University of Amsterdam

UvA-DARE (Digital Academic Repository)

INSIGHT in risk factors and treatment of inhibitors in nonsevere hemophilia A

van Velzen, A.S.

Link to publication

CHAPTER 2

VARIATION IN BASELINE FACTOR VIII CONCENTRATION IN A RETROSPECTIVE COHORT OF MILD AND MODERATE HEMOPHILIA A PATIENTS CARRYING THE SAME *F8* MUTATION

J.I. Loomans, A.S. van Velzen, C.L. Eckhardt, M. Peters, A. Mäkipernaa, M. Holmstrom, P.P. Brons, N. Dors, S. Haya, J. Voorberg, J.G. van der Bom, and K. Fijnvandraat on behalf of the INSIGHT and RISE study group

Abstract

Introduction

In patients with mild and moderate hemophilia A (MHA) the bleeding phenotype is inversely associated with the residual plasma concentration of factor VIII (FVIII). Within a group of patients with the same *F8* missense mutation the FVIII baseline plasma concentration (FVIII:C) may still vary, as other determinants also influence baseline FVIII:C. In healthy individuals von Willebrand factor (vWF) levels, ABO blood group and age are known to influence baseline FVIII:C. In patients with MHA, our pathophysiological understanding on how the causative genetic event leads to reduced baseline FVIII:C is still limited.

Aim

This study aimed to estimate the variation and determinants of baseline FVIII:C among MHA patients with the same *F8* missense mutation.

Methods

In total 346 patients carrying mutations that were present in at least 10 patients were selected from the INSIGHT and the RISE study, cohort studies together including data of 3534 MHA patients from Europe, Canada and Australia. The baseline FVIII:C used for this analysis was measured by one-stage clotting assay. Levene's test, univariate and multivariate linear regression analysis were used to analyse the variance in the lowest baseline FVIII:C of all patients. A mixed model was constructed to address the determinants of inter- and intra-individual variation of all baseline FVIII:C measurements that were available.

Results

In this retrospective cohort of 346 patients with 13 different *F8* missense mutations we found that the observed variation in lowest baseline FVIII:C was explained for 59% by age and genotype. Nine *F8* mutations significantly associated with lowest baseline FVIII:C, but the other four mutations did not significantly associate with lowest baseline FVIII:C. Intra-individual variation explained 45% of the observed variance in baseline FVIII:C among patients with the same *F8* missense mutation.

Conclusion

Although in this cohort age and genotype explain 59% of the observed lowest baseline FVIII:C variance in patients with MHA, for four out of 13 *F8* missense mutations other factors were stronger determinants of baseline FVIII:C. Our results indicate that baseline FVIII:C levels are not exclusively determined by *F8* genotype in MHA. These findings emphasize the need for individual patient-tailored treatment in mild and moderate hemophilia A.

Introduction

Hemophilia A is a rare X-linked coagulation disorder caused by a broad spectrum of mutations in the *F8* gene that lead to a deficiency of clotting factor VIII (FVIII). Patients are classified based on residual FVIII concentration (FVIII:C) as severe (FVIII:C<1 IU/dL), moderate (1–5 IU/dL) or mild 5–40 IU/dL).[1] The bleeding phenotype in hemophilia A patients correlates inversely with FVIII plasma concentration. Severely affected patients present with spontaneous bleeding in joints and muscles. Patients with moderate hemophilia A may bleed spontaneously, and have pronounced bleeding after trauma, whereas in mild patients bleeding is usually restricted to traumatic events.

In this study, we focus on mild and moderate hemophilia A patients. The bleeding tendency among mild and moderate hemophilia A patients varies and is inversely associated with the residual plasma concentration of FVIII. An 18% reduction of joint bleed frequency has been observed with every IU/dL increase in residual FVIII:C in mild and moderate hemophilia A patients who are treated on demand.[2] Moderate hemophilia A patients have the highest risk for joint bleeds, whereas in mild patients with a baseline FVIII:C >15 IU/dL joint bleeds rarely occur.[2]

The underlying gene mutation is the most important determinant of the residual level of FVIII:C in hemophilia A.[3] Mild and moderate hemophilia A is usually caused by missense mutations. Currently about 700 different missense mutations have been reported.[3-7] However, within a group of patients with the same *F8* missense mutation, FVIII:C plasma levels may still be variable, as the *F8* genotype is not the only determinant of baseline FVIII:C level. FVIII is protected against degradation in the circulation by its carrier molecule von Willebrand Factor (vWF).[8,9] Consequently, vWF plasma levels are associated with the plasma concentration of FVIII. Furthermore, blood group and age indirectly influence the plasma concentration of FVIII by their effect on VWF levels. Individuals with blood group O have significantly lower vWF levels compared to individuals with blood group non-O and vWF levels increase throughout life with advancing age.[10-12]

