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BRIEF REPORT

Characterization of the anti-factor VIII immunoglobulin profile in patients with hemophilia A by use of a fluorescence-based immunoassay

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Summary. *Background:* The development of neutralizing antibodies, referred to as inhibitors, against factor VIII is a major complication associated with FVIII infusion therapy for the treatment of hemophilia A (HA). Previous studies have shown that a subset of HA patients and a low percentage of healthy individuals harbor non-neutralizing anti-FVIII antibodies that do not elicit the clinical manifestations associated with inhibitor development. *Objective:* To assess HA patients' anti-FVIII antibody profiles as potential predictors of clinical outcomes. *Methods:* A fluorescence immunoassay (FLI) was used to detect anti-FVIII antibodies in 491 samples from 371 HA patients. *Results:* Assessments of antibody profiles showed that the presence of anti-FVIII IgG_1, IgG_2 or IgG_4 correlated qualitatively and quantitatively with the presence of an FVIII inhibitor as determined with the Nijmegen–Bethesda assay (NBA). Forty-eight patients with a negative inhibitor history contributed serial samples to the study, including seven patients who had negative NBA titers initially and later converted to being NBA-positive. The FLI detected anti-FVIII IgG_1 in five of those seven patients prior to their conversion to NBA-positive. Five of 15 serial-sample patients who had a negative inhibitor history and had anti-FVIII IgG_1 later developed an inhibitor, as compared with two of 33 patients with a negative inhibitor history without anti-FVIII IgG_1. *Conclusions:* These data provide a rationale for future studies designed both to monitor the dynamics of anti-FVIII antibody profiles in HA patients as a potential predictor of future inhibitor development and to assess the value of the anti-FVIII FLI as a supplement to traditional inhibitor testing.

Keywords: factor VIII; factor VIII deficiency; hemophilia A; immunoassay; inherited blood coagulation disorders.

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Introduction

Hemophilia A (HA) is an X-linked inherited bleeding disorder in which coagulation factor VIII is absent or dysfunctional, and is most commonly treated by infusion of plasma-derived or recombinant FVIII. A major complication associated with FVIII infusion therapy is that up to 30% of patients develop antibodies that inhibit the function of and/or induce immune-dependent clearance of the infused product [1,2]. Anti-FVIII antibodies, referred to as inhibitors, diminish the effectiveness of infusion therapy, and, in the case of high-titer inhibitors, necessitate the use of FVIII-bypassing agents [3] or immune tolerance induction therapy [4,5]. Patients who develop FVIII inhibitors face an increased risk of bleeding complications [6] and present substantial financial and patient management challenges to the healthcare system [7].

The Bethesda assay [8] for measurement of FVIII inhibitors was developed in 1975, and modified in 1995 to the Nijmegen–Bethesda assay (NBA) [9], which is the gold standard method in use today. The NBA utilizes the degree

to which HA patient plasma inhibits the *in vitro* clotting reaction of healthy donor plasma as a means to assign FVIII inhibitor titers. More recently, assays utilizing chromogenic substrates [10], ELISA [11,12], surface plasmon resonance (SPR) [13,14] and fluorescent immunoassays (FLIs) [15–19] have been developed to detect anti-FVIII antibodies in HA patients. Many previous studies have observed that there is some discrepancy between the results obtained with functional assays, such as the NBA, and those obtained with other testing methods [11,12,18]. Although the assortment of FVIII inhibitor assays all share the common goal of identifying the presence of anti-FVIII antibodies, they have key fundamental differences that contribute to the generation of discrepant results. The NBA and chromogenic inhibitor assay (CBA) attempt to simulate *in vivo* conditions in order to detect FVIII-specific functional inhibition of the clotting process. For the purpose of these assays, functional inhibition of FVIII-dependent clotting is reflected in decreased extent or kinetics of an *in vitro* clotting reaction [8,9] or the cleavage of a chromogenic substrate as a surrogate for clotting activity [10], but there is no direct measurement of FVIII-specific immunoreactivity. Alternatively, SPR, ELISAs and anti-FVIII FLIs (αFVIII-FLIs) directly detect anti-FVIII antibodies, but do so without any means to assess the detected antibody's ability to inflict functional inhibition on FVIII. These differences, as well as the lack of uniformity among laboratories in the methods used to determine what constitutes a positive reaction, make it difficult to integrate the various test results in order to reach a definitive diagnosis of a clinically significant inhibitor.

