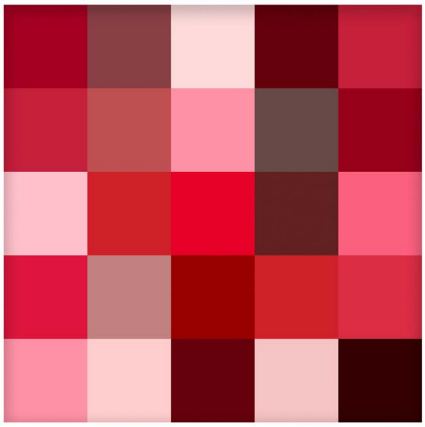
Innovative drug monitoring of factor VIII and emicizumab in haemophilia A



Anouk Donners

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COLOPHON

The work presented in this thesis was performed at the Department of Clinical Pharmacy in collaboration with the Van Creveldkliniek at the University Medical Center Utrecht, Utrecht, the Netherlands.

Provided by thesis specialist Ridderprint, ridderprint.nl
Printing: Ridderprint
Cover design: David Veldhuizen
Layout and design: Eduard Boxem, persoonlijkproefschrift.nl
ISBN: 978-94-6483-066-8

Innovative drug monitoring of factor VIII and emicizumab in haemophilia A

Innovatieve geneesmiddelmonitoring van factor VIII en emicizumab in hemofilie A

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de
Universiteit Utrecht
op gezag van de
rector magnificus, prof. dr. H.R.B.M. Kummeling,
ingevolge het besluit van het college voor promoties
in het openbaar te verdedigen op

donderdag 1 juni 2023 des middags te 12.15 uur

door

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geboren op 16 februari 1991
te Heerlen

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Ars longa,
vita brevis,
occasio praeceps,
experimentum periculosum,
iudicium difficile.

The art is long,
and life short,
opportunity fleeting,
experiment treacherous,
judgment difficult.

– *Hippocrates*

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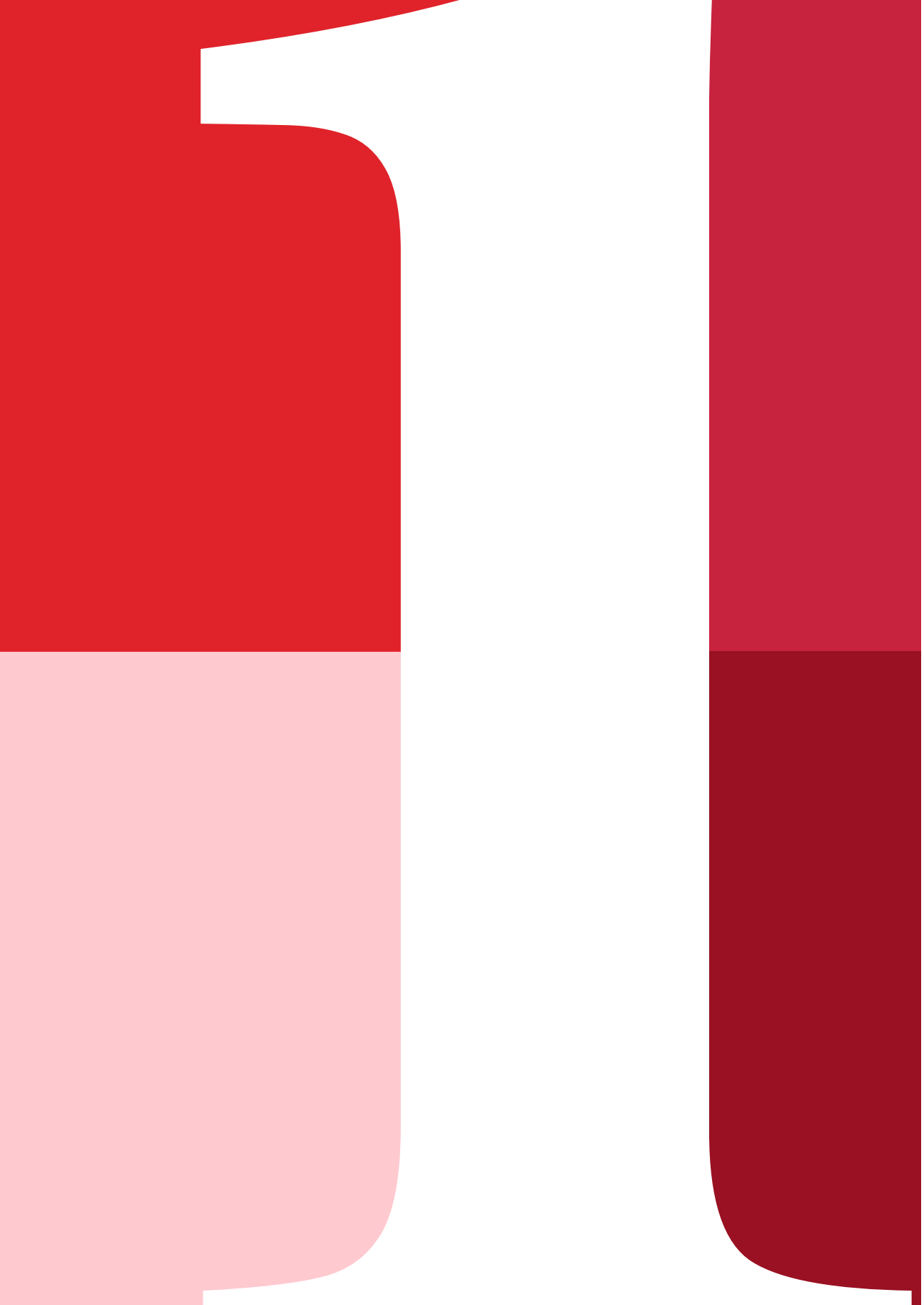

CHAPTER

General Introduction

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Carin M.A. Rademaker
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Haemophilia A

Haemophilia A is an X-linked congenital bleeding disorder caused by a deficiency of coagulation factor VIII (FVIII). The diagnosis is based on a person's endogenous FVIII activity and is classified into severe (<1%), moderate (1 to 5%) or mild (>5 to 40%) haemophilia A [1]. Approximately eight out of 100,000 people have haemophilia A (PwHA) in the Netherlands [2]. These PwHA receive care at six dedicated Haemophilia Treatment Centers, of which Van Creveldkliniek, established in 1964, is the oldest. PwHA experience bleeding, predominantly into major joints, such as ankles, knees and elbows, leading to painful and disabling arthropathy. Intracranial bleeds and bleeding into internal organs may also occur among PwHA, which can be life-threatening. When left untreated, the bleeds occur spontaneously among those suffering from severe haemophilia. In contrast, persons with moderate and mild haemophilia present a milder phenotype in which trauma and surgical interventions can provoke uncontrolled bleeding. The life expectancy of PwHA has been extended from 10–15 years a century ago to a nearly normal life expectancy and quality of life today [3].

Pharmacotherapy for people with haemophilia A

Haemophilia therapy has progressed remarkably throughout the twentieth century, progressing from no available therapy to complete blood and plasma infusions. The cornerstone of therapy for PwHA is the substitution of missing FVIII [4, 5]. In the 1960s, the discovery of cryoprecipitate, which involves concentrating FVIII in a pellet, led to the industrial manufacturing of plasma-derived FVIII (pdFVIII) in the 1970s [6]. This breakthrough provided the first efficacious treatment of bleeds and marked the beginning of home care and self-infusion. The success was overshadowed in the 1980s by the outbreak of serious and fatal blood-borne viral infections such as hepatitis and HIV/AIDS in a large proportion of PwHA due to a lack of screening methods [7, 8]. Fortunately, safer products became available in the 1990s due to new DNA technology that provided recombinant FVIII (rFVIII) and manufacturing advancements in viral inactivation and virus removal, which provided safer pdFVIII [6, 9]. PwHA were burdened by frequent weekly intravenous injections due to the short half-lives (SHL) of rFVIII products. This drove the development of the extended half-life (EHL) rFVIII products in the 2010s, although maximum half-lives of only 20 hours were achieved [10, 11]. The improvements of EHL products included Fc-infusion, conjugation of polyethylene glycol or shortening of the protein sequence to increase stability [12].

Currently, 15 FVIII products are available on the Dutch market [13]. Substitution therapy with these FVIII products can be given prophylactically to prevent bleeds (especially for severe PwHA) or on-demand to treat bleeds. Prophylactic FVIII replacement therapy has effectively reduced the average treated bleeds from 20–30 to 1–4 per person per year [1, 14]. Moreover, prophylaxis can convert a severe phenotype into a moderate phenotype

when maintaining FVIII activity above 1% [15, 16]. The main challenges of FVIII therapy are the FVIII-inhibitor development in 30% of PwHA, which renders the treatment ineffective, the burden of intravenous administration and the costs. Financial restraints lead to poor access and health inequalities worldwide, as the cost of prophylaxis therapy is estimated at ~150,000 euros per person per year [17, 18].

A new therapeutic strategy became available with the introduction of non-factor replacement therapies. Emicizumab, which was approved in 2018, was the first therapy offering prophylaxis in PwHA with and without FVIII inhibitors. This recombinant, bispecific, monoclonal antibody (mab) effectively mimics the FVIII function to an equivalent of 10–20% FVIII activity [19-21]. Because there is no sequence homology to FVIII, the PwHA with FVIII inhibitors have an effective prophylactic option for the first time. Compared to FVIII products, the other revolutionising benefits of emicizumab are the subcutaneous instead of intravenous administration and the monthly instead multiple-weekly dosing frequency. Side effects, which rarely occur, include thrombotic events with concomitant use of activated prothrombin complex concentrate [22] and <0.6% immunogenicity with declining plasma concentrations [23]. While most PwHA in the Netherlands wish to start emicizumab prophylaxis, patient access is limited due to the financial impact on healthcare budget. Public pricing of emicizumab therapy is higher than FVIII therapy and is set at ~400,000 euros per person per year, although non-public pricing is probably close to the costs of the current FVIII therapies [24-26]. Another disadvantage of emicizumab is that it does not fully correct coagulation, and is therefore not suitable for the management of acute bleeds or major surgery [5].

Several other non-factor replacement products are currently under clinical investigation and are expected to be approved in the near future [27]. Their mode of action is either mimicking the FVIII function (i.e., similar to emicizumab) or targeting the natural coagulation inhibitors (i.e., antithrombin, tissue factor pathway inhibitor, or activated protein C). Rare thromboembolic events have been reported and warrant continuous post-marketing surveillance, although the overall safety profile looks promising [28]. The ultimate goal of haemophilia treatment is a phenotypical cure, which is achievable today, as Roctavian® valoctocogene roxaparvovec, the first gene therapy product of its kind, was approved in June 2022 [29]. Gene therapy allows PwHA to avoid the fears and obligations of haemophilia A treatment for a number of years [30, 31].

Monitoring

Prescribing drugs involves more than writing a prescription and following the drug label [32, 33]. It also includes monitoring of the individual by continuous evaluation of the benefit-risk balance of pharmacotherapy and optimisation. Monitoring can involve the clinical observation of the individual or actually measuring markers that indicate a disease status. Many of these markers are biomarkers measured in a laboratory or by the

individual at home (point-of-care testing). In fact, it has been shown that for a substantial part of the commonly used drugs, the summary of product characteristics (SmPC) (i.e., the drug label) or the clinical guidelines advise ("prescribe") the measurement of laboratory markers [34].

The aim of drug dosing is to reach a so-called therapeutic window. The balance between benefit and risk (i.e., desired effect and toxicity) is considered optimal for an individual in this window [35] (see *Figure 1*). Subtherapeutic effects are expected under the window and supratherapeutic due to maximum effect resulting in toxic or financial toxicity. For some drugs (e.g., aminoglycosides, lithium, digoxin), the therapeutic window is narrow, and strict monitoring is of the essence. Monitoring can involve the measurement of either an endogenic biomarker (e.g., glucose, INR, anti-Xa) or the measurement of the drug in plasma, serum, blood or other specimens. The latter is called therapeutic drug monitoring (TDM).

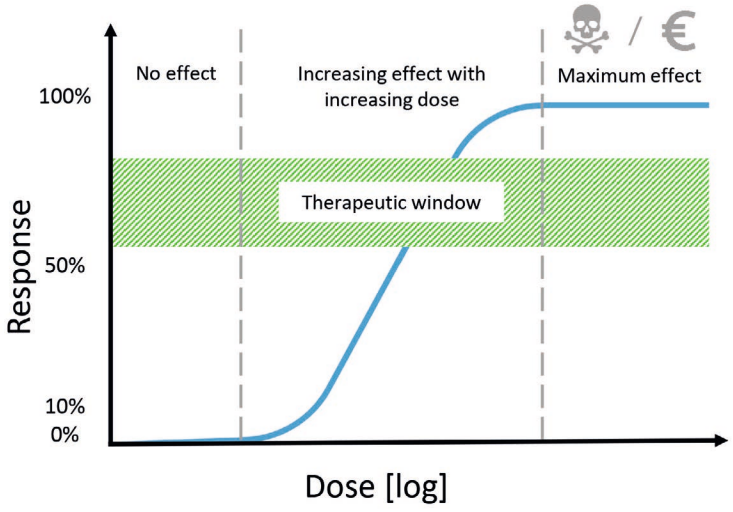

Figure 1. Dose-response curve with a therapeutic window.

Monitoring of FVIII

The standard monitoring approach in haemophilia management does not involve the measurement of a drug concentration; instead, the biomarker 'FVIII activity' is used. This FVIII activity is monitored to diagnose haemophilia A, to dose the FVIII products and to provide clinical support to PwHA (e.g., during surgery or bleeding) (see *Figure 2*). FVIII activity is used for diagnosing purposes to classify the severity of haemophilia A. The frequency of monitoring and treatment strategy are based on the severity classification.

People with severe haemophilia A are monitored closely and receive prophylaxis early in life, while people with moderate and mild haemophilia A are monitored less often and mostly receive on-demand treatment. For dose monitoring, a minimal effective FVIII activity is the goal, and this goal differs between clinical situations. For instance, ≥1% of FVIII activity is required for prophylaxis, 30% for treating a mild bleed, or around 100% during surgery. High FVIII activity is not necessarily toxic but merely unattractive from a financial perspective, and high variability for FVIII products has been reported [4, 10]. Thus, monitoring for dose purposes is important to ensure sufficient efficacy.

FVIII activity is currently measured with different clotting assays, mainly by the one-stage clotting assay (OSA) or the chromogenic-substrate assay (CSA). These assays are routinely available in many clinical laboratories worldwide and are relatively fast and cheap to perform using an automated coagulation analyser. These assays demonstrate discrepancies in some cases, for instance, between the mild and moderate severity, between different FVIII-product types or between different laboratories [36]. Assay variability has been related to the use of different reagents, assay settings, assay interference (e.g., anti-drug antibodies [ADA] or non-factor products) and some lack of standardisation [36-38]. Ascertaining the FVIII activity is, however, critical in haemophilia A, particularly in the lower area of 0–6%. Measurement errors may worsen a person's bleeding prognosis due to misdiagnosis of the severity classification, which results in postponed prophylaxis, or by suboptimal dosing of the FVIII products, which results in insufficient efficacy or financial "toxicity".

Monitoring of emicizumab

Emicizumab is a so-called biopharmaceutical. The standard monitoring approach for biopharmaceuticals is TDM and the determination of anti-drug antibodies (ADA). The general purpose of monitoring is to guide treatment decisions to optimise treatment and cost-effectiveness. The dose selection of biopharmaceuticals is often set at the upper end of the dose–response curve, given the absence of a maximum tolerated dose. Moreover, the most important toxicity, immunogenicity, is not dose related [35, 39, 40]. For emicizumab specifically, its drug label does not mention laboratory monitoring or measurement of concentrations during treatment. However, guidelines on emicizumab therapy do recommend monitoring its concentration when suspecting ADAs [41]. Moreover, the monitoring of emicizumab might be useful in order for clinicians to recognise therapy non-adherence and for researchers to investigate its clinical pharmacology. Lastly, cost-efficient monitoring might be appealing when efficacy is maintained with lower doses of emicizumab, leading to healthcare savings and improved patient access [42]. Thus, the role of TDM for emicizumab is helpful in clinical support, research and potentially in cost-efficient dosing (see *Figure 2*).

The standard method for measuring the concentration of emicizumab in clinical practice is based on a calibrated, modified clotting assay. This assay is not commonly adopted in clinical laboratories, and moreover, assay interference by high concentrations of FVIII concentrates or anti-emicizumab antibodies has been reported [43].

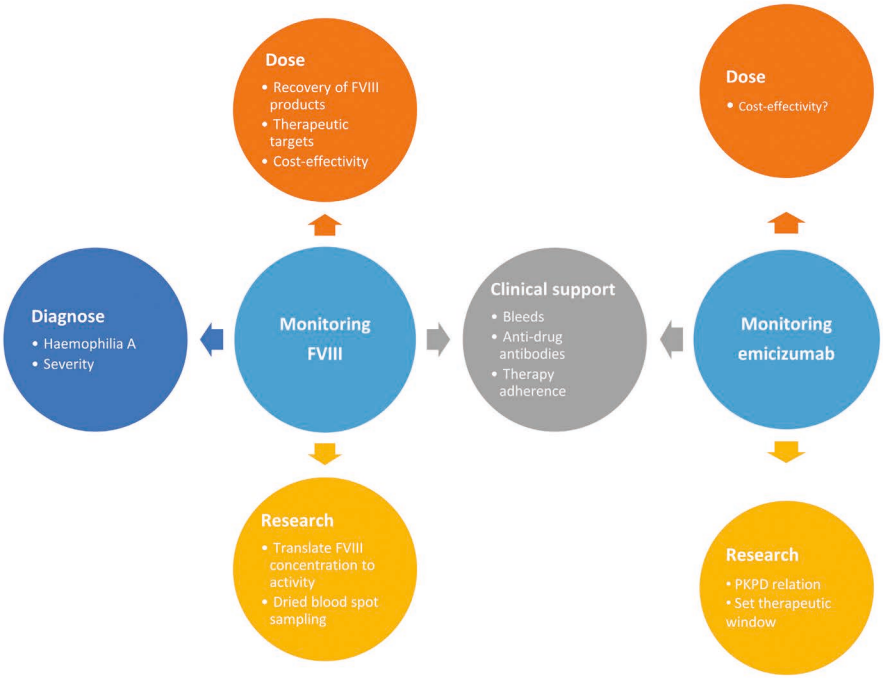

Figure 2. Monitoring purposes of factor VIII and emicizumab.

Mass spectrometry: A novel technique in haemophilia A

In the last decennium, liquid chromatography (LC) coupled with tandem mass spectrometry (MS/MS) has rapidly become the state-of-the-art technique for TDM in the laboratory landscape. Advantages of this technique over other techniques, such as clotting assays or enzyme-linked immunosorbent assays, include high sensitivity, speed, resolution, accuracy and reproducibility. In addition to these analytical benefits, LC-MS/MS methods offer the potential of multiplexing, leading to more efficient measurement runs. Especially advantageous is the low sampling volume, which offers minimal blood drawing for children and the potential of dried-blood-spot measurements. Additionally, LC-MS/MS analysis will likely continue to grow as one of the leading techniques for the quantification of proteins (i.e., proteomics) in a clinical setting.

Knowledge gaps

Measuring the concentrations of FVIII and emicizumab with LC-MS/MS has not been done before. This LC-MS/MS technique may be of interest to treating clinicians because of its many advantages in comparison to other assays. The potential role of bioanalysis with LC-MS/MS in haemophilia A is schematically presented in *Figure 3*. For FVIII, the dose, the FVIII activity and the bleed outcomes are well correlated, but the role of the FVIII concentration measured with LC-MS/MS in the dose–concentration–response relation is unknown. For emicizumab, no biomarker has yet been identified, and the entire dose–concentration–outcome relation, established in the pre-approval studies, is unclear. Bioanalysis with the LC-MS/MS technique could be applied to start filling these knowledge gaps and to further optimise the monitoring of FVIII and emicizumab in PwHA in the future. Two questions arise from these knowledge gaps:

- How is the relationship between FVIII concentration and FVIII activity best described?
- How low can we dose emicizumab without sacrificing the desired clinical response?

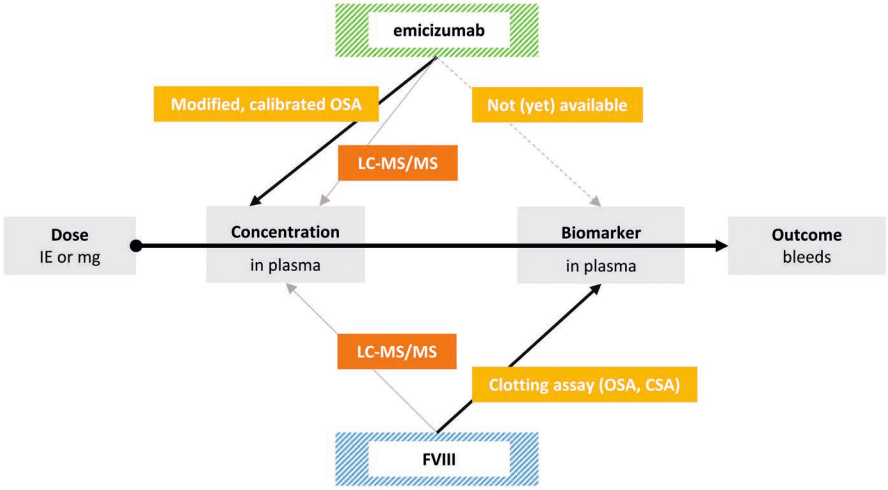

Figure 3. Dose–concentration–response relationship with bioanalysis of FVIII and emicizumab in PwHA.

Thesis objectives

This thesis is designed to optimise the drug monitoring of FVIII and emicizumab in PwHA. To achieve this objective, I will:

1) Develop and validate the LC-MS/MS methods that quantify FVIII and emicizumab in human plasma;
2) investigate the dose–biomarker relationship of FVIII and the dose–concentration–response relationships of emicizumab; and
3) propose and evaluate a cost-efficient dosing strategy for emicizumab.

Outline

In **Chapters 2, 3 and 5,** I provide a framework for measuring therapeutic proteins, such as FVIII and emicizumab, in human plasma using LC-MS/MS bioanalysis. In **Chapter 4**, I investigate the concentration–biomarker relationship of FVIII, and in **Chapters 5–7**, I investigate the dose–concentration–response relationship for emicizumab. In **Chapters 6–8,** I conduct studies to support a cost-efficient approach to emicizumab treatment, which may inspire others to dose biopharmaceuticals more affordable. An overall discussion in **Chapter 9** is presented in the last chapter of this thesis to put the lessons learned into a broader perspective. I also deliberate on the potential future applications of monitoring FVIII concentrations in PwHA and a framework for the cost-efficient dosing of biopharmaceuticals. This thesis can be summarised as the groundwork for measuring and monitoring FVIII and emicizumab with LC-MS/MS bioanalysis in PwHA.

Author's contribution

AD conceived the idea and set-up the general introduction. AD conducted literature review, outlined and wrote the general introduction. Throughout the process, AD asked and implemented input and feedback from the supervision team.

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CHAPTER

Six-step workflow for the quantification of therapeutic monoclonal antibodies in biological matrices with liquid chromatography mass spectrometry: A tutorial

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Anouk A.M.T. Donners
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Anal Chim Acta 2019; 1080: 22-34

ABSTRACT

The promising pipeline of therapeutic monoclonal antibodies (mAbs) demands robust bioanalytical methods with swift development times for pharmacokinetic studies. Over the past decades ligand binding assays were the methods of choice for absolute quantification. However, the production of the required anti-idiotypic antibodies and ligands limits high-throughput method development for sensitive, accurate, and reproducible quantification of therapeutic mAbs. In recent years, high-resolution liquid chromatography-tandem mass spectrometry (LC-MS) systems have enabled absolute quantification of therapeutic mAbs with short method development times. These systems have additional benefits, such as a large linear dynamic range, a high specificity and the option of multiplexing. Here, we briefly discuss the current strategies for the quantification of therapeutic mAbs in biological matrices using LC-MS analysis based on top-down and middle-down quantitative proteomics. Then, we present the widely used bottom-up method in a six-step workflow, which can be used as guidance for quantitative LC-MS/MS method development of mAbs. Finally, strengths and weaknesses of the bottom-up method, which currently provides the most benefits, are discussed in detail.

SCAN ME

INTRODUCTION

Therapeutic monoclonal antibodies (mAbs) nowadays are widely accepted as valuable treatment options for patients suffering from a variety of diseases, particularly in the areas of oncology and immune diseases. At present, 76 mAbs have been granted market authorization by the Food and Drug Administration (FDA) and European Medicines Agency (EMA) and are now commercially available for therapeutic use [1]. Judging from drug pipelines this number is set to grow considerably in the near future [2]. Therapeutic mAbs target pathological processes with high specificity and concomitantly lead to fewer side effects compared to conventional small molecule based therapies [3]. Furthermore, due to the high molecular weight of mAbs, the clearance pathway is not by renal elimination after hepatic enzyme metabolism but rather by proteolytic catabolism, receptor-mediated uptake and degradation, and sometimes by the catabolic pathway of their molecular target. Two thirds of mAbs are salvaged from degradation by binding to the protective neonatal Fc-receptor (FcRn) particularly on endothelial cells, which extends their elimination half-life to ~18-21 days [4]. MAb production and design has made great strides from the early discovery in 1975 by Kohler and Milstein [5]. The progression from murine mAbs (1975) using hybridoma technology to chimeric mAbs (1984) using recombinant DNA techniques to humanized mAbs (1988) using complementary determining region (CDR) grafting and finally to fully human mAbs (1994) using phage display or transgenic mice took less than 20 years [6-9]. These steps were essential to reduce the risk of anti-drug antibodies (ADA) development and allergic reactions associated with first generation mAbs [10-12]. In fact, additional requirements from the EMA, Food and Drug Administration (FDA) and World Health Organization (WHO) for the evaluation and monitoring of immunogenicity of new biopharmaceuticals were mandated as part of regulatory approval, together with a rigorous post-authorization pharmacovigilance with product-level traceability for of all biopharmaceuticals [13-17]. The discovery of new therapeutic targets and the high treatment efficacy of biopharmaceuticals accelerated the development of novel mAb-based therapies [16, 18]. For this purpose, bioanalytical methods were necessary to facilitate the required preclinical pharmacokinetic (PK) studies. In addition, therapeutic drug monitoring of mAbs concentrations can highlight accelerated drug clearance in patients which is indicative of ADA development and loss of drug response. Traditional bioanalytical methods such as ligand-binding assays rely on an anti-idiotypic antibody or a ligand with high avidity towards the therapeutic protein of interest. However, the development of such antibodies is notoriously difficult and time consuming [19-21]. Therefore, advances in analytical techniques were essential to attain shorter method development times, which is why liquid chromatography tandem mass-spectrometry (LC-MS/MS) has received increasing interest as an alternative method for quantification over the last decade. Following strength and weaknesses analysis of ligand binding assays, this tutorial systematically addresses bioanalytical methods to quantify therapeutic mAbs in biological matrices using LC-MS. Three main branches of quantitative proteomics

using top-down (intact), middle-down (semi-intact) and bottom-up (signature peptide) strategies are briefly explored. Finally, the most widely used bottom-up quantification strategy via signature peptide is chronologically discussed in a general workflow where strengths and weaknesses of each step are extensively explained.

Ligand binding assay

Enzyme-linked immunosorbent assay (ELISA) is arguably the most widely used form of ligand binding assay (LBA), because of its high sensitivity, ease of use, and low instrumental costs [22, 23]. Moreover, due to the spectrophotometric detection principle, ELISA offers a safer alternative with a high ease of use compared to historically used radio immunoassays which require special facilities and operators trained to handle radioactive material [24]. In general, ELISA methods are based on the quantification of the target protein (antigen) using strategies, such as direct, indirect or sandwich type ELISA (*Figure 1*) [25-27]. In the final step, quantification is performed by adding a substrate solution, often tetramethylbenzidine (TMB), which is gradually oxidized by horseradish peroxidase (HRP) to a colored product. To improve sensitivity the use of a polyclonal secondary antibody in indirect or in sandwich type ELISA can help boost the signal intensity [28]. Alternatively, polymerized HRP can be used to increase sensitivity. Background staining is a very common challenge in these assays, and is often caused by non-specific interactions of either the primary or the secondary polyclonal antibody used [29]. The choice of binding strategy can help minimize the risk of non-specific interactions. For example, in sandwich type ELISA's, an additional anti-idiotypic antibody binding the antigen at a different epitope provides higher specificity [30]. The reliance of ELISA methods on these specific anti-idiotypic antibodies or ligands can lead to lengthy method development times [31] and higher consumables costs compared to LC-MS/MS methods. Furthermore, ELISA's allow for one component analysis in one specific bio-matrix, while LC-MS/MS methods allows for multiplexed measurement of multiple therapeutic and endogenous proteins in various bio-matrices [32-34] and due to the narrow linear dynamic range in ELISA, accurate quantification may require multiple sample dilutions thus limiting sample throughput. Importantly, the validation acceptance criteria for ELISAs are less stringent in comparison to LC-MS/MS methods [35, 36], mainly because ELISA methods cannot incorporate internal standards to correct for binding efficiencies influenced by sample matrix or component loss due to binding and washing steps. Finally, different results can be obtained with different ELISA assays as was demonstrated by Vande Casteele by comparison of three commercially available ELISA kits for infliximab and anti-infliximab quantification, stating that comparison of drug levels and ADA monitoring is hampered by lack of standardization [37]. Inter-assay variability makes it difficult for clinicians to compare results from other centers and invalid measurements affects decisions made in patient's diagnosis and treatment.

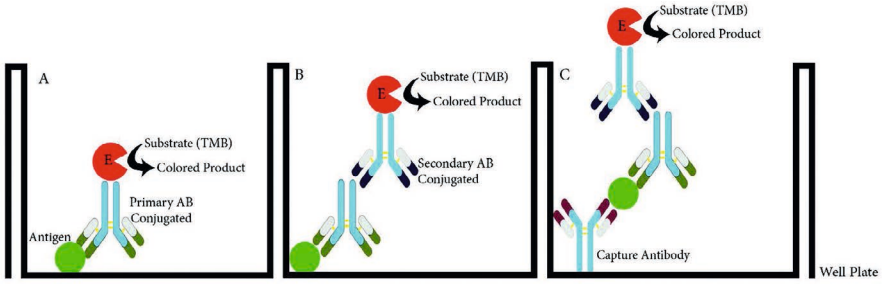

Figure 1. A) Direct ELISA, B) Indirect ELISA and C) Sandwich ELISA.

Top-down, middle-down and bottom-up quantitative proteomics

Quantification with LC-MS offers various advantages over LBA and over the years various LC-MS strategies have been explored and reported. Three main strategies are discussed in detail namely topdown, middle-down and bottom-up proteomics. The flow chart in *Figure 2* depicts the decision making process for the preferred strategy for quantification based on factors, such as the intended assay specifications in terms of sensitivity and selectivity and the applied instrumentation and materials. For therapeutic drug monitoring of trough levels, in most cases a LLOQ of 1 mg/L would suffice [38]. This would allow for the quantification of the peptide using LC-MS/MS or intact after selective purification using various instruments such as LC-HRMS, LBA or LC-FLD. However, for pharmacokinetic applications more sensitive assays are regularly required (e.g., 100 µg/L or less) to characterize the terminal elimination phase. Therefore, in most cases only LC-MS/MS using bottom-up proteomics to quantify the signature peptide or LBA after selective purification with anti-idiotypic antibodies would be suitable (*Figure 2*).

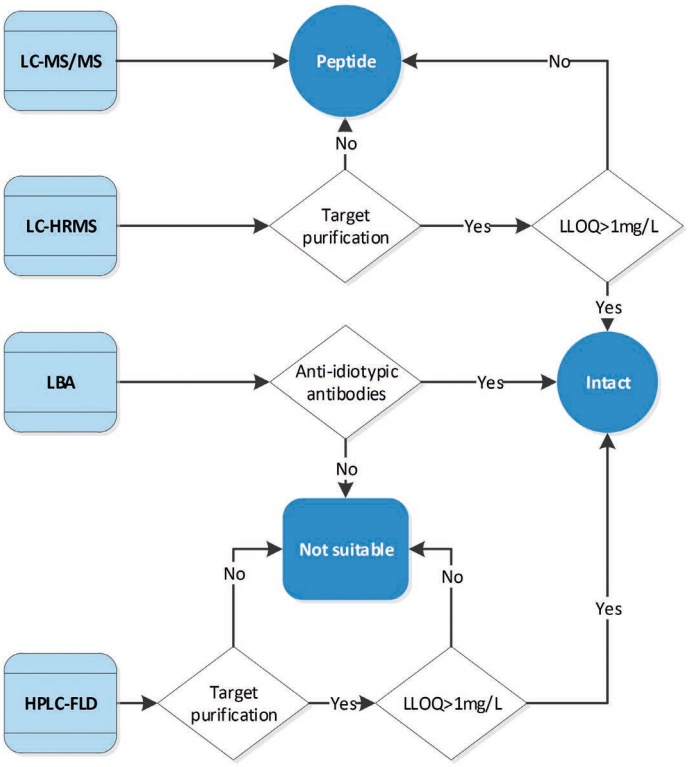

Figure 2. General recommendation for therapeutic mAb quantification depending on instruments and materials available and on assay requirements; Quantification on the basis of peptide (bottom-up) using LC-MS/MS or LC-HRMS and intact on the basis of middle-down and top-down strategies with LC-HRMS or intact on the basis of LBA, HPLC-FLD.

Bottom-up quantitative proteomics is preceded by denaturation and enzymatic digestion of the therapeutic mAb which releases numerous peptides of different chain lengths. Peptides that are unique for the mAb (signature peptides) are selected for measurement. These peptides are easily and efficiently separated using a standard reverse-phase HPLC system and thereafter quantified on a standard triple quadrupole mass spectrometer. In general, the chromatographic peak shape of the peptides is more symmetrical compared to the peak shape of proteins due to fewer secondary interactions on the stationary phase.

Top-down and middle-down quantitative proteomics is based on the measurement of intact or semi-intact proteins. Large proteins such as mAbs can shift to high charge states during electrospray ionization yielding a mass to charge (m/z) ratio within the working range ~1800 – 4000 of a high resolution mass spectrometers (HRMS) such as Orbitrap or Time-Of-Flight (TOF). These methods do not require protein unfolding and enzymatic digestion which can be challenging and time-consuming to optimize.

However, top-down and middle-down methods do have some limitations. Firstly, targeted sample purification with anti-idiotypic antibodies or ligands is necessary due to structural similarities between the therapeutic mAb and endogenous IgG. Secondly, the required HRMS apparatus is expensive compared to the triple quadrupole mass spectrometer. Finally, attaining the required lower limit of quantifications (LLOQ) can be challenging because of wider precursor charge distribution, broadened chromatographic peak shape of large proteins, and mAb glycoform heterogeneity [39-41]. Some additional steps can be implemented to gain higher signals and thus lower LLOQ's. For example, human immunoglobulin G1 in rat serum was successfully quantified with LC-HRMS after target specific purification with anti-idiotypic antibodies followed by deglycosylation [32]. Here, a remarkable LLOQ of 0.1 mg/mL was achieved through this strategy by utilizing a high sample volume (50 mL) in combination with high volume injection (60 mL) and 1mm diameter analytical column. Top-down quantification can also be performed by HPLC coupled to fluorescence detector (FLD). For example, intact trastuzumab, bevacizumab and infliximab in human serum were successfully quantified by HPLC-FLD after targeted purification [42, 43]. Unlike HRMS methods, FLD methods are not affected by signal dilution caused by charge distributions. However, fluorescents measurements have a low specificity. Most proteins have similar excitation and emission spectra which result in noisy and overlapping chromatographic peaks. Furthermore, because of the lower sensitivity, a higher sample volume (~100 mL) is required which limits its applicability. Middle-down strategies can also be used to reduce precursor charge distribution found in intact analysis. Here, only a portion of the mAb is measured such as the light chains after dithiothreitol (DTT) reduction, or Fab regions after limited Lys-C digestion [44, 45]. In contrast to intact mAb measurement, these regions are smaller and are usually free from glycan chains, leading to fewer precursor ions resulting in an increase in signal intensity of the mAb. As can be seen from *Figure 2*, peptide level quantification via LC-MS/MS or LC-HRMS instruments have low requirement and are thus frequently employed. This approach has been extensively used for numerous mAbs with great success. However, for some fully human/humanized therapeutic mAbs, quantification via signature peptide can be challenging because of the human polyclonal serum background [45]. In these situations, a targeted purification followed by intact or peptide level quantification might be preferred.

Bottom-up quantification

Currently, bottom-up quantification of mAbs in biological matrices using signature peptides is the most common approach. This principle offers fast, easy and flexible method development with high detection sensitivity using standard triple quadrupole mass spectrometers.

Table 1. Published methods for peptide level quantification of therapeutic mAbs with LC-MS/MS.

Sample type			**Internal standard used**				
Rodent	Monkey	Human	Analogue protein	SIL peptide	SIL protein	Extended SIL peptide	Dimethyl labeled
[33, 47-53]	[21, 54-64]	[34, 65-85]	[47, 63, 76, 79-81, 83]	[34, 49, 53, 54, 57, 60, 64, 67-71, 73, 75, 78, 85]	[21, 33, 48, 51, 52, 56, 59, 62, 72, 74, 77, 82]	[58, 61, 65, 73]	[55]
Sample purification							
None (whole digest)	Albumin depletion	Pellet Digestion	Protein A	Protein G	Anti-human FC antibody	Anti-Idiotypic antibody	Ligand
[21, 47, 55, 60, 73, 78]	[33, 54]	[49-52, 57, 58, 61, 63, 64, 66, 67, 75, 85]	[45, 53, 68-71, 80, 84]	[65, 76, 81]	[48, 56, 59, 60, 62]	[34, 65]	[72, 74, 77, 79, 82, 83]
Digestion by denaturation				**Digestion by reduction & alkylation**			
Guanidine	Urea	Surfactant	TFE	None	TCEP and IAA	DTT and IAA	DTT
[47]	[21, 33, 48, 54-56, 59, 74, 82]	[34, 50, 60, 62, 73, 78, 85]	[66]	[49, 51, 57, 64, 67, 71, 75, 82, 83]	[33, 48, 60, 65, 77]	[21, 34, 47, 50, 52-56, 58, 59, 61-63, 66, 73, 76, 78, 80, 81, 85]	[72, 79]
Post digest SPE cleanup		**Mass spectrometer**			**LLOQ method performance**		
RP	Ion exchange	Triple Quad	Q-Trap	HRMS	≤0.5mg/L	≤1 mg/L	>1mg/L
[33, 47, 48, 75, 78]	[47, 52, 60, 61, 63, 73]	[21, 34, 47, 50, 51, 53, 55, 56, 58-62, 64-68, 71-76, 79-81, 85]	[33, 48, 49, 54, 57, 63, 78, 82]	[52, 77, 83]	[34, 47, 48, 50, 53, 56, 59, 62, 65, 67, 71, 77, 84, 86]	[49, 52, 55, 57, 65, 68, 74, 75, 81-83, 85]	[21, 33, 51, 54, 58, 60, 63, 64, 66, 73, 76, 78, 80]

Abbreviations: SIL: stable isotopically labeled; TFE: trifluoroethanol; TCEP: tris(2-carboxyethyl)phosphine; IAA: iodoacetamide; DTT: dithiothreitol; SPE: solid phase extraction; RP: reversed phase.

