THROMBOSIS AND HEMOSTASIS

Correlation between phenotype and genotype in a large unselected cohort of children with severe hemophilia A

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Key Points

- Previously untreated patients with severe hemophilia A caused by F8 null mutations show a more severe phenotype than previously untreated patients with non-null mutations.
- The phenotypic differences are modest, and as such not likely to affect decisions regarding when and how to start prophylaxis.

Phenotypic variability is well recognized in severe hemophilia A. A few studies, mainly in adults treated lifelong on demand, suggest that bleeding phenotype correlates with factor VIII gene (F8) mutation type. Because treatment regimens influence outcomes to a large extent, examining bleeding phenotype during the first years of life may be the most suitable way to define this variability. We set out to analyze the very early phenotypic expression of severe hemophilia A in 621 consecutively enrolled, well-characterized previously untreated patients and to correlate this with patients' F8 mutation. Detailed information was collected on bleeds and treatment of the first 75 exposure days or until inhibitor development. F8 mutation type was known for 531 patients; 402 had null mutations and 129 had non-null mutations. Considering only patients who had not started prophylaxis or developed an inhibitor before select bleeding events, we found that patients with null mutations experienced their first bleed and first joint bleed at younger median ages than patients with non-null mutations (9.7 vs 10.9 months and 13.8 vs 16.1 months, respectively). We conclude that F8 mutation type accounts for only a small component of the significant phenotypic variability found among patients with severe hemophilia A. (*Blood*. 2013;121(19):3946-3952)

Introduction

Bleeding phenotype in patients with hemophilia A is generally related to the residual factor (F) VIII level in plasma, and FVIII gene (F8) mutation is the main determinant of such levels.[1] However, even in the presence of the same clotting factor activity, considerable phenotypic variability exists, and a number of studies have determined that 10% to 15% of patients with severe hemophilia A seem to have a much milder disease phenotype.[2-6]

In the past, bleeding phenotype has been described using several indicators such as annual bleeding frequency (including joint and nonjoint bleeds), annual factor concentrate consumption, and markers of joint status (radiologic and clinical joint scores).[2]

Nowadays, the widespread use of prophylaxis started at very young ages has changed the natural history of severe hemophilia A by reducing bleeding frequency and making factor consumption no longer a reliable predictor of hemophilia phenotype. Because of this, the period of life from birth to the implementation of prophylaxis may represent the most suitable period to define the intrinsic bleeding phenotype of patients with severe hemophilia.

There are a variety of factors that are thought to influence bleeding tendency in patients with severe hemophilia A. These include F8 mutation, coinheritance of other bleeding or clotting disorders, pharmacokinetic handling of factor, physical activity patterns, and most important, different treatment regimens. F8 mutations are categorized as null or non-null mutations, taking into account that a certain level of FVIII synthesis is possible for the latter even if it is not detected by routine laboratory assays. Null mutations include intron 22 inversion mutations, which are the most common mutation responsible for severe hemophilia A, accounting for between 42% and 45% of all cases.[7,8]

Some retrospective studies in patients with severe hemophilia treated life-long on demand have suggested that patients with F8 null mutations have a more severe phenotype than patients with F8 non-null mutations.[6,9] However, no systematic evaluation of the role of F8 genotype as a determinant of bleeding phenotype in young children with severe hemophilia A has been performed to date.

F8 mutation can be determined early in life and before the child is started on therapy; moreover, in familial cases it is often already known when a newborn is diagnosed with hemophilia. The determination of F8 mutation may enable prediction of the bleeding pattern in newborn children with severe hemophilia, and in doing so may affect clinical decision making. For example, in countries

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that cannot afford to place all patients on prophylaxis, this might guide clinicians in deciding which patients should be placed on prophylaxis early and which patients might be able to start prophylaxis much later. Moreover, such a selection may allow the tailoring of prophylaxis regimens to individual patients and might also allow better justification for early insertion of a central venous line in those patients predicted to have a more severe phenotype, while delaying or avoiding a central venous line in those patients predicted to have a less severe phenotype.

