blood®
Letters to *Blood*

TO THE EDITOR:

Timing of inhibitor development in more than 1000 previously untreated patients with severe hemophilia A

H. Marijke van den Berg,[1] Kathelijn Fischer,[2] Manuel Carcao,[3] Hervé Chambost,[4,5] Gili Kenet,[6] Karin Kurnik,[7] Chris Königs,[8] Christoph Male,[9] Elena Santagostino,[10] and Rolf Ljung,[11,12] on behalf of the PedNet Study Group

[1]PedNet Haemophilia Research Foundation, Baarn, The Netherlands; [2]Van Creveld Kliniek, University Medical Center Utrecht, Utrecht, The Netherlands; [3]Division of Haematology/Oncology, Hospital for Sick Children, Toronto, ON, Canada; [4]Centre de Recherche en Cardiovasculaire et Nutrition (C2VN), Aix Marseille University, Institut National de la Recherche Agronomique (INRA), INSERM, Marseille, France; [5]Centre for Bleeding Disorders, La Timone Children Hospital, Assistance Publique–Hopitaux de Marseille (APHM), Marseille, France; [6]National Hemophilia Center, Ministry of Health, Sheba Medical Center, Tel Hashomer, Israel; [7]Dr V. Haunersches Kinderspital, University of Munich, Munich, Germany; [8]Clinical and Molecular Hemostasis, Department of Pediatrics and Adolescent Medicine, J .W. Goethe University Hospital, Frankfurt, Germany; [9]Department of Paediatrics, Medical University Hospital of Vienna, Vienna, Austria; [10]Angelo Bianchi Bonomi Hemophilia and Thrombosis Center, Fondazione, Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS) Ca' Granda, Ospedale Maggiore Policlinico, Milan, Italy; and [11]Department of Clinical Sciences and [12]Department of Pediatrics, Malmö-Lund University, Lund, Sweden

Inhibitory antibodies (inhibitors) against factor VIII (FVIII) develop in 25% to 35% of previously untreated patients (PUPs) with severe hemophilia A (SHA). It is the most serious complication of classic hemophilia treatment.[1-3] Most inhibitors develop during the first 50 exposure days (EDs) to FVIII, with 50% of inhibitors already present after 14 to 15 EDs.[2-4] After 50 EDs, inhibitor development is rare and is reported in number of new inhibitors per 1000 person-years. A recent systematic review reported an overall inhibitor incidence of 2.06 per 1000 person-years.[5] A definition specifically designed to separate a priori high-risk and low-risk category patients was established after the outbreaks of 2 product-specific inhibitors in the 1990s.[6-8] According to this definition, previously treated patients (PTPs), considered tolerant to exogenous FVIII, are patients with >150 EDs.[8]

Hemophilia treatment has changed considerably over the last decades due to the development of many new therapies.[9] Prophylaxis, first only practiced in a few countries, has become standard of care and is started at increasingly earlier ages and with higher dosing and frequencies.[10,11]

The PedNet cohort study prospectively includes all newly diagnosed patients with hemophilia of the participating centers.[12]

The aim of this study was to define the risk periods for inhibitor development until 1000 EDs and to refine the definition of PTPs and the age at which patients have reached this "near-zero" risk situation.

All PUPs with SHA (FVIII activity <0.01 IU/mL) born from 1 January 2000 onward, and diagnosed in 1 of the participating centers of the PedNet Study Group (supplemental Appendix, available on the *Blood* Web site), were enrolled into the PedNet study protocol (this trial was registered at www.clinicaltrials.gov as #NCT02979119). The study design is an unselected birth cohort that included over 90% of all newly diagnosed patients in the participating centers. Detailed data on each ED was collected until 75 EDs for patients born between 2000 and 2009 and until 50 EDs for patients born from 2010 onward. Approval for inclusion in the registry was obtained from every center's institutional review board. Written informed consent was obtained from the parents/guardians of all participants.

For the present analysis, patients were followed until inhibitor development, or censored based on the number of EDs at their last follow-up as of January 2018.

The primary outcome of the study was development of a clinically relevant inhibitor, defined as at least 2 positive inhibitor titers in combination with decreased in vivo FVIII recovery. The secondary outcome was development of a high-titer inhibitor, defined as the occurrence of a clinically relevant inhibitor with a peak titer of at least 5 Bethesda units (BU)/mL. Positivity was defined according to the cutoff level in each individual center's laboratory, the highest cutoff level used being 0.6 BU/mL. The number of EDs at the time of inhibitor development was defined as the last ED before the first positive titer was reported. All laboratory results for inhibitor tests in all patients were collected.

