Comparison among three different bleeding scores and the thrombin generation assay to assess the different hemorrhagic phenotypes in patients with FVII deficiency

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ABSTRACT

Defining the bleeding risk in patients with FVII-deficiency is not easy. Aim of this study is to define correlation and differences between three different scores and the thrombin generation assay (TGA) in correctly evaluating the hemorrhagic phenotype in a group of FVII-deficient patients. Fifty-seven patients patients with FVII-deficiency whose hemorrhagic phenotype was assessed by Mariani, ISTH/SSC- Bleeding Assessment Tool (BAT) and Di Minno scores, and by the TGA, were enrolled in this study. TGA parameters (LagTime, Peak, ttPeak, ETP - endogenous thrombin potential) highlighted how both LagTime and ttPeak can discriminate major bleeders from the others, while the same conclusion could not be reached by the ETP and Peak. However, no TGA parameter was found to be useful in separating the mild hemorrhagic phenotype from the moderate one. Scores and TGA were found to be able to only define the severe hemorrhagic phenotypes. None of the methods was able to exactly discriminate the other phenotypes. Given these results, there is therefore a risk of either underestimating or overestimating the potential bleeding risk in patients with non-severe FVII-deficiency.

INTRODUCTION

The FVII deficiency is the most frequent among rare congenital bleeding disorders, estimated at 1 in 500.000, apparently without any sex, racial or ethnic predilection.[1,2] The prevalence of FVII deficiency among the general population is probably high due to large number of asymptomatic or poorly symptomatic subjects. This is an inherited, autosomal recessive defect. The factor VII protein is part of the initiating complex of the extrinsic coagulation pathway, laboratory diagnosis is easy, in fact FVII deficiency is the only congenital bleeding disorder diagnosed by prolonged prothrombin time (PT), while activated partial thromboplastin time (aPTT) is within normal range. FVII deficiency may be mild, moderate, or severe,[3,4] based on the plasmatic level of the coagulation factor, but bleeding phenotype could be different among subjects with similar levels of plasma FVII. The subjects presenting low levels of FVII may have symptoms like hemophilia patients. Women with FVII deficiency can have severe menorrhagia, bleeding during delivery or post-partum.[5,6]

The published reports highlighted as in some cases the severity of FVII deficiency cannot correlate with the bleeding symptoms.[7] This observation is often based on the hemophilia categorization. This classification has been considered not correct by the ISTH-SSC and replaced in 2012,[8] based on the results of the European Network of Rare Bleeding Disorders (EN-RBD) registry.[9] In the Seven Treatment Evaluation Registry (STER) most major bleeds occurred in patients with plasmatic FVII <3%, while minor bleeds were more frequent in subjects with a moderate/mild disease. Patients presenting major bleeding at enrolment resulted also at high risk for severe hemorrhagic recurrences.[10] Intracranial bleeding was usually considered restricted to a few areas in which consanguineous marriages are frequent and most patients are homozygous for the FVII defect, but the data reported in the IF7 Registry also showed a high incidence of major bleeding in patients with severe FVII at early onset.[11] Spontaneous major bleeds in patients presenting FVII level >20% were practically null, but also in this population differentiation between bleeders and not bleeders could be useful to avoid during surgery useless or harmful replacement therapy.

With this background the management of patients presenting FVII deficiency can be very difficult, a correct assessment is needed to predict the bleeding risk of each patient. Different scores have been created to define the hemorrhagic phenotype: Mariani score;[10] ISTH/SSC-BAT bleeding assessment tool;[13,14] or Di Minno classification.[10] All these tools are equally easy to use, but not all have the same accuracy. Bleeding classifications may therefore not be exactly superimposable.

In addition to bleeding scores, the use of Thrombin Generation Assay (TGA) to define the hemorrhagic phenotype of FVII deficiency patients can be a valuable option for clinicians. TGA evaluates thrombin generation and its disappearance, thus assessing the balance between these two different moments.[15,16]

Objective

The purpose of this study is to define whether exists a correlation between three different bleeding scores and TGA in the prediction of the hemorrhagic phenotype in subjects with FVII deficiency.

MATERIALS AND METHODS

Patients

This is a spontaneous, prospective, multicenter study that included all patients of any age with FVII deficiency referred to four Italian Hemophilia Centers. Data collection started in January 2019 and ended in December 2020.

The study protocol was approved by each institution's Ethical Committee and was conducted in accordance with the principles of the Declaration of Helsinki and with local laws and regulations. All patients provided written informed consent. In the case of minor patients, the informed consent was signed by parents.