Generally, these published methods share similarities as they all include steps depicted in *Figure 3*. The major differences between these methods are the selection of internal standard, sample processing and digestion conditions (*Table 1*). The merits and drawbacks of various options in each steps of method development will be discussed in next sections.

Figure 3. General workflow to develop quantitative LC-MS/MS method to measure therapeutic mAbs.

Signature peptide selection

The first step in method development is selection of unique signature peptides to serve as surrogate for quantification. The peptide sequence of the therapeutic mAb is essential for this step. Sequences of approved therapeutic mAb can be found in the Immunogenetics Information system® (http://www.imgt.org/) or in Drugbank (http://www.drugbank.ca). For the quantification of chimeric mAbs in human serum, tryptic peptides from the entire variable region can be chosen and targeted. However, for human or humanized mAbs the choice is limited to the complementarity-determining regions (CDRs) of which there are six in the variable light and heavy chains. In silico tryptic digestion can be performed manually or by using an online tool 'Protein Prospector' (http://prospector.ucsf.edu). The peptides generated can be screened online with protein Blast® software (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The peptide sequence is compared against the target organism (biological matrix of the analyte) from an appropriate database such as UniProtKB/Swiss-prot. Any peptide scoring under 100% for either the query cover or the positive identification, represents a unique peptide and as such a potential quantifier. Thereafter, a list of potential signature peptides can be screened for amino acid stability. Amino acids such as cysteine and methionine are susceptible to oxidation leading to mass shifts of +16, +32, +48 Da depending on the number of oxidation reactions. Asparagine followed by a smaller amino acid such as glycine can readily be deamidated into aspartic acid, isoaspartic acid and succinimide during tryptic digestion, causing a mass shift of +0.98, +0.98, and -17.03 Da respectively. Also N terminal glutamine cyclization can occur at alleviated pH and prolonged digestion time. Here, the loss of ammonia leads to a mass shift of -17.03 Da [87]. Preferably, these amino acids should be avoided. However, when stable alternatives are unavailable, these peptides can still be used if a stable isotopically labeled (SIL) internal standard is included. For example, infliximab, somatropin and nivolumab quantification with signature peptides containing a methionine were previously reported [47, 66, 68]. Also, endogenous insulin-like growth factor 1 was successfully quantified in serum with a signature peptide containing two cysteines that were protected from oxidation by attaching a carboxymethyl group on the thiol moiety through iodoacetamide alkylation and acid hydrolysis following disulfide bond

reduction [88]. Nevertheless, care must be taken when these peptides are used since *in vivo* degradation of the therapeutic mAb via the above mentioned pathways can lead to an underestimation of circulating mAb as was demonstrated by Bults and colleagues in their research into deamidation of trastuzumab in human plasma [67]. After a list of potential signature peptide candidates is composed, a tryptic digest of the stock standard is preformed and run on LC-MS/MS to identify the various eluting peptides. An example of this is illustrated in *Figure 4* where a number of infliximab peptides were identified using a shallow gradient (5-50% acetonitrile 0.1% formic acid in 20 min). It is important to note that due to the slow scan speed of the triple quadrupole mass spectrometer a high stock standards concentration (~100 mg/mL) should be digested.

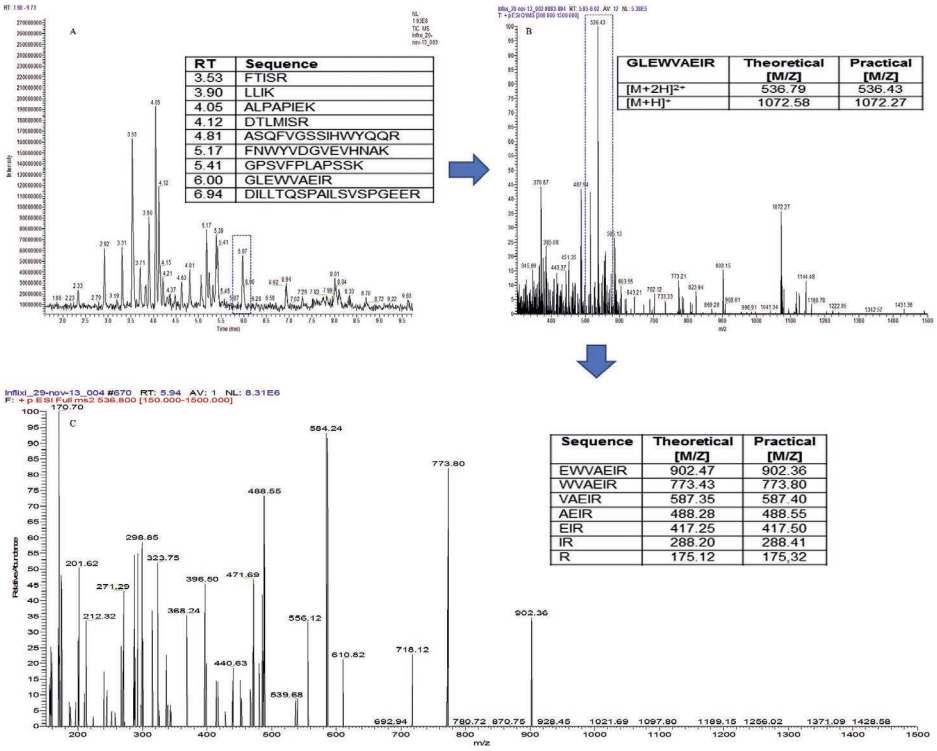

Figure 4. Signature peptides identification of infliximab tryptic digest with a triple quadrupole operating in full scan MS (300-1500 m/z) (A), GLEWVAEIR precursor elucidation though comparison with theoretical mass (B) and finally, conformation of the precursor sequence through fragmentation (20 eV) of [Mþ2H]2þ and product ion scan (150-1500 m/z) (C).

Internal standard selection

Methods that include an IS are able to correct for various factors causing variability during sample analysis, such as component losses during sample preparation as well as

instrument related factors such as injection, ionization and fragmentation. Depending on the IS used, different levels of corrections can be obtained (*Table 2*). Also in ELISA there have been attempts to incorporate IS in the assays [89]. However, the correction ability here is limited to dilution corrections only. In LC-MS/MS, SIL proteins can correct for the entire sample pretreatment and analysis because of their matching amino acid sequence and conformational folding and are considered the gold standard in quantitative proteomics [21, 90]. Unfortunately, these SIL variants of therapeutic proteins are often very expensive and only a limited number are commercially available. As an alternative, Nouri-Nigjeh and colleagues have shown that hybrid calibration, which use the therapeutic mAb of interest as 'calibrator' in combination with a SIL peptide or extended-SIL peptide as IS, can obtain accurate and precise results in whole sample digestion methods [90]. This observation was also supported by Prasad and Unadkat stating that SIL peptides can be used when maximum trypsin digestion is ensured [91]. The largest source of variability in this type of work-up originates from ionization suppression due to sample complexity. In contrast to the calibrator protein, flanking SIL peptides and extended peptides are easily dissolved in the sample matrix and due to the lack of structural folding and S-S bonds, provide easier access to the cleavage site. Therefore, the correction for digestion efficiency is expected to be very low using this approach. Furthermore, experiments performed in house using a regular SIL peptide and a SIL extended peptide showed that SIL peptide performed better than SIL extended peptide since the SIL extended peptide produced additional variability during digestion that was not correlated to variability found in calibrator protein digestion. Dimethyl labeling was used by Ji and coworkers and was found to be a cheap way of generating multiple labeled peptides from the protein calibrator [55]. However, reaction conditions need to be carefully optimized to obtain maximum labeling efficiency. This principle has not gained a lot of ground since cheap SIL labeled peptides with high purity can easily be obtained. Analogue proteins are also frequently used to correct for sample purification and digestion (*Table 1*). However, the peptides generated from these analogue proteins are not identical to the signature peptides of the target mAb. Therefore, differences in charge and or hydrophobicity could lead to suboptimal correction for clean-up and enrichment steps. Moreover, differences in protein folding, solubility and disulfide bond location between the calibrator protein and the analogue may only result in moderate correction for digestion if preceding protein reduction, alkylation and denaturation was suboptimal. Matrix effect correction for ionization relies on the elution order of the signature peptide and the IS. So, depending on the elution similarities between the signature peptide and the analogue peptide, varying levels of corrections can be achieved. This is also true for fragmentation correction, here similarities in amino acid sequences between the signature peptide and the analogue determine the levels of correction. Nevertheless, Li and colleagues have shown that when a selective purification is used, analogue proteins can perform better than SIL peptide or a SIL flanking peptide [56]. Here, sample recovery was the major contributor to the method error and therefore, by including an analogue protein that can experience the same losses as the calibrator protein, correction was achieved. Furthermore, variability in LC-MS/MS analysis is

expected to be low since reproducible ionization and fragmentations can be achieved as a result of the clean sample extract.

Table 2. Performance of various internal standards during sample workup and analysis.

	Sample preparation			LC-MS/MS Analysis		
Internal Standard	Sample Purification	Digestion	Clean-up and Enrichment	Injection	Ionization	Fragmentation
SIL Protein	++	++	++	++	++	++
SIL Peptide	–	–	++	++	++	++
Dimethyl Label	–	–	++	++	++	++
Flanking SIL (Extended) Peptide	–	–	++	++	++	++
Analogue Protein	++	+	+	++	+	+

Notes: ++: optimum correction; +: moderate correction, –: no correction.
Abbreviation: SIL: stable isotopically labeled.

LC-MS/MS optimization

Chromatographic separation and MS/MS optimization are critical steps in method development and if done properly can lead to higher assay sensitivity. LC separation and MS/MS optimization are firstly performed on a digested mAb standard and after sample purification and digestion conditions are optimized, LC separation is re-evaluated with a mAb spiked sample of the biological matrix of interest. When sample purification methods are used that result in clean extracts, such as targeted sample purification, short LC runtimes (~5 min) can successfully be achieved [34, 48, 56, 62, 72]. Nevertheless, columns with higher plate numbers and longer gradient times might be required to separate isobaric interferences and matrix effects when generic sample purification methods are used. High resolution instruments such as the Orbitrap, time of flight (TOF) or ion trap (QTap) can also be used and may provide the necessary selectivity. However, matrix effects can only be eliminated through sample cleanup and sufficient LC separation. Detection limits can be lowered by selecting the optimal signature peptide but also by monitoring the most abundant precursor and product-ions. Signature peptides with chain lengths of around 20 amino acids produce precursors consisting of single, double, triple and quadruple charged states. Therefore, a precursor mass-scan needs to be performed to determine the most abundant charged state for quantification (*Figure 4B*). Consecutively, the most intense product-ion can be found by performing a mass-scan after collision-induced dissociation (CID) of the most abundant precursor peak (*Figure 4C*). In contrast to triple quadrupole MS, HRMS instrumentations have a higher full mass scan rate of >12 Hz and can easily obtain precursor and product-ion scans of the desired signature peptide with high accuracy and sensitivity using low amount of sample [92]. This combination of most abundant precursor and subsequent product-ion is termed selected reaction monitoring (SRM) and can further be optimized for

collision gas pressure and collision energy. This can easily be done through infusion of a synthesized peptide directly into the MS or alternatively, can be optimized by performing multiple injections of a digested protein, each time with a different collision setting. Unfortunately, different MS instrumentation can generate different precursor charge ratios and product ion profiles. Therefore, SRM settings are not interchangeable between different instruments. This is demonstrated in *Figure 5* where GLEWVAEIR fragments were scanned by 2 different MS instruments. Here, CID with a triple quadrupole we see that fragment 773.80 was higher than fragment 488.55, while this was the opposite with higher energy collision dissociation (HCD) with the orbitrap. Nevertheless, three most intense product ions per signature peptide should be monitored and only after complete validation, the best performing signature peptide with its concomitant product ion can be selected as quantifier. Other signature peptides that have successfully passed validation can be selected as qualifiers and can be used for quality assurance. Here, sample results from the quantifier peptide can be compared with those of the qualifier peptide during routine analysis. Large variations (>15%) between results could be caused by isobaric interferences which can be present in one signature peptide but not necessarily the other and would require further investigation. However, for each signature peptide a SIL internal standard equivalent is required for quantification this would lead to increased costs when SIL peptides are used.

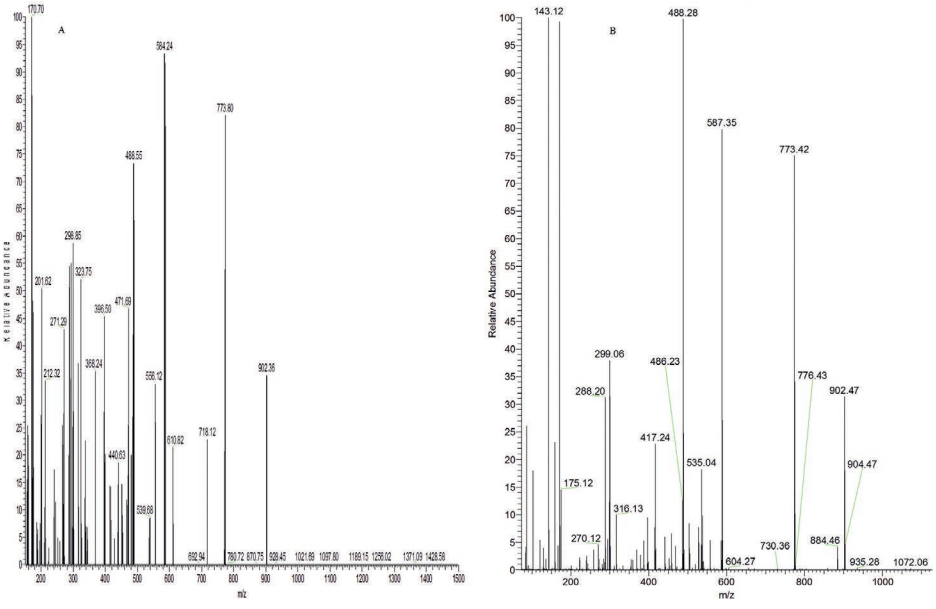

Figure 5. Product ion scan of GLEWVAEIR peptide with Quantum Access Max triple quadrupole (A) and with Q Exactive Orbitrap (B).

Sample purification

Arguably, the most laborious step in the development of a successful bioanalytical method is sample purification. Sample purification is necessary to eliminate interfering proteins and reduce sample complexity (*Table 3*) and over the last decades a myriad of strategies have been reported. Target-specific sample purification in a human bio-matrix utilizes an anti-idiotypic antibody or ligand fixed to a solid support such as a magnetic bead or 96 well plate (*Figure 6A*). Here, only the active therapeutic mAb fraction with at least one free epitope can be purified. This principle was used by our group to purify active infliximab in human serum with its antigen tumor necrosis factor alpha bound to a 96-well plate by means of biotin-streptavidin interaction [72]. A similar principle was reported for the purification of bimagrumab in human serum using activin receptor type 2B which was cross-liked to sepharose magnetic beads by means of NHS (Nhydroxysuccinimide) reagent [77]. The use of anti-idiotypic antibodies, which bind the complementarity-determining regions (CDRs) of the therapeutic mAb, is exemplified with the purification of trastuzumab in human serum with anti-trastuzumab idiotypic antibodies [42]. Purifications with anti-human Fc antibodies in animal bio-matrices can also be considered 'targeted'. Here, only the human/humanized therapeutic antibody will be captured, resulting in the quantification of total therapeutic antibody [48, 56, 59]. When combining a targeted sample pre-treatment with LC-MS/MS measurement, low detection levels (<0.5 mg/L) can be achieved through background noise reduction (*Table 3*). In contrast, a generic sample work-up aimed to capture the entire IgG fraction in serum can be used. Protein A and G are bacterial cell wall proteins that bind IgG via their Fc region, thus protecting it in vivo from the immune system. These proteins achieve relatively clean extracts and have been used with success to purify the total therapeutic mAb (*Figure 6B*) [45, 53, 65, 68-71, 76, 80].

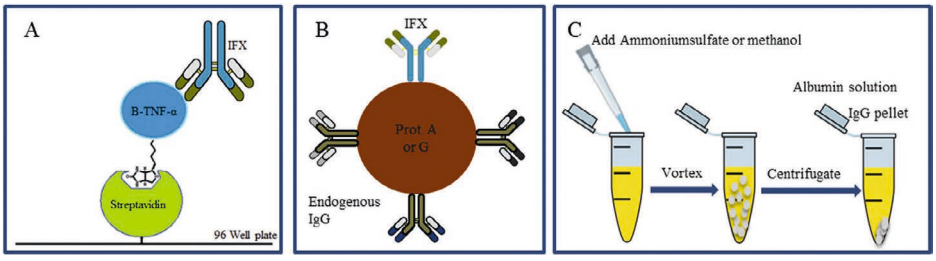

Figure 6. Sample purification methods using; TNF alpha to selectively capture infliximab (A), Protein A or G to capture FC portion of the antibodies (B) and IgG pellet precipitation with ammoniumsulfate or methanol (C)[72].

Experiments performed in-house and others have shown that the ammonium sulfate (AS) precipitation method is highly efficient in the removal of albumin fraction, which comprises of around 60% of total plasma protein (*Figure 6C*) [85, 94]. Proteins with low solubility (usually large proteins), are precipitated first at increasing AS concentration, leaving the highly soluble, smaller proteins and molecules in solution [95]. This is a cost

effective and fast way to remove unwanted proteins and was successfully employed for dinutuximab and infliximab quantification in human serum with LC-MS/MS [66, 85]. The major advantage of AS versus the widely used MeOH pellet digestion method (*Table 1*), is that the protein pellet retains its tertiary conformation allowing for fast re-solvation for reduction and alkylation [95]. Each of the above-mentioned methods has its merits and challenges. The targeted assay tends to be more time consuming and requires ligands or antibodies which can be scarce and expensive. However, the targeted assay provides lower detection limits because of clean extracts and measures the 'active and free' mAb fraction which likely associates with loss of treatment efficacy [72]. In contrast, generic sample purification methods are easy and fast to perform and measure total mAb fraction.

Importantly, total and free fraction results can show poor correlation, especially when ADA are present in the sample [21, 96]. Therefore, correlation studies must be performed when switching from one assay to another. Nevertheless, there are numerous reports of good agreements between free fraction measured by ELISA and total fraction measured by LC-MS/MS [48, 56, 60, 78, 97], even in subsets consisting of ADA positive samples, as was demonstrated by Willrich and colleagues in the quantification of infliximab in human serum [66].

Table 3. Estimated and theoretical levels of purifications of human plasma proteins using different sample preparation strategies. Theoretical data for protein A and G purification obtained from Thermo Scientific [93].

Plasma Components	Concentration in plasma [g/L]	Targeted Purification	Protein A	Protein G	AS Precipitation	MeOH Pellet digestion
Albumin (60 kDa)	45	++	++	++	++	+
IgG (150 kDa)	10	++	–	–	–	–
Fibrinogen (340 kDA)	2.5	++	++	++	–	–
Transferrin (80 kDa)	2.5	++	++	++	++	+
IgA (320 kDa)	2	++	+	++	–	–
Alpha-1 Anti-Trypsin (54 kDa)	1.5	++	++	++	++	+
Phospholipids (<1 kDa)	1	++	++	++	++	++
IgM (900 kDa)	1	++	+	++	–	–
IgD (180 kDa)	0.02	++	++	++	–	–
IgE (200 kDa)	0.0002	++	++	++	–	–

Notes: ++: efficiently eliminated; +: moderately eliminated; –: not eliminated.
Abbreviations: AS: ammonium sulfate; MEOH: methanol.

Digestion conditions

After sample purification, the therapeutic mAb needs to be denatured (unfolded) to allow, in the subsequent step, the digestive enzyme easier access to the cleavage sites. The protein tertiary structure is maintained by hydrophobic, ionic, hydrogen and disulfide bonds. Thus, abrogation of these interactions and bonds can achieve faster and efficient digestion. Disulfide bonds can be reduced with 5mM DTT or tris (2-carboxyethyl)phosphine (TCEP). TCEP is a stronger and more stable reducing agent compared to DDT. However, DTT is most frequently used due to its neutral pH being more compatible with downstream trypsin digestion. Usually, a reduction is carried out under heating conditions (around 60 °C) to speed up the reaction process and to aid in protein denaturation. Urea concentrations >6M can also unfold the protein structures, but sample dilution or dialysis is then required to lower the urea concentration <1M prior to trypsin digestion. Furthermore, undesirable physiochemical reactions can occur when using urea at elevated temperatures. Recent publications focus mainly on unfolding the protein via heating >70 °C with or without MS compatible surfactants such as RapiGest™ [73, 76, 78]. The use of sodium dodecyl sulfate (SDS) surfactant has also been reported [50, 85]. However, when SDS is left in the buffer solution prior to trypsin digestion, the proteolytic enzyme would denature and subsequent MS analysis would suffer from ionization suppression. As proteolytic enzyme, trypsin is mostly preferred for bottom up proteomics, because it cleaves the peptide bonds following arginine (R) and lysine (K), two basic amino acids that are easily ionized during electrospray ionization. Trypsin is active in a buffered solution with low ionic strength <0.1M with pH 7-9 [72]. Digestion efficiency is dependent on factors such as trypsin to protein ratio, temperature, time, protein accessibility and the presence of trypsin inhibitors such as alpha-1 antitrypsin [50]. Digestion efficiency can further be improved by incorporating methylated trypsin which can retains its activity during digestion and can thus be used in lower amounts [98] or by addition 1mM calcium ions to the solution which aids in trypsin stability [99]. Treatment with 6-(1-tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK) is also required to disable chymotrypsin activity in native trypsin [100], since untreated chymotrypsin would cleave the protein at different locations (tyrosine, tryptophan and phenylalanine) affecting signature peptide recoveries. Immobilized trypsin is increasingly being implemented, since it offers fast and complete digestion and can be used in combination with high temperatures [68, 71, 78, 101, 102]. Immobilization secures trypsin on a silica bead or agarose resin and retains its active conformation during high temperature sample denaturation which is necessary for fast protein digestion. Moreover, efficient digestion is promoted by immobilizing excess mounts of trypsin thus favourable enzyme to protein ratio is achieved. Combinations of enzymes can also be incorporated to improve digestion efficiency such as the inclusion of Enzyme LysC, which tolerates high urea concentrations, in combination with trypsin [33]. Since these combination of enzymes, immobilized trypsin and methylated trypsin are significantly more expensive than TPCK trypsin it is recommended to test these enzymes side by side to determine their added value.

Method validation

After optimization of assay conditions the method is subjected to validation following FDA or EMA guidelines for bioanalytical method validation [36, 103]. Parameters, such as LLOQ, accuracy, precision, matrix effect, linearity, stability and carry-over should comply with requirements as states in these guidelines. Furthermore, it is strongly recommended to carry out a cross-validation against an established method to determine whether the methods are strongly correlated. In regards to the latter, it should be stressed that measurement of different therapeutic mAb fractions, e.g., free or total, can result in inter-assay differences. Moreover, inter-assay variation can occur, evenwhen the same fraction is measured. This is exemplified by the comparison of active infliximab quantification in human serum with LC-MS/MS versus ELISA-based assay [72]. Here, the sandwich type ELISA required two free mAb paratopes, one paratope for fixation and the other for detection, while the LC-MS/MS assay only required one free paratope. This meant that the infliximab fraction with only one free paratope could not be quantified with the ELISA assay which may have resulted in an underestimation of free infliximab in serum.

CONCLUSION AND DISCUSSION

An overview of state-of-the-art LC-MS/MS methods used for quantification of therapeutic mAbs in biomatrices is provided. Current literature on peptide level quantification is summarized in six workflow steps, and benefits and drawbacks in each step of method development have been critically evaluated. We conclude that LC-MS/MS instruments offer fast method development and multiplexing capabilities and will continue to replace ligand-binding assays as these instruments get cheaper, improve in terms of sensitivity and mass accuracy with each generation. Innovations and improvements in materials, such as the immobilization of trypsin and magnetic beads conjugated with various ligands, will aid in speeding analysis times while providing high recoveries and sensitivities. Also, with increasing availability of stable isotopically labeled mAbs, method robustness, precision and accuracy will further improve.

Top-down and middle-down quantitative proteomics are expected to become more important as newly developed mAb are mostly fully human or humanized. Improvement to instrument hardware and software are needed to facilitate the growth in this area. As more awareness in the scientific community is growing to the possibilities that these methods have to offer, LC-MS/MS methods have the potential to become the technique of choice for mAb quantification in preclinical and clinical settings.

Author's contribution

AD helped the first author with study design, retrieved and checked data, assisted in the comprehensiveness of the literature search, drafted the first versions of the manuscript together with the first author, helped in implementation of significant contribution from co-authors up to the final publication.

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CHAPTER

Quantification of coagulation factor VIII in human plasma with liquid chromatography tandem mass spectrometry using a selective sample purification with camelid nanobodies

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J Pharm Biomed Anal 2019; 175: 112781

ABSTRACT

Introduction

Patients with haemophilia A are currently diagnosed and monitored by measuring the activity of coagulation factor VIII (FVIII) in plasma mostly with the one-stage clotting assay (OSA). Although the OSA is routinely available in many clinical laboratories, it has in some circumstances relatively low sensitivity and specificity. Therefore, the FVIII activity as a biomarker does not always correlate with the bleeding phenotype. Therefore, we have developed a liquid chromatography-tandem mass spectrometry method to quantify the concentration of coagulation FVIII in plasma which would allow us to investigate the relation between FVIII plasma concentration, FVIII activity and bleeding tendency in future studies.

Methods

LC-MS/MS method was set up by firstly dissociation Von Willebrand factor (VWF) from coagulation factor VIII by triggering the coagulation cascade to occur thus generating active factor VIII (FVIIIa). FVIIIa was then selectively extracted by means of immunoaffinity interaction using anti-FVIII camelid nanobody, after which FVIIIa was eluted, heat denatured and trypsin digested. Finally, a FVIII specific peptide was used as a surrogate for quantification by mass spectrometry. Critical method parameters such as antibody amount, incubation time, sample volume and type of streptavidin 96 well plate were optimized.

Results

The method was validated according to European Medicines Agency (EMA) guidelines where an LLOQ of 1 ng/mL was obtained using 50 µL of citrate plasma sample. Within-run and between-run accuracy and precision for quality control (QC) samples, LLOQ (1 ng/mL), QC Low (5 ng/mL), QC Med (150 ng/mL), QC High (300 ng/mL) were within the threshold of 15% relative standard deviation (RSD) and Bias. The selective immunoaffinity method which was used in combination with a highly sensitive mass spectrometer allowed for an unpresented LLOQ of 1 ng/mL utilizing 50 µL plasma sample.

Conclusion

This method will be used to investigate the beneficial value of FVIII plasma concentration which may be used in conjunction with FVIII activity for patient diagnosis and dosage optimization.

INTRODUCTION

Congenital haemophilia A is a clotting disease caused by a defect in the factor VIII (FVIII) gene located on the X-chromosome and therefore predominantly affects the male population. The prevalence of haemophilia A is 1 in 5.000 male persons. Haemophilia A can lead to serious complications such as bleeding after surgery, bruising, disabling arthropathy, gastrointestinal and urological bleeding and intracranial hemorrhage [1-5]. Acquired haemophilia A is rare and has been estimated to affect 0.2 – 1 in 1 million persons per year [4]. Acquired haemophilia A is caused by the development of auto-antibodies directed against FVIII [4, 6-8]. The majority of the patient population (60%), which consists predominantly of men around the age of 60 years, have a severe type of haemophilia A with FVIII activity usually below 3%. The current treatment requires infusion with recombinant or plasma-derived FVIII [9]. Furthermore, patients with congenital haemophilia are also at risk of developing inhibitors against FVIII. Some of these patients also require high dose FVIIa, which may increase the risk of thrombosis. Patients with either congenital or acquired haemophilia A are diagnosed and treated depending on the activity of FVIII in plasma and therefore careful monitoring of these patients is critical [10]. Currently, there are three commercially available assay types for measuring FVIII activity: a one-stage assay, a two-stage assay, and a chromogenic (Amidolytic) assay [11-14]. The most commonly used assay is the one-stage assay, which is based on activated partial thromboplastin time (aPTT) [15, 16]. This assay measures the time required for patient plasma to form a fibrin clot after it has been combined with a FVIII deficient plasma and reagents to initiate clotting have been added [17, 18]. The one-stage assay is relatively fast and cheap to perform on automated coagulation analyzers. However, this assay has been shown to have higher %CV for elevated FVIII values in comparison to the other methods [14, 19]. Depending on the endpoint detection method, the one stage-assay may be more sensitive for lipemic plasma samples, and the assay may also have varying sensitivity and specificity to anticoagulant medication and to endogenous inhibitors such as antibodies against FVIII or lupus anticoagulant. Depending on the dilution factor and the activator used in the one-stage assay, both of these types of antibodies may lead to a prolonged aPTT value, which would result in a low FVIII activity, hence complicating diagnosis [20, 21]. Furthermore, due to the lack of standardization in instrumentations and reagents the intra-laboratory variation is the highest in this type of assay [11]. Finally, some patients with mild or moderate haemophilia A will show correct lower activity in the chromogenic assay and two-stage assay but will show false normal activity with the one stage assay possibly leading to misdiagnosis [11, 22]. Aside from these technical limitations in the one-stage assay, activity-based assays in general are sensitive to sample condition. Citrate plasma kept at room temperature (25 °C) or at 4 °C needs to be analyzed within 2 to 6 hours after sample collection, due to the rapid degradation of FVIII, alternatively, the samples can be quickly processed and frozen at -80 °C [23-27]. In contrast to mass-spectometry methods, ligand binding assays pose other challenges such as cross reactivity and limited linearity.

Therefore, in order to eliminate the drawbacks mentioned above, we have investigated the suitability of liquid chromatography-tandem mass spectrometry (LC-MS/MS) in the measurement of FVIII concentration in plasma. The method proposed is based on immunoaffinity purification in combination with tryptic digestion and LC-MS/MS analysis. Potentially, LC-MS/MS may be complementary to the current activity-based assay due to its ability to measure the absolute plasma concentration. Furthermore, pharmacokinetic and pharmacodynamics studies can be performed with FVIII plasma concentration which might be a better predictor for the bleeding phenotype compared to the current activity based assay. In addition, the stability issue of plasma samples, which has been proven to influence the activity results, could be circumvented by measuring FVIII concentration. This could bring home monitoring for haemophiliacs one step closer by allowing mail-in samples. Finally, other coagulation factors can also be added to the existing method at a later stage thus allowing for multiplexed LC-MS/MS analysis.

MATERIALS AND METHODS

Chemicals and reagents

Octocog alfa (Advate®) was obtained from Baxter (Lessines, Belgium) as lyophilized powder and was reconstituted in LC-MS grade water to a final concentration of 500 IU/mL equivalent to 94 µg/mL FVIII; 40 µL aliquots of this solution were pipetted in Eppendorf LoBind Microcentrifuge tubes and stored at -80 °C. Stable isotope labeled peptide internal standard (IS) GELNEHLGLLGPYIR[$^{13}C_6$, $^{15}N_4$] was synthesized by Pepscan (Lelystad, the Netherlands) as a 1 mg lyophilized powder and was dissolved in 1 mL elution solvent (0.5% trifluoroacetic acid (TFA) in 50 % methanol, 50% water). Biotinylated Anti FVIII conjugate, reference number 7102862100 was obtained from Thermoscientific (Waltham, MA, USA) as a 1 mg/mL solution and was stored in -20 °C. Streptavidin high binding capacity coated 96 well plates were obtained from Thermo Fisher (Waltham, MA, USA). MS grade modified trypsin was obtained from Promega (Madison, WI, USA) and was dissolved to 0.1 µg/µL in 50 mM acetic acid and aliquoted in Eppendorf LoBind microcentrifuge tubes. Aliquots were stored at -80 °C. FVIII deficient human plasma was obtained from Precision BioLogic Inc. (Dartmouth, NS, Canada). All other reagents and LC-MS grade mobile phase solvents were obtained from Sigma (Saint Louis, MO, USA).

Preparation of standards, Internal standard and QCs

The FVIII working solution (500 ng/mL) was prepared fresh from 94000 ng/mL stock solution by diluting in FVIII deficient human plasma. Standards at concentrations of 500, 200, 80, 40, 16, 4 and 1 ng/mL were prepared from the working solution by serial dilution in FVIII deficient human plasma. Before use, the IS solution (1 µg/µL) was diluted to 5 ng/mL in 0.1% formic acid (FA) and 0.005% zwittergent 3-16. Quality Control samples (QCs) where prepared at 4 levels namely; at lower limit of quantification (LLOQ) (1 ng/mL), QC low (5 ng/mL), QC med (150 ng/mL) and QC high (300 ng/mL). Aliquots were stored at -80 °C.

Instrumentation and chromatographic conditions

Sample purification was performed on a vibramax 100 plate shaker (Heidolph Instruments, Schwabach, Germany). Sample digestion was performed on a ThermoMixer (Eppendorf, Nijmegen, the Netherlands). All experiments were performed on an Vanquish UHPLC coupled to a TSQ Altis (Thermo Fisher, Waltham, MA, USA). The analytical column was Acclaim™, RSLC 120, C18, 2.1 x 100 mm, 2.2 µm particle size obtained from Thermo Fisher and was maintained at 50 °C. The mobile phases were: (a) 0.1 % formic acid in water; (b) 0.1 % formic acid in ACN. The LC gradients in minutes per percentage of mobile phase B were 0.0 (min)/10 (% B), 8/25, 8.1/80, 10/80, 10.1/10 and 12/10. The flow rate was 0.6 mL/min and the run time was 9 min. The MS was operated in positive mode with spray voltage of 2.5 kV, Ion Transfer Tube Temperature 400 °C, vaporizer temperature 350 °C, aux gas pressure 20 Arb, sheath gas pressure 40 Arb, sweep gas pressure 0 Arb and collision gas pressure 2.5 mTorr. The precursor ions, product ions, collision energy and radio frequency (RF) lens settings are listed in *Table 1* for FVIII signature peptide and for the stable isotopic labeled internal standard.

Table 1. TSQ Altis Mass Spectrometry Conditions for SRM transitions for the signature peptide liberated from FVIII after digestion with trypsin and the internal standard stable isotopic labelled FVIII peptide.

Peptide sequence	Precursor charge	Precursor (m/z)	Product charge	Product (m/z)	Product-Ion type	CE (V)	RF (V)	Dwell time (ms)
GELNEHLGLLGPYIR	3+	560.97	2+	747.93	Y5	16	65	340
GELNEHLGLLGPYIR[$13C_6$,$15N_4$], (IS)	3+	564.31	2+	752.93	Y5	16	65	60

Notes: both sequences had a retention time of 7.2 min.
Abbreviations: CE: collision energy; RF: radio frequency lens; IS: internal standard.

Sample preparation

Sample preparation was based on immunoaffinity purification where the light chain of FVIIIa was captured by means of a biotinylated camelid nanobody (b-anti-FVIII) which in turn was bound to a streptavidin coated 96 well plate (*Figure 1*). In day 1, b-anti-FVIII was coupled to a streptavidin coated 96 well plate by pipetting 200 µL b-anti-FVIII (1 ng/µL) dissolved in PBS, 0.05% Tween-20, 0.1% BSA in each well, followed by 3 hour binding on a plate shaker (300 rpm) at room temperature. The plate was washed 3 times with 200 µL PBS (0.1% Tween-20, 0.1% BSA) and stored upside down in a zip lock bag at -80 °C.

On day 2, 100 uL(micro symbool) PBS (0.05% Tween-20, 0.1% BSA) and 50 uL(ook microsymbool hier) sample (standard or QC) were pipetted to the antibody coated 96 well plate. Then, VWF was dissociated from FVIII by adding 2.5 uL(ook weer micro symbool) (Ca 2.5 M + thrombin 500 IE/mL) followed by an overnight incubation at 400 rpm on a plate shaker.

The next day, the wells were washed three times with 200 µL PBS (0.05% Tween-20, 0.1% BSA). Then, 100 µL IS solution (5 ng/mL) in elution solvent (0.1% FA with 0.005% zwittergent 3-16 dissolved in water) was added to each well and mixed for 5 min at

1200 rpm on a plate shaker. The sample extracts were transferred to a 500 µL lobind eppendorf 96-well plate and heat denatured at 80 °C for 60 min. The samples were neutralized with 10 µL Tris (1M). Then, 5 µL trypsin (0.1 µg/µL) was added to each well and the sample plate was placed in a ThermoMixer set at 37 °C for 3 hour digestion at 800 RPM. Trypsin activity was stopped by adding 20 µL 10% formic acid dissolved in 100 % acetonitrile and finally, 50 µL was injected on a LC-MS/MS.

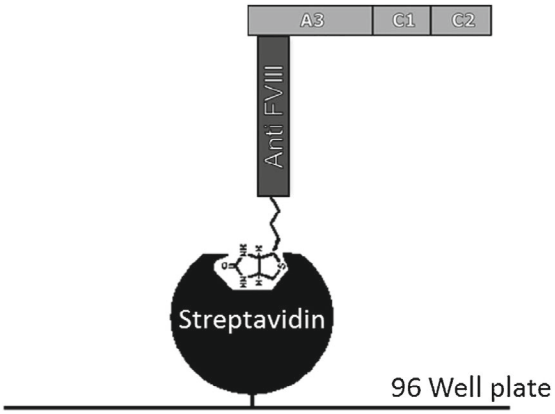

Figure 1. Sample purification of FVIII in plasma. The light chain of active factor FVIII is captured by means of immunoaffinity interaction using anti FVIII camelid nanobody fixed onto a 96 well plate by means of biotin-streptavidin interaction.

Camelid nanobody amount and signature peptide signal intensity

This experiment was performed to determine the amount of biotinylated camelid nanobody required to retain 75 µL of the highest standard (500 ng/mL) Octocog alfa in FVIII deficient plasma. The plate was coated according to sample-preparation section with 10, 40, 100, 250 and 500 ng antibody per well each in triplicate. The highest standard was purified with a 4 hours incubation time and analyzed.

Binding time and signature peptide signal intensity

Citrate plasma sample from a volunteer was used to determine the time needed for the dissociation of VWF, conversion of FVIII to FVIIIa and for optimum sample binding during immunoaffinity interaction. Incubation times of 1, 2, 3, 4, 5 and 24h were evaluated in triplicate and the sample was run using the procedure described in sample-preparation section.

