Acid Treatment of FVIII-Containing Plasma Samples Unmasks a Broad Spectrum of FVIII-Specific Antibodies in ELISA

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Abstract

During routine treatment, plasma samples of patients with hemophilia A or acquired hemophilia A are frequently analyzed for the presence of FVIII-specific antibodies. While only inhibitory antibodies can be detected by the Bethesda assay, inhibitory and non-inhibitory antibodies can be detected by ELISA. However, plasma samples of patients frequently contain endogenous or substituted FVIII, hence interfering with both types of analyses. One option for the inactivation of FVIII is heat denaturation, which unfortunately has been shown to lead to high background signals complicating the discrimination of negative and positive plasma samples. In the current study, we developed a method of acid denaturation for FVIII-containing plasma samples that can help identify samples containing FVIII-specific antibodies and compared the effects of heat and acid denaturation on the detection of FVIII–antibody interactions in a monoclonal setting. The aim of our study was to establish an analysis that allows safer treatment decisions in the context of tolerance to FVIII.

Keywords

- hemophilia A/B
- factor VIII inhibitors
- inherited coagulation disorders

Introduction

Reduced amounts of FVIII or nonfunctional FVIII in plasma result in hemophilia A (HA). The gene of FVIII resides on the X chromosome; thus, HA almost exclusively affects males, having an incidence of 1 in 5,000.[1] HA patients receive replacement therapy for prophylaxis or treatment of bleeds to reestablish hemostasis. However, up to 30% of the patients treated with FVIII develop inhibitory antibodies specific for FVIII.[2] Furthermore, acquired HA is a rare autoimmune disorder with an autoimmune response against endogenous FVIII. Hemophilia patients with inhibitory antibodies can receive prophylactic treatment with non-replacement therapy but need bypassing agents for the treatment of bleeds. Thus, immune-tolerance induction (ITI) is applied in patients with congenital HA and inhibitors to allow effective treatment with FVIII again. Here, frequent injections of FVIII aim to induce tolerance toward this protein. Inhibitors are routinely diagnosed by the Bethesda assay. In addition, inhibitory and non-inhibitory antibodies can also be quantified by ELISA. However, patients receiving replacement therapy especially undergoing ITI can have residual amounts of FVIII in plasma at the time point of blood draw, which can interfere with the detection of FVIII-specific antibodies and may result in false-negative results. These may negatively influence treatment decisions during ITI and also when patients change from replacement to non-replacement therapy, only receiving FVIII on occasion. Previously, it was shown that a restricted incubation of plasma samples at 56 °C results in the destruction of FVIII activity and can help detect FVIII-specific antibodies in plasma samples containing FVIII with the help of the Bethesda assay as well as fluorescence immunoassays and ELISAs.[3–5] However, it has been known for decades that heat treatment of plasma

received
February 12, 2024
accepted after revision
May 15, 2024

Georg Thieme Verlag KG,
Rüdigerstraße 14,
70469 Stuttgart, Germany

DOI https://doi.org/10.1055/a-2329-1781.
ISSN 0720-9355.

samples can lead to false-positive signals in ELISA, as, for example, shown for the detection of antibodies binding to HIV or phospholipids, and recently as well for FVIII.[6–8] Furthermore, heat inactivation abolishes FVIII activity and therefore changes the read out in Bethesda assays, but does not fully destroy the FVIII antigen.[5] Though, especially antibodies that cannot be detected in the Bethesda assay are either masked by FVIII or of low affinity but often subsequently develop into inhibitory antibodies.[9,10] This observation produces a need to develop ELISA procedures that can also detect low amounts of FVIII-specific antibodies that might be masked by residual FVIII in plasma samples.

In this study, we first compared the influence of heat and acid treatment on FVIII-specific ELISA signals of different plasma sample controls and continued to analyze the feasibility of using acid denaturation of FVIII to identify plasma samples containing FVIII-specific antibodies in the presence of FVIII in plasma.