Methods

All clinical and anamnestic information of the patients have been collected through the compilation of a paper case report form (CRF) at enrolment.

The severity of FVII deficiency was classified following the "Consensus Definitions in Rare Bleeding Disorders of the Factor VIII/Factor IX Scientific and Standardization Committee (SSC) of the International Society on Thrombosis and Hemostasis (ISTH)" and based on the results of the European Network of Rare Bleeding Disorders (EN-RBD) Registry.[8,9] Severe disease: FVII level <10% (high risk for spontaneous major bleeding); moderate disease: FVII level 10-20% (risk for mild spontaneous or traumatic bleeding); mild disease FVII level >20 (usually asymptomatic patients).

Bleeding scores

Bleeding risk was assessed for each patient by three different scores:

i. Mariani score: the patients have been classified as a) severe bleeders if reported at least one intracranial hemorrhage (ICH) and/or gastrointestinal (GI) bleeding and/or hemarthrosis in association with other bleeding events; b) moderate bleeders if reported three or more bleeding events excluding ICH, GI bleeding and hemarthrosis; c) mild bleeders if reported one or two bleeding symptoms excluding ICH, GI bleeding and hemarthrosis; d) non-bleeders.[12]
ii. ISTH/SSC-BAT score: the patients have been classified with (abnormal/positive) or without (normal/negative) bleeding risk based on the bleeding score (BS) obtained by the questionnaire, and different for age and sex. Normal/negative BS: children <3; males <4; and female <6.[13,14]
iii. Di Minno classification: the patients have been classified as a) asymptomatic, b) minor bleeders or c) major bleeders based on the characteristic of the first bleeding event (no bleeding; minor bleeding: epistaxis, ecchymosis, gum bleeds, hematuria, menorrhagia, umbilical cord bleeding, hemorrhoidal bleeding muscle or subcutaneous hematoma; major bleeding: ICH, GI bleeding and hemarthrosis).[10]

Each score then divided the patients into different groups based on their bleeding risk. These groups were subsequently compared with the results obtained by the thrombin generation assay.

Thrombin generation assay

The TGA analysis was performed following the Hemker protocol.[17]

Thrombin generation curves were calculated in comparison to a calibration curve measured in the same sample with a dedicated software (Thrombinoscope™, Thrombinoscope BV, Maastricht, The Netherlands).

All reagents for TGA were supplied by Diagnostica Stago (Asnières-sur-Seine, France).

A negative and a positive control, Cryocheck Normal Reference Plasma – Ref. Plasma (HIT), and Factor VII Deficient Plasma – FVII DP (Affinity biological), respectively, were included in the assay to assess the correct thrombogram for each patient. A first set-up of the TGA method was then carried out on these controls to determine the most suitable Tissue Factor (TF) concentration to trigger the coagulation reaction. Each sample (patient/control) was analyzed in duplicate (each duplicate represented by n=3 technical replies, applying an acceptability criterion ≤15%, as a coefficient of variation (CV) between the replicates.

Using the Thrombin Generation Assay (TGA) method we have analyzed:

i. LagTime: time needed to detect the thrombin formation onset
ii. ETP: endogenous thrombin potential; it detects the amount of thrombin formed in a defined period, which corresponds to the area under the curve (AUC)
iii. Peak Thrombin: concentration of the thrombin peak; corresponding to the maximum amount of thrombin reached during the thrombogram formation
iv. ttPeak: time to Peak; corresponding to time needed to reach the maximum amount of thrombin

All the obtained data have been compared with those obtained from twenty healthy subjects equally divided between males and females and collected at the participating Centers.

We have subsequently evaluated the derived parameter Velocity Index, defined by the peak thrombin concentration divided by the difference between time to peak and lag time.

Statistics

Descriptive statistical analyses were performed using SAS statistical software version 9.2 (SAS, Cary, NC) in Windows 7 professional environment.

The TGA statistical results analyses were performed using Minitab 17 software. The ANOVA test (α=0.05) with the Tukey Pairwise comparison was performed to analyze all the parameters with normal data distribution, while the non-parametric Kruskal-Wallis test was used for to analyze the remaining parameters with non-normal distribution.