Effect of sample volume on signal intensity

Variable sample volumes were evaluated to determine the matrix effect on sample recovery. Citrated plasma from a volunteer was used for this test. The procedure in sample-preparation section was used with variable sample volumes (5, 10, 25 and 50 µL; each in duplicate).

Streptavidin 96 well plate brand and capacity test

Two high capacity streptavidin 96 well plates were compared. One plate was obtained from Sigma-Aldrich (SigmaScreen) and the other from Thermo Scientific (Streptavidin Coated High Capacity Plates). The test was performed according to the procedure described above using 25, 50 and 75 µL standards (500 ng/mL) in duplicate on both plates.

Validation of FVIII LC-MS/MS method

The validation was performed according to EMA guidelines which requires the evaluation of LLOQ, linearity, accuracy and precision, carry-over, auto sampler stability, freeze and thaw stability and matrix effect [28]. The acceptance criterion for LLOQ was that the signal of the QC LLOQ level (1 ng/mL) should be at least 5× that of the blank sample which consisted of FVIII deficient human citrated plasma. The calibration curve used to establish linearity consisted of 7 standards ranging from 1 to 500 ng/mL and was analyzed on 3 separate days. Within run and between run accuracy expressed as %bias and precision expressed as %CV were validated by measuring four QC levels (LLOQ (1 ng/mL), QC low (5 ng/mL), QC med (150 ng/mL) and QC high (300 ng/mL)) in five-fold during three days. The overall bias was calculated from the mean concentration and the within-run and between-run %CV was calculated from one-way ANOVA derived mean squares. Carry-over effect was tested by injecting a blank sample after the highest standard and comparing the signal intensity at the analyte retention time to the signal intensity of the LLOQ. Auto-sampler stability was evaluated by re-injecting the samples on the next day. Overnight stability was evaluated on QC low and QC high sample. Matrix effect was evaluated by spiking random samples with FVIII and calculating the spike recovery.

RESULTS AND DISCUSSION

Method Development

Human FVIII consists of a light chain and a heavy chain held together by calcium ion and by the stabilizing protein von Willebrand factor that is bound to the light chain of FVIII. The antibody used for sample purification targets the light chain of FVIII and therefore dissociation of von Willebrand factor was necessary to obtain a high recovery. This was achieved by triggering the coagulation cascade to occur through the addition of calcium chloride and thrombin to the citrated plasma sample. The amount of antibody used per well to capture FVIII was 200 ng providing around 100× molar excess in relation to the highest standard used. This was based on previous work with similar interaction where a therapeutic antibody was purified by means of a ligand [29]. However, FVIII concentration range in plasma is around 100× lower compared to therapeutic monoclonal antibodies, and therefore, the selection of the signature peptide was primarily based on the peptide that had low background interference and delivered the highest signal to noise ratio to meet assay requirements. The peptide 'GELNEHLGLLGPYIR' was found to meet this criterion and was found to be unique to

FVIII. A stable isotopic labeled peptide GELNEHLGLLGPYIR[$13C_6$, $15N_4$] was synthesized and was used as internal standard to correct for MS ionization variability. Sample handling procedure was based on our previous work where we have compared various denaturation conditions for simultaneous quantification of adalimumab and infliximab [30]. Using the 80 °C denaturation procedure we found that most signature peptides provided similar digestion efficiency as the commercial Smart Digest kit and was superior to denaturation and reduction with DTT at 60 °C. Finally, remaining steps in the method were evaluated and optimized to ensure a sensitive, repeatable and accurate result.

Camelid nanobody amount and signature peptide signal intensity

The optimum amount of biotinylated camelid nanobody needed for efficient purification of activated FVIII light chain was investigated. Camelid nanobody consist of an antibody variable domain fragment with a molecular mass of 13kDa targeting the light chain of FVIII. This is an important advantage over traditional full length antibodies derived from other mammals which are 10 times bigger. More camelid nanobody can fit in each streptavidin coated well thus providing increased binding sites for factor FVIII. The saturation curve in *Figure 2* showed there was no significant (p = 0.075) change in signal intensity between 100 and 500 ng camelid nanobody. Therefore, 200 ng (15 pmol) camelid nanobody per well was used for all experimentations.

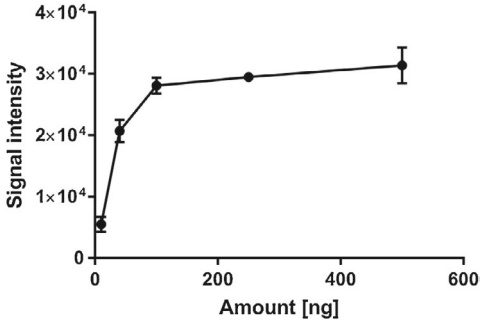

Figure 2. On the x-axis, coated amount of biotinylated camelid anti FVIII nanobodies are plotted versus signal intensity of the signature peptide of the highest standard (500 ng/µL) on the y-axis, error bars represent SD with *n* = 3.

Binding time and signature peptide signal intensity

Another important parameter is the time required for the dissociation of VWF, the conversion of FVIII to FVIIIa and the binding of FVIIIa light chain to the 96 well plate coated camelid nanobodies. This experiment was performed with human derived FVIII plasma, since octocog alfa in the standard solution is not pre-conjugated with VWF. At 24 hours the signal intensity was significantly higher (p = 0.0004) than the signal intensity obtained at 5 hours *(Figure 3)*. However, the difference in signal intensity between 1 and

24 hours was only 30%. Since the signal intensity at 24 hour was significantly higher than 5 hours, an incubation period of 24 hours was chosen. An even longer incubation period than 24 hour was not deemed necessary since the difference in signal intensity between 5 and 24 hours was only 15%.

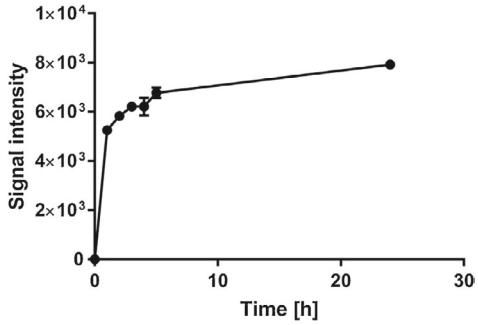

Figure 3. On the x-axis binding time between camelid nanobodies and the light chain fragment of FVIII is plotted versus signature peptide signal intensity from a volunteer sample on the y-axis, error bars represent mean with SD, *n* = 3.

Effect of sample volume on signal intensity

This experiment was set-up to determine if sample matrix interferes with FVIII binding or LC-MS/MS signature peptide measurement. The signal intensity obtained after sample purification and measurement was corrected for sample volume. *Figure 4* shows that volume 50 and 25 µL produced similar (p = 0.051) signal intensities when corrected for volume. However, when sample volume lower than 25 µL was purified, lower signal intensity was obtained than expected. Internal standard signal was stable for all samples indicating that no ionization differences was present due to matrix. A possible reason for the diminished signal intensity could be due to other coagulation factors being present in lower levels which might have affected FVIII activation. FVIIIa light chain is smaller and can easily be captured compared to intact FVIII. Since a low detection level were required, 50 µL sample was used. The fibrin blood clot obtained after overnight incubation was too big with higher >50 µL sample volumes. This made sample handling such as decanting and washing difficult to perform.

96 well plate brand and capacity test

Streptavidin and biotin interactions are amongst strongest biological interactions known with binding strengths equivalent to covalent bonds. Furthermore, biotin and streptavidin bind selectively with each other, thus limiting cross-reactivity.

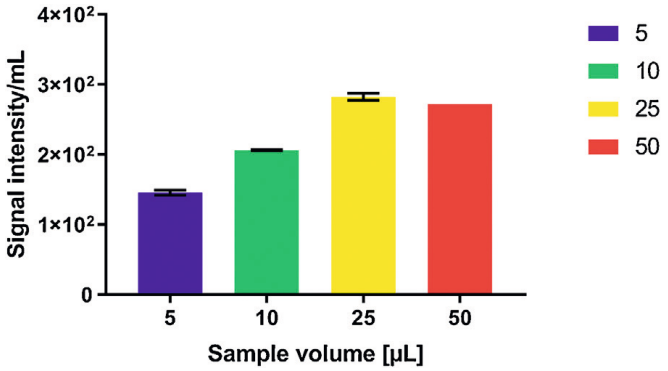

Figure 4. On the x-axis sample volumes from one volunteer is plotted versus the signal intensity corrected for volume on the y-axis; error bars represent mean with SD, *n* = 2.

Even though the Sigma plate had the highest capacity (>300 pmol/well) compared to the Thermo Scientific ~125 pmol/well, the results show that both plates performed equally well (*Figure 5*). The linearity was maintained even at the highest volume of 75 µL (500 ng/mL) and the variations in duplicates were similar between plates and low. The reason for this good agreement is because only 0.1 pmol FVIII needed to be purified in the highest standard and both plates had more than 1000× molar access streptavidin per FVIII available. Thermo Scientific plates were chosen because they were significantly cheaper and were available in 8-well strip format.

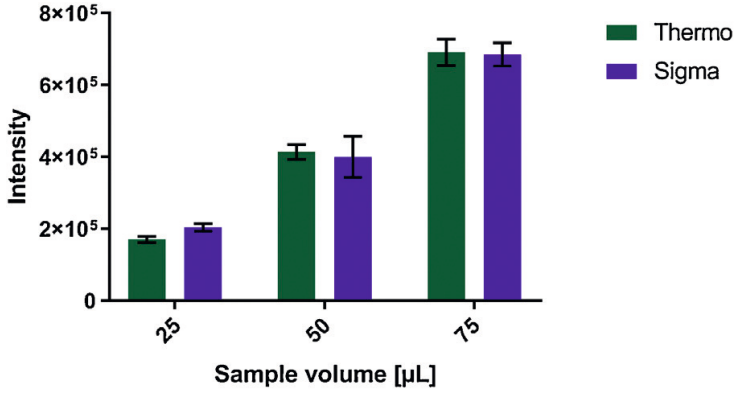

Figure 5. Signal intensity obtained with different streptavidin coated 96 well plates, namely the SigmaScreen from Sigma and Thermo Scientific Pierce Streptavidin Coated High Capacity Plates using various sample volumes of the highest standard 500 ng/mL, errors bars representing SD with *n* = 2.

Validation

First, a test was performed to determine whether the LLOQ level of 1 ng/mL was achievable. After performing the sample purification as described above, LLOQ sample was analyzed together with a blank (FVIII deficient plasma). The signal to noise (S/N) ratio at the retention time of the surrogate peptide "GELNEHLGLLGPYIR" was obtained. Here a S/N ratio of 27 was found which is above the acceptance threshold of 5 (*Figure 6*). The accuracy and precision of the calculated concentrations of standards 1 to 7 were also in agreement with guidelines (*Table 2*). Within-run and between-run precision and accuracy were validated in three days using four QC levels in fivefold. The overall precision and accuracy for LLOQ, QC low, med and high were found to be within acceptance criteria of <20% and 15%, respectively (*Table 3*). Combined standard uncertainty, which consists of the bias and CV, was calculated through the Nordtest approach and can be used to derive uncertainty values of sample measurements between de validated calibration range 1 – 500 ng/mL (*Table 4*). Matrix effect was investigated by spiking known amounts of FVIII to citrate plasma from 3 different volunteers. No matrix effect was observed; all calculated values corresponded well with spiked values (*Table 5*).

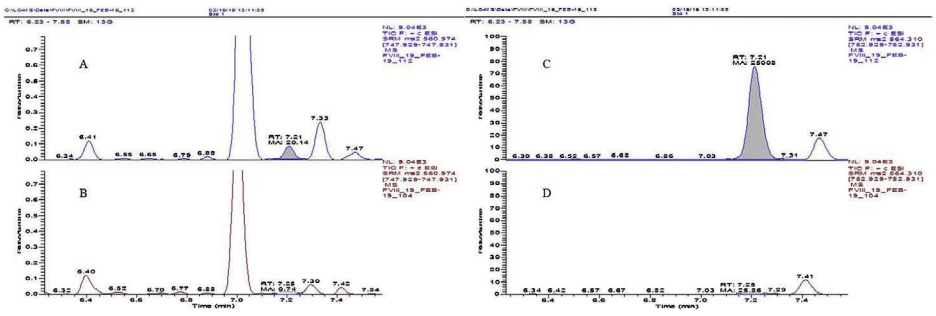

Figure 6. Left side, chromatogram of standard 1 ng/mL (A) and chromatogram of blank depleted FVIII plasma (B) both measuring GELNEHLGLLGPYIR SRM transition 561→747.93. Right side, chromatogram of internal standard (C) and chromatogram of blank (D) measuring GELNEHLGLLGPYI(R*) SRM transition 564→752.93.

Table 2. Statistics of the back calculated concentrations of the standard curve analyzed during three days.

	Nominal concentration [ng/mL]						
	500	**200**	**80**	**40**	**16**	**4**	**1**
Mean	506.97	194.42	74.81	39.16	15.76	4.34	1.00
Stdev	9.45	3.43	0.56	1.14	0.44	0.26	0.04
Accuracy[a]	1.39	-2.8	-6.5	-2.1	-1.5	8.5	0.1
Imprecision[b]	1.9	1.8	0.8	2.9	2.8	6.1	4.2

[a] Accuracy: determined as (measured conc. – nominal conc.) / nominal conc. × 100%.
[b] Imprecision: expressed as co-efficient of variation.

Table 3. Accuracy and precision validation data for QC's at LLOQ, Low, Medium and High levels. Within-run data were based on 5 replicates and between-run data on 3 different days.

	Precision (% CV)			Accuracy (% bias)
QC	**Within- run**	**Between-run**	**Overall**	**Overall**
LLOQ	14.5	13.5	19.8	3.3
Low	7.4	5.3	9.1	-4.3
Med	3.2	5.7	6.5	-4.5
High	2.2	4.6	5.1	-4.7

Table 4. QC data of within-run and between run accuracy and precision for the calculation of the combined standard uncertainty through the Nordtest approach.

Nominal value [ng/mL]	**Day**	**#1**	**#2**	**#3**	**#4**	**#5**	**CV [%]**	**Bias [%]**
LLOQ	1	0.995	0.680	1.075	1.097	0.900		
1	2	1.351	1.148	1.106	1.179	1.275	18.46	3.33
	3	1.187	0.839	1.045	0.824	0.800		
QC Low	1	4.807	4.975	5.136	5.217	5.508		
5	2	4.70	4.85	4.36	4.80	4.27	8.62	-4.30
	3	3.88	4.44	4.84	5.06	4.95		
QC Med	1	154.43	142.24	145.94	151.86	143.06		
150	2	125.20	136.42	132.64	136.18	137.59	5.79	-4.48
	3	147.09	152.21	146.18	152.05	146.01		
QC High	1	289.01	277.16	275.32	271.04	276.14		
300	2	281.57	280.50	265.73	287.29	278.00	4.51	-4.66
	3	303.80	304.81	302.93	301.59	295.20		
RMS_{CV}		10.83	%	$RMS_{CV} = \sqrt{\frac{\sum(CV_i)^2}{n}}$				
RMS_{bias}		4.23	%	$RMS_{bias} = \sqrt{\frac{\sum(bias_i)^2}{n}}$				
Combined Standard Uncertainty		11.62	%	$Combined\ Standard\ Uncertainty = \sqrt{(RMS^2{}_{CV} + RMS^2{}_{bias})}$				

Table 5. Matrix effect, each sample spiked at 3 different concentration levels.

Sample	Spike [ng/mL]	Expected [ng/mL]	Measured [ng/mL]	Recovery [%]
# 1	290	564.8	570.5	101.0
	145	419.8	431.7	102.8
	72.5	347.3	351.2	101.1
	0	274.8	274.8	100.0
# 2	290	543.2	521.2	96.0
	145	398.2	395.7	99.4
	72.5	325.7	325.3	99.9
	0	253.2	253.2	100.0
# 3	290	578.8	551.1	95.2
	145	433.8	421.9	97.2
	72.5	361.3	346.7	95.9
	0	288.8	288.8	100.0

CONCLUSION

Here we describe for the first time the use of LC-MS/MS for the quantification of FVIII in citrated plasma. Critical method parameters were optimized and the resulting method was subjected to validation according to EMA guidelines. All parameters were found to be well within predefined acceptance criteria. The method, which utilizes camelid anti FVIII nanobodies for sample purification and LC-MS/MS for measurement is highly selective. The removal of interfering plasma proteins lowered the detection threshold significantly, resulting in similar sensitivity as the one-stage activity assay. The lower limit of quantification of FVIII was 1 ng/mL which corresponds to 3.6 fmol/mL, which to the best of our knowledge has never been reported before in the analysis of biopharmaceuticals with LC-MS/MS. This was mainly achieved by using an easy and robust sample processing method which consisted of an efficient immunoaffinity purification in combination with a highly sensitive mass spectrometer. This method was developed for an ongoing study to investigate the pharmacokinetics of FVIII and its added value to existing activity-based diagnosing and monitoring. Furthermore, LC-MS/MS method enables 'telemonitoring' of patients by means sampling at home using dried blood spot sampling for instance. Finally, multiplexing capabilities of LC-MS/MS would allow for other coagulation factors to be included in the same assay thus providing a way to quantitate multiple coagulation proteins in patient plasma in one analysis.

Author's contribution

AD helped the first author in analysis of data, drafted the first versions of the manuscript together with the first author, helped in implementation of significant contribution from co-authors up to the final publication.

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CHAPTER

Comparison between coagulation factor VIII quantified with one-stage activity assay and with mass spectrometry in haemophilia A patients: Proof of principle

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Int J Lab Hematol 2020; 42: 819-826

ABSTRACT

Introduction

Haemophilia A is a hereditary bleeding disorder caused by a factor VIII (FVIII) deficiency. As biomarker, FVIII activity is used to classify disease severity and to monitor treatment. The one-stage clotting assay (OSA) is performed to measure FVIII activity, but OSA's limitations may result in misclassification of disease severity or suboptimal monitoring of treatment. Measurement of FVIII plasma concentration with liquid chromatography-tandem mass spectrometry (LC-MS/MS) might overcome these challenges. The objective is to investigate the correlation between FVIII activity and concentration, and determinants for differences between the two methods.

Methods

In this cross-sectional study, all haemophilia A patients receiving standard-of-care were eligible for inclusion. Within the activity categories of <1 IU/dL, 1–5 IU/dL, >5–40 IU/dL, >40–150 IU/dL, and >150–600 IU/dL we randomly selected 15–20 plasma samples, and compared FVIII concentration (LC-MS/MS) to FVIII activity (OSA) with linear regression and Bland-Altman analysis. Potential determinants for differences were analysed with linear regression.

Results

Inclusion was 87 samples. Bland-Altman analysis demonstrated an overall mean difference of -1% with an SD of 64% between the two methods. Large differences were correlated with the presence of anti-FVIII antibodies (133% [95% CI 81, 185] n = 5) and use of exogenous FVIII products (-37% [95% CI -65,-9] n = 58), e.g., plasma-derived and B-domain modified FVIII products.

Conclusion

Despite good overall correlation between the two methods, relative differences were large, especially for samples with anti-FVIII antibodies or exogenous FVIII products. These differences may have clinical impact. More research is needed to determine the value of FVIII plasma concentration in comparison with FVIII activity.

INTRODUCTION

Haemophilia A is a hereditary bleeding disorder resulting from a deficiency or dysfunction of endogenous coagulation factor VIII (FVIII) with a prevalence of 1:5,000 male live births [1-3]. The International Society of Thrombosis and Haemostasis classifies the severity of haemophilia A based on the endogenous FVIII activity as severe (<1 IU/dL), moderate (1–5 IU/dL), or mild (>5–40 IU/dL), all three with a specific phenotype [4]. Patients with severe haemophilia (approximately 40% of haemophilia patients) have spontaneous or provoked bleeds in soft tissue and joints, causing arthropathy, impaired quality of life, and higher risks of intracranial haemorrhage or early death. Patients with moderate haemophilia, in contrast, are less affected but suffer from, for example, prolonged bleeding or easy bruising. Patients with mild haemophilia only experience bleeding problems during and after major trauma or surgery [5-7]. The standard of care in the developed regions with access to costly FVIII products, preferably entails an intravenous substitution of exogenous FVIII products based on disease severity and bleeding phenotype. Typically, severe patients receive regular prophylactic infusions with FVIII, and mild or moderate patients are treated in case of bleeding only (on-demand). The dose is often based on an individualised pharmacokinetic profile of a patient's FVIII activity. To minimise bleeding risk and to prevent bleedings, many protocols aim at maintaining minimum trough levels of FVIII activity (>1 IU/dL) in patients with severe haemophilia [8].

The FVIII activity (>1 IU/dL) is currently used as a biomarker to assess disease severity and for monitoring treatment with FVIII products which is dependent on an accurate and precise quantification. The FVIII activity can be measured in clinical laboratories with the one-stage clotting assay (OSA) and/or the chromogenic assay (CSA). The OSA is based on the activated partial thromboplastin time (aPTT), making it easily automated, simple, fast, and inexpensive compared to CSA. The CSA is perceived to be more complex and technically challenging as a consequence of the two-stage principle with factor X activation and an additional chromogenic substrate step [9]. For diagnosing, it is recommended to perform multiple OSA measurements, to combine both OSA and CSA to ascertain the absence of discrepancies, or to evaluate the mutation profile of a patient [10].

Unfortunately, FVIII activity measuring has several limitations. Not only can these limitations result in misclassification of disease severity leading to under- or overestimation of the bleeding phenotype in specific subgroups, but also can result in suboptimal treatment monitoring of patients receiving FVIII replacement products [11, 12]. Both OSA and CSA are hampered by interference of different drugs (e.g., heparin, direct oral anticoagulants) and endogenous inhibitors such as lupus anticoagulant. Results from the assays are also affected by inter-laboratory variability, caused by the use of a wide variety of instruments, reagents, standards, and dilution algorithms [14, 15].

We have recently developed and published a novel method to determine the human FVIII plasma concentration with liquid chromatography-tandem mass spectrometry (LC-MS/MS) [13]. The LC-MS/MS technique enables quantification of the FVIII molecule with a high sensitivity and specificity. Although this method is further upstream than activity measurements and has its shortcomings as well, the new method might also have some advantages. For example, sampling could be done by patients themselves at home using the dried blood spot technique. The primary objective of this proof of principle study is therefore to investigate the correlation between FVIII activity measured with OSA compared to FVIII plasma concentration measured with LC-MS/MS in patients with haemophilia A, and to identify determinants for differences between the two methods.

MATERIALS AND METHODS

Setting and participants

This cross-sectional study was conducted at the University Medical Center Utrecht, and specifically at the laboratories of the Department of Clinical Chemistry and Haematology and the Department of Clinical Pharmacy. All haemophilia A patients or female carriers receiving standard-of-care treatment were eligible for inclusion. Their remnant material was stored in accordance with the local opt-out procedure. Within each of the clinically used FVIII activity categories (<1 IU/dL, 1–5 IU/dL, >5–40 IU/dL, >40–150 IU/dL, and >150–600 IU/dL) 15–20 samples were randomly selected in the period of August 2017 to March 2018. The FVIII plasma concentration was measured with LC-MS/MS and compared to the FVIII activity measured with OSA. Per patient, a maximum of one sample was included per category. The local institution's ethics committee approved the procedure and provided a waiver for patients' consent. This study was conducted in accordance with the current revision of the Declaration of Helsinki as revised in 2013.

FVIII activity with OSA

After blood had been drawn for usual care, the FVIII activity was measured at the ISO15189-certified Laboratory of Clinical Chemistry and Haematology and reported as a percentage of FVIII activity compared to reference plasma. The FVIII activity was measured on a STA-Rack evolution coagulation analyser using STA CK Prest aPTT reagent (Diagnostica Stago, Asnières-sur-Seine, France). The FVIII-deficient plasma and reference plasma were obtained from Precision Biologic (Dartmouth, NS, Canada). The assay had a within-run %CV of 3.8% at a FVIII activity of 1.3 IU/dL, a between-run %CV of 4.6% at a FVIII activity of 34 IU/dL, a between run %CV of 5.6% at a FVIII activity of 88 IU/dL, a linearity range <1–600 IU/dL, a recovery of 100.7%, and a 1.0 IU/dL lower limit of quantification. Robust internal and external quality assessment schemes were performed on the assay, and local performance characteristics were within the pre-defined limits stated by the manufacturers.

FVIII plasma concentration with LC-MS/MS

The FVIII plasma concentration was measured using the LC-MS/MS method at the ISO15189-certified Laboratory of Clinical Pharmacy. The LC-MS/MS method development is extensively described in the previously published manuscript of El Amrani *et al.* and was validated in accordance with European Medicines Agency guidelines on bioanalytical methods [16]. In brief, the method starts with the dissociation of the Von Willebrand factor from FVIII by triggering the coagulation cascade, which generates an unbound FVIII molecule. Subsequently, FVIII is selectively extracted by immunoaffinity interaction using monoclonal anti-FVIII camelid nanobodies. After washing, the FVIII is eluted, heat denatured, and trypsin digested. Finally, a specific peptide sequence from the A3 active domain of the FVIII molecule is selected as a surrogate for quantification by mass spectrometry. This signature peptide has a sequence that is unique for FVIII proteins, both endogenous and exogenous, and the sequence is not present in other human proteins. The method had a mean precision (%CV) within-run of 6.8% and between-run of 7.2%, a mean accuracy (bias%) of -2.6%, a linearity range 1–500 ng/mL, and 1 ng/mL was the lower limit of quantification.

Determinants and patient characteristics

Patient-, disease-, and treatment characteristics, such as gender, age, weight, exogenous FVIII products, anti-FVIII antibodies (also termed inhibitors), and co-medication interacting with the coagulation cascade, were identified as potential determinants for differences between the two methods. Anti-FVIII antibodies were measured with the Bethesda assay (Nijmegen modification) for which the clinical cut-off ≥0.6 Bethesda Units (BU) per millilitre was used [14]. Samples were carefully checked for presence of exogenous FVIII products by (1) all comments linked to the assay orders (2) data on active medication obtained from the Research Data Platform (also taking drug half-life into account) (3) multiple measurements of activity and inhibitors per patient in time. Based on this information the samples were labelled as 'exposed' or 'unexposed' to exogenous FVIII product, independent of the presence of endogenous FVIII or procoagulant co-mediation. The information regarding the determinants was obtained from medical records using the Utrecht Patient Oriented Database, a local data platform [15].

Data analysis

To enable statistical analyses on laboratory results expressed in different units and with categories of different interval size, the correlation between FVIII activity and plasma concentration was evaluated with linear regression and a Bland-Altman analysis. The potential determinants of relative differences were analysed with linear regression analysis. The differences in the Bland-Altman analysis and in the linear regression of the determinants were expressed as relative differences not only to compare the two parameters of different units but also to compare the differences over the complete activity range (since an absolute difference in the lower range would weigh less compared to an absolute difference in the higher activity category). For the interpretation of the

latter argument an ln transformation of the linear regression is performed. Relative differences were calculated as follows:

$$\text{Relative difference (\%)} = \frac{(\text{plasma concentration} - \text{activity})}{((\text{plasma concentration} + \text{activity})/2)} \times 100\%$$

Since the units of both parameters are different, the values of the relative differences can only be used for the statistical interpretation of the result and cannot be interpreted clinically. Univariable linear regression analysis was used to assess the correlation of relative difference with various FVIII products (using non-exposed samples as a reference), patient characteristics (age, weight, presence of anti-FVIII antibodies) and co-medication. In the multivariable regression analysis, differences associated with exogenous FVIII products were adjusted for anti-FVIII antibodies and vice versa. The application IBM SPSS Statistics for Windows (version 25.0. Armonk, NY: IBM Corp.) was the software used for statistical analysis.

RESULTS

In the study 87 samples were included, from 70 patients (54 patients with 1 sample, 15 patients with 2 samples, and 1 patient with 3 samples) as patients were allowed to be included in multiple categories. The study population consisted primarily of men (98%) with a mean age of 37 years and a mean body mass index of 23 kg/m^2. Of the included samples, 6% had anti-FVIII antibodies ≥0.6 BU/mL and 67% had exogenous FVIII product present (see *Table 1* for more patient characteristics). Fifty-eight samples contained one or two FVIII products: mostly one FVIII product, four samples contained two FVIII products and two samples contained an unspecified FVIII product.

The correlation between the FVIII activity and FVIII plasma concentration demonstrated an overall R^2 of 0.81 (*Figure 1*). For better interpretation of the lower range, a linear regression figure with ln transformation was made available in Supplemental Figure 1.

Table 1. Patient characteristics.

	Number	Percentage of total
Total	*n* = 87	100%
Gender, male	*n* = 85	98%
Anti-FVIII antibodies (≥0.6 BU/mL)	*n* = 5	6% (0.8-5.3 BU/mL)
Samples 'exposed' to exogenous FVIII products	*n* = 58	67%
Samples 'unexposed' to exogenous FVIII products	*n* = 29	33%
Plasma-derived FVIII, full-length - Aafact®	*n* = 7	8%
Recombinant FVIII, full-length - octocog alfa, Advate® - octocog alfa, Helixate®/Kogenate®	*n* = 26 - *n* = 8 - *n* = 18	30% - 9% - 21%
Recombinant FVIII, B-domain modified - turoctocog alfa, NovoEight® - efmoroctocog alfa, Elocta ®	*n* = 27 - *n* = 22 - *n* = 5	31% - 25% - 6%
	Mean	**SD**
Age (years)	37	22
Height (cm) (*n* = 72)	167	33
Body weight (kg) (*n* = 75)	69	31
Body Mass Index (kg/m^2) (*n* = 72)	23	6

Abbreviations: FVIII: factor VIII; BU: Bethesda Units.

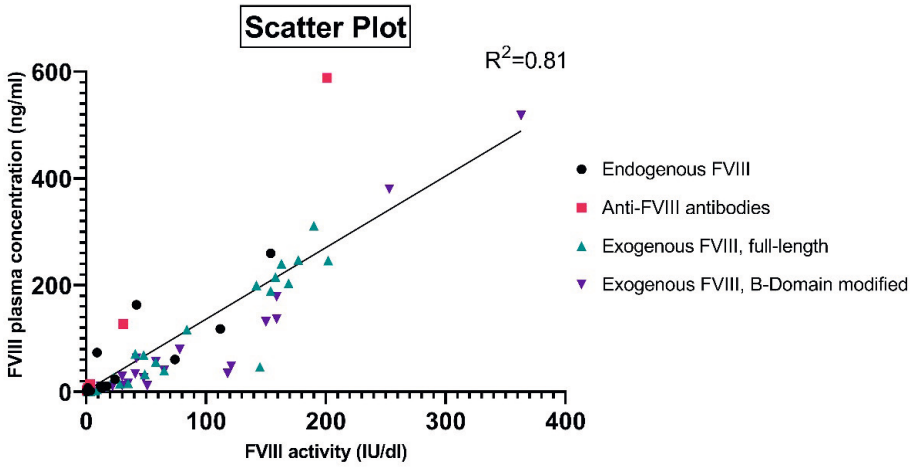

Figure 1. Scatter plot of linear regression between FVIII plasma concentration (measured with LC-MS/MS) compared to FVIII activity (measured with OSA). R^2 of 0.81. Subgroups are illustrated with different symbols, see legend.

The Bland-Altman analysis showed an overall mean difference of -1% between FVIII activity and plasma concentration, with an SD of 64% (limits of agreement are -127% to 125%) and more variability in the lower measurement range. The relative differences were normally distributed. When cut off at a mean of 40% (the upper activity limit for haemophilia A diagnosis), the mean difference was -13% in the lower range (mean <40%) and a mean difference of 20% was found in the upper range (mean >40%). In *Figure 2*, Bland-Altman plots are demonstrated for four different subgroups, all with the overall mean and limits of agreement.

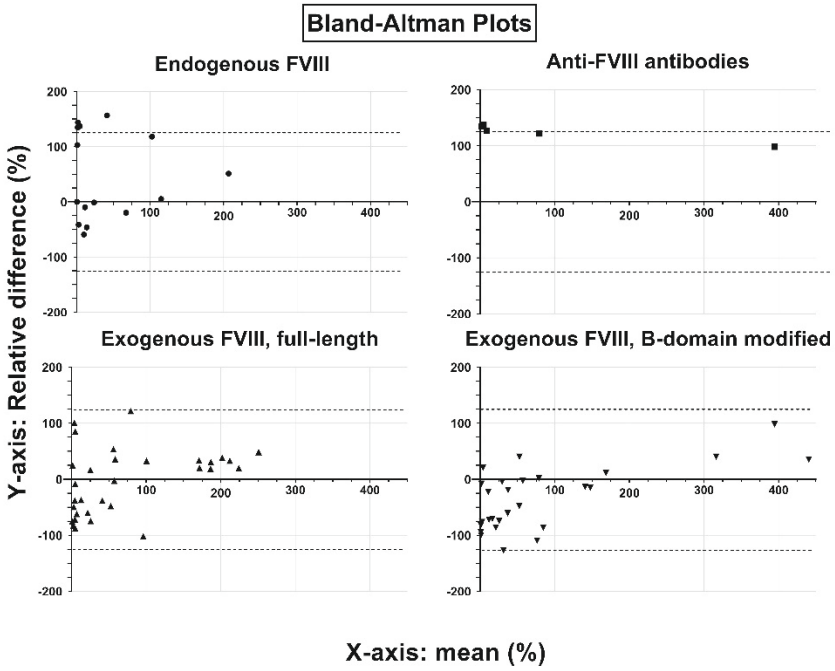

Figure 2. Bland-Altman plots of relative difference compared to mean (FVIII plasma concentration – FVIII activity)/2). Subgroups are illustrated per panel and with different symbols, see subtitles. Overall mean of -1%, limits of agreement are -127% to 125%.

Linear regression analyses demonstrated the correlation between determinants and the relative differences of the results from LC-MS/MS and OSA. Relevant results are demonstrated in *Table 2* and two significant results are illustrated in the boxplots of *Figure 3*. The relative differences of samples with anti-FVIII antibodies (≥0.6 BU/mL, *n* = 5) were 133% (95% CI: 81; 185%) higher compared to the relative differences of samples without anti-FVIII antibodies. The relative differences of samples with exogenous FVIII products (*n* = 58), the 'exposed' group, were -37% (95% CI: -65; -9%) when compared to samples without exogenous FVIII (*n* = 29), the 'unexposed' group. When multivariable linear regression was performed, the found correlation between differences and

exogenous FVIII products was independent of anti-FVIII antibodies and vice versa (both $p < 0.01$).

Table 2. Linear regression analysis on the correlation between the determinant and relative differences.

Univariable linear regression analysis	B% (95% CI)	p-value
Age	-0.3 (-0.9; 0.3)	0.372
Weight	-0.2 (-0.7; 0.3)	0.498
Anti-FVIII antibodies (≥0.6 BU/mL)	132.6 (80.7; 184.5)	<0.001
Exogenous FVIII products (exposed)[a]	-36.7 (-64.8; -8.5)	0.011
Full-length FVIII products[a] - pdFVIII - rFVIII	-29.3 (-60.0; 1.3)	0.060
pdFVIII, Aafact®[a]	-64.1 (-113.8; -14.5)	0.013
Full-length rFVIII[a] - octocog alfa, Advate® - octocog alfa, Helixate®/Kogenate®	-21.2 (-53.4; 11.1)	0.194
B-domain modified rFVIII products[a] - turoctocog alfa, NovoEight® - efmoroctocog alfa, Elocta®	-57.5 (-88.7; -26.4)	<0.001
Efmoroctocog alfa, Elocta®[a]	-76.7 (-136.1; -17.3)	0.013
Turoctocog alfa, NovoEight®[a]	-53.2 (-86.2; -20.2)	0.002
Co-medication		
Desmopressin	9.3 (-45.1; 63.7)	0.735
Tranexamic acid	-8.9 (-52.1; 34.3)	0.683
Heparin	24.9 (-10.3; 60.1)	0.163
Comorbidities		
Any heart disease	24.9 (-29.2; 79.1)	0.363
Hepatitis B or C	-8.9 (-42.2; 24.4)	0.597

[a]reference group is 'unexposed to exogenous FVIII products (*n* = 29)'.
Abbreviations: B: the influence of determinant (slope in linear regression); 95%-CI: 95%-confidence interval; FVIII: factor VIII; pd/rFVIII: plasma-derived/recombinant factor VIII.

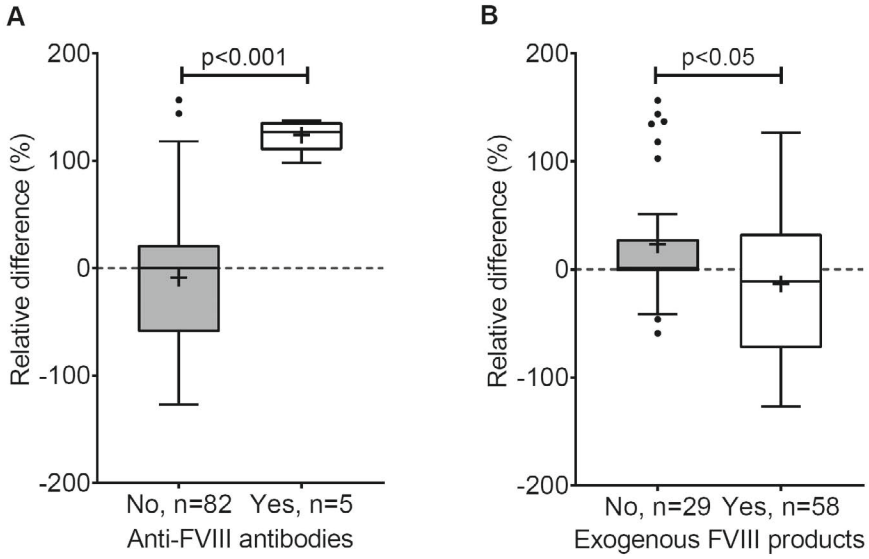

Figure 3. Boxplots comparing the relative differences of the determinant group with the non-determinant group. The '+' illustrates the mean. Boxplot 3A, determinant is anti-FVIII antibodies ≥0.6 BU/mL with a mean relative difference 133% (95% CI 81, 185%). Boxplot 3B, determinant is the exposed group compared to unexposed group with a mean relative difference -37% (95% CI -65, -9%).

Also, specific FVIII product subgroups were compared to the unexposed group. Plasma-derived FVIII product (*n* = 7) had a relative difference of -64% (95% CI: -114; -15%). The two B-domain modified products included in this study were turoctocog alfa (NovoEight® *n* = 22) and efmoroctocog alfa (Elocta® *n* = 5). The relative differences of samples with B-domain modified products (*n* = 27) were -58% (95% CI: -89; -26%) lower compared to the relative differences in the unexposed group. Linear regression was also performed on turoctocog alfa and efmoroctocog alfa separately and significantly demonstrated their relative differences were -53% (95% CI: -86; -20%) and -77% (95% CI: -136; -17%) lower when compared to the relative differences of the unexposed samples. In general, all exogenous FVIII products resulted in negative relative differences (*Table 2*), meaning that the FVIII plasma concentration is lower compared to the FVIII activity.

