Nonneutralizing FVIII-specific antibody signatures in patients with hemophilia A and in healthy donors

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Key Points

- Isotypes and IgG subclasses of nonneutralizing FVIII-specific antibodies are similar in hemophilia A patients and healthy subjects.

- Prevalences, titers, and affinities of nonneutralizing anti-bodies, however, differ significantly between patients and healthy subjects.

Previous studies identified nonneutralizing FVIII-specific antibodies in the circulation of severe and nonsevere hemophilia A (sHA and nsHA) patients without FVIII inhibitors and also in some healthy individuals. To gain a better understanding of the nature of these nonneutralizing antibody responses, we analyzed and compared anti-FVIII antibody signatures in 3 study cohorts: previously treated sHA as well as nsHA patients without FVIII inhibitors, and healthy donors. FVIII-binding IgM, IgG1-4, and IgA antibodies were differentiated, FVIII-specificity was assessed, and associated apparent affinity constants were determined. Our results indicate that the nonneutralizing FVIII-specific antibody response in all study cohorts is dominated by IgG1 and IgA. Prevalences, titers, and affinities of these nonneutralizing antibodies were higher in the hemophilia A cohorts than in healthy donors. Stratification for the anti-hepatitis C virus (HCV) antibody status demonstrated the presence of FVIII-specific IgA with elevated titers in sHA patients with an active or past HCV infection when compared with HCV antibody-positive nsHA patients or HCV antibody-negative patients and healthy donors. Increased titers and affinities of FVIII-specific IgG1 antibodies were observed in a considerable number of hemophilia A patients as opposed to healthy subjects independently of the patients' anti-HCV antibody status. Overall, our findings support the hypothesis that the generation of nonneutralizing anti-FVIII antibodies in healthy individuals and in noninhibitor hemophilia A patients might be based on similar immune mechanisms. However, differences in prevalences, titers, and affinities of these antibodies indicate distinct differences in the antibody evolution between healthy individuals and patients.

Introduction

Congenital hemophilia A is a rare, X-linked bleeding disorder affecting 1 in 5000 to 10 000 newborn males in the United States.[1] The severity of the disease is classified by the residual plasma concentration of functionally active factor VIII (FVIII).[2] Severe hemophilia A (sHA) patients (FVIII clotting activity [FVIII:C] <1%) experience spontaneous bleeds in joints and muscles, with a high risk for progressive joint disease from an early age on. Nonsevere hemophilia A (nsHA) patients (FVIII:C 1% to 40%), who account

The full-text version of this article contains a data supplement.

for 50% to 60% of all hemophilia A patients, suffer from prolonged bleeding episodes upon trauma or in a perioperative setting.[3,4] Based on disease severity, treatment approaches are different.[5] Although minor bleeds in nsHA patients are often successfully treated with 1-Deamino-8-D-ArgininVasoPressin (desmopressin; DDAVP), a substantial number of bleeding events demand exogenous FVIII supply or a combination of both. In 2017, Batty and colleagues reported that 79% of 377 nsHA patients treated in 7 London hemophilia centers over the course of 2 years required at least 1 hemostatic treatment with FVIII concentrate.[6] In contrast, the majority of sHA patients receive intravenous FVIII replacement prophylactically already from an early age on.[5,7-9] Although prophylactic nonfactor replacement therapies have recently been approved (eg, emicizumab) or are currently in development, experience with respect to their use and associated risks is still limited. Furthermore, exogenous FVIII supplementation remains particularly important for treating breakthrough bleeds.[10]

The major complication associated with FVIII replacement therapy is the development of neutralizing anti-FVIII antibodies (FVIII inhibitors), rendering the treatment less effective or even ineffective. FVIII inhibitors, which are quantified by their potential to inhibit FVIII activity in the Bethesda and the Nijmegen-modified Bethesda assay,[11,12] are induced in approximately 25% to 30% of sHA and in up to 13% of nsHA patients.[13-15] While sHA patients develop neutralizing anti-FVIII antibodies predominantly within the first 50 exposure days (EDs), their risk of developing a new inhibitor at a later time point is less than 1%.[16-18] NsHA patients, on the other hand, seem to carry a lifelong risk of developing a FVIII inhibitor. Apart from endogenous risk factors such as *F8* gene mutations, patient age, high-dose FVIII treatment, high treatment frequency, surgical interventions, and administration of FVIII in the presence of danger signals are reported as hazards for inhibitor development in nsHA patients.[15,17,19-23] With the occurrence of FVIII inhibitors, morbidity, mortality rates, and cost of care increase substantially.[24-27]

Not only neutralizing but also nonneutralizing anti-FVIII antibodies can have a clinical impact for hemophilia A patients. Dazzi et al described an enhanced FVIII clearance in a hemophilia A patient with high-titer, nonneutralizing anti-FVIII antibodies.[28] In 2016, Hofbauer and colleagues demonstrated a significant correlation between nonneutralizing, FVIII-specific IgG in hemophilia A patients without FVIII inhibitors and FVIII half-life reduction.[29] Furthermore, significantly reduced FVIII in vivo recoveries were observed in noninhibitor hemophilia A patients with nonneutralizing anti-FVIII antibodies upon comparison with noninhibitor patients without anti-FVIII antibodies.[30]

By combining Bethesda assays with enzyme-linked immunosorbent assays (ELISAs) or Luminex-based approaches, anti-FVIII antibodies were detected in hemophilia A patients with inhibitors, but also in hemophilia A patients without inhibitors and in healthy donors.[31-35]

In 2013, Whelan et al identified distinct anti-FVIII antibody signatures in healthy donors and sHA patients with and without inhibitors.[31] In a follow-up study, Hofbauer and colleagues reported that antibodies in sHA-inhibitor patients bind to FVIII with an approximately 100-fold higher apparent affinity than antibodies detected in noninhibitor patients and healthy individuals.[32] In the recently published Hemophilia Inhibitor Previously Untreated Patient (PUP) study (HIPS), we demonstrated differential longitudinal antibody kinetics for PUPs with sHA developing a persistent FVIII inhibitor during their

first 50 EDs to a single source of recombinant human full-length FVIII and for study participants developing a transient or no inhibitor at all.[36] When comparing these distinct anti-FVIII antibody signatures and associated characteristics in inhibitor and noninhibitor PUPs after 50 EDs, similar FVIII-specific immunoglobulin (Ig) profiles and apparent affinity constants were observed as Hofbauer and colleagues described in their retrospective analysis of sHA patients with and without inhibitors.[32,36]

In order to gain a better understanding of the nature of nonneutralizing antibody responses against FVIII, we analyzed and compared anti-FVIII antibody signatures in 3 study cohorts: sHA patients as well as nsHA patients without FVIII inhibitors who had received FVIII replacement therapy at least once in the past, and healthy donors. Overall, our results indicate that Ig isotypes and IgG subclass profiles of nonneutralizing FVIII-specific antibodies are similar in hemophilia A patients and in healthy subjects. However, differences in prevalences, titers, and affinities of these nonneutralizing antibodies might be the outcome of a distinct evolution in healthy donors and hemophilia A patients.