Previous studies utilizing direct antibody detection methods [11–13,20,21] have shown that the Ig subtype and subclass composition of the anti-FVIII antibody response may be critical in assessing the clinical implications of the immune response. These studies implicated IgG_1 and IgG_4 as the most common anti-FVIII antibody subclasses present in NBA-positive patient samples. The current study investigated the composition of the antibody response in 371 HA patients, the largest group of patients studied to date, using an αFVIII-FLI. The study examined the prevalence of anti-FVIII antibodies in HA patient plasma, evaluated the make-up of the antibody response by IgG subclass, and assessed the clinical relevance of antibody subtype by evaluating the extent of correlation between FLI results and those obtained with the NBA.

Materials and methods

Subjects

The study included 491 plasma samples from 371 HA patients (median age, 13 years; mean age, 18.5 years) enrolled in the Hemophilia Inhibitor Research Study [22]. Of the patients, 20.5% (n = 76) were NBA-positive, and of the samples, 24.8% (n = 122) were NBA-positive. Inhibitor measurements were performed with a modified version [23] of the NBA [9]. The investigational review boards of the Centers for Disease Control and each participating site approved the protocol, and all participants or parents of minors gave informed consent. Control samples were obtained from 56 paid healthy donors.

FLI

The αFVIII-FLI is a modified version of our previously described method [18]. Briefly, plasma samples diluted 1 : 30 in phosphate-buffered saline containing 1% dried milk were incubated with SeroMAP beads (Luminex Corporation, Austin, TX, USA) coupled to Kogenate FS (Bayer Healthcare, Tarrytown, NY, USA). Anti-FVIII antibodies were detected by use of serial incubations with biotinylated anti-human Ig (anti IgG_1, A-10650; anti IgG_2, 05-3540; anti IgG_3, MH1532; anti IgG_4, A-10663; anti IgM, H15015; Life Technologies, Carlsbad, CA, USA) and R-phycoerythrin-conjugated streptavidin (Jackson ImmunoResearch, West Grove, PA, USA) with a Bio-Plex 200 suspension array system (Bio-Rad Laboratories, Hercules, CA, USA). Results are expressed as median fluorescence intensity (MFI). The threshold for positivity was set at two standard deviations above the mean MFI of the results obtained for healthy donors.

Statistical analyses

Comparisons of FLI and NBA results on individual plasma samples were made by the use of GRAPHPAD PRISM (GraphPad Software, San Diego, CA, USA) to generate

Table 1 Summary of positive fluorescence immunoassay (FLI) results for anti-factor VIII antibodies segregated by Ig subclass

		% Positive for anti-FVIII by FLI				
	n	IgG_1	IgG_2	IgG_3	IgG_4	IgM*
Healthy donors	56	5.4	5.4	1.8	1.8	7.1
All HA specimens	491	40.5	17.3	6.1	26.5	3.9
NBA-negative HA specimens	369	23.3	8.9	3	6	3.3
NBA-positive HA specimens	122	92.6	42.6	15.6	88.5	5.9
Correlation of FLI and NBA		0.5438, $P < 0.0001$	0.3411, $P < 0.0001$	0.2829, $P < 0.0001$	0.5766, $P < 0.0001$	0.0643, $P = 0.1589$

HA, hemophilia A; NBA, Nijmegen–Bethesda assay. *n = 482 HA specimens: 364 NBA-negative, and 118 NBA-positive.