Methods

Plasma Samples and Monoclonal Antibodies

Plasma samples used in this study were residuals from citrated plasma from HA patients taken as part of routine care to detect inhibitors. Bethesda unit (BU) values for inhibitor-positive samples are given in the "Result" section. High-positive control (HPC) and low-positive control (LPC) plasmas were obtained from Gen-Probe (Wiesbaden, Germany). FVIII-negative plasma was obtained from Siemens (Marburg, Germany). Murine monoclonal GMA antibodies were purchased from Green Mountain Antibodies (Burlington, Vermont, United States). Human monoclonal BO2C11 was obtained from the Centre for Molecular and Vascular Biology (Leuven, Belgium).

FVIII–Antibody ELISA

Dilutions of plasma samples or monoclonal antibodies were incubated on microtiter plates coated with 1 IU of recombinant full-length FVIII (Kovaltry; Bayer Healthcare AG, Leverkusen, Germany). Plasma was diluted 1:10, except for the HPC sample, which was diluted 1:50. Samples were either left untreated, heat-denatured for 50 minutes at 56 °C, or treated with 0.1 M glycine-HCl, pH 2.5 for 30 minutes, followed by neutralization with 1 M Tris buffer. Volumes of samples were adjusted with PBS. In case of competition experiments, FVIII was added to the samples at a concentration of 12 IU/mL. Bound antibodies were detected with a horseradish-peroxidase–labeled anti-human IgG (Fc) antibody (Merck KGaA, Darmstadt, Germany) or anti-murine IgG (H + L) antibody (Jackson ImmunoResearch, Ely, United Kingdom). ELISAs were developed with o-phenylenediamine for 6 minutes before stopping the reaction with H_2SO_4 (0.5 M). Absorption was measured at 492 nm (650 nm as reference).

Bethesda Assay

FVIII inhibitor titers were measured with the Bethesda assay.[11] The cutoff for a positive inhibitor titer was set at 0.6 BU/mL.

Data Analysis

ELISA results were plotted and analyzed using statistical Software R (version 4.2.0) or GraphPad Prism (version 9.5.0). As neither the initial dataset nor any transformations showed a Gaussian distribution, as tested by Shapiro–Wilk test, nonparametric methods were used in the analysis. Friedman test with post hoc group comparison and Holm correction was used to compare the groups. The linear correlation analysis was performed using the Spearman method. All tests were two sided and p-values <0.05 were considered statistically significant.

Results

Feasibility of Heat and Acid Treatments for Unmasking of FVIII-Specific Antibodies Present in Plasma

In a previous study we reported that heat denaturation of plasma samples may result in false-positive results.[8] We therefore first compared the influence of heat and acid treatment on FVIII-specific ELISA signals of plasma samples (►Fig. 1A). While ELISA signals of acid-treated and untreated samples were identical, heat treatment resulted in higher signals for all analyzed samples, including FVIII-negative plasma and a normal plasma (NP) pool, both not containing FVIII-specific antibodies. Thus, acid treatment did not increase the signal in the FVIII-specific ELISA for antibody-positive samples, but it also did not result in false-positive results. Of note, our inhibitor-positive HPC and LPC plasma samples did not contain FVIII. Thus, an increase in ELISA signal was not expected. Because of the observed stable signal following acid treatment and as heat treatment did result in false-positive results, we subsequently concentrated on the analysis of acid treatment and next analyzed the possibility of using acid treatment for the detection of FVIII-specific antibodies in inhibitor-positive plasma samples containing FVIII (►Fig. 1B). Addition of FVIII to those initially FVIII-deficient and inhibitor-positive plasma samples diminished ELISA signals for all samples. For patients 1 and 2, signals could be partly restored by acid treatment. Interestingly, also after acid treatment, signals for patient 3 and the monoclonal antibody BO2C11 remained low.

Thus, our data show that acid treatment of plasma samples allows the detection of some FVIII-specific antibodies in the presence of soluble FVIII which were otherwise missed. However, this treatment does not always help decipher the presence of FVIII-specific antibodies.