RESULTS

Descriptive analyses

Overall, fifty-seven total patients were enrolled in four different Italian Hemophilia Centers (Padua, Palermo, Pavia and Rome), 63.2% of them were females, only one patient was black, the others were all Caucasian. Mean age at diagnosis was 26 years (range 9 months – 66 years). Family history for FVII deficiency was found in the 45.6% of cases. 31.6% of subjects had a severe disease, 14.0% moderate, and the remaining 54.4% mild. Mean FVII plasma level was 23.0% (range <1-50%). Eight patients (14.0%) presented a cardiovascular disease at diagnosis, arterial hypertension was present in 7/8 of them, while the remaining presented an ischemic cardiopathy (IC). The same patient with IC also presented a previous thrombosis in the upper left limb.

Mean age of twenty healthy controls was 31 years (range 19-57 years), and mean plasma FVII level was 83% (range 72-126%).

Bleeding scores

The three scores were applied to all 57 patients. For each score the following groups were identified in which the patients were thus distributed:

i. Mariani score: 13/57 (22.8%) severe, 11/57 (19.3%) moderate, 33/57 (57.9%) mild
ii. Di Minno classification: 13/57 (22.8%) major, 40/57 (70.2%), 4/57 asymptomatic (7.0%)
iii. ISTH/SSC-BAT: 27/30 (47.4%) abnormal, 30/57 (52.6%) normal

In 34/57 patients (59.6%) the bleeding risk obtained by each different score was in agreement with the severity of disease based on FVII plasma level. Conversely, in 12/57 patients (21.1%) with severe or moderate disease one or more bleeding scores showed a hemorrhagic risk lower than expected; while in the remaining 11/57 patients (19.3%), almost all mild, one or more of the bleeding scores were higher than supposed.

Considering only the ISTH/SSC-BAT bleeding score (BS) a statistically significant difference ($p<0.05$) was obtained comparing severe patients (median score 11.0) with mild (median score 3.0) or moderate (median score 3.5) patients respectively. No difference in BS between mild and moderate was highlighted.

TGA assay

Complete assessment was performed in 53 subjects; four patients (PV009, PV010, PV014 and PD005) were excluded from TGA analyses. PV009 and PV014 were excluded due to a Coefficient of Variation (CV) <15%, lower than the acceptability criterion of method; while

PV010 and PD005 were excluded due to the generation of a low amount of thrombin, not sufficient for analysis.

TGA *vs* FVII plasma level

The LagTime and the ttPeak since they have a normal distribution were analyzed with ANOVA test with Tukey Pairwise Comparisons. In this case both, LagTime and ttPeak showed a difference statistically significant ($p<0.05$) comparing to healthy control, while only for LagTime this difference was found comparing different phenotypes. Graphically, in almost all the patients analyzed, those with the most severe hemorrhagic phenotype have a higher curve, a situation not highlighted with ETP and Peak. These last two parameters, not presenting a normal distribution, were analyzed with the non-parametric Kruskal-Wallis test. A significant difference was found for both parameters by comparing the different groups with increasing hemorrhagic phenotype, $p=0.002$ for Peak and $p=0.001$ for ETP.

Compared to healthy control the ttPeak was increased of 2.3-fold in severe patients, 1.4 in cases of moderate, and 1.3 in case of mild ones; while the LagTime was increased 3.2-fold in severe subjects, 1.5 in both, moderate and mild ones. The Peak was reduced by 38.9% in patients with severe phenotype, by 14.8% for moderate, and by 13.2% for mild; while ETP was reduced by 27% compared to healthy control, while by 16.8% and by 17.9% for moderate and mild subjects, respectively. Representative TGA curves of severe, moderate, and mild bleeding phenotypes, compared to healthy controls were shown in Figure 1.

All the TGA results were subsequently compared with the three different scores (Mariani score – Di Minno classification – ISTH/SSC-BAT score).

TGA vs Mariani score

The comparison carried out between the four different parameters of the TGA, and the different groups of patients based on the Mariani score allowed us to establish that only the LagTime and the ttPeak are able to discriminate subjects with mild hemorrhagic phenotype from those with severe hemorrhagic phenotype, according to the Mariani score. On the other hand, no parameter was effective in discriminating subjects with a moderate phenotype who are therefore not distinguishable from either mild or severe patients.

The box plot comparing the TGA data with the hemorrhagic phenotypes obtained with the Mariani score is shown in Figure 2.

TGA vs Di Minno classification

LagTime and ttPeak were found to be able to discriminate between major bleeders versus minor bleeders and major bleeders versus asymptomatic ones. On the other hand, no parameter could differentiate the minor bleeders from the asymptomatic ones.