Finally, there is an increased awareness that the assay method used to measure FVIII:C level may explain some of the variability of FVIII:C levels in moderate and mild

hemophilia A patients.[13-15] In 40% of the patients, one-stage clotting assay is found to be up to twice as high when compared to two-stage clotting or chromogenic clotting assays. It has been proposed that chromogenic assays may better predict bleeding phenotype.[13,16] If only a one-stage assay is performed in the diagnostic work up, then FVIII:C levels may be overestimated and the bleeding risk may be underestimated in these patients.[16]

Our pathophysiological understanding of the way in which a causative genetic event leads to a reduced FVIII:C level is incomplete.[16] This lack of knowledge contributes to ongoing diagnostic uncertainties. Estimates of the variability of FVIII:C plasma levels in patients with the same *F8* genotype and identification of the determinants that influence FVIIII:C levels in mild and moderate hemophilia A is essential for optimizing both diagnostic and individual treatment strategies and to understand the molecular basis of the development of hemophilia A.

In this article we present the observed variation in baseline FVIII:C among mild and moderate hemophilia A patients with the same *F8* mutation. Furthermore, we assessed the determinants of lowest baseline FVIII:C. Finally, we analyzed the determinants of inter- and intra-individual variation of all baseline FVIII:C measurements that were available in patients with the three most prevalent *F8* missense mutations.

Patients and methods

Study population
Patients were selected from the INSIGHT and the RISE study. The INSIGHT study is an international study on the etiology of inhibitors in patients with a moderate or mild form of hemophilia A (baseline FVIII:C of 2-40 IU/dL).[17-20] This large international retrospective cohort study contains clinical data on 2,711 patients. RISE is a study that investigates the response to Desmopressin (DDAVP) in mild and moderate hemophilia A patients. Data have been collected from 1376 patients, 598 of them have also been included in the INSIGHT cohort.

In these studies all patients with mild and moderate hemophilia A patients that were treated with DDAVP and/or FVIII concentrates were included from 19 Hemophilia Treatment Centers (HTCs) between 1980 and 2012. These centers (see Appendix A) participated in both studies and were located in Europe and Australia. The institutional review boards of all participating centers approved the study. Since this project involves retrospective data collection, all review boards indicated that informed consent was not required. This study was conducted in accordance with the Declaration of Helsinki.

Patient selection
To reduce the risk of selection bias, we only included patients from centers in which the majority (70%) of the patients was genotyped for routine care. The threshold of 70% was arbitrarily chosen. We only included patients with mutations that were present in at least 10 patients. For the present analysis we excluded patients aged <10 years to reduce selection bias as DDAVP/FVIII administration at younger age may be associated with a more severe phenotype (Figure 1a).

First, we analyzed the lowest measured baseline FVIII:C level of 346 patients derived from 8 HTCs, located in Europe and Australia (Figure 1b).

Second, we analyzed data of 208 patients with the three most prevalent *F8* missense mutations (Arg2169His;n=55, Arg612Cys;n=85 and Asn637Ser;n=68), and analyzed all available 454 one-stage FVIII:C measurements by mixed model analyses (Figure 1b).

Data collection

We collected the following data from medical records using a standardized electronic case report form: date of birth, body mass index (BMI), ethnicity, baseline FVIII:C, vWF antigen and activity (vWF:Ag, vWF:Act), *F8* mutation, family history of hemophilia A, and blood group. BMI was recorded within 5 years from the lowest baseline FVIII:C measurement for adults and within 1 year for children. Each *F8* mutation is reported using the Human Genome Variation Society (HGVS)-type numbering.

The number of baseline FVIII:C measurements available for a patient varied from one to five. Patients included in the INSIGHT study (those who were ever treated with FVIII concentrate) had a maximum of two baseline FVIII:C recorded. Patients included in the RISE study (those who were ever treated with DDAVP) had a maximum of three baseline FVIII:C recorded. Thus, patients included in both studies had a maximum of five baseline FVIII:C measurements recorded.

FVIII:C measurements

The baseline FVIII:C levels used for this analysis were the plasma FVIII coagulant activities measured locally by the one-stage clotting assay. We collected all baseline FVIII:C measurements that were available without treatment with DDAVP or FVIII concentrate in the 72 preceding hours.

Statistical analyses

Figure 1b shows an overview of the statistical analyses. Levene's test for equality of variances was used to compare the observed variation in lowest baseline FVIII:C levels among different mutation groups.

Inter-individual variance in lowest baseline FVIII:C was addressed by univariate analyses to explore associations of *F8* mutation, vWF and age with lowest baseline FVIII:C. We conducted a sensitivity analysis for patients in whom vWF levels were known at the exact date of the lowest baseline FVIII:C measurement. Unpaired t-test was used to compare lowest baseline FVIII:C in patients with blood group O with lowest baseline FVIII:C in patients with blood group non-O.

Multiple linear regression analysis was performed with significant predictors of lowest baseline FVIII:C, adjusting for HTC. The adjusted R^2 represents the percentage of variance in lowest baseline FVIII:C levels explained by this model.