In our study, we addressed the question of how predictive the F8 mutation type might be with respect to bleeding phenotype in a large unselected cohort of previously untreated patients (PUPs) with severe hemophilia A, hypothesizing that children with null mutations would have a more severe bleeding tendency than those with non-null mutations.

Methods

Patients

We included all PUPs with severe hemophilia A (FVIII activity <1%) born between January 1, 2000, and January 1, 2010, who were diagnosed and followed in 1 of the 29 hemophilia treatment centers participating in either the PedNet Registry or the RODIN Study. For the present study, we used data updated as of May 1, 2012. Approval for anonymous data collection was obtained from each of the 29 centers' institutional review boards, and written informed consent was obtained from the parents or guardians of all participants in accordance with the Declaration of Helsinki.

On the basis of F8 mutation type, patients were allocated into 3 groups:

Group 1, referred to as F8 null mutations, included patients with intron 22 and intron 1 inversions, nonsense mutations, large deletions, small deletions/insertions outside poly-A runs, or splice-site mutations involving conserved nucleotides.

Group 2, referred to as F8 non-null mutations, included patients with missense mutations, small deletions/insertions within poly-A runs, or splice-site mutations involving nonconserved nucleotides.

Group 3 included those patients with F8 mutations that were unknown as a result of (1) genetic testing not having been done, (2) of complete gene sequencing having been undertaken but no mutation being found, or (3) 2 centers only testing for inversion mutations.

Setting

Patients with severe hemophilia A in the PedNet Hemophilia Registry database (www.pednet.nl) and the Research on Determinants of Inhibitors (RODIN) Study database (www.rodinstudy.eu) were included. The 2 databases constitute a joint research effort among 29 hemophilia centers in Europe, Israel, and Canada. The RODIN study is a satellite study of the PedNet Hemophilia Registry, the aim of which is to prospectively evaluate risk factors for inhibitor development in PUPs with severe hemophilia A. Separate analyses regarding various aspects of the Rodin/PedNet study (eg, FVIII product type and inhibitor development and intensity of FVIII treatment and inhibitor development) have been recently published.[10,11]

Data collection

From 2004 on, anonymized data were collected by the participating centers by means of specially designed patient log books. The data were submitted to the centralized PedNet and Rodin databases through Web-based case report forms. In addition to patients' demographics, detailed data (including dates of infusion, doses and types of FVIII product, reasons for treatment, type of bleeds, and surgery) were collected for all FVIII administrations up to 75 exposure days (an exposure day was defined as a calendar day during which 1 or more infusions of FVIII were given) or until inhibitor development.

For patients born between 2000 and 2003, data were collected retrospectively. However, only patients in whom complete records were available were included in the study.

Genotyping was performed locally at each hemophilia center. Complete genotyping results were provided to the central coordinating center and entered into the database.

Outcomes

The date and type of each bleeding episode until either inhibitor development or 75 exposure days, whichever came first, was recorded. The following 6 variables were considered markers of bleeding phenotype: age at diagnosis (only for patients who had a negative family history of hemophilia), age at first bleed, age at first joint bleed, age at second joint bleed, time elapsed between first and second joint bleed, and age at prophylaxis start.

Because the implementation of regular prophylaxis could have changed the bleeding tendency, ages at first bleed, at first joint bleed, and at second joint bleed were evaluated by censoring patient population on the basis of start of prophylaxis. All of these outcomes were also censored for inhibitor development.

Statistical analysis

Continuous variables were expressed as median values and interquartile ranges (IQRs) and compared by Mann-Whitney *U*-test. Categorical variables were expressed as frequencies and percentage values and compared by χ^2 test or Fisher's exact test. Survival analysis was used to evaluate the occurrence of first bleed, first joint bleed, and second joint bleed on the basis of the presence of a certain type of F8 mutation. Kaplan-Meier curves were plotted and log-rank test performed to compare the 3 groups of patients.

All *P* values reported are 2-sided, and a value <.05 was considered statistically significant. All analyses were performed with SPSS software (release 16.0; SPSS Inc).

Results

Patient characteristics

The Pednet and RODIN study databases included 621 patients with severe hemophilia A; F8 mutation was known in 531 patients (85.5%).

