UNIVERSITÀ DEGLI STUDI DI MILANO

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Global coagulation assays and hemophilia arthropathy scores for monitoring emicizumab prophylaxis in patients with hemophilia A

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Abstract

Background: Non-replacement therapies are emerging as valuable treatments in hemophilia patients. However, despite promising findings, there is no monitoring technique. The first non-replacement product to be approved for people with severe hemophilia A (HA) was emicizumab. Despite its remarkable efficacy in the reduction of bleeding rate, some patients still suffer from spontaneous joint hemorrhages and no objective method able to monitor drug efficacy or to stratify patient's hemorrhagic risk is available.

Objectives: To perform a comprehensive investigation of two global coagulation assays, namely non-activated thromboelastography (NATEM) and thrombin generation assay (TGA) in their ability to monitor the effects of emicizumab therapy and their ability to correlate with the bleeding phenotype. The clinical and instrumental assessment of joint involvement was evaluated as well.

Methods: All HA patients on emicizumab prophylaxis referring to our Centre were enrolled in a prospective study (from the 1st of January 2021 to the 1st of October 2022). TGA was assessed according to Hemker with a homemade method upon addition of tissue-factor (1pM) to plasma and synthetic phospholipids (1µM). Emicizumab concentration was measured by the modified calibrated one stage FVIII assay. NATEM was performed in citrated whole blood within 30 minutes from sampling, after addition of CaCl2 (100mM). Plasma levels of the prothrombin fragment F1+2, a reliable marker of in vivo thrombin generation, were measured with an ELISA (Enzygnost F1+2; Siemens Healthcare Diagnostics™). A trained physiotherapist collected the hemophilia joint health score (HJHS), based on the physical examination on six index joints (elbow, knee and ankle pairs) and an expert rheumatologist performed the hemophilia early arthropathy detection ultrasound (HEAD-US) score.

Spearman's Rho correlation coefficient was used to assess the association between the variables. Then a Cox proportional hazards model was used to evaluate the association between each laboratory parameter with the bleeding outcome (annualized bleeding rate [ABR]). A model capable of predicting the spontaneous bleeding risk was investigated with ROC analysis.

Results: Thirty-eight patients with HA on emicizumab prophylaxis were enrolled, 36 adults and two children. Of these, 26 started emicizumab during the study and 12 were already on maintenance at study entry. The reported median total ABR throughout the study was 0.70 (95%CI 0.51 - 0.89). In particular, the median post-traumatic ABR was 0.34 (95%CI 0.21 - 0.47) and the median spontaneous joint ABR was 0.30 (95%CI 0.18 - 0.43). Nine out of the 38 patients developed spontaneous joint bleeding.

Correlations between global coagulation assays and emicizumab plasma concentration were assessed. The endogenous thrombin potential was increased after emicizumab treatment but still lower than the normal range, in association with an increased *in vivo* thrombin generation, as indicated by increased F1+2 plasma levels.

TGA parameters (namely peak thrombin, time to peak and velocity index) showed differences between patients with or without spontaneous bleeding, while NATEM parameters and F1+2 levels did not.

ROC analysis revealed that the total synovitis and HEAD-US scores were predictive parameters of developing spontaneous joint bleeding (AUC 0.85).

Conclusion: Patients on emicizumab prophylaxis demonstrated an increased thrombin generation both as potential and actual. Global coagulation assays could represent promising tools for monitoring emicizumab therapy and the degree of hemophilic arthropathy might help predicting the spontaneous joint bleeding risk.

Abstract

Introduzione: Le terapie non sostitutive stanno emergendo come valido trattamento nei pazienti affetti da emofilia. Tuttavia, nonostante i risultati promettenti, non esiste una tecnica di monitoraggio. La prima terapia non sostitutiva approvata per le persone affette da emofilia A grave (HA), con o senza inibitori, è stato emicizumab. Nonostante la sua notevole efficacia nella riduzione del tasso di sanguinamento, alcuni pazienti soffrono ancora di emartri spontanei e non è ad oggi disponibile un metodo di laboratorio in grado di monitorare l'efficacia del farmaco o di stratificare i pazienti per il rischio emorragico.

Obiettivi: Effettuare un'indagine completa su due test di coagulazione globale, ovvero la tromboelastografia non attivata (NATEM) e il test di generazione della trombina (TGA), per quanto riguarda la loro capacità di monitorare gli effetti della terapia con emicizumab e la loro capacità di correlarsi al fenotipo di sanguinamento. Anche il coinvolgimento articolare (il grado di artropatia emofilica) è stata valutata con un modello di previsione degli emartri spontanei.

Metodi: Tutti i pazienti affetti da HA in profilassi con emicizumab afferenti al nostro centro sono stati arruolati in uno studio prospettico (dal primo gennaio 2021 al primo ottobre 2022). La TGA è stata valutata secondo Hemker con un metodo home-made con l'aggiunta di fattore tissutale (1pM) al plasma e fosfolipidi sintetici (1µM). La concentrazione di emicizumab è stata misurata con il metodo one-stage per FVIII modificato e calibrato. Il NATEM è stato eseguito in sangue intero citrato entro 30 minuti dal prelievo, dopo l'aggiunta di CaCl2 (100mM). I livelli plasmatici del frammento di protrombina F1+2, un marcatore affidabile della generazione di trombina in vivo, sono stati misurati con un ELISA (Enzygnost F1+2; Siemens Healthcare Diagnostics). Un fisioterapista esperto ha raccolto lo score clinico di salute articolare dell'emofilia (HJHS score), basato sull'esame fisico di sei articolazioni indice (gomiti, ginocchia e caviglie) e un reumatologo esperto ha raccolto lo score ecografico che attesta l'artropatia emofilica sulle medesime articolazioni (HEAD-US score).

Il coefficiente di correlazione Rho di Spearman è stato utilizzato per valutare l'associazione tra le variabili. Quindi è stato utilizzato un modello di Cox per valutare l'associazione tra ciascun parametro di laboratorio e l'esito del sanguinamento (tasso di sanguinamento annualizzato [ABR]). Con l'analisi ROC è stato studiato un modello in grado di prevedere il rischio di sanguinamento spontaneo.

Risultati: Sono stati arruolati trentotto pazienti con HA in profilassi con emicizumab, 36 adulti e due bambini. Di questi, 26 hanno iniziato emicizumab durante lo studio e 12 erano già in fase di mantenimento all'ingresso nello studio. L'ABR totale mediano riportato nel corso dello studio è stato di 0,70 (95%CI 0,51 - 0,89). In particolare, l'ABR mediano post-traumatico è stato di 0,34 (95%CI 0,21 - 0,47) e l'ABR degli emartri spontanei è stato di 0,30 (95%CI 0,18 - 0,43). Nove dei 38 pazienti hanno sviluppato un'emorragia articolare spontanea.

Sono state valutate le correlazioni tra i test di coagulazione globale e la concentrazione plasmatica di emicizumab. Il potenziale trombinico endogeno è risultato aumentato dopo il trattamento con emicizumab, ma ancora inferiore al range di normalità, in associazione a un'aumentata generazione di trombina in vivo, come indicato dall'aumento dei livelli plasmatici di F1+2.

I parametri del TGA (il picco di trombina, il tempo al picco e l'indice di velocità) hanno mostrato differenze tra i pazienti con o senza emorragia spontanea, mentre i parametri NATEM e i livelli di F1+2 non hanno mostrato differenze.

L'analisi ROC ha rivelato che i punteggi di sinovite e HEAD-US erano parametri predittivi dello sviluppo di emorragie articolari spontanee (AUC 0,85).

Conclusioni: I pazienti sottoposti a profilassi con emicizumab hanno dimostrato un aumento della generazione di trombina sia potenziale che reale. I test di coagulazione globale potrebbero rappresentare strumenti promettenti per il monitoraggio della terapia con emicizumab e il grado di artropatia potrebbe aiutare a prevedere il rischio di sanguinamento articolare spontaneo.

1. Background

Hemophilia A is a rare chromosome X-linked recessive bleeding disorder characterized by the deficiency of factor VIII (FVIII), with the more severe forms being associated with lower natural clotting factor plasma concentrations. Severe hemophilia A (HA) is characterized by residual clotting factor level below 1% and spontaneous bleeding, hemarthrosis and potentially fatal bleeding, such as intracranial hemorrhages (1). The prevalence of hemophilia A is 1 in 5,000-10,000 newborn males.

The life-expectancy and quality of life of patients with hemophilia have dramatically improved in the last years thanks to the development of novel therapeutical strategies, from extended plasma half-life coagulation factors to non-replacement therapies and gene transfer with viral vectors (2-6). Nonetheless, despite the huge advances in therapeutical strategies that occurred mostly in the last 20 years, hemophilic patients still suffer from disability, lower quality of life and reduced life expectancy.

Coagulation factor replacement prophylaxis has markedly increased patients' quality of life by easing the burden of intravenous injections, and the more recent introduction of non-replacement therapies, such as subcutaneous emicizumab injections, has enhanced treatment adherence and ensued increased protection of HA patients. Despite these advancements, degenerative arthropathy is still a major issue. The introduction of point-of-care ultrasound has improved the diagnosis of acute hemarthrosis and allowed the early recognition of hemophilic arthropathy, as well as the monitoring of progressive joint damage (7). Nevertheless, spontaneous subclinical joint bleeding (the so-called "microbleeding") might still occur, leading to progressive joint damage despite optimal adherence and proper prophylactic treatment (8).

One of the most fearsome complications of hemophilia treatment and prophylaxis is the development of neutralizing alloantibodies against FVIII, called inhibitors. The appearance of inhibitors breaks treatment efficacy and is associated with treatment complications, increased bleeding, worsening

arthropathy and increased mortality. Approximately 30% of patients affected with HA will develop inhibitors (9). In severe HA the risk of developing inhibitors is highest within the first 50 exposures to FVIII products, occurs typically in childhood, and the probability is highest in patients carrying null mutations, such as large deletions, nonsense mutations and intron 22 inversion (10).