Materials and methods

Human plasma samples

Blood samples from adult healthy donors and patients were collected and processed after each subject gave written informed consent in accordance with the approval of the respective local ethical committees (Ethics Commission of the Medical University of Vienna, Institutional Review Board [IRB] of the Medical University of Innsbruck, IRB of the University Hospital St. Poelten, IRB of the Upper Austrian Red Cross).

Citrated human plasma samples were stored at $\leq -65°C$ until analysis.

Study cohorts

Our study comprised 2 hemophilia A cohorts without FVIII inhibitors and 1 healthy donor cohort. Of note, all patients from both, sHA and nsHA, cohorts had a negative history of FVIII inhibitors, and all had received at least 1 treatment with exogenous FVIII at study inclusion.

Patients with nsHA without FVIII inhibitors. Samples of 81 patients with mild (n = 63 [77.8%]; baseline FVIII:C of 5% to 40%) and moderate (n = 18 [22.2%]; baseline FVIII:C of 1% to <5%) hemophilia A without FVIII inhibitors were collected at the Medical University of Vienna, the Medical University of Innsbruck, the University Hospital St. Pölten, and by the Upper Austrian Red Cross. For disease severity assessment, the lowest FVIII:C level ever measured was considered. Discrimination between mild, moderate, and sHA was performed as recommended by the Scientific and Standardization Committee (SSC) of the International Society on Thrombosis and Haemostasis (ISTH).[2] The median age of patients in the nsHA without inhibitor cohort at sample collection was 50.0 years (36.5-61.0 years [interquartile range (IQR)]). Blood samples were collected during a routine visit. Additional patient-specific clinical information is provided in the supplemental data (supplemental Table 1).

Table 1. Estimated prevalence of pooled FVIII-binding Igs

Cohort	Sample size (n)	Prevalence of pooled FVIII-binding Igs with titers ≥1:20 % (95% CI)	Prevalence of pooled FVIII-binding Igs with confirmed FVIII specificity* % (95% CI)
nsHA	81	67.9 (57.1-77.1)	40.7 (30.7-51.6)
sHA	39	53.8 (38.6-68.4)	25.6 (14.6-41.1)
healthy	90	37.8 (28.5-48.1)	12.2 (7.0-20.6)

CI, confidence interval (acc. Wilson EB40); FVIII, factor VIII; Ig, immunoglobulin; nsHA, nonsevere hemophilia A patients; sHA, severe hemophilia A patients; healthy, healthy donors.
*Only patients with FVIII-specific IgG1, IgG3, and IgA titers ≥1:40 and with FVIII-specific IgG2, IgG4, and IgM titers ≥1:80 were considered.

Patients with sHA without FVIII inhibitors. Thirty-nine samples of patients with sHA (baseline FVIII:C <1%) without FVIII inhibitors were collected at the Medical University of Vienna and the University Hospital St. Pölten. All patients had more than 100 EDs to FVIII replacement products. The median age of patients in the sHA without inhibitor cohort was 30.5 years (24.0-42.5 years [IQR]). Blood samples were collected during a routine visit. Additional patient-specific clinical information is provided in the online supplement (supplemental Table 2).

Healthy donors. Ninety healthy men, age-matched with the nsHA cohort, were recruited at the Medical University of Vienna. The median age of subjects in the healthy donor cohort (healthy) at sample collection was 47.0 years (31.5-56.0 years [IQR]). All healthy controls had a negative bleeding history.

FVIII activity measurement

FVIII activity was determined using both, a coagulometric and/or a chromogenic, method in the respective study center or in the central laboratory at the Medical University of Vienna.

Detection of FVIII-binding antibodies

Antibodies binding to FVIII and associated titer levels were identified using Ig isotype-/IgG subclass-specific direct binding ELISAs as described by Whelan et al.[31] Assay outlines are provided in the supplemental data.

Confirmation of FVIII specificity and assessment of apparent affinity of FVIII-binding antibodies

FVIII specificity and apparent affinity constants (K_A [M-1]) of FVIII-binding antibodies for IgG subclasses and IgA were determined as described by Whelan et al and Hofbauer et al.[31,32] Assay validation

activities were reported by Hofbauer and colleagues.[32] Technical assay details and the test principles are summarized in the supplemental data. In short, a competition-based ELISA approach was used to confirm FVIII specificity and apparent K_As for up to 2 distinct antibody affinity populations per sample for each IgG subclass and for IgA. Nonlinear regression modeling, according to Stevens and Bobrovnik, was employed to calculate the apparent K_As.[37,38] This model enabled K_A determination for up to 2 distinct antibody affinity populations per sample and IgG subclass/IgA isotype as well as identification of the dominant antibody population. For determination of the dominant antibody affinity population, the model considered for which of the 2 distinct antibody affinity populations per IgG subclass or IgA the nonlinear regression function fitted more accurately (≥50%).

Prevalences of anti-FVIII antibodies

Prevalences were calculated and compared between individual FVIII-binding Ig isotypes and IgG subclasses as well as pooled FVIII-binding Igs prior to and after FVIII specificity confirmation. For pooled FVIII-binding Ig prevalence calculations, all patients with FVIII binding (prior to FVIII specificity confirmation), respectively FVIII-specific (after FVIII specificity confirmation), antibodies of any isotype or IgG subclass (IgG1, IgG2, IgG3, IgG4, IgA, and/or IgM) were considered, as described by Abdi et al.[39]

Determination of hepatitis C virus (HCV) status

HCV status was assessed in 70 patients of the nsHA and 34 patients of the sHA cohort at the time of enrollment. Anti-HCV antibodies were detected in 25 nsHA (35.7%) and 20 sHA patients (58.8%) using an anti-HCV antibody chemiluminescent microparticle immunoassay. Five out of 25 anti-HCV antibody-positive patients in the nsHA cohort and 7 out of 20 anti-HCV antibody-positive

Table 2. Estimated prevalence of FVIII-binding Ig isotypes and IgG subclass antibodies with confirmed FVIII specificity