Due to the promising results of patients 1 and 2, we continued to utilize acid denaturation for the analysis of ITI samples, which are known to sometimes contain FVIII due to limited wash-out phases during ITI treatment (►Fig. 1C). Analyzed samples were inhibitor negative, except for "ITI 1–tp3," "ST 2," and "emicizumab." Acid treatment did not influence the binding of FVIII-specific antibodies within our control plasma (HPC), plasma of patients treated with emicizumab or under substitution therapy (ST), as well as for most ITI samples. However, for ITI patient 1, we saw an increase in FVIII-specific ELISA signals by acid treatment for 9

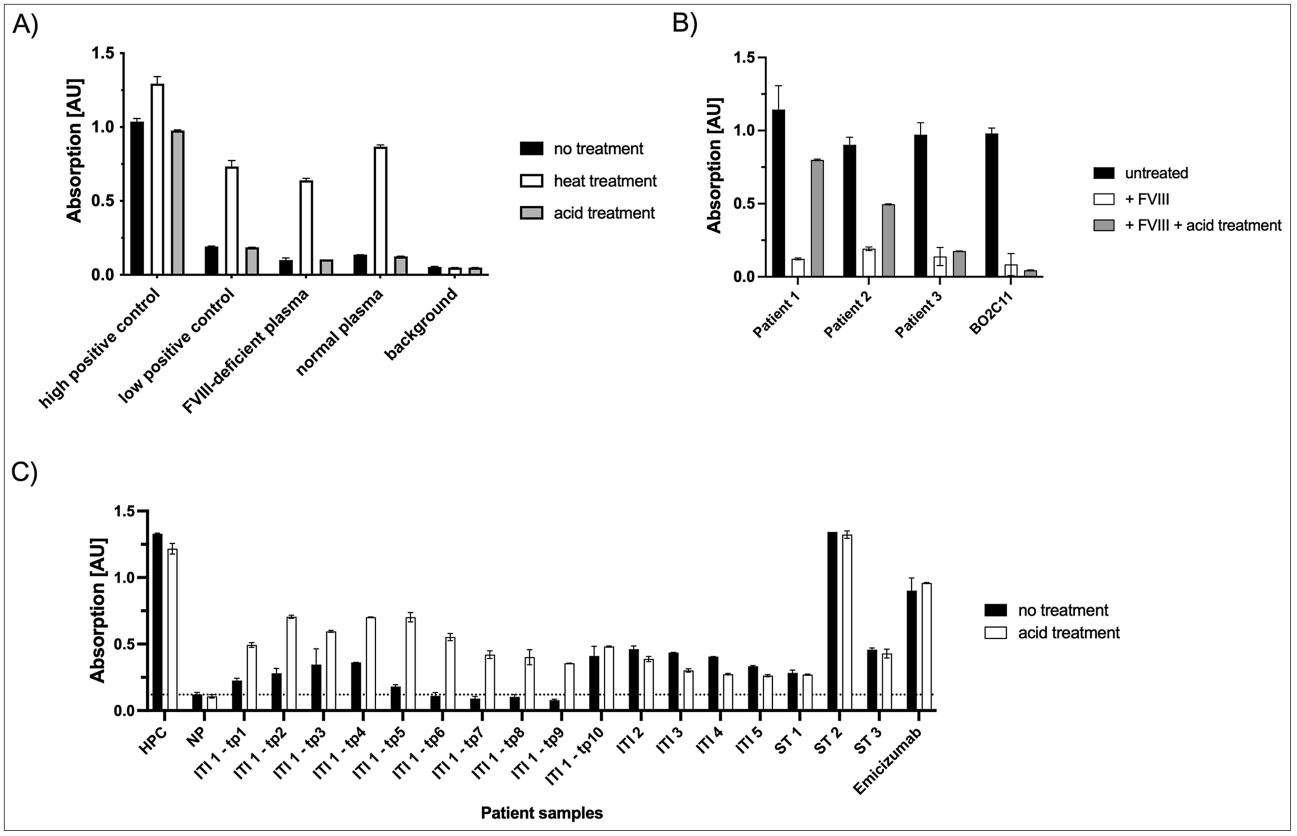

Fig. 1 Feasibility of acid denaturation for the detection of FVIII-specific antibodies in plasma samples. Plasma samples or monoclonal antibodies were incubated on FVIII-coated microtiter plates. (**A**) Plasma samples were either incubated without or with heat or acid treatment to compare the impact of these treatments on the FVIII signal in ELISA. (**B**) Patient samples or monoclonal human BO2C11 were directly added to the wells or following the addition of FVIII with and without acid treatment. Inhibitor titers for plasma samples: patient 1, 6.3 BU/mL; patient 2, 18.0 BU/mL; patient 3, 9.3 BU/mL. (**C**) Plasma samples were incubated on FVIII-coated microtiter plates without prior treatment or following acid treatment. Samples were inhibitor-negative except for ITI 1–tp3, 0.8 BU/mL; ST 2, 18.0 BU/mL; emicizumab, 5.6 BU/mL. HPC, high-positive control (diluted 1:50); LPC, low-positive control; FVIII-neg, FVIII-negative; NP, normal plasma; ST, substitution therapy.