Emicizumab (Hemlibra®; F. Hoffmann-La Roche Ltd) was the first non-replacement therapy approved for the treatment of HA patients with or without inhibitors. Emicizumab is a bispecific humanized monoclonal antibody, able to bridge activated factor IX (aFIX) close to factor X, allowing the activation of the latter without the missing cofactor (activated FVIII). Emicizumab was found to increase the thrombin generation in patients affected with HA with an effect comparable to FVIII levels of around 10% (11). The efficacy and safety of emicizumab were assessed in international clinical trials (12-17) and real-world evidence. Nevertheless, there is no standardized monitoring method nowadays for emicizumab prophylaxis monitoring. As reported in previous studies, activated partial thromboplastin time (aPTT) shortens to approximately normal limits after the very first doses of emicizumab (18). Since the normalization of aPTT was found to occur at subtherapeutic emicizumab concentrations in patients who may still experience bleeding (19), aPTT is not considered as accurate marker of efficacy during emicizumab prophylaxis. In the presence of bleeding, only marked increase of aPTT could be an indicator of anti-emicizumab antibodies development (20).

Aiming to monitor emicizumab prophylaxis, several studies have investigated Thrombin Generation Assay (TGA) as a possible global coagulation test able to assess safety and efficacy of non-replacement therapies (20). TGA is an ex vivo approach that can be used to estimate the ability of plasma to produce thrombin upon activation of coagulation in vitro, expressing the balance between anticoagulants and procoagulants.

In HAVEN-1 study the Thrombin Peak Height was measured after four loading doses of emicizumab with a median of 108.8 nM (95% CI 29.7 – 187.0), corresponding to the thrombin generation of approximately 20-30% of healthy volunteers. TGA parameters on emicizumab prophylaxis were

lately found to be equivalent to the TGA of FVIII activity level of 10-40% (11). Furthermore, Peak Height demonstrated a good correlation with emicizumab levels up to concentrations of 80 to 100 μg/mL (16).

However, TGA has never been standardized as a monitoring test for patients on emicizumab prophylaxis, nor it has been shown to predict the hemorrhagic or thrombotic risk (21).

Besides TGA, prothrombin fragment 1 + 2 (F1+2), a short peptide generated when prothrombin is converted to thrombin by the prothrombinase complex, is an in vitro marker of coagulation activation. An enhanced rate of prothrombin to thrombin conversion leads to an increase in plasma F1+2 concentration. Due to its relatively short half-life (about 90 minutes), plasma levels of F1+2 are regarded as accurate estimates of in vivo thrombin production at the time of blood sampling, and a prolonged time to analysis may diminish the F1+2 concentration measured (22). F1+2 levels were measured during clinical trials to evaluate the thrombotic risk of emicizumab (12), but have not been assessed in clinical practice.

Recently, a few authors evaluated Non-Activated Thromboelastometry (NATEM) in a small number of patients before and after the initiation of emicizumab, demonstrating a good correlation between the NATEM curve parameters and emicizumab concentrations (23, 24).

In early 2022, Nakajima et al. investigated the NATEM parameters in 63 patients affected by HA on emicizumab prophylaxis, comparing the curve parameters between severe hemophilic A patients developing bleeding and patients without bleeding. Nineteen out of sixty-three patients experienced break-through bleedings, and the authors found a slight difference in the Clotting Time (CT) between severe hemophilic A patients developing bleeding and patients without bleeding, but the others curve parameters did not differ between the two groups. In addition, the median plasma levels of emicizumab did not differ between the two groups (25). The authors concluded that their findings were consistent with the concept that spontaneous bleeding in HA patients on emicizumab prophylaxis is uncommon, and these patients usually experience bleeding episodes only after trauma or severe injuries. However, as this study was conducted in a population of children and adolescents,

it is difficult to extend these findings to adults with hemophilic arthropathy. Non replacement therapies such as emicizumab or other new molecules at different stages of clinical trials (such as Mim8®, Fitusiran®, Concizumab®, Marstacimab®, etc.) (26) differ from factor replacement therapies in that there is no standardized technique for monitoring their biological effects.

Notwithstanding the "one size fits all" paradigm, over the last few years some doubts about the safety of such dosing for non-replacement therapies have emerged, such as Concizumab® or Fitusiran®, and adjustment in the dosages of drugs according to laboratory monitoring have been recently applied in both treatments' clinical trials, indeed (27).

The identification of laboratory or clinical parameters able to predict long-term efficacy could help to optimize the clinical management of patients on prophylaxis with non-replacement therapies.

Given this background, my project aims to investigate if NATEM and TGA measurable parameters correlate with the hemorrhagic risk of HA patients on emicizumab prophylaxis.

To fulfill my aims I will also investigate whether clinical and ultrasound markers of hemophilic arthropathy could aid in better determining the efficacy of emicizumab prophylaxis.

Moreover, the usefulness of a model based on clinical and laboratory characteristics able to predict spontaneous bleeding risk in hemophilic patients on emicizumab prophylaxis will be investigated.

2. Project aims

The current clinical and scientific scenario is characterized by several efforts to identify a method capable of monitoring emicizumab prophylaxis and other non-replacement therapies prophylaxis, so the identification and validation of an assay that can adequately monitor such promising therapies is of utmost importance. This project aims to conduct an in-depth exploration of global coagulation assays, as NATEM, and clinical or laboratory parameters that could help monitor emicizumab prophylaxis. Furthermore, we also aim to investigate a model that might identify at an early-stage individuals who are at a greater risk of experiencing spontaneous joint bleeding.

Namely, the aims of the current project are:

1) To contribute to the validation of NATEM as a global coagulation assay in hemophilic patients on emicizumab prophylaxis
2) To evaluate whether the NATEM and TGA curves parameters show a relationship with emicizumab plasma concentration
3) To evaluate whether TGA or NATEM parameters correlate with the bleeding phenotype
4) To investigate a model based on clinical and laboratory characteristics able to predict patients at major risk of spontaneous joint bleeding

3. Materials and methods

3.1 Study design

This is a prospective follow-up study, enrolling all patients with severe HA with and without inhibitors against FVIII on emicizumab prophylaxis followed at the Angelo Bianchi Bonomi Hemophilia Centre, Milan.

This study was conducted from January 1st, 2021 until October 1st, 2022.

All patients provided written informed consent. This study was conducted in accordance with the principles of the Declaration of Helsinki and approved by the Ethical Committee of the Hospital.

3.2 Population

Inclusion criteria

- Body weight over 20 Kg (for blood sampling)
- Patients or patient's parents' (in the case of children) willingness to participate in the study after signing a written informed consent
- Patients starting emicizumab during the study period or patients already on emicizumab prophylaxis but with detailed bleeding information since their emicizumab start
- Patients with at least 6 months of follow-up

Exclusion criteria

- Patients without detailed information on bleeding since the start of prophylaxis with emicizumab
- Patients referred to our Centre after the beginning of emicizumab prophylaxis
- Patients lost to follow-up

For each patient, information was collected on sports activities, previous medical history, and bleeding history.

Sports activities were divided into 4 subgroups in relation to the intensity of exertion (i.e., 0 = no sport; 1 = passive movements; 2 = sports without body contact; 3 = sports with body contact).

3.3 Laboratory

At each time point patients underwent an antecubital vein sampling at rest; a total amount of 15 mL of blood in citrated vials (5 vials) was collected per each participant.

Patients not yet on emicizumab were evaluated at baseline (week 0), after loading (week 5) and during maintenance (week 20 and 50).

Patients already on maintenance with emicizumab had their blood collected at the time of study entry, defined as maintenance.

For NATEM, whole blood in citrate (300 µL) was added with 20 µL of CaCl2 (100 mM) in the absence of activators (NATEM), followed by monitoring of the viscosity of clot formation at 37 °C using the ROTEM Delta® instrument (Werfen). The coagulation process was recorded for 9000 s and analyzed using standardized parameters. The clotting time (CT) was measured as the time from the start of the test until a clot of 2 mm of width was observed. Clot formation time (CFT) was defined as the time period between the 2 mm amplitude and the 20 mm amplitude. The α angle (Alpha) was defined as the angle between the baseline and a tangent to the clotting curve through the 2 mm time point. The maximum clot firmness (MCF) was considered the maximum amplitude reached during the test. For TGA, blood was drawn into vacuum tubes containing 1/10 volume of trisodium citrate 10^9 mmol/L, then centrifuged at 3000 g for 15 minutes. Platelet deficient plasma (PPP) was aliquoted into plastic tubes, frozen by immersion in liquid nitrogen, and kept at 80°C until the day of the test. Thrombin Generation was assessed according to Hemker (28) with a homemade method, as previously described elsewhere (29). PPP was induced by 1 pmol/L human recombinant tissue-factor (Recombiplastin; Werfen) and 1.0 µM/L phospholipid mixture (1:1:1 phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine; Avanti Polar Lipids, Alabaster, AL), in the presence or absence of 2nM rabbit thrombomodulin (Haematologic Technologies, Essex, VT). Lag time was defined as the lag-phase from the addition of trigger to the initiation of thrombin generation; thrombin peak was the maximum thrombin generation achieved and the time to peak (TTPeak) was the time to reach the peak. The velocity index (Vel index) is defined as the peak height divided for

the difference between the time to peak and the lag-phase. The endogenous thrombin potential (ETP) measures the area under the curve and correlates to total amount of thrombin produced (30).
Prothrombin fragment F1+2 levels (F1+2) were measured with an ELISA (Enzygnost F1 + 2; Siemens Healthcare Diagnostics™). The detection limit of the assay was 20-1200 pmol/L.

Plasma concentration of emicizumab was measured at weeks 5, 20 and 50 for new starters or at their entry in the study if already on emicizumab prophylaxis.
Emicizumab plasma concentration was measured with the modified calibrated one stage FVIII assay (31) and anti-drug antibodies (ADA) were searched using a previously described Western blot approach (32).

3.4 NATEM validation

Since NATEM is a non-validated global coagulation test, we performed tests to evaluate the intra- and inter- individual variation. Hospital employees were selected as controls. Thromboelastometry parameters were evaluated as the mean of duplicate measurements performed on each sample at the same timepoint, as previously described (33). The parameters of each NATEM test were calculated as the mean between the two simultaneous tests. Although the results of activated ROTEM assays (INTEM, EXTEM and FIBTEM) remain unaltered in blood samples held for up to 2 hours, the majority of NATEM parameters are considerably influenced by the time between blood collection and sample analysis, due to citrate degradation and the subsequent increase of free calcium (34). We therefore assessed multiple analyses at different time-points [within 30 minutes from sampling (T0); 30 minutes after T0 (T30), 60 and 90 minutes after T0 (T60 and T90)], both in hemophilic patients and in healthy controls, to establish the best time-point to analyze fresh blood samples (35).