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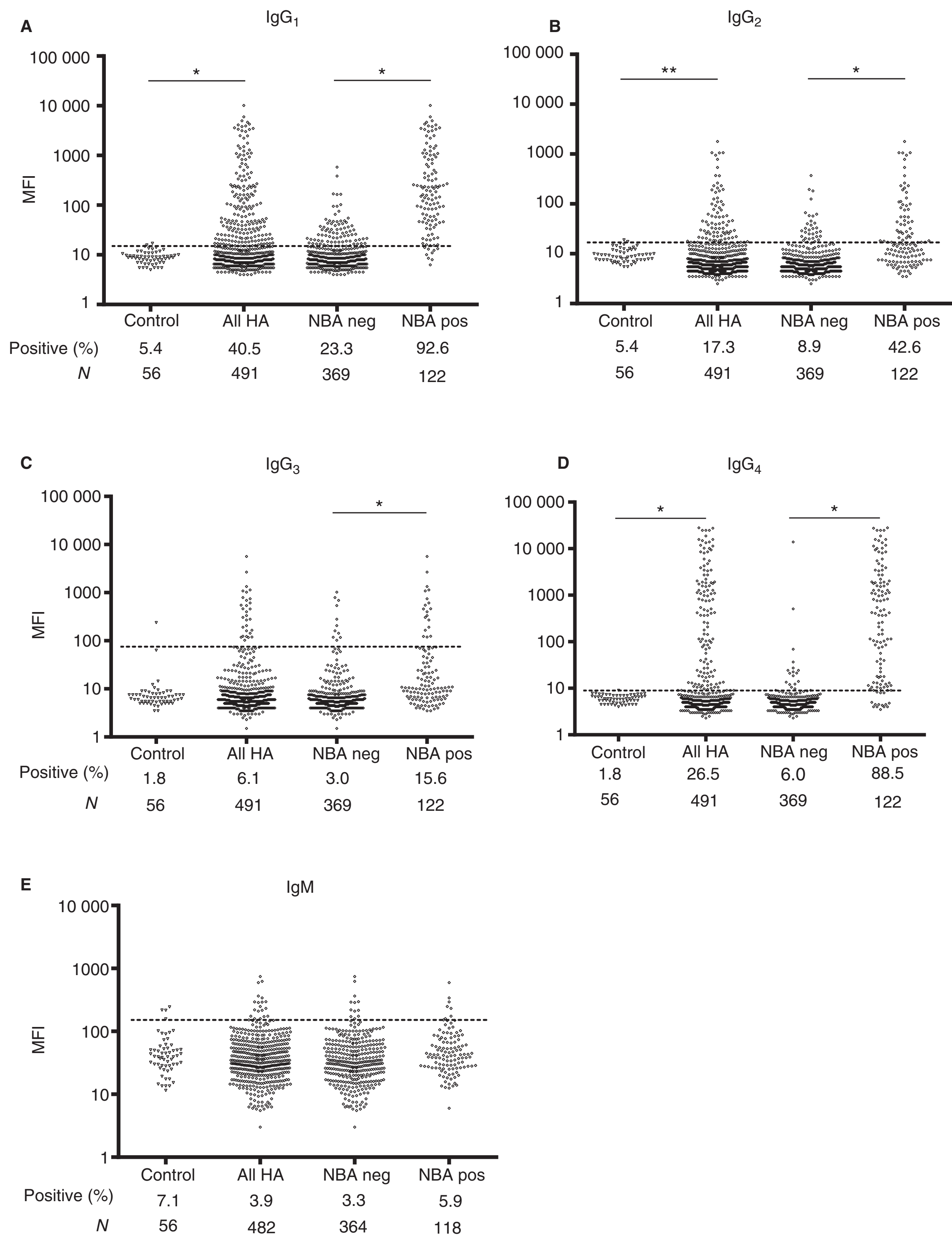

Fig. 1. Fluorescence immunoassay results for anti-FVIII antibodies in plasma from hemophilia A (HA) patients and healthy controls. Individual data points represent plasma samples assayed for anti-FVIII IgG_1 (A), IgG_2 (B), IgG_3 (C), IgG_4 (D), and IgM (E). Results are displayed on a log-scale for control plasmas from healthy donors, all HA patient samples, and the subsets of HA patient samples with negative or positive Nijmegen–Bethesda assay results for each Ig measured. The dashed line, which represents the assay's positive threshold, is two standard deviations above the mean median fluorescence intensity of 56 control samples from healthy donors. The number of samples (*N*) and the percentage of the samples that tested positive are as indicated. $^{*}P < 0.0001$; $^{**}P = 0.02$.

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Spearman's correlation coefficient and two-tailed P-values. Fisher's exact test was used to evaluate differences in categorical data.