In a second approach, we addressed both inter- and intra-individual variation

including all baseline FVIII:C measurements that were available for patients with one of the three most prevalent *F8* missense mutations. We used linear mixed models to analyze determinants of all available baseline FVIII:C measurements. The linear mixed model allows for the investigation of group differences while controlling for the non-independency of data (i.e. more than one baseline FVIII:C level was measured in one patient, resulting in related measurements within individuals). All available baseline FVIII:C measurements, *F8* gene mutation and the age at time of baseline FVIII:C measurement were included in the model. We included random intercepts for the Hemophilia Treatment Centers (HTCs) and patients. We used the method proposed in equation 26 by Nakagawa et al. to calculate the adjusted R^2 for mixed-effects models.[21] Subsequently, the same linear mixed model analysis was performed in each of the three largest mutation groups in order to quantify the explained variation of age at time of baseline FVIII:C measurement specifically per mutation group.

Finally, Estimated Marginal (EM) means are presented to compare the main effects of the different missense mutations contributing to baseline FVIII:C.

Statistical analyses were performed with SPSS for Windows, version 22.0 (SPSS Inc, Chicago, IL, USA). A p-value <0.05 was considered statistically significant.

Missing data were imputed if original data showed an association with baseline FVIII:C.

Figure 1b Flowchart of analyses performed in patient groups.

| # patients	# measurements	Variables investigated	Analyses
346 with 13 different mutations	346	Outcome: lowest FVIII:C (interindividual variation) Determinants: mutation, vWF, age, blood group, HTC	Levene's test Univariate Multivariate regression analyses
208 with 3 largest mutation groups	454	Outcome: all FVIII:C measurements (inter- and intraindividual variation) Determinants: mutation, age, adjusted for HTC	Mixed model analyses

The variables in green are included in the multivariate regression analyses.

Results

Patient Characteristics

We selected 346 patients aged 10 years or older that carried missense mutations that were present in at least 10 patients (Figure 1a). Patient characteristics are displayed in Table 1. The 346 genotyped patients had 13 different missense mutations and a total of 759 one-stage FVIII:C measurements had been performed. The variation in lowest baseline FVIII:C observed in patients with the same *F8* missense mutation is depicted in Figure 2. This boxplot shows the median and Inter Quartile Range of the lowest measured FVIII:C per *F8* mutation present in at least 10 patients. We observed a significant difference in the variances in lowest measured baseline FVIII:C levels between the different mutation groups (Levene's test p<0.001).

Figure 1a Flowchart of patients included in the analyses.
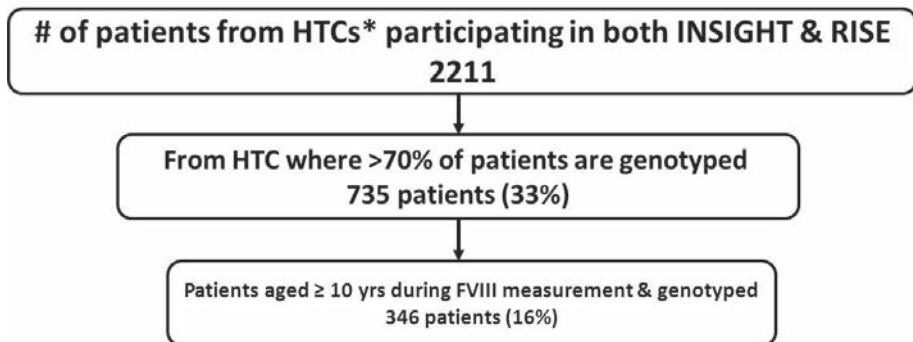
*Hemophilia Treatment Center.

Table 1 Patient characteristics.

Parameter	Genotyped patients (n=346)	
	n or median	% or (IQR)
Baseline FVIII:C all measurements*, IU/dL	15	*(9-23)*
Baseline FVIII:C lowest measurement, IU/dL	14	*(7-21)*
Age at baseline FVIII:C measurement	38	*(22-53)*
VWF:Act	98	*(75-120)*
VWF:Ag	107	*(86-136)*
Body Mass Index (BMI)	25	*(22-28)*
Blood group known	155	*45*
Blood group:		
O	71	*21*
Non-O	84	*24*
# of different missense mutations**	**13**	
Mutation groups:		
Pro149Arg	11	*(3.2)*
Val502Gly	10	*(2.9)*
Tyr530His	11	*(3.2)*
Arg550Cys	13	*(3.8)*
Arg550His	13	*(3.8)*
Arg612Cys	85	*(24.6)*
Asn637Ser	68	*(19.7)*
Leu644Val	23	*(6.6)*
Arg717Trp	11	*(3.2)*
Arg1960Gln	12	*(3.5)*
Arg2169His	55	*(15.9)*
Arg2178Cys	17	*(4.9)*
Gln2265Arg	17	*(4.9)*
Total	**346**	**100%**

*The 346 patients displayed 759 FVIII:C one-stage measurements. **HGVS nomenclature is used.