To calculate cumulative inhibitor incidence according to the number of EDs, survival analysis was performed with EDs up to 1000 as the time variable. We calculated the median age and interquartile range (IQR) in years at ED 1 and ED 75. To assess inhibitor rate in person-years, we included all patients who had reached 75 EDs and calculated the time in years between the date of ED 75 and the date of their last follow-up. Analyses were performed using SPSS 24.

A total of 1038 PUPs with SHA were eligible, 943 of whom (91%) were followed until 50 EDs; 899 (87%) were followed until 75 EDs (Table 1). The median (IQR) age at first exposure was 1.1 years (0.8-1.5 years); 75 EDs were reached at a median (IQR) age of 2.3 years (1.7-2.8 years), only 1.2 years after the first ED. A total of 869 patients (83.7%) were of white ancestry. Almost all inhibitors (298 of 300; 99.3%) developed within the first 75 EDs. Seventy-nine percent of all inhibitors (N = 236, 173 high-titer and 63 low-titer) developed within 20 EDs, 18% (N = 53,

Table 1. Inhibitor development from ED 1 until 1000 EDs for all and high-titer inhibitors

EDs	Patients at risk	Patients with inhibitors	High-titer inhibitors	All inhibitors, cumulative %
0	1038	0	0	0
1-10	897	109	78	36
11-20	753	236	173	79
21-30	708	268	196	89
31-40	688	282	203	94
41-50	654	289	208	96
51-60	646	294	210	98
61-75	601	298	212	99.3
76-150	568	298	212	99.3
151-250	524	299	212	99.7
251-500	430	300	212	100
501-1000	214	300	212	100

The cumulative inhibitor incidence for all inhibitors is reported during follow-up.

35 high-titer and 18 low-titer) between 21 and 50 EDs, and 3% (N = 9, 4 high-titer and 5 low-titer) between 51 and 75 EDs (Table 1).

At this point, inhibitor development reached a plateau. No inhibitors developed between 75 and 150 EDs, whereas another 2 low-titer inhibitors (0.7%) developed at ED 249 and 262 EDs, respectively (Figure 1). Based on survival analysis, the cumulative inhibitor incidence was 28.9% at 50 EDs and 29.9% at 75 EDs. Total cumulative inhibitor incidence reached 30.2% at 1000 EDs (Table 1).

In newborns with severe hemophilia A, inhibitor development is of great concern to parents and physicians. Knowing the time period of greatest risk is important as there is evidence that intense exposure to FVIII (as occurs with surgery) should be avoided during this period as this might increase the inhibitor risk.[2,13,14] Our study shows that children on prophylaxis reach a near-zero risk plateau of inhibitor development at 75 EDs only 1.2 years after the first ED.

We were interested in determining inhibitor risk after 75 EDs. To calculate this, we included all patients who reached at least

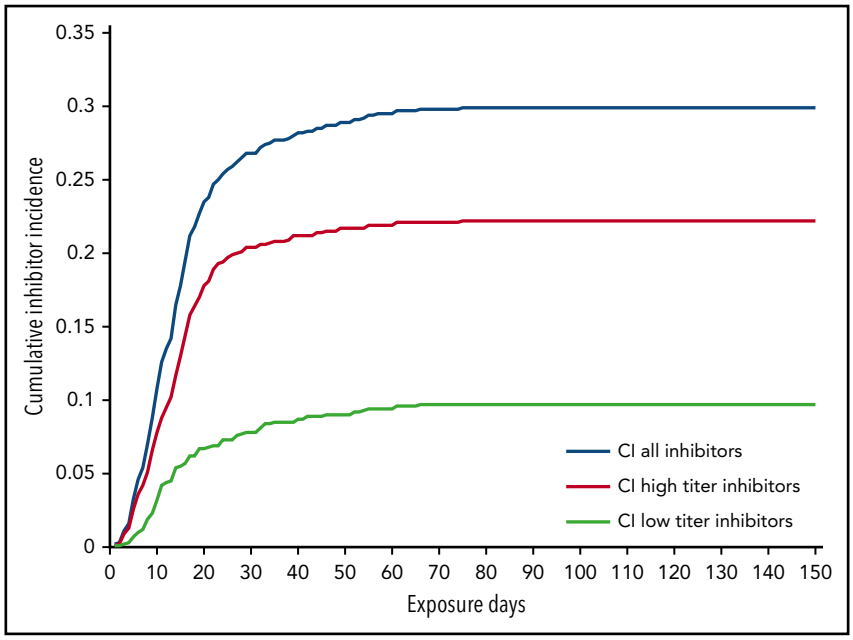

Figure 1. Kaplan-Meier curve of 1038 patients with SHA followed from ED 1 onward until ED 150. After 75 EDs, only 2 low-titer inhibitors developed, at 249 and 264 EDs.

