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Influence of factor 5 rs6025 and factor 2 rs1799963 mutation on inhibitor development in patients with hemophilia A - an Israeli-German multicenter database study

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ABSTRACT

Objective: The present cohort study was performed to investigate the impact of the factor 5 rs6025 [F5] and the factor 2 rs1799963 [F2] mutations on high-titer inhibitor development [HRI] in patients with severe/moderate-severe hemophilia A [HA].
Patients and Methods: 216 patients with F8 < 2% born between 1980 and 2011 were followed after initial HA diagnosis over the first 200 exposure days. The first HA patient per family who presented for diagnosis was included in the present study.
Results: 32 of 216 children [14.8%] tested for F5/F2 carried either the F5 or the F2 variant. HRI occurred in 14 out of 32 F5/F2-carriers compared with 40 of 184 without F5/F2. Multivariate analysis adjusted for F8 genotype, treatment intensity, first-line use of plasma derived FVIII versus recombinant FVIII concentrates revealed that the presence of F5/F2 independently increases the risk of HRI development to odds [OR] of 3.4. Large deletions in the F8 gene [OR: 5.10], patients from Israel [OR: 4.0], the increase of FVIII per one IU/kgbw [OR: 1.05] and birth year [OR: 1.12] were significantly associated with the risk to develop HRI.
Conclusion: Data presented here suggest that HRI development is of multifactorial origin and that F5 and F2

Introduction

The development of inhibitor antibodies against factor VIII [FVIII] is one of the most important clinical challenges for patients with hemophilia A and their treating physicians, with important implications with respect to patient's health and socio-economic burden [1]. Apart from non-modifiable risk factors for inhibitor development, such as underlying severity [FVIII level at baseline], the genetic background [type of F8 gene mutation], and a positive family history of inhibitor development [in part related to inherited mutation type], research has emphasized the role of modifiable risk factors, with treatment-related risks as the most important candidates [2–5]. Among treatment-related risk factors, the use of recombinant FVIII [rFVIII] concentrates or high dose FVIII administration have been controversially debated as risk factors for inhibitor development [2,4,5]. In a recent systematic

Abbreviations: BU, Bethesda units; ED, exposure days; F2, Factor 2 rs1799963; F5, Factor 5 rs6025; FVIII, Factor VIII; F8, Factor 8 genotypes; HA, hemophilia A; HRI, high-titer inhibitor; HR/CI, hazard ratio/confidence interval; ICH, intracranial hemorrhage; IFS, inhibitor-free survival; IQR, Interquartile range; IT, thrombophilic risk factor; OR/CI, Odds ratio/confidence interval; PUP, previously untreated patient; pdFVIII, plasma-derived factor VIII product; rFVIII, recombinant FVIII product.

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review on risk factors of high-titer inhibitor [HRI] development [4], it has been shown that possible confounders such as Factor VIII products must be carefully considered when drawing conclusions from the analysis of observational data, while awaiting results of prospective randomized and adequately powered multicenter studies [6].

The clinical phenotype of hemophilia A is not always explained by its underlying F8 genotype, and it has been controversially discussed if the phenotype of severe hemophilia A [HA] is influenced by co-inheritance of the factor 5 rs6025 [F5] mutation [7–15]. In a German cohort study we demonstrated that the first symptomatic bleeding onset in children with severe HA carrying the F5 or the factor 2 rs1799963 [F2] variant was significantly later in life than in non-carriers [10] In the latter cohort a protective effect of thrombophilic risk factors [IT] was shown for the annual bleeding frequency and the severity of the hemophilic arthropathy] [12]. In contrast, however, in a further adequately powered adult HA cohort this association could not be completely confirmed: [15] in 100 adolescent and adult patients with hemophilia A or B from Sweden Shulman and colleagues found that the clinical severity of hemophilia measured by a hemophilia risk score appeared to be modified by the F2 mutation but not by coinheritance of the F5 variant. Furthermore, in an animal model the effect of the F5 polymorphism to improve the hemophilic phenotype was restricted at the microcirculation level followed by vascular injury [14].

The present cohort study was performed to investigate the impact of the F5 and F2 mutations on clinical meaningful high responding inhibitor development [outcome variable] in white children with severe/moderate-severe HA.