3.5 Bleeding

Aiming to record each bleeding event, patients starting emicizumab were evaluated with outward medical visits at weeks 0, 1, 5, 20, and 50 of emicizumab prophylaxis. Patients who initiated emicizumab prior to the first of January 2021 were only enrolled if complete data on bleeding events were available from the very beginning of their emicizumab prophylaxis. Additionally, all patients were instructed to contact a 24-hour phone line to report any bleeding suspicions to the on-call physician.

Data on bleeding were collected since the beginning of the prophylaxis. Patients who developed spontaneous joint bleeding during emicizumab prophylaxis were considered as "spontaneous bleeders" (25). As the injected FVIII and/or consumption of coagulation factors may unexpectedly influence the results, global coagulation tests were never performed during an acute hemorrhagic episode.

When distinguishing between spontaneous and post-traumatic bleeding events, patients who developed bleeding only after traumas were considered together with "non-spontaneous bleeders", since traumatic events may occasionally occur by chance in each patient.

Each bleeding event was characterized for type (spontaneous *versus* post-traumatic) and location.

The Annualized Bleeding Rate (ABR) was calculated by dividing the total number of bleeds for the duration of observation and then normalizing the result for one year, as previously reported.

3.6 Hemophilic Arthropathy evaluation

The Haemophilia Joint Health Score (HJHS) is a validated outcome instrument designed for assessing joint health in individuals with hemophilia. The HJHS score evaluates nine criteria across six index joints (elbows, knees, and ankles) and gait assessment. HJHS is collected at each check-up visit in all patients referring to our centre by a trained physiotherapist.

Martinoli et al. developed the Hemophilia Early Arthropathy Detection with Ultrasound (HEAD-US) score aiming to detect early indicators of joint involvement in hemophilic patients and to assess

disease progression and treatment efficacy (36). The HEAD-US is characterized by three domains investigated with joint ultrasound (synovitis, cartilage, and subchondral bone) on the six main index joints (knees, elbows, and ankles), with a maximum score of 8 points per joint (synovitis 0-2 points; cartilage 0-4 points; subchondral bone 0-2 points). In order to prevent underestimation of patients with severe hemophilic arthropathy requiring orthopedic surgery, we assigned prosthetic joints a maximum osteochondral damage score of 6. The HEAD-US was performed by the same expert rheumatologist on each patient. One single US machine equipped with a 5-13 MHz linear probe was used.

3.7 Statistical analyses

Statistical analysis was performed with R version 4.2.1 and IBM SPSS Statistics (version 25.0; IBM Corp., USA).

The differences between global coagulation assays before and after emicizumab prophylaxis were explored. In order to determine the intra-individual variability of emicizumab plasma levels, the coefficient of variation was calculated.

Then we assessed the degree to which NATEM/TGA parameters correlated with emicizumab levels in several ways. Firstly, we calculated Spearman's rank correlation coefficient, both in the standard way and adjusting for multiple measurements. Secondly, we fitted two regression models, one standard linear regression model and one linear random-intercept regression model (in this model, a separate intercept was estimated for each group of observations from a single patient instead of one general intercept for all).

Then, we assessed if NATEM/TGA parameters were significantly different in patients with high emicizumab levels (which we defined as having emicizumab blood concentration of > 45 mcg/kg).

The differences between patients expressing ADA (rare outcome) and patients negative for ADA were also explored.

Thus, we assessed if NATEM/TGA parameters were associated with the bleeding outcomes. The main outcome was the spontaneous joint annualized bleeding rate. In order to evaluate the association between each NATEM/TGA parameter and clinically relevant variables and spontaneous joint ABR, we fitted a Cox proportional hazards model. To adjust for multiple measurements from the same patient, we used the mean value of each variable for a given patient. Using these models, we calculated the P-value and the AUC of the model.

Subsequently, a model capable of predicting the spontaneous bleeding risk was investigated with the ROC analysis.

4. Results

4.1 NATEM validation

Since NATEM is a non-validated global coagulation test, we performed multiple analyses to explore the intra- and inter- individual variation. A population of healthy subjects, laboratory employees and physicians working at our department, were tested to obtain a normal range.

Our analyses confirmed that NATEM changes towards a procoagulant state with time as previously described (37), both in hemophilic patients and in healthy controls. This is the first study describing the variation of NATEM with time in a population of patients affected with severe hemophilia A on emicizumab prophylaxis. Based on the analyses conducted, we performed the NATEM tests within the first 30 minutes after sampling.

Figure 1: Intra-individual variation of NATEM curve parameters within time. The red line refers to baseline measurement, T0 (within 30 minutes from sampling). The green line refers to T30. The blue line refers to T60. The pink line refers to T90.

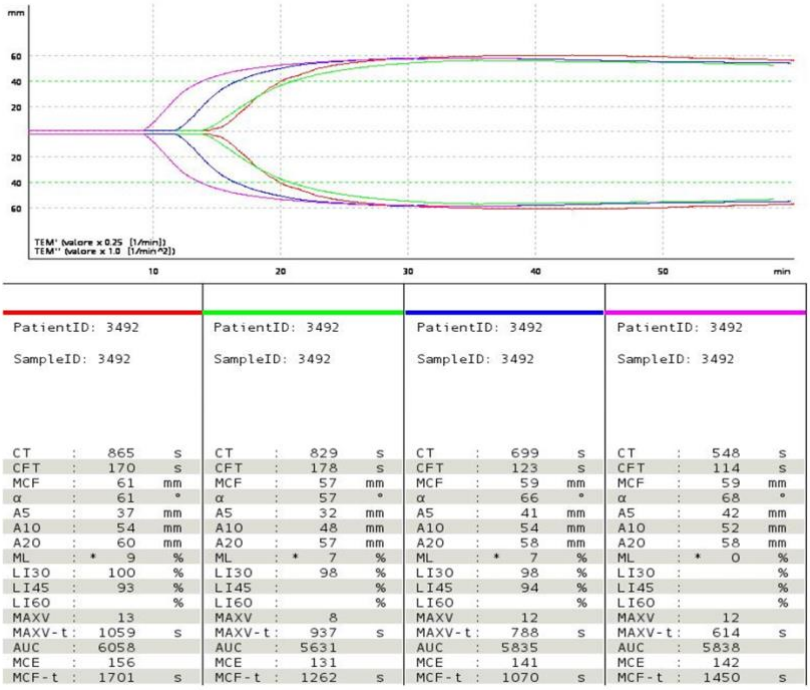
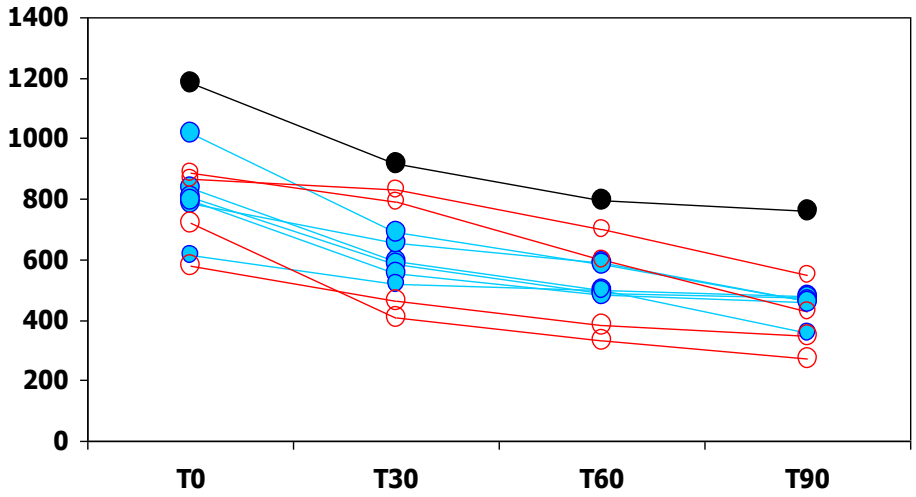


Figure 2: Inter-individual variation of NATEM CT within time. On the y-axis: CT in seconds. On the x-axis T0 (analysis within 30 minutes from sampling); T30 (30 minutes after T0); T60 and T90. Red dots represent patients affected with severe hemophilia A on emicizumab prophylaxis. Black dots represent on emicizumab prophylaxis who developed ADA. Blue dots represent healthy controls.



4.2 Study population

A total of 38 patients affected with HA on emicizumab prophylaxis referring to our Centre were enrolled (as shown in **Figure 3**). Thirty patients started emicizumab prophylaxis during the study period. Of these, four had a follow-up of less than 6 months and were therefore excluded from the study. Twelve patients were already on prophylaxis with emicizumab at the time of study entry, and complete information on their bleeding history was available.

The median follow-up study time of our cohort was 20 months (range follow-up time 6 – 70 months).

Figure 3: Study flow-chart.