75 EDs and calculated the time between the date of ED 75 and the date of last follow-up. The total follow-up time was 4031 person-years. Because only 2 low-titer inhibitors occurred in this period, the calculated inhibitor risk was only 0.5 per 1000 person-years. Earlier studies reported much higher risks for PTPs. In 1 of the first studies, a 4-year prospective study from the United States, 31 new inhibitors were detected in 1306 patients. The study concluded that inhibitor risk was 8 per 1000 person-years; this risk was used as an estimate to evaluate neoimmunogenicity in new products.[15] However, that study included patients with <50 EDs (ie, PUPs), causing the denominator to be a mixture of PTPs and PUPs. Studies reporting only on PTPs (using the definition of >150 EDs) over the last decades estimated the inhibitor risk at ~2 to 4 per 1000 person-years.[4,5,16,17]

Recently, investigators suggested that clinical trials in PUPs at 20 EDs be stopped, arguing that most inhibitors develop very early, whereas longer follow-up was too time-consuming and limited by patients being lost to follow-up.[18] Our data on 1038 PUPs with SHA show that such an approach would be inappropriate, as 21% of inhibitors occurred between 21 and 75 EDs. Clinical trials of factor concentrates in PUPs undertaken by manufacturers have generally followed patients only until 50 EDs or until 3 years on study. Differences in follow-up period and, as a consequence, the lack of information regarding late inhibitors have beenimportant limitations in comparing inhibitor risks between studies.[19,20] Our data demonstrate that the age to reach 75 EDs was at a median of 2.3 years. Frequent testing for inhibitors until 75 instead of 50 EDs, therefore, is feasible and should be recommended for all PUPs.

It should be noted that our results are applicable to children who receive early prophylaxis, as almost all of our patients were started on prophylaxis very early in life.[11] In countries where this is not the case, the timing of inhibitor development could potentially be different.

In conclusion, we have strong evidence from the largest prospective cohort study of PUPs that virtually all inhibitors develop by ED 75. Consequently, we propose that 75 EDs should to be the cutoff to distinguish PUPs from PTPs.

Acknowledgments

The authors thank all of the data registrars of the PedNet Study Group for providing data, and Ella van Hardeveld and Marloes de Kovel for managing data, verifying sources, and running quality checks.

Authorship

Contribution: All authors were members of the writing committee.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

A complete list of the members of the PedNet Study Group appears in the supplemental Appendix, available on the *Blood* Web site.

ORCID profiles: H.M.v.d.B., 0000-0002-2553-2324; K.F., 0000-0001-7126-6613; R.L., 0000-0003-3999-8747.

Correspondence: H. Marijke van den Berg, PedNet Haemophilia Research Foundation, Mollerusstraat 1, 3743 BW Baarn, The Netherlands; e-mail: h.marijke.vandenberg@pednet.eu.

Footnotes

Data collected in the frame work of the PedNet cohort studies are available for all members of the PedNet study group.

The online version of this article contains a data supplement.

REFERENCES

1. Calvez T, Chambost H, d'Oiron R, et al; for FranceCoag Collaborators. Analyses of the FranceCoag cohort support differences in immunogenicity among one plasma-derived and two recombinant factor VIII brands in boys with severe hemophilia A. *Haematologica*. 2018;103(1):179-189.

2. Gouw SC, van den Berg HM, Fischer K, et al; PedNet and Research of Determinants of INhibitor development (RODIN) Study Group. Intensity of factor VIII treatment and inhibitor development in children with severe hemophilia A: the RODIN study. *Blood*. 2013;121(20):4046-4055.

3. Iorio A, Halimeh S, Holzhauer S, et al. Rate of inhibitor development in previously untreated hemophilia A patients treated with plasma-derived or recombinant factor VIII concentrates: a systematic review. *J Thromb Haemost*. 2010;8(6):1256-1265.