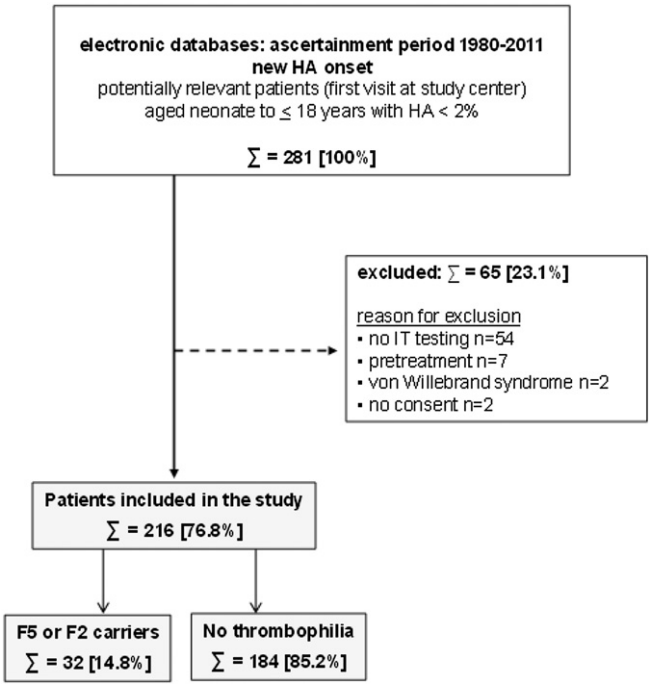

Fig. 1. Flow chart: study population break-down by inclusion and exclusion criteria.

Methods

Ethics

The present multicenter database study in consecutively recruited pediatric patients with HA which were prospectively followed for the development of HR inhibitor development by the participating centers was performed in accordance with the ethical standards laid down in a relevant version of the 1964 Declaration of Helsinki and was approved by the Medical Ethics Committee of the University of Münster, Germany. The present cohort study was reported in accordance to STROBE guidelines for observational studies [16].

Inclusion/Exclusion Criteria

Inclusion and exclusion criteria are shown in Fig. 1. Previously untreated patients [PUPs] with severe/moderate-severe HA aged neonate to ≤18 years, who had been admitted to the University Children's Hospitals of Frankfurt, Halle, the MVZ Duisburg, Kiel-Lubbock, Munich, Münster, Germany and the Hemophilia Treatment Center Tel-Hashomer, Israel, at first symptomatic onset of the disease were enrolled [5,10,12,17]. Patients born before 1980, pediatric patients with HA additionally carrying von Willebrand disease, children with HA ≥ 2%, and HA patients not tested for the factor 5 and F2 mutation [no parental/patient consent] were not included in this cohort study. In addition, children pretreated with transfusion of red blood cell concentrate or fresh frozen plasma before the first administration of FVIII concentrate, were not enrolled. To avoid family cluster effects in both countries only the first HA patient within a given family who presented for diagnosis at the treatment center was included in the present study.

Outcome Measures

Inhibitor-free survival time [IFS: first 200 exposure days (ED)] related to presence or absence to F5 or F2 mutations: HA patients carrying the F5 or 2 mutation were compared with subjects not carrying the above mentioned F5 or F2 variants. Further debated variables were F8 gene mutations, first-line use of plasma-derived [pd] versus rFVIII concentrates [2–5] and individual median single FVIII dosage [per IU/kg bodyweight] administered over the first three months of treatment as a proxy for treatment intensity. In addition adjustment was performed for treatment periods [year of birth] and country of patient origin, i.e. Israel or Germany. From 1980 to 2011, 281 consecutive pediatric PUPs of Caucasian origin with a first symptomatic onset of HA < 2% residual FVIII activity were ascertained: From these patients 65 individuals were excluded because of *i)* non-testing for thrombophilia, *ii)* pretreatment with blood products, *iii)* co-expression of von Willebrand syndrome or *iv)* non-consent. Off note: 54 of 281 children which were not tested for thrombophilia were equally distributed within the study centers and did not differ with respect to inhibitor development [n = 13]. The final study cohort included 216 unrelated children [Fig. 1].