of 10 time points analyzed. Interestingly, for time points (tp) 6 to 9, where the patient samples were inhibitor negative, the ELISA signal without acid treatment was below the signal of our negative control (NP), and samples were positive for FVIII-specific antibodies after acid treatment. Thus, here FVIII-specific antibodies could be unmasked by acid treatment.

The Impact of Acid Treatment on Monoclonal FVIII-Specific Antibodies

As acid treatment only resulted in the additional detection of FVIII-specific antibodies for one of five ITI patients analyzed and acid treatment could not restore binding of one patient plasma sample and monoclonal antibody BO2C11 after competition with FVIII, results raised the question which FVIII-specific antibody entities can be detected by the use of acid treatment. We, therefore, aimed to analyze the impact of acid treatment at a monoclonal level using murine FVIII-specific antibodies. Prior to FVIII competition and acid treatment, antibodies were titrated to identify antibody concentrations within the linear range of binding. These titrations (►**Supplementary Fig. S1** [available in the online version only]) were also used for the calculation of concentrations for half-maximal binding (B50) to quantify binding to FVIII (►**Supplementary Table S1**, available in the online version only). Appropriate dilutions of the antibodies were then added directly to FVIII-coated microtiter plates (control) or competed with FVIII with or without a following acid treatment (►**Fig. 2A**). FVIII competition of antibodies reduced binding to coated FVIII to a median of 38.48% compared with the untreated control. Additional acid treatment led to a median binding of 82.74%. Binding differences between the three conditions were statistically significant. Looking at the signal reduction produced by FVIII competition, we observed a wide range of normalized binding, ranging from 7 to 107% (see ►**Supplementary Table S1** [available in the online version only]). Acid treatment following FVIII competition resulted in a range of normalized binding from 17 to 119%. Interestingly, acid treatment restored the binding of competed antibodies for 18 out of 23 analyzed antibodies (►**Fig. 2B**). Only for antibodies F147, B25, G32, I160, and GMA-8014, acid treatment resulted in an additional reduction of binding (►**Fig. 2C**). We looked for linear correlations that might explain our observations and found that the B50 value quantifying the binding strength of the antibodies correlated with the percentage of FVIII binding under competing conditions (linear correlation after Spearman: 0.53, $p < 0.05$, ►**Fig. 2D**). A linear correlation