Univariate analyses of determinants for lowest baseline FVIII:C

We analyzed the effect of *F8* mutation, vWF plasma level, blood group and age on the lowest baseline FVIII:C. Eleven of the included 13 *F8* missense mutations were associated with baseline FVIII:C (p=0.031 for Arg550Cys and p<0.001 for the other ten mutations). For two of the mutations (Arg1960Gln and Tyr530Cys) no association with baseline FVIII:C could be demonstrated (p=0.404 and p=0.063 respectively).

We could not demonstrate an association between baseline FVIII:C and vWF:Ag or vWF:Act measured on the same day (available in 42 and 35 patients respectively). As the *F8* mutation may be a more important determinant of baseline FVIII:C than vWF level, we also analyzed the association between lowest baseline FVIII:C and vWF level within the subgroup of patients with the Arg612Cys mutation. In this mutation group, we did find a significant association between baseline FVIII:C and vWF:Act (p=0.003). For all mutations grouped together we did not observe a difference in baseline FVIII:C between patients with blood group O and blood group non-O (available in 155 patients). Older age was associated with an increase in baseline FVIII:C (supplemental Figure 1).

Determinants of lowest measured FVIII:C

A multiple linear regression model was constructed to predict lowest baseline FVIII:C based on *F8* mutation and age, adjusted for HTC. The adjusted R^2 represents the percentage of variance in lowest baseline FVIII:C explained by this model. A significant regression equation was found (p<0.001), with an R^2 of 59%. Eight of the 13 included mutations, age, and 1 HTC were significant predictors of lowest baseline FVIII:C (supplemental Table 1). Using Arg612Cys as the reference group, patients with Pro149Arg, Val502Gly, Arg550Cys, Arg2169His and Gln2265Arg mutations had significantly lower lowest baseline FVIII:C and patients with Arg550His, Asn637Ser, and Arg717Trp had significantly higher lowest baseline FVIII:C (Figure 2). Four mutations were not found to be significant predictors of lowest baseline FVIII:C in this model (Tyr530His, Leu644Val, Arg1960Gln, Arg2178Cys; Figure 2). Figure 3 displays the mutations and the associations with lowest measured FVIII:C grouped per domain of the FVIII protein.

Baseline FVIII:C increased 0.066 IU/dL for every year increase in age. Patients with lowest baseline FVIII:C levels measured in one of the HTCs had a lowest baseline FVIII:C that was 6 IU/dL lower than patients with baseline FVIII:C levels measured in the reference group.

Figure 2. Lowest measured FVIII:C per *F8* mutation present in at least 10 patients.
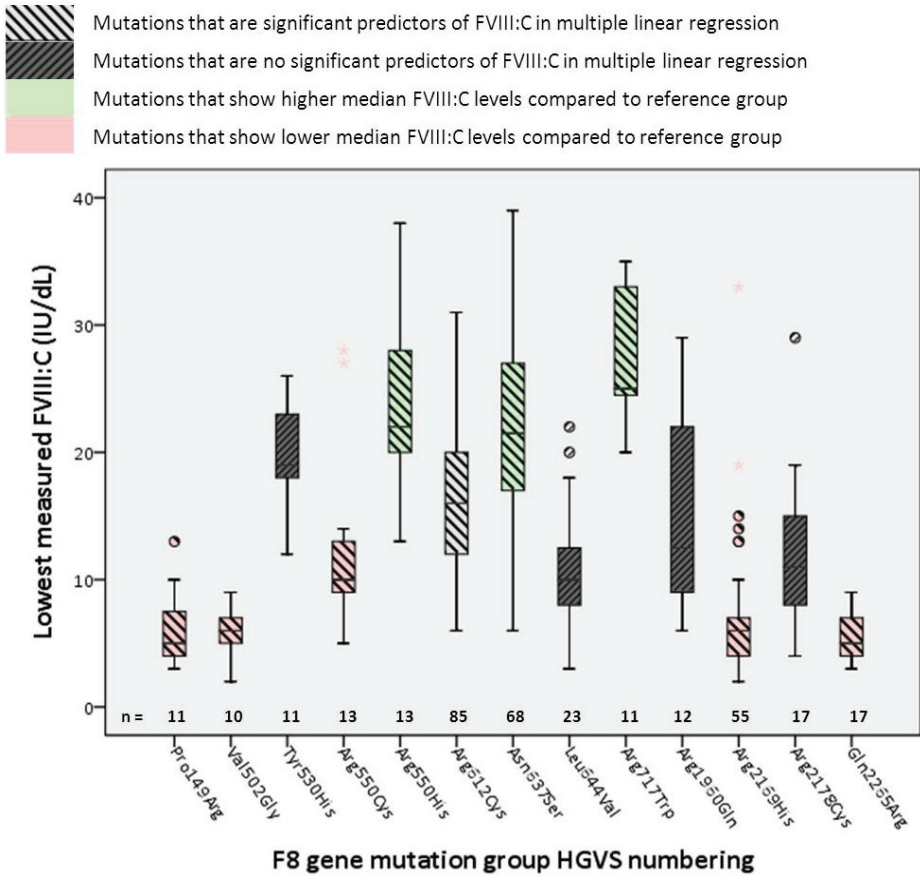
The variance in baseline FVIII:C was significantly different between mutation groups (Levene's test $p<0.001$). Arg612Cys is used as reference group for the multiple linear regression analysis.