Treatment

At the discretion of the participating centers and according to standard of care in the years of patient enrollment children were either treated with primary prophylaxis or with secondary prophylaxis. The opportunity of primary prophylaxis was offered to all newly diagnosed patients independent from age at presentation. The treatment regimens were maintained as standard over time and the treatment regimens were administered without knowledge of the individual thrombophilia status, with no difference between carriers and non-carriers of F5/F2 [18–20]. For patients presenting with severe soft tissue bleeding at HA onset an intensified treatment protocol was introduced in the mid-1990s. These children received a primary prophylactic treatment regimen following the first symptomatic hemorrhage [three times per week]. In cases of trauma-associated or large spontaneous hemorrhage two to three daily FVIII infusions were administered for a minimum of five to seven days. The latter treatment episodes were classified as "intensified treatment moments".

Data Collection

baseline FVIII, F8 genotype, age at first FVIII infusion, FVIII brand, median single dosage administered over the first three months of

treatment, frequency of weekly factor administration, clinical bleeding situations requiring intensive FVIII administration, such as intracerebral hemorrhage [ICH], liver rupture or surgery, ethnicity, family history of inhibitor development, country of patient origin, results of inhibitor measurements, and FVIII ED and carrier status of thrombophilia, i.e. factor 5 and factor 2 variants and antithrombin, protein C and protein S deficiency were collected.

Laboratory Analysis

Plasma levels of FVIII were determined by one-stage clotting assays using standard laboratory methods. Inhibitor testing was performed at least monthly when on therapy using the Bethesda method or its modification [Nijmegen]: The lower detection limit was set according to the inhibitor assay used in each study center, and a peak inhibitor titer of >5 BU was defined as high-titer antibody. Inhibitor positivity was stated when an inhibitor was measured at least in two independent follow-up visits.

Statistics

Statistical analyses were performed with the MedCalc software [version 12.3.0] and the StatView 5 software package [SAS Institute Inc.]. Continuous data were presented as median/interquartile range [IQR] or minimum-maximum values, and evaluated by non-parametric statistics using the Wilcoxon-Mann–Whitney U test. Frequency distributions of adverse outcome and possible interactions within independent variables were compared with chi-square test or, if necessary, Fisher's exact test. IFS, defined as the number of cumulative ED until inhibitor development, was calculated by Kaplan-Meier method, and compared between groups by Cox proportional hazard modeling with calculation of hazard ratios [HRs]/95% confidence intervals [CIs]. The effect of variables possibly associated with HRI development and variables of interest in bivariate analysis [first-line use of pd- versus rFVIII concentrates, F8 genotypes [one categorical variable with five options] [21], individual median single FVIII dose administered over the first three months, year of birth [continuous variable; proxy for different treatment periods], country of patient origin] within the observation period of 200 ED was assessed by multivariate analyses [logistic regression]: Odds ratio [ORs] and 95% CIs were calculated. P-values < 0.05 were considered significant. The quality of the logistic regression model was tested with the Hosmer-Lemeshow goodness-of-fit test. Off note: As HRI generally develops in a short time period after FVIII substitution time to inhibitor development is negligible: therefore logistic regression rather than Cox proportional-hazards regression was chosen in this study design [median of 22 EDs]. In addition, since we have recently shown that Israeli and German HA patients with FVIII activity <1% did not differ from HA children with FVIII activities between 1% to <2% with respect to underlying HA genotypes and clinical phenotypes patients with a remaining FVIII activity <2% were analyzed together [17].

Results

Study Population

The characteristics of the final study population are shown in Tables 1a and 1b. According to inclusion and exclusion criteria the final study cohort ascertained from 1980 to 2011 included 216 PUPs with HA < 2%. In the HA patients investigated, the overall heterozygous F5 [n = 22] or F2 [n = 10] carrier frequency was 14.8%: 19.6% in the Israeli cohort and 12.9% in Germany [p = 0.3]. In the present cohort no patient was a homozygous or double heterozygous F5/F2 carrier. None of the children with HA <2% carried antithrombin-, protein C– or protein S-deficiency. The median [IQR] single FVIII administered over the first three months of treatment and the median weekly substitution intervals on prophylaxis prior to inhibitor development are shown in Table 1a. For trauma-associated hemorrhage an intensified FVIII administration was documented.

HR Inhibitor Development - Descriptive & Bivariate Analysis

Within a median [minimum-maximum] time period of 22 [8–172] EDs 54 of 216 children [25%] developed HRI. The distribution of underlying F8 gene mutations is shown in the suppl. material [Fig. 1]. Four out

Table 1a
Patient characteristics and rates of HR inhibitor development by country is shown.