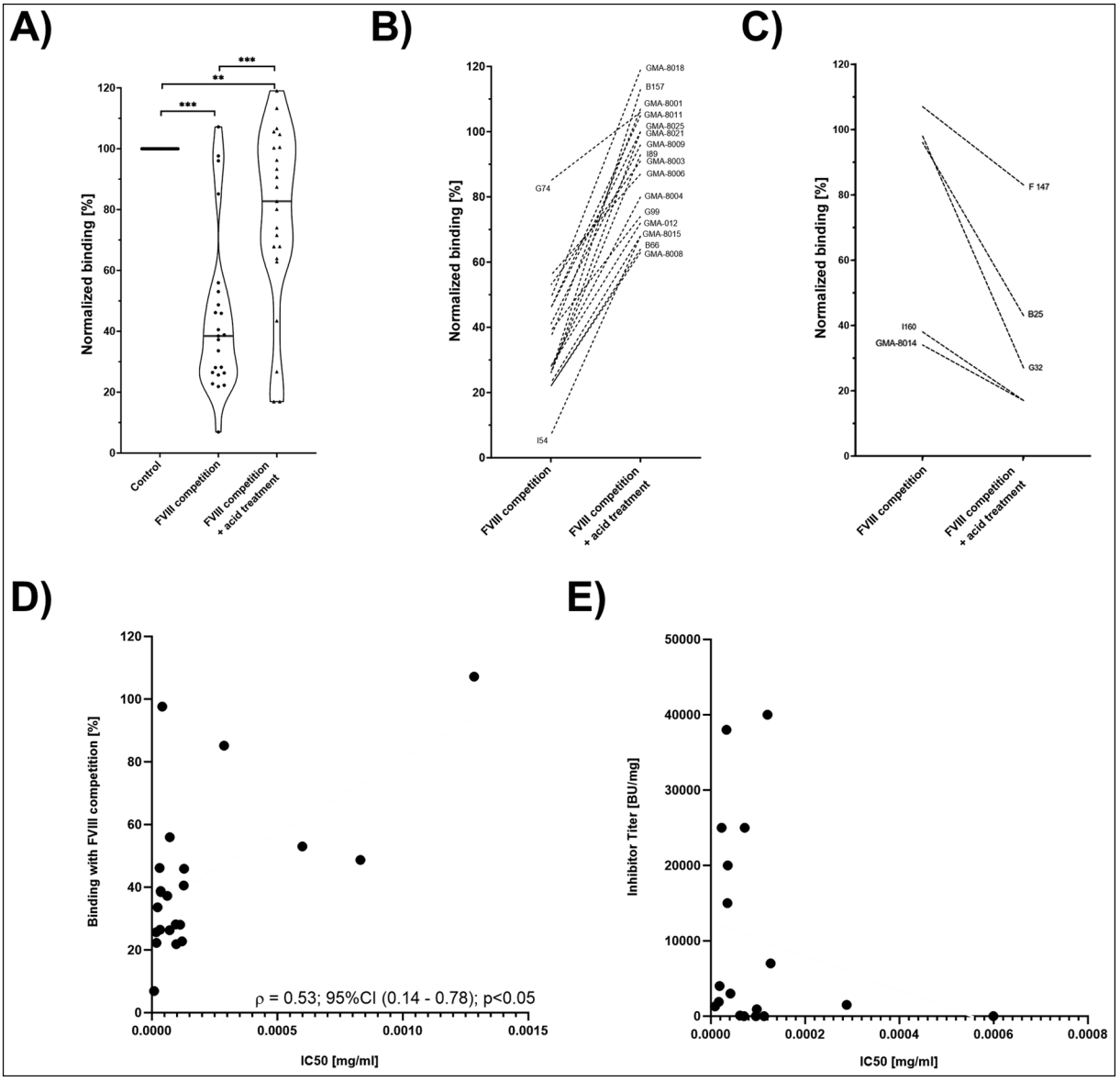

Fig. 2 Analysis of binding of monoclonal FVIII-specific antibodies in the presence of competing FVIII with and without acid treatment. (**A**) A total of 23 antibodies were analyzed for binding to FVIII-coated microtiter plates under different conditions and results were normalized for binding to FVIII without further treatment (control). Statistical analysis was performed with statistical software R. (**B**) Individual view on monoclonal antibodies that show an increase in binding following acid treatment. (**C**) Individual view on monoclonal antibodies that show further reduction of binding following acid treatment. (**D**) B50 values and corresponding normalized binding percentages for FVIII competition were plotted for 22 antibodies to display the linear correlation between these two antibody characteristics. (**E**) B50 values and corresponding inhibitory titers were plotted for 18 antibodies. (**D, E**) Linear Spearman correlation coefficients ρ are given with their 95% confidence intervals (CI) and p-values were significant.

was also found for the B50 value and binding after FVIII competition and acid treatment (linear correlation after Spearman: 0.37, $p < 0.05$, not shown). However, after looking for partial correlations, this finding was found to be a spurious correlation due to the strong effect of FVIII competition on the binding after FVIII competition and acid treatment. Interestingly, the B50 value did not correlate with the specific inhibitory activity of analyzed antibodies (►Fig. 2E). Thus, although we found an explanation for differences in the antibody response to competition with FVIII, we could not find any explanations for binding behavior following acid treatment.