F8 null mutations were present in 402 (75.7%) (group 1), and F8 non-null mutations were present in 129 (24.3%) of 531 patients (group 2). The detailed distribution of F8 null and non-null mutations in group 1 and 2 is reported in Table 1. In the remaining 90 patients (group 3), no F8 mutation was known at time of data review (May 1, 2012). In 16 patients, mutation analysis had not yet been undertaken at time of data review; in 37 patients, no mutation was found after complete gene sequencing, and in 37 cases, patients were only tested for inversion mutations and were found to be negative for them.

Family history of hemophilia was positive in only 275 patients (44.3%). At the time of data analysis, the median age of patients was 7.2 years (IQR, 4.9-9.9 years; range, 2.3-12.3 years).

Overall, the median age at diagnosis in patients with a negative family history of hemophilia was 8.8 months (IQR, 4.2-12.2 months).

Bleeding tendency was evaluated considering the occurrence of a patient's first bleed, first joint bleed, and second joint bleed. Because the introduction of prophylaxis would modify the bleeding phenotype, we evaluated these bleeding events until the start of

Table 1. Distribution of F8 mutations in the 531 patients for whom molecular characterization was available

Mutation group	N (%)
Group 1 (F8 null mutations)	
Inversions	287 (71)
Nonsense mutations	55 (14)
Small deletions/insertions outside poly-A runs	35 (9)
Splice site mutations of conserved nucleotides	12 (3)
Large deletions	10 (2)
Mutations of the promoter	3 (1)
Group 2 (F8 non-null mutations)	
Missense mutations	70 (54)
Small deletions/insertions within poly-A runs	56 (43)
Splice site mutations of non-conserved nucleotides	3 (3)

prophylaxis or the development of an inhibitor. Seventy-four patients (11.9% of the entire cohort) either developed an inhibitor or were started on prophylaxis before experiencing a first bleed. Some of these patients would have been exposed to factor in the context of trauma or surgery. Censoring for start of prophylaxis or inhibitor development, a first bleed was experienced by 549 (88.4% of the entire cohort) patients at a median age of 9.6 months (IQR, 4.2-13.3 months), 342 (55.1%) patients experienced their first joint bleed at a median age of 14.8 months (IQR, 10.5-22.6 months), and 179 (28.8%) patients experienced their second joint bleed (in any joint) at a median of 20.2 months (IQR, 13.8-29.1 months). For these patients, the median time elapsed between the first and second joint bleed was 2.3 months (IQR, 0.9-6.5 months).

A total of 197 patients (31.7% of the entire cohort) developed inhibitors after a median of 15 exposure days (IQR, 10-21 exposure days), with 112 developing inhibitors without ever having been placed on prophylaxis and 85 developing inhibitors after having been started on prophylaxis. The details of inhibitor development and correlation with factor VIII product type and with intensity of treatment and start of prophylaxis can be found in 2 recently published papers.[10,11]

Regular prophylaxis was started in 439 patients (70.7% of entire cohort) at a median age of 16.7 months (IQR, 11.9-24.9 months). Excluding the 112 patients who developed an inhibitor before ever starting on prophylaxis, 86.2% of the remaining 509 patients started prophylaxis.

Markers of bleeding phenotype

Age at diagnosis of hemophilia, age at first bleed, age at first joint bleed, age at second joint bleed, time elapsed between first and second joint bleed, and age at prophylaxis start for the 3 groups of patients distinguished on the basis of F8 mutation type are shown in Table 2.

Children with F8 null mutations were diagnosed at a median of 1.8 months earlier ($P = .04$), experienced their first bleed at a median of 1.2 months earlier ($P = .009$), and experienced their first joint bleed at a median of 2.3 months earlier ($P = .05$) than children with F8 non-null mutations. All 3 of these markers of bleeding phenotype were statistically different between those children with null and those children with non-null mutations, with the largest temporal difference (2.3 months) being in age at first joint bleed. No statistically significant difference was observed with respect to the occurrence of the second joint bleed (21.2 vs 21.3 months) or the time elapsed between the first and the second joint bleed (2.2 vs 2.7 months) between patients with null and non-null mutations (Table 2). The time between patients experiencing their first and second joint bleeds was reasonably short for the majority of patients (median, 2.4 months) and was not different between the different mutation types. About 75% of patients not started on prophylaxis (ie, who continued to receive treatment on demand) after a first joint bleed experienced a second joint bleed within 6 months of the first. There were, however, 3 children who went more than 3 years between their first joint bleed and their second.