	Israel N = 61	Germany N = 155	Total N = 216
Years of birth	1980 – 2011	1980 – 2011	1980 – 2011
Ethnicity			
Caucasian (%)	100	100	100
Factor concentrates used [n]			
- pdFVIII	34	100	134
- rFVIII	27	55	82
Median [IQR] single dose FVIII [IU/kg/bw]	30 [17]	38 [30]	35 [25]
Thrombophilia status: number [%]			
- F 5	9 [14.7]	13 [8.4]	22 [10.2]
- F 2	4 [6.6]	6 [3.9]	10 [4.6]
Persistent high responding inhibitor [>5 BU]	23 [37%]	31 [20%]	54 [25%]
Indications for intensified treatment prior to HR inhibitor development:			
Total: number	12	16	28
- neonatal ICH	2	4	6
- cephalohematoma	1	5	6
- liver rupture	-	1	1
- head/spinal trauma	4	-	4
- knee or ankle bleed	1	3	4
- tongue bleed	1	3	4
- appendectomy	1	-	1
- meatotomy	1	-	1
- nephroblastoma	1	-	1

Abbreviations: BU: Bethesda Units; F2: Factor 2 rs1799963; F5: Factor 5 rs6025; ICH: intracranial hemorrhage; IQR: interquartile range kgbw: kilogram bodyweight; IT: inherited thrombophilia; pd: plasma derived; r: recombinant.

Table 1b
Patient characteristics by inhibitor development is shown.

	No inhibitor N = 162	High-titre inhibitor N = 54	Total N = 216
Years of birth:			
Median [IQR]	1990 [10]	1994 [17]	1991 [11]
Factor concentrates used: number			
- pdFVIII	106	28	134
- rFVIII	56	26	82
Median [IQR] single dose FVIII [IU/kg/bw]	32 [20]	50 [36]	35 [25]
Median [minimum-maximum] weekly substitution interval	3 [1–3]	3 [1–4]	3 [1–4]
Thrombophilia status: number	18	14	32
- F5	11	11	22
- F2	7	3	10
High risk gene mutations: number	49	30	79
Exposure days to HRI development: median [IQR]	-	22 [30]	-

Abbreviations: F2: Factor 2 rs1799963; F5: Factor 5 rs6025; HRI: high-titre inhibitor; IQR: interquartile range; kgbw: kilogram bodyweight; pd: plasma derived; r: recombinant.

of 54 children [7.4%] developed their inhibitor during on-demand therapy [EDs: 12, 18, 22 & 23], 50 patients developed HRI while on primary or secondary prophylaxis. In 10 of 28 children [35.7%] with intensified FVIII administration due to trauma-associated or spontaneous hemorrhage HRI were detected. In this subgroup one neonate with ICH out of 10 children with HRI receiving intensified FVIII substitution carried additionally the F5 mutation. Higher factor VIII doses administered in the latter 10 children contributed to the higher factor VIII doses administered in children with HRI (Table 1b). HRI occurred in 14 [F5: n = 11; F2: n = 3] out of 32 carriers of IT [44%] compared with 40 of 184 [22%] without IT [P = 0.009]. The IFS in patients carrying F5 or F2 mutations was decreased as compared to patients without IT [Fig. 2]. The difference in cumulative IFS was statistically significant [HR/95%CIs: 3.07/1.4-6.9].

No statistically significant interactions were found between F5/F2 carriers and *i)* the presence of F8 genotypes [p = 0.93; data are shown in suppl. Fig. 2] or *ii)* children treated with intensified FVIII administrations [p = 0.73].

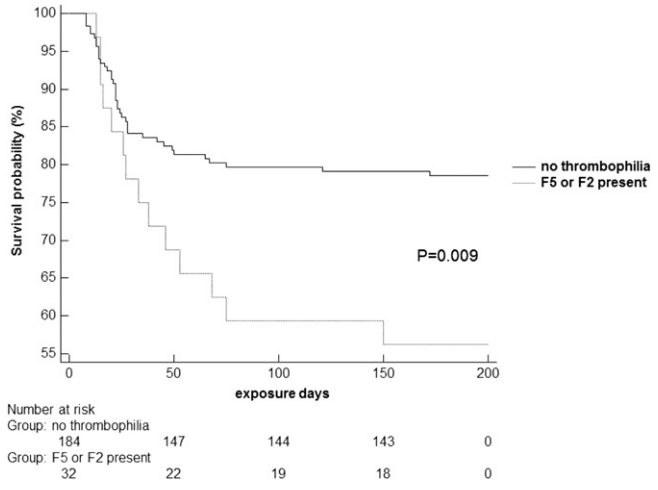

Fig. 2. Cumulative inhibitor-free survival of patients with HA < 2% in children with and without F5 or F2 variant.