Cohort	Sample Size (n)	IgG1* (%)	IgG2† (%)	IgG3* (%)	IgG4† (%)	IgA* (%)	IgM† (%)
nsHA	81	**32.1**	0.0	**1.2**	1.2	**21.0**	0.0
sHA	39	**17.9**	0.0	**5.1**	0.0	**10.3**	0.0
healthy	90	**5.6**	0.0	**2.2**	0.0	**5.6**	0.0

See Table 1 for definitions.
Estimated prevalences of FVIII-binding IgG1, IgG3, and IgA antibodies with proven FVIII specificity are highlighted in bold.
*Only patients with FVIII-specific IgG1, IgG3, and IgA titers ≥1:40 were considered.
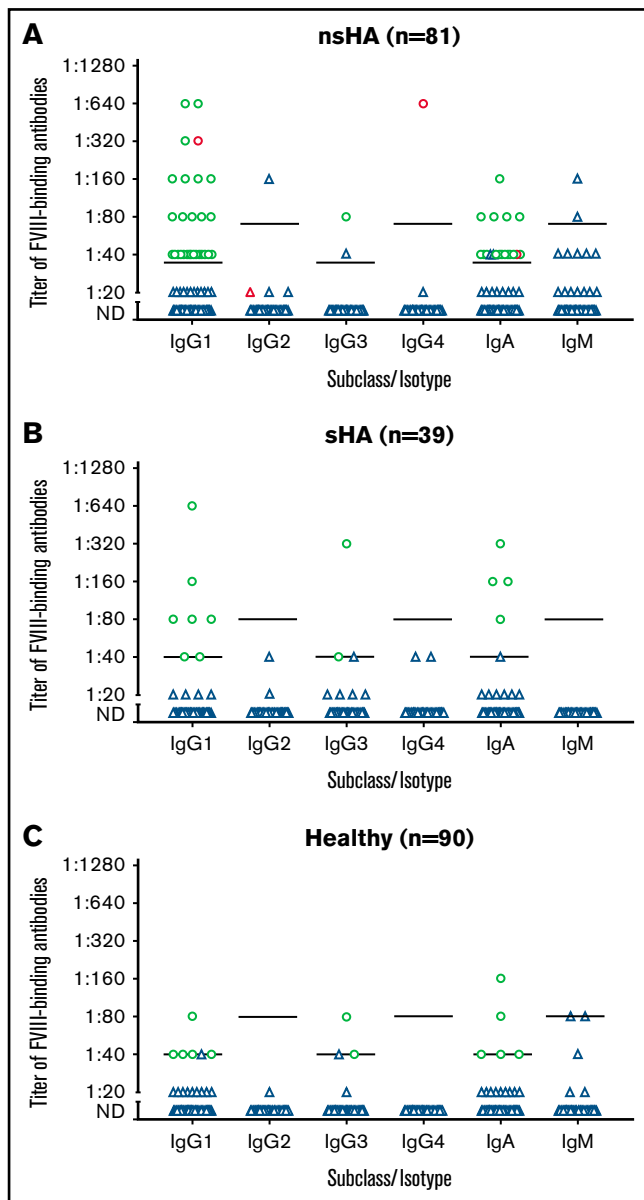†Only patients with FVIII-specific IgG2, IgG4, and IgM titers ≥1:80 were considered.

Figure 1. Titers of FVIII-binding antibodies assessed for individual Ig isotypes and IgG subclasses. Presented are the results for FVIII-binding antibodies for all plasma samples. The screening cutoff of the methods to differentiate positive from negative samples is at a titer of 1:20. The lines at a titer of 1:40 respectively 1:80 represent the lower limit of quantification (LLOQ) for confirmed FVIII specificity of the different Ig isotype/IgG subclass antibodies based on successful FVIII-competition.[31,32] Circles depict positive results for FVIII-binding antibodies with confirmed FVIII specificity; triangles represent results below the FVIII-specific LLOQ. (A) Thirty-three of 81 patients with nsHA, (B) 10 of 39 patients with sHA, and (C) 11 of 90 healthy donors contained FVIII-binding antibodies with confirmed FVIII specificity. Titers highlighted in red belong to patient 58 (nsHA). FVIII, factor VIII; Ig, immunoglobulin; ND, not detectable.

patients in the sHA cohort had an active HCV infection at sample collection, which was confirmed by quantitative real-time polymerase chain reaction (qRT-PCR). The HCV status was not assessed in the healthy cohort, as only healthy men without active liver disease were

included in this study. Anti-HCV antibody and qRT-PCR analytics were performed in the central laboratory at the Medical University of Vienna.

Evaluation and statistical analyses

The prevalence of FVIII-binding antibodies was estimated along with 95% confidence intervals (CIs) calculated according to Wilson.[40] For cohort comparison of FVIII-specific antibody prevalences, χ-squared (X^2) and Fisher's exact tests (FET) were performed using IBM SPSS Statistics Version 23. In order to analyze and compare in-depth antibody characteristics, medians and IQRs for titers and dominant K_As of IgG1 and IgA antibodies with confirmed FVIII specificity were calculated, and cohorts were compared with Mann-Whitney U tests using GraphPad Prism 8.4.3. In addition, the absolute numbers of patients and healthy donors with FVIII-specific Ig titers and associated K_As were considered for cohort comparison. In case cohort members with elevated titers and/or K_As were reported, these presented with Ig titers or associated K_As higher than the largest Ig titer or K_A value observed in the comparison cohort. For all analyses, a P value ≤.05 was considered statistically significant. Means, medians, and IQRs were used to describe data.

Results

Prevalence and specificity of FVIII-binding antibodies

We investigated plasma samples from 81 nsHA patients without inhibitors, 39 sHA patients without inhibitors, and 90 healthy donors (healthy) for the presence of FVIII-binding antibodies. A 67.9% (55 of 81) prevalence of pooled FVIII-binding Igs with titers ≥1:20 was identified in nsHA patients, 53.8% (21 of 39) in sHA patients, and 37.8% (34 of 90) in healthy donors (Table 1). The prevalence of pooled FVIII-binding Igs with titers ≥1:40 (IgG1, IgG3, and IgA) respectively ≥1:80 (IgG2 and IgG4) and confirmed FVIII-specificity was 40.7% (33 of 81) in the nsHA, 25.6% (10 of 39) in the sHA, and 12.2% (11 of 90) in the healthy cohort (Table 1).