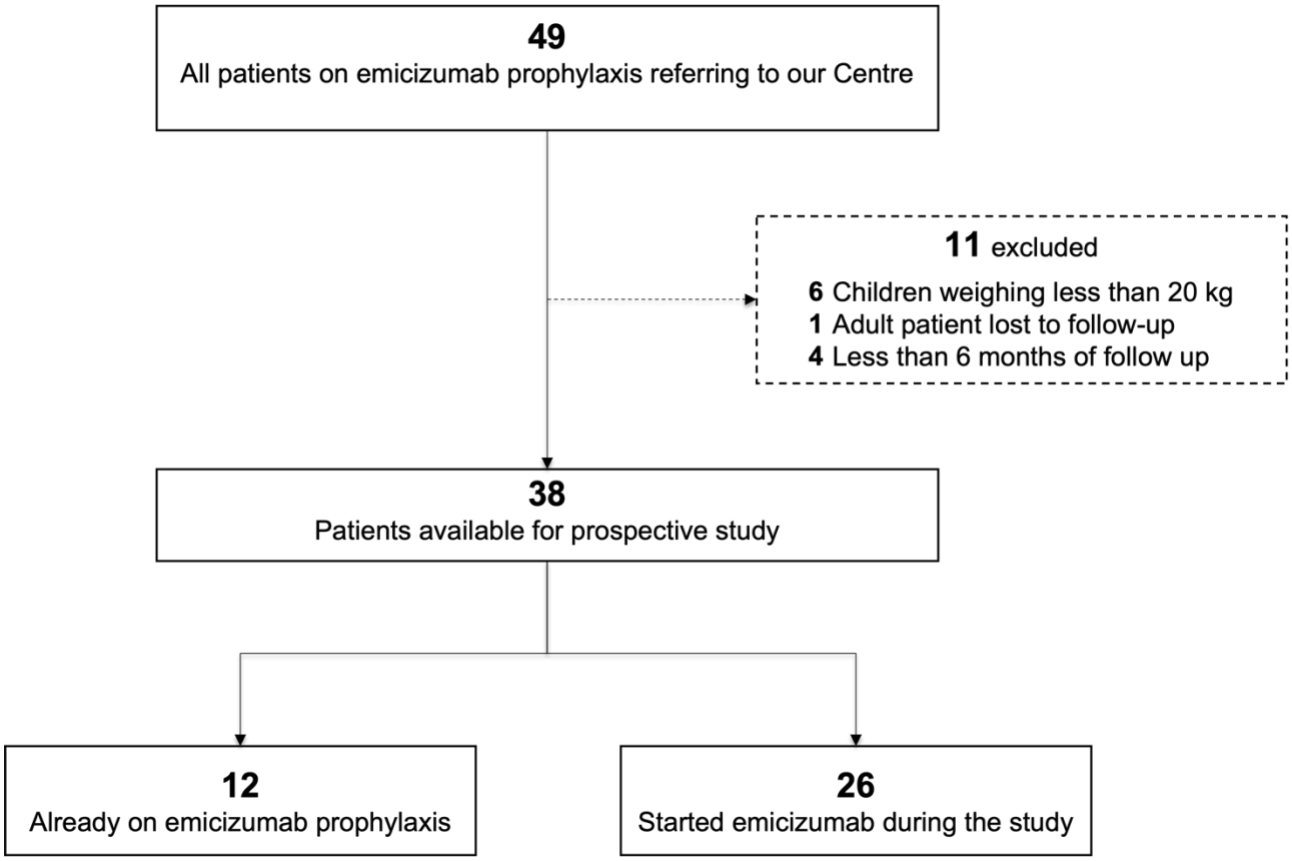


Table 1 shows the clinical and laboratory characteristics of the study population; moreover, it shows the differences of global coagulation assays between patients enrolled and healthy subjects.

The median age of the patients enrolled was 45 years; they were all adults except for one child of 11 and one adolescent of 16 years old. Fifteen out of 38 patients had a history of previous inhibitor, and 8 out of these 15 still expressed antibodies against FVIII. Furthermore, three out of 38 patients developed anti-drug antibodies (ADA) against emicizumab during follow-up.

Table 1: Demographic, clinical and laboratory characteristics of the enrolled population are shown. A population of healthy controls was enrolled to define NATEM parameters. Median and interquartile range are shown (IQR) for each variable, except for the normal values of Fibrinogen and aPTT ratio. *Values are drawn from Capecchi et al. (22). **Fibrinogen and aPTT ratio normal values for the reference laboratory, expressed as range. *** TGA normal range from laboratory healthy control pool.

	38 HA patients	**38 Healthy controls**
Age (years)	45.0 (27.0 – 57.0)	47 (37.5 – 51.5)
Emicizumab (ug/mL)	48.0 (37.2 – 60.5)	
BMI	23.6 (20.6 – 25.5)	–
History of inhibitor	15/38	–
Actual inhibitor	8/38	–
ADA	3/38	–
Artropathy		
HJHS score	14.0 (6.0 – 28.0)	–
HEAD-US score	14.0 (5.0 – 25.0)	–
Synovitis score	1.0 (0.0 – 2.0)	–
Lab characteristics		
F1+2 (pmol/L)	269 (191 – 332)	159 (124 – 202) *
Fibrinogen (mg/dL)	296 (262 – 359)	[min 165 – max 350] **
aPTT ratio	0.73 (0.70 – 0.78)	[min 0.86 – max 1.20] **
NATEM		**NATEM healthy controls**
CT (s)	860 (743 – 994)	593 (543 – 656)
CFT (s)	201 (163 – 260)	148 (120 – 177)
MCF (mm)	58 (54 – 62)	54 (50 – 58)
MCF.t (s)	1710 (1555 – 1848)	1426 (1236 – 1546)
Alpha (°)	56 (49 – 61)	62 (58 – 67)
MaxV (mm/min)	8 (7 – 9)	11 (9 – 12)
MaxV.t (s)	1046 (886 – 1224)	692 (622 – 787)
AUC (mmx100)	5876 (5530 – 6309)	5522 (5062 – 5923)
CT+CFT (s)	1054 (903 – 1265)	758 (662 – 814)
TGA		**TGA normal range*** **
Lag time (min)	8.3 (7.6 – 9.9)	6.0 (5.3 – 6.7)
ETP (nM · min)	978 (771 – 1212)	2026 (1816 – 2242)
Peak thrombin (nM)	70 (50 – 84)	307 (270 – 353)
TTPeak (min)	19.5 (17.7 – 21.1)	9.2 (8.4 – 10.1)
Vel Index (nM/min)	6.4 (4.2 – 8.9)	100 (77 – 128)

Nine out of 38 patients developed spontaneous joint bleeding. Of these, 100% developed post-traumatic bleeding, also. Of the remaining patients, only 5 out of 29 developed post-traumatic bleeding (**Table 2**). Median total ABR was 0.70 (95%CI 0.51 – 0.89) in the entire cohort. Median post-traumatic ABR was 0.34 (95%CI 0.21 - 0.47). Median spontaneous joint ABR was 0.30 (95%CI 0.18 - 0.43).

Table 2: Bleeding profile of the study population.

	Spontaneous Joint bleeders	**Non-spontaneous joint bleeders**
Number of patients	9	29
Age	55 (41 - 61)	41 (28 - 52)
Actual INH	56%	14%
Previous INH	22%	17%
No INH	22%	69%
ADA	1 out of 9%	2 out of 29%
BMI	23.6 (20.1 - 28.2)	23.7 (21.4 - 24.7)
Emicizumab (ug/mL)	37.7 (35.4 - 46.0)	51.2 (41.0 - 63.1)
aPTT ratio	0.74 (0.73 - 0.77)	0.72 (0.69 - 0.77)

4.3 Emicizumab concentration, NATEM, TGA and F1+2 over time

Since patients not yet on emicizumab prophylaxis were evaluated at baseline and then at weeks 5, 20 and 50, a comparison between baseline global coagulation assays and their curve parameters at emicizumab steady state was performed. The non-parametric Wilcoxon-Mann-Whitney test was performed between variables at different timepoints (**Table 3**). FVIII levels at baseline were also measured since patients were still on FVIII prophylaxis.

Table 3: Emicizumab concentration, FVIII and global coagulation assays at baseline (before emicizumab prophylaxis) and at steady state. Data are expressed as Median and IQR. *Wilcoxon-Mann-Whitney was performed.

	Baseline	**Steady state (at least 5 EDs)**	**p-value***
Emicizumab (ug/mL)	-	48.0 (37.2 – 60.5)	
FVIII (%)	2.7 (1.1 – 4.0)	-	
F1+2 (pmol/L)	115 (89 – 149)	269 (191 – 332)	<0.01
NATEM			
CT (s)	2027 (1444 – 2302)	860 (743 – 994)	<0.01
CFT (s)	486.7 (380.2 – 522.1)	201 (163 – 260)	<0.01
MCF (mm)	48.5 (42.2 – 50.6)	58 (54 – 62)	<0.01
MCF.t (s)	2098 (1833 – 2393)	1710 (1555 – 1848)	<0.01
Alpha (°)	29.75 (28.6 – 36.38)	56 (49 – 61)	<0.01
MaxV (mm/min)	3.5 (3.0 – 5.0)	8 (7 – 9)	<0.01
MaxV.t (s)	1724 (1414 – 2443)	1046 (886 – 1224)	<0.01
AUC (mmx100)	4776 (3793 – 5010)	5876 (5530 – 6309)	<0.01
CT+CFT (s)	2643 (1797 – 3573)	1054 (903 – 1265)	<0.01
TGA			
Lag time (min)	9.0 (7.1 – 9.9)	8.3 (7.6 – 9.9)	0.3
ETP (nM · min)	723.5 (499.0 – 937.0)	978 (771 – 1212)	<0.05
Peak thrombin (nM)	53.3 (26.9 – 63.0)	70 (50 – 84)	<0.05
TTPeak (min)	19.4 (16.7 – 22.3)	19.5 (17.7 – 21.1)	0.6
Vel Index (nM/min)	5.7 (3.5 – 6.8)	6.4 (4.2 – 8.9)	0.1

Then, the coefficient of variation (CV) was used to determine the intra-individual variability of emicizumab. This analysis was performed to assess the variability of emicizumab plasma concentration, considering several steady-state measurements per subject. In our cohort, the within-subject CV for the plasma concentration of emicizumab was estimated to be 10%. Therefore, at any given time, the plasma concentration of emicizumab per patient may vary from minus 10 ug/mL to plus 10 ug/mL in each random measurement. This expresses how each patient responds consistently to the dosage of emicizumab, which, although the same for all patients (1.5 mg/kg per week), is clearly influenced by subjective pharmacokinetics. As a result, given the same dosage, the emicizumab plasma concentration measured in our population varied from 17.90 ug/mL to 92.30 ug/mL, but tended to be around the same value with a variation of 10% for each patient at the different time-points.