Figure 3. Mutations and associations with lowest measured FVIII:C grouped per domain of the FVIII protein.
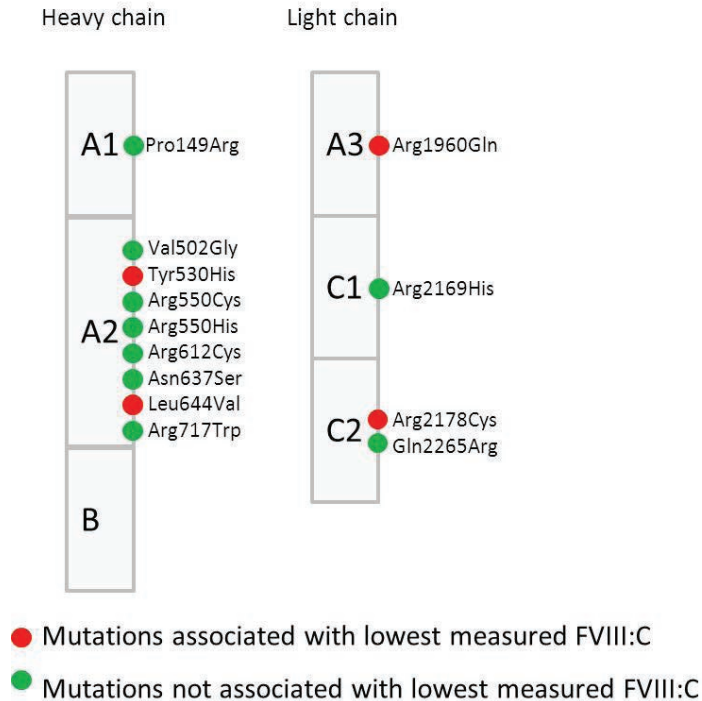
Construction of a model to explain variation in all FVIII:C measurements

To address intra- and inter-patient variation, we analyzed patients from the three largest *F8* gene mutation groups (Arg612Cys (n=85), Asn637Ser (n=68) and Arg2169His (n=55)), using data on all baseline FVIII:C measurements and age, adjusted for HTC. There were 454 baseline FVIII:C measurements from 208 patients available.

We observed a significant difference between the variances in baseline FVIII:C measurements between the three largest mutation groups (Levene's test p<0.001). The mutations Arg612Cys and Arg2169His showed reduced baseline FVIII:C levels compared to Asn637Ser (point estimates of -7,04 and -16.70 respectively with p<0.001, Table 2).

The baseline FVIII:C variation within an individual explained 45% of the total observed variance in FVIII:C measurements adjusted for *F8* mutation, age and HTC (supplemental Table 2). Baseline FVIII:C increased 0.09 IU/dL for every year a patient was older ($p<0.001$). Estimated Marginal Means of all baseline FVIII:C measurements in the three largest mutation groups adjusted for age and HTC are displayed in Table 2. Subsequently, the linear mixed model for each separate mutation group showed differences in the effect of age, that was strongest in patients with the Arg612Cys mutation compared to patients with the Arg2169His or Asn637Ser mutation (point estimate of annual increase in baseline FVIII:C is 0.132, 0.0708 and 0.0399 respectively). Age at time of baseline FVIII:C measurement explained 0.5%, 5% and 13% of the observed variation in Asn637Ser, Arg2169His and Arg612Cys respectively (supplemental Table 3).

Table 2

F8 gene mutation group	Mean	95% Confidence Interval	
		Lower Bound	Upper Bound
Arg2163His	9.004	6.571	11.437
Arg612Cys	18.293	15.797	20.79
Asn637Ser	25.701	23.189	28.212

Estimated Marginal Means of all FVIII:C measurements in the three largest mutation groups, adjusted for variation within an individual, age and HTC.

Discussion

In this retrospective cohort of 346 patients we found that among groups of at least 10 patients with the same *F8* missense mutation, large inter- and intra-individual variance in baseline FVIII:C is observed.

Although in this cohort age and genotype, adjusted for HTC, explain 59% of the observed inter-individual variation in lowest baseline FVIII:C, for four out of 13 *F8* missense mutations other factors than the genotype were stronger determinants of lowest baseline FVIII:C.

We could not find an association between lowest baseline FVIII:C and vWF, which is probably due to a lack of power as data on VWF levels were only available for a small group of patients because we did not have information on the exact date of vWF levels measurement in patients from the INSICHT study.