Inhibitor Development - Multivariate Analysis

Multivariate analysis [logistic regression] adjusted for F8 genotype, first-line use of pdFVIII [n = 134] versus rFVIII concentrates [n = 82], patient's individual median single FVIII dose, the year of birth and country of origin revealed that the presence of F5/F2 independently increased the risk of inhibitor development, with an odds ratio of 3.4 [CI: 1.33-8.7]. In addition, patients carrying the F8 genotype "large deletion" [OR/95%CI: 5.10/1.12-23.17], children enrolled from Israel [OR/95%CI: 4.0 [1.45-11.0], the increase of FVIII per one IU/kgbw [OR/95%CI: 1.05/1.02-1.08] and the year of birth [OR/95%CI: 1.12/1.05-1.20] were significantly associated with the risk to develop a HRI. Children born in the nineties towards 2011 [latest year of birth] showed a higher rate of inhibitor development compared to children treated in the eighties. There was a weak but statistically significant correlation between each increasing year of birth [1980 – 2011] and occurrence of HRI [rho = 0.297; 95% CIs: 0.17-0.41]. Findings of bi- and multivariate analyses are summarized in Table 2 [the variables are adjusted for each other]. The Hosmer-Lemeshow goodness-of-fit P-value was 0.88. Results of Cox proportional-hazards-regression analysis are shown in suppl. Table 1. Results calculated for the subgroup of 192 children with HA < 1% were comparable to the entire study group [Data- logistic regression & Cox proportional hazards regression analysis- are shown in suppl. Table 2].

Discussion

The data reported in the present cohort study demonstrate that the development of a HRI in children with HA < 2% is of multifactorial origin. In addition to the known risk factors associated with inhibitor development, we have shown that the presence of F5 or F2 mutations in the heterozygous state did not only modify the clinical phenotype of HA [7,8] but have been shown to be independent risk factors for inhibitor development. Of note, the prevalence of both mutations was within prevalence rates reported in the general Israeli and German population [22–24]. Here we speculate, that the carrier status of the F5 or F2 mutation contributes to HRI development in the cohorts investigated.

The mechanism by which patients with HA < 2% additionally carrying the F5 or F2 mutation are prone to a higher risk to develop inhibitors

Table 2
Unadjusted [univariate] and adjusted [multivariate] odds ratios [OR] and 95% confidence intervals [CIs] are shown. Variables in multivariate analysis are adjusted for each other.

Parameter investigated	Unadjusted OR/CIs	Adjusted OR/CIs
Comparator: no F5/F2		
F5 or F2 carrier status	2.9 [1.32-6.3]	3.4 [1.33-8.7]
Comparator: intron 22 inversion		
Large deletions	3.61 [1.01-12.86]	5.10 [1.12-23.17]
Nonsense mutations	0.9 [0.24-3.42]	1.47 [0.30-7.19]
Missense mutations	0.33 [0.13-0.77]	0.27 [0.09-0.85]
Small deletions/insertions/splice-site mutations	0.08 [0.02-0.30]	0.06 [0.01-0.30]
Increase per one IU/kgbw		
Median single FVIII dose	1.05 [1.03-1.07]	1.05 [1.02-1.08]
Comparator: pdFVIII		
rFVIII	1.75 [0.97-3.3]	1.92 [0.73-4.99]
Per year of birth [1980 towards 2011]		
Year of birth	1.09 [1.05-1.14]	1.12 [1.05-1.20]
Comparator Germany		
Country of origin	2.5 [1.3-4.8]	4.0 [1.45-11.01]

Abbreviations: F2: Factor 2 rs1799963; F5: Factor 5 rs6025; kgbw: kilogram bodyweight.
* inversions, large or small deletions, nonsense, missense, splice and frameshift mutations.