No significant differences were found for age, body weight, the subgroup full-length FVIII products (*n* = 32), octocog alfa (recombinant full-length FVIII: Advate® *n* = 8; Helixate®/ Kogenate® *n* = 18), co-medications desmopressin (*n* = 6), heparin and low molecular weight heparins (*n* = 16), tranexamic acid (*n* = 10), and co-morbidities heart disease (*n* = 6) or hepatitis B and C (*n* = 19). The determinants gender, other co-medications (such as oral anticoagulants, antiplatelet drugs, immunosuppressive drugs, and corticosteroids), and other co-morbidities (such as HIV, lung disease, hypertension) could not be studied because of low variability and numbers.

DISCUSSION

This study demonstrates that regardless of an overall strong correlation, there is a large variability between FVIII activity measured by OSA and the FVIII plasma concentration measured with LC-MS/MS. Significant differences between the two methods were independently correlated with the presence of anti-FVIII antibodies or use of exogenous FVIII products.

Correlation and variability

To our knowledge, this is the first clinically established comparison between a FVIII activity assay and a FVIII plasma concentration method using LC-MS/MS. A strong correlation was expected, however, we did not expect this large variability. Explanations for the large variability could be (i) the influences of the determinants and (ii) the OSA result variability. We found that the FVIII plasma concentration measurements are evidently higher compared to the FVIII activity in the high measurement range (from >40). As only haemophilia A patients were included, activity results >40 IU/dL (cut-off for diagnosis) could be indicative for the presence of FVIII product. When hypothesizing, a potential false estimate of FVIII activity in this range could result in a misleading drug half-life, which might be interesting to investigate with efficacy outcome measurements in new upcoming research [8]. Another explanation for a higher plasma concentration than activity is that LC-MS/MS method may measure dysfunctional FVIII, with no activity, in so called cross-reacting material (CRM) positive patients [16]. In contrast, in the range of <1–40IU/dL FVIII activity, most measurement points were under the regression line or relative difference line (indicating a higher activity than plasma concentration). In other studies, OSA demonstrated a significantly higher activity than CSA in approximately 30% of the moderate and mild patients with haemophilia A [17, 18]. To prevent misclassification in the diagnostic phase, but especially to prevent underestimation of the bleeding risk, it is preferred to use more than one OSA activity measurement, to combine OSA with CSA, or to identify the FVIII gene mutation [19, 20]. Whether a FVIII plasma concentration measurement is a better representation of the clinical effect than the FVIII biological coagulation activity cannot be demonstrated with the results of this exploratory study. The presented data support the supposed overestimation of OSA in selected samples, which might indicate that LC-MS/MS could be a useful predictor for classification of disease severity and therapeutic monitoring in these specific patients, but this needs to be confirmed with further research.

Determinants

Currently, the major complication in haemophilia A treatment is the development of neutralising anti-FVIII antibodies, rendering endogenous and exogenous FVIII ineffective. Anti-FVIII antibodies neutralise the FVIII activity by forming FVIII-antibody complexes that accelerate clearance of FVIII or by sterically hindering the interaction of FVIII with other coagulation factors [21, 22]. For the samples with anti-FVIII antibodies (in the range

0.8–5.3 BU/mL, *n* = 5) present, we found a substantial higher FVIII plasma concentration than activity. Due to the small sample size of anti-FVIII antibodies in this study, the proportional correlation between antibody level and relative difference could not be confirmed. This result might indicate that for some patients the specific FVIII-antibody complexes are still present in the circulation, not being cleared by the immune system, and that non-functional FVIII is still being measured by the LC-MS/MS method. This phenomenon deserves further clinical exploration, but could not be included in the present study as bleeding data were unavailable for this retrospective study on remnant material.

Another essential finding of this study is the trend seen in exogenous FVIII products of a higher FVIII activity than plasma concentration. This result is consistent with the study of Barrowcliffe *et al.* mentioning an overestimation of FVIII activity by unbound FVIII, since FVIII may become activated during sample collection [23]. Other reasons for overestimation of FVIII activity are that OSA is simply not validated for innovative new products such as shortened (turoctocog alfa) or deleted (efmoroctocog alfa) B-domain modified products or Fc-fusion (also efmoroctocog alfa) products, and also the discrepancy seen in mild and moderate patients with haemophilia A [5, 12, 24]. The LC-MS/MS method is less affected by the new types of FVIII products since the camelid antibodies have a high affinity for the specific FVIII epitope. It should be mentioned that the full-length FVIII product group was on borderline of significance, which might make this group interesting as well to investigate in a study with more power.

Limitations and strengths

The study was limited by the retrospective design and the lack of an appropriate gold standard to compare to the new LC-MS/MS method. Although OSA is a functional assay with result variability, OSA was used as a reference in this study, since it is the most used method worldwide, and corrected for by using relative differences in this study [25]. Bias might have been introduced by including the same patient in another category, but a sensitivity analysis demonstrated no differences in results. We performed this study as a proof of principle experiment to commence validated further clinical research on FVIII quantified with mass spectrometry, therefore undoubtedly making the strength of this study its first clinical contribution.

Recommendations

Further research regarding the quantification of FVIII with OSA, CSA, and LC-MS/MS is indicated and is expected to start at our center to make a well-founded recommendation for the potential correcting steps per different subgroups of patients and products and for correcting steps to simplify the interpretation of the FVIII plasma concentration. A first recommendation for future research would be to conduct a prospective study where the FVIII activity (OSA and CSA) and FVIII plasma concentration (LC-MS/MS) is measured and compared in multiple samples over time, in a curve, after administrating

FVIII product to a haemophilia A patient, or after a high stress test in a volunteer. This would allow investigating the intra-patient variability, potential loss of activity over time and mutated FVIII molecules. Another recommendation for future research is to conduct a study with different types of FVIII products and different batches per FVIII product that are reconstituted, not only with FVIII-deficient plasma (as human material might contain FVIII fragments) but also with solvents (e.g., NaCl 0.9%) to compare OSA, CSA and LC-MS/MS results over time. Since the LC-MS/MS method was development with one FVIII product, the method would benefit from studying multiple product types and batches e.g., to compare different activation rates.

Major advantages of LC-MS/MS measuring is the possibility to elucidate the discrepancies between OSA and CSA in different patient populations, to distinguish between neutralizing and clearing anti-FVIII antibodies, and to determine the FVIII concentration variation in the normal population. Other advantages with more clinical implications for the future, are that LC-MS/MS offers the opportunity to patient-friendly telemonitoring (with dried blood spot) facilitating blood sampling from home and a patient-friendly sampling volume, which could be used in neonatal diagnostic screening as 50µl would suffice. In the future, the possibility of mass spectrometry-based techniques to measure multiple samples in one run could be exploited by the combined measurement of multiple clotting and anticlotting factors, thus constructing a personal haemostasis profile and bleeding score [26].

CONCLUSION

Despite a strong overall correlation between the two methods, the relative differences between measured FVIII activity and FVIII plasma concentration in individual samples were large, especially in case of the presence of anti-FVIII antibodies or use of exogenous FVIII products. These differences may have impact on clinical decision making regarding diagnosing disease severity and monitoring the treatment of FVIII products. Further research is needed to determine the value of FVIII plasma concentration measurements in comparison with the FVIII activity measurements.

Author's contribution

AD designed the study, performed data management, analysis and validation, prepared the first draft of the manuscript, and implemented significant contribution from co-authors up to the final publication. Throughout the process, AD asked and implemented input and feedback from supervision team and co-authors, who performed critical review of the manuscript and provided significant contributions to the study.

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CHAPTER

Quantification of emicizumab by mass spectrometry (LC-MS/MS) in plasma of heamophilia A patients: A method validation study

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Res Pract Thromb Haemost 2022; 6: e12725

ABSTRACT

Introduction

Emicizumab is a new treatment option for patients with haemophilia A. Emicizumab was approved with a body-weight-based dosage regimen, without laboratory monitoring requirements. Guidelines, however, recommend measuring emicizumab concentrations when suspecting the presence of anti-drug antibodies. Furthermore, drug monitoring can be useful in clinical decision-making, in adherence checking, and for research purposes. Therefore we developed a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for quantifying emicizumab. We performed a validation study on this LC-MS/MS method quantifying emicizumab in the plasma of patients with haemophilia A.

Methods

Sample preparation for LC-MS/MS analysis included ammonium sulphate protein precipitation and trypsin digestion. A signature peptide of emicizumab and a matching stable isotope-labeled internal standard were used to quantify emicizumab by LC-MS/MS analysis. Validation was performed in accordance with the 'Guideline on bioanalytical method validation' of the European Medicines Agency (EMA). The LC-MS/MS method was cross-validated against a modified and calibrated (r^2 Diagnostics) one-stage clotting assay (OSA).

Results and Conclusion

The LC-MS/MS method demonstrated linearity over a wide range of emicizumab concentrations, far exceeding the concentrations observed in patients with haemophilia A. Precision and accuracy were excellent and all other validation parameters were also within the acceptance EMA criteria. Cross-validation showed that the LC-MS/MS method and the OSA-based method can be used interchangeably for drug monitoring of emicizumab, without the application of a correction factor.

INTRODUCTION

Haemophilia A is a congenital bleeding disorder resulting from a deficiency or malfunction of coagulation factor VIII (FVIII) [1]. This cofactor is required to bridge activated factor IX (FIXa) and factor X (FX) for adequate hemostasis. The recommended treatment to prevent bleeding for patients with FVIII levels of ≤1 IU/dL is FVIII replacement therapy on a regular basis [2, 3]. Despite its efficacy, prophylaxis is burdensome due to frequent intravenous injections [4, 5]. In addition, a major complication is the formation of anti-FVIII antibodies (called inhibitors), which renders treatment with FVIII products less effective [6].

Emicizumab (ACE910, Hemlibra®; by Roche and Chugai) is the first licensed non-factor replacement product. The European Medicines Agency (EMA) and the US Food and Drug Administration approved emicizumab for the prophylaxis of patients with haemophilia A in 2018 [7, 8]. This recombinant, humanized, and bispecific immunoglobulin G (IgG)-4 antibody binds both FIXa and FX, and mimics the function of activated FVIII in coagulation reactions [9]. The advantages of emicizumab compared with FVIII products are subcutaneous instead of intravenous administration, longer dosing intervals, and lack of interference by anti-FVIII antibodies [10]. Emicizumab has been approved with a body-weight-adjusted regimen without the requirement of drug monitoring [11, 12]. Guidelines recommend, however, measuring the emicizumab plasma concentration when suspecting the presence of anti-drug antibodies (ADAs) against emicizumab [13-17]. In addition, drug monitoring of emicizumab can be useful in clinical decision-making, in detecting lack of adherence, and for research purposes [18, 19].

Consequently, efforts have been made to determine emicizumab concentrations in human plasma. An enzyme-linked immunosorbent assay (ELISA) was used for this purpose in the HAVEN premarket approval studies, but is not commercially available [20-23]. Instead, the manufacturer supplies emicizumab-specific calibrators and controls to use in combination with a modified activated partial thromboplastin time (APTT)-based one-stage clotting assay (OSA), commonly used in a clinical setting [18, 24]. The modified, calibrated OSA (mcOSA) has shown agreement with the non-commercial ELISA [25]. However, disadvantages of the mcOSA are interference by FVIII or by ADAs and its availability at specialized hematologic laboratories [19, 26, 27].

A novel liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for quantification of emicizumab was developed by our research group [28]. The objective was to perform a validation study on this LC-MS/MS method quantifying emicizumab in the plasma of patients with haemophilia A.

MATERIAL AND METHODS

Liquid chromatography-tandem mass spectrometry method

The development of the LC-MS/MS method was reported previously [28]. Here, the protocol and validation procedures are described in detail.

Chemicals and reagents

The vials containing emicizumab (batch # B2002) at a concentration of 150 µg/µL were obtained from F. Hoffmann-La Roche Ltd. (Basel, Switzerland). A stable isotope-labeled (SIL) internal standard (IS) was used to correct for variations during sample preparation and to eliminate the matrix effect. The amino acid sequence of this SIL-IS, matching the signature peptide, was SGGSIYNEEFQD(R*), where (R*) = Arg ($^{13}C_6$,$^{15}N_4$). The SIL-IS was synthesized by and obtained from Pepscan (Lelystad, the Netherlands). The tosyl phenylalanyl chloromethyl ketone (TPCK)-trypsin was supplied by Thermo Scientific (Breda, the Netherlands) as a lyophilized powder and was dissolved in acetic acid (50 mM) to a concentration of 10 mg/mL; aliquots were stored in Eppendorf LoBind® microcentrifuge tubes at -80 °C. The methanol mobile phase solvent (LC-MS grade) and all remaining reagents were obtained from Sigma-Aldrich (Saint Louis, MO, USA).

Standard working solution, calibration standard, internal standard and quality controls

The working emicizumab standard solution was prepared by pipetting 10 µL stock solution of Hemlibra® (150 mg/mL) and 140 µL pooled human plasma in a LoBind® tube (10 mg/mL). Calibration standard solutions with concentrations of 512, 256, 128, 64, 32, 16, 8, and 4 µg/mL were prepared freshly from the working standard solution by serial dilution in pooled human plasma and aliquots were stored at -80 °C. The working IS solution (50 µg/mL) was prepared in tris(hydroxymethyl)aminomethane (Tris) buffer pH 8.5, 100 mM, containing 0.5% octyl glucoside (OG). The following quality control (QC) samples were prepared in pooled human plasma: lower limit of quantification (LLOQ; 4 µg/mL), low (10 µg/mL), medium (200 µg/mL), and high concentration (400 µg/mL). Aliquots of QC samples were stored at -80 °C.

Sample preparation for LC-MS/MS

An ammonium sulphate (AS) protein precipitation method was chosen for simplicity and fast workflow [29]. From the plasma sample, 10 µL was taken and diluted with 5 µL IS solution and 85 µL Tris buffer (50 mM, pH 8, 0.5% OG) in a 1 mL LoBind® 96 Deep-well plate and mixed for 1 min at 1350 rpm. Then, 100 µL saturated AS solution was added to each sample and mixed for 1 min at room temperature at 1350 rpm to precipitate both therapeutic and endogenous immunoglobulins from the plasma samples. The 96-well plate was centrifuged at 4000 G for 5 min to collect the IgG pellet at the bottom. The supernatant containing albumin was decanted, and the pellet was re-dissolved in 50 µL Tris buffer (100 mM, pH 8.5, 6 M guanidine chloride, 20 mM 1,4-dithiothreitol [DTT]).

Then, the 96-well plate was placed in a ThermoMixer at 60 °C, at 1000 rpm for 30 min to denature the proteins and enable the DTT to reduce the disulfide bonds. The thiol groups were alkylated by adding 20 µL iodoacetamide (IAA) solution (100 mM) and placed on the ThermoMixer at 37 °C for 30 min of mixing in the dark. Subsequently, 150 µL ultrapure water was added and mixed for 1 min to dilute guanidine and IAA. After mixing, 400 µL methanol was added to precipitate the IgG fragments, and the 96-well plate was centrifuged at 4000 G for 5 min. The supernatant, containing guanidine and IAA, was decanted. Subsequently, 90 µL Tris buffer (pH 8.5, 50 mM) with 0.5% OG was added to the pellet, followed by addition of 10 µL TPCK-trypsin solution (2 mg/mL). Samples were placed on the ThermoMixer for overnight digestion at 37 °C at 1000 rpm. Trypsin activity was stopped by adding 20 µL 10% formic acid in acetonitrile (pH 3) and centrifugation at 4000 G for 5 min. Finally, a 5 µL sample was injected into the LC-MS/MS system.

Instrumentation and chromatographic conditions

As described previously [28].

Signature peptide selection

The amino acid sequence of emicizumab was obtained from the International Immunogenetics Information System (http://imgt.org). From *in silico* (tryptic) digestion of emicizumab, potential signature peptides within the variable chains with amino acids $6<n<20$, were identified with the Skyline® software (University of Washington, Seattle, WA, USA). These peptides were screened for absence from the human genome using the basic local alignment search tool (Blast) (https://blast.ncbi.nlm.nih.gov/Blast). Finally, the retention time and the signal intensity of peptides were assessed with Skyline®. Three stable, unique signature peptides on the heavy chain were identified: the SGG peptide had the smallest isobaric interferences, a high signal-to-noise ratio and was selected as the quantifier; the remaining QAP and ASG peptides were adequate to function as qualifiers (*Table 1*).

Table 1. Optimized SRM transition information for signature tryptic peptides and SIL-IL of emicizumab.

Signature peptide sequence	Analyte	Function	Precursor (m/z)	Product (m/z)	Product ion	Charge	CE (V)
SGGSIYNEEFQDR	EMI	Quantifier	751.331	1100.46	y_8	1+	23.8
QAPGQGLEWMGDINTR	EMI	Qualifier	886.923	787.375	y_{14}	2+	26.4
ASGYTFTDNNMDWVR	EMI	Qualifier	888.886	1150.50	y_9	1+	28.5
SGGSIYNEEFQDR*[$^{13}C_6$,$^{15}N_4$]	IS	SIL-IS	756.335	1110.47	y_8	1+	23.8

Abbreviations: CE: optimized collision energy; EMI: emicizumab; SIL-IS: stable isotope-labeled internal standard; SRM: selected reaction monitoring.

Analytical validation study

The analytical validation was performed in accordance with the EMA guideline on bioanalytical method validation [30]. The selectivity and matrix effect were investigated

with 12 blank human plasma samples from 12 different individuals. The linearity of the standard curve was assessed with 1/*x* weighting. The within-run and between-run accuracy values and precision were evaluated for the quality control (QC) samples of LLOQ, QC low, QC medium, and QC high, corresponding to concentrations of 4, 10, 200 and 400 µg/mL, respectively. Stability was tested using QC low and high samples in the autosampler (after sample preparation, at 10 °C) and for three freeze (-80 °C)-and-thaw cycles. Samples were analyzed in quintuplicate on three different days.

Cross-validation study

Patient sampling

The cross-validation study had a cross-sectional design and was performed on patients from the Van Creveldkliniek (University Medical Center Utrecht, the Netherlands) in accordance with our local institutional Medical Ethics Review Board-approved, opt-out procedure (study approval number 21-77/C). Patients with haemophilia A received emicizumab loading doses of 3 mg/kg/week for 4 weeks, followed by maintenance doses of 6 mg/kg/4 weeks with varying dosing intervals (from 7 to 28 days) using entire vials according to local clinical protocol [31]. Samples were taken in loading and maintenance phases during clinical visits (usually at week 1, 2, 4, and month 3, then annually) in the period between June 2018 and February 2021. All peripheral blood samples from patients receiving emicizumab were collected through venipuncture in 4.5 mL tubes (BD vacutainer®), containing 1/10 volume of 105 mM trisodium citrate. Plasma samples were prepared from blood samples by two subsequent centrifugation steps at 2000 G for 5 min at room temperature. Samples were aliquoted, stored at -80 °C and analyzed with mcOSA and LC-MS/MS.

Modified and calibrated one-stage clotting assay

The emicizumab concentration was measured with the mcOSA on a Sysmex CS2500, a coagulation analyzer (TOA Medical Electronics Co., Ltd., Hamburg, Germany) with Actin FS APTT reagent (Siemens, Marburg, Germany). Standard dilutions for CS2500 were applied, and were followed by an extra dilution 1:8 with Owren's Veronal Buffer (CA system buffer) to minimize FVIII interference, then FVIII-deficient plasma, ActinFS and $CaCl_2$ were added (Siemens, Marburg, Germany). Emicizumab concentrations were deduced from an emicizumab calibration curve, based on the plasma calibrator (R^2 Diagnostics; catalog #152-401-RUO, 102 µg/mL, LOT #EC0140). The plasma controls (R^2 Diagnostics; catalog #152-401-CE) of Level 1 (26.6 µg/mL, LOT #E10310) and Level 2 (73.4 µg/mL, LOT # E20410) were used as internal quality controls. The calibration curve was linear over a concentration range of 10–200 µg/mL with an R^2 of 0.9985. The within-run and between-run precision (relative standard deviation [RSD], %) of the control samples ranged between 3.5% and 5.7%. The RSDs of the two control samples were similar after four freeze-and-thaw cycles. The lower limit of quantification was 2 µg/mL.

Cross-validation parameters

Plasma samples from patients with haemophilia A were measured with mcOSA and LC-MS/MS. The following EMA criterion for cross-validation was applied "*the difference between the two values obtained should be within 20% of the mean for at least 67% of the repeats*" [30]. Samples with >20% difference were re-analyzed with LC-MS/MS method. Cross-validation results were analyzed with weighted Deming regression and Bland-Altman analysis. The regression was performed with jackknife based calculation of 95% confidence intervals (CI) according to Linnet's method and a Pearson's correlation coefficient, with a validated web-based tool [32]. The Bland-Altman analysis of absolute and relative differences included mean bias (in µg/mL or %, respectively) with standard deviation (SD) and 95% Limits of Agreement (LoA). Relative differences (%) were calculated as:

$$\text{Relative difference (\%)} = \frac{(\text{plasma concentration} - \text{activity})}{((\text{plasma concentration} + \text{activity})/2)} \times 100\%$$

The influence of covariates on absolute differences was assessed with an unpaired Student's t-test (dichotomous). Anti-FVIII antibodies and FVIII in samples were scored based on lab results and reviewing the Electronic Patient Records. Titers of anti-FVIII antibodies were determined when indicated by the local protocol with the Bethesda assay (Nijmegen modified chromogenic assay with bovine reagents) for which the clinical cut-off ≥0.6 Bethesda Units (BU) per milliliter was used [16]. No FVIII activity was measured during emicizumab therapy in our clinic. Statistics were performed in GraphPad Prism (GraphPad Software LLC, Version 8.3.0, San Diego, CA, USA).

DISCUSSION

Analytical validation study

Linearity of the LC-MS/MS method was established from 4 to 512 µg/mL with an R^2 of 0.999. The RSD of the within-run precision ranged from 2.1% to 4.9% and the RSD of the between-run precision ranged from 2.8% to 7.4%. The accuracy (%bias) ranged from -4.1% to 6.1%. All other validation parameters were also well within the acceptance criteria of the EMA guideline (*Table 2*). The validated LLOQ was 4 µg/mL and had a signal-to-noise ratio of 88, which indicated an even lower LLOQ can be achieved. Emicizumab in QC samples was stable during three freeze-and-thaw cycles. The QC low and high samples remained stable after sample preparation for one week at 10 °C.

Table 2. Summary of LC-MS/MS method validation performance[a].

Validation parameter	Sample	Expressed as	Result	Acceptance[b]
Within-run precision	QC LLOQ	RSD (%)	4.9	<20
	QC Low		4.2	<15
	QC Medium		2.4	<15
	QC High		2.1	<15
Between-run precision	QC LLOQ	RSD (%)	7.4	<20
	QC Low		4.5	<15
	QC Medium		2.8	<15
	QC High		3.4	<15
Accuracy	QC LLOQ	Bias (%)	6.1	<20
	QC Low		-4.1	<15
	QC Medium		-3.8	<15
	QC High		1	<15
Selectivity in plasma	Human samples #1–12	Max relative to LLOQ (%)	0.2	<20
LLOQ	LLOQ of 4 µg/mL	Signal/noise	88	>5×
Linearity	Standards 4, 8, 16, 32, 64, 128, 256, 512 µg/mL	R^2	0.999	>0.99
Freeze-and-thaw stability	QC Low	Bias (%)	-4.5	<15
	QC High		4.3	<15
Spiked recovery in plasma	Human samples #1–12 low	Min/max Bias (%)	-1.1/11.8	<15
	Human samples #1–12 high	Min/max Bias (%)	-6.3/1.2	<15
Autosampler stability	Day 1 reinjected after 7 days	Min/max Bias (%)	-9.6/8.8	<15
Carry over	Blank after highest standard	Relative to LLOQ (%)	0.3	<20
Zero sample	Pool human plasma with IS	Relative to LLOQ (%)	0.2	<20

[a]SGG as signature peptide for SRM transition of 751.33 → 1100.46.
[b]In accordance with 'Guideline on bioanalytical method validation' of the European Medicines Agency [29].
Abbreviations: IS: internal standard; LLOQ: lower limit of quantification; max: maximum; min: minimum; RSD: relative standard deviation; QC: quality control.

In addition to the analytical validation results, two plasma control samples from R^2 Diagnostics were measured with the LC-MS/MS method. The assigned values of these controls were 26.6 and 78.3 µg/mL, and LC-MS/MS results were 25.9 and 79.2 µg/mL. Also, a sample with an unknown amount of emicizumab from a pilot External Quality Assessment study of the WFH (UK-NEQAS, Sheffield, UK. sample: WFH EMI 21:01, July 2021) was tested. The LC-MS/MS result was 59.0 µg/mL for a median of 57.5 µg/mL derived from 11 laboratories. Both these findings corroborate the results of the analytical performance of the LC-MS/MS method.

Cross-validation study

A total of 77 samples obtained from 41 patients were used for cross-validation (*Table 3*). Most patients were male with a diagnosis of severe congenital haemophilia A. The mean age at sampling was 28 years (range 0-78 years) and the mean treatment week at sampling was 20 weeks (range 1-133 weeks). The mean plasma concentration of emicizumab measured with LC-MS/MS was 49 µg/mL (range 11–106 µg/mL), and also 49 µg/mL (range 8–104 µg/mL) when measured with mcOSA.

Table 3. Patient characteristics from samples in cross validation.

Total number of patients = 41	**Number of patients**				
Severe congenital HA	38[a]				
Male	40				
Total number of samples = 77	**Number of samples**	**mean**	**min**	**max**	**SD**
Emicizumab concentration (µg/mL)[b]	77	49	11	106	23
Age at sampling (year)	77	28	0	79	26
Treatment week of sampling	77	20	1	133	29
Albumin concentration		42.0	32.1	47.8	4.3
- Measured (g/L)	39				
- Not measured	38				
aFVIII titer		549	0.6	2790	951
- >0.5 BU/mL	19				
- ≤0.5 BU/mL	58				
FVIII in sample					
- Present[c]	19				
- Absent	58				

[a] Remaining patients: one woman with acquired HA (three samples); two men with moderate HA (two samples).
[b] Measured with LC-MS/MS.
[c] FVIII:C was not quantified in presence of emicizumab.
Abbreviations: aFVIII: anti-FVIII antibodies (inhibitors); BU: Bethesda Units; FVIII: coagulation factor VIII; FVIII:C: factor VIII activity; HA: haemophilia A; SD: standard deviation.

The correlation between observations of the emicizumab concentrations measured with mcOSA and the LC-MS/MS method, using weighted Deming regression, is depicted in *Figure 1*. The slope of the regression line was 1.02 (95% CI 0.891–1.144) with an intercept of -1.61 (95% CI -7.18–3.95) (Pearson's r = 0.986). The line of identity, with a regression slope of 1, lies within the 95% CI of the weighted Deming regression line (*Figure 1*).

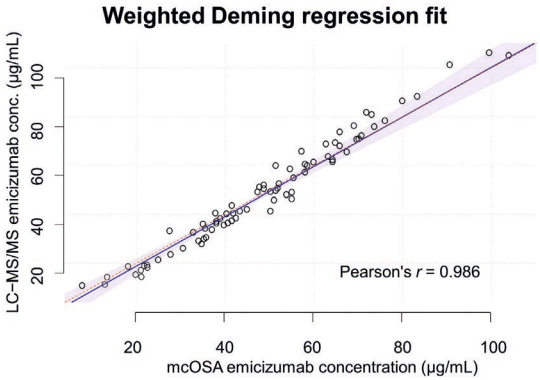

Figure 1. Weighted Deming regression for cross-validation. Emicizumab concentrations using the modified, calibrated one-stage clotting assay (mcOSA) are plotted against emicizumab concentration using liquid chromatography-tandem mass spectrometry (LC-MS/MS) method in patient samples (*n* = 77). Purple line is the regression fit (-1.61 + 1.02*X; Pearson's *r* = 0.986); purple area represents the 95%-confidence interval (jackknife method) of the fit; dashed red line is line of identity.

A Bland–Altman analysis was performed on absolute and relative differences. The absolute differences had a mean bias of 0.03 µg/mL (SD = 4) with 95% LoAs ranging from -9 to 9 µg/mL (*Figure 2A*). No trends or outliers were observed. The relative differences (*Figure 2B*) had a mean bias of 2% (SD of 11), with 95% LoAs from -20 to 25%. The mean difference between methods was <20% in 71 of 77 samples (92%), which is well within the acceptance criterion of >67% of samples. The six samples with >20% difference had a mean emicizumab concentration ranging between 4 and 35 µg/mL ; the mean absolute difference of these six samples was 3.9 µg/mL. Four of six samples retained a difference of >20% after re-analysis with LC-MS/MS.

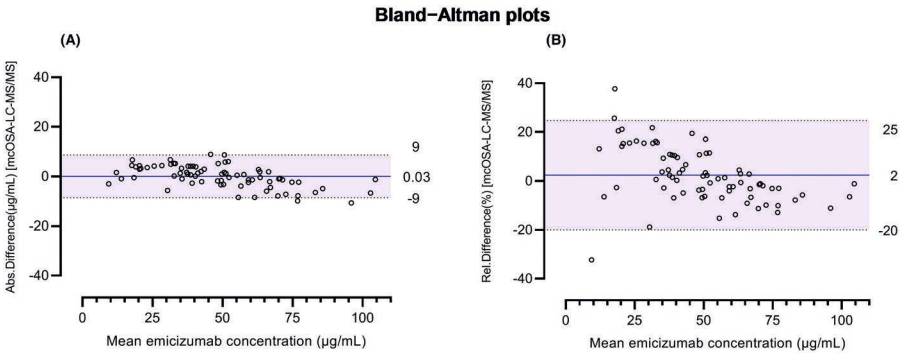

Figure 2. Bland–Altman difference plots for cross-validation. Absolute (A) and relative (B) differences in emicizumab concentrations obtained by modified, calibrated one-stage clotting assay (mcOSA) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) method in patient samples (*n* = 77) were plotted against the corresponding mean emicizumab concentration (µg/mL). Straight line is mean bias; purple area (between the dotted lines) represents 95%-limits of agreement.

The influence of anti-FVIII antibodies and FVIII on the emicizumab concentration differences obtained by both methods was assessed. The absolute differences were similar (p = 0.30) for samples in presence (n = 19) and absence (n = 58) of anti-FVIII antibodies. The absolute differences were also similar (p = 0.17) for samples in presence (n = 19) and absence (n = 58) of FVIII. The presence of both covariates resulted in minor increases of mcOSA results (positive absolute differences), but these were neither statistically significant nor clinically relevant. This is in line with reported spike experiments; despite a 1:80 dilution, mcOSA remains sensitive to the presence of replacement FVIII or endogenous FVIII and cannot be made completely specific to emicizumab by using higher dilutions [33]. Especially in a clinical setting, patients receive large amounts of FVIII products during bleeding episodes or peri-operative periods during emicizumab therapy [34]. Fortunately, the LC-MS/MS method is unaffected by FVIII interference owing to its principle, which is one of the strengths of this method.

The ideal comparator for the LC-MS/MS method would be a total ELISA; that is, an ELISA with a preceding dissociation step to release the drug from any other potential binding target. This classical cross-validation approach cannot be applied in our study, because the sole existing ELISA, which was used in the HAVEN studies [20-23], detects only the free, dual-binding competent drug and cannot detect emicizumab in complex with either FIXa or FX. Nevertheless, a cross-validation can still be of value to determine whether the data obtained are reliable and can be compared between laboratories. As the ELISA from the HAVEN studies was not commercially available, the LC-MS/MS method was compared with the standard mcOSA. The principle of this type of OSA-based assay relies upon measuring emicizumab activity as a factor VIII mimetic and is based on clotting (enzymatic) reactions in FVIII-deficient plasma [24]. In contrast, the principle of the LC-MS/MS method relies upon measuring the exact amount of a signature peptide of emicizumab per sample using SIL-IS for quantification. These different principles explain the slight negative trend in absolute differences above 50 µg/mL (*Figure 2A*). Despite the fundamental differences, a very strong correlation between the methods was found. The 95% CI of the intercept contained 'zero' in the weighted Deming regression fit, and the 95% CI of the slope contained 'one' (*Figure 1*). The relative differences were well within the EMA's acceptance criteria for cross-validation (*Figure 2B*). Therefore, the application of a correction factor for interchangeable method use is not required.

The six samples with relative difference of >20% had emicizumab concentrations below 34 µg/mL. These six samples were obtained during the loading phase, because concentrations in maintenance phase range between 38 and 67 µg/mL [11, 35]. While relative differences are of analytical importance, they have low clinical value. The absolute differences were small, especially from a clinicians' perspective, and relevant outliers or trends were lacking. The EMA criterion was evidently met, making this finding not clinically relevant.

Strengths and limitations

This is the first report on clinical use of an LC-MS/MS method quantifying emicizumab in plasma, further building on our previous work measuring FVIII in plasma with LC-MS/MS [36, 37]. The strengths of this LC-MS/MS method over the mcOSA method are the lack of interference, a high-throughput and easy-to-implement design, and the opportunity to multiplex with other therapeutic monoclonal antibodies. In addition, the sampling volume for this LC-MS/MS method is only 0.25 mL (minimal required volume of tube), which is particularly beneficial to the pediatric population. Furthermore, the LC-MS/MS-based methods have become the standard for measuring drug concentrations in clinical laboratories worldwide [38]; making this method for emicizumab quantification accessible to routine practice.

Another form of assay-interference might result from the formation of ADAs against emicizumab. This immune response generally enhances drug clearance and removal from the circulation but might also form neutralized emicizumab–ADA complexes that remain in the circulation [39, 40]. These neutralized complexes could potentially lead to falsely high emicizumab concentrations using the LC-MS/MS method. The occurrence of such complexes remaining in the circulation has rarely been reported for therapeutic monoclonal antibodies and has not been reported for emicizumab as well. Unfortunately, no robust assays for ADA detection or neutralized complexes are commercially available. The presence of ADAs in our study samples is, however, highly unlikely, as it is extremely rare (reported incidence of <0.8% [41, 42]) and the clinical response of our patients was excellent [31]. This validation study was not powered for the development of emicizumab–ADA. Therefore, future studies should further investigate the impact of potential interference by this phenomenon, especially for the mcOSA and the ELISA, and to demonstrate the complementary role of LC-MS/MS.

A limitation of the LC-MS/MS method is the sample preparation time of 24 hours, due to the overnight trypsin digestion step, and an analysis run-time of 13 min per sample. Fast drug monitoring of emicizumab is not required according to clinical guidelines, but might be supportive in an acute bleeding setting [15, 34]. Consequently, the work-flow may need to be optimized.

In conclusion, the LC-MS/MS method for the quantification of emicizumab in the plasma of patients with haemophilia A was performed successfully in this validation study. The strong correlation between the current reference method and the LC-MS/MS method allows interchangeable use. This LC-MS/MS method can be implemented for drug monitoring of emicizumab.

Author's contribution

AD designed the study, assisted in analytical analysis, performed data management and analysis, prepared the first draft of the manuscript, and implemented significant

contribution from co-authors up to the final publication. Throughout the process, AD asked and implemented input and feedback from supervision team and co-authors, who performed critical review of the manuscript and provided significant contributions to the study.

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CHAPTER

Pharmacokinetics and associated efficacy of emicizumab in humans: A systematic review

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Clin Pharmacokinet 2021; 60: 1395-1406

ABSTRACT

Introduction

Emicizumab is an effective, new treatment option for people with haemophilia A (PwHA). The approved dosing regimens are based on body weight without the necessity for laboratory monitoring. This assumes a clear dose-concentration-response relationship, with acceptable variability due to factors other than body weight. To investigate this assumption, a systematic review on the pharmacokinetics (PK) and associated efficacy of emicizumab in humans was conducted.

Methods

The databases Embase, Pubmed and CENTRAL were systematically searched up to November 2020 to identify studies on PK data of emicizumab in humans. Data on the study, population, PK and efficacy (annualized bleeding rate of treated [joint] bleeds) were extracted and synthesized, and exposure effects modelling was performed using non-linear least squares regression in a maximum effect (E_{max}) model.

Results

The 15 studies included reported data for 140 volunteers and 467 PwHA, including children (0 to <12 year), adolescents and adults (≥12 year), both with and without factor VIII (FVIII) inhibitors. Emicizumab demonstrated dose-linear PK. The inter-individual variability of trough concentrations was moderate (32%) and similar across various subgroups, such as FVIII inhibitor status, age group and dosing interval. The control of bleeds did not further improve above emicizumab concentrations of 30 µg/mL, potentially enabling lower dosing in a substantial proportion of PwHA.

Conclusion

This review supports body-weight-based dosing, although individualized monitoring of emicizumab concentrations may allow for more cost-effective dosing.

INTRODUCTION

Haemophilia A is an inherited bleeding disorder caused by an absence or dysfunction of coagulation factor VIII (FVIII) [1]. Without adequate treatment, people with haemophilia A (PwHA) who are severely affected suffer from recurrent bleeds, predominantly in joints, which results in crippling arthropathy, functional limitations and a significantly reduced life expectancy [2, 3]. The primary goal in the management of these PwHA is to prevent bleeds, preferably through regular coagulation factor replacement therapy (prophylaxis) [1, 4]. Prophylaxis with plasma-derived and, later, recombinant FVIII products has effectively reduced episodes of bleeding from an annual average of 20–30 to 1–4 [5-8]. However, replacement therapy with FVIII products has some disadvantages. This treatment is invasive requiring intravenous administration every 24–48 hours, usually starting before the age of 2 years [9]. Additionally, neutralizing antibodies against FVIII (known as inhibitors) develop in 30% of severely affected PwHA, rendering treatment with FVIII products ineffective [4, 10].

Emicizumab (Hemlibra®) is the first non-factor replacement product and was approved in 2018 by the U.S. Food and Drug Administration and the European Medicines Agency as prophylaxis for PwHA, both with and without FVIII inhibitors. This humanized, IgG4, bispecific monoclonal antibody (mAb) effectively restores the hemostatic function of missing FVIII by bridging activated factor IX and factor X. Moreover, the subcutaneous administration and the less frequent dosing interval of once per 1–4 weeks offer dosing convenience especially for (pediatric) PwHA with difficult venous access. Emicizumab has limited toxicity, although concomitant use of high doses activated prothrombin complex concentrate increases the thrombotic risk, and should be avoided [11, 12].

A body-weight-based standard dosing regimen for emicizumab (1.5 mg/kg per week, 3 mg/kg per two weeks, 6 mg/kg per four weeks) without the requirement of dose adjustments based on laboratory monitoring has been approved by the regulatory authorities [13]. This dosing recommendation assumes clear dose–concentration (pharmacokinetics [PK]) and concentration–response (pharmacodynamics [PD]) relationships with acceptable variability due to factors other than body weight. Unexpected variability (e.g., resulting from anti-drug antibodies or population characteristics) should be absent, as differences in concentration and even response require monitoring and individualized dose tailoring [14, 15]. Therefore, the objective of this study was to investigate these basic principles by conducting a systematic review on the PK and associated efficacy of emicizumab in humans.