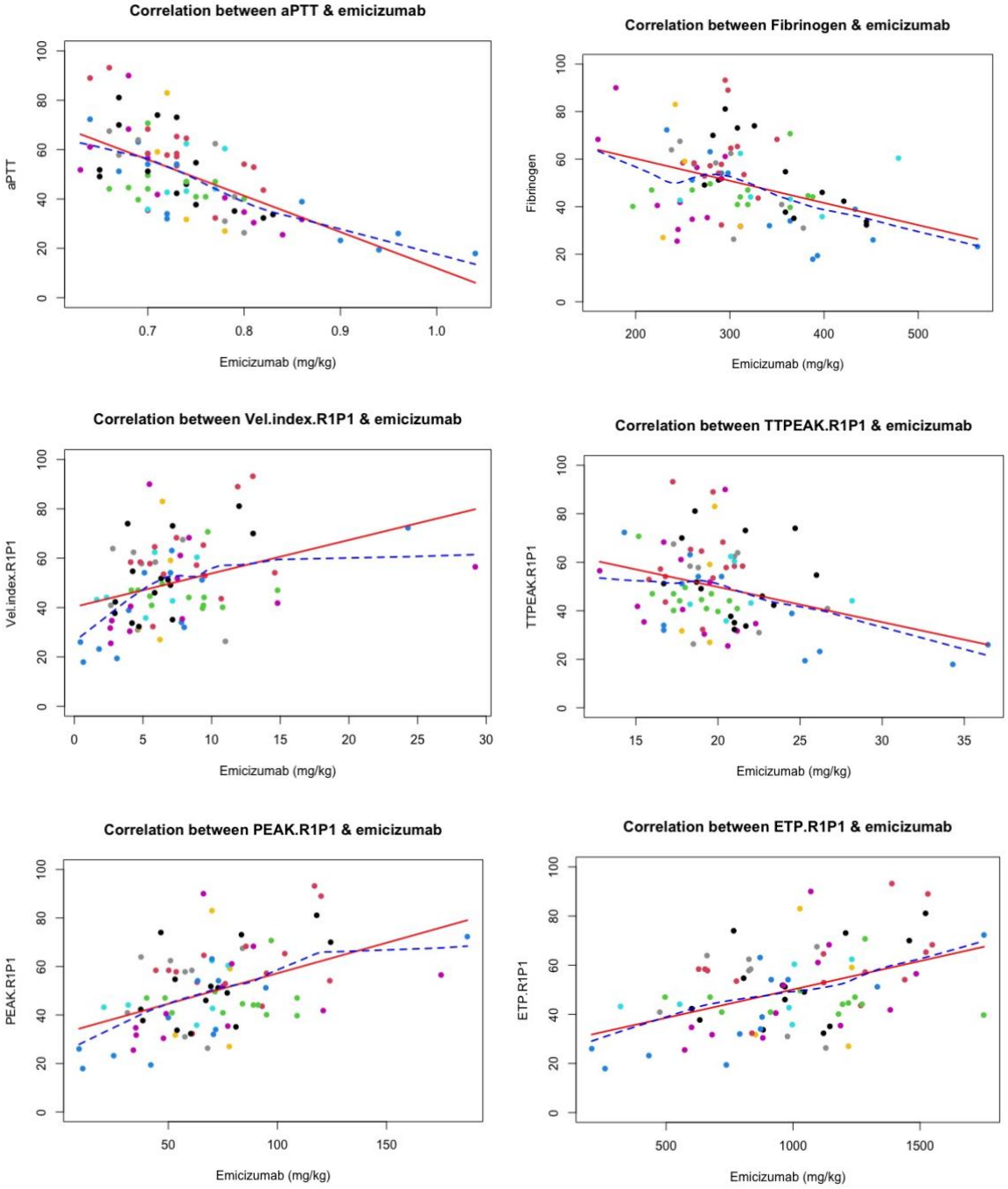
4.4 Association between emicizumab and global coagulation assays

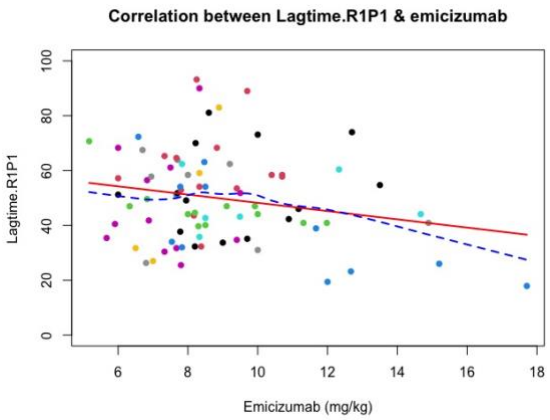
To address the first aim, the association between the global assay's parameters and emicizumab plasma concentration was investigated with two regression models. The first model was a standard linear regression model, the second model was a linear regression model where the dependent variable (i.e. the assay parameter of interest) was modeled using a quadratic function.

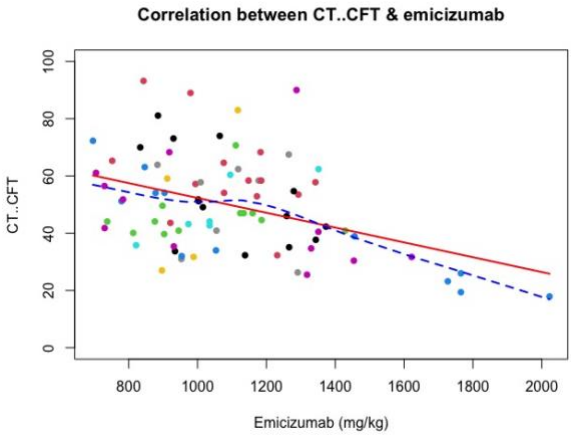
Using this data, a series of scatterplots were generated. The dots shown in **Panel 1** represent the observed values, whereas the lines show the predicted values derived from the regression models.

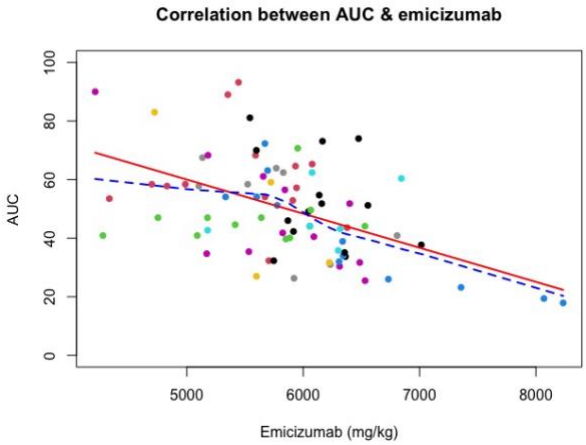
The same results are also shown in **Table 4**. Since we collected 82 measurements for the 38 patients enrolled in our study, the models were adjusted with the linear mixed model, to correct the analyses for multiple observations per person.

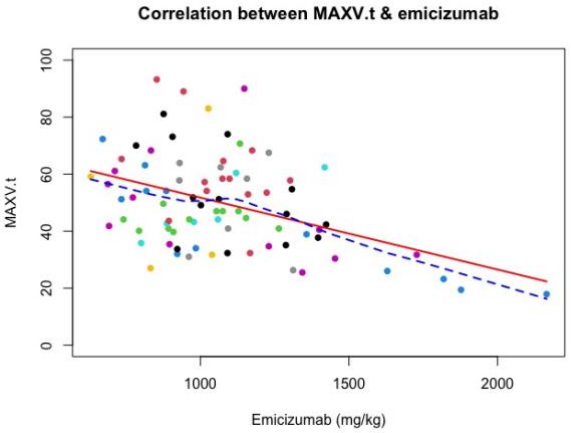
Panel 1: Scatterplots of association between emicizumab plasma concentration and TGA and NATEM curves parameters. The dots are observed values. The red lines represent the fit from a standard linear regression model and the blue dashed line represents the fit from a linear mixed model.

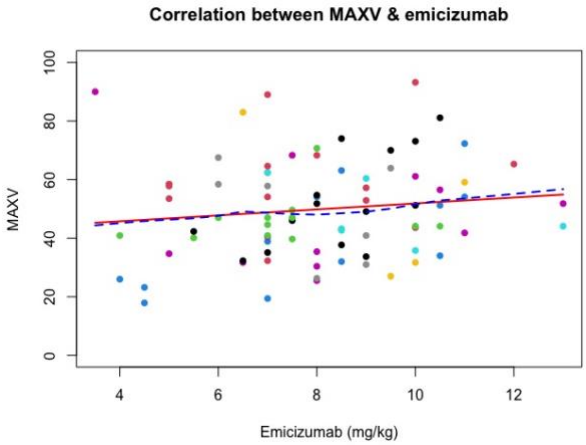


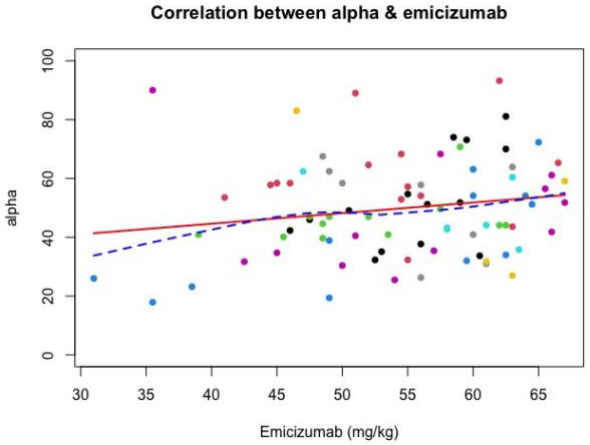

Correlation between Lagtime.R1P1 & emicizumab
Lagtime.R1P1
Emicizumab (mg/kg)

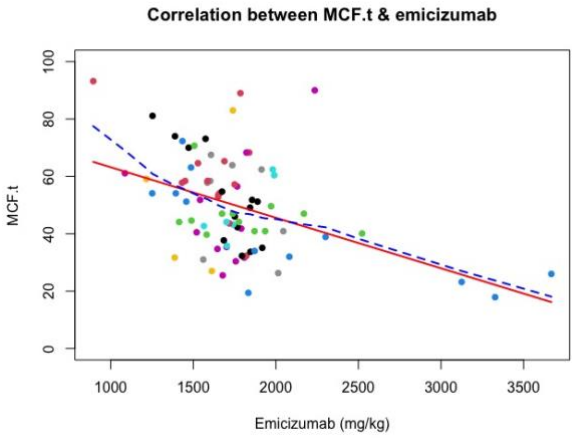

Correlation between CT..CFT & emicizumab
CT..CFT
Emicizumab (mg/kg)

