). In contrast, patient H conquered higher t½ (11.930h) but lower values of CL than patient F and I (1.7 dL/h and 7.3dL/h).

Importantly, Elocta® and Adynovi® are EHL and present lower t½ than ReFacto AF® and ADVATE®, which are SHL.

Chapter 5- Discussion

HA population is described as heterogenic since the course of the disease and the treatment response differ among them. In addition, FVIII shares a particular pharmacokinetic profile that has been reported to be influenced by individual characteristics. Therefore, the present study aimed to identify characteristics of HA patients that have influence in FVIII pharmacokinetics and hence on efficacy of the treatment.

This was a retrospective study including only HA patients with more than 17 years old and treated with at least one FVIII concentrate between 1st January of 2018 and 30th June of 2020 at the immune-hemotherapy service of CHUC, EPE. All the data (demographic, clinical, pharmacological, and biochemical) was collected, described and analyzed.

The incidence and prevalence of HA worldwide (24.6 cases per 100,000 male birth patients; 17.1 cases per 100,000 male patients, respectively) explains the rareness of the disease. Moreover, since the number of patients in Portugal surrounds an incidence of 22 cases per 100,000 males' births (Café *et al.,* 2019), the fact that this study was able to accomplish 46 patients is a positive outcome of this study.

Since the inclusion criteria required patients older than 17 years old, the sample of the study is described as diversified in terms of the age which ranged from 20 to 78 years old. In terms of their weight, there were limitations regarding the lack of data in some patients (n=5), so the minimum (58Kg) and the maximum (148Kg) only accounted for 41 patients. Furthermore, patients' height was not registered preventing from IMC calculation.

Secondly, the mostly predominant severity grade identified amongst the patients was severe (n=34) with patients having a mean age of 39.9 years old. Curiously, one of the patients classified as severe had reported a mild phenotype, suggesting the coexistence variability within the manifestation of the disease between patients with the same FVIII <1IU/dL. As evidenced by *Nogami* and *Shima,* in 2015, patients with same level of FVIII (<1IU/dL) may experience mild stymptoms whereas, moderate patients may present more severe (Nogami and Shima, 2015). As a result, many researchers are debating for a reformulation in the classification of the HA patients regarding disease severity. Namely, some authors classified severe patients as "intensely hemorrhagic and not intensely hemorrhagic" based on ABR and on the concentrates consumption; others classified severe patients when they had more than 3 hemarthroses per annum, moderate patients when presenting 1 or 2 hemarthroses and, finally, mild patients when suffering from less than 1 hemarthroses (Jayandharan and Srivastava, 2008). No consensus has been attained and further investigations remain necessary to conquer this specific aim.

Still related to HA severity, there was no moderate HA patients in the population herein studied, even though the severity of HA was not reported for two patients. Ten patients had mild severity (21.7%) and presented a mean age of 54 years old. The absence of HA severity grade and the non-existing of moderate patients are restraints of the study, making not possible to correlate the factors of variability with the state of HA severity.

It was noteworthy the very high (93.5%) percentage of patients with no inhibitors. Among the others (6.5%), they were classified as low titer, which means that concentration of inhibitors was less than 5 BU. These characteristics support the hypothesis that variability in pharmacokinetics on these population will hardly be due to the presence of inhibitors very low.

Regarding the comorbidities or complications associated to HA, most of the patients were immunized against viral infections (HAV, HIV, HCV, HVB). Therefore, a small percentage (13.1%) developed viral activations. The reasons behind the prevalence of this infections in HA remain unknown, although in literature higher number of infections has been justified by the viral contamination through blood donors (Zhubi *et al.*, 2009). Even though this may not be the case, literature is lacking regarding the justification for this prevalence on hemophilia patients. Nonetheless, the influence of HCV on the decreasing FVIII levels has been reported as a consequence of underlying inflammation as well as a risk factor to develop chronic liver disease or hepatocellular carcinoma (Pradhan-Sundd *et al.*, 2021).