Results and discussion

Characterization of anti-FVIII antibodies in the plasma of HA patients

HA patient plasma samples were examined for the presence of anti-FVIII IgG_1, IgG_2, IgG_3, IgG_4 and IgM with an αFVIII-FLI (Table 1; Fig. 1). IgG subclass-specific analysis of plasma samples showed that 40.5%, 17.3%, 6.1% and 26.5% of the 491 patient samples were positive for anti-FVIII IgG_1, IgG_2, IgG_3, and IgG_4, respectively, as compared with 5.4% (IgG_1 and IgG_2) or 1.8% (IgG_3 and IgG_4) of healthy donor samples (IgG_1 and IgG_4, $P < 0.0001$; IgG_2, $P = 0.02$; IgG_3, $P = 0.353$). Evaluation of the IgG subclass-specific FLI results segregated by NBA status revealed that NBA-positive samples had significantly higher rates of positivity than NBA-negative samples for anti-FVIII IgG_1, IgG_2, IgG_3, and IgG_4 ($P < 0.0001$) (Table 1; Fig. 1). Rates of anti-FVIII IgM positivity were not significantly different between patients (3.9%) and healthy donors (7.1%) ($P = 0.285$).

In order to assess the relative importance of each subclass of anti-FVIII IgG in patients with FVIII inhibitors, we analyzed the IgG subclass-specific FLI results to determine the composition of the FVIII antibody response in NBA-positive samples. The results showed that 98.4% of the NBA-positive samples had positive FLI titers for one or more subclasses of anti-FVIII IgG, including 13.9% that were positive for a single subclass of anti-FVIII IgG, and 84.4% that were positive for multiple subclasses of anti-FVIII IgG; the remaining 1.6% had no FLI-detectable anti-FVIII antibodies (Table 2). All of the 120 NBA-positive samples that also tested positive by FLI contained anti-FVIII IgG_1 and/or IgG_4, and 101 (84.2%) were positive for both anti-FVIII IgG_1 and anti-FVIII IgG_4. Both of the NBA-positive/FLI-negative results were obtained in samples with low-titer inhibitors (0.7 and 0.8 NBU), and one of these samples was previously reported to a be a false positive, owing to the negative result by CBA [18].

Linear correlations were calculated according to Spearman to evaluate the relationship between titers obtained from the αFVIII-FLIs and the NBA. The αFVIII-FLI results for anti-FVIIIIgG$_1$ and IgG_4, which were positive in 92.6% and 88.5% of samples, respectively, showed a strong positive correlation with NBA titers ($r[IgG_1] = 0.5438$, $r[IgG_4] = 0.5766$; $P < 0.0001$). Correlations between FLI and NBA results were weak, but significant for anti-FVIII IgG_2 ($r = 0.3411$; $P < 0.0001$) and anti-FVIII IgG_3 ($r = 0.2829$; $P < 0.0001$), whereas anti-FVIII IgM did not show a quantitative correlation with NBA results (Table 1).

Table 2 Fluorescence immunoassay (FLI) results in 122 Nijmegen–Bethesda assay (NBA)-positive samples

FLI result	NBA-positive samples, % (n)	Number of FLI-positive samples			
		IgG_1	IgG_2	IgG_3	IgG_4
Negative	1.6 (2)	0	0	0	0
Positive for one subclass of IgG	13.9 (17)	10	0	0	7
Positive for two subclasses of IgG	40.2 (49)	49	1	1	47
Positive for three subclasses of IgG	32.0 (39)	39	37	2	39
Positive for four subclasses of IgG	12.3 (15)	15	15	15	15

Anti-FVIII IgG composition in serial samples from individual HA patients

Sixteen patients showed a change in NBA inhibitor status over the course of specimen collection. Seven of these patients (patients 1–7) had negative NBA titers in their initial study specimen, but later developed a positive NBA reaction following FVIII infusion therapy for the indicated exposure days (Table 3). Examination of FLI results in plasma samples from these seven patients revealed that five of them harbored one or more classes of anti-FVIII Ig in samples prior to developing an inhibitor detectable by the NBA (Table 3, patients 1–5). All of these five patients were positive for anti-FVIII IgG_1 prior to their conversion from NBA-negative to NBA-positive; one was also positive for anti-FVIII IgG_4 (patient 5) and one for anti-FVIII IgM (patient 4). Analysis of the FLI results in 201 samples from all 81 patients who contributed multiple specimens (data not shown) showed that five of 15 (33.3%) patients with a negative inhibitor history and a positive anti-FVIII IgG_1 result later developed an inhibitor, as compared with two of 33 (6.1%) patients with a negative inhibitor history without anti-FVIII IgG_1 antibodies ($P = 0.0239$). Patients 8–16 (Table 3) all have a history of inhibitors, and are of interest because of the transitory nature of their NBA positivity. It is important to note that whereas, overall, the FLI results for anti-FVIII IgG_1, IgG_2, IgG_3 and IgG_4 showed significant positive correlations with the NBA, FLI and NBA results in serial samples from individual patients did not necessarily change proportionally with time. The lack of intrapatient consistency is probably attributable to the differing role of kinetics in the two assays, and may also reflect changes in the patient's immune response over time.