METHODS

Sources

The literature search was conducted in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines [16]. The databases of Embase, PubMed and Cochrane Central Register of controlled trials (CENTRAL) were searched from the start of these databases through April 28th 2020, and was updated on November 13th 2020. The search terms included 'emicizumab' and 'pharmacokinetics' with their associated synonyms; see Supplemental Data SD1 for an example of the search algorithm of Embase. In the Embase database, the search was conducted using the corresponding EMtree terms, and for PubMed and CENTRAL databases the National Library of Medicine (MeSH) search terms were used. Finally, the included studies were manually searched for relevant references and the European Public Assessment Report (EPAR) of emicizumab was used as a cross-reference for possible missed studies [12].

Study selection

The search results from the three databases were merged in Endnote X9 (Clarivate Analytics, Version 19.3.3.13966), and duplicate records (title, abstract or both) were removed automatically. Hereafter, the records were imported into the web-based tool Rayyan (https://rayyan.qcri.org/[17]) and were screened and categorized. First, the records and, secondly, the full-text articles were screened and categorized in duplicate by two reviewers (AD, LB). The following inclusion criteria were applied: emicizumab studies providing (i) data on humans, (ii) original PK data or modelled PK data or PK–PD relationships, and (iii) access to the abstract and to the full text in English. In the event of doubt about eligibility, the records or articles were included. Disagreements were discussed until consensus was reached, and when necessary a third reviewer (TE) was consulted.

Data extraction

The following data were extracted from the included studies: (i) study characteristics (authors, year of publication, number of subjects, phase, design, dosing regimen, follow-up, funding); (ii) population characteristics (population [volunteer/PwHA], anti-FVIII antibodies, age group, haemophilia A severity, ethnicity); and (iii) evaluated PK and efficacy data. Relevant primary PK parameters included the absorption rate constant (K_a), apparent clearance (CL/F), and apparent volume of distribution (V_d/F); and secondary (exposure) PK parameters included terminal half-life ($t_{1/2}$), the area under the plasma concentration–time curve extrapolated to infinity (AUC_{inf}), maximum plasma concentration (C_{max}), trough plasma concentration in steady-state conditions ($C_{trough,ss}$) and time to reach maximum plasma concentration (t_{max}). The relevant efficacy parameter was expressed as the annualized bleeding rate (ABR) of different bleeding types (i.e., treated or treated joint bleeds). When relevant data were missing from text, a data request was sent to the corresponding authors or sponsors.

Trough concentrations with corresponding errors (standard deviation [SD] and 95% confidence interval [95%-CI]) from steady-state conditions per study subgroup were primarily extracted from text. However, when data were presented in graphs only and the data requests remained unanswered, the data were extracted from the graphs [18-20]. To minimize bias, first, two independent and blinded reviewers (AD, LB) visually scored the data with magnification and a set square. Secondly, the data were scored using WebPlotDigitizer, a reliable and validated web-based tool for extracting underlying numerical data from plots (https://automeris.io/WebPlotDigitizer [21]) [22]. Both the visually and digitally extracted values were compared and if necessary reassessed. The data were entered (LB/AD) into a database for systematic data extraction and were double-checked by a second reviewer (AD/LB).

Data synthesis

Extracted data relating to study and population characteristics were categorized and summarized. The PK data ($C_{trough,ss}$) were plotted according to doses of the multiple-injection regimens in PwHA and for the single-injection regimens in volunteers (primary and secondary parameters). The coefficient of variation (%CV, or the variability) of trough concentrations was calculated as (SD/mean)×100%. If unavailable, the SD was derived from the 95%−CI using the formula √n×((upper limit − lower limit)/3.92), or from the interquartile range (IQR) using (IQR_{max}–IQR_{min})/1.35 [23].

Exposure effects modelling was conducted with weighted (study size) non-linear least squares regression using a standard inhibitory maximum effect (E_{max}) model. The ABRs of treated bleeds and treated joint bleeds were used as efficacy parameters if calculated by means of negative binominal regression (model-based). As data for the extremes of the curve were lacking, values for baseline ABR and E_{max}, derived from other sources, were assumed and the Hill coefficient was fixed to 1. The baseline ABRs for treated bleeds and treated joint bleeds were assumed at values of 28.0 and 21.6, respectively, as reported for severe PwHA treated with on demand-therapy in a real-world setting [6]. These values were in line with the reported baseline ABRs for treated bleeds of 21.9, 23.3 and 38.2, and for treated joint bleeds of 6.7 and 26.5 in severe PwHA without prophylactic treatment [18, 19, 24]. The E_{max} for treated bleeds was set at a value of 0.96 (i.e., 96% drug effect at infinite exposure, corresponding to an ABR of 1) and for treated joint bleeds the E_{max} was set at 0.98 (corresponding to an ABR of 0.5). This was done to account for the occurrence of traumatic bleeding (based on clinical experience) and the lack of complete of coagulation by emicizumab (based on mouse and primate models). In addition, the FVIII-equivalent (hemostatic) activity of emicizumab was hypothesized at only 10–20% in humans [24-26]. An ABR of 1 was, therefore, perceived as more realistic than an ABR of 0, and is also in line with the reported ABRs range of 0.2–5.1 (Supplemental Table ST2). The ABR baseline values were also used to calculate the Relative Risk Reduction (RRR). Sensitivity analysis was performed to assess validity of the ABR baseline and E_{max} assumptions.

Summary statistics and graphics were performed using GraphPad Prism (GraphPad Software LLC, Version 8.3.0), and modelling was performed using R (R Core Team, version 4.0.2).

RESULTS

A total of 368 records were identified with the computerized search. After duplicate removal (*n* = 83), 285 titles and abstracts and 77 full-text articles were assessed for eligibility. The search update in November 2020 yielded 15 studies meeting the inclusion criteria (Supplemental Figure SF1).

Study characteristics

The 15 included studies were published between 2016 and 2020 in *Table 1*. During the drugs' development, phase I (*n* = 4), simultaneous phase I/II (*n* = 1) and phase III (*n* = 6) clinical studies were conducted in combination with two pharmacometric analyses reporting on the dose–response relationship with data from the previously published studies. Two studies were conducted after market approval. The majority of the studies included were global multi-center (*n* = 8), open-label (*n* = 12), non-randomized (*n* = 8), non-controlled (*n* = 9), industry-funded (*n* = 13) clinical studies with a follow-up period of at least 16 weeks (*n* = 11). Eight studies had a parallel comparative design, including six reporting on an intra-individual comparison of emicizumab with previous treatment. The multiple-dose injection studies most often included the weekly dosing interval (QW; 75%), followed by the once per four weeks regimen (Q4W; 14%) and once per two weeks regimen (Q2W; 11%).

Table 1. Study characteristics. In order of study phase.

1st Author (study name)	Year of publication	*n* =	Study phase	Study country	Multi-center	Open-label	Random-ized	Con-trolled	Parallel comparison	Study design	Population	FVIII inhibitor	Dosing regimen	Follow-up period	Industry funded[a]
Uchida N.	2016	64	I	Japan	-	-	+	+	-	RCT, dose-escalation	Volunteer	NA	SSI	4, 16, 20 or 24 weeks	+
Shima M.	2016	18	I	Japan	+	+	-	-	+, Intra-individual	CT, dose-escalation	PwHA	+, -	QW	12 weeks	+
Shima M.	2017	16	I/II	Japan	+	+	-	-	+, Intra-individual	CT, consecutive study	PwHA	+, -	QW	Until median >2 year	+
Kotani N.	2019	60	I	Japan	-	+	+	-	+	RCT	Volunteer	NA	SSI, SII	16 weeks	+
Li H.	2020	16	I	China	-	+	-	-	-	CT	Volunteer	NA	SSI	16 weeks	+
Yoneyama K.	2018	60	NA	NA	NA	NA	NA	NA	NA	PK Analysis	PwHA, Volunteer	+, -	SSI, QW	NA	+
Oldenburg J. (HAVEN 1)	2017	109	III	Global	+	+	+	+	+, Intra-individual	RCT	PwHA	+	QW	≥24 weeks	+
Schmitt C. (HAVEN 1)	2020	112	III	Global	+	+	+	+	+, Intra-individual	RCT, consecutive study	PwHA	+	QW	61 weeks (median)	+
Young G. (HAVEN 2)	2019	88	III	Global	+	+	-	-	+, Intra-individual	CT	PwHA, child	+	QW, Q2W, Q4W	≥52 weeks	+
Mahlangu J. (HAVEN 3)	2018	152	III	Global	+	+	+	+	+, Intra-individual	RCT	PwHA	-	QW, Q2W	≥24 weeks	+
Pipe S.W. (HAVEN 4)	2019	48	III	Global	+	+	-	-	-	CT, two-stage	PwHA	+, -	Q4W	≥24 weeks	+
Shima M. (HOHOEMI)	2019	13	III	Japan	+	+	-	-	+	CT	PwHA, child	-	Q2W, Q4W	≥24 weeks	+

Table 1. Continued.

1st Author (study name)	Year of publication	*n* =	Study phase	Study country	Multi-center	Open-label	Random-ized	Con-trolled	Parallel comparison	Study design	Population	FVIII inhibitor	Dosing regimen	Follow-up period	Industry funded[a]
Retout S.	2020	389	NA	NA	NA	NA	NA	NA	NA	PK Analysis	PwHA	+, -	QW, Q2W, Q4W	NA	+
Hooimeijer H.L.	2020	1	IV	Nether-lands	-	NA	NA	NA	NA	Case report	PwHA	+	QW, once every 10 days	1 year	-
Barg A.A.	2020	40	IV	Israel	-	NA	NA	NA	NA	Prospective cohort study	PwHA, child	+, -	QW	45 weeks (median)	-, conflict of interest

[a]Industry-funded by F. Hoffman-La Roche and/or Chugai Pharmaceutical.
Abbreviations: (R)CT: (randomized) clinical trial; n: number of subjects; NA: not applicable; PK: pharmacokinetic; PwHA: people with haemophilia A; QW: weekly dose interval; Q2W: two weekly dose interval; Q4W: four weekly dose interval; SSI: single subcutaneous injection; SII: single intravenous injection.

Population characteristics

Eleven studies [18, 19, 27-34] provided original PK data of emicizumab in humans and yielded 607 subjects (*Table 2*) after excluding four studies [24, 35-37] with double-reported subjects. These 607 unique subjects were either volunteers (*n* = 140) or PwHA (*n* = 467). The PwHA included adults and adolescents (*n* = 328) and children younger than 12 years (*n* = 139). The PwHA with and without FVIII inhibitors were similarly represented across studies. Severe haemophilia A and the Asian and Caucasian races were predominant across the studies.

Table 2. Population characteristics. Eleven studies providing original PK data on emicizumab in unique subjects.

	Total = 607 (*n* [%])
Population	
Volunteer	140 (23%)
Haemophilia A	467 (77%)
FVIII inhibitor	234 (39%)
Age group	
Child with haemophilia A (<12 years)	139 (23%)
Adult or adolescent (≥12 years)	468 (77%)
Volunteer	140 (23%)
Haemophilia A	328 (39%)
Haemophilia A severity	
Severe	436 (75%)
Moderate	6 (1%)
Mild	5 (1%)

Dose-concentration relationship

The original PK data were used to investigate the dose–concentration relationship, therefore excluding two pharmacometric analysis studies (re-used data), two preliminary studies (shorter follow-up period than their consecutive studies) and a case report (no steady state conditions). The PK data from the PwHA (*n* = 469) demonstrated a linear dose–concentration relationship when the $C_{trough,ss}$ was plotted according to increasing doses of emicizumab per week (*Figure 1*). The longer dose intervals (Q2W and Q4W) were associated with a lower $C_{trough,ss}$.

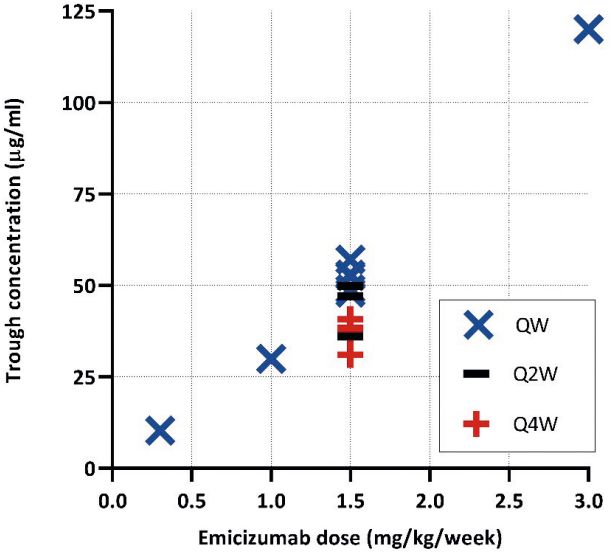

Figure 1. Linear dose–concentration relationship of emicizumab in PwHA. The mean or median $C_{trough,ss}$ according to increasing doses of emicizumab per week (mg/kg/week). Intervals Q2W and Q4W shown per week. Data from PwHA (*n* = 469) receiving multiple doses regimens were included (see 'a' for 15 study subgroups in Supplemental Table ST2).

The PK data from the volunteers (*n* = 112) in the single-dose injection studies with increasing doses (0.1, 0.3 and 1 mg/kg), demonstrated constant primary PK parameters and a dose-linear increase in the exposure metrics C_{max} and AUC_{inf}, with an exponential decay over time. These PK parameters obtained per study subgroup are presented in Supplemental Figure SF2 and Supplemental Table ST1 and were similar across ethnicities.

The study size weighted variability (%CV) of the $C_{trough,ss}$ was similar across FVIII inhibitor status (present or absent) and the various dosing intervals (QW, Q2W or Q4W), whereas children had slightly less variability than adults and adolescents (*Figure 2)*. The overall weighted %CV of the $C_{trough,ss}$ in PwHA was 32% and ranged from 17% to 44% (Supplemental Table ST2).

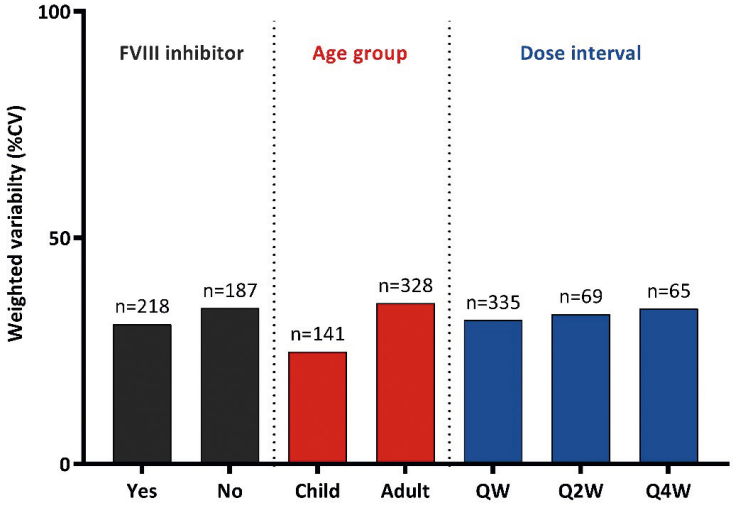

Figure 2. Trough concentrations of emicizumab with variability across various subgroups in PwHA. The weighted variability (%CV) of the $C_{trough,ss}$ of emicizumab was similar across FVIII inhibitor status and across various dosing intervals, whereas children had slightly less variability than adults/adolescents. The overall weighted %CV was 32%. Data from PwHA (*n* = 469) receiving multiple dose regimens were included (see 'a' for 15 study subgroups in Supplemental Table ST2).

Concentration–response relationship

Table 3 shows the relevant parameter estimates from the published population PK models on emicizumab. The model by Yoneyama *et al.* was based on PK data from the phase I and I/II studies to establish the dosing regimens, and the model by Retout *et al.* was based on the long-term phase I/II and the phase III HAVEN 1–4 studies [24, 36]. The total PK inter-individual variability (approximately 60% [36]) could be explained primarily by the covariates body weight (BW), neutralizing anti-drug antibodies (ADA) against emicizumab, age >30 years and, to a lesser extent, abnormal albumin (ALB) levels and African race.

Table 3. Summary of parameter estimates from published population PK models.

Study	Population	Parameter estimates	Inter-individual variability
Yoneyama *et al.* (2018)[a]	Japanese volunteers (*n* = 24) Caucasian volunteers (*n* = 18) Dose finding study in PwHA - 0.3mg/kg QW (*n* = 6) - 1 mg/kg QW (*n* = 6) - 3 mg/kg QW (*n* = 6)	PK parameters: Cl/*F* 0.222 L/day V_d/F 10.2 L $t_{1/2,\ abs}$ 1.56 days $t_{1/2,eli}$ 31.8 days for volunteer $t_{1/2,eli}$ 30.1 days for PwHA Response parameters: λ 21.9 events/year EC_{50} 1.19 µg/mL	Variance: Cl/*F* 0.0737 V_d/F 0.0455 $t_{1/2,\ abs}$ 0.502
Retout *et al.* (2020)[b]	Dose finding study in PwHA (*n* = 16) Phase III trials in PwHA with approved dosing regimens - HAVEN 1 (*n* = 112) - HAVEN 2 (*n* = 63) - HAVEN 3 (*n* = 148) - HAVEN 4 (*n* = 48)	PK parameters: Cl/*F* 0.272 L/day[b] V_d/F 10.4 L[b] K_A 0.536 1/day[b] $t_{1/2,\ abs}$ 1.61 days $t_{1/2,eli}$ 26.8 days	Variability (%): Cl/*F* 28.7 V_d/F 25.9 K_A 72.5

[a]Standardized for a volunteer of 70 kg, without anti-drug antibodies.
[b]Standardized for a subject of 70 kg, albumin 45 g/L, age <30 years.
Abbreviations: PK: pharmacokinetic, PwHA: people with haemophilia A; QW: weekly dose interval; CL/F: apparent clearance; V_d/F: apparent volume of distribution; EC_{50}: half maximal effective concentration; K_a: absorption rate constant; $t_{½,abs}$: terminal half-life of absorption; and $t_{½,abs}$: terminal half-life of elimination.

Eleven PwHA study subgroups within five studies [18-20,31,32], provided the model-based mean ABRs of treated bleeds. The $C_{trough,ss}$ was the only exposure metric that could be extracted in combination with these ABRs. The ABRs were fitted with corresponding $C_{trough,ss}$ values in an E_{max} model (*Figure 3)*. The EC_{50} (i.e., 50% of maximum drug effect) was estimated at 1.47 µg/mL (standard error [SE] 0.90) assuming ABR baseline of 28.0 and E_{max} of 0.96. The effectiveness plateau of the concentration–response relationship was clearly established, and all ABR observations resided herein. Based on this model, a $C_{trough,ss}$ of 30 and 50 µg/mL would result in ABRs of treated bleeds of 2.4 and 1.9, respectively, and with an RRR of 91% and 93%, respectively. A second E_{max} model was fitted with the ABRs of treated joint bleeds, instead of treated bleeds, with corresponding $C_{trough,ss}$ values (Supplemental Figure SF3). The EC_{50} was estimated at 1.09 µg/mL (SE 0.36, assuming baseline ABR 21.6 and E_{max} 0.98, and $C_{trough,ss}$ values of 30 and 50 µg/mL would result in ABRs of treated joint bleeds of 1.1 and 1.0, respectively, and with an RRR of 94.9% and 95.4%, respectively.

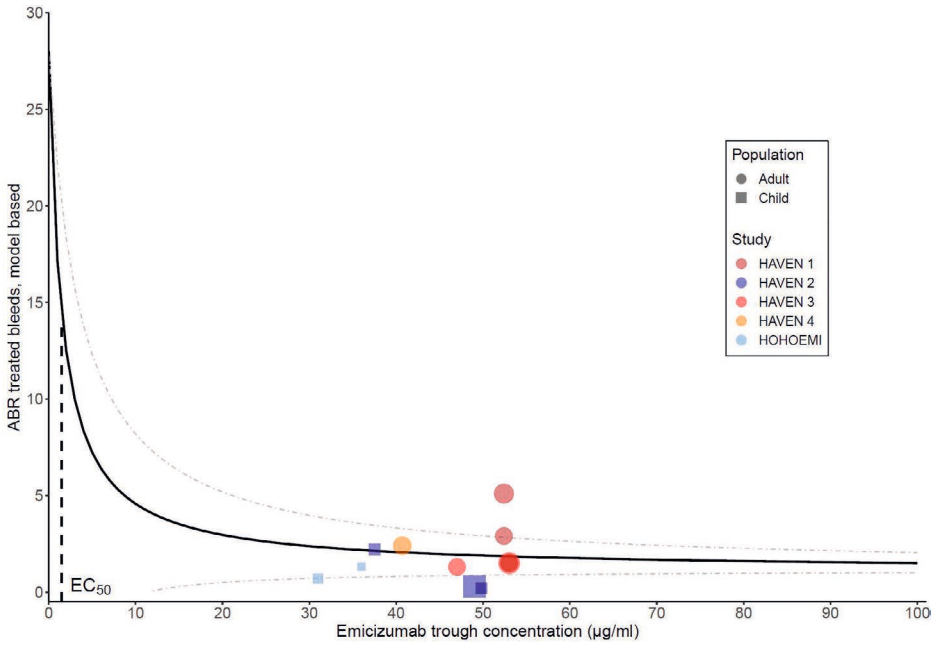

Figure 3. E_{max} model with concentration-response relationship of emicizumab in PwHA. Fit of an E_{max} model of ABRs of treated bleeds according to $C_{trough,ss}$ of emicizumab in PwHA (*n* = 349), described by $ABR = ABR_{baseline} * (1 - (\frac{(C_{trough,ss} * E_{max})}{(C_{trough,ss} + EC_{50})})$. Included ABRs were model-based, estimated using negative binominal regression. The EC_{50} was estimated at 1.47 µg/mL (SE 0.90) and effectiveness plateau was established. Dashed line is 95%-confidence interval and symbol size equals study size. Data from PwHA receiving maintenance were included, see 'b' for 11 study subgroups in Supplemental Table ST2.

For $C_{trough,ss}$ of 30 and 50 µg/mL, sensitivity analyses showed ABRs of treated bleeds of 2.9 and 1.9, respectively, with a conservative setting (baseline ABR 21.9 and E_{max} 1 [24]) and ABRs of respectively 1.9 and 1.9, respectively, with a more liberal setting (baseline ABR 38.2 and E_{max} 0.90 [18]), see Supplemental Table ST3. In addition, for ABRs of treated joint bleeds of respectively 1.4 and 0.9 with a conservative setting (baseline ABR 6.7 and E_{max} 1 [18]) and ABRs of respectively 1.0 and 0.9 with a more liberal setting (baseline ABR 26.5 and E_{max} 0.97 [19]). $ABR_{baseline}$

DISCUSSION

For emicizumab in humans, this systematic review demonstrated a linear dose–concentration relationship with moderate inter-individual variability, with all ABR observations residing in the effectiveness plateau of this concentration–response relationship.

Dose-concentration relationship

With increasing doses of emicizumab, the primary PK parameters remained constant, while the exposure metrics exhibited a linear dose increase. These findings are in line with the results of the model by Retout *et al.*, who showed a dose-proportional AUC increase due to constant clearance [36]. Compared to the primary PK parameters of other therapeutic IgG4-based mAbs, the clearance of emicizumab was comparable (within 0.2–0.5 L/day), but distribution volumes were increased (10 vs 6 L) and, consequently, the reported half-life of emicizumab is longer [38]. This higher volume of distribution is notable, indicating distribution to other compartments or binding of emicizumab, possibly to its targets in the blood circulation. In general, therapeutic mAbs demonstrate linear PK at high doses when the target-saturated concentration is achieved [39, 40], and this was observed already at doses of ≥ 0.1 mg/kg for emicizumab (Supplemental Figure SF2).

The sources for variability of the PK parameters were explored in the two published population PK modelling studies, in which BW and neutralizing ADA against emicizumab were identified as influential covariates [24, 36]. A body-weight-based dosing regimen was justified, because including BW in the model reduced the inter-individual variability in CL/F from 56.4% to 30.0% and on V/F from 60.7% to 28.1% [36]. Additionally, Retout *et al.* reported that age and albumin concentration were significantly correlated to primary PK parameters in their model. For age >30 years, bioavailability gradually decreased, and, for age >65 years, bioavailability strongly decreased (e.g., a 31% lower exposure for a PwHA aged 77 years than for PwHA aged 30 years), which is uncommon for therapeutic mAbs [41]. Although the authors did not report a relationship with clinical response, PwHA older than 65 years may be more susceptible to lower emicizumab concentrations, potentially even reducing bleeding control in a small proportion of PwHA. A low albumin concentration of 33 g/L was associated with a 16% decrease in exposure [36].

The PK variability across several studies was described in modelling studies before, but this review is the first to investigate variability across seven studies. We have reported slightly higher %CV for adults and adolescents compared to children. This finding is in line with the considerable influence of the covariate 'age' on exposure identified by Retout *et al.* [36]. Therefore, we recommend to investigate the bioavailability and exposure in this patient subgroup (i.e., aged >30 years, especially >65 years) in future studies.

Concentration–response relationship

Our search did not yield individual patient data, making it unfeasible to use a complex model, such as repeated-time-to-event. However, the estimated EC_{50} of 1.47 µg/mL for treated bleeds was close to the previously reported values of 1.19 µg/mL [24] and 3.58 µg/mL [42] obtained by more sophisticated models, endorsing our model. The $C_{trough,ss}$ of 51.1 µg/mL obtained by the approved standard dosing regimens is 35-fold this EC_{50}, which is abundant [13]. Our E_{max} model visualized the concentration–response relationship and revealed a clear effectiveness plateau from 30 µg/mL, beyond which no further increase in beneficial drug effect was achieved. All the bleeding rate observations of the included studies were well within this plateau, potentially enabling lower dosing for a substantial proportion of the PwHA.

Unquestionably, setting other values for E_{max} and baseline ABRs would result in other predictions. A sensitivity analyses was performed (see Supplemental Table ST3) to show the impact of the ABR baseline and E_{max} assumptions on the estimates of EC_{50} and the ABRs for $C_{trough,ss}$ of 30 µg/mL and 50 µg/mL. In this regard the estimates remained approximately the same, indicating robustness. Also, the EC_{50} decreased fivefold when the ABRs (outliers) from HAVEN 1 were excluded from the model. We hypothesizes that many subjects from this study had long-term inhibitors and by definition had severe arthropathy, which is something to keep in mind for physicians. The ABR of treated joint bleeds was explored as well, because treated joint bleeds are generally better defined, reducing misclassification [43-46]. In the E_{max} model of treated joint bleeds, the effectiveness plateau was reached at even lower concentrations of 20 µg/mL.

Dosing and monitoring considerations

The question that remains is to what extent are we overdosing our PwHA? Based on our E_{max} models, a $C_{trough,ss}$ of 30 and 50 µg/mL would result in ABRs of treated bleeds of 2.4 and 1.9, respectively (RRRs of 91% and 93%, respectively) and ABRs of treated joint bleeds of 1.1 and 1.0, respectively (RRRs of 94.9% and 95.4%, respectively). Are the theoretic ABR differences of 0.5 and 0.1 clinically relevant while the RRRs remain essentially equal? Understandably, manufacturers design dosing regimens without laboratory monitoring, because this is user-friendly and robust. Moderate inter-individual variability in PK without monitoring necessitates higher dosing to guarantee efficacy for all users. In combination with an absence of toxicity at high drug concentrations, which is the case for most therapeutic mAbs, this makes overdosing clinically not problematic. Nonetheless, concerns were expressed by Hooimeijer *et al.*, who reported joint pain episodes in a PwHA at high emicizumab concentrations of 90 µg/mL, which resolved after dose reduction to concentrations of approximately 30 µg/mL [33]. Furthermore, when drugs are costly (the wholesale acquisition cost of emicizumab is approximately US$482,000 for the first year of treatment and US$448,000 for following years), monitoring and subsequent dose reduction may lead to substantial savings in health care [47]. The weighted overall variability of the $C_{trough,ss}$ was 32%, which is common

for therapeutic mAbs. Notably, individual $C_{trough,ss}$ ranged widely from 2.8 to 148 µg/mL [18]. No specific loss of efficacy nor side effects for these extreme individual cases were described. As more studies presented subjects with plasma concentrations <20 µg/mL (Supplemental Table ST2), we propose a beneficial role for Therapeutic Drug Monitoring to increase the dose to an efficacious concentration. In addition, we recommend to conduct a prospective, individual PK-guided dosing study to target an efficacious trough concentration of 30 µg/mL and to collect data on both inter-individual variability and inter-occasion variability in the real world setting to optimize efficacy, safety and cost-effectiveness of emicizumab treatment.

Laboratory monitoring with PD biomarkers has been unsuccessful, and functional assays should be interpreted with caution and as relative indications of the procoagulant potential [34, 48]. Until proper functional tests become available, the emicizumab concentration appears to be the best predictor for bleeding risk [24]. The concentration is not routinely monitored in clinical practice, although monitoring may be useful for research purposes, to check adherence, or in case of suspected neutralizing ADA against emicizumab [49]. The emicizumab concentration was measured in PK samples during phase I–III clinical studies using an enzyme-linked immunosorbent assay (ELISA) and during phase IV studies using a modified, calibrated one-stage clotting assay (OSA). Unfortunately, Roche has not provided access to the ELISA while the OSA is available only at specialized laboratories, as it needs to be calibrated by a standardized kit of two reference values [50]. An assay capable of measuring emicizumab concentrations in human plasma on routinely available platforms, such as liquid chromatography-tandem mass spectrometry (LC-MS/MS), would be valuable. An LC-MS/MS method for the quantification of emicizumab has been developed and validated at the University Medical Center Utrecht [51].

Limitations & Strengths

This systematic review was limited by the number of available studies, the inclusion of funded studies, a heterogeneous population, limited study sizes and lack of studies with a blinded, placebo-controlled, head-to-head design owing to the rarity and severity of haemophilia A. There is some uncertainty in our findings, particularly in the estimated ABRs at $C_{trough,ss}$ of 30 µg/mL and 50 µg/mL, as a consequence of limited availability of data at lower and higher emicizumab concentrations. A search in the ClinicalTrials.gov database in January 2021 identified at least three relevant ongoing studies on the PK of emicizumab (i.e., HAVEN 5–7), of which two are still recruiting PwHA. Therefore, it is certain that the present review will require an update in the near future. Another difficulty was the risk of bias assessment in the studies included due to a lack of standardized tools for such PK studies, in contrast to the quality assessment tools used for (randomized) clinical studies. However, the data used were considered methodologically sound, as PK parameters are objective measurements, studies were included using a systematic PRISMA search approach, and we only used for the E_{max} model negative binomial mean

ABRs (excluding the median ABRs [28, 34, 35]) and excluded the studies with baseline imbalances [28, 35].

The ABR is not an entirely objective outcome parameter. Misinterpretation of bleeds might have occurred, as verification by the physician and complementary imaging were often missing. Subjective assessments, combined with follow-up periods of <12 months and small study sizes, may have affected the calculated ABRs. Moreover, clinically unstable disease leads to numerous (spontaneous) bleeds, especially in the first weeks of emicizumab treatment, leading to overestimation of ABRs in shorter studies [43-46]. Recently, the analysis of pooled bleeding data from HAVEN 1–4 reported ABRs maintaining <1 in 24-week intervals and an increase in the proportion of PwHA without treated bleeds from 70.8% in the first 6 months to 80.2% after one year of emicizumab treatment [52]. Consequently, predicted ABRs may be overestimated in our model.

The strength of this review was the large amount of information that has been summarized in tables and graphs. The novelty of this review was the critical appraisal by an independent research group, the dosing and monitoring considerations and the proposed role for TDM in relation to low concentrations and cost-effectiveness. This information may offer guidance in clinical decision-making and in future study designs assessing (cost-)effectiveness, safety and PK/PD modelling studies [53].

CONCLUSION

This systematic review provided a comprehensive overview of PK and associated efficacy data for emicizumab in humans. Emicizumab demonstrated a clear linear dose–concentration profile with moderate inter-individual variability. The control of bleeds did not further improve above emicizumab concentrations of 30 µg/mL, potentially enabling lower dosing in a substantial proportion of PwHA. In conclusion, this review supports body-weight-based dosing, although individualized monitoring of emicizumab concentrations may allow for more cost-effective dosing.

Author's contribution

AD designed the study, performed data management, conducted data curation, analysis, and validation, prepared the first draft of the manuscript, and implemented significant contribution from co-authors up to the final publication. Throughout the process, AD asked and implemented input and feedback from supervision team and co-authors, who performed critical review of the manuscript and provided significant contributions to the study.

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CHAPTER

The efficacy of the entire-vial dosing of emicizumab: Real-world evidence on plasma concentrations, bleeds and drug waste

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Res Pract Thromb Haemost 2023; 7: e100074

ABSTRACT

Introduction

Prophylaxis with emicizumab provides effective bleeding protection in persons with haemophilia A (PwHA) but pressures healthcare budgets. The body-weight adjusted dosing at 7-, 14- or 28-day intervals, according to the label, often mismatches the vial content. Entire-vial dosing resulted in therapeutic concentrations according to pharmacokinetic simulations and was introduced to avoid waste. The aim was to evaluate the efficacy of entire-vial dosing of emicizumab by investigating real-world evidence of plasma concentrations, bleeds and drug waste.

Methods

Single-center, observational study with PwHA receiving emicizumab in mg/kg doses according to label but dosing interval extrapolated to nearest vial size. Patient characteristics and bleeds were compared one year before starting emicizumab, and during emicizumab until January 2022. Concentrations were assessed at Weeks 4, 12, and annually. The mean (95%-confidence interval [CI]) annualized bleed rates (ABR) were compared using negative binomial regression. Drug waste between label-based dosing and entire-vial dosing was compared.

Results

A total of 112 individuals (94% severe phenotype and 9% positive FVIII inhibitors) were followed for a median of 56 (interquartile range [IQR] 52–68) weeks before and 51 (IQR 29–75) weeks after starting emicizumab. The median emicizumab dose was 5.9 (IQR 5.5–6.2) mg/kg/4 weeks with median concentrations of 63 (IQR 51–80) µg/mL. The ABR of treated bleeds before emicizumab was 3.6 (95%-CI 2.9–4.4) and was 0.8 (95%-CI 0.6–1.1) during emicizumab (p-value < 0.001). Drug waste was reduced by 9%.

Conclusion

The entire-vial dosing of emicizumab is an attractive treatment option for PwHA leading to therapeutic plasma concentrations, good bleeding control and drug waste avoidance.

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INTRODUCTION

Emicizumab (Hemlibra®) prophylaxis provides effective bleeding prevention in persons with haemophilia A (PwHA) [1]. This humanized, bispecific FVIII-mimicking antibody was approved by the European and U.S. regulatory authorities for PwHA with and without FVIII inhibitors in 2018. The advantages of using emicizumab over the traditional factor VIII (FVIII) concentrates are subcutaneous administration instead of intravenous administration, longer dosing intervals with more continuous bleeding protection and no interference with FVIII inhibitors [2]. Emicizumab reduces the treatment burden for PwHA on prophylaxis, especially for those with FVIII inhibitors on bypassing agents (BPA) or with difficult venous access, and may enhance treatment adherence. Although many PwHA are candidates for emicizumab therapy, access to this therapy is limited due to the financial impact on healthcare budget of hospitals [3-5].

Emicizumab is available as injection vials for single use in four different vial sizes: 30 mg/1.0 mL, 60 mg/0.4 mL, 105 mg/0.7 mL and 150 mg/1.0 mL [6]. The maintenance dosage regimens, according to the drug label, are 1.5 mg/kg weekly, 3 mg/kg every 2 weeks or 6 mg/kg every 4 weeks (i.e., the dose per administration varies with body weight, but the dosing intervals are fixed). Given an individual's weight, the dose is unlikely to exactly match the content of the vial size suggested by the online HEMLIBRA® calculator that is provided by the manufacturer, which often forces the prescribers to either overdose or discard the unused remainder of a vial. This introduces the risk of administration errors and leads to expensive drug waste, which are two topics of concern in the haemophilia community and society in general [7].

Entire-vial-based dosing could be used to tackle these two issues. While maintaining the mg/kg dose according to the registered label, the prescriber could extrapolate the dosing interval to the nearest vial size. For example, a PwHA with a body weight of 13 kg who receives 39 mg every 14 days according to the drug label (discarding 21 mg of a 60 mg/0.4mL-vial), could instead be given 60 mg every 21 days according to the entire-vial dosing. Until now, no studies on the efficacy outcomes of entire-vial dosing for PwHA in daily clinical practice were reported. Entire-vial dosing can be justified by the long elimination half-life of emicizumab (i.e., ~30 days), and the linear relationship across the three available dosing regimens suggests that alternative dosing combinations will result in similar plasma concentrations [7]. Furthermore, entire-vial dosing has been suggested to result in therapeutic plasma concentrations (~55 µg/mL) in two reports on pharmacokinetic modelling simulations [8, 9]. Therefore, we introduced entire-vial dosing to PwHA who receive emicizumab therapy in our clinic. The objective was to evaluate the efficacy of the entire-vial dosing of emicizumab by investigating real-world evidence of the plasma concentrations, bleeds and drug waste.

MATERIALS AND METHODS

Design and setting

This single-center, retrospective observational study on prospectively registered data was conducted at the Van Creveldkliniek, University Medical Center in Utrecht, the Netherlands. All PwHA (adults and children) who started emicizumab between July 2018 and January 26th 2022 were eligible. The inclusion criteria were a diagnosis of congenital haemophilia A, at least one plasma emicizumab concentration measurement available and no objection against the usage of clinical data for research in the Electronic Health Record.

The PwHA were switched from prophylaxis or on-demand therapy with either FVIII concentrates (standard half-life [SHL] or extended half-life [EHL]) or bypassing agents (BPA; i.e., activated factor VII [rFVIIa] or activated prothrombin concentrate complex [aPCC]) to emicizumab therapy during a regular medical visit. All PwHA received emicizumab loading doses of 3 mg/kg per week for 4 consecutive weeks according to the drug label. Subsequently, the maintenance dose (≥28 days after the first loading dose) was an equivalent of the registered dose of 6 mg/kg/4 weeks, but with varying dosing intervals that were rounded to the highest frequency and the nearest vial size [8, 9]. These dosing intervals ranged between 7 and 28 days and were based on shared decision-making between the PwHA and their own treating clinician. Previous prophylaxis was continued for 1 week during the emicizumab loading phase, except for individuals with inhibitors or frequent bleeding, who then continued their regular prophylaxis for 2 weeks after starting emicizumab. The PwHA were instructed to contact the center in case of suspected bleeds. Bleeding episodes were treated with regular doses of FVIII or rFVIIa. This study was evaluated and approved by the Medical Ethics Review Board of UMC Utrecht with study number 21/825. Individual informed consent was waived.