The box plot comparing the TGA data with the hemorrhagic phenotypes obtained with the Di Minno classification is shown in Figure 3.

TGA vs ISTH/SSC-BAT score

Comparative statistical analyzes showed that LagTime, ttPeak and Peak were able to discriminate between normal and abnormal subjects according to the ISTH/SSC-BAT score. Only the ETP does not appear to be useful in differentiating the two groups.

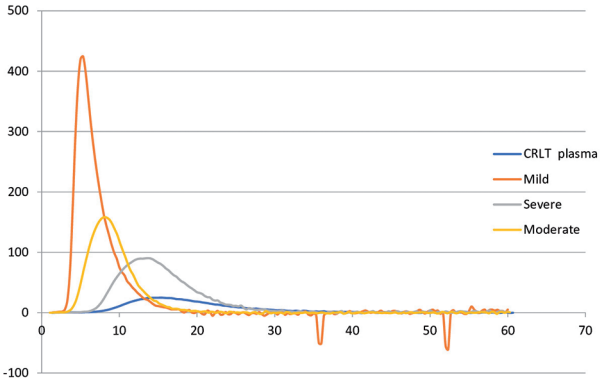

Figure 1. TGA curves of severe, moderate and mild bleeding phenotypes, compared to healthy controls (CRLT).

Table 1. Statistical comparison among TGA (all the 4 parameters) and the 3 different bleeding scores.

	Plasmatic FVII (%)	Mariani score	Di Minno classification	ISTH/SCC-BAT score
Lag-Time	Semi-discriminative (severe *vs* others)*	Semi-discriminative (severe *vs* mild/no-bleeders)*	Semi-discriminative (major *vs* minor)*	Semi-discriminative (abnormal *vs* normal)*
ETP	Non-discriminative	Non-discriminative	Non-discriminative	Non-discriminative
Peak	Non-discriminative	Non-discriminative	Non-discriminative	Semi-discriminative (abnormal *vs* normal)*
ttPeak	Semi-discriminative (severe *vs* others)*	Semi-discriminative (severe *vs* mild/no-bleeders)*	Semi-discriminative (major *vs* minor)*	Semi-discriminative (abnormal *vs* normal)*

**Discriminative results.*

The box plot comparing the TGA data with the hemorrhagic phenotypes obtained with the ISTH/SSC-BAT score is shown in Figure 4.

A summary of the results has been proposed in Table 1.

The derived parameter Velocity Index (VI) was subsequently obtained for each patient. Mean VI of mild subjects was 63.65 (±56.59) nM/min, higher than moderate or severe ones in which this parameter was 37.70 (±26.17) nM/min and 31.79 (±18.27) nM/min, respectively. Compared with FVII plasma level, this index resulted be useful in discriminating severe patients from mild, but not from moderate ones. In no case, the VI was able to discriminate the different patients presenting different hemorrhagic phenotype as classified by the three different scores

DISCUSSION

The plasmatic FVII level cannot correlate with the bleeding phenotype.[7] With this background it is very difficult to manage the people with FVII deficiency correctly, for this reason, clinicians try to find reliable hemorrhagic scores and laboratory assays that can help them in clinical practice.

In a recent article published by Toret *et al.,*[18] 27 children with plasmatic FVII <35% were included in a study designed to compare the global assays, thromboelastography (TEG) and TGA, with the ISTH/SSC-BAT score in assessing their bleeding phenotype. The FVII level resulted negatively correlated with the LagTime, of TGA, and positively with the ttPeak, while no significant correlation was showed between the FVII level and any TEG parameter, similar as observed in our patients in which the same TGA parameters seem to be able to discriminate between the major bleeders and the others. The medians of BS reported by Toret *et al*. were 8.3 and 2.9 in the severe and mild/moderate group, respectively, and the difference was statistically significant. Also, in our case the difference between the median BS in severe patients (11.0) and the median BS in mild (3.0) or moderate (3.5) subjects, was statistically significant (<0.05); and only in about half of them, FVII level correlate with the results of all three hemorrhagic scores. The ISTH/SSC-BAT