When we analyzed the variance of all available baseline FVIII:C measurements in a subgroup of 208 patients with the three most prevalent missense mutations, it appeared that the intra-individual variation explained 45% of the total observed variance in all baseline FVIII:C measurements. The effect of age on baseline FVIII:C was different for each of the three mutations. The strongest increase in FVIII:C for advancing age was observed in patients with the Arg612Cys missense mutation.

Limitations & Strengths

This large, international cohort of mild and moderate hemophilia A patients provides data on the association between *F8* missense mutations and baseline FVIII:C. Our study is unique in its large size. Due to the broad spectrum of missense mutations associated with MHA, knowledge on the association between *F8* mutation and baseline FVIII:C is difficult to obtain as smaller studies lack the statistical power to analyze this. The high number of patients in our study could only be included by participation of multiple HTCs. As we used the locally measured baseline FVIII:C, assay discrepancies between HTC may increase the observed variance in baseline FVIII:C. Fortunately, all HTCs used the same type of assay, the one stage clotting assay. To account for assay discrepancies between HTCs we adjusted for HTC in the multiple linear regression model and in the linear mixed model analysis. Moreover, the high variety in missense mutations hampered more specific analyses of the effect of the different mutations. We reduced selection bias by restricting our analysis to all patients from centers that

had genotyped at least 70% of their patients and by excluding patients <10 years.

Clinical significance

Baseline FVIII:C is multifactorial in origin and not only determined by the *F8* mutation. This is important to understand for correct diagnosis and in order to achieve a more personal approach in mild and moderate hemophilia A care.

We observed four *F8* missense mutations that were not significantly associated with lowest baseline FVIII:C in this study population. This implies that other factors had a stronger effect on baseline FVIIII:C. This awareness is essential in diagnostic patient management, since patients with the same mutation might have a different baseline FVIII:C.

Older age was associated with higher baseline FVIII:C and the effect of age was different in patients from the three most prevalent mutations.

As baseline FVIII:C increases with age, it is questionable whether the phenotype becomes milder with advanced age. Baseline FVIII:C also increases in healthy individuals.[22] The increase of baseline FVIII:C in older individuals might be required to maintain hemostasis in the more fragile circumstances of old age. Coagulation factor levels above the "normal" range may reflect a physiological response to the increased hemostatic requirement of old age, in analogy to higher physiological levels of FVIII:C at the end of pregnancy.[23]

Future studies

To investigate the relation between mutation, baseline FVIII:C and bleeding phenotype, data on the severity and frequency of bleeding in mild and moderate hemophilia A needs to be collected. With this information it is possible to investigate whether baseline FVIII:C is a valid indicator of bleeding phenotype. It is important to stress that both one-stage and chromogenic FVIII:C assays should be applied, since there is ongoing discussion which assay most appropriately reflects the clinical phenotype in patients with discrepancies between the two assays.[17] Processes from mutation to protein formation might be more complex than we think. Moreover, alterations of genes other than the *F8* gene, for instance the fibrinolytic pathway, may also influence the bleeding phenotype, and should be further investigated as potential indicators of bleeding risk for mild and moderate hemophilia A patients.[17]

Conclusion

Age and genotype explain 59% of the observed variance in lowest baseline FVIII:C in this large cohort of patients with moderate and mild hemophilia A. However, other factors were stronger determinants for four out of 13 *F8* missense mutations. Our results indicate that FVIII:C levels are not exclusively determined by *F8* genotype. These findings emphasize the need for individual patient-tailored treatment in mild and moderate hemophilia A.

References

1. White GC, Rosendaal F, Aledort LM, Lusher JM. Scientific and Standardization Committee Communication Definitions in Hemophilia Recommendation of the Scientific Subcommittee on Factor VIII and Factor IX of the Scientific and Standardization Committee of the International Society on Thrombosis and Haem. 2001;2012.

2. den Uijl IEM, Fischer K, Van Der Bom JG, et al. Analysis of low frequency bleeding data: the association of joint bleeds according to baseline FVIII activity levels. *Haemophilia*. 2011;17(1):41–4.

3. Margaglione M, Castaman G, Morfini M, et al. The Italian AICE-Genetics hemophilia A data base: results and correlation with clinical phenotype. *Haematologica*. 2008;93(5):722–8.

4. Rallapalli PM, Kemball-Cook G, Tuddenham EG, Gomez K PS. Manuscript under Preparation. 2014.

5. Vidal F GD. Hemobase Molecular basis of hemophilia.

6. CDC Hemophilia A Mutation Project (CHAMP).

7. Sengupta M, Sarkar D, Ganguly K, et al. In silico analyses of missense mutations in coagulation factor VIII: identification of severity determinants of haemophilia A. *Haemophilia*. 2015;1–8.

8. Terraube V, O'Donnell JS, Jenkins P V. Factor VIII and von Willebrand factor interaction: biological, clinical and therapeutic importance. *Haemophilia*. 2010;16(1):3–13.