Variable and outcome analyses

The data sources were health diaries, telephone calls to attending clinicians and clinical visits. The data on outcomes and variables were prospectively registered in the Electronic Health Records and extracted by performing retrospective chart reviews. The data were collected preferably 1 year (but at least 12 weeks) before starting with emicizumab therapy until the study's end date of January 26th, 2022 (see *Figure 1* for a schematic study timeline per individual).

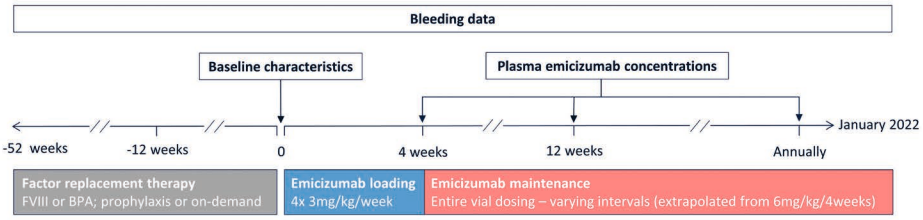

Figure 1. Schematic study timeline per individual.

Baseline characteristics

The following baseline characteristics were collected: haemophilia A severity, FVIII inhibitor status, age, weight, Body Mass Index (BMI), previous factor-replacement regimen and emicizumab regimen. The haemophilia severity was classified on endogenous FVIII activity as severe (<1%), moderate (1–5%), or mild (>5–40%) [10]. The FVIII inhibitor status was classified as present if the inhibitor titre at baseline was ≥0.3 BU/mL. All maintenance doses of emicizumab were converted to a 4-week dosing frequency for comparison (i.e., each mg/kg dose was divided by the dosing interval and multiplied by 28 days).

Plasma concentrations of emicizumab

According to the local protocol, plasma samples for emicizumab concentration measurements were usually assessed before the 4th loading dose (i.e., after receiving 3 loading doses), at 3 and 12 months after starting emicizumab and at least once a year thereafter. All concentrations were combined with monitoring of complete blood count and renal function.

A (cross-)validated liquid chromatography-tandem mass spectrometry (LC-MS/MS) method was used to quantify the concentration of emicizumab in human plasma [11, 12]. The plasma concentration-time curve was fitted using a weighted nonlinear least squares regression with a one-phase association model, and the fit was presented with 90%-prediction bands of points. The between- and within-individual variabilities of plasma emicizumab concentrations during the maintenance phase (defined from ≥28 days) were expressed as the percentage coefficient of variation (%CV), which was calculated as (SD/mean) × 100%. The within-individual variability was calculated for both ≥2 and ≥3 concentrations per individual without dose changes during the studied period.

Bleeds

The PwHA were monitored closely over time and it was mandatory to contact a 24/7-available attending clinician at any suspicion of bleeding. During those calls or visits, the need for FVIII/BPA treatment was evaluated and all cases of suspected joint- or muscle bleeds were evaluated at the clinic. Adverse events (e.g., thrombotic) were

documented in patient files as well. Bleeding events were defined according to the definitions of the ISTH as pain and/or swelling, only bleeds treated with additional FVIII/BPA were considered in this study [13]. Bleeds were classified as all, joint and/or muscle bleeds. The mean annualized bleeding rate (ABR) and the mean annualized joint bleeding rate (AJBR) with 95% CI were modelled by using a negative binomial regression with a log link to account for variations in follow-up times and the skewness of bleeding data. The ABRs before and during emicizumab therapy were compared using this regression model.

The proportion of PwHA with 0 bleeds, 1–3 bleeds and >3 bleeds during the 24-week intervals of 0–24, 25–48, 49–72 and 73–96 weeks after starting emicizumab were estimated to enable a comparison with the data from the HAVEN studies [1]. The individuals were only included in a 24-week interval if they completed the entire interval.

Drug waste

The drug waste (i.e., the difference between the prescribed dose and the dispensed drug in the vial(s)) was calculated by subtracting the label-based dose from the vial size suggestion, which were both obtained by entering every individual's body weight in the HEMLIBRA® Dosing Calculator (http://www.hemlibra-hcp.com/dosing-administration/dosing-calculator). This drug waste was expressed as a proportion, or was calculated as waste during follow-up or as annualized waste. For individuals with a maintenance-dosing interval of 21 days, the mean of the label-based doses at 14- and 28-day intervals was used. The closest label-based interval was taken for other alternative intervals.

Statistical methods

The continuous variables were presented as means with standard deviations (SD) if they were normally distributed or as medians with interquartile ranges (IQR) and categorical variables were presented as individual counts with percentages. The last emicizumab concentration that was observed for each individual during maintenance therapy (steady state condition) and the ABRs were compared across subgroups using respectively non-parametric tests and multivariable regressions. Subgroups included age categories (adults/adolescents [≥12 years] versus children [<12 years]), dosing interval categories (label-based [7, 14, 28 days] versus alternative dosing intervals), BMI categories (BMI <18 versus 18–25 versus >25 kg/m^2), FVIII inhibitor status at baseline (present versus absent) and adherence categories (adherent versus non-adherent). For this last category, the individuals who self-reported skipping injections of emicizumab repeatedly were defined as non-adherent. When self-reported, non-adherence was quantitated by comparing the pharmacy expenditure records with prescribed dose [14]. Additionally, the ABRs and AJBRs were compared across the subgroups of concentrations (<40 versus 40–80 versus >80 µg/mL). These concentration subgroups were based on the mean ±standard deviation of 6mg/kg every 4 weeks (highest peak-trough fluctuation), as stated in the drug label [6].

Two-tailed *p*-values of less than 0.05 were considered statistically significant. Statistical analyses were performed using IBM SPSS Statistics (IBM Corp. Released 2019. IBM SPSS Statistics for Windows, Version 26.0. Armonk, NY: IBM Corp), and graphics were made using GraphPad Prism (GraphPad Software LLC, Version 8.3.0, San Diego, CA, USA).

RESULTS

Baseline characteristics

A total of 115 individuals treated with emicizumab were studied from July 2018 to January 2022. Three individuals were excluded, including one individual with type III Von Willebrand Disease and two individuals with acquired haemophilia A. The baseline characteristics are presented in *Table 1*; 112 PwHA were included in the studied cohort. Most (94%) of these PwHA had severe haemophilia A, 10 (9%) had FVIII inhibitors at baseline, and the median age was 24 (IQR 10–49) years. The cohort consisted of 80 adults/adolescents and 32 children, baseline characteristics according to age subgroup are presented in Supplemental Table ST1. Ten children started emicizumab therapy before the age of 3, including five previously untreated patients (PUPs), who started with emicizumab prophylaxis before FVIII exposure.

Table 1. Baseline characteristics (*n* = 112)

	median (IQR) or *n* (%)
Haemophilia A severity, *n*	
- Severe	105 (94%)
- Moderate	5 (4%)
- Mild	2 (2%)
FVIII inhibitor present, *n*	10 (9%)
Age in median years	24 (10–49)
Body weight in median kg	70 (34–89)
BMI in median kg/m^2	24 (17–27)
Previous therapy, *n*	
PwHA without inhibitors:	
- FVIII-SHL prophylaxis	50 (45%)
- FVIII-EHL prophylaxis	47 (42%)
- FVIII-SHL and -EHL on demand	5 (4%)
PwHA with inhibitors:	
- rFVIIa prophylaxis	1 (1%)
- aPCC prophylaxis	2 (2%)
- rFVIIa and aPCC on demand	2 (2%)
Previously untreated persons:	5 (4%)

Abbreviations: aPCC: activated prothrombin complex concentrate; BU: Bethesda Units; EHL: extended half-life; FVIII: factor VIII; IQR: interquartile range; n: number; PwHA: persons with haemophilia A; SHL: standard half-life; rFVIIa: recombinant, activated factor VII; U: dosage units.

The median follow-up time before starting emicizumab was 56 (IQR 52–68 and range 12–166) weeks per individual. The individuals had a follow-up time of at least 1 year, except for four children who were less than a year old, who had 12–15 weeks, one 3-year-old child with 36 weeks, and one adolescent (age 14) with 47 weeks of follow-up time. Most PwHA (*n* = 97, 87%) were on FVIII prophylaxis before starting emicizumab therapy. The median prophylactic dose of the FVIII-SHL products was 44 units/kg/week and was higher for the FVIII-EHL products at 60 units/kg/week. Most of the adults received FVIII-SHL prophylaxis, while most of the children received FVIII-EHL prophylaxis; five were PUPs, as demonstrated in Supplemental Table ST1.

Emicizumab therapy

The reasons for switching from the previous treatment to emicizumab were breakthrough bleeds/ineffectiveness (*n* = 34, 30%), difficult venous access (*n* = 34, 30%), user-friendliness/individual preference (*n* = 17, 15%), non-adherence (*n* = 12, 11%) or FVIII-inhibitor development (*n* = 10, 9%), additionally five individuals (4%) were PUPs. The reasons to start emicizumab according to age subgroup are represented in Supplement Table ST2.

The median follow-up time during emicizumab therapy was 51 (IQR 29–75 and range 1–190) weeks. The median dose of emicizumab at the initiation of the maintenance phase was 5.9 (IQR 5.5–6.2) mg/kg/4 weeks with dosing intervals ranging between 7 and 28 days, as demonstrated in *Figure 2*. While most adult/adolescent PwHA (*n* = 74) were treated with the registered dosing intervals of 7 or 14 days with entire-vial dosing, most children (*n* = 17) had alternative dosing intervals, usually 21 days (*n* = 12, 38%). Six adolescents self-reported non-adherence to emicizumab treatment; their median age was 17 (IQR 15–20 and range 13–24) years. Their emicizumab consumption was only 53% (range 31–81%) of the prescribed amount.

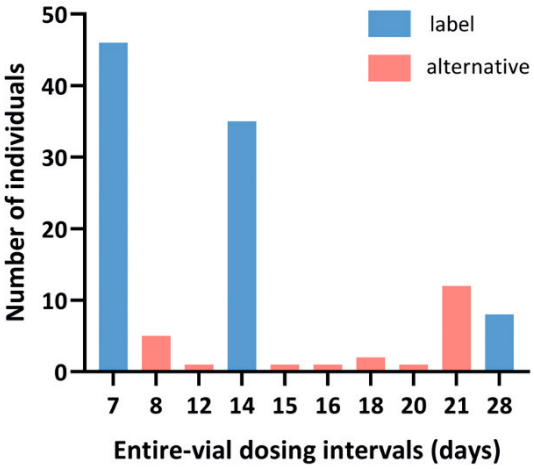

Figure 2. The number of individuals per entire-vial dosing interval of the initial maintenance dose of emicizumab (*n* = 112).

Plasma concentrations of emicizumab

A total of 264 plasma concentrations of emicizumab during the loading phase (*n* = 90) and the maintenance phase (*n* = 174) were available. These emicizumab concentrations according to the time after the first injection with emicizumab are presented in *Figure 3*. The concentrations from the six (self-reported) non-adherent individuals (red squares) were comparable to those from the adherent individuals (blue points) during the loading phase, but their concentrations declined during the maintenance phase. In the adherent individuals only (88 of 106 had a concentration during maintenance phase available), the between-individual variability (%CV) increased with time and was 38%, whereas the within-individual variability was 15% for individuals with at least two concentrations (*n* = 42) and was 22% for individuals with at least three concentrations (*n* = 15).

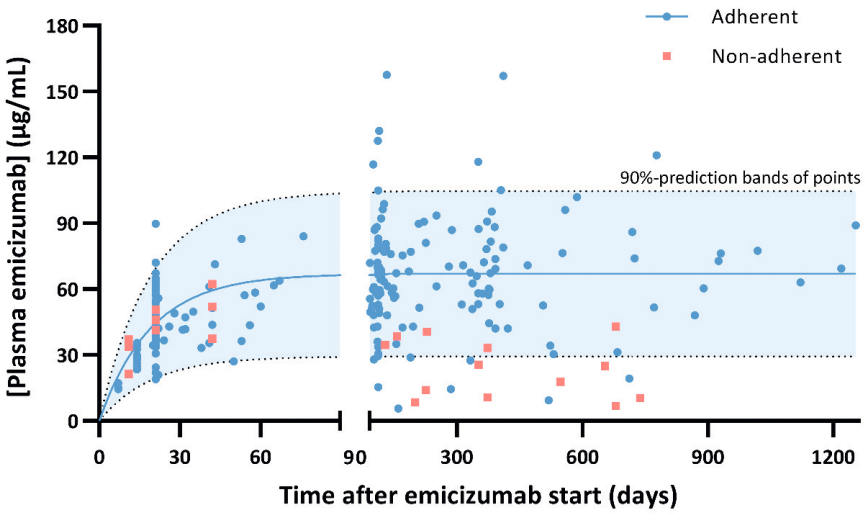

Figure 3. Plasma concentrations of emicizumab according to the time since starting emicizumab with adherence.

The subgroup analyses on the last observed concentration per individual in the maintenance phase demonstrated a significant difference in the adherence subgroups: non-adherent individuals (*n* = 6) had a median concentration of 18 (IQR 8–30) µg/mL, while adherent individuals (*n* = 88) had a median concentration of 63 (IQR 51–80) µg/mL (*p*-value <0.001), which included eight individuals (9%) with last concentrations of <40 µg/mL. Therefore, the concentrations from non-adherent individuals were excluded from further analyses of the age, dosing interval and BMI subgroups. The adults/adolescents had median concentrations of 69 (IQR 51–87) µg/mL, while the children had median concentrations of 58 (IQR 51–66) µg/mL (*p*-value 0.017). The concentrations were similar across the dosing interval and BMI subgroups, as presented in Supplemental Table ST3.

Bleeds

The data on treated bleeds with an overall follow-up in of 131 person-years before and 96 person-years during the emicizumab therapy are summarized in *Table 2*. The total number of treated bleeds observed decreased from 442 before to 74 during the emicizumab therapy, with a concomitant significant reduction of ABRs from 3.6 (95% CI 2.9–4.4), to 0.8 (95% CI 0.6–1.1) (*p*-value <0.001). The number of treated joint bleeds decreased from 271 before to 34 during the emicizumab therapy, with a concomitant significant reduction of AJBRs from 2.2 (95% CI 1.7–2.7) to 0.4 (95% CI 0.2–0.6) (*p*-value <0.001). The number of treated muscle bleeds decreased from 72 before to 13 during the emicizumab therapy. As presented in *Table 2*, the bleed rates before emicizumab were higher in adults/adolescents than in children. Concomitantly, the reduction in bleed rates after switching to emicizumab therapy appeared more pronounced in adults (ABR 80%, AJBR 86%) than in children (ABR 70%, AJBR 50%).

Table 2. Bleeds before and during emicizumab therapy (n = 112) according to age subgroups.

	All (n = 112)		Adults/adolescents (n = 80)		Children (n = 32)	
	Before	**During**	**Before**	**During**	**Before**	**During**
Total follow-up in person-years	131	96	81	67	37	29
Follow-up in median (IQR) weeks	56 (52–68)	39 (19–58)	53 (52–64)	37 (17–56)	67 (53–77)	50 (29–63)
Total number of bleeds	442	74	360	55	82	19
Mean ABR (95% CI)	3.6 (2.9–4.4)	0.8 (0.6–1.1)	4.0 (3.2–5.3)	0.8 (0.6–1.2)	2.3 (1.5–3.5)	0.7 (0.4–1.2)
Total number of joint bleeds	271	34	257	29	14	5
Mean AJBR (95% CI)	2.2 (1.8–2.8)	0.4 (0.2–0.6)	2.9 (2.3–3.9)	0.4 (0.3–0.7)	0.4 (0.2–0.7)	0.2 (0.1–0.5)
Total number of muscle bleeds	72	13	57	11	15	2

Notes: Only treated bleeds were considered.
Abbreviations: ABR: annualized bleed rate; AJBR: annualized joint bleed rate; CI: confidence interval; IQR: interquartile range.

To allow comparison with previous reports, the proportion of individuals without (joint) bleeds during emicizumab were calculated in 24-week intervals. A total of 78 PwHA had a follow-up time of ≥24 weeks after starting emicizumab therapy (i.e., the first 24-week interval), of which the proportion without any bleeds was 73.1%; the proportion with 1–3 bleeds was 24.4%, and the proportion with >3 bleeds was 2.6% (see Supplemental Figure SF1). Ten of the total 32 bleeds (31%) in the first 24-week interval occurred during the loading phase. Only two individuals had >3 bleeds during this first 24-week interval: the first one had five bleeds during the loading phase and one during the maintenance phase, and the second individual had four bleeds during the maintenance phase. The zero-bleed proportion of all individuals was 75.0%, 73.3% and 80.0%, respectively, in the consecutive 24-week intervals. After the first 24-week interval, the proportion with >3 bleeds remained at 0% for all the consecutive 24-week intervals.

Due to pain experienced during infusion of emicizumab, most children (*n* = 21, 66%) used a local anaesthetic cream (EMLA®) prior to the injection. Except for local pain, no adverse events of changes in the complete blood count and the renal function, nor thromboses or losses of response were observed.

As presented in *Figure 4*, bleed rates (*p*-values 0.997 and 0.863) and joint bleed rates (*p*-values 0.354 and 0.148) were similar across the concentration subgroups of <40, 40–80 and >80 µg/mL. Additionally, similar bleed rates were observed across the adherence, age, dosing interval, BMI and FVIII inhibitors subgroups, which are presented in Supplemental Table ST3.

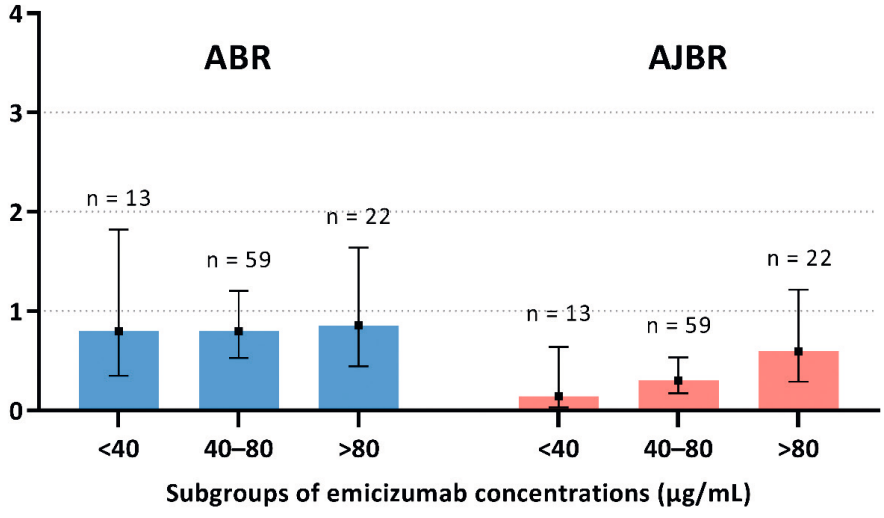

Figure 4. The mean annualized bleed rates (ABRs) and annualized joint bleed rates (AJBRs) (95% CI) of only treated bleeds across the subgroups of the plasma concentration of emicizumab.

Drug waste

Compared to label-based dosing, the total drug waste avoided was 28,533 mg of emicizumab in the period studied, corresponding to a mean annualized drug waste of ~260 mg per adult/adolescent or ~200 mg per child. The mean relative drug waste was 9% per individual with a minimum of 0% and a maximum of 40%. The 18 individuals with a drug waste of ≥15% had a median body weight of 16 (13–23) kg, and median age of 4 (IQR 2–7) years. These individuals (*n* = 8) were mostly prescribed dosing intervals of 21 days for entire-vial dosing.

DISCUSSION

We demonstrated the efficacy of entire-vial dosing of emicizumab in 112 PwHA during 96 person-years. Real-world evidence demonstrated therapeutic plasma concentrations and good bleeding control. The drug waste was reduced by 9% after introduction of entire-vial dosing in our clinic, which was equal to a mean annualized drug waste of ~260 mg per adult per year.

When emicizumab is dosed with 6mg/kg every 4 weeks according to the drug label, the mean trough concentration is 38 µg/mL, midway 54 µg/mL and peak 67 µg/mL, while weekly-dosing intervals result in almost constant concentrations varying between 51 and 55 µg/mL [6]. Due to the retrospective study design, the concentrations could not be classified as trough, midway or peak concentrations. Nevertheless, the observed median concentration was 63 µg/mL, and was 69 µg/mL in adults/adolescents, which indicates higher concentrations in this study than in the drug label [6]. Possibly entire vial dosing may have resulted in higher concentrations due to overfilling of vials. Indeed, regulators have recommended overfilling vials of liquid drug products because it may be difficult or impossible to remove 100% of the content from a vial. Regulators are concerned that manufacturers overfill vials without appropriate justification, which is not clear or made public for emicizumab vials [15]. Thus, the entire-vial dosing may have led to the administration of overfilled vials in our cohort, explaining the higher concentrations achieved in comparison to concentrations from the registration studies, and we recommend to measure a concentration when applying the entire-vial dosing strategy.

The within-individual variability observed (15% and 22%) is in line with previous studies [16] and may have originated from the undefined sampling times (trough-peak fluctuations) or/and (not reported) adherence issues. Based on clinical observation and emicizumab concentrations, the presence of ADAs against emicizumab was not suspected in our cohort. This is in concordance with the low reported immunogenicity risk [17] and the current guidelines recommending emicizumab's concentration measurement in the absence of ADA-detection assays [18].

The bleed rates were reduced substantially before and during the emicizumab therapy and were more reduced in adults/adolescents than in children due to baseline imbalances (as presented in Supplemental Table ST1).

Most children (*n* = 21, 66%) used a local anaesthetic cream (EMLA®) prior to the injection, which has not been reported in existing literature. Except for local pain, there were no documented side effects: no cases of a declining PK profile or suspected ADAs, sudden or gradual loss of response or thrombosis/thrombotic microangiopathy were reported at our center.

In several European countries, the drug waste of emicizumab accounted for 6% of its total cost for adolescents/adults and 26% for children [4]. The costs of emicizumab originate from the exorbitant acquisition costs/list pricing costs that are based on separate deals that countries have with the manufacturer, which is why even low drug waste has a considerable financial impact. We demonstrated 9% drug waste in this study, which is in line with previous studies that reported 8.4% for emicizumab and 7–9% for comparable drugs [19, 20]. Furthermore, the 9% drug waste that was observed during this study was calculated based on the manufacturer's calculator and is probably higher in clinical practice. Especially since the manufacturer prohibits combining two different strengths in one injection syringe, which often leads to a suggestion of at least two injections instead of one injection. As this is very inconvenient for the recipient, the manufacturer's instructions are probably not adhered to in daily practice, which would implicate that larger vial sizes are used and even more of the drug is wasted, as has been suggested before [4].

Almost all self-reported non-adherent PwHA were teenagers. The injection avoidance might result from a combination of factors, including independence, risky behaviour at that age and painful experiences that might be linked to the high drug viscosity and/or to a lack of adipose tissue in male teenagers. Injection pain seemed an important issue as children had a low BMI and 66% used EMLA prior to injection [15]. The manufacturer's prohibition to combine two different strengths in one injection syringe, even when the maximum volume for subcutaneous administration (2 mL) is not exceeded, is pointless from a pharmacological perspective and leads to more painful injections per dose, and further reduces the user-friendliness of emicizumab. Fortunately, the forgiveness of poor adherence is higher for emicizumab than for factor replacement therapy due to the drug's long half-life and the high exposure that comes from label-based dosing. The absence of bleeding on emicizumab might encourage the PwHA to adopt new behaviours that may increase their risk profiles and potentially later lead to more trauma-related bleeds. Treating clinicians should be aware of this potentially changing profile, and inquire about the individual's pain experience to assist with adequate treatment adherence.

The limitations of this study are typical to those of most retrospective observational studies, although prospectively registered data were used. For instance, ethnicity was not available as determinant in analysis. A limitation might be that the bleed rates that we calculated during emicizumab in this study were overestimated. Selection bias might have been introduced because the need to start emicizumab prophylaxis quickly could have been higher in PwHA with insufficient bleeding control on previous therapy, which could have led to more bleeds for the early switchers in comparison to the later switchers. To overcome this effect, state-of-the-art bleed modelling was performed using a negative binomial regression to account for some of the channelling effects (skewness of bleed data) [21], although long-term data will be more accurate on bleeds during emicizumab. Furthermore, the bleed rates might have been overestimated as some PwHA experience pain and start episodic treatment immediately without an actual bleed occurring [22], and not all of the bleeds were verified by a clinician or were confirmed by imaging. Nevertheless, all the included bleeds were treated with FVIII/BPA. This is relevant from a financial perspective regardless of whether the bleed occurred. Therefore, this study does reflect the real-world setting after emicizumab's market entry.

For future research, we recommend investigating the role of the cost-efficient monitoring of emicizumab. Firstly, the effectiveness plateau was set at >30 µg/mL, while the majority of PwHA in this study (81%) had concentrations of >40 µg/mL. Secondly, similar bleed rates were demonstrated in this study across the concentration subgroups of <40, 40–80 and >80 µg/mL. These two study findings suggest a highly variable dose–response relationship. When considered in this context, entire-vial dosing seems non-controversial and a more liberal dose range might be considered to allow whole-weekly intervals, without the need for monitoring emicizumab concentrations. Furthermore, these two study findings support dosing lower than 6mg/kg/4 weeks (or an equivalent mg/kg with shorter intervals) in a substantial proportion of PwHA. An intervention study is needed to confirm this hypothesis.

In conclusion, we evaluated the efficacy of entire-vial dosing of emicizumab in a large Dutch cohort of PwHA and observed therapeutic plasma emicizumab concentrations, good bleeding control and a 9% reduction of drug waste. This real-world evidence supports entire-vial dosing as an attractive and practical option for clinicians who treat PwHA with emicizumab therapy.

Author's contribution

AD designed the study, performed data management, conducted data curation, analysis, and validation, prepared the first draft of the manuscript, and implemented significant contribution from co-authors up to the final publication. Throughout the process, AD asked and implemented input and feedback from supervision team and co-authors, who performed critical review of the manuscript and provided significant contributions to the study.

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CHAPTER

DosEmi study protocol: A phase IV, multicenter, open-label, crossover study to evaluate noninferiority of pharmacokinetic-guided reduced dosing compared with conventional dosing of emicizumab in people with haemophilia A

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ABSTRACT

Introduction

Emicizumab effectively prevents bleeding in people with haemophilia A (PwHA), but is a burden for national healthcare budgets and consequently may limit access. According to the drug label, dosing of emicizumab is based on body weight with fixed intervals of 7, 14 or 28 days, which leads to mean plasma concentrations of 55 µg/mL (SD 15 µg/mL). However, a moderate variability of concentrations and a minimal effective concentration of 30 µg/mL have been suggested in studies. Therefore, a dose of emicizumab that targets a trough concentration of 30 µg/mL is hypothesized to be equally effective as conventional dosing in the prevention of bleeding.

Methods

We designed a phase IV, multicenter, open-label, crossover study to evaluate noninferiority of bleed control of ≥6 months on conventional dosing in comparison to ≥6 months on dose intervention. This dose intervention consists of reducing the dose of emicizumab to target a trough concentrations of 30 µg/mL using individual pharmacokinetic (PK) parameters. Ninety five PwHA aged > 1 years who received conventional dosing of emicizumab for ≥12 months with good bleeding control during the last 6 months will be recruited from all Dutch haemophilia treatment centers. The study is powered to detect a clinically relevant decrease (risk difference) of 15% in the proportion of patients without treated bleeds during both follow-up periods. Secondary endpoints are spontaneous joint- or muscle bleeds, and annualized treated bleeding rates (using negative binomial regression). Cost-effectivity between conventional dosing and individualized PK-guided dosing of emicizumab will be compared.

Ethics and dissemination

The DosEmi Study was approved by the Medical Ethics Review Committee NedMec of the University Medical Center of Utrecht, the Netherlands. Study results will be communicated through publications in international scientific journals and presentations at (inter)national conferences. Trial registration number: EUCTR2021-004039-10-NL at https://trialsearch.who.int.

INTRODUCTION

People with haemophilia A (PwHA) have a deficiency of coagulation factor VIII (FVIII) and present with spontaneous or provoked bleeds, predominantly into major joints leading to painful and chronic arthropathy [1, 2]. The cornerstone in the management of haemophilia A is still self-administration of FVIII concentrates by intravenous injections [3]. These injections are 2–7-times weekly to prevent bleeds (prophylaxis), or at the time of bleeding (on demand) when prophylaxis is unavailable. Prophylaxis with FVIII concentrates has effectively reduced the number of treated bleeds from an annual mean of 20–30 to 1–4 [4-7]. Additionally, anti-FVIII antibodies (known as inhibitors) render therapy with FVIII concentrates ineffective and develop in 30% of PwHA, who then require alternative, suboptimal therapies [8].

The first globally approved non-factor therapy is the bispecific, FVIII-mimicking antibody, emicizumab (Hemlibra®) [9]. Emicizumab became available for PwHA with severe haemophilia A in the Netherlands in July 2020. This novel drug promotes effective haemostasis, regardless of inhibitor status, achieving complete eradication of treated bleeds in around 80% of PwHA (*n* = 374) during the second 24-week interval of treatment [10]. More benefits of emicizumab are the subcutaneous and less frequent injections every 1, 2 or 4 weeks. Reported side-effects are thrombotic microangiopathy or thrombotic events when concomitantly using activated prothrombin complex concentrates in doses over 100IU/kg/day, which have not been observed since a change in guidelines for treatment of breakthrough bleeding [10]. Remaining side effects are the development of neutralizing or clearing anti-drug antibodies (ADA) against emicizumab (<1%) and injection-site reactions [10, 11]. Although many PwHA are candidate for prophylaxis with emicizumab, access is limited due to the financial impact on healthcare budgets.

Emicizumab was approved with a loading dose of 3 mg/kg/week for four weeks and a maintenance dose of 1.5 mg/kg/week, 3 mg/kg/2 weeks or 6 mg/kg/4 weeks [12]. These dosing regimens were simulated in a pharmacometric approach, instead of a traditional dose-finding study, targeting a trough concentration (C_{trough}) of 45 µg/mL [13]. Dosing according to drug label leads to mean concentrations of 55 µg/mL with 66% of observations between 40 and 70 µg/mL (SD of 15 µg/mL) [12, 14]. Additional real-world evidence from the Netherlands demonstrated that entire-vial dosing led to even higher concentrations of 63 µg/mL with 81% having concentrations >40 µg/mL [15]. In the meantime, the long-term bleed data from the phase III and IV studies were included in pharmacokinetic (PK) and pharmacodynamic (PD) modelling studies, and the minimal effective C_{trough} was suggested at 30 µg/mL [14, 16-18]. Although this new target is substantially lower than the previous target, dosing regimens have not been adjusted.

Concomitantly, reduced dosing of emicizumab, by 20–96%, without treated bleeds has been reported [19]. Consequently, we hypothesized that reducing the dose of emicizumab to target a Ctrough of 30 µg/mL using individual pharmacokinetic (PK) is equally effective in the prevention of bleeding as conventional dosing. We designed the DosEmi study to investigate this hypothesis in a large cohort of adult and paediatric PwHA. Additional benefits from this intervention are less frequent injections or lower injection volumes. This is especially beneficial for children, as 66% used a local anaesthetic prior to injection to prevent pain at the injection site [15]. The DosEmi study will be conducted with intensive clinical and laboratory monitoring and is expected to result in significant healthcare savings and improved cost-effectiveness without loss of bleeding control. In this report, we publish the study protocol of the DosEmi study registered as EUCTR2021-004039-10-NL at https://trialsearch.who.int. This study is supported with a grant (Transformatiegelden) by the Dutch federation of academic hospitals (Nederlandse federatie van universitaire medische centra [NFU]).

METHODS AND ANALYSIS

Primary objective

The primary objective of the DosEmi study is to determine whether individualized PK-guided dosing of emicizumab targeting a C_{trough} of 30 µg/mL is noninferior to conventional dosing of emicizumab in the prevention of treated bleeds in people with congenital haemophilia A.

Eligibility

A participant must meet the following inclusion criteria: confirmed diagnosis of congenital haemophilia A with a baseline FVIII activity of <6 IU/mL, aged >1 year, receiving conventional dosing of emicizumab dosed according to label (± 6 mg/kg per 4 weeks, rounded to entire vials) at 1–4 weekly intervals for a duration of ≥12 months prior to inclusion and demonstrating good bleeding control defined as (i) no spontaneous joint/muscle bleeds in the previous 6 months, and (ii) a maximum of two treated (traumatic) bleeds in the previous 6 months.

Study design and setting

The DosEmi study is a multi-center, prospective, open-label, crossover study. It was designed as a noninferiority study that was powered to detect a clinically relevant decrease of 15% (risk difference) in the proportion of patients without treated bleeds during follow-up (see Sample size). The crossover intervention was chosen to account for potential imbalanced baseline characteristics, which might occur at treatment start (e.g., instable joint health), and to ensure comparability in an open-label setting [20].

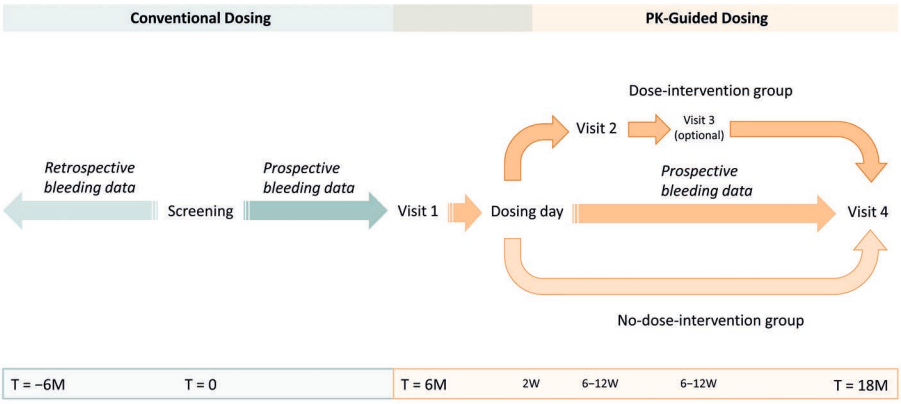

Figure 1. DosEmi study design.

A schematic summary of the study design is shown in *Figure 1* and in more detail in Supplementary Figure 1. The different phases depicted are a retrospective Clinical Phase, and a total prospective study duration of 18 months, which includes 6 months of bleeding assessment on conventional dosing, 6 months of PK-guided dosing and 6 months of dose continuation. The first participant was enrolled in September 2022 and total inclusions with sufficient follow-up time are expected to be reached in 2026.

The study is investigator-driven with the UMC Utrecht as study sponsor. The UMC Utrecht acts as coordinating center and participants will be recruited from all Dutch haemophilia treatment centers, which are located in Amsterdam, Den Haag, Groningen, Leiden, Maastricht, Rotterdam, Utrecht, Nijmegen, and Eindhoven. All sites were involved in the design of the study and have personnel with the expertise to assess bleeds, examine joint health, assist in questionnaires and perform clinical and laboratory monitoring. Day to day activities of the study are performed by the principal investigator (study supervision and medical responsibility), study coordinator (trial registration, coordinates study visits, annual safety reports), study physicians (identify potential participants, obtain informed consent, ensure follow-up according to protocol) and data manager (supports in data capture, safeguards quality of data). The study team of the coordinating center meets weekly. A multidisciplinary study steering committee has been installed, consisting of (but not limited to) haemophilia clinicians, pharmacists, representative from the Dutch Haemophilia Patients Organisation and the SYMPHONY consortium for valued based health care support and PK guidance [21]. Study monitoring will be performed by a professional CRO (Julius Clinical, Zeist, the Netherlands). The monitoring plan is available upon request. Furthermore, the Board of Dutch Haemophilia Treaters (NVHB) and the Steering Committee will act as Scientific Advisory Committee and periodically review study results and safety data. Unblinding and randomization procedures are not

applicable due to the type of intervention, self-treatment, and the crossover design of the study.

Patient and public involvement

A representative from the Dutch Haemophilia Patients Organisation (NVHP) has been involved in grant applications, study design and protocol drafting. As a member of the multidisciplinary study steering committee, he will be a full author on any publications originating from this project. This study is supported by the Dutch federation of academic hospitals.

Intervention

The following will be compared within each participant:

- Comparator: 6 months of conventional dosing of emicizumab (i.e., 6mg/kg per 4 weeks at individualized 1–4-weekly intervals);
- Intervention: 12 months of individualized PK-guided dosing of emicizumab to achieve a target C_{trough} of 30 µg/mL (range 25–35 µg/mL).

The research pharmacists of the SYMPHONY consortium will provide a PK-guided dose advice based on a maximum a posteriori (MAP) Bayesian analysis of the individual observed concentration using an online platform https://opticlot.nl [21, 22]. MAP Bayesian will be performed using NONMEM software (v 7.4.1, Icon Development Solutions, Gaithersburg, Maryland, United States). Population PK parameters will used as reported by Retout [18]. The patient variables (i.e., body weight, height, age, and serum albumin) will be included in the PK simulations. A dose advice consists of: a lag-time period providing a restart date for new dosing, a dose in entire vials and a dosing interval. The research pharmacists will also provide a window for the next C_{trough} measurement, to check if the target C_{trough} is reached following the intervention. In general, this will be done after one or two half-lives (30–60 days) based on the C_{trough}. Both a target C_{trough} between 25–35 µg/mL, as well as a varying dosing interval between 7 to 42 days, are allowed to enable use of entire vials only. The maximum dose-reduction at each step will be 50%, and doses will be increased if the new $C_{troughs}$ is less than 25 µg/mL. The target C_{trough} between 25–35 µg/mL reflects the target for the PK model; while during the dose intervention, C_{trough} between 25–39 µg/mL in individual participants will be accepted. The dose advice also takes individual preference in dosing interval and maximum injection volume of 2mL/dose into account.

Outcomes

Primary outcome

The proportion of patients without treated bleeds during 6 months on conventional dosing (comparator) compared to 6 months on individual PK-guided dosing (intervention).

Secondary outcomes
The secondary outcomes on bleeds are:

- The proportion of patients without treated bleeds in the follow-up periods of 12 months on conventional dosing in comparison to 12 months on individual PK-guided dosing;
- The proportion of patients without spontaneous joint- or muscle bleeds in the periods of 6 and 12 months on conventional dosing in comparison to the 6 and 12 months on individual PK-guided dosing respectively;
- Annualized Bleed Rates (ABRs) of treated bleeds, including joint bleeds and sports induced bleeds in the periods of 6 and 12 months on conventional dosing in comparison to the 6 and 12 months on individual PK-guided dosing respectively.

Other secondary outcomes are: Health Related Quality of Life (HR-QoL), maintenance of stable joint health and sports participation will be compared before and after the dose intervention, reduced pain due to emicizumab injections, the performance of the population PK model, the cost-effectiveness, thrombin generation parameters as pharmacodynamic (PD) biomarkers for emicizumab treatment efficacy.