Of the 402 patients with F8 null mutations 287 (71.4%) had inversion mutations. Among patients with inversion mutations, the median ages at diagnosis, at first bleed, at first joint bleed, and at second joint bleed were almost identical to the overall group of F8 null mutations. This was not surprising, as the 287 children with inversion mutations represented the majority of children with null mutations in this study.

Figure 1 shows the Kaplan-Meier curves of age at first bleed (A), age at first joint bleed (B), and age at second joint bleed (C) plotted for the 3 groups of patients. Survival analyses were performed by censoring patients who started prophylaxis before first bleed, first joint bleed, and/or second joint bleed. The Kaplan Meier curves did show remarkable similarities between the 3 groups, although a significant difference was found in the age at first bleed, which was younger for patients with F8 null mutations ($P = .028$).

Table 2. Clinical markers of disease severity in the cohort of 621 patients divided in 3 groups according to F8 mutation type

	Group 1 (null mutations)	Group 2 (non-null mutations)	Group 3 (unknown mutations)	*P* value
No. of patients (%)	402 (64.7)	129 (20.8)	90 (14.5)	
Median age at diagnosis*, mos (IQR)	8.3† (4.1-11.9)	10.1† (6.4-13.0)	8.4 (0.9-12.8)	.04†
Median age at 1st bleed,‡ mos (IQR)	9.7† (5.8-13.2)	10.9†,§ (7.7-15.0)	8.8§ (2.1-13.5)	.009†; .007§
Median age at 1st joint bleed,‡ mos (IQR)	13.8† (10.0-21.0)	16.1† (10.8-26.7)	14.8 (8.4-23.3)	.05†
Median age at 2nd joint bleed,‡ mos (IQR)	21.2 (13.4-29.9)	21.3 (12.9-36.5)	16.9 (11.7-21.8)	ns
Median time elapsed between 1st and 2nd joint bleed‖, mos (IQR)	2.2 (0.8-8.1)	2.7 (0.9-6.9)	2.2 (0.5-6.7)	ns
Median age at prophylaxis start, mos (IQR)	16.2 (11.8-23.7)	19.8 (12.6-27.6)	17.2 (12.2-24.9)	ns

Only significant *P* values are reported. ns, not statistically significant.

*Age at diagnosis was evaluated only in the 340 patients without a positive family history for hemophilia.

†Refers to which groups are being compared.

‡Ages at first bleed, at first joint bleed, and at second joint bleed were calculated only for those patients who had not started prophylaxis or who developed an inhibitor before first bleed, first joint bleed, or second joint bleed (n = 549, 342, and 179, respectively).

§Refers to which groups are being compared.

‖Time elapsed between first and second joint bleed was calculated only for patients who had not started prophylaxis before the second joint bleed (n = 179).