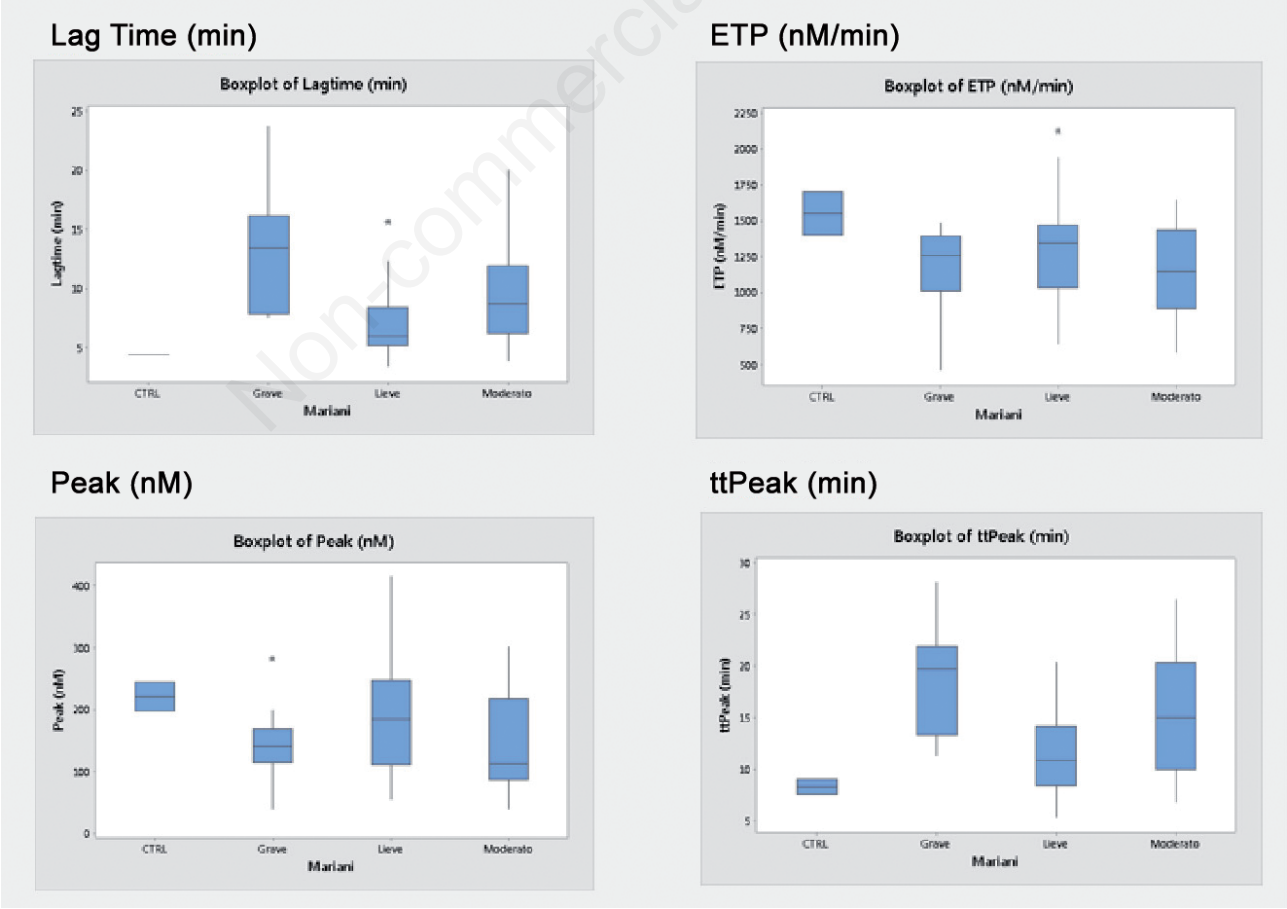

Figure 2. Box plot comparing TGA parameters and hemorrhagic phenotypes obtained by Mariani score.

score was in fact created to assess the bleeding phenotype in von Willebrand patients,[18] it is not always useful to define the real hemorrhagic phenotype of patients with rare bleeding disorders, such as FVII deficiency. In 2016 Palla *et al*.[20] developed a new Bleeding Score to specifically determine the hemorrhagic phenotype in subjects affected by RBDs and to distinguish them from healthy people, based on clinical history. The importance of BS in clinical practice is therefore proven, but no tool has been found to be superior to others in accurately classifying the bleeding risk of each individual patient. In our study we therefore used, in addition to the ISTH/SSC-BAT, also the Mariani score, and the Di Minno classification and we correlated them to the data obtained through the Thrombin Generation Assay. As in case of the plasmatic FVII level, the LagTime and the ttPeak resulted able to discriminate the severe patients from the others.