Assessments

The study design is shown in *Figure 1*, Supplementary Figure 1 and the schedule of all assessments over time is presented in Supplementary Table 1. If inclusion criteria are met and informed consent is signed, the retrospective data on bleeds during the six months prior to inclusion will be collected (Clinical Phase [nonstudy, used for inclusion criterion]). Upon inclusion in the DosEmi study, participants will be monitored for a period of six months to prospectively assess bleeds (Bleeding Assessment Phase). During the following phase, participants are categorized into the 'dose-intervention group' when plasma emicizumab concentrations at visit 1 are ≥40 µg/mL, or the 'No Dose Intervention Group' when emicizumab plasma levels are <40µg/mL.

The dose-intervention group undergoes individualized PK-guided dosing of emicizumab at 2-3 weeks after Visit 1 (Dosing Day). The emicizumab concentration is checked again on Visit 2, and if the target concentration was not reached the (optional) Visit 3 is planned to check emicizumab concentration after the second dose adjustment. Participants will be followed for six months after Visit 1 (PK-guided Dosing Phase), after which the participants continue with their PK guided dosing regimen for another six months (Dose Continuation Phase). Thus, a total of 12 prospective months of follow-up on PK-guided dosing regimen per participants are obtained.

The No-dose-intervention group includes two groups for observational data collection. Participants with an emicizumab plasma concentration 25-39 µg/mL at Visit 1 will continue on their current dose regimen and will be followed according to the same

assessment schedule as the Intervention Group, except for Visit 2. These participants will be followed for 12 months in total to collect additional data on bleeding according to emicizumab concentrations. Participants with emicizumab C_{trough} concentrations <25 µg/mL at Visit 1 will be monitored closely by their treating physician, and may receive increased emicizumab dosing at the discretion of their treating physician. Since treatment of these PwHA (emicizumab C_{trough} <25 µg/mL) is outside the scope of this study (i.e., dose reduction intervention), these PwHA will not perform Visit 2 through Visit 4. However, to follow-up on safety, we will continue to collect available selective safety data, such as bleed assessment and the presence of anti-drug antibodies (ADA), for these PwHA for a period of 12 months.

The study is designed with two age-based cohorts consisting of participants aged ≥16 years (Cohort 1) and aged <16 years (Cohort 2). After a total of 25 participants from Cohort 1 have completed six months of follow-up on PK-guided dosing, data on bleed control will be analysed and the power calculation will be repeated to provide a more precise estimate of the number of participants required for the study. Data of this interim analysis will be reviewed by the Scientific Advisory Committee (NVHB and Steering Committee). Enrolment of paediatric participants of Cohort 2 can start if bleed control in these 25 participants of Cohort 1 participants is good, according to definitions in the inclusion criteria, combined with a maximum of one additional bleed in six months.

Discontinuation of study

Criteria for discontinuation are withdrawal of consent (at any time for any reason), development of a medical condition that precludes participation and/or is associated with increased bleeding risk (e.g., other bleeding disorders), formation of neutralizing or clearing ADA against emicizumab, occurrence of a spontaneous joint- or muscle bleed or >2 treated bleeds during 6 months as determined by the investigator or haemophilia treating physician, persistent non-adherence to protocol requirements, or loss to follow-up. Withdrawn participants during the Bleeding Assessment Phase may be replaced to reach the required sample size. All efforts will be made to complete and report the protocol-defined study observations up to the time of the participant's withdrawal as completely as possible. No further data will be collected after the moment of withdrawal, except for withdrawal due to ADAs after which selective safety data will be collected, and discontinuation of emicizumab treatment, after which one last contact moment will be arranged.

Participant recruitment and retention

As haemophilia is a lifelong condition, PwHA remain in care at their haemophilia treatment centers. Recruitment is based on both information provided in ongoing conversations with their haemophilia treating team and information provided by the patient society (NVHP) which is represented in the Steering Committee. No specific measures for patient retention during follow up are in place.

Sample size

The power calculations are based on the ability to detect a clinically relevant difference between the groups before and after the dose intervention in the proportion of participants without treated bleeding. The treatment centers and study group reached consensus on an expected response of 80% without treated bleeds in both groups before and after dose intervention and a noninferiority margin of 15% (i.e., risk difference) [10]. A required inclusion of 88 participants results from sample size calculations with settings of a noninferiority test, crossover design, binary data, 80% power, one-sided alpha 0.05, noninferiority margin 0.15 and expected response in both groups 0.8 https://app.sampsize.org.uk. To account for possible drop-out, we will aim for inclusion of 95 participants. The power calculation will be repeated after the first 25 participants have completed 6 months of follow-up after the dose intervention.

Recruitment

Potential participants, who meet the inclusion criteria, will be informed about the DosEmi study by their treating physician. To allow sufficient time for consideration, the informed consent procedure will be executed after a minimum of one weeks' delay of the formal invitation and opportunity to ask questions regarding the Informed Consent.

Data collection and management

All study data will be entered in the Good Clinical Practice (GCP) compliant eCRF system Castor [23]. The medical data will be collected during study visits and monthly contact. The primary source for medical data is the electronic medical record system of the hospitals. Blood samples for routine checks will be measured locally at the laboratories of the hospitals. The blood samples for emicizumab concentration and thrombin generation will be measured centrally in the UMC Utrecht by ISO-certified laboratories. The emicizumab concentration will be measured using a validated liquid-chromatography-tandem mass spectrometry (LC-MS/MS) method [24, 25]. Plasma coagulation potential will be measured using thrombin generation tests as a potential read-out for pharmacodynamics. Joint status will be measured by physical examination (Haemophilia Joint Health Score [HJHS]) [26-28], ultrasound (if available, according to the HEAD US score). Health related quality of life (QoL) will be assessed with EQ5D(Y)-3L [29-31], and PROMIS instruments (Physical Function/mobility and Pain Interference short forms) [32-34]. Assessment of pain during emicizumab administration will be scored with the Visual Analogue Scale (VAS). Sports participation (type, duration, frequency) will be assessed with Modifiable Activities Questionnaire (MAQ) [35, 36]. These QoL-questionnaires (i.e., total of 34 questions per visit) will be sent out electronically via Castor.

A data management plan (DMP) is generated to describe data collection, handling, storage and back-up, analysis, archiving, and sharing. Participants will be assigned a unique study number, stored according to GCP requirements. All data will be reported at group level.

Statistical methods

All analyses will be performed by within-patient comparisons using paired tests. All participants receiving dose reduction will be included in the statistical analyses, merging data of Cohort 1 and Cohort 2. Primary and secondary outcomes on proportion of bleeds will be analysed with McNemar test one sided *p*-value threshold 0.05. The treated A(J)BRs (mean and 95% confidence intervals) will be modelled with negative binomial regression.

Data from withdrawn participants with at least 1 concentration sample available can be included in the analysis. Treatment adherence will be assessed by the percentage of vials distributed by the hospital pharmacy versus prescribed by physician. The percentage of vials taken will be calculated as: 100 × (total number of vials administered) / (total number of vials prescribed). The percentage of vials taken will be summarized descriptively as quantitative variables. The number and percentage of participants whose treatment compliance is <80% or ≥80% will be summarized. Available data of non-adherent participants will be included in the analysis of bleeding according to emicizumab concentration. Missing data on emicizumab concentrations or bleeding will not be imputed nor analyzed.

Safety

The collection of AEs will be limited to AEs of special interest (AESI). The AESIs include bleeds (i.e., trauma-related or spontaneous), haemophilia-related events (such as inhibitor development or pain), thromboembolic events (arterial, venous, catheter related and thrombotic microangiopathy [TMA]) and development of neutralizing ADAs. Recording of serious adverse events (SAE) will be restricted to SAEs of special interest (SAESI) as well. The SAESI will be immediately reported to the coordinating investigator and announced to the Medical Ethics Review Committee of the UMC Utrecht.

ETHICS AND DISSIMINATION

The protocol of the DosEmi Study was approved by the MERC NedMec of the UMC Utrecht (local study registration number NL81112.041.22) on July 2022. Approval by the local MERCs of participating centers is requested/pending. All substantial amendments to the protocol will be notified to the MERC and competent authority. Non-substantial amendments will be recorded and filed by the coordinating investigator. All participants or their guardians will be asked to provide written informed consent to participate in the study. All study procedures will be performed in accordance with the ethical standards of the Declaration of Helsinki (Fortaleza, Brazil, October 2013) and the Medical Research Involving Human Subjects Act (WMO).

Results from this study will be analysed and submitted for publication in peer-reviewed international scientific journals and presented at scientific meetings. The coordinating investigator will initiate these scientific activities. There are no restrictions regarding

the public disclosure and publication of the research data. The study was registered in the public trial registries of EUDRACT (included in the WHO registry and accepted by all major international medical journals) and the competent authority, prior to inclusion of the first participant.

DISCUSSION

Individualized dosing of emicizumab based on a target C_{trough} of 30 µg/mL is hypothesized to be equally as effective in the prevention of bleeds as conventional dosing. Besides the benefits for participants (i.e., less frequent injections and/or with lower volume), this PK-guided dosing is expected to result in significant healthcare savings and improved cost-effectiveness without loss of bleeding control. The DosEmi Study has enrolled its first participant in September 2022.

Our study is supported by several reports on reduced dosing of emicizumab without loss of efficacy. Reported first was a case of a boy in whom higher emicizumab concentrations of ~90 µg/mL were associated with more episodes of pain in muscles and joints [37]. The dose was reduced to result in emicizumab concentrations of ~24 µg/mL, after which the pain resolved and no bleeds occurred during the following six months. Subsequently, emicizumab was given in lower doses in 11 PwHA from Finland and 6 PwHA from Thailand without loss of efficacy [19, 38]. Additionally, real-world evidence from our center demonstrated similar bleed rates across the concentration subgroups of <40 µg/mL (*n* = 13), 40–80 µg/mL (*n* = 59) and >80 µg/mL (*n* = 22) [15]. We assume that, in clinical practice, many others dose emicizumab in a reduced form without publishing the results, especially as global access is limited [39].

There are limitations and strengths to the DosEmi Study. The sample size is relatively large for a rare disorder, requiring a labour-intensive multicenter design. Furthermore, recruitment of paediatric participants may be difficult [40] and potential participants may be reluctant to reduce dosing of an effective treatment. Nevertheless, the study provides a unique opportunity to evaluate alternative dosing strategies in a safe and well-controlled clinical setting. Additionally, the opportunity for patients (especially children) to receive fewer painful emicizumab injections is provided. This study can eventually provide meaningful conclusions that benefit the global application of reduced dosing.

Author's contribution

AD helped in study design, prepared the first draft of the manuscript, and implemented significant contribution from co-authors up to the final publication. Throughout the process, AD asked and implemented input and feedback from supervision team and co-authors, who performed critical review of the manuscript and provided significant contributions to the study.

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CHAPTER

General Discussion

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Background

People with haemophilia A (PwHA) have a deficiency of coagulation factor VIII (FVIII) [1]. These individuals present with recurrent bleeding, predominantly into major joints, eventually resulting in chronic arthropathy [2]. The first effective treatment of bleeding became available with the discovery of cryoprecipitate in the 1960 [3] followed by the development of plasma-derived concentrates of FVIII (pdFVIII) in the 1970s, enabling home therapy [4]. Subsequently, the first recombinant FVIII (rFVIII) concentrates were introduced in the 1990s, eliminating the virus-related problems associated with pdFVIII [5, 6]. In recent decades, rFVIII products with extended plasma half-lives have been bioengineered, non-factor replacement products (e.g., emicizumab) were introduced [4, 5], and gene therapy was recently regulatory approved [7].

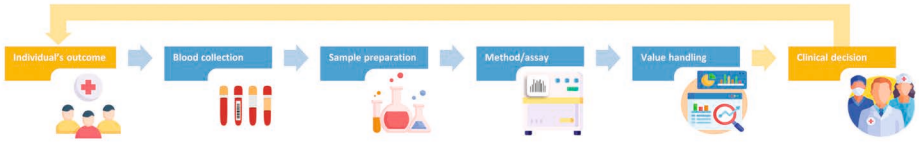

Figure 1. Process of measuring (blue) and monitoring (yellow) in a clinical setting.

Measuring and monitoring in a clinical setting (*Figure 1)* are important, as described in the general introduction. *Measuring* involves assigning a numerical or categorical value to a certain parameter, whereas *monitoring* involves clinically interpreting these (potentially changing) values to make clinical decisions regarding diagnosis or therapy. The process of measuring and monitoring FVIII activity is key in the care management of people with haemophilia A [8]. This FVIII activity is measured using a clotting assay and expressed as a percentage compared with the FVIII activity in the pooled human plasma of people without a FVIII deficiency. This biomarker directly correlates with the likelihood of bleeding and is used for diagnosis, clinical support and dose optimisation (see *Figure 2* of general introduction) [9]. On the other hand, measuring and monitoring emicizumab is not yet standard care. For instance, emicizumab was regulatory approved with a body-weight-based dose regimen without requiring dose adjustments based on laboratory monitoring [10]. In the pre-approval clinical trials, the emicizumab concentration was measured for pharmacokinetic purposes with an enzyme-linked immunosorbent assay (ELISA) [11-14]. In current clinical practice, this concentration is infrequently measured (see *Figure 2* of general introduction) using a modified, calibrated clotting assay [15]. The liquid chromatography (LC) coupled with tandem-mass spectrometry (MS/MS) analysis is a relatively novel technique for quantifying protein concentrations in human plasma. Prior to this thesis, the potential value of concentration measurements with LC-MS/MS analysis for monitoring PwHA in clinical practice was unclear. The knowledge gaps regarding this process concerned the relationship between the FVIII concentration

(measured using LC-MS/MS) versus the FVIII activity (measured using clotting assay) and emicizumab's concentration in routine daily practice.

Thesis objectives

The overarching objective of this thesis was to optimise drug monitoring of FVIII and emicizumab in PwHA via the following sub-objectives:

1) developing and validating LC-MS/MS methods that quantify the concentrations of FVIII and emicizumab in human plasma
2) investigating the concentration–biomarker relationship of FVIII and the dose–concentration–response relationship of emicizumab
3) proposing and evaluating a cost-efficient dosing strategy for emicizumab

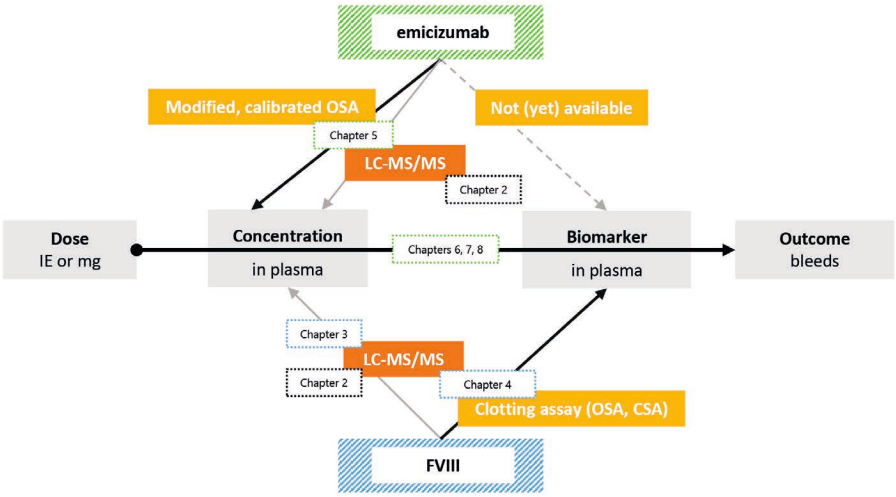

Figure 2. Dose–concentration–response relationship with bioanalysis of FVIII and emicizumab in PwHA including the chapters of the thesis.

The key lessons and recommendations from this thesis are as follows:

- Bioanalysis with LC-MS/MS is advantageous for **measuring plasma proteins** (e.g., FVIII and emicizumab) in a clinical setting. In this thesis, the LC-MS/MS method development for measuring FVIII plasma concentration proved challenging and required an additional purification step to dissociate FVIII from the Von Willebrand factor. However, the LC-MS/MS method development measuring emicizumab was straightforward and could be multiplexed with other biopharmaceuticals. Cross-validation against a standard method is highly recommended for the external validity of the method.

- The relationship between the FVIII concentrations measured using LC-MS/MS analysis and the FVIII activity measured using a clotting assay revealed an unexpectedly large variability. Consequently, the role of **monitoring FVIII** with LC-MS/MS analysis remains unclear, and the added value must still be determined before the method is introduced to the clinic. However, **monitoring emicizumab** with LC-MS/MS analysis can be implemented in a clinical setting for clinical support, cost efficiency and research.

- A **cost-efficient dosing strategy** for biopharmaceuticals can be developed based on the four studies on emicizumab in this thesis. This strategy involves (i) developing a measurement method to quantify a biopharmaceutical in human plasma; (ii) critically evaluating the literature to identify a minimum effective target of the drug; (iii) evaluating real-world evidence in the target population; and eventually (iv) designing a clinical study comparing cost-efficient dosing with conventional dosing.

This general discussion chapter provides a broad perspective on the following two themes:

1. Potential future applications of LC-MS/MS **monitoring** of FVIII concentrations in haemophilia A
2. Framework for the **cost-efficient dosing** strategies of biopharmaceuticals

This perspective includes clinical implications, future perspectives and recommendations. As outlined below, both themes begin with background and rationale and end with critical notes and conclusion. This general discussion ends with concluding remarks on the thesis.

Potential future applications of LC-MS/MS monitoring of FVIII concentrations in haemophilia A

Background and rationale

The FVIII activity plays an important role in managing haemophilia A. This thesis investigated but did not determine how the FVIII concentration relates to the FVIII activity. Nevertheless, this work is a first step towards explaining the relationship. This step included developing and validating an LC-MS/MS method to quantify the FVIII concentrations in human plasma (**Chapters 2 and 3**). The LC-MS/MS method was then used in a proof-of-principle study (**Chapter 4**) that described the initial comparison between the FVIII concentration (using LC-MS/MS analysis) and the FVIII activity (using a clotting assay). This comparison revealed significant variability, which was partially explained by the neutralising FVIII inhibitors and the modified exogenous FVIII product. Most of this variability could not be explained, although it may have been introduced by plasma samples of PwHA, which can contain exogenous, endogenous, inactive FVIII molecules or FVIII peptide fragments. The LC-MS/MS method cannot discriminate between endogenous or exogenous FVIII molecules, nor between active or inactive FVIII fragments. These unknown proportions in the heterogeneous samples added to the uncertainties in the comparison between the FVIII activity and the FVIII concentration, potentially overestimating the functional FVIII concentration. One could further investigate both the person and product variability of FVIII – first separately, then combined – to explain fully the observed variability and to determine the necessary corrections. A follow-up study could obtain pharmacokinetic (PK) profiles by measuring the FVIII activity using different clotting assays, and the FVIII concentration via an LC-MS/MS analysis. Such a study would preferably be performed for people with severe, mild and moderate haemophilia A and people with normal FVIII activity. This study should also include various modified FVIII products to compare activation rates. These data could then be modelled using PK simulations to explain the total variability and the concentration–biomarker relationship of FVIII. The FVIII concentration's role must also be thoroughly investigated and established for diagnostic and therapeutic monitoring before implementing the FVIII concentrations with LC-MS/MS analysis in a clinic.

The LC-MS/MS technique has become an indispensable tool in proteomics. Analysis using LC-MS/MS is the standard method for determining amino acid sequences and peptides, characterising post-translational modifications, determining absolute and relative protein quantities, and identifying and quantifying multiple proteins in one measurement run (i.e., multiplexing) [16-18]. This technique has been employed to measure small molecules and therapeutic proteins in many laboratories worldwide and will probably become one of the leading techniques in the clinic [19]. The possibilities of this analysis seem endless when adopting mass spectrometry. Four potential applications of LC-MS/MS analysis in haemophilia A are now outlined.

Potential future applications

1. Multiplexing in haemophilia A and beyond

Regarding haemophilia A measuring and monitoring, the LC-MS/MS method could be used to multiplex. For instance, our research group developed a multiplex analysis of seven therapeutic monoclonal antibodies, including emicizumab, in human plasma or serum with a generic sample preparation [20]. Multiplexing can simultaneously measure multiple proteins in one run, which is helpful for diagnosing not only haemophilia A, but also other bleeding disorders (e.g., haemophilia B, VWF disease). This analysis could generate an entire coagulation profile or a 'coagulation passport' for an individual, which is especially advantageous for those with difficult venous access (e.g., children) because LC-MS/MS requires only 10 µL of plasma. A coagulation profile could also help individuals without a bleed history who present with unexpected and uncontrolled bleeding in the emergency room, although faster result reporting of LC-MS/MS is first required. Multiplexing could also be used to design an LC-MS/MS method to measure the entire therapeutic panel of drugs prescribed daily by a clinician. Thus, a specific 'therapeutic haemophilia run' could be developed that would be efficient from a logistical perspective in a clinical setting. The coagulation profile and the haemophilia A drug panel testing could even be combined in one run. However, the concentration-biomarker relationship of all the peptides in this run should be established because a quantifiable protein (i.e., concentration) does not necessarily mean that protein is functional (i.e., effect). This thesis began work on this topic in **Chapters 3 and 4**.

2. Cases of discrepant FVIII activity

The monitoring moments and the treatment strategies are based on the traditional classification of severe, moderate and mild haemophilia A [1, 21]. This classification system was first described by Biggs and MacFarlane in 1958 and is still used in the ISTH SSC definition [22, 23]. The underlying FVIII gene mutation is often responsible for subnormal endogenous FVIII activity. Null mutations typically prevent FVIII-molecule synthesis and are associated with an undetectable FVIII activity, whereas non-null mutations permit the synthesising of some of the molecule and are usually associated with a residual FVIII activity [24]. The clinical distinction between severe and non-severe haemophilia A was confirmed in 2011 in a similar manner [9]. Although the classification generally relates to the frequency of bleeding symptoms, 10–15% of PwHA with the severe classification bleed less than expected [25-27]. This heterogenic phenotype in the severe classification is only partially explained by genetic and acquired factors [24]. About one-third of people with the non-severe classification demonstrate a discrepancy in the FVIII activity measurements between the one-stage clotting assay (OSA) and chromogenic substrate assay (CSA) [28, 29]. Most of these people display higher FVIII activity based on OSA when compared with CSA (standard discrepancy), whereas the opposite is true in a smaller group (inverse discrepancy) [30]. Depending on the specific

mutation affecting the assays, neither OSA nor CSA perfectly predict patients' bleeding phenotypes in people with non-severe haemophilia A [31].

The second future application is to use LC-MS/MS bioanalysis in cases of discrepancy between the FVIII activity and the clinical phenotype. As mentioned, the non-null mutations partially synthesise FVIII, and LC-MS/MS analysis can quantify these mutated peptides (non-functional or partially functional) in human plasma. As many mutated peptides as desired can be included (multiplexing), beginning with the most common. The entire mutated-peptide profile could eventually be screened. The FVIII concentration measurements using an LC-MS/MS method could add value to the existing clotting assays, identifying genetic factors in PwHA at risk of discrepancies, and thus misdiagnosis, when using common clotting assays. Measurements using LC-MS/MS could eventually be included in the first steps of diagnosing of haemophilia A.

3. Neonatal screening

A third future application of LC-MS/MS could be the inclusion of haemophilia A in a neonatal screening programme. The Dutch neonatal screening programme aims to detect 26 treatable and severe disorders early in life [32]. The selection of these disorders was based on Wilson and Jungner's principles of screening, published in 1963, which are still consulted regarding the inclusion of new disorders [33]. Haemophilia A is a potential candidate for the screening programme when applying these principles because half of new-borns have no family history and it being a serious and severe disorder that can be treated with access to accurate diagnostic tools and adequate therapies in the Netherlands.

Why is this application of value? Diagnostic tests are currently performed for new-borns with a known family history of haemophilia A [34, 35]. Therefore, neonatal screening would help new-borns without a known family history of haemophilia A (±50% of cases), because this diagnosis rarely occurs until later in life, usually when a bleed occurs. Overall, approximately 80% of PwHA are at risk of a bleed during their first two years of life [36]. The most serious bleed, the intracranial haemorrhage (ICH), is also most likely to occur in the first two years of life [36, 37] and has been estimated to occur in 3–4% of new-borns [38]. Including haemophilia A in neonatal screening with LC-MS/MS analysis could facilitate earlier diagnosis, allowing clinicians to begin prophylaxis with FVIII concentrates sooner and preventing bleeds in the first days, weeks or years of life.

4. Dried blood-spot sampling

The second benefit of using LC-MS/MS analysis in neonatal screening is the sampling approach [39]. Sampling for neonatal screening involves collecting heel-prick blood drops from a neonate in the first week after delivery on a screening filter paper card. The dried blood spots (DBS) are then punched and analysed within a week [32, 40]. The LC-MS/MS approach has very high sensitivity with the ability to measure very low FVIII

concentrations in DBS, which is impossible with existing clotting assays. An additional benefit is the LC-MS/MS method's robustness, lowering the interlaboratory variability present in neonatal screening [41].

Measuring FVIII in DBS can be also used for remote health monitoring (or 'telemonitoring') [42]. This usage would benefit PwHA who need to travel long distances for monitoring, such as in large countries with a low density of healthcare facilities or in low-income countries where diagnostic tools are unavailable. Although access to expensive FVIII therapy is limited in low-income countries, an accurate diagnosis could help PwHA to make the necessary lifestyle changes and begin physiotherapy. Telemonitoring might also be convenient for PwHA during a pandemic lockdown, as hospital visits may be reduced [43].

Critical notes

A critical note for these future applications of LC-MS/MS analysis is the analysis run time. For instance, an overnight incubation step is required during sample preparation to dissociate FVIII from VWF. Thus, method optimisation is required to shorten the run time when this method is used in clinical practice because clinicians are accustomed to the rapid reporting of clotting assay results, usually within four hours, or within one hour if urgent. The current costs for LC-MS/MS analysis are only somewhat higher than other assays in Dutch hospitals. Since most clinical laboratories have adopted or will soon adopt this technique, the method costs will decrease. The competition of point-of-care devices with cartridges [44] is another aspect to consider. These devices can generate instant results from a single drop of 25 µL capillary blood by using a device the size of a mobile phone [45, 46]. However, these devices are not yet ready for the market, but their potential value is high, and they might take over a substantial part of the market after being launched.

Certain critical notes regarding the ethical and timing issues of neonatal screening must also be addressed. For instance, few reports have been published on the ethical standpoints of the haemophilia A community towards screening. Two reports on this attitude in the UK have mentioned that most participants considered haemophilia a 'liveable' disability, and that affected families could be offered support by neonatal screening programmes rather than reducing the birth rate of affected children based on prenatal screening [47, 48]. In the rapidly changing treatment landscape of haemophilia A, patient- and clinician organisations should evaluate the need for neonatal screening. Therefore, patient and clinician organisations in the Netherlands could form focus groups and conduct survey research to study this topic. Another issue is the timing of screening. Neonatal screening (i.e., post-delivery) is too late to prevent birth-related bleeds, suggesting a need for prenatal haemophilia A screening. More research is needed to determine whether haemophilia A is better suited for neonatal or prenatal screening. The ethics and timing issues should be clear before developing a DBS screening method

with LC-MS/MS analysis. Therefore, I recommend early diagnostic screening as a topic on the agendas of patient/clinician organisations (NVHP, WFH), to form a work or focus group at a haemophilia congress (WFH) or to conduct a systematic review on this topic, including expert opinions.

The possibilities for LC-MS/MS analysis regarding monitoring haemophilia A are promising due to the method's multiplex potential, sensitivity and small sampling volume. The studies in this thesis provide the first steps for adopting FVIII concentrations with LC-MS/MS analysis in haemophilia A care. Although additional research must be conducted before clinical implementation, LC-MS/MS analysis has the potential to optimise the monitoring of FVIII in several ways.

Framework for the cost-efficient dosing strategies of biopharmaceuticals

Background and rationale

Biopharmaceuticals are drug products with an active substance extracted or produced from a biological source through recombinant-DNA technology [49]. Biopharmaceuticals have rapidly emerged as important medical tools that highly specifically target their therapeutic receptors, offering a broad therapeutic window, usually without a ceiling of clinical toxicity [50]. The general challenges of therapy with biopharmaceuticals are immunogenicity (i.e., anti-drug antibodies [ADAs]), immune-mediated adverse effects, and the high costs resulting from drug development and industrial manufacturing [51, 52]. The dose selection for biotherapeutics is frequently set at the upper end of the dose–response curve in the absence of a maximum tolerated dose (MTD), and clinical toxicity is often not dose related [53-55]. Dosing far above a minimal effective target (overdosing) is generally not clinically relevant for biopharmaceuticals but may cause financial toxicity. Due to escalating healthcare costs, global overpopulation and an increasingly older population, a critical approach to drug dosing is vital.

This thesis outlines how to establish a cost-efficient dosing strategy for emicizumab (**Chapters 4–8**). The strategy begins with an idea but could produce actual cost-savings and can be presented as a cycle (*Figure 3).* Generating this process quickly is crucial from a financial perspective, because manufacturers make drug deals for a specific period with health insurance companies, hospitals and governments. The period opens a window of opportunity for researchers to develop a cost-efficient strategy before the deal ends. The quicker this cycle is generated, the greater the financial benefit, eventually improving patient access to a formerly expensive biopharmaceutical. This strategy can also be beneficial from a medical perspective, as lower injection volumes or less-frequent injections are often part of a cost-efficient strategy.

Figure 3. Cycle for the cost-efficient dosing strategy development of biopharmaceuticals.

Building a framework

1. Idea

Every innovation begins with an idea that trigger an hypothesis regarding cost-efficient dosing for a specific drug. This hypothesis can be checked with a quick literature scan. I recommend scanning and selecting biopharmaceuticals with high cost-efficient potential based on the following criteria:

- Pricing of either >€40 million per year, or >€50,000 per treatment and >€10 million per year: This criterion follows the pricing criteria adopted for the *Sluisgeneesmiddelen* in the Netherlands [56], which are drugs with a substantially high financial effect on healthcare.

- Limited or alternative dose-finding studies were performed in the Phase I/II clinical trials. This criterion can be checked in the literature or in the European Public Assessment Report. Theoretical assumptions are frequently employed in alternative dose-finding models, introducing greater uncertainty, so higher dosing is often

required to guarantee effectiveness. No assumptions are necessary in traditional dose-finding models, because the dose is related directly to concentration and effect.

- Having chronic, broad or rare disease indications: For instance, the indications of inflammatory bowel disease, rheumatoid arthritis and malignant haematologic or oncologic disorders have chronic and expensive biopharmaceuticals in their medicinal arsenal prescribed to many patients. Additionally, rare and orphan indications (e.g., bleeding disorders or metabolic diseases, such as Fabry or Pompe) often have high priced biopharmaceuticals due to small patient groups.

- Preferably exhibiting pharmacology with a linear dose–concentration relationship and an effectiveness plateau at conventional dosing: that is, the drug-target binding is saturated, and the maximum effect of the drug is achieved, respectively.

2. Research team creation

If the hypothesis is verified via a quick literature scan, a multidisciplinary research team can be developed based on it. The members of this team should have research experience and preferably perform more than one role. The team should include one PhD candidate, who is the driving force for continuous output; a clinician involved in the regular care of the affected individuals who receive the biopharmaceutical; and a pharmacist with experience of using the required laboratory techniques and clinical pharmacology. Other team members who could prove beneficial to the research team include experts in the disorder (i.e., other clinicians, medical staff or researchers), the clinical laboratory (e.g., laboratory analyst), health or pharmaco-economics, and academic supervisors (e.g., post-doc, co-promotor or promotor). Furthermore, patient and public involvement (PPI) is recommended and often required by subsidising parties or journals. The PPI significantly contributes to research and probably increases its quality [57-61].

3. Stakeholder involvement

Stakeholder involvement should begin early, preferably during the conceptualisation of the hypothesis. The primary stakeholders to involve are the research departments of the hospital, university, institution, clinic or pharmacy where the research team members are employed. The members must engage with their colleagues to gain knowledge and awareness, and to use the infrastructure. Furthermore, hospital financial departments often have internal financial programmes for supporting cost-efficient research because these efforts directly benefit the hospital.

The secondary stakeholders for clinical support are the hospitals, universities, institutions, clinics or pharmacies of external colleagues and the patient and caregiver organisations. It is important to engage these stakeholders to use existing infrastructures, such as the SYMPHONY consortium, or to promote study inclusion [62]. Lower dosing can be difficult from a patient's perspective, so a national standpoint from patient and caregiver organisations can help when explaining the risk–benefit ratio during informed consent as part of study enrolment.

The secondary stakeholders for financial support are healthcare insurance companies, drug regulators and governments. Research on cost-efficient dosing and personalised medicine are pertinent topics for these organisations' research agendas. For instance, in the Netherlands, the ZonMW and the NFU have programmes such as *GoedGebruikGeneesmiddelen* and *Transformatiegelden Dure Geneesmiddelen*, respectively. Funding research is highly attractive for these parties because successful research results in a high return on investment (e.g., one million euros invested in a study could result in hundreds of millions of euros of savings per year), generating additional savings for healthcare and new research projects. A manufacturer may even be interested in a funding role under specific agreements and conditions, such as the optimisation of user-friendly administrations to obtain a better product with increased sales and commercial value.

4. PKPD-relation investigation

The next step to developing a cost-efficient dosing strategy is to investigate the clinical pharmacology of the biopharmaceutical to set the therapeutic window. A descriptive study can be conducted on the pre-approval studies in the literature, although PKPD-modelling these data is usually more insightful. In this step, both the dose–concentration (PK) and concentration–response (PD) relationships should be investigated (**Chapter 6**). The PK can be linear (dose proportional) for saturated targets or nonlinear for target-mediated distributions, each requiring a different dosing strategy. The PD should be investigated to determine the concentration in which all target receptors are occupied (i.e., E_{max}) and also to determine the potency of the drug, which is the required concentration for a given response (i.e., concentration at half of response [EC_{50}]). The response can be a disease outcome, such as a clinical event, or a surrogate outcome parameter, such as a biomarker. The variability of the PK and PD parameters should also be evaluated to determine whether dose adjustments are required for the specific characteristics of an individual (**Chapter 6**).

Beyond the data from the literature, real-world data from a clinic can also be evaluated to investigate the PKPD relations (**Chapter 7**). Such data better reflect a real-world setting than data from the literature because a Phase IV setting is different and less

controlled than the strict Phase I–III trials. Real-world studies (e.g., on remnant material or by retrospective chart review) are currently popular and an excellent approach for evaluating potential overdosing and drug waste. Non-adherence to therapy contributes to variability and can also be assessed in a real-world study. The real-world data can be compared with the literature data to quantify the cost-efficient potential in the target population. For instance, emicizumab's dose could be halved because concentrations from the real-world study (**Chapter 7**) were ~60 µg/mL while the effectiveness plateau began already at ~30 µg/mL (**Chapter 6**).

5. Dosing strategy and measurement

A dose strategy can be simple (e.g., 'halve every dose' or 'give only one vial to an individual regardless of body weight') or straightforward calculated on the back of a napkin (e.g., 'give a ratio of a dose'), but can also be more complex and then dependent on PKPD modelling simulation. Additionally, dose-PK modelling of multiple cost-efficient strategies within a virtual population offers a rapid approach to determine the most optimal strategy. Examples of different strategies to consider include entire-vial dosing, dose-capping, stratified dosing (e.g., on BMI or age groups) or fixed dosing (i.e., everyone the same dose) in case of a body-weight-based dosing or vice versa (i.e., body-weight dosing if dosing was fixed). Additionally, several initiatives exist to combine a single-use vial for multiple individuals (e.g., when young siblings with haemophilia inject simultaneously) to avoid drug waste. Combining vials should occur in a controlled environment to minimise the risk of contamination and can be included in PK simulations as well. Moreover, the Phase IV experience can yield new user preferences that could be included in PK simulations to explore optimised user convenience. For example, this PK study simulated alternative dosing for emicizumab to minimise the burden of injection pain by extending the dosing intervals and lowering injection volumes [63].

Laboratory measurements of exposure (plasma concentration) or effect (biomarker) can be combined with the dosing strategy or can be mandatory for clinical evaluation (next step). Assays or methods for these measurements are often unavailable due to the novelty of biopharmaceuticals, meaning a method set-up is required. Ligand-binding assays have been the preferred choice for many years, although these have the disadvantages of interference, no multiplex capacity and expensive reagents with long production times [64-66]. The quantification of biopharmaceuticals with LC-MS/MS analysis has the advantages of high sensitivity, speed, resolution, accuracy, reproducibility and multiplex potential, as well as a short development time and small sampling volume. I recommend following the proposed six-step workflow to develop an LC-MS/MS method with bottom-up quantification, entailing the use of a signature peptide, with a multiplex run (**Chapter 2**).

6. Clinical evaluation

Cost-efficient dosing is often off-label. Efficacy and safety must be demonstrated in a clinical evaluation with well-designed, prospective clinical studies. Designs with blinding, randomisation and a (placebo-)control are favoured to exclude bias. However, to evaluate the cost-efficient dosing of biopharmaceuticals, blinding is impractical due to self-injections, randomising requires a large sample size, and using a placebo is unethical because of the drug's proven effectivity. Therefore, I recommend a crossover, noninferiority design with intensive monitoring for the clinical evaluation of cost-efficient dosing for biopharmaceuticals (**Chapter 8**). A crossover approach has the advantage of comparing treatment effects on one person, which increases study power and lowers the required sample size [67]. In addition, noninferiority tests whether cost-efficient dosing is not unacceptably less efficacious than conventional dosing [68]. However, testing for noninferiority makes the design and interpretation less straightforward than testing for superiority [69]. Therefore, choosing the noninferiority margin is crucial and should be based on both statistical reasoning and clinical judgement. Consulting a statistical expert and achieving a broad clinical consensus when setting noninferiority margins are highly recommended [68].

Conducting a clinical trial to evaluate cost-efficient dosing is labour intensive. Increasing technical innovations mean big data analytics and common data models herald a new era in the generation of evidence for clinical evaluations [70, 71]. A common data model is a standardised model that allows data exchange between multiple healthcare data sources and applications, such as different hospital systems, electronic healthcare records, clinical registries, databases and healthcare apps [72]. These models standardise the observational, real-world data to provide reliable, real-world evidence rapidly [73]. This process is especially beneficial for the clinical evaluation of rare indications that require multi-center or multinational studies.

7. Clinical-practice implementation

Cost-efficient dosing, combined with monitoring, can be implemented in clinical practice following a successful clinical evaluation. Funding parties generally require extensive implementation plans before funding is granted. Such plans often include commitment of the support base, set-up of measurement methods in laboratories, upscaling requirements, and revising protocols and guidelines. This plan should be executed in Step 7 of the cost-efficient dosing strategy in *Figure 3*. Another important effort to make, is a drug label change by including the new cost-efficient dosing strategy in the Summary of Product Characteristics (i.e., the drug label). A drug label change is the highest achievable goal for the research team because the drug label reaches clinicians and patients globally,

potentially increasing awareness, acceptance and drug access. The drug label is kept by the manufacturer, and lower profits would not be of direct interest. Therefore, a lucrative return for the manufacturer might be necessary to motivate such initiatives.