The results of the study herein carried on also evidenced the development of HA complications such as arthropathy (6.5%), several local hemorrhages (10.9%) and orthopedic replacement surgeries (6.5%). These complications are usually developed in the presence of lower concentrations of FVIII, probably resulting from the regimen applied, lack of adherence or lack of a proper monitoring treatment. For instance, arthropathy usually is more prone to be evolved in patients that are under the on-demand treatment, as it was confirmed in one comparative study between the on-demand and prophylaxis in severe patients with more irreversible damages (such hemophilic arthropathy) (Aznar *et al.*, 2012). Herein, two of the three patients were prescribed with concentrates on-demand and had severe HA. The other patient reported with arthropathy was in prophylaxis, suggesting no-compliance. Although no definition has been reached yet to the adherence in hemophilia prophylaxis, one study aimed to correlate adherence to bleedings episodes in 56 severe HA patients and the conclusions were a positive effect of a good adherence in reducing bleeding episodes and possible complications in HA (Dover *et al.*, 2020).

Between the 1st January of 2018 and 30th June of 2020, herein tested patients (n=20) switched the treatment. Patients that were severe and on-demand (n=17) changed to prophylaxis (n=27). This is a positive outcome since, as previously explained, on-demand regimens do not allow to a proper control of the disease (Aznar *et al.*, 2012). Nonetheless, the percentage of the patients remaining on-demand with severe HA was significant (32.6%) and, even tough, the reasons for this clinical choice were not described, it can be hypothesized that on-demand regimen enhances patient compliance. Another reason can be the price of the rFVIII concentrates in prophylaxis as it constitutes 46% of the overall costs per year while on-demand costs approximately 35% (Café *et al.*, 2019).

ADVATE® (SHL) was the most prescribed third generation rFVIII although, after *switch*, Adynovi® (EHL PEGlyted) started to be more frequently prescribed. It is not possible to accurately know the reasons of this swap but, it may be because Adynovi® promotes a better compliance since, EHL has an extended half-life time and so, less infusions are required to attain the same levels (Escobar *et al.*, 2019).

Monitoring FVIII levels is important step to establish the efficiency and safety of the treatments. The results of its monitoring in this study population were scattered. More blood samples were expected to be collected for each patient (at least, three blood samples). Instead, among all the monitored patients (n=40), 41.3% had only one blood sample. In addition to that, nine (19.6%) were diagnosed with severe HA within a prophylactic regimen while, seven (15.2%) were severe patients but on-demand.

Ten patients with severe HA presented C_{trough} values lower than 1 IU/dL. One-half were on prophylaxis with one blood sample to be monitored (n=3) whilst the other half was under on-demand regimen and had only one blood sampling. These results may be the explanation for the appearance of hemorrhages, knees replacement surgery or, worse, the development of ankle hemarthroses. Also, they demonstrate how crucial it is to monitor all patients regardless the regimen. Lower levels of FVIII prevent irreversible damages in joints and also reduce the consequences of clinical futures in patients life (Davari *et al.*, 2019).

In addition to these features regarding the monitoring scheme at CHUC, EPE, two of the patients with one blood sample for FVIII monitoring had a clinical report of recurrent physical activity (cycling and table tennis) and were diagnosed with severe HA. As these patients are more prone to bleedings and injuries than sedentary patients (Morfini and Farrugia, 2019), they are not advisable to be under on-demand regimen, which is the scenario for the patient that does cycling for physical activity. Predictably, this patient has developed hemophiliac arthropathy alongside with hemorrhages on the ankle of the right foot. In opposition, the other patient (table tennis) was under a prophylaxis regimen (two times *per* week) with no

reports regarding the complications. As these two patients were poorly monitored (only one blood sample), it was not possible to assess the time to attain peak levels or the AUC, as these two parameters are associated to the risk of joint and non-joint bleeding in a *post hoc* study (Valentino *et al.*, 2016). For instance, an individual with HA that frequently practices sports is more prone to the risk of bleeding and injuries than sedentary patients (Morfini and Farrugia, 2019). Therefore, assessing the time to attain peak levels is more relevant to define the exact time of the next infusion (Escobar *et al.*, 2019; Morfini and Farrugia, 2019). The utility of AUC will remain on the evaluation of the total exposure to understand the overall protection and preventing, then, subclinical bleeds and target joints (Delavenne and Dargaud, 2020; Escobar *et al.*, 2019).