Tran *et al*.[16] analyzed the global assays to define whether TEG and TGA could predict the hemorrhagic phenotype in patients with severe FVII deficiency. In case of TGA parameters, ETP was reduced to 30% of the healthy controls in platelet poor plasma, while the LagTime and the ttPeak were increased threefold compared with those of the controls. Like what was observed in our study, in which the ETP of severe patients was reduced by 27% compared to controls, the ttPeak was increased twofold, while the Lag-Time was also increased threefold. Although differences were observed between moderate/mild subjects and healthy controls, it was not possible to discriminate accurately between mild or moderate patients. The derived parameter Velocity Index was able only to discriminate severe patients from mild, when compared with FVII plasma level.

CONCLUSIONS

Our study showed that the bleeding tendency of severe patients affected by inherited Factor VII deficiency can be equally discriminated from that of others using both, bleeding scores or TGA, while no discrimination was possible in patients with moderate or mild disease. The correct determination of the hemorrhagic phenotype of patients with FVII deficiency therefore remains an unknown factor, a difficult to interpret variable, which often makes it difficult to manage by clinicians.

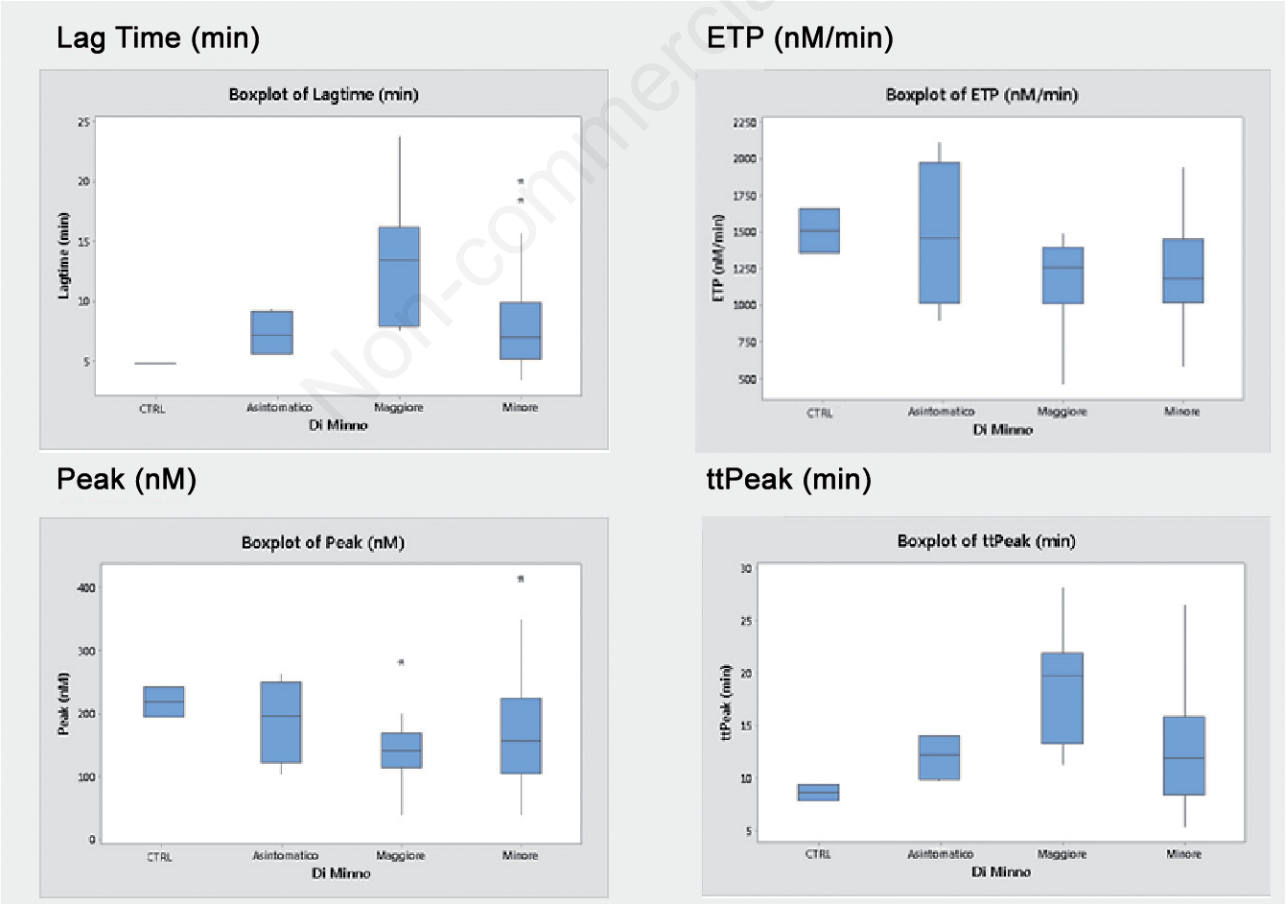

Figure 3. Box plot comparing TGA parameters and hemorrhagic phenotypes obtained by Di Minno classification.