is not fully elucidated yet. However, based on previous findings that children with the F5 or F2 mutation had a later bleeding onset [10] and that both thrombophilic mutations may protect from blood loss in adults [11] and reduce joint damage in children [11], we hypothesize that in patients with the aforementioned thrombophilic changes in case of a clinically relevant bleeding *i)* a more severe exogenous trigger is needed to let the patient bleed, with *ii)* a higher peak dose of factor FVIII to be used to stop the hemorrhage [25]. In addition, children who bleed less severely do frequently show up later in the hemophilia treatment centre with a larger untreated hemorrhage. Thus, a more severe trigger along with a larger amount of blood, possibly leading to a higher degree of cell and tissue damage, consequently may lead to a more intensive activation of the so called "danger signals" [26–29]. It has been reported that in individuals with thrombophilia increased thrombin generation is observed [30–32]. In addition, Skupsky and colleagues demonstrated in an animal model that thrombin formation through the procoagulant activity of FVIII is necessary to induce co-stimulation for the immune response to FVII treatment [33]. As thrombin is a potential "danger signal", the children who are treated with higher FVIII doses due to occurrence of larger bleeds as previously explained, are more susceptible to inhibitor formation. Danger signals, first discussed in 1994 [26], can be induced by bleeding associated tissue or cell-damage and stimulate inflammatory responses of the immune system, thereby up-regulating antibody responses, with the here speculated consequence of HR development against FVIII.

In our multivariate analyses we also showed that the individual FVIII dose administered over the first three months of treatment and the year of birth did play a role in the HR inhibitor development. In contrast, the controversially discussed risk of rFVIII over the use of pdFVIII concentrate did not reach significance in the present analyses. This finding is in line with data of the CANAL and RODIN cohort studies [3,21,34] and a recently published meta-analysis on observational trials [4]. Notably, inclusion of "year of birth" in the analytic model was responsible for the decrease of the odds of type of FVIII concentrate, underlining the importance of concurrent comparison between product types.

Limitations of this multicenter study include the long ascertainment period from 1980 to 2011. This latter characteristic increases the potential for time-period effects linked to changes in clinical practice that may in turn impact risk for HRI development. However, similar to the reported Canadian hemophilic cohorts [20], our patients have been on treatment protocols that remained unchanged with respect to treatment indications. In the Canadian cohort and our cohort, a similar increasing preference of prophylactic treatment regimens was observed since the late eighties/mid-nineties. Since the treatment regimens were administered without knowledge of the individual F5/F2 status [5,10,12], with no difference between carriers and non-carriers of thrombophilia, our observation gives evidence that the thrombophilic gene mutations truly contribute to the higher inhibitor frequency in the children reported. An additional potential limitation is the restriction of the cohort data to a binational sample. In particular, to the extent that the prevalence rates of the F5 and F2 variants in Israel and Germany differ from those in other countries, caution should be exercised in generalizing the findings to other nationalities. Finally we are aware that although the study cohort is small, it is one of the largest continuously recruited pediatric HA patient cohort. Thus, based on the small sample size as further study limitation we have to discuss the lack of power to detect significant study results. This mainly affects a type II error, i.e. the mistake not to see an association between F5/F2 status and inhibitor development which, however, is not the case in the present study because we could show a statistically significant association also in multivariate analysis.

In conclusion, data presented here suggest that development of HR inhibitors is of multifactorial origin in which, apart from a positive family history of inhibitors, presence of F5 and F2 mutations should be investigated.

Table 3
Contribution of the present study to the understanding of inhibitor development in children with hemophilia A.

What is known about the topic
• Genetic and treatment related variables play a role in high titer inhibitor development in patients with hemophilia A
What does the paper add
• Multivariate analysis adjusted for F8 genotype, treatment intensity, first-line use of plasma derived FVIII versus recombinant FVIII concentrates revealed that the presence of F5 rs6025 or F2 rs1799963 independently increases the risk of HRI development to odds of 3.4.
• Data presented here suggest that high titer inhibitor development is of multifactorial origin and that F5 rs6025 or F2 rs1799963 may contribute to this risk.

Contribution

GK and UNG designed the study and analyzed the data. NB performed F8 genotyping. GK, SH2, NG and UNG wrote the paper, CB, CEE, SH1, VJ, SG, GK, KK and UNG recruited patients and had full access to the data and took part in the design, execution and data analysis, discussion, and in writing the report.

Conflict of Interest Statement

The authors declare no competing financial interests.

Acknowledgement

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Appendix A. Supplementary Data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.thromres.2014.01.005.

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