Ig isotype/IgG subclass distribution and associated prevalence of FVIII-binding antibodies with confirmed FVIII specificity

The isotype and IgG subclass profiles of FVIII-binding antibodies with titers ≥1:40 (IgG1, IgG3, and IgA) respectively ≥1:80 (IgG2 and IgG4) and confirmed FVIII specificity in hemophilia A patients were comparable to those of healthy donors (Figure 1). FVIII-specific IgG1 and IgA were identified as predominant IgG subclass/Ig isotypes in all study cohorts. FVIII-specific IgG3 was observed in only a few study participants. Apart from 1 patient within the nsHA cohort (patient nsHA 37), who is described in detail in the supplemental data, FVIII-specific IgG2 and IgG4 were absent in all other patients and healthy individuals (Table 2; Figure 1).

Statistical comparison of pooled FVIII-binding Ig, IgG1, and IgA antibodies

The prevalences of pooled FVIII-binding Igs, IgG1, and IgA antibodies with confirmed FVIII specificity were statistically compared using FET and X^2 tests. The prevalences of pooled FVIII-specific Igs ($p_X^2 = 0.000$), IgG1 ($p_X^2 = 0.000$), and IgA ($p_X^2 = 0.003$) antibodies were significantly higher in the nsHA than in the healthy

Table 3. Prevalence comparisons of FVIII-binding Ig isotypes and IgG subclass antibodies with confirmed FVIII specificity

IgG subclass/Ig isotype	Cohort comparison (patients with FVIII-specific Igs)	Statistical test	P value
Pooled Igs	healthy (11) vs nsHA (33)	X	**.000*****
	healthy (11) vs sHA (10)	X	.058
	nsHA (33) vs sHA (10)	X	.106
IgG1	healthy (5) vs nsHA (26)	X	**.000*****
	healthy (5) vs sHA (7)	FET	**.043***
	nsHA (26) vs sHA (7)	X	.104
IgA	healthy (5) vs nsHA (17)	X	**.003****
	healthy (5) vs sHA (4)	FET	.452
	nsHA (17) vs sHA (4)	X^2	.147

See Table 1 for definitions; X^2, χ-squared test; FET, Fisher's exact test; P value, level of significance ($P \leq .050$).
Significant P values are indicated in bold and marked with asterisk(s): *$P \leq .050$; **$P \leq .010$; ***$P \leq .001$.

cohort. Furthermore, FVIII-specific IgG1 ($p_{FET} = 0.043$) antibodies occurred more frequently in sHA patients than in healthy donors, while there was also a trend for an increased prevalence of pooled FVIII-specific Igs ($p_X^2 = 0.058$). No difference in the prevalences of pooled FVIII-specific Igs, IgG1, and IgA antibodies was observed when comparing nsHA and sHA patients (Table 3). To sum up, differences in the prevalences of nonneutralizing antibodies with confirmed FVIII-specificity were detected between hemophilia A patients and healthy donors, but not between the 2 hemophilia A cohorts with different disease severities.

In-depth characterization of FVIII-binding IgG1 and IgA antibodies with confirmed FVIII specificity

In addition to the differences in the prevalences of FVIII-specific Ig isotype and IgG subclass antibodies, the question arose whether the anti-FVIII antibody response also differed qualitatively (with respect to antibody titers and apparent K_As) between the study cohorts. By comparing FVIII-binding antibody titers with confirmed FVIII specificity, 8 out of 26 nsHA and 2 out of 7 sHA patients presented with higher IgG1 titers than healthy donors (Figures 1 and 4). Upon evaluation of apparent K_As by nonlinear regression modeling,[32] we observed that most samples with FVIII-specific antibodies contained 2 IgG1 and/or IgA antibody populations with distinct K_As (Figure 2). The K_As of the dominant antibody affinity populations are depicted in Figure 3.

For statistical comparison of FVIII-specific IgG1 and IgA titers as well as their dominant K_As, medians and IQRs were calculated, and Mann-Whitney U tests were performed. FVIII-specific IgG1 titers and associated dominant K_As were not significantly different between the 3 study cohorts (Figure 4). Nevertheless, a trend toward increased dominant K_As of FVIII-specific IgG1 antibodies was observed between healthy donors and nsHA patients ($P = .082$). Furthermore, 3 out of 7 sHA patients with FVIII-specific IgG1 presented with higher dominant K_As than healthy donors (Figure 4A). Differences in FVIII-specific IgA titers and associated dominant K_As were not seen between the nsHA and the healthy donor cohort. There was a trend for a difference between FVIII-specific IgA titers of sHA patients and healthy donors, but the apparent difference was not statistically significant ($P = .071$). FVIII-specific IgA titers of nsHA and sHA patients ($P = .003$) were significantly different. In addition, dominant K_As of FVIII-specific IgAs showed significant differences between the sHA

and the healthy ($P = .032$) as well as the nsHA and the sHA cohort ($P = .039$) (Figure 4B).

Comparison of FVIII-binding IgG1 and IgA antibody characteristics with confirmed FVIII specificity in hemophilia A patients stratified by HCV antibody status

For a considerable number of hemophilia A patients, the anti-HCV antibody status was known. Therefore, we asked if prevalences or characteristics of FVIII-specific antibodies were associated with a past or active HCV infection. First, changes in prevalences of pooled FVIII-binding Igs, IgG1, or IgA antibodies with confirmed FVIII specificity were investigated, taking the anti-HCV antibody status into account. For this purpose, hemophilia A patients stratified by their anti-HCV antibody status as well as anti-HCV antibody positive and negative patients stratified by their cohort affiliation were statistically compared using FET or X^2 tests. Only nsHA patients with anti-HCV antibodies showed a significantly increased prevalence of pooled FVIII-specific Igs upon comparison with anti-HCV antibody-negative patients of the same study cohort ($p_X^2 = 0.019$) (supplemental Table 4). Furthermore, the FVIII-specific IgG1 and IgA antibody titers and associated dominant K_As were statistically compared by Mann-Whitney U tests between patients with an active or a past HCV infection and healthy donors (Figure 5A-B). Five of 15 hemophilia A patients without anti-HCV antibodies showed increased FVIII-specific IgG1 titers, while 4 out of 15 hemophilia A patients without anti-HCV antibodies presented with increased dominant IgG1-K_As as opposed to healthy donors (Figure 5B). With respect to FVIII-specific IgA, a significant difference ($P = .036$) in titers was observed between HCV antibody-positive nsHA and sHA patients (Figure 5C). In contrast, FVIII-specific IgA antibodies were completely absent in anti-HCV antibody-negative patients of the sHA cohort. Moreover, no difference in FVIII-specific IgA titers and associated dominant K_As between anti-HCV antibody-negative nsHA patients and healthy donors was detected (Figure 5D).