4. Fischer K, Lassila R, Peyvandi F, et al; EUHASS participants. Inhibitor development in haemophilia according to concentrate. Four-year results from the European HAemophilia Safety Surveillance (EUHASS) project. *Thromb Haemost*. 2015;113(5):968-975.

5. Hassan S, Cannavò A, Gouw SC, Rosendaal FR, van der Bom JG. Factor VIII products and inhibitor development in previously treated patients with severe or moderately severe hemophilia A: a systematic review. *J Thromb Haemost*. 2018;16(6):1055-1068.

6. White GC II, Rosendaal F, Aledort LM, Lusher JM, Rothschild C, Ingerslev J; Factor VIII and Factor IX Subcommittee. 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 Haemostasis. *Thromb Haemost*. 2001;85(3):560.

7. Rosendaal FR, Nieuwenhuis HK, van den Berg HM, et al; Dutch Hemophilia Study Group. A sudden increase in factor VIII inhibitor development in multitransfused hemophilia A patients in The Netherlands. *Blood*. 1993;81(8):2180-2186.

8. European Medicines Agency. Guideline on the clinical investigation of recombinant and human plasma-derived factor VIII products. https://www.ema.europa.eu/documents/scientific-guideline/guideline-clinical-investigation-recombinant-human-plasma-derived-factor-viii-products-revision-2_en.pdf. Accessed 14 June 2019.

9. Arruda VR, Doshi BS, Samelson-Jones BJ. Novel approaches to hemophilia therapy: successes and challenges. *Blood*. 2017;130(21):2251-2256.

10. Nilsson IM, Berntorp E, Löfqvist T, Pettersson H. Twenty-five years' experience of prophylactic treatment in severe haemophilia A and B. *J Intern Med*. 1992;232(1):25-32.

11. Nijdam A, Altisent C, Carcao MD, et al; PedNet and CANAL study groups. Bleeding before prophylaxis in severe hemophilia: paradigm shift over two decades. *Haematologica*. 2015;100(3):e84-e86.

12. Fischer K, Ljung R, Platokouki H, et al. Prospective observational cohort studies for studying rare diseases: the European PedNet Haemophilia Registry. *Haemophilia*. 2014;20(4):e280-e286.

13. Gouw SC, van der Bom JG, Auerswald G, Ettinghausen CE, Tedgård U, van den Berg HM. Recombinant versus plasma-derived factor VIII products and the development of inhibitors in previously untreated patients with severe hemophilia A: the CANAL cohort study. *Blood*. 2007;109(11):4693-4697.

14. Marcucci M, Mancuso ME, Santagostino E, et al. Type and intensity of FVIII exposure on inhibitor development in PUPs with haemophilia A. A patient-level meta-analysis. *Thromb Haemost*. 2015;113(5):958-967.

15. McMillan CW, Shapiro SS, Whitehurst D, Hoyer LW, Rao AV, Lazerson J. The natural history of factor VIII:C inhibitors in patients with hemophilia A: a national cooperative study. II. Observations on the initial development of factor VIII:C inhibitors. *Blood*. 1988;71(2):344-348.

16. Kempton CL, Soucie JM, Abshire TC. Incidence of inhibitors in a cohort of 838 males with hemophilia A previously treated with factor VIII concentrates. *J Thromb Haemost*. 2006;4(12):2576-2581.

17. Xi M, Makris M, Marcucci M, Santagostino E, Mannucci PM, Iorio A. Inhibitor development in previously treated hemophilia A patients: a systematic review, meta-analysis, and meta-regression. *J Thromb Haemost*. 2013;11(9):1655-1662.

18. Peyvandi F, Mannucci PM, Garagiola I, et al. A randomized trial of factor VIII and neutralizing antibodies in hemophilia A. *N Engl J Med*. 2016;374(21):2054-2064.

19. Wight J, Paisley S. The epidemiology of inhibitors in haemophilia A: a systematic review. *Haemophilia*. 2003;9(4):418-435.

20. Keipert C, Jonker CJ, van den Berg HM, Hilger A. Clinical trials and registries in haemophilia: opponents or collaborators? Comparison of PUP data derived from different data sources. *Haemophilia*. 2018;24(3):420-428.