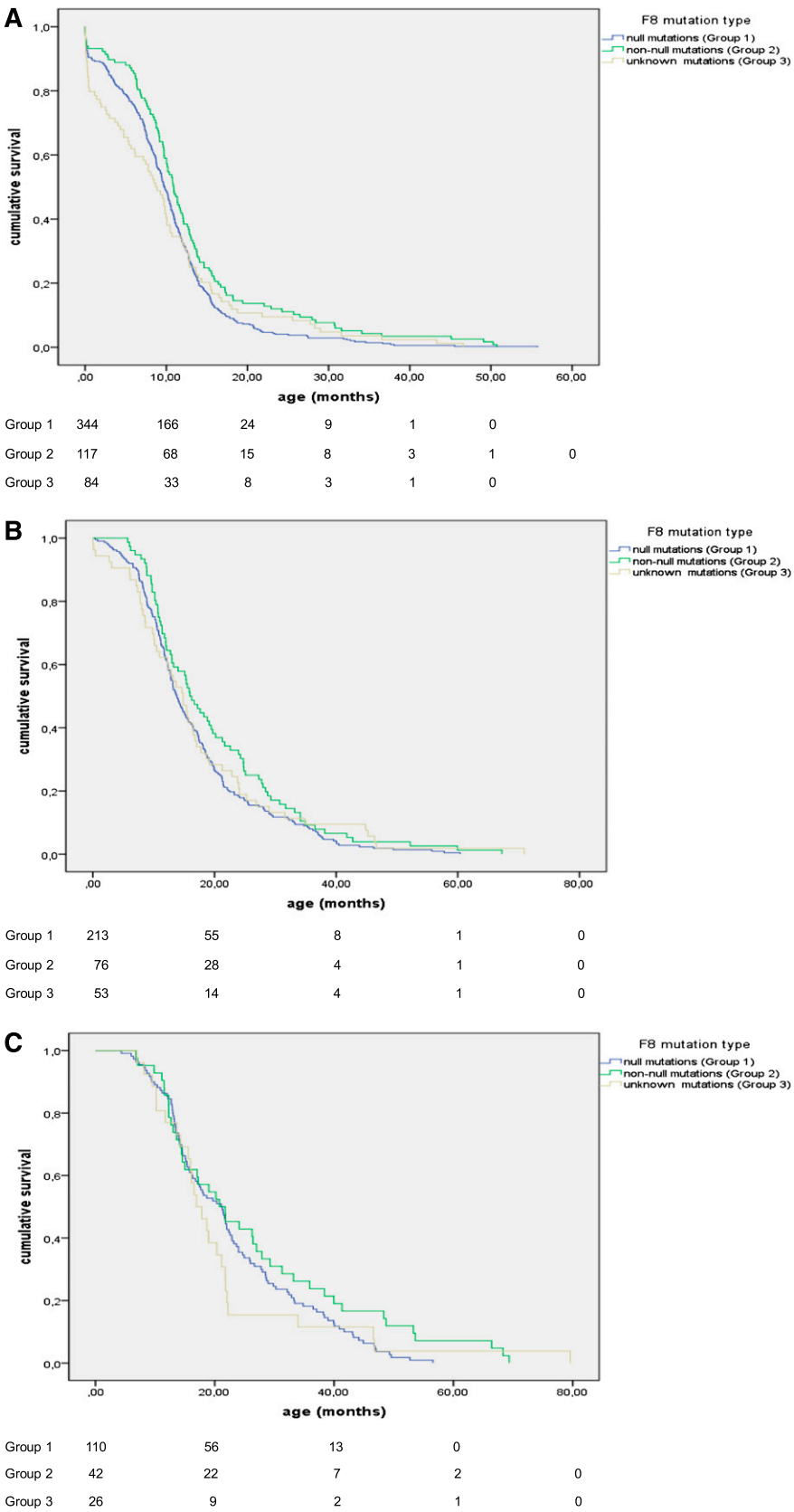

Figure 1. Kaplan-Maier curves showing the percentage of patients not having experienced these occurrences, according to mutation groups. First bleed (A), first joint bleed (B), and second joint bleed (C). Shown below each of the panels is the number of patients censored step by step at each of the age groups shown on the *x*-axis. In each analysis, patients who started prophylaxis before the bleeding event were censored. The Log-rank test showed a significant difference ($P = .028$) in age at first bleed between patients with null mutations and those with non-null mutations. For age at first joint bleed and age at second joint bleed, the Log-rank test was not statistically different between the 3 groups with P values of 0.18 and 0.26, respectively.

Patients with F8 null mutations started prophylaxis at an earlier median age (16.2 months) in comparison with those with F8 non-null mutations (19.8 months); however, this difference was not statistically significant. Patients with inversion mutations also started prophylaxis at a median of 16.2 months, which is identical to the overall group of children with F8 null mutations.

Within each mutation group, there was considerable variability in all of these phenotypic markers of disease severity (age at diagnosis, age at first bleed, age at first joint bleed, and age at second joint bleed), as noted by the wide IQRs within each of the genotype groups. Furthermore, there were outliers for all of these phenotypic markers of disease severity. While continuing to receive on-demand therapy, some patients did not experience a first bleed until 4 years of age, some patients did not experience a first joint bleed until 5.9 years of age, and some did not experience a second joint bleed until 6.6 years of age.