Discussion

The antibody response against FVIII in hemophilia A patients has been investigated for decades. Although research activities in the past predominantly focused on neutralizing anti-FVIII antibodies in
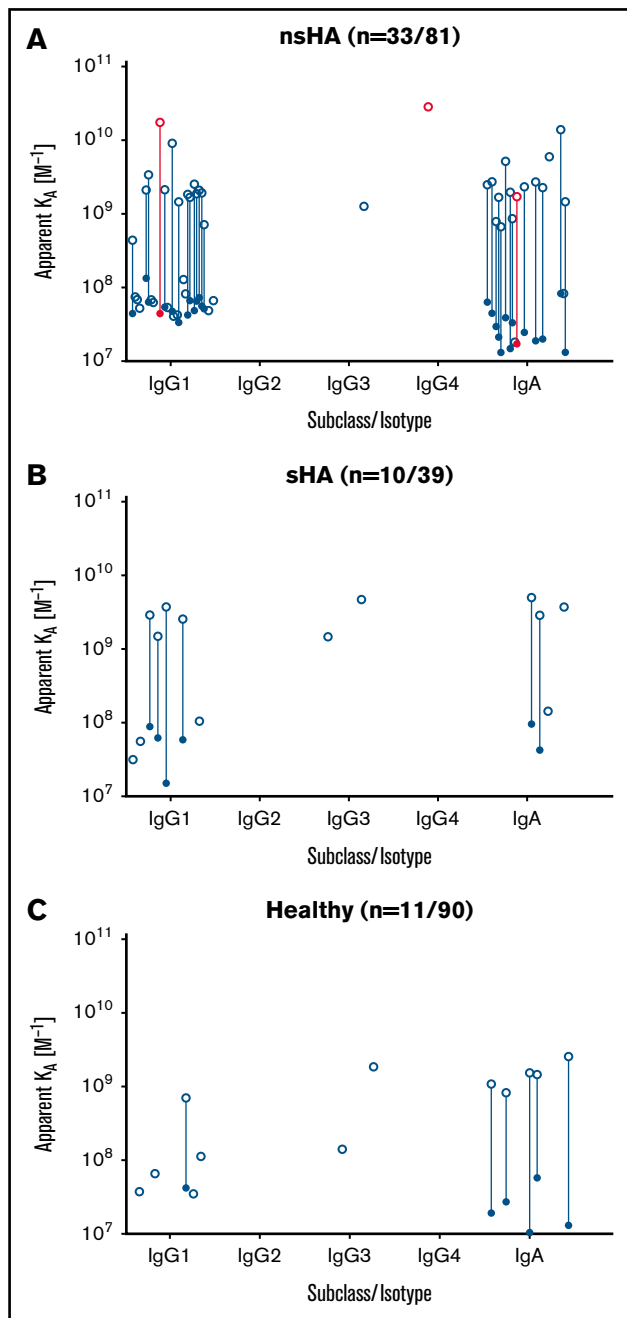
Figure 2. Apparent affinity constants of FVIII-binding antibodies with confirmed FVIII specificity assessed for individual Ig isotypes and IgG subclasses. Presented are the apparent affinity constants (K_A [M^{-1}]) of FVIII-binding antibodies with confirmed FVIII specificity found in the different study cohorts presented in Figure 1. All samples containing FVIII-binding antibodies with a titer of 1:40 (IgG1, IgG3, and IgA) respectively 1:80 (IgG2 and IgG4), for which FVIII-specificity was confirmed, were included in the analysis. (A) Patients with nsHA. (B) Patients with sHA. (C) Healthy donors. Some samples in each study cohort contained 2 populations of FVIII-binding antibodies with confirmed FVIII specificity with distinct K_As. These 2 populations present in the same sample are indicated by an open and a closed circle connected by a straight line. K_As and connecting lines highlighted in red belong to patient 58 (nsHA).

sHA patients, FVIII-specific antibodies were also detected in severe and nonsevere patients without inhibitors and in healthy donors.[31-35] In order to evaluate and compare these nonneutralizing antibody signatures, we characterized FVIII-specific antibody profiles in previously treated nsHA and sHA patients without inhibitors and healthy donors in a retrospective study.

In our study, we observed increased prevalences of pooled FVIII-specific Igs (including IgG1, IgG2, IgG3, IgG4, IgA, and/or IgM) not only in previously treated sHA patients, but also in previously treated nsHA patients compared with healthy donors. This observation extends the findings recently published by Abdi and colleagues in a meta-analysis. In their manuscript, the authors reported on a pooled nonneutralizing anti-FVIII antibody prevalence of 25% (95% CI, 16% to 38%) in previously treated hemophilia A patients. For the most part, the study populations investigated by Abdi et al were composed of noninhibitor patients with sHA. IgG1 was identified as the predominant FVIII-specific antibody isotype.[39] In all of our study cohorts, we identified comparable FVIII-specific Ig isotype/IgG subclass-profiles. In addition to IgG1, IgA antibodies with confirmed FVIII specificity were detected in some of the nsHA and sHA patients as well as in some healthy donors.

Only 1 nsHA patient, patient nsHA 37 (supplemental Table 3), presented with an exceptional FVIII-specific antibody signature. Besides low titer, low-affinity IgA, the patient had high titer, high-affinity IgG1, and IgG4 antibodies with confirmed FVIII specificity, which were previously described as typical characteristics for inhibitor patients.[31,32] Up to sample collection, the patient had never presented with a FVIII inhibitor. One reason for this unique antibody fingerprint in the absence of any inhibitor could be the epitope profile of his nonneutralizing antibodies. While neutralizing antibodies have been shown to target functional FVIII epitopes, nonneutralizing antibodies were revealed to interact predominantly with nonfunctional epitopes.[28,41-48] Another reason might be that this patient is already developing a FVIII inhibitor. Hofbauer and colleagues reported on the development of neutralizing antibodies in 2 noninhibitor patients with sHA who presented with FVIII-specific IgG1 and/or IgG4 antibodies up to 543 days before an inhibitor was detected.[29,32] Together with our observations, these findings highlight that intense monitoring and longitudinal antibody profiling, particularly for noninhibitor hemophilia A patients with such unique nonneutralizing anti-FVIII antibody signatures, should be considered.

In-depth characterization of FVIII-specific antibodies demonstrated differences in our study cohorts with respect to Ig isotype/IgG subclass: (1) prevalence, (2) titers, and (3) associated apparent K_As.

Pooled FVIII-binding Igs and IgG1 antibodies with confirmed FVIII specificity occurred with a higher prevalence in nsHA and sHA patients than in the healthy donor cohort. On the other hand, IgA prevalence was significantly increased in the nsHA, but not in the sHA cohort compared with healthy donors. Prevalences in pooled FVIII-specific Igs, IgG1, and IgA were not different between nsHA and sHA patients.