9. Fijnvandraat K, Peters M, Ten Cate JW. Inter-individual variation in half-life of infused recombinant factor VIII is related to pre-infusion von Willebrand factor antigen levels. *Br. J. Haematol*. 1995;91(2):474–476.

10. O'Donnell J, Laffan MA. The relationship between ABO histo-blood group, factor VIII and von Willebrand factor. *Transfus. Med*. 2001;11(4):343–351.

11. Conlan M, Folsom A, Finch A, et al. Associations of factor VIII and von Willebrand factor with age, race, sex, and risk factors for atherosclerosis. The Atherosclerosis Risk in Communities (ARIC) Study. *Thromb. Haemost*. 1993;70(3):380–385.

12. Sanders Y V, Giezenaar M a, Laros-van Gorkom B a P, et al. von Willebrand disease and aging: an evolving phenotype. *J. Thromb. Haemost*. 2014;12(7):1066–75.

13. Duncan EM, Rodgers SE, McRae SJ. Diagnostic testing for mild hemophilia a in patients with discrepant one-stage, two-stage, and chromogenic factor VIII:C assays. *Semin. Thromb. Hemost*. 2013;39(3):272–82.

14. Bowyer AE, Van Veen JJ, Goodeve AC, Kitchen S, Makris M. Specific and global coagulation assays in the diagnosis of discrepant mild hemophilia A. *Haematologica*. 2013;98(12):1980–7.

15. Peyvandi F, Oldenburg J, Friedman KD. A critical appraisal of one-stage and

chromogenic assays of factor VIII activity. *J. Thromb. Haemost.* 2015;

16. Pavlova A, Oldenburg J. De fi ning Severity of Hemophilia : More than Factor Levels. *Semin Thromb Hemost*. 2013;39:702–710.

17. Eckhardt CL, Van Velzen AS, Peters M, et al. Factor VIII gene (*F8*) mutation and risk of inhibitor development in nonsevere hemophilia a. *Blood*. 2013;122:1954–1962.

18. Eckhardt C, Loomans J, van Velzen A, et al. Inhibitor development and mortality in nonsevere hemophilia A. *J. Thromb. Haemost*. 2015;13:1217–1225.

19. van Velzen AS, Eckhardt CL, Streefkerk N, et al. The incidence and treatment of bleeding episodes in nonsevere haemophilia A patients with inhibitors. *Thromb. Haemost*. 2015;115(3.):

20. Velzen AS Van, Eckhardt CL, Hart DP, et al. Inhibitors in nonsevere haemophilia A : outcome and eradication strategies. *Thromb. Haemost*. 2015;(114/1):46–55.

21. Nakagawa S, Schielzeth H. A general and simple method for obtaining R 2 from generalized linear mixed-effects models. *Methods Ecol. Evol*. 2013;4(2):133–142.

22. Mari D, Coppola R, Provenzano R. Hemostasis factors and aging. *Exp. Gerontol*. 2008;43(2):66–73.

23. Stoof SCM, Cnossen MH, de Maat MPM, Leebeek FWG, Kruip MJH a. Side effects of desmopressin in patients with bleeding disorders. *Haemophilia*. 2016;22(1):39–4

Supplemental figure 1
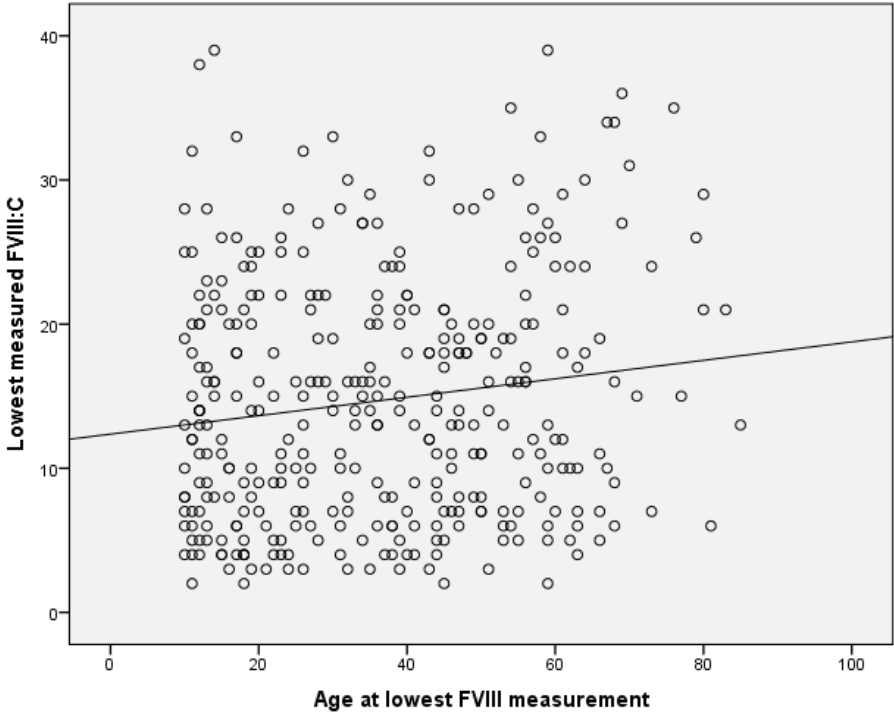
We observed an association between lowest measured FVIII:C and age during that measurement (n=345, p=0.008, adjusted R2=2%)

Supplemental table 1 Multiple linear regression analyses of lowest measured FVIII: C predicted by *F8* mutation, age and HTC.