Positive FLI results in samples with a corresponding negative NBA result were present in a low percentage of samples tested for anti-FVIII IgG_{2-4}, occurring in 3–9%, whereas disparities for anti-FVIII IgG_1 were more common, with positive FLI results occurring in 23.3% of NBA-negative samples. These discrepant results may be caused by the presence of anti-FVIII antibodies that are

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Table 3 Anti-factor VIII fluorescence immunoassay (FLI) results on serial plasma draws from hemophilia A patients who exhibited a change in Nijmegen-Bethesda assay (NBA) status over the course of sample collection. Positive results are in bold

				Median fluorescence intensity units (MFI)						
	Pt.	Severity	Draw date	IgG_1	IgG_2	IgG_3	IgG_4	IgM	NBU	Exposure days
No history of inhibitor	1	Mild	12/5/07	5.5	4.5	5	6	11.5	0.1	0–20
			9/10/08	11.5	7	7	6	84.3	0	–
			9/16/09	**25.5**	5.5	6	5	17	0	–
			4/21/10	**1093.3**	7	**191.5**	8	38	**1.7**	0–20
			6/9/10	**4646.8**	**46**	**332.3**	**85**	60.8	**1.3**	–
			9/21/10	**386.5**	7.5	20.5	**96.8**	29	**1.8**	–
	2	Severe	8/9/10	**22**	5	4.5	3.3	39.5	0.1	0–20
			10/11/10	**4111.8**	**42.3**	**612**	**1921**	34	**3.2**	21–50
			11/18/10	**827**	10	28.8	**1109**	83.5	**18.7**	–
			2/8/11	**3352**	**43.5**	44	**1277**	25.5	**7.2**	–
			3/9/11	**234.5**	7.5	10.3	**262**	25	**1.4**	–
	3	Severe	10/1/08	**75.3**	5	8	4.5	90.5	0	0–20
			9/22/09	**441.3**	15.5	8.3	**1592**	85.3	**13.6**	0–20
	4	Severe	7/23/08	**37.5**	8.3	5.8	6.5	**746.5**	0.2	0–20
			7/8/09	**16.8**	5.8	5.8	4	69	0	–
			6/2/10	**240.5**	9	8	**792.3**	**173.8**	**3.9**	21–50
	5	Severe	8/6/08	**33**	6	3.5	**9**	25.5	0.3	21–100
			8/12/09	**48.5**	12.8	6.5	**14.5**	53.8	**1.4**	>150
			8/14/09	11	6	3.5	**10.5**	46	**1.4**	–
			6/30/10	6	6.8	4	3.5	58.8	0	–
	6	Mild	3/3/10	10.5	4.3	6	4.5	109.3	0.1	0–20
			5/27/10	**504.8**	11.8	73.5	**12.3**	**597.5**	**1.4**	0–20
			6/14/10	**3914.5**	**111.3**	**746.8**	**114.5**	103	**1.7**	–
			11/14/12	7.5	5.5	5	4.5	70.8	0.1	–
	7	Severe	2/5/07	7	4.5	3.5	4	ND	0	101–150
			6/18/08	**34**	6.8	4.8	**1193.8**	39	**6.5**	101–150
			6/17/09	**51.5**	7	6.8	**1276.5**	**248.8**	**3.8**	–
Previous history of inhibitor	8	Severe	7/5/06	**249.5**	12	13	**8548.3**	32	**19.3**	ND
			7/23/08	7	5.5	5	5	42	0.2	–
	9	Severe	3/15/06	10	5.3	4	**11.5**	**231**	**0.5**	ND
			5/7/08	14.5	6.3	6.5	**18.5**	110.3	0	–
			5/6/09	9	8	5.8	7	93.8	0	–
	10	Severe	9/5/07	**157.3**	11	6.8	**27**	26.5	**1.1**	ND
			9/5/12	**41**	6.3	5.5	**27.3**	16	0.4	–
	11	Severe	6/17/08	**35.8**	5.8	6.5	**39.5**	42.5	**0.5**	ND
			6/17/09	**38.5**	10.5	8.5	**21**	15.8	0.3	–
			6/16/10	**19.5**	4.5	5.5	**22.5**	25	0.3	–
	12	Severe	4/12/06	**15.5**	4	4.5	4	54.8	**0.5**	ND
			4/23/08	**16.5**	6	4	6	15.5	0.4	–
			4/29/09	8	8.5	5	4	46.5	0	–
	13	Mild	12/15/08	**66.3**	**96.5**	12	**542**	37	**0.8**	ND
			3/4/09	10.8	**58.3**	6.5	**14**	40.5	0	–
	14	Severe	11/16/07	**85.5**	10	5.8	**1527**	15	**24.6**	ND
			9/25/09	14.5	5	4	**9.8**	37	0.3	–
			6/2/10	**337.5**	**92.3**	**398.5**	**145.8**	95.5	**3.3**	–
	15	Severe	2/6/08	**240.8**	**55.5**	**1341.5**	**85.3**	41.5	**3.9**	ND
			4/8/09	**16**	6.5	14.5	**69**	23.5	0.2	–
	16	Severe	10/10/07	**48**	**25**	31	**510**	20.8	0.3	ND
			12/5/08	13.3	9	7	**207.5**	26.5	**0.6**	–
			Threshold for positivity	14.6*	16.1*	75.5*	8.3*	153.6*	0.5	–