A considerable number of hemophilia A patients presented with increased titers and associated dominant K_As when compared with healthy donors. In contrast, titers and K_As of FVIII-specific IgA were
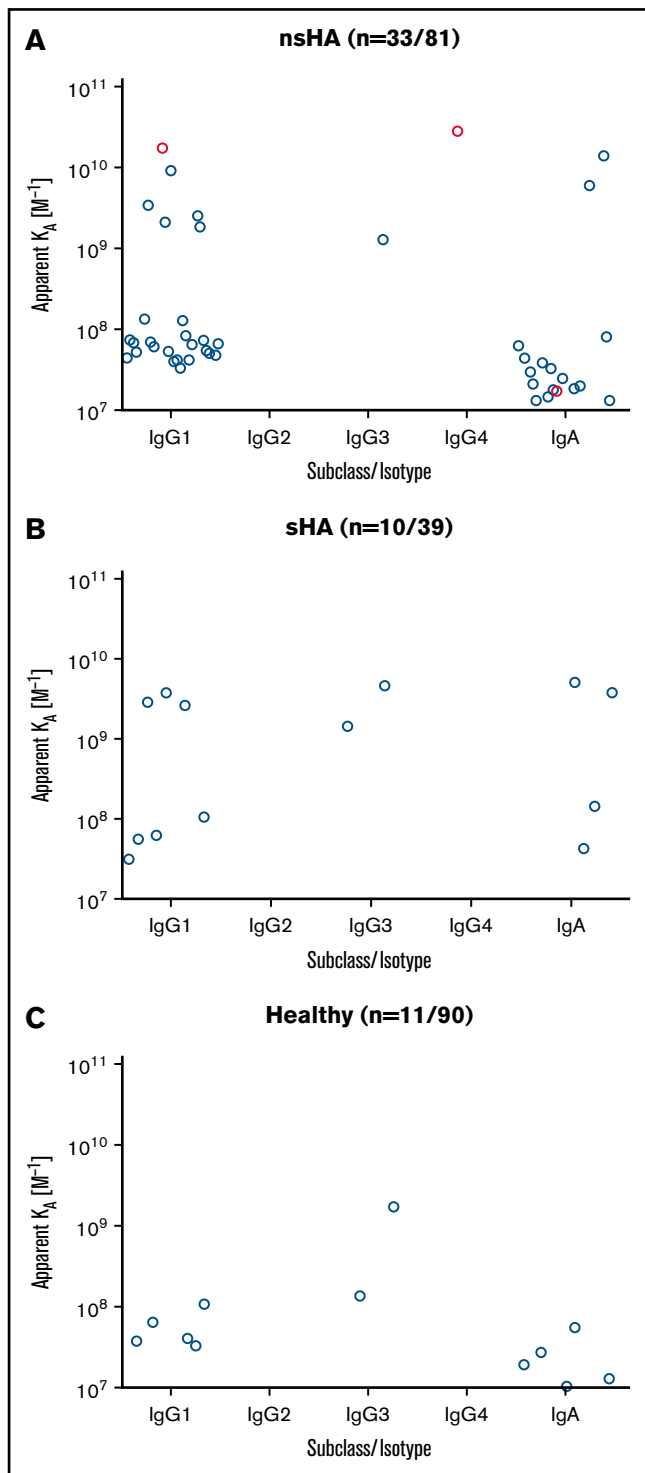
Figure 3. Affinity constants of the dominant antibody affinity populations of FVIII-binding antibodies with confirmed FVIII specificity assessed for individual Ig isotypes and IgG subclasses. Presented are the apparent affinity constants of the dominant antibody affinity populations (K_A [M^{-1}]) of FVIII-binding antibodies with confirmed FVIII specificity, as described in the Materials and methods section. (A) Patients with nsHA. (B) Patients with sHA. (C) Healthy donors. K_As highlighted in red belong to patient 58 (nsHA).

only increased in sHA patients. A Mann-Whitney U test based comparison of the medians of both IgA parameters confirmed a statistically significant difference between the nsHA and the sHA cohort.

A large proportion of the hemophilia A patients received plasma-derived FVIII concentrates before efficient virus inactivation steps were implemented in industrial fractionation processes. Therefore, many of them were anti-HCV antibody-positive, indicating an active or past infection with HCV. FVIII-specific antibody characteristics were reanalyzed after stratification for the HCV antibody status. The general observation of increased FVIII-specific IgG1 titers and associated dominant K_As in both hemophilia A cohorts, as opposed to healthy donors, was not affected by comparing anti-HCV antibody-negative participants only. Interestingly, FVIII-specific IgA titers and associated dominant K_As were identical in healthy donors and nsHA patients without anti-HCV antibodies. On the other hand, FVIII-specific IgA antibodies were completely absent in the anti-HCV antibody-negative fraction of the sHA cohort. The same observation was recently reported in sHA PUPs that either did not develop a FVIII inhibitor or developed only a transient inhibitor throughout their first 50 EDs to recombinant, human full-length FVIII (see subgroups 2 and 3 in Reipert et al).[36] In sHA patients with anti-HCV antibodies, FVIII-specific IgA antibodies were not only present but their titers were significantly increased compared with nsHA patients. Based on these results, we hypothesize that an HCV infection might foster FVIII-specific IgA antibody development.

The question arises if there might be shared underlying immune mechanisms linking the development of natural FVIII-specific autoantibodies in healthy donors to similar nonneutralizing, anti-FVIII antibody fingerprints in hemophilia A patients. In our study, comparable Ig isotype-/IgG subclass distributions of FVIII-specific antibodies, predominantly of the IgG1 subclass and the IgA isotype, were identified in all cohorts. Recently, Reipert et al reported that the development of FVIII-specific IgG1, but not of any other isotype respectively IgG subclass with confirmed FVIII specificity, was observed in sHA PUPs, who remained FVIII inhibitor-negative throughout their first 50 EDs to a recombinant, human full-length FVIII.[36]