The cycle in *Figure 3* is based on a plan–do–check–act approach, enabling change implementation and continuous improvements in healthcare [74]. Many lessons will presumably be learned by the research team during the seven steps of the cycle. I recommend including these valuable lessons, following the Standards for Reporting Implementation Studies (StaRI), in the dissemination plan and to execute this plan in step 7 [75]. Dissemination of the research is highly recommended to inspire others, because every innovation begins with a new idea.

Critical notes

Biopharmaceuticals would not be on the market or even developed without their manufacturers. The pharmaceutical industry follows a profit-driven model to bring a drug to the market, which can create tension between the industry and healthcare. Potential ways to overcome this tension include price arrangements or greater public-sector involvement [76], both at an early stage of research and development (R&D) and between manufacturers, government, health insurance companies and hospitals. Pharmaco-economic evaluations, for instance, based on quality-adjusted life-years, may assist in setting a valid price for a biopharmaceutical. However, one disadvantage of these ideas is that the incentive to invest in R&D might decrease as investigators lose interest due to lower potential rewards. Although it may seem unethical to profit from a person's health, manufacturers have high expenses, such as obligations during expensive clinical studies, demanding regulatory requirements, risky R&D-development phases, patent constraints, high production-facility and marketing costs, as well as a need to generate a return on investment [77, 78]. Pharmaceutical companies and their investors take significant financial risks to gain high rewards. However, until government or health insurance companies also take such risks, it is crucial to find the right balance between financial profits and patient health. Therefore, researchers and clinicians must begin the cycles from *Figure 3* to provide counterweight to the current profit-driven model.

The four studies on emicizumab yielded a basis for designing a cost-efficient dosing framework for the biopharmaceuticals described in Theme 2. Our research team is currently on step 6 (Clinical evaluation) of the cycle in *Figure 3*. The results of the prospective clinical study are expected in 2026. These results will demonstrate whether there is a future role for the cost-efficient dosing and monitoring of emicizumab and whether the research team can proceed to the final measure, step 7 (Implementation in practice). If so, the team must perform the final step before the deal between the manufacturer and health insurance companies ends.

Concluding remarks

This thesis investigated how to optimise monitoring FVIII and emicizumab in PwHA. This thesis provides a framework for measuring therapeutic proteins, such as FVIII and emicizumab, in human plasma using LC-MS/MS bioanalysis (**Chapters 2, 3 and 5**). The concentration-biomarker relationship of FVIII and the dose–response relationship of emicizumab were investigated, yielding more questions than answers regarding FVIII (**Chapter 4**), but mainly answers for emicizumab (**Chapters 5–7**). Subsequently, I conducted studies to support the cost-efficient dosing of emicizumab, which could inspire others to dose other biopharmaceuticals more cost efficiently (**Chapters 6–8**). This thesis adds knowledge to the optimisation of monitoring FVIII and emicizumab in PwHA by addressing some issues while simultaneously raising others, and now future researchers, pharmacists and clinicans must further build on the findings of this thesis.

Author's contribution

AD conceived the idea and set-up the general discussion. AD conducted literature review, outlined and wrote the general discussion. Throughout the process, AD asked and implemented input and feedback from the supervision team.

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CHAPTER

Summary

Introduction

In **Chapter 1** a general introduction with background and rationale for the thesis was provided. Herein is explained that haemophilia A is a bleeding disorder caused by a deficiency of coagulation factor VIII (FVIII). The endogenous FVIII activity is used to classify severe (<1%), moderate (1 to 5%) or mild (>5 to 40%) haemophilia A. People with haemophilia A (PwHA) experience recurrent bleeding, predominantly into major joints, such as ankles, knees and elbows, leading to painful and disabling arthropathy. Currently, effective protection against bleeding is provided by replacement therapy using FVIII products or by emicizumab. Because FVIII products and emicizumab are costly, they can pressure healthcare budgets and limit patient access.

Measuring and monitoring are key in haemophilia A care. The FVIII activity is measured for diagnosing, but also to determine the dose of FVIII products. Monitoring emicizumab is not routinely performed, although guidelines recommend to measure its concentration in suspicion of anti-drug antibodies (ADA). The liquid chromatography (LC) coupled with tandem-mass spectrometry (MS/MS) analysis is a beneficial technique for quantifying therapeutic protein concentrations in human plasma. Prior to this thesis, the potential value of concentration measurements with LC-MS/MS analysis for monitoring PwHA in clinical practice was unclear. The knowledge gaps regarding this process concerned the relationship between the FVIII concentration (measured with LC-MS/MS) versus the FVIII activity (measured with clotting assays) and a rationale for emicizumab's concentration in routine daily practice.

In this thesis, the objective was to optimise drug monitoring of FVIII and emicizumab in PwHA by:

- developing and validating LC-MS/MS methods for their quantification in plasma;
- investigating the concentration–biomarker relationship of FVIII and the dose–concentration–response relationship of emicizumab; and
- proposing and evaluating a cost-efficient dosing strategy for emicizumab.

General LC-MS/MS method development

In recent years, LC-MS/MS methods have enabled absolute quantification of therapeutic proteins. These methods have additional benefits, such as a large linear dynamic range, a high specificity and the option of multiplexing. In **Chapter 2** we discussed the strategies for the quantification of therapeutic proteins in several biological matrices using LC-MS analysis based on top-down and middle-down quantitative proteomics. Then, we presented the widely used bottom-up method in a six-step workflow as a tutorial for quantitative LC-MS/MS method development. Considerations for signature peptide selection were provided and critical method parameters were discussed for

internal standard selection, chromatographic separation, sample purification, digestion, and method validation.

Measuring and monitoring FVIII using LC-MS/MS

Then, the LC-MS/MS technique was used to quantify FVIII in human plasma. The development and validation of this method were described in **Chapter 3**. Samples were prepared by first triggering the coagulation cascade, which freed FVIII from Von Willebrand factor, and then a selective immunoaffinity step was performed to selectively purify the active FVIII with camelid nanobodies. After heat denaturation and trypsin digestion, a FVIII specific peptide was used as a surrogate for quantification. The validation results were all well within the acceptance criteria of the current European Medicines Agency (EMA) guideline on bioanalytical method validation. This validated method allowed us to further investigate the relationship between the FVIII plasma concentration and the FVIII activity in PwHA in **Chapter 4**. A proof of principle, cross-sectional study was conducted on remnant plasma samples (n = 87) from PwHA from our clinic. The FVIII concentrations using LC-MS/MS analysis and the FVIII activities using a clotting assay were compared. An overall mean relative difference of -1% with an SD of 64% was demonstrated. Despite a good overall correlation between the two methods, several relative differences were large. Large differences between concentration and activity were correlated with the presence of anti-FVIII antibodies and use of exogenous FVIII products (e.g., plasma-derived and modified FVIII products). The clinical impact thereof is yet unclear. Therefore, more research is needed to determine the value of FVIII plasma concentration in comparison with FVIII activity.

Measuring and monitoring emicizumab using LC-MS/MS

Similarly, the LC-MS/MS technique was used to quantify emicizumab's concentration. A method validation study was performed on a generic sample preparation method with LC-MS/MS analysis in **Chapter 5**. The method demonstrated linearity over a wide range of emicizumab concentrations, far exceeding the concentrations observed in PwHA. Precision and accuracy were excellent and all other validation parameters were also well within the acceptance criteria of the current EMA guideline on bioanalytical method validation. A cross validation against a common clotting-based method demonstrated that both methods can be used interchangeable for drug monitoring of emicizumab, without the application of a correction factor.

Emicizumab has been approved with body-weight-based dosing without the necessity for laboratory monitoring. This assumes a clear dose–concentration–bleed relationship, with acceptable variability due to factors other than body weight. To investigate this assumption, a systematic review on the pharmacokinetics (PK) and associated efficacy of emicizumab in humans has been reported in **Chapter 6**. Data from 15 clinical studies

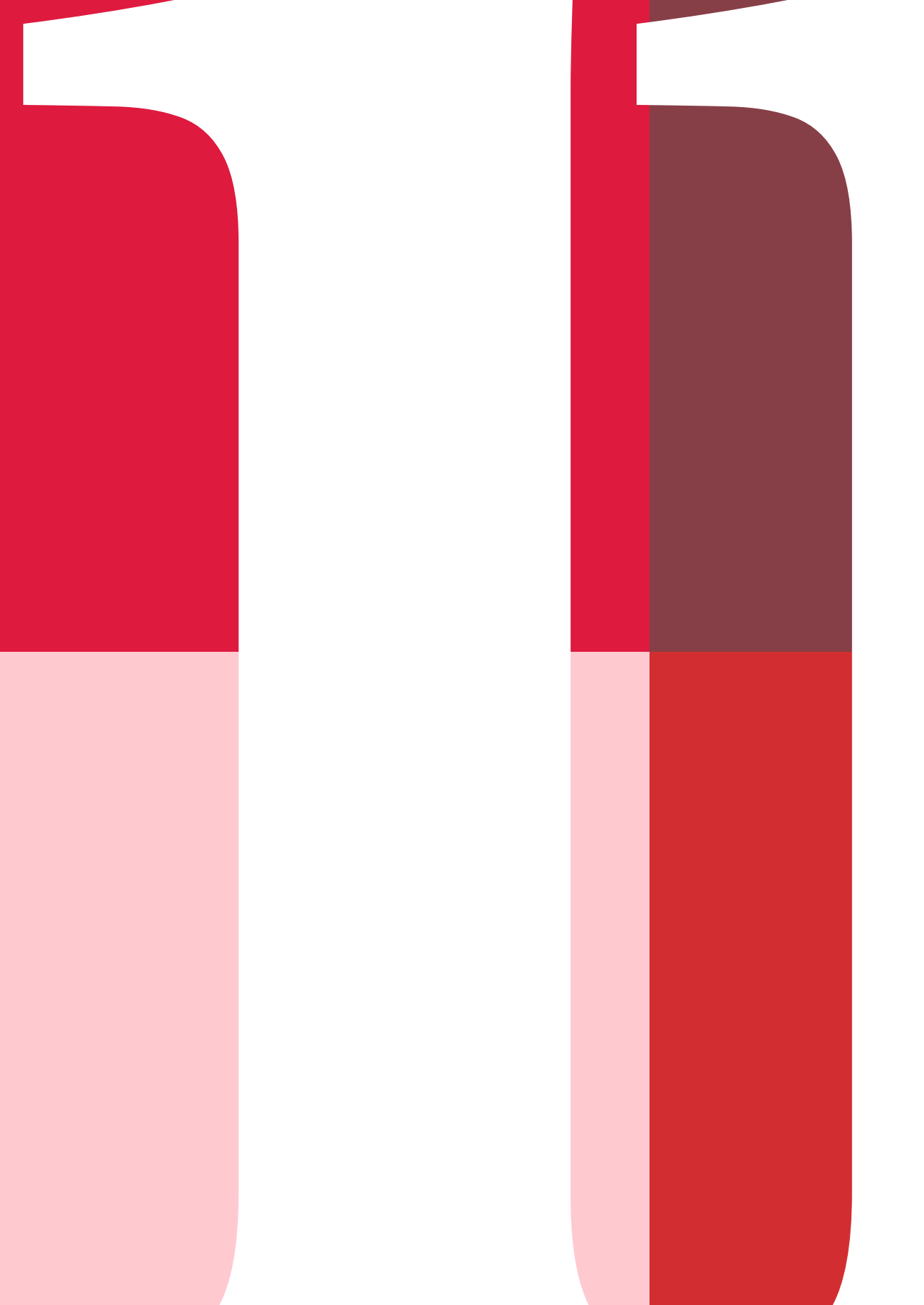
demonstrated dose–linear PK and a moderate (32%) inter-individual variability of trough concentrations (C_{trough}). The control of bleeds did not further improve above emicizumab concentrations of 30 µg/mL, potentially enabling lower dosing in a substantial proportion of PwHA. Therefore, the study supported body-weight-based dosing but suggested individualized monitoring of the emicizumab concentrations to dose more cost-efficiently.

The LC-MS/MS method (Chapter 5) and PK knowledge (Chapter 6) were then used to conduct a single-center, observational study, described in **Chapter 7.** Emicizumab is dosed on body-weight at 7-, 14- or 28-day intervals which often mismatches the vial content. To avoid drug waste, PwHA at our clinic received emicizumab doses of entire vials, but with variable intervals. Real-world evidence on concentrations, bleeds and drug waste was collected and evaluated. A total of 112 individuals (94% severe phenotype and 9% FVIII-inhibitor positive) were followed for a year before and a year during emicizumab therapy. Therapeutic concentrations of emicizumab were observed. The annualized treated bleeds reduced significantly from 3.6 before to 0.8 during emicizumab. Drug waste was reduced by 9%. The entire-vial dosing of emicizumab seemed an attractive treatment option for PwHA leading to therapeutic concentrations, good bleeding control and drug waste avoidance.

Dosing according to label resulted in C_{trough} of 55 µg/mL, while moderate variability and an effective C_{trough} of 30 µg/mL have been reported. This statement in combination with the three thesis Chapters 5, 6 and 7 formed the groundwork for a study protocol described in **Chapter 8**. The DosEmi study has a phase IV, multicenter, open-label, crossover design. The study will evaluate noninferiority of bleed control of ≥6 months on conventional dosing in comparison to ≥6 months on dose intervention. The dose intervention consists of reducing the dose to target a C_{trough} of 30 µg/mL using individual-PK parameters. The DosEmi study was approved by the local Medical Ethics Review Committee and is currently including participants to further investigate the findings from this thesis.

Discussion and conclusion

In **Chapter 9** general discussion, two themes further built upon the results from the thesis. The first theme included four potential future applications for monitoring FVIII concentrations in haemophilia A using LC-MS/MS analysis: multiplexing, discrepant FVIII activity, neonatal screening and dried-blood-spot sampling. In the second theme, a framework for cost-efficient dosing strategies of biopharmaceuticals was provided from idea to clinical-practice implementation. This thesis added knowledge to the optimisation of monitoring FVIII and emicizumab in PwHA by addressing some issues while simultaneously raising others, and now future researchers, pharmacists and clinicians must further build on the findings of this thesis.



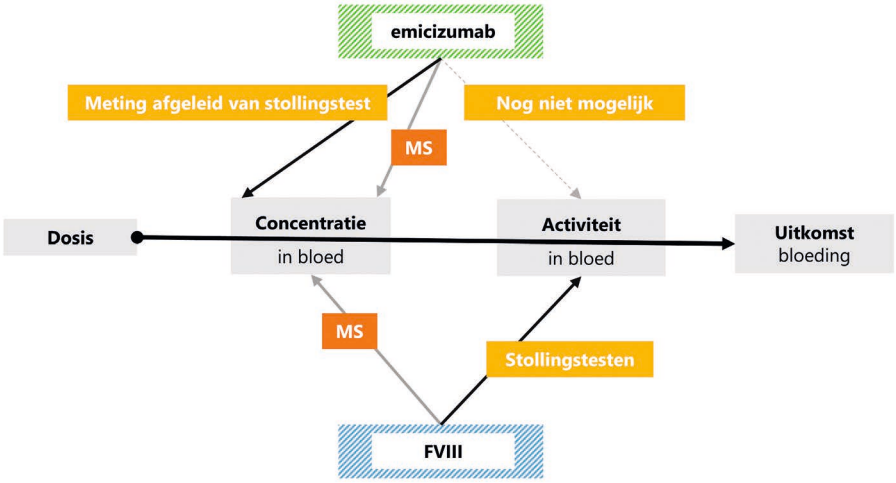
CHAPTER

Nederlandse lekensamenvatting

Achtergrond

Een algemene introductie wordt gegeven in **Hoofdstuk 1**. Hierin wordt uitgelegd dat hemofilie A een stollingsstoornis is, die wordt veroorzaakt door een tekort aan het stollingseiwit factor VIII (hierna 'FVIII'). Personen met hemofilie A hebben daardoor last van bloedingen. Bloedingen treden voornamelijk op in de grote gewrichten zoals enkels, knieën en ellebogen. Uiteindelijk leiden die bloedingen tot invaliderende gewrichtspijn. Momenteel wordt effectieve bescherming tegen bloedingen gegeven door geneesmiddelen toe te dienen in een bloedvat. Deze geneesmiddelen, ook wel FVIII producten, zijn therapeutische eiwitten die vrijwel gelijk zijn aan het lichaamseigen stollingseiwit FVIII. Deze FVIII producten worden verkregen uit donoren of worden biologisch geproduceerd. Daarnaast is in 2018 een nieuw geneesmiddel op de markt gekomen voor hemofilie A, genaamd emicizumab. Dit is ook een therapeutisch eiwit dat de functie van FVIII nabootst, maar het heeft een andere eiwitstructuur. Het voordeel van emicizumab ten opzichte van de FVIII producten is de toedienweg onder de huid (in plaats van in een bloedvat) en het minder vaak toedienen namelijk wekelijks, tweewekelijks of vierwekelijks (in plaats van 2–7 keer per week). De FVIII producten en emicizumab kosten grofweg tussen de €100.000 tot €400.000 per patiënt per jaar. Daardoor zetten deze geneesmiddelen het ziekenhuisbudget onder druk en is de beschikbaarheid wereldwijd, en zeker in lage-inkomenslanden, beperkt.

Het meten en monitoren van de FVIII activiteit (mate van stollen, hierna 'activiteit') bij personen met hemofilie A is belangrijk, zie onderstaande *Figuur 1*. Om hemofilie A te diagnosticeren, wordt de activiteit van een persoon in het bloed gemeten. De activiteit wordt ook routinematig gemeten voor het vaststellen van de dosis van de FVIII producten. De stollingstesten voor het meten van de activiteit hebben als nadeel dat ze niet altijd de juist waarde afgeven door verstorende stoffen (zoals andere geneesmiddelen). Monitoren van emicizumab in het bloed wordt niet routinematig gedaan, hoewel richtlijnen aanbevelen om de emicizumab concentratie te meten bij verdenking op antistoffen die emicizumab ineffectief maken.



Figuur 1. Dosis–concentratie–activiteit–bloedingsrelatie van factor VIII en emicizumab.

In veel laboratoria wordt wereldwijd een analysemethode gebruikt, die met behulp van massaspectrometrie (hierna 'MS') de absolute concentratie van therapeutische eiwitten in het bloed kan meten. De MS methode heeft als bijkomende voordelen: het meten van extreem lage concentraties en weinig last van storende stoffen. Er bestond echter nog geen MS methode voor het meten van de concentraties van FVIII en emicizumab. Het was onduidelijk of het opzetten van MS methodes hiervoor zinvol was. Vragen die bij ons opkwamen, waren 'Wat is de relatie tussen de FVIII concentratie en de FVIII activiteit bij personen met hemofilie A?' en 'Is het zinvol om de emicizumab concentratie te meten in de dagelijkse praktijk van de zorg voor personen met hemofilie A?'.

Het doel van dit proefschrift was optimalisatie van het monitoren van FVIII en emicizumab in het bloed van personen met hemofilie A door:

- het ontwikkelen en valideren van MS methoden voor het meten van FVIII en emicizumab concentraties in het bloed van personen met hemofilie A;
- het onderzoeken van de relatie tussen de FVIII concentratie en FVIII activiteit, en de relaties tussen de dosis, concentratie en bloedingen van emicizumab; en
- het evalueren van een kostenefficiëntere doseringsstrategie voor emicizumab ten opzichte van de huidige manier van doseren.

Algemene massaspectrometrie methodeontwikkeling

Traditioneel gezien waren immunologische testen de eerste en soms zelfs de enige mogelijkheid voor het meten van concentraties of activiteiten van therapeutische eiwitten in bloed. Echter, de methodeontwikkelingsfase was vaak lang, bijvoorbeeld door

de afhankelijkheid van complexe en dure hulpstoffen. De afgelopen jaren hebben MS gebaseerde methoden daar verandering in gebracht. Deze methodes kunnen relatief snel worden ontwikkeld en gevalideerd. Ze hebben ook andere voordelen, zoals het meten van extreem lage concentraties, weinig last van storende stoffen en de mogelijkheid om verschillende eiwitten tegelijkertijd te meten, ook wel multiplexen genoemd. In **Hoofdstuk 2** introduceren we een 6-stappen-handleiding voor het opzetten van een MS methode voor concentratiemetingen van therapeutische eiwitten in het algemeen. In dit hoofdstuk leggen we de verschillende strategieën uit voor het kwantificeren van therapeutische eiwitten in menselijk materiaal. *Top-down*, *middle-down* en *bottom-up* eiwitkwantificatie worden uitvoerig besproken en tot slot wordt de meest gebruikte techniek op basis van *bottom-up* eiwitkwantificatie beschreven met voor- en nadelen.

Meten en monitoren van factor VIII met massaspectrometrie

Vervolgens hebben we een MS methode voor het meten van de FVIII concentratie in het bloed opgezet. De ontwikkeling en validatie hiervan staan beschreven in **Hoofdstuk 3**. De voorbehandeling van de bloedmonsters begint met initiëren van de stollingscascade. Hierdoor werd FVIII vrijgemaakt van Von Willebrand factor en kwam actief FVIII beschikbaar om te meten. Vervolgens werd een selectiestap met zogenaamde *nanobodies* uitgevoerd om het actieve FVIII uit het bloedmonster te trekken en te isoleren. Hierna volgt een hitte behandeling om de eiwitten uiteen te laten vallen en wordt trypsine toegevoegd om de eiwitten in nog kleinere stukjes te knippen. Tot slot worden de monsters in het MS apparaat ingevoerd en gemeten. Alle validatieparameters waren ruim binnen de acceptatiecriteria van de vigerende Europese richtlijn voor bioanalytische methodevalidatie. De methode was succesvol opgezet en gevalideerd. Deze gevalideerde MS methode stelde ons vervolgens in staat om de relatie tussen de FVIII concentratie en FVIII activiteit te onderzoeken in **Hoofdstuk 4**. Een *proof of principle* onderzoek werd uitgevoerd op 87 bloedmonsters (restmateriaal) van personen met hemofilie A uit onze kliniek. In elk bloedmonster werd zowel de FVIII concentratie gemeten met behulp van de MS methode (Hoofdstuk 3) als de FVIII activiteit met behulp van een standaard stollingstest. De FVIII concentratie werd vergeleken met de FVIII activiteit. Een gemiddeld relatief verschil van -1% met een spreiding van 64% werd tussen de concentratie en activiteit aangetoond. Ondanks een goede algemene correlatie vertoonden enkele bloedmonsters een relatief groot verschil tussen de concentratie en de activiteit. Deze grote verschillen tussen concentratie en activiteit waren gecorreleerd aan de aanwezigheid van anti-FVIII antistoffen en het gebruik van FVIII producten (bijvoorbeeld uit plasma verkregen of gemodificeerde FVIII producten). De impact van deze grote verschillen is nog onduidelijk. Daarom is meer onderzoek nodig om de meerwaarde van FVIII concentratie in vergelijking met FVIII activiteit vast te stellen.

Meten en monitoren van emicizumab met massaspectrometrie

De wereldwijde standaard methode voor het meten van de emicizumab concentratie in bloed is een gemodificeerde en gekalibreerde stollingstest. De 'emicizumab stollingsactiviteit' wordt in deze meting omgezet naar de emicizumab concentratie. Deze standaard methode voldoet redelijk, maar kan verstoring ondervinden en heeft niet alle voordelen van de MS methode. Wij hebben daarom ook een MS methode voor het meten van emicizumab in het bloed opgezet. Deze methode maakt gebruik van een simpele en generieke voorbehandeling van de bloedmonsters. In **Hoofdstuk 5** is het validatieonderzoek van de MS methode beschreven. Alle validatieparameters waren ook ruim binnen de acceptatiecriteria van de vigerende Europese richtlijn voor bioanalytische methodevalidatie. Er werd tevens een kruisvalidatie uitgevoerd tegenover de standaard methode. Het vergelijk toonde aan dat beide methoden uitwisselbaar kunnen worden gebruikt voor het monitoren van de emicizumab concentratie, zonder toepassing van een correctiefactor.

Emicizumab is geregistreerd met een dosering op basis van lichaamsgewicht zonder de noodzaak van laboratoriumcontroles. Dit veronderstelt een duidelijke dosis–concentratie–bloedingsrelatie (zoals zichtbaar in *Figuur 1*), met aanvaardbare variabiliteit als gevolg van andere factoren dan het lichaamsgewicht. Om deze veronderstelling te onderzoeken is in **Hoofdstuk 6** een systematisch literatuuroverzicht gepresenteerd betreffende de farmacokinetiek (hierna 'PK') en de daarmee samenhangende werkzaamheid van emicizumab. De PK beschrijft wat het lichaam met het geneesmiddel doet en de werkzaamheid de preventie van bloedingen. We hebben de PK- en werkzaamheidgegevens van 15 grote onderzoeken in mensen gecombineerd. Vervolgens vonden we dat emicizumab een lineair verband toonde tussen de dosis en de concentratie in het bloed, omdat de concentratie dosisproportioneel toenam. Ook werd een matige (32%) variabiliteit van concentratie tussen personen gevonden. We vonden daarnaast dat de bescherming tegen bloedingen niet toenam bij een concentratie boven 30 µg/mL terwijl de meesten rond 55 µg/mL zaten. Hierdoor is het mogelijk om ongeveer de helft van de dosering te geven bij een aanzienlijk deel van de personen. Het onderzoek ondersteunde daarom het doseren op basis van lichaamsgewicht, maar opperde om de emicizumab concentratie per individu te controleren om zo lager te kunnen doseren.

De MS methode (Hoofdstuk 5) en de PK kennis (Hoofdstuk 6) worden vervolgens gebruikt om een observationeel onderzoek in één centrum uit te voeren, beschreven in **Hoofdstuk 7**. Emicizumab wordt gedoseerd op basis van lichaamsgewicht met intervallen van 7, 14 of 28 dagen, wat vaak niet overeenkomt met de exacte inhoud van een injectieflacon. Om verspilling van emicizumab te voorkomen, ontvingen patiënten in onze kliniek de volledige inhoud van de injectieflacons en werd zo nodig het doseerinterval hierop aangepast. We verzamelden gegevens over de concentraties, bloedingen en spillage van half 2018 tot en met januari 2022. In totaal werden 112 personen gevolgd. Elke persoon

volgende we een jaar vóór en een jaar tijdens de emicizumab therapie. Vervolgens vonden we therapeutische concentraties van emicizumab. We vonden dat het jaarlijks aantal bloedingen aanzienlijk verminderde van 3,6 vóór tot 0,8 tijdens emicizumab therapie. De verspilling verminderde met 9%. We concludeerden dat het doseren op basis van volledige injectieflacons een aantrekkelijke behandelingsoptie was die leidde tot therapeutische concentraties, goede bloedingscontrole en het vermijden van spillage.

Doseren volgens de bijsluiter resulteert in een concentratie van 55 µg/mL, terwijl matige variabiliteit en een effectieve concentratie van 30 µg/mL zijn gerapporteerd. Deze veronderstelling in combinatie met de drie Hoofdstukken 5, 6 en 7 vormden de basis voor het opstellen van een onderzoeksprotocol dat we in **Hoofdstuk 8** beschreven. Het onderzoek heet DosEmi en zal worden uitgevoerd in zes verschillende ziekenhuizen in Nederland. De bloedingen die optreden gedurende 6 maanden op de standaard dosering worden vergeleken met de bloedingen gedurende 6 maanden op een lagere dosering. We onderzoeken of de lagere dosering in ieder geval niet slechter is, ook wel non-inferioriteit genoemd. De lagere dosering wordt bepaald aan de hand van de emicizumab concentratie in het bloed van de individuele onderzoeksdeelnemer. We passen de dosering zodanig aan, dat de onderzoeksdeelnemer precies op een concentratie van 30 µg/mL zal uitkomen. We hebben immers aangetoond, dat er geen meerwaarde in effectiviteit is door hogere concentraties van emicizumab. Het DosEmi onderzoek is al goedgekeurd door de plaatselijke Medisch Ethische Toetsingscommissie en is gestart met het includeren van onderzoeksdeelnemers om de bevindingen van dit proefschrift verder te onderzoeken.

Discussie en conclusie

In de algemene discussie, **Hoofdstuk 9**, wordt in twee thema's voortgebouwd op de resultaten van het proefschrift. Thema 1 betreft vier potentiële toekomstige toepassingen van MS metingen van FVIII concentraties bij hemofilie A: multiplexen, afwijkende FVIII activiteit, neonatale screening en bemonstering door middel van gedroogde bloeddruppels op een papiertje. Daarnaast wordt in Thema 2 een platform voor kostenefficiënte doseringsstrategieën van therapeutische eiwitten gepresenteerd; van idee tot implementatie in de praktijk. De maatschappelijke relevantie van geneesmiddelbesparing kwam aan bod. Dit proefschrift voegde kennis toe aan de optimalisatie van het monitoren van FVIII en emicizumab bij personen met hemofilie A door sommige vraagstukken op te lossen en tegelijkertijd nieuwe vraagstukken aan de orde te stellen. Nu is het aan de toekomstige onderzoekers, apothekers en artsen om verder te bouwen op de bevindingen van dit proefschrift.

APPENDICES

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About the author

Anouk Donners was born on the 16th of February 1991 in Heerlen, the Netherlands, as the first of three daughters. In 2009, she graduated from secondary school (Gymnasium) at Trevianum Scholengroep in Sittard. She obtained both her Bachelor's (2013) and Master's degree (2015) in Pharmacy at Utrecht University. She participated actively in several student committees and organizations during her studies.

As part of her Master program, she performed a research internship about perceived immune status and sleep at the Henry Ford Sleep Research Center in Detroit, USA. Additionally, she performed two internships at the hospitals of Maastricht and Leiden, because of her affinity with an academic hospital setting. These three internships highly motivated her to combine research and care, due to the complexity and the potential clinical impact on a patient's therapy.

After graduating as a pharmacist, Anouk started her career in the clinical pharmacy of the University Medical Center Utrecht (UMCU) in 2016. Here, she was accepted for a pharmacy residency program in combination with a PhD project. From the start thereof, she was fascinated by innovative drugs, such as biopharmaceuticals and advanced therapy medicinal products (ATMPs). Consequently, she focused on the pharmaceutical laboratory and protein analysis, manufacturing of ATMPs and was trained as a Qualified Person during her residency program.

After completing her residency program in 2019, she continued with her PhD project at the department of clinical pharmacy (prof. dr. Toine Egberts and dr. Karin Rademaker) in close collaboration with the Van Creveldkliniek (prof. dr. Roger Schutgens and dr. Kathelijn Fischer). During her PhD project, she worked on measuring and monitoring of factor VIII and emicizumab and the clinical implications in people with haemophilia A. Currently, she works as a hospital pharmacist and as a Qualified Person (with a focus on ATMPs) in the department of clinical pharmacy of the UMCU.

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Dankwoord

Terugkijkend op de laatste 7 jaren, heb ik veel om dankbaar voor te zijn. Promoveren wordt vaak vergeleken met topsport. Dit inspireerde me om het dankwoord te schrijven naar analogie van een grote wielerronde: 'Tour de Promo'. Deze grote ronde leidde mij door uitdagende weersomstandigheden en langs fenomenale vergezichten, door diepe dalen en over prachtige pieken. De finish van de slotetappe genaamd 'hora est' is eindelijk in zicht.

Het is onmogelijk om een grote ronde alleen te rijden. Je hebt een team van ploegleiders, mechaniekers en verzorgers nodig om je optimaal voor te bereiden en begeleiden. Voor de proloog, het startschot van mijn promotie, vond ik in Toine Egberts en Karin Rademaker de ideale ploegleiding. Ik kreeg de strategische opdracht: "Rijd de ronde die jij wilt". Jullie gaven mij de vrijheid en het vertrouwen om mijn eigen koers te kleuren. Niet veel later breidde het team uit met de twee gerenommeerde ploegleiders Roger Schutgens en Kathelijn Fischer. Vanaf daar gold: Het is koers! Samen steggelden jullie over tactieken en strategieën. Jullie vormden en slepen me, zodat ik in optimale wedstrijdconditie aan de start kon verschijnen.

Allereerst, wil ik graag **Toine** bedanken voor je betrouwbaarheid, betrokkenheid en academische vorming. Ik bewonder hoe je een antwoord wist op iedere vraag, je elke afspraak nakwam en je 24/7 voor me klaar stond, ondanks een overvolle agenda en alle promovendi die je begeleidde. Je hebt op een bepaald moment de rol van zowel promotor als copromotor aangenomen, waardoor ik me enorm gesteund voelde. Daarnaast tilde je mijn onderzoek naar een hoger niveau met inspirerende gedachtewisselingen en een scherp oog voor detail. Wat ik koester is dat ik me onder jouw vleugels heb kunnen ontwikkelen tot een allround onderzoeker. **Karin**, jou wil ik graag bedanken voor je sturing en zorgzaamheid. Je was zowel copromotor als opleidster, twee rollen die je met warmte en daadkracht vervulde. Als geen ander kende je mijn valkuil om te hard te gaan. Je zorgde ervoor dat ik niet uit bocht vloog. Als mijn hoofdmecanicien stelde je continu mijn versnellingen en remmen af én leerde je me dat zelf te doen. Daarnaast heb je me meermaals opgelapt als ik ten val kwam en ik de rit even niet meer zag zitten. Samen maakten we een actieplan in jouw kamer in het WKZ, waardoor ik weer met frisse moed opstapte en verder kon. **Kathelijn**, aan jouw reactiesnelheid op mailtjes, appjes, telefoontjes kon ik afleiden dat je steevast in de eerste volgauto zat. Vroeg ik om feedback op een conceptversie, dan kwam dat vrijwel per ommegaande (rood) terug. Van jou heb ik geleerd dat een deadline je beste vriend is. Door jouw klinische inbreng en jouw bekendheid in én met het onderzoeksveld was je de ideale aanvulling als copromotor. Specifiek wil ik je bedanken voor je eerlijke mening. De eerste meeting was het voor mij even wennen, maar al snel ben ik je mening enorm gaan waarderen. Het bracht scherpte en duidelijkheid in de discussie waardoor ik kon groeien. **Roger**, jou wil ik graag bedanken voor de ploegleidersrol op de achtergrond. Met een tweede promotor als jou, kon ik

erop vertrouwen dat iemand een oogje in het zeil hield. Ik wil je ook bedanken voor je scherpe klinische inbreng, je visionaire ideeën en enthousiasme. **Erik**, ook jou wil ik graag bedanken. Je geloofde in me en je was een trotse en enthousiaste copromotor met een visie. Je was een legende in het veld. Uiteraard wil ik je ook bedanken voor de naam van onze grote klinische studie: DosEmi. Ik had graag dit proefschrift aan je overhandigd.

De ploegensamenstelling is meermaals uitgebreid en gewisseld. Graag wil ik op de eerste plaats mijn belangrijkste meesterknechten **Yrea**, **Lisanne** en **Eveline** bedanken. Gedurende de opleiding tot ziekenhuisapotheker, heb ik lang in jullie wiel mogen rijden. Mede door jullie hoge wattages heb ik de slotetappe gehaald. Dank voor de noeste arbeid tijdens jullie onderzoeksstages, maar ook voor de hilarische momenten en gezelligheid. Graag wil ik daarnaast mijn trouwe trainingsmaten **Mohsin**, **Konrad**, **Rolf**, **Albert** en **Alwin** bedanken. Jullie waren ontzettend fijne co-auteurs, waarvan ik veel heb geleerd. Ik hoop nog vaak met jullie samen te werken in de toekomst. Veel dank aan **Evelyn** en **Marieke**, jullie stonden bij elke vraag voor me klaar. In het bijzonder dank aan **Cees** voor je inspirerende inbreng en verbinding.

Beste lezer, een vraag die mogelijk bij u opkwam tijdens het lezen van dit dankwoord: Is er doping gebruikt in deze Tour de Promo? Er wordt beweerd dat promoveren op topniveau niet zonder farmacologische ondersteuning kan. Het antwoord op de vraag is simpelweg 'ja' en sterker nog: Zonder de doping van mijn collega's in het UMC Utrecht, had ik het niet gered. Graag wil ik mijn onderzoeks- en opleidingsbuddy's **Laurent**, **Heshu**, **Bastiaan**, **Heleen**, **Laura**, bedanken voor de goede adviezen en diepgaande gesprekken waarin we menige worstelingen (en successen) deelden. Dank aan de **collega's V&K** die tijdens periodes van 'oranje' of 'rood' de nodige cafeïne doping bij de Pitstop faciliteerden. Dank aan **Gera** voor je "preken", die me steeds weer opbeurden. Dank aan **Ingeborg** voor je persoonlijke manier van opleiden met oog voor het individu. Ook veel dank aan **Kim** en **Matthijs** voor jullie lab expertise gedurende het onderzoek. Bij jullie was alles mogelijk en jullie dachten altijd mee met een onderzoeker. Dank aan **Yves** voor je check-ins onderweg. Dank voor de overige verboden middelen die ik kreeg van de Squaties (**Laura** en **Laurent**) en de UMC AIOS crew in de vorm van feestavonden die mijn gedachten op nul zetten. In het bijzonder dank aan **Jeroen** en **Erin** voor de mechanische doping door middel van Strava lead-outs. Als laatste heb ik bemoedigende woorden voor de jonge collega's in witte trui die hun eigen Tour de Promo nog rijden: Velen gingen jullie voor en Parijs is nog ver, maar ik vertrouw erop dat jullie het redden tot de meet.

Tijdens de Tour de Promo, was ik gezegend met een laaiend enthousiast publiek en toegewijd thuisfront. Liefste **Louies**, **Ladies** en **Vaartjes** jullie waren het beste publiek dat ik me kon wensen. Dank voor jullie geduld, luisterend oor en welkome afleiding als ik dat nodig had. Lieve zusjes, **Simone** en **Kristel**, ik kan me niemand beter aan mijn zijde voorstellen. Jullie vervullen je rol als paranimf met verve waardoor ik met een gerust hart kan uitkijken naar de finish. Daarbij speciaal dank aan Simone voor de (late)

trainingssessies in de toren en aan Kristel voor je onderhandel en organisatie skills. Lieve **papa** en **mama**, ik draag dit proefschrift op aan jullie. Dank voor de onvoorwaardelijke en warme thuisbasis waar ik altijd naartoe mag terugkeren. Speciaal dank aan Piet voor de hoogtestages met kuitenbijters in het zonnige zuiden en aan Margie voor de beste koerssoep, haute cuisine pasta's en overige foeragering. Dierbaar thuisfront jullie weten het: Alles. Lieve **David**, dankjewel voor je zorgzaamheid, geduld en enthousiasme voor mijn tour. In het bijzonder wil ik je bedanken voor het design ervan, alleen met jou kon ik het concept van de opgedane kennis zo toepasbaar vormgeven tot dit eindresultaat. Ik kijk uit naar de volgende tours met jou.


V D R C M L I A V Q
D Y Y L Q E Q A Q V
A M Y L I W W S P Q
R Q L R E V V G G L
F S G F L S R F G V
P D A C S S Q T S E
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