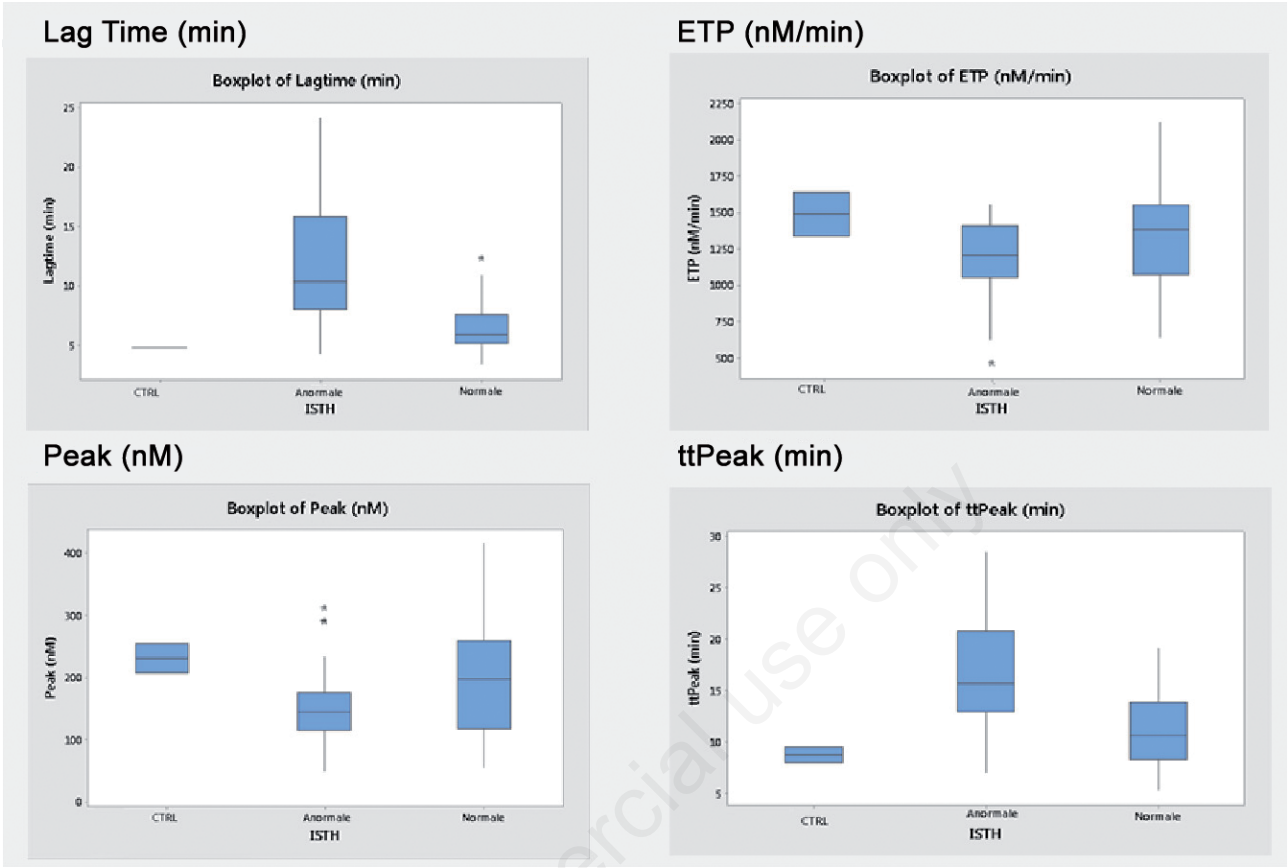

Figure 4. Box plot comparing TGA parameters and hemorrhagic phenotypes obtained by ISTH/SCC-BAT score.

Limitation

First limitation of this study is due to a low number of patients, and to a non-homogeneous distribution among the various severity degrees of deficiency. Second limitation is due to lack of genetic mutation for each patient still under laboratory assessment.