DOI 10.1182/blood.2019000658

TO THE EDITOR:

Simple, reliable detection of amyloid in fat aspirates using the fluorescent dye FSB: prospective study in 206 patients

Masayoshi Tasaki,[1-4] Paolo Milani,[1,2] Andrea Foli,[1,2] Laura Verga,[2,5] Laura Obici,[1,2] Marco Basset,[1,2] Margherita Bozzola,[1,2] Giovanni Ferraro,[1,2] Mario Nuvolone,[1,2] Patrizia Morbini,[2,5] Gianluca Capello,[2,5] Mitsuharu Ueda,[4] Konen Obayashi,[3] Marco Paulli,[2,5] Yukio Ando,[4] Giampaolo Merlini,[1,2] Giovanni Palladini,[1,2] and Francesca Lavatelli[1,2]

[1]Amyloidosis Research and Treatment Center, Fondazione Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS) Policlinico San Matteo, Pavia, Italy; [2]Department of Molecular Medicine, University of Pavia, Pavia, Italy; [3]Department of Morphological and Physiological Sciences, Graduate School of Health Sciences and [4]Department of Neurology, Graduate School of Medical Sciences, Kumamoto University, Kumamoto, Japan; and [5]Unit of Pathology, Department of Molecular Medicine, IRCCS San Matteo, Pavia, Italy

Diagnosis of amyloidosis, with the possible exception of cardiac transthyretin amyloidosis (ATTR) in patients without monoclonal components,[1] requires demonstration of tissue amyloid deposits and identification of the amyloidogenic protein.[2,3] The gold standard for amyloid detection is Congo red (CR) staining, based on amyloid's congophilia and birefringence under polarized light (PL; CR-PL).[4] When systemic amyloidosis is suspected, subcutaneous abdominal fat, acquired by fine-needle aspiration or punch biopsy, is considered the tissue of choice, given the high sensitivity (up to 80% to 90%)[5-7] and low risk of major complications.[5]

Although birefringence under PL is a defining feature of amyloid,[2] CR-PL interpretation critically depends on equipment quality, the pathologist's expertise, and connective tissue abundance.[8,9] This may translate into low specificity in non-specialized centers and unnecessary second-level testing. In our experience, 85% of the >300 putatively CR$^+$ fat specimens sent to our Center for amyloid typing were reclassified as negative after performing and evaluating CR staining in-house and with immuno–electron microscopy (IEM). Novel amyloid-binding compounds were developed as possible complements to CR in histology.[10-14] A major challenge is to achieve the clinical performances of CR, while allowing for easier staining and results interpretation. Examples of new dyes include luminescent-conjugated oligothiophenes,[10-12] and the fluorescent CR analogs (E,E),-1-bromo-2,5-bis-(3-hydroxycarbonyl-4-hydroxy)styrylbenzene (BSB)[13,14] and (E,E)-1-fluoro-2,5-bis-(3-hydroxycarbonyl-4-hydroxy) styrylbenzene (FSB).[14,15] In particular, FSB was shown to bind amyloid specifically in tissues and in vivo[16] and to stain various amyloid types, with stronger fluorescence than CR and BSB. In pilot studies, FSB showed higher sensitivity than CR.[14,15,17] These dyes, however, have not yet reached the clinical routine, and systematic studies on large patient cohorts are lacking. Herein, we prospectively assessed the usefulness of FSB to detect amyloid in fat aspirates from individuals with suspected systemic amyloidosis, through comparison of FSB results against CR-PL.

Consecutive individuals referred to the Pavia Amyloidosis Center (December 2017 to July 2018) were enrolled. Written informed consent was obtained for use of biological samples and clinical data for research, according to the institutional review board guidelines. Patients underwent clinical, instrumental, and genetic examination as described.[6] In cases without evidence of amyloid deposits in fat but persistent clinical suspicion of amyloidosis, biopsy of affected organs was performed to confirm or exclude diagnosis. Subcutaneous periumbilical fat was acquired by fine-needle aspiration; samples (~50 mg) were split into 3 comparable parts to be examined by IEM,[6] FSB, and CR-PL (the latter was stained and evaluated as described[4,6] by 2 expert physicians blinded to FSB results). For FSB staining, samples were immediately washed 3 times with cold phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde (1 hour), and immersed in FSB working solution (0.01% vol/vol in PBS; 2 hours) (Sigma-Aldrich/Merck). Specimens were washed 4 times with PBS (5 minutes each; agitation), placed on histology slides, overlaid with ProLong Gold (Thermo Fisher Scientific), and squeezed under a cover slide. Fluorescence was visualized using a Olympus Fluoview FV10i-LIV confocal microscope (excitation, 405 nm; emission, 420-520 nm). Negative and positive controls were used to set microscope parameters that allowed visualization of the FSB signal without background autofluorescence.