NBU, Nijmegen-Bethesda units; ND, No data collected; *Mean + 2 standard deviations of 56 healthy donors.

of insufficient titer to have an inhibitory effect on coagulation in the NBA, the presence of anti-FVIII antibodies that recognize epitopes that are insignificant for the functional integrity of the FVIII molecule, or non-specific or indirect antibody binding to the FVIII-coupled beads. Our data on serial samples drawn from 81 patients support the first hypothesis. Although it is important to note that patients harboring non-neutralizing antibodies may never progress to developing an inhibitor, one-third of 15 patients who had a negative inhibitor history and were

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positive for IgG_1 converted from NBA-negative to NBA-positive over the course of the sample collection, as compared with only 6.1% of patients with a negative inhibitor history without anti-FVIII IgG_1. These findings, although preliminary, suggest that NBA-negative patients with anti-FVIII IgG_1 are more likely to develop inhibitors detectable by the NBA than patients without such antibodies, and that these patients may merit closer scrutiny (e.g. patients undergoing surgical procedures) or more frequent follow-up testing (e.g. patients receiving initial FVIII infusions) to facilitate prompt clinical intervention.

The identification of anti-FVIII antibodies in HA patients is an important clinical development, but the results presented here and by others have shown that the mere presence of antibodies does not always correlate with the clinical manifestations of FVIII inhibition [11,12,16–19,24,25]. Identifying the underlying features that distinguish cases of benign and/or transient anti-FVIII antibodies from those that are clinically relevant anti-FVIII inhibitors is an important area of research. Although it remains unclear why the presence of certain antibody subclasses may be predictive of a worse clinical outcome, the data presented herein support those from a recently published study by Whelan *et al.* [12], in which the authors used an ELISA to show that anti-FVIII IgG_1 and IgG_4 were present in 19 of 20 inhibitor-positive HA patients. They also found that anti-FVIII IgG_4 was completely absent in 77 non-inhibitor patients and 600 healthy individuals, and that anti-FVIII IgG_1 was present in 19% and 6% of non-inhibitor HA patients and healthy individuals, respectively [12]. Whelan *et al.* hypothesized that their data could indicate the presence of variations in immune regulatory pathways in the different study cohorts. Previous studies that examined the potential link between single-nucleotide polymorphisms in immune response genes and a predisposition to inhibitor development [26–30] and the results from the current study, with a larger patient population using a different methodology, support this hypothesis. In addition, our data illustrate that anti-FVIII IgG_4 may be present in a low percentage of patients lacking inhibitors, as measured with the NBA, including 2.5% (7/283) of patients with a negative inhibitor history (data not shown), and that anti-FVIII IgG_1 production may be an early checkpoint in inhibitor development. Taken together, these data provide a rationale for future clinical studies designed to monitor the dynamics of HA patients' anti-FVIII antibody profiles in order to assess their value as predictors of the future development of clinically relevant inhibitors and to determine the usefulness of the αFVIII-FLI as a supplement to traditional inhibitor testing methods.