REFERENCES

1. Perry DJ. Factor VII Deficiency. Br J Haematol 2002;118:689-700.
2. Napolitano M, Siragusa S, Mariani G. Factor VII Deficiency: Clinical Phenotype, Genotype and Therapy. J Clin Med 2017;6:38.
3. Peyvandi F, Duga S, Akhavan S, et al. Rare coagulation deficiencies. Haemophilia 2002;8:308–21.
4. Lapecorella M, Mariani G; International Registry on Congenital Factor VII Deficiency. Factor VII deficiency: defining the clinical picture and optimizing therapeutic options. Haemophilia 2008;14:1170-5
5. Mariani G, Herrmann FH, Dolce A, et al. International Factor VII Deficiency Study Group. Clinical phenotypes and factor VII genotype in congenital factor VII deficiency. Thromb Haemost 2005;93:481-7.
6. Napolitano M, Di Minno MN, Batorova A, et al. Women with congenital factor VII deficiency: clinical phenotype and treatment options from two international studies. Haemophilia 2016;22:752-9.
7. Iwaniec T, Zdziarska J, Musiał J, Sanak M. A Retrospective Analysis of Clinical and Laboratory Data of Patients with Factor VII Deficiency: A Single Centre Experience. Hamostaseologie 2019;39:368-76.
8. Peyvandi F, di Michele D, Bolton-Maggs PHB, et al. Project on Consensus Definitions in Rare Bleeeding Disorders of the Factor VIII/Factor IX Scientific and Standardisation Committee of the International Society on Thrombosis and Haemostasis. Classification of rare bleeding disorders (RBDs) based on the association between coagulant factor activity and clinical bleeding severity. J Thromb Haemost 2012;10:1938-43.
9. Peyvandi F, Palla R, Menegatti M, et al. European Network of Rare Bleeding Disorders Group. Coagulation factor activity and clinical bleeding severity in rare bleeding disorders: results from the European Network of Rare Bleeding Disorders. J Thromb Haemost 2012;10:615-21.
10. Di Minno MN, Dolce A, Mariani G; STER Study Group. Bleeding symptoms at disease presentation and prediction of ensuing bleeding in inherited FVII deficiency. Thromb Haemost 2013;109:1051-9.
11. Mariani G, Herrmann FH, Bernardi F, et al. Clinical mani-

festations, management, and molecular genetics in congenital factor VII deficiency: the International Registry on Congenital Factor VII Deficiency (IRF7). Blood 2000;96:374.
12. Mariani G, Herrmann FH, Dolce A, et al. Clinical phenotypes and factor VII genotype in congenital factor VII deficiency Thromb Haemost 2005;93:481-7.
13. Rodeghiero F, Tosetto A, Abshire T. ISTH/SSC-BAT bleeding assessment tool: a standardized questionnaire and a proposal for a new bleeding score for inherited bleeding disorders. J Thromb Haemost 2010;8:2063-5.
14. Elbatarny M, Mollah S, Grabell J, et al. Normal range of bleeding scores for the ISTH-BAT: adult and pediatric data from the merging project. Haemophilia 2014;20:831–5.
15. Tripodi A. Thrombin Generation Assay and its application in the clinical laboratory. Clin Chem 2016;62:699-707.
16. Tran HT, Tjønnfjord GE, Holme PA. Use of thromboelastography and thrombin generation assay to predict clinical phenotype in patients with severe FVII deficiency. Haemophilia 2014;20:141-6.
17. Hemker HC, Giesen P, Al Dieri R, et al. Calibrated automated thrombin generation measurement in clotting plasma. Pathophysiol Haemost Thromb 2003;33:4-15.
18. Toret E, Ay Y, Karapinar TH, et al. Evaluation of Bleeding Phenotype of Inherited Factor VII Deficiency in Children With a Bleeding Assessment Tool and Global Assays. J Pediatr Hematol Oncol 2020;42:e527-30.
19. Tosetto A, Rodeghiero F, Castaman G, et al. A comparison between two semi-quantitative bleeding scales for the diagnosis and assessment of bleeding severity in type 1 von Willebrand disease. Haemophilia 2011;17:165–6.
20. Palla R, Siboni SM, Menegatti M, et al. European Network of Rare Bleeding Disorders (EN-RBD) Group. Establishment of a bleeding score as a diagnostic tool for patients with rare bleeding disorders. Thromb Res 2016;148:128–34.