Pharmacokinetic parameters were assessed and divided according to the monitoring process (within the same day or in different days). Starting with patients monitored in the same day (n=2), they were under prophylaxis three times *per* week. The collected blood samples were different regarding their quantity (three samples, four or even five) and also the time at post-infusion (e.g. 30 or 40 minutes, 3 hours, 4 hours or 24 hours). Even though it was possible to compare the highest and the lowest values for C_{max} between patients, it was not achievable to assess the relationship between prescribed dose or frequency rFVIII. Besides this external variability in blood sampling scheme, it was possible to focus on C_{trough} in a way that, patient A presented rFVIII levels under 1 IU/dL (0.8%) and reported stomach hemorrage and knee replacement. Comparing with patient C, both have the same rFVIII concentrate and the same dose was administered, but C_{trough} levels were different, proving, once again, the inter-variability that coexists in this population and treatment.

Another interesting aspect found within patients A and C was the variation of half-life and CL values with patient's age. Comparing both, patient A had lower values of half-life and higher CL, being older than patient C, which does not match the studies found of the literature on this aspect (Hermans and Dolan, 2020; Turecek *et al.*, 2020). The same tendency was observed within patients B and D even though they were prescribed with a frequency of two times *per* week. As explained in section **1.7.5.4 Age**, the levels of vWF rises as the age increases. FVIII is, hence, protected, prolonging half-life and decreasing CL (Hermans and Dolan, 2020; Turecek *et al.*, 2020). However, it is not possible to have vWF:Ag levels nor the blood type to understand the influence of these individual factors in treatment. So, the only explanation for this contradiction could be the fact that, the samples were heterogenic and limited to a maximum of 24 hours. In fact, International Society on Thrombosis and Haemostasis (ISTH) offer guidelines for pharmacokinetic studies of FVIII in clinical practice (M. Lee, M. Morfini, S. Schulman, 2001). They purpose ten to eleven samples for adults, four at the distribution phase

(0, 10 min-15min, 30 min, 1hour) and seven at elimination phase (3, 6, 9, 24, 28, 32, 48 or 72 hours in case of being an EHL) (M. Lee, M. Morfini, S. Schulman, 2001; McEneny-King *et al.*, 2016).

Even thought, these classic pharmacokinetic studies purpose from ISTH are valid, in clinical practice they have limitations. Firstly, a washout period is required, putting the patient at risk of bleedings (Iorio *et al.*, 2018). Secondly, these studies are driven in homologous population of males, not taking in consideration the variability or the need for individualization (McEneny-King *et al.*, 2016). Finally, the number of required samples are immense and a burden for adults but, mostly for pediatric (Iorio *et al.*, 2018; McEneny-King *et al.*, 2016). Therefore, it is possible to reduce the number of samples to three but never under the 48 hours post infusion, which according to studies made by *Björkman* sampling under 48 hours leads to underestimation of half-life whereas clearance is overestimated (Mceneny-king, 2020).

Considering the patients monitored in different days, different rFVIII concentrates, SHL (ADVATE® and ReFacto AF®) and EHL (Adynovi® and Elocta®), were prescribed and it was possible to compare all C_{max} on the same post-infusion time (30 minutes). These are strengths of the present study. However, there was patients with five samples and other with nine, evidenced that the blood sample scheme applied in CHUC, EPE was not standardized to everyone. Once again, it was found another correlation between the C_{trough} levels and the worst clinical consequences, namely in patient F as levels of FVIII (0.4%) were translated in ankle hemarthroses during the time of the study.

In addition, patient H with Elocta® had lower half-life than when prescribed with ReFacto AF®, which contradicts the definition of EHL about extending the half-life of FVIII. Again, this may be justified by the samples taken or by other factors such vWF:Ag or phenotypes on LRP1 that are still unknown (explained **1.7.3 Elimination**). As there was no other inter-individual factor to establish with PK, the age factor between patient F patient G, patient H and patient I. Comparing patient G to patient H (older), with the same FVIII concentrates of the same generation (ReFacto AF® *VS* ADVATE®), the half-life of patient G was lower as expected in literature (Hermans and Dolan, 2020; Turecek *et al.*, 2020). However, comparing patient G with patient H', the half-life was once again higher which contradicts the literature (Hermans and Dolan, 2020; Turecek *et al.*, 2020). As no more inter-individual factors were possible to attain, this variation is not possible to be justify.