Addendum

B. Boylan designed and performed the research, analyzed results, and wrote the paper. A. S. Rice performed the research. A. L. Dunn, M. D. Tarantino, D. B. Brettler, and J. C. Barrett provided patient samples and contributed to the manuscript. C. H. Miller analyzed results and wrote the paper.

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Disclosure of Conflict of Interests

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Appendix
Study Contributors

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References

1 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**: 2576–81.

2 Kruse-Jarres R. Inhibitors: our greatest challenge. Can we minimize the incidence? *Haemophilia* 2013; **19**(Suppl. 1): 2–7.

3 Leissinger CA. Prevention of bleeds in hemophilia patients with inhibitors: emerging data and clinical direction. *Am J Hematol* 2004; **77**: 187–93.

4 Waters B, Lillicrap D. The molecular mechanisms of immunomodulation and tolerance induction to factor VIII. *J Thromb Haemost* 2009; **7**: 1446–56.

5 DiMichele DM. Immune tolerance in haemophilia: the long journey to the fork in the road. *Br J Haematol* 2012; **159**: 123–34.

6 Colowick AB, Bohn RL, Avorn J, Ewenstein BM. Immune tolerance induction in hemophilia patients with inhibitors: costly can be cheaper. *Blood* 2000; **96**: 1698–702.

7 Guh S, Grosse SD, McAlister S, Kessler CM, Soucie JM. Healthcare expenditures for males with haemophilia and employer-sponsored insurance in the United States, 2008. *Haemophilia* 2012; **18**: 268–75.

8 Kasper CK, Aledort L, Aronson D, Counts R, Edson JR, van Eys J, Fratantoni J, Green D, Hampton J, Hilgartner M, Levine P, Lazerson J, McMillan C, Penner J, Shapiro S, Shulman NR. Proceedings: a more uniform measurement of factor VIII inhibitors. *Thromb Diath Haemorrh* 1975; **34**: 612.

9 Verbruggen B, Novakova I, Wessels H, Boezeman J, van den Berg M, Mauser-Bunschoten E. The Nijmegen modification of the Bethesda assay for factor VIII:C inhibitors: improved specificity and reliability. *Thromb Haemost* 1995; **73**: 247–51.

10 Blanco AN, Alcira PA, Grosso SH, Gennari LC, Perez BR, Lazzari MA. A chromogenic substrate method for detecting and titrating anti-factor VIII antibodies in the presence of lupus anticoagulant. *Haematologica* 2002; **87**: 271–8.

11 Gilles JG, Arnout J, Vermylen J, Saint-Remy JM. Anti-factor VIII antibodies of hemophiliac patients are frequently directed towards nonfunctional determinants and do not exhibit isotypic restriction. *Blood* 1993; **82**: 2452–61.

12 Whelan SF, Hofbauer CJ, Horling FM, Allacher P, Wolfsegger MJ, Oldenburg J, Male C, Windyga J, Tiede A, Schwarz HP, Scheiflinger F, Reipert BM. Distinct characteristics of antibody responses against factor VIII in healthy individuals and in different cohorts of hemophilia A patients. *Blood* 2013; **121**: 1039–48.

13 Lewis KB, Hughes RJ, Epstein MS, Josephson NC, Kempton CL, Kessler CM, Key NS, Howard TE, Kruse-Jarres R, Lusher JM, Walsh CE, Watts RG, Ettinger RA, Pratt KP. Phenotypes of allo- and autoimmune antibody responses to FVIII characterized by surface plasmon resonance. *PLoS ONE* 2013; **8**: e61120.

14 Nguyen PC, Lewis KB, Ettinger RA, Schuman JT, Lin JC, Healey JF, Meeks SL, Lollar P, Pratt KP. High-resolution mapping of epitopes on the C2 domain of factor VIII by analysis of point mutants using surface plasmon resonance. *Blood* 2014; **123**: 2732–9.

15 Lavigne-Lissalde G, Tarrade C, Lapalud P, Chtourou S, Schved JF, Granier C, Villard-Saussine S. Simultaneous detection and epitope mapping of anti-factor VIII antibodies. *Thromb Haemost* 2008; **99**: 1090–6.

16 Krudysz-Amblo J, Parhami-Seren B, Butenas S, Brummel-Ziedins KE, Gomperts ED, Rivard GE, Mann KG. Quantitation of anti-factor VIII antibodies in human plasma. *Blood* 2009; **113**: 2587–94.