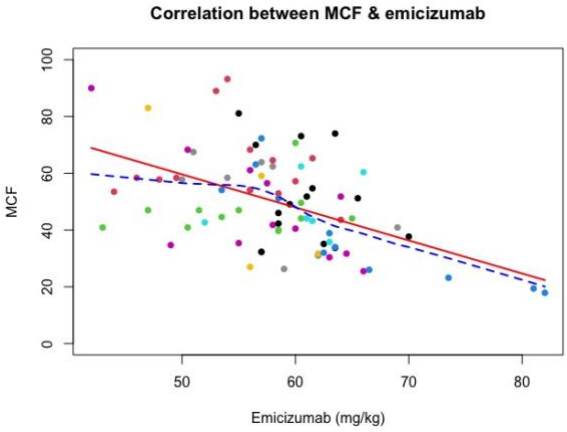

Correlation between AUC & emicizumab
AUC
Emicizumab (mg/kg)

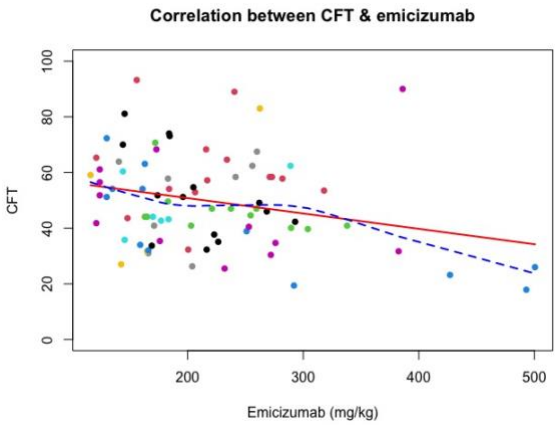

Correlation between MAXV.t & emicizumab
MAXV.t
Emicizumab (mg/kg)


Correlation between MAXV & emicizumab
MAXV
Emicizumab (mg/kg)


Correlation between alpha & emicizumab
alpha
Emicizumab (mg/kg)


Correlation between MCF.t & emicizumab
MCF.t
Emicizumab (mg/kg)


Correlation between MCF & emicizumab
MCF
Emicizumab (mg/kg)


Correlation between CFT & emicizumab
CFT
Emicizumab (mg/kg)

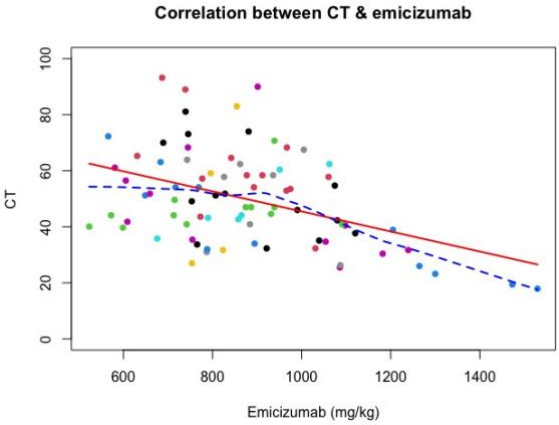

Correlation between CT & emicizumab
CT
Emicizumab (mg/kg)

Table 4: Spearman correlation coefficient (standard and repeated measures version) and the p-value from a linear regression model (both in the standard way and using a linear mixed model to correct for multiple observations per person).

Association between laboratory parameters and emicizumab plasma concentration				
Variable	**Correlation standard**	**Correlation repeated**	**p-value standard**	**p-value mixed**
aPTT	-0.64	-0.37	<0.01*	<0.01*
NATEM				
CT (s)	-0.36	-0.13	<0.01*	0.06
CFT (s)	-0.23	-0.10	0.02*	0.27
MCF (mm)	-0.47	-0.21	<0.01*	<0.01*
MCF.t (s)	-0.39	-0.26	<0.01*	0.03
Alpha (°)	0.18	0.09	0.11	0.52
MaxV (mm/min)	0.14	0.07	0.26	0.55
MaxV.t (s)	-0.33	-0.14	<0.01*	0.02*
AUC (mmx100)	-0.46	-0.21	<0.01*	<0.01*
CT+CFT (s)	-0.33	-0.12	<0.01*	0.07
TGA				
Lag time (min)	-0.10	-0.03	0.06	0.40
ETP (nM · min)	0.40	0.23	<0.01*	<0.01*
Peak thrombin (nM)	0.44	0.18	<0.01*	<0.01*
TTPeak (min)	-0.25	-0.05	<0.01*	0.14
Vel Index (nM/min)	0.40	0.12	<0.01*	0.01*

4.5 Differences between higher and lower emicizumab plasma concentration

We also assessed if NATEM/TGA parameters were significantly different in patients with higher emicizumab levels (which we defined as having emicizumab blood concentration of > 45 mcg/kg). The cut-off of 45 ug/mL was selected based on previous literature (38) and based on the median emicizumab level in our population (48 ug/mL). **Table 5** shows the distribution of the parameters among patients with emicizumab levels below and above 45 ug/mL. For each patient, a mean of all observed parameters was generated, aiming to correct for the multiple observations per patient.

Table 5: Wilcoxon-Mann–Whitney test was performed between the population of patients with less than 45 ug/mL of emicizumab plasma concentration and the rest of the population. Median and interquartile range (IQR) are shown.

Variable	Emi<45 ug/mL	Emi≥45 ug/mL	p-value
Number of patients	18	20	
Number of spontaneous joint bleeders	**6**	**3**	
Age	48.0 (34.5 – 11.0)	41.5 (27.7 – 50.2)	0.24
F1+2	252.7 (84.6-404.7)	289.8 (121.1-561.5)	0.04*
Fibrinogen	331.5 (224.7-445.8)	288.5 (169.5-395.1)	0.06
aPTT	0.8 (0.7-0.9)	0.7 (0.6-0.8)	<0.01*
NATEM			
CT (s)	861.5 (676-1354.4)	804.8 (607.2-1006.8)	0.16
CFT (s)	180.2 (142.5-392.7)	192.4 (116-286)	0.55
MCF (mm)	60.8 (46.8-73.2)	57.5 (46.2-63.2)	0.05*
MCF.t (s)	1721.8 (1387.5-2850.9)	1643.9 (1216-2028.5)	0.06
Alpha (°)	58 (40.6-63.5)	57.1 (44.1-67)	0.70
MaxV (mm/min)	8.5 (5.4-13)	8.8 (5-11.5)	0.68
MaxV.t (s)	1034.6 (800.5-1769.3)	992.8 (631-1278.1)	0.20
AUC (mmx100)	6097.9 (4706.8-7346.1)	5772.8 (4697.2-6460.2)	0.05*
CT+CFT (s)	1035 (821.5-1747.1)	1005.2 (729.8-1244)	0.23
TGA			
Lag time (min)	8.4 (5.7-14.9)	7.9 (5.2-12.1)	0.36
ETP (nM · min)	905 (320.5-1413.7)	1114.9 (680.2-1460.2)	<0.01*
Peak thrombin (nM)	62.9 (20.5-94)	77.9 (51.1-148)	<0.01*
TTPeak (min)	20.1 (15.5-29.3)	18.9 (13.9-24.2)	0.11
Vel Index (nM/min)	5.8 (1.6-9.9)	7.5 (4.2-22)	0.03*

4.6 Bleeding analyses

The differences between spontaneous joint bleeders and non-spontaneous bleeders were then analyzed. **Figure 4** shows the annualized bleeding rate (ABR) plotted against emicizumab levels.

Figure 4: Scatterplot graph showing the annualized bleeding rate (ABR) for each patient who had spontaneous or post-traumatic bleeding and their emicizumab plasma concentration.

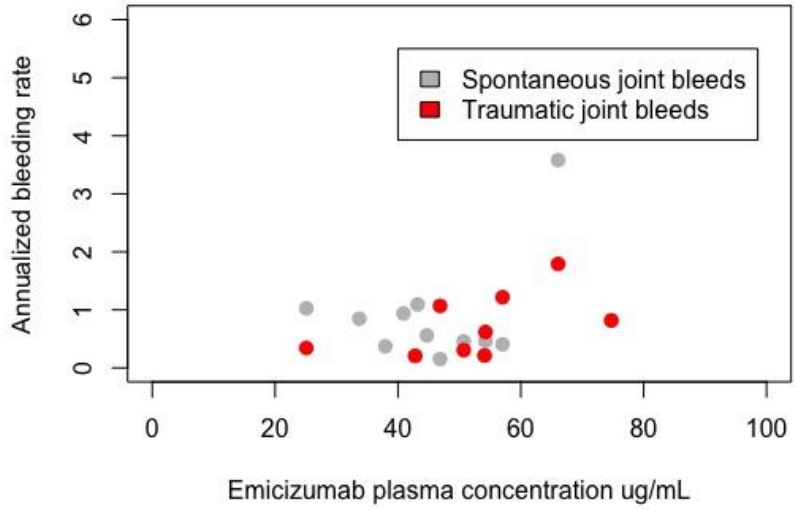


Table 6 shows the distribution of various parameters in spontaneous joint bleeders and non-spontaneous joint bleeders and the p-value and AUC extracted from a Cox proportional hazards model (considering both the outcome and the observational time).