Chapter 6- Conclusions

HA is a rare bleeding disorder caused by the lack of the FVIII in coagulation cascade. As there are lower amounts of the FVIII, the treatment is based on the replacement of the normal levels through FVIII concentrates such rFVIII (either SHL or EHL). However, this population is heterogenic regarding their clinical status as well as their treatment response.

Therefore, this research aimed to identify the factors of variability in clotting FVIII concentrates centralized on HA patients at the University Hospital Centre of Coimbra (CHUC; EPE).

From all the 46 enrolled patients, it was possible to describe their demographic data (age and weight), clinical data (severity, inhibitors, infections and HA complications) and the treatment data before and after *the switch* (regimen applied, concentrate prescribed, dose, frequency of infusion). Some limitations were found at this specific objective, starting by demographic data as five patients had no weight reported on hospital software. Furthermore, height was not reported for any patient, hampering the calculation of IMC and consequently, not using this as a variability factor for pharmacokinetics. Furthermore, severity classification and treatment specificities were not consistently registered, consequently influencing the observations in terms of the concentrates prescribed.

In terms of the assessment of FVIII levels, majority of the patients was monitored. However, most of them had only one blood sample scheme. The ones with more than one blood sample had either a poor clinical data to relate with, or a not standard sample scheme to establish valid comparisons. This is a result of a non-implemented protocol at the CHUC, EPE but, even with established well-stablished protocol, it would not be possible to go further when regards the identification of variability factors, since neither the blood type or vWF levels were measured and they are essential for determination of t½ and CL.

It is also noteworthy that this retrospective study was conducted from 2018 to 2020, which also caught the pandemic times, leading some of these patients to stay in quarantine and not go to the hospital facilities to check the levels properly. Aside from the pandemic, the results are the reflection on how important is to have a well-defined protocol in HA field to give the patients a better clinical outcome.

Since the monitoring levels at immune-hemotherapy service of CHUC, EPE was not carried on under a pre-defined monitoring pharmacokinetic protocol, it is recommended to establish internal guidelines, to successfully characterize patient's pharmacokinetics and individualize their therapeutic regime.

As ISTH guidelines purposes, ten to eleven blood samples should be collected in adults (in pediatric population) (M. Lee, M. Morfini, S. Schulman, 2001). This is extremely burden for the patients, and the ideal protocol should only require collection of three samples (after infusion) since these could mimic the extensive PK profile (Barnes, 2013; Björkman, 2010). Studies of this sparse sample protocols advice to collect samples between 4 hours and 48 hours spaced at least 12 hours (Barnes, 2013; Björkman, 2010). Therefore, it is proposed to measure the pre-infusion (baseline FVIII) level, which is usually in mornings, and then, the first post infusion should be at 4 hours in the same afternoon. In the following days, it should be collected at 24 hours and 48 hours post infusion (Barnes, 2013; Björkman, 2010).

To tailor the treatment, it should be collected data of variability such predictable variability presented at **Annex 1** and, additionally, as this involved reduced samples, it is crucial to record the exact time of the samples as well as the date precisely **(Annex 2)**.

Annexes

Annex I- Example of the variability data gathering of the HA patient.

Demographic data collection		
Patient Process number		
Date of Birth		
Weight (kg)		
Height (m)		
Clinic data collection		
Severity (Severe/Moderate/Mild+ level de FVIII)		
Blood Type (A, B, AB, O)		
von Willbrand Factor (levels of vWF:Ag)		
Orthopedic HA complications (hemophilic arthropathy, hemorrhages, surgeries…)		
Viral Infections (HAV,HBV,HCV,HIV,etc)		
Liver Disease (Acute, chronic, cirrhose)		
Inhibitors (Yes or No + amount)		
Treatment data collection		
Regimen (Prophylaxis, on-demand)		
FVIII concentrate (market named)		
Days (Monday, Tuesday…) and time for administration		
Dose (IU)		
Historic of the last three infusions (Question the patient)	Time of infusions (hours: minutes)	
	Dates of infusions (DD/MM/YY)	
	Doses (IU)	

Annex 2- Suggestion of a scheme to collect and monitor FVIII levels.

Identification	
Patient Process number	
Date (DD/MM/YY)	
Time (hours)	

Sampling scheme		
Time aims	**Time of samples (hours)**	**Notes:**
Baseline		
4h post infusion		
24 post infusion		
48 post infusion		

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