Cohen hypothesized that the natural autoimmune antibody repertoire is important for the maintenance of immune homeostasis and termed it immunologic homunculus.[49] One lymphoid organ involved in autoantibody formation is the spleen.[50,51] A defined area within the extrafollicular space of the spleen, the marginal zone (MZ), is populated by macrophages, B cells, dendritic cells, and neutrophils, which were described to closely interact in the regulation of T-cell-independent antibody responses.[52-58] MZ B cells are not only able to differentiate into plasmablasts or memory B cells but were observed to perform limited class switch recombination resulting in the expression of IgG respectively IgA antibody isotypes. Furthermore, MZ B cells were shown to undergo limited somatic hypermutations, which are essential for antibody affinity maturation.[56,58-65] Together with our data, these findings would support the hypothesis that the formation of nonpathogenic autoantibodies against FVIII in healthy donors and the formation of nonneutralizing anti-FVIII antibodies in hemophilia A patients might share underlying immune mechanisms. Differences in prevalences, titers, and affinities of the cohorts' anti-FVIII antibody signatures might be the result of a distinct evolution. Therefore, we hypothesize that FVIII-specific
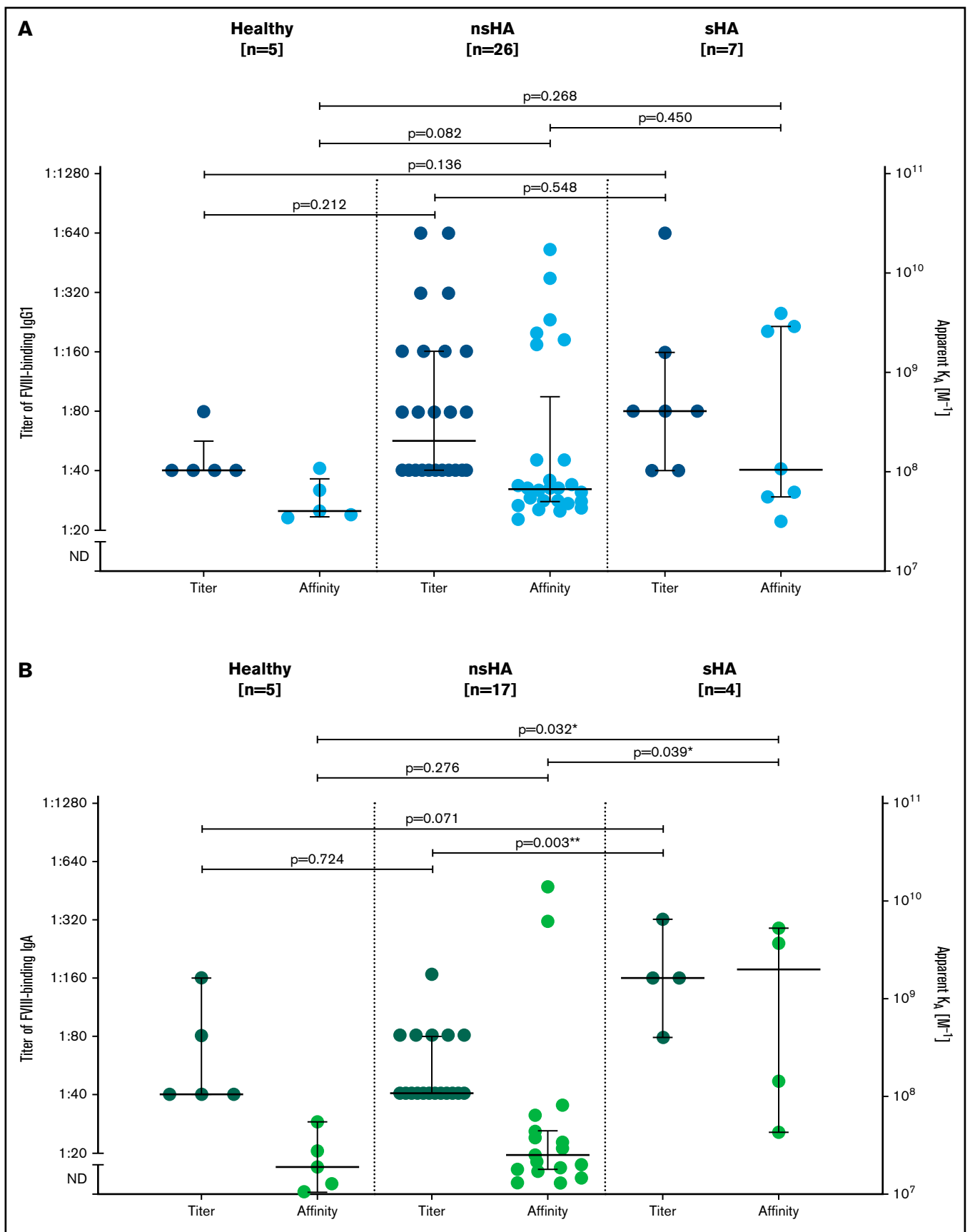
Figure 4. Study cohort comparison of titers and dominant apparent affinity constants for FVIII-binding IgG1 and IgA antibodies with confirmed FVIII specificity by Mann-Whitney *U* tests. Presented are scatter dot blots of individual IgG1 (A) and IgA (B) titer values (dark blue and dark green dots) as well as associated dominant apparent affinity constants (K_A [M^{-1}]; light blue and light green dots) of FVIII-binding antibodies with confirmed FVIII specificity. Medians are indicated by bold horizontal lines. The vertical bars represent corresponding IQRs. Mann-Whitney *U* test-based cohort comparisons were performed. *P* values for group comparisons are indicated. Statistically significant *P* values are highlighted in bold and marked with asterisk(s): *$P \leq$ .050; **$P \leq$ .010.
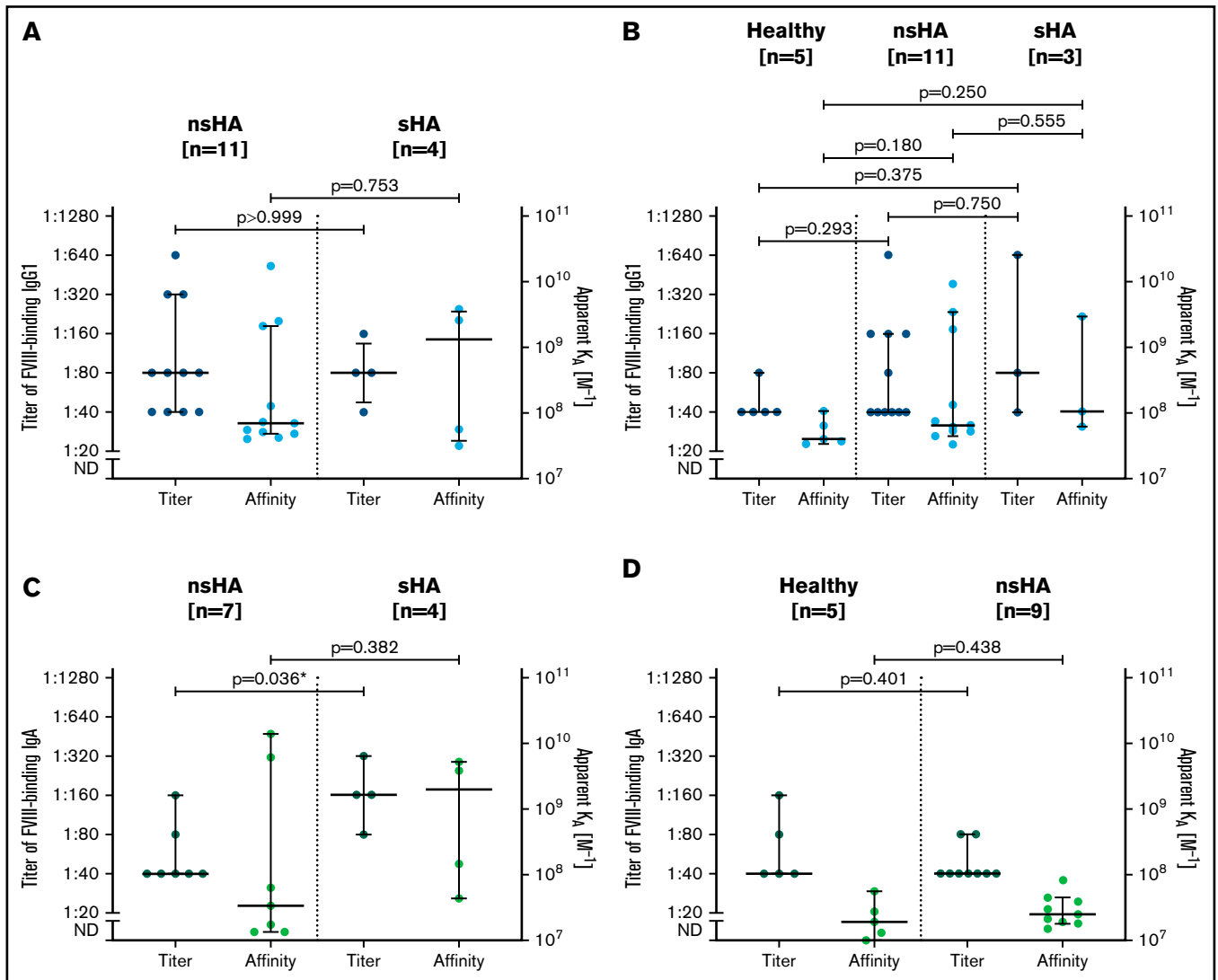
Figure 5. Titers and dominant apparent affinity constants of FVIII-specific IgG1 and IgA antibodies in hemophilia A patients stratified by HCV antibody status and in healthy donors. Presented are scatter dot blots for individual titers and dominant apparent affinity constants (K_{AS} [M^{-1}]) for FVIII-specific IgG1 (A-B; titers: dark blue; affinities: light blue) and FVIII-specific IgA (C-D; titers: dark green; affinities: light green) in patients with (A,C) or without (B,D) anti-HCV antibodies and in healthy donors (B,D). Bold horizontal lines indicate the medians of titers and dominant K_{AS}. Vertical bars indicate corresponding IQRs. *P* values for group comparisons by Mann-Whitney *U* tests are indicated. Statistically significant *P* values are highlighted in bold: **p = 0.036** and marked with an asterisk: *$P \leq .050$.