17 Zakarija A, Harris S, Rademaker AW, Brewer J, Krudysz-Amblo J, Butenas S, Mann KG, Green D. Alloantibodies to factor VIII in haemophilia. *Haemophilia* 2011; **17**: 636–40.

18 Miller CH, Rice AS, Boylan B, Shapiro AD, Lentz SR, Wicklund BM, Kelly FM, Soucie JM. Comparison of clot-based, chromogenic and fluorescence assays for measurement of factor VIII inhibitors in the US Hemophilia Inhibitor Research Study. *J Thromb Haemost* 2013; **11**: 1300–9.

19 Lebreton A, Lapalud P, Chambost H, Biron-Andreani C, Morange PE, Combescure C, Marques-Verdier A, Berger C, Schved JF, Granier C, Lavigne-Lissalde G. Prevalence and epitope specificity of non-neutralising antibodies in a large cohort of haemophilia A patients without inhibitors. *Thromb Haemost* 2011; **105**: 954–61.

20 Towfighi F, Gharagozlou S, Sharifian RA, Kazemnejad A, Esmailzadeh K, Managhchi MR, Shokri F. Comparative measurement of anti-factor VIII antibody by Bethesda assay and ELISA reveals restricted isotype profile and epitope specificity. *Acta Haematol* 2005; **114**: 84–90.

21 van Helden PM, van den Berg HM, Gouw SC, Kaijen PH, Zuurveld MG, Mauser-Bunschoten EP, Aalberse RC, Vidarsson G, Voorberg J. IgG subclasses of anti-FVIII antibodies during immune tolerance induction in patients with hemophilia A. *Br J Haematol* 2008; **142**: 644–52.

22 Soucie JM, Miller CH, Kelly FM, Payne AB, Creary M, Bockenstedt PL, Kempton CL, Manco-Johnson MJ, Neff AT. A study of prospective surveillance for inhibitors among persons with haemophilia in the United States. *Haemophilia* 2014; **20**: 230–7.

23 Miller CH, Platt SJ, Rice AS, Kelly F, Soucie JM. Validation of Nijmegen-Bethesda assay modifications to allow inhibitor measurement during replacement therapy and facilitate inhibitor surveillance. *J Thromb Haemost* 2012; **10**: 1055–61.

24 Klintman J, Hillarp A, Berntorp E, Astermark J. Long-term anti-FVIII antibody response in Bethesda-negative haemophilia A patients receiving continuous replacement therapy. *Br J Haematol* 2013; **163**: 385–92.

25 Ling M, Duncan EM, Rodgers SE, Street AM, Lloyd JV. Low detection rate of antibodies to non-functional epitopes on factor VIII in patients with hemophilia A and negative for inhibitors by Bethesda assay. *J Thromb Haemost* 2003; **1**: 2548–53.

26 Hay CR, Ollier W, Pepper L, Cumming A, Keeney S, Goodeve AC, Colvin BT, Hill FG, Preston FE, Peake IR. HLA class II profile: a weak determinant of factor VIII inhibitor development in severe haemophilia A. UKHCDO Inhibitor Working Party. *Thromb Haemost* 1997; **77**: 234–7.

27 De Barros MF, Herrero JC, Sell AM, De Melo FC, Braga MA, Pelissari CB, Machado J, De Souza SS, De Souza HL, Visentainer JE. Influence of class I and II HLA alleles on inhibitor development in severe haemophilia A patients from the south of Brazil. *Haemophilia* 2012; **18**: e236–40.

28 Astermark J, Oldenburg J, Pavlova A, Berntorp E, Lefvert AK. Polymorphisms in the IL10 but not in the IL1beta and IL4 genes are associated with inhibitor development in patients with hemophilia A. *Blood* 2006; **107**: 3167–72.

29 Pavlova A, Delev D, Lacroix-Desmazes S, Schwaab R, Mende M, Fimmers R, Astermark J, Oldenburg J. Impact of polymorphisms of the major histocompatibility complex class II, interleukin-10, tumor necrosis factor-alpha and cytotoxic T-lymphocyte antigen-4 genes on inhibitor development in severe hemophilia A. *J Thromb Haemost* 2009; **7**: 2006–15.

30 Astermark J, Oldenburg J, Carlson J, Pavlova A, Kavakli K, Berntorp E, Lefvert AK. Polymorphisms in the TNFA gene and the risk of inhibitor development in patients with hemophilia A. *Blood* 2006; **108**: 3739–45.

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