Discussion

In this study, we observed that F8 mutation type accounts for a small difference in the bleeding phenotype of young children with severe hemophilia A. Children with F8 null mutations were diagnosed at a statistically earlier age (8.3 vs 10.1 months) and experienced their first bleed (9.7 vs 10.9 months) and their first joint bleed (13.8 vs 16.1 months) at a statistically younger age than those with F8 non-null mutations. These differences in markers of disease severity at very young ages between the F8 mutation groups were, however, relatively minor (in the range of only 1.2 to 2.3 months). As such, awareness of F8 mutation is unlikely to significantly affect clinical decisions regarding when and how to start prophylaxis in children with severe hemophilia A.

The bleeding phenotype in older children and adults with severe hemophilia is largely influenced by treatment (prophylaxis vs on-demand; primary vs secondary prophylaxis; full-dose vs less-intense prophylaxis). As a consequence, it is difficult to evaluate the intrinsic bleeding phenotype of older patients because of the considerable influence of treatment variables, and in particular prophylaxis. Differentiating patients' intrinsic bleeding phenotypes is more likely to be effective at the start of life than later in life, when treatment regimens, traumas, and lifestyle have had their effect. This led us to focus on phenotypic variability at the start of life in previously untreated children.

Age at first bleed and, in particular, age at first joint bleed have been identified as good markers of clinical phenotype, and the latter was found to be inversely related to the degree of joint damage and annual clotting factor consumption in a cohort of Dutch patients with severe hemophilia.[12] Moreover, the amount of time elapsed between a patient's first and second joint bleed is an important variable to evaluate, as many clinicians will hold off starting prophylaxis until a patient experiences a second joint bleed. Therefore, in this study we decided to evaluate these markers of disease severity.

In our study, median age at first joint bleed (14.8 months) was slightly younger than that reported in previous retrospective studies.[6,9,12] This is partially explained by the youngest patients in our study still being only 2.4 years of age. A few of these patients have not, as yet, had a joint bleed, and when they do, if the analysis were to be redone, the median age at first joint bleed would likely be slightly higher. An additional reason for the younger age at first joint bleed in our study is the fact that the PedNet/RODIN study data collection was prospective and, as such, less likely to miss bleeds, which is a problem common to retrospective studies.

Somewhat surprisingly, the prevalence of a positive family history of hemophilia was lower than expected in our very large cohort of mainly European patients (44.3%). We speculate that this might reflect small family sizes in Europe that result in patients having few older siblings who might have hemophilia. For those patients with a negative family history of hemophilia, the median age at diagnosis was in keeping with what we expected at 8.8 months.

In this study, 55.0% of all patients (n = 342) experienced a first joint bleed, but only 28.8% (n = 179) experienced a second joint bleed without either developing an inhibitor or having been started on prophylaxis. For those 179 patients who did not start prophylaxis or develop an inhibitor after a first joint bleed, the median time to a second joint bleed was only 2.3 months. Overall, 75% of these 179 patients experienced a second joint bleed within 6 months of the first, indicating that waiting to start prophylaxis until a second joint bleed occurs only postpones the start of prophylaxis by a few months while causing patients to incur a second joint bleed.

We speculate that the slightly lower severity of severe hemophilia A that we observed in patients with F8 non-null mutations may arise from such patients having very low circulating levels of FVIII that are not detected by current laboratory FVIII assays. Such very low levels of FVIII may be able to induce thrombin generation, which is at variance with patients carrying F8 null mutations, and as such lessen the severity of severe hemophilia A in patients with non-null mutations.[13]

Our data clearly show that there is considerable interpatient phenotypic heterogeneity within each of the F8 mutation groups, as evidenced by the large IQRs for all 6 disease severity markers we evaluated (Table 2). This variation within mutation groups is, of course, not explained by F8 mutation and suggests that there are factors other than F8 mutation that affect hemophilia phenotype, even at these very early ages.