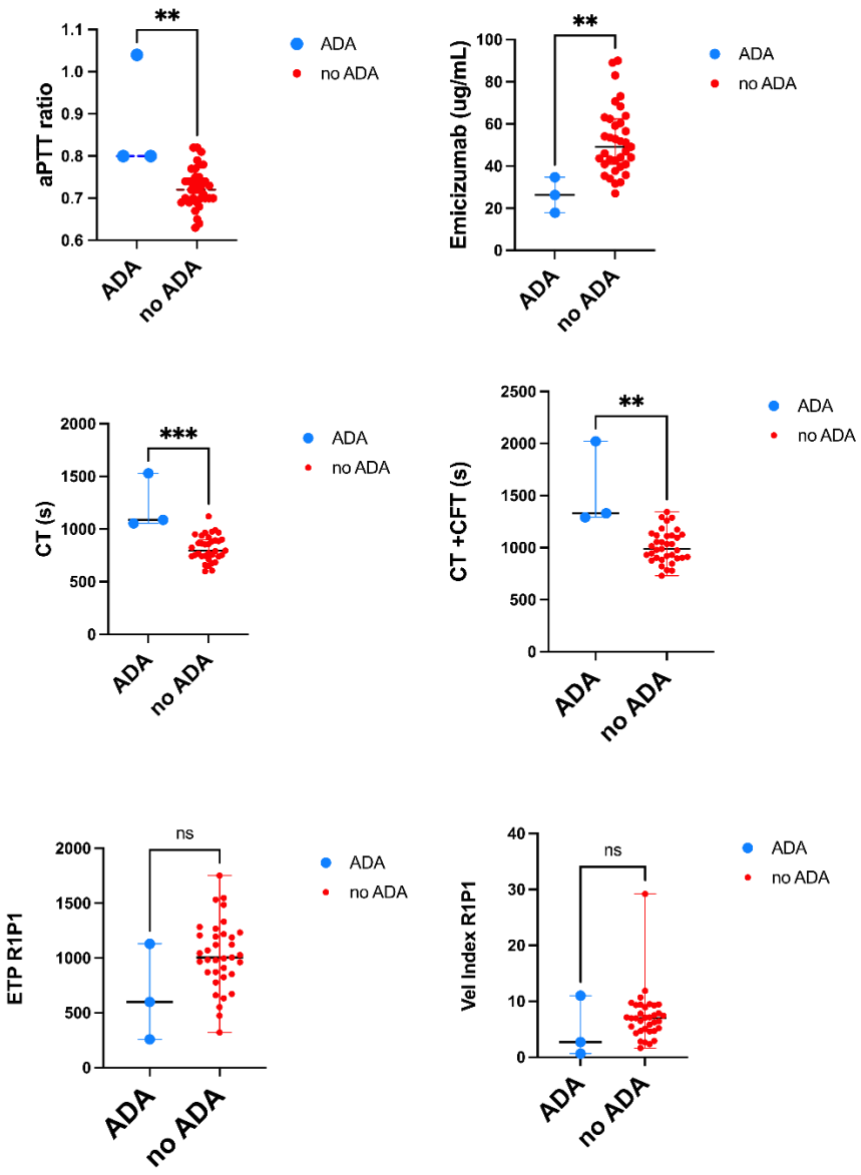
Table 6: Distribution of parameters in spontaneous joint bleeders (SJB) and non-spontaneous joint bleeders (NSB). A Cox proportional hazards model was applied. P-values and AUC are shown per each model.

Variable	SJB median(IQR)	NSB median(IQR)	p-value Cox regression	AUC Cox regression
Number	9	29		
Age	55 (23-67)	41 (11-75)	0.59	0.58
BMI	23.6 (17.9-36.7)	23.7 (17.7-27.5)	0.51	0.52
Sport grade	1 (0-2)	2 (0-3)	0.28	0.65
HJHS	25 (7-38)	10 (0-48)	0.19	0.66
HEAD-US	30 (9-39)	11 (0-39)	0.06	0.78*
Synovitis	2 (0-3)	1 (0-3)	0.03*	0.79*
Emicizumab	37.7 (25.1-57)	50.7 (28.6-91.1)	0.23	0.62
F1+2	296.6 (248.0-339.6)	262.8 (195.9-298.97)	0.14	0.69
Fibrinogen	322 (229-445.8)	295 (169.5-419.3)	0.79	0.52
aPTT	0.7 (0.7-0.9)	0.7 (0.6-0.8)	0.61	0.50
NATEM				
CT (s)	865 (676-1354.4)	823.5 (607.2-1131.9)	0.26	0.62
CFT (s)	176 (135-392.7)	185 (116-283.1)	0.49	0.55
MCF (mm)	60.5 (46.9-73.2)	58.5 (46.9-73.2)	0.73	0.45
MCF.t (s)	1684.5 (1251-2850.9)	1739.5 (1216-2143.5)	0.94	0.41
Alpha (°)	57 (40.6-64)	58 (46.5-67)	0.42	0.58
MaxV (mm/min)	8.5 (5-13)	8.5 (5.5-11.5)	0.56	0.61
MaxV.t (s)	1059 (800.5-1769.3)	995.2 (631-1430.8)	0.26	0.65
AUC (mmx100)	6058 (4712.4-7346.1)	5841.8 (4697.2-6807)	0.75	0.48
CT+CFT (s)	1035 (821.5-1747.1)	1032.3 (729.8-1415)	0.31	0.66
TGA				
Lag time (min)	8.3 (5.7-14.7)	8.3 (5.2-14.9)	0.71	0.41
ETP (nM · min)	785.1 (502.7-1218.5)	1052.5 (320.5-1460.2)	0.07	0.65
Peak thrombin (nM)	52.8 (27.4-77.9)	77 (20.5-148)	0.06	0.65
TTPeak (min)	20.7 (15.5-29.3)	18.6 (13.9-26.7)	0.52	0.57
Vel Index (nM/min)	5.1 (2-7.9)	7.6 (1.6-22)	0.07	0.63

4.7 Differences between patients with and without partially neutralizing ADA

To further investigate the possible clinical relevance of global coagulation tests, an explorative analysis was made to detect possible differences between ADA and ADA negative patients. Wilcoxon-Mann-Whitney non-parametric test was applied.

Panel 2: Box and whisker graph showing median and range per each parameter, in ADA and non-ADA patients. The asterixis expresses the Wilcoxon-Mann-Whitney test.



4.8 Early spontaneous joint bleeding prediction model

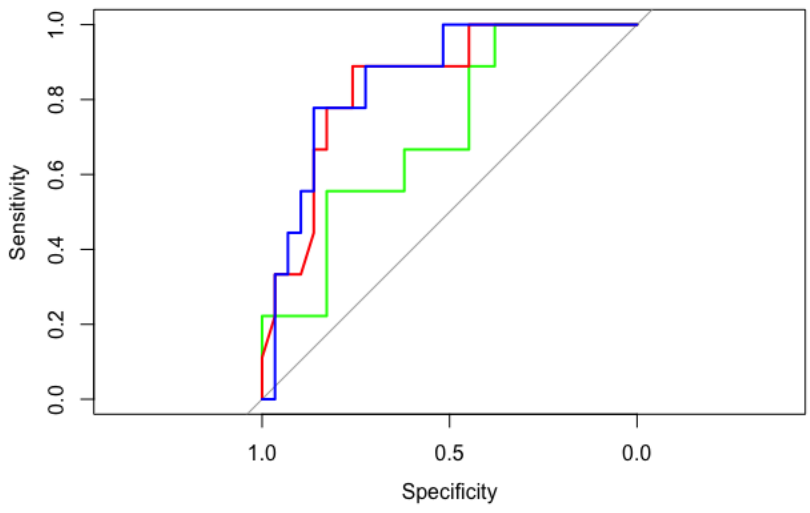
A model capable of predicting the spontaneous bleeding risk was investigated through the ROC analysis. Different variables expected to have relevance in determining the bleeding risk were identified from the Cox proportional hazard model previously reported (section **4.6**) and from expected clinical relevance variables.

The best prediction was found using a model considering the HEAD-US score and the synovitis score together (AUC 0.85).

Figure 5 shows the AUC of three different models. As shown, emicizumab plasma concentration didn't add gain to the prediction model. Moreover, no gain in the AUC was shown by adding age (AUC 0.58) and/or sports activities (AUC 0.64) to the prediction model.

Figure 5: ROC analysis.

Model 1 (green line): emicizumab plasma concentration was considered in the prediction model (AUC 0.70)
Model 2 (red line): HEAD-US score plus synovitis score were considered in the prediction model (AUC 0.84)
Model 3 (blue line): HEAD-US score plus synovitis score plus emicizumab plasma concentration were considered in the prediction model (AUC 0.85)



5. Discussion

Emicizumab effectively reduce bleedings and severe HA patients during prophylaxis show a milder phenotype, in which trauma accounts for most of the bleeding (39). Nevertheless, spontaneous joint bleeding might still occur, and a thrombotic risk was evidenced in association with bypassing agents administration during clinical trials.

The present study explored the chance to use global coagulation tests in association with clinical characteristics in monitoring and/or predicting the risk of spontaneous joint hemorrhages in patients on emicizumab prophylaxis.

NATEM was recently proposed as a variation-sensitive method for emicizumab monitoring in a few studies (23, 25, 40). Despite this, NATEM has not been validated as a global coagulation test in HA patients on emicizumab. A contribution to the standardization and validation of the NATEM test was therefore explored in the present study. We determined that the optimal period for analysis is within 30 minutes from sampling, to avoid heat activation. Moreover, this is the first study exploring the variation of NATEM within time in patients on emicizumab prophylaxis, which did not differ from the activation observed in healthy subjects (Figure 2).

After the identification of the best conditions to perform NATEM, the two global assays (NATEM and TGA) were performed on the same samples.

The differences in global coagulation tests between HA patients and healthy controls are outlined in Table 1, where the 82 samples were considered as independent samples, therefore without statistic correction. In a preliminary descriptive study, fibrinogen did not differ between healthy controls and emicizumab-treated patients. The median CT, CFT, and MCF.t differed between HA patients and healthy controls, whereas the maximum clotting firmness (MCF), Alpha angle and NATEM AUC did not differ. It is therefore plausible, based on our exploratory analysis, to state that emicizumab normalizes the clot properties of NATEM assay but not the time-period in which clotting starts.

Considering TGA, all parameters were different between patients and healthy controls. HA patients on emicizumab prophylaxis showed diminished thrombin generation potential and a delayed onset. The Vel index, representing the speed between the beginning of thrombin generation and its peak, was strongly different between patients and controls.

Prothrombin fragment F1+2 was found to be higher in patients on emicizumab prophylaxis then in healthy controls. A previous study analyzing the safety profile of Hemlibra® found no differences in F1+2 generation in HA patients before and after starting emicizumab (16). However, the median of F1+2 found in our population of hemophilic patients deeply differs from that of the healthy population previously described by Capecchi et al (22), applying the same laboratory methods.

In addition, when assessing the global coagulation changes upon achieving steady-state, all NATEM parameters and F1+F2 diverged significantly from baseline to steady-state, but TGA parameters differed to a lesser extent. This difference could be due to the need for coagulation activators for TGA performance, expressing a potential and not an in-vivo process.