Model	B	Sig.	95.0% Confidence Interval for B	
			Lower Bound	Upper Bound
(Constant)	12.706	<0.001	10.606	14.805
Pro149Arg	-8.183	<0.001	-11.819	-4.547
Val502Gly	-10.418	<0.001	-14.042	-6.794
Arg550Cys	-3.773	0.031	-7.208	-0.337
Arg2169His	-9.097	<0.001	-11.570	-6.625
Gln2265Arg	-11.085	<0.001	-15.071	-7.099
Arg550His	10.460	<0.001	6.899	14.021
Asn637Ser	6.771	<0.001	4.769	8.773
Arg717Trp	11.044	<0.001	7.190	14.898
Tyr530His	2.595	0.243	-1.769	6.959
Leu644Val	2.100	0.350	-2.313	6.513
Arg1960Gln	-1.146	0.503	-4.510	2.217
Arg2178Cys	0.894	0.658	-3.078	4.866
Age at lowest FVIII measurement	0.066	<0.001	0.034	0.097
HTC A	-6.064	0.002	-9.881	-2.247
HTC B	1.793	0.065	-0.113	3.698
HTC C	1.617	0.703	-6.713	9.947
HTC D	1.577	0.326	-1.576	4.730
HTC E	-0.993	0.412	-3.374	1.388
HTC F	2.738	0.403	-3.699	9.175
HTC G	1.545	0.264	-1.171	4.261

Predicted lowest FVIII:C measurement is equal to 12.706-9.097(Arg2169His)-3.773(Arg550Cys)-11.085(Gln2265Arg)-8.183(Pro149Arg)-10.418 (Val502Gly)+10.460(Arg550His)+11.044(Arg717Trp)+6.771 (Asn637Ser)+0.066(age)-6.064(HTC A), where age is measured in years. For *F8* mutations, Arg612Cys was reference group. One center from The Netherlands was used as reference group for HTCs.

Supplemental table 2 Mixed model analyses in three largest mutation groups.

Estimates of Fixed Effects

Parameter	Estimate	Sig.	Lower Bound	Upper Bound
			95% Confidence Interval	
Intercept	22.191097	<0.001	19.348534	25.03366
Arg2169His	-16.696960	<0.001	-19.398850	-13.99507
Arg612Cys	-7.407439	<0.001	-9.577252	-5.23762
Asn637Ser	0			
Age at time of measurement	0.090542	<0.001	0.049913	0.13117

Estimates of Covariance Parameters

Parameter		Estimate
Residual		24.505215
Patient number	Variance	21.497932
HTC	Variance	2.468683

Descriptive Statistics

	N	Variance
Fixed Predicted Values	454	39.674

R2 added by multiple measurements in 1 person: 39.674/(39.674+24.5+21.5+2.5)=45%.

Supplemental table 3 Mixed model analyses per largest mutation group.

Estimates of Fixed Effects

F8 gene mutation group		Estimate	Sig.	95% Confidence Interval	
				Lower Bound	Upper Bound
Arg2169His	Intercept	5.891272	<0.001	3.030130	8.752414
	Age at time of measuremen	0.070826	0.020	0.011324	0.130328
Arg612Cys	Intercept	13.402724	<0.001	10.428762	16.376686
	Age at time of measurement	0.132323	<0.001	0.076331	0.188315
Asn637Ser	Intercept	24.147545	<0.001	16.817762	31.477329
	Age at time of measurement	0.039852	0.450	-0.064584	0.144288

Estimates of Covariance Parameters

Parameter			Estimate
Arg2169His	Residual		16.576496
	Patient #	Variance	17.747471
	HTC	Variance	0
Arg612Cys	Residual		26.602202
	Patient #	Variance	12.011442
	HTC	Variance	1.314060
Asn637Ser	Residual		29.028203
	Patient #	Variance	38.548679
	HTC	Variance	14.468510

Descriptive Statistics

F8 gene mutation group		N	Variance
Arg2169His	Fixed Predicted Values	135	1.809
Arg612Cys	Fixed Predicted Values	197	5.862
Asn637Ser	Fixed Predicted Values	122	0.485

R2 added by multiple measurements in 1 person in:
Arg2169His: 1.809/(1.809+16.576+17.747)=5%
Arg612Cys: 5.862/(5.862+26.602+12.01+1.31)=13%
Asn637Ser: 0.485/(0.485+29.03+38.55+14.47)=0.5%