antibodies detected in healthy donors might be natural autoantibodies, while the nonneutralizing anti-FVIII antibody response of sHA patients might be dominated by induced antibodies against exogenous FVIII. Consequently, we would conclude that the FVIII-specific antibody repertoire observed in nsHA patients might be composed of a mix of natural autoantibodies and induced antibodies to exogenous FVIII. Similar considerations regarding natural anti-FVIII autoantibodies and induced antibodies to exogenous FVIII were raised by Lacroix-Desmazes et al.[66]

Another question that arises relates to the opposing FVIII-specific IgA profiles when stratifying nsHA and sHA patients for their anti-HCV antibody status. In the past, several research groups reported on both anti and proinflammatory properties for IgA.[67-70] The underlying phenomenon has been linked with differential binding properties of IgA to the Fc-α receptor I (FcαRI, CD89). Whereas binding

of monomeric IgA to FcαRI expressed on myeloid cells (eg, macrophages, neutrophils, and dendritic cells) inhibits proinflammatory responses, FcαRI crosslinking caused by multimeric IgA (eg, IgA immune complexes), triggers proinflammatory cell activation.[71-73]

In summary, we present the first comparative Ig isotype-/IgG subclass-specific, in-depth characterization of nonneutralizing antibodies with confirmed FVIII-specificity in nsHA and sHA patients and in healthy donors. We believe that the similarity of nonneutralizing anti-FVIII antibody signatures found in the different study cohorts might indicate shared underlying immune mechanisms that could be linked to T-cell-independent antibody development (eg, driven by B cells in the MZ of the spleen). FVIII-specific IgA antibodies might counterbalance this effect due to their anti-inflammatory properties, whereas they might become proinflammatory drivers in patients with an active or past HCV infection. We suggest designing prospective clinical trials in previously

treated noninhibitor hemophilia A patients of all severity levels to further explain the immunological mechanisms and epitope signatures underlying the formation of nonneutralizing antibodies against FVIII.

Authorship

Contribution: H.S. designed the immunological part of the study, performed antibody analytics, created statistical analyses and figures, reviewed and interpreted the immunological data, and wrote the manuscript; J.R. collected and provided plasma samples of patients, cared for study participants, reviewed and interpreted the immunological data, and reviewed and edited the manuscript; C.J.H. performed antibody analytics, reviewed and interpreted the immunological data, and reviewed and edited the manuscript; V.B. created figures, reviewed and interpreted the immunological data, and reviewed and edited the manuscript; P.A. coordinated the research project and reviewed and edited the manuscript; K.Z. collected and provided patient samples, cared for study participants, and reviewed and edited the manuscript; C.F. collected and provided patient samples, cared for study participants, and reviewed and edited the manuscript; G.S. collected and provided patient samples and reviewed and edited the manuscript; C.A. collected and provided plasma samples of healthy donors and patients, cared for study participants, reviewed and interpreted the immunological data, and reviewed and edited the manuscript; B.M.R. designed the immunological part of the study, reviewed and interpreted the immunological data, and wrote and reviewed the manuscript; and I.P. designed the clinical part of the study, collected and provided plasma samples of healthy donors and patients, cared for study participants, reviewed and interpreted the immunological data, and wrote and reviewed the manuscript.

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