In determining what other factors might account for the significant heterogeneity between patients, it should be appreciated that although all patients with severe hemophilia A have by definition FVIII levels lower than 1%, variations may occur in the level and/or activity of all the other procoagulant/anticoagulant factors, including fibrinolytic factors as well as platelet function. Indeed, in the study by Santagostino et al,[6] the authors show that the thrombin generation assay, reflecting the ensemble of plasmatic procoagulant and anticoagulant activities, allowed identification of mild bleeders in a series of patients with severe hemophilia, suggesting that the clinical phenotype is influenced by determinants other than FVIII levels.

Coexisting bleeding disorders such as von Willebrand disease or clotting disorders (eg, Factor V Leiden, prothrombin mutation, etc) might have an effect on bleeding phenotype. Several retrospective studies have looked at the effect of coinheritance of prothrombotic mutations, with contradictory results.[6,9,14-25] Some studies have found that severe hemophiliacs carrying a prothrombotic mutation have a milder phenotype characterized by older age at diagnosis,[25] older age at first bleed,[15,17,26] lower annual bleeding frequency, and less arthropathy.[21,22] In contrast, other studies failed to find significant differences in severity of disease attributable to the presence of a prothrombotic mutation.[6,14,23,24] These conflicting results, together with the low prevalence of inherited prothrombotic mutations in hemophiliacs, is such that it is unlikely that such prothrombotic risk factors will account for much of the heterogeneity in disease severity seen in patients with hemophilia.

In our study, there was no systematic evaluation of patients for coexisting bleeding or clotting disorders. Some patients, nevertheless, probably on account of being perceived as having a more (or less) severe phenotype, were arbitrarily screened by their treating physicians, with 5 found to have von Willebrand disease and 3 found to be positive for a Factor V Leiden mutation. These 8 patients were too few to make any meaningful conclusions regarding the effect of these coexisting conditions on hemophilia severity. A follow-up study to systematically screen all patients for thrombophilia might allow us

to make some conclusions regarding the effect of coexisting inherited thrombophilias on hemophilia disease severity, as reflected at these very young ages. This would be particularly important in studying patient outliers (those patients who appeared to have a much less severe phenotype) to determine whether coexisting thrombophilic disorders contributed to their apparently milder phenotype.

Still other reasons to explain the interpatient phenotypic variability at these very young ages (before starting on prophylaxis) may not have anything to do with laboratory measures of hemostasis but, instead, may relate to patients' behavior (eg, related to patients' activity patterns, level of caution, parental precautions, etc). At older ages, considerable differences between patients in their pharmacokinetic handling of FVIII might also have significant effects on their disease phenotype.

One of the limitations of our study is that for purposes of analysis and sample size, we had to group mutations into 2 broad categories (null vs non-null), but it could be that there might be select mutation types that might be either more severe or less severe than the overall larger group of null mutations or non-null mutations. We did analyze patients with inversion mutations and found that they had phenotype markers almost identical to those of the broad group of patients with null mutations. We also looked specifically at patients with large deletions ($n = 10$) and found that although they had a high incidence of inhibitor development (70%), their markers of bleeding severity (ages at first bleed, first joint bleed, and second joint bleed) were no different than those of the overall group of patients with null mutations.

In conclusion, our study shows that F8 mutation does correlate to differences in disease phenotype in very young patients with severe hemophilia A. Patients with F8 non-null mutations do have a slightly milder bleeding phenotype (as noted by older age at diagnosis, older age at first bleed, and older age at first joint bleed) when compared with those with F8 null mutations. However, as differences between mutation groups were modest, it is unlikely to influence clinical decision-making (eg, when and how to start prophylaxis). It is possible, however, that the relatively modest differences in disease phenotype seen in children with severe hemophilia A at very young ages might become more accentuated as these patients age, and thus lead to a greater differentiation in disease severity between those patients with F8 null mutations in comparison with those with F8 non-null mutations. Still, we speculate that the largest contributor to disease phenotype later in life, as demonstrated by many studies, is most likely a reflection of the effect of different treatment regimens and not the F8 mutation or intrinsic differences in coagulation between patients.

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Authorship

Contribution: All authors conceived the study. M.E.M. performed statistical analysis. M.D.C. wrote the initial manuscript. M.D.C, H.M.v.d.B, R.L., and M.E.M. revised the final version of the manuscript.

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A complete list of the members of the PedNet and the Rodin Study Groups appears in "Appendix."

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