Emicizumab is a bispecific monoclonal antibody able to bind the epidermal growth factor (EGF)-like domain 1 of FIX/FIXa and the EGF-like domain of FX/FXa, bridging the two complex and accelerating significantly FIXa-mediated FX activation, thus enhancing thrombin production (41).

The increase in thrombin generation induced by emicizumab could explain the lower thrombin generation potential and the higher F1+2 fragments measured in hemophilic patients respect to what observed in healthy controls. Further research is needed to investigate the differences in F1+2 production in HA patients on FVIII prophylaxis and HA patients before and during emicizumab prophylaxis.

After the descriptive analysis, we assessed the degree to which NATEM/TGA parameters were associated with emicizumab plasma levels. In our cohort, the aPTT ratio showed a good correlation with the plasma concentration of emicizumab, nevertheless aPTT is known to reach normal values

also with low emicizumab plasma concentration; therefore, it is not thought to be a good monitoring assay. Moreover, it was recently shown that dose up-titration of emicizumab had no effect on aPTT ratio (42).

Most of the parameters of both NATEM and TGA curves also showed strong association with emicizumab concentration, but only MCF, MaxV.t and AUC for NATEM and ETP, Peak thrombin and Vel Index for TGA remained deeply correlated after linear mixed model correction. A good correlation between NATEM CT+CFT and emicizumab plasma concentration and between Peak and emicizumab levels were already described (16, 23), but the two tests have never been studied in the same population as we did.

Previous simulations, conducted before phase III clinical trials, revealed that emicizumab plasma concentrations of ≥ 45 ug/mL should result in zero bleeding events for at least one year in at least fifty percent of patients (38). Emicizumab was administered in our entire population at the same dosage (1.5 mg/Kg/weekly) and in one child only at 3 mg/Kg/every 2 weeks. When we assessed the differences between HA patients with higher and lower emicizumab plasma concentration, MCF and AUC (for NATEM) and ETP, Peak and Vel Index (for TGA) were found to be different among the two groups. The AUC of NATEM, defined as the first derivative curve from start until MCF is reached, depends on MCF. The MCF of the NATEM test reflects clot quality and stability; therefore, our findings suggest that patients with a higher plasma emicizumab concentration (>45 ug/mL) have a greater capability to form clots. This could be reflected by the higher proportion of patients developing spontaneous joint bleeding in the lower concentration of plasma emicizumab (6 out of 9) than in the higher concentration group (3 out of 9).

Our findings are consistent with a recent study revealing that up-titration of emicizumab dosage improved ABR in patients with inadequately controlled bleeding tendency (42).

When we further investigated the usefulness of global coagulation assays to distinguish between spontaneous joint bleeders and non-spontaneous joint bleeders, only ETP, Peak, time to peak and Vel Index for TGA showed differences between spontaneous joint bleeders and non-spontaneous bleeders. Thrombin generation parameters are known to show a greater extent of thrombin generation in a lessen time with age, due to the increased pro-thrombotic phenotype (43). Nevertheless, our patients developing spontaneous joint bleeding were older but showed a lower thrombin generation than that of the non-spontaneous joint bleeders (Table 6).

The presence of hemophilic arthropathy was found to be the strongest predictor of future spontaneous joint bleeding. Notwithstanding the well-known relationship between hemophilic arthropathy and joint bleeding, this is the first study demonstrating the predictive power of the ultrasound score in patients treated with emicizumab. A HEAD-US score of 21.5 was found to be a possible cut-off with a sensibility of 83% and a specificity of 75% in predicting spontaneous joint bleeding. This finding might help future clinicians in recognizing patients at higher risk for spontaneous joint bleeding development.

However, since the degree of sports activities did not add any gain to the predictive model, the probability of spontaneous bleeding does not appear to be related to physical exertion as much as to the basal joint condition. This result could be of great importance for patients coping with everyday life and for the physicians caring for them.

In contrast to FVIII, emicizumab does not require activation by thrombin. As a result, the aPTT-shortening effect caused by the antibody is more significant than that of FVIII (44). Consequently, aPTT clotting assay is deemed to be inadequate to monitor emicizumab activity. Global coagulation assays have been suggested as suitable alternatives for monitoring hemostasis. While NATEM studies the achievement of clotting with minimal activation and considers the cellular contribution, TGA requires coagulation activators and demonstrates the in vitro ability to generate thrombin. This is the

first study showing an increased release of F1+2 in patients on emicizumab prophylaxis when compared both to their baseline levels and to healthy controls.

Notwithstanding the same dosage per Kg, patients of our cohort showed different plasma emicizumab concentration ranging from 17 to 90 ug/mL, with low intra-individual variation over time (within-subject CV for emicizumab plasma concentration was estimated to be 10%). Global coagulation assays performed in our population showed that thrombin generation and NATEM clotting parameters changed toward a pro-coagulant phenotype at the increasing of emicizumab plasma concentration. When we stratified the analysis between concentration higher or lower than 45 ug/mL, we found that most of the spontaneous joint bleeders were in the lowest concentration group (6 out of 9). Nevertheless, emicizumab concentration was only slightly different among the spontaneous and non-spontaneous joint bleeders (p-value 0.07), and this effect could be partially explained by the different distribution of age among the two groups (median age of spontaneous joint bleeders 55 years *versus* 41 in the non-spontaneous bleeders, Table 2).

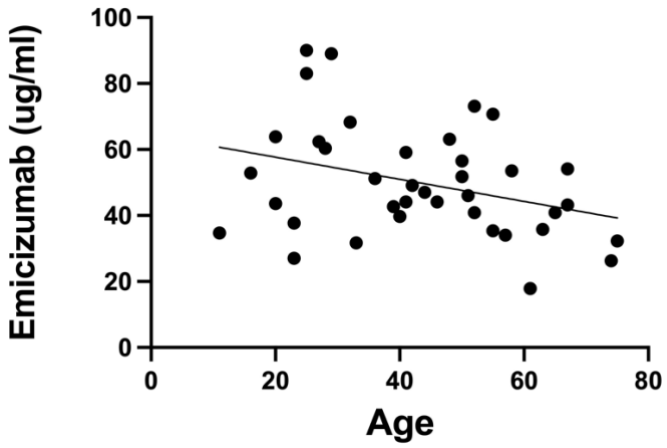


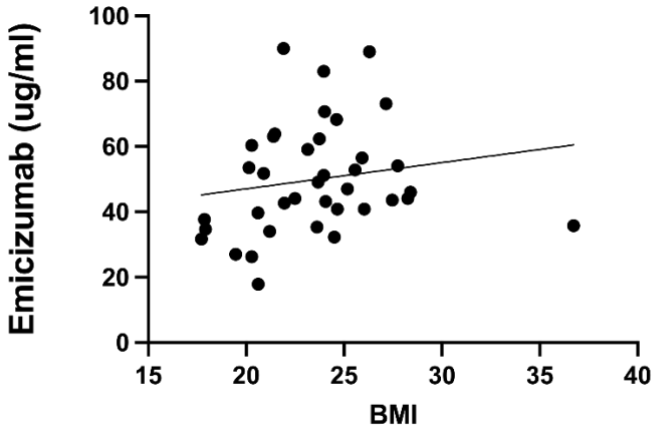
Figure 6: Emicizumab plasma concentration (ug/mL) plotted against age (years).

Age was previously described to be a determinant factor of emicizumab PK, indeed. In our cohort, emicizumab showed a negative correlation with age (Emicizumab = -0,3352*Age + 64,40; $p<0.05$),

as shown in Figure 7. This is consistent with previous findings showing that the bioavailability of emicizumab declined steadily for patients with hemophilia older than 30 years, resulting in a 31% drop in steady-state exposure for a 77-year-old compared to a 30-year-old patient (45). This conclusion is thought to be due to an age-related decrease in the thickness of the hypodermis. However, the decreased exposure in older patients was not found to decrease efficacy, with ABRs close to 0 found in both elderly and younger patients. In humans, the thickness of the hypodermis grows with body mass and declines with age, influencing monoclonal antibodies absorption and variability (46).

Moreover, Body Mass Index (BMI) was not found to modify emicizumab plasma concentration in our cohort (Y = 0,8037*X + 31,00; p=0.3), as shown in Figure 7.

Figure 7: Emicizumab plasma concentration (ug/mL) plotted against BMI (Kg/m2).



The HEAD-US score was the most effective predictor of the risk of spontaneous joint hemorrhage. This finding is consistent with what has been documented earlier in the setting of hemophilic arthropathy, where the presence of synovitis facilitates intra-articular bleeding due to inflammation and neoangiogenesis (7). Furthermore, our results confirm a recent paper that highlights a higher risk of developing spontaneous joint bleeding in older patients (47).

6. Limits and strengths

The follow-up of our single-centre cohort, the availability of real-world data and the expertise of our laboratory employees are the strengths of our study. The limitations include the lack of information about untreated bleeding, since each hemophilic patient might underreport bleeding (48). Nevertheless, our centre directly prescribes emergency amount of FVIII replacement to patients on emicizumab prophylaxis, it was therefore easy to check data on reported or diagnosed bleeds together with information on FVIII supply. Moreover, our patients could call a 24-hour physician's phone number, and they are instructed to refer all the bleeding events.

A major limitation of the present study was the small sample size, given the monocentric nature of our study regarding a rare disease.

7. Conclusion

In conclusion, our study explored if NATEM and TGA could be used as methods for emicizumab prophylaxis monitoring.

Correlations between global coagulation assays and emicizumab plasma concentration were observed. The endogenous thrombin potential increased during emicizumab treatment but was found to be lower than the normal range, in association with an increased *in vivo* thrombin generation, as indicated by augmented F1+2 plasma levels. TGA parameters (namely peak thrombin, time to peak and velocity index) showed differences between spontaneous and non-spontaneous bleeders, while NATEM parameters and F1+2 levels did not. Finally in our cohort we demonstrated that the degree of hemophilic arthropathy might predict the spontaneous joint bleeding risk of patients on emicizumab prophylaxis. Indeed, the degree of hemophilic arthropathy at the beginning of emicizumab prophylaxis may aid in the proper management of patients at a higher risk of developing

spontaneous joint bleeding and, in the future, could help determining which patients might require an emicizumab dosage increase.

Further research is needed to investigate the differences in F1+2 production in HA patients on FVIII prophylaxis and HA patients before and during emicizumab prophylaxis.

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