Influence of Heat and Acid Treatment on FVIII and Antibodies

Using monoclonal antibodies to test FVIII competition with and without acid treatment, we could also see that for some antibodies binding to coated FVIII could not be restored by acid treatment (►Fig. 2C). However, restoration of binding was possible for most antibodies, pointing out that acid treatment indeed can be an option to decipher the existence of FVIII-specific antibodies in FVIII-containing samples. As acid and heat treatment of samples not only has an impact on the monoclonal antibodies but also FVIII itself, we analyzed the binding of the same set of murine monoclonal antibodies

in an additional ELISA. Here, we analyzed the binding of (1) untreated antibody to untreated FVIII, (2) heat-treated antibody to untreated FVIII, (3) heat-treated antibody to heat-treated FVIII, (4) acid-treated antibody to untreated FVIII, and (5) acid-treated antibody to acid-treated FVIII. While conditions (2) and (4) aimed to decipher the influence of plasma treatment on the antibodies alone, conditions (3) and (5) aimed to understand the impact of plasma treatment on antibody–FVIII complexes present in the sample (►Fig. 3A).

Heat treatment of antibodies led to a median reduction of 47.65% of binding to FVIII-coated wells. In comparison, the effect of acid treatment seems to be less abrasive, as it only resulted in a median reduction of 28.79% of binding. Thus, either the acid treatment applied did not lead to a high extent of denaturation or the neutralization step was possible to restore a proper antibody folding for a high proportion of antibodies. An additional acid treatment of FVIII prior to immobilization to the microtiter plate did not result in a further median reduction of the ELISA signal. In contrast, additional heat treatment of FVIII led to a drastic reduction of median antibody binding pointing out that not only a high proportion of the antibodies but also of FVIII were irreversibly denatured by heat treatment. Looking at heat (►Fig. 3B) and acid treatment (►Fig. 3C) on a monoclonal level, treatment of antibodies or antibodies and FVIII with heat had a more homogeneous effect than acid treatment. Interestingly, additional heat treatment of FVIII had a reducing effect on binding for all monoclonal antibodies except for GMA-012, where binding stayed constant, and GMA-8018 as well as GMA-8025, where heat treatment of FVIII led to an increase in binding compared with heat treatment of the antibodies alone. These three antibodies are specific for the A2 domain (GMA-012), the light chain (GMA-8018), and the activation peptide (GMA-8025). Probably these three antibodies bind to non-conformational epitopes, which are easier to access after the denaturation of FVIII. For GMA-8018 and GMA-8025, the same trend can be observed after acid treatment, although the effect of additional acid treatment of FVIII is generally lower, as already stated. To summarize, acid treatment in fact corresponds to the less abrasive technique, but might not be able to secure the identification of FVIII-specific antibodies, as FVIII present in plasma samples seems to properly refold during the neutralization step of the protocol. However, heat treatment does not only produce unspecific background binding in ELISA but also results in a profound destruction of FVIII-specific antibodies. Thus, acid treatment of plasma samples certainly is superior to heat treatment for the detection of masked FVIII-specific antibodies in FVIII-containing plasma samples.

Next, we looked for linear correlations within the binding behavior of antibodies under the described conditions (►Fig. 4). The strongest correlation (linear correlation after Spearman: 0.75, $p < 0.0005$) was found for the binding of antibodies after acid treatment alone and of antibodies and FVIII (►Fig. 4A). This again shows that additional acid treatment of FVIII has a rather low impact on antibody binding. In addition, binding after acid treatment of antibodies and FVIII correlated with binding after competition and acid treatment (linear correlation after Spearman: 0.59, $p < 0.005$, ►Fig. 4B). This linear correlation implies that both treatments have a similar effect on binding of FVIII-specific antibodies. Thus, the amount of antibodies binding to FVIII-coated wells is comparable when either coated FVIII was also treated with acid or coated FVIII was untreated, but FVIII present in the sample to mimic FVIII-containing plasma samples was treated with acid. This observation might be explained by a rather temporary denaturation of FVIII provoked by acid treatment. This way, the binding of acid-treated antibodies to acid-treated FVIII on the plate is hardly influenced by the treatment of FVIII. On the other hand, a temporary denaturation of FVIII in solution results in a dissociation of antibody–FVIII complexes, and when the sample is added to the microtiter wells there are still unbound antibodies available that are now able to bind to coated FVIII.

The finding of a correlation between binding after acid treatment of the antibody and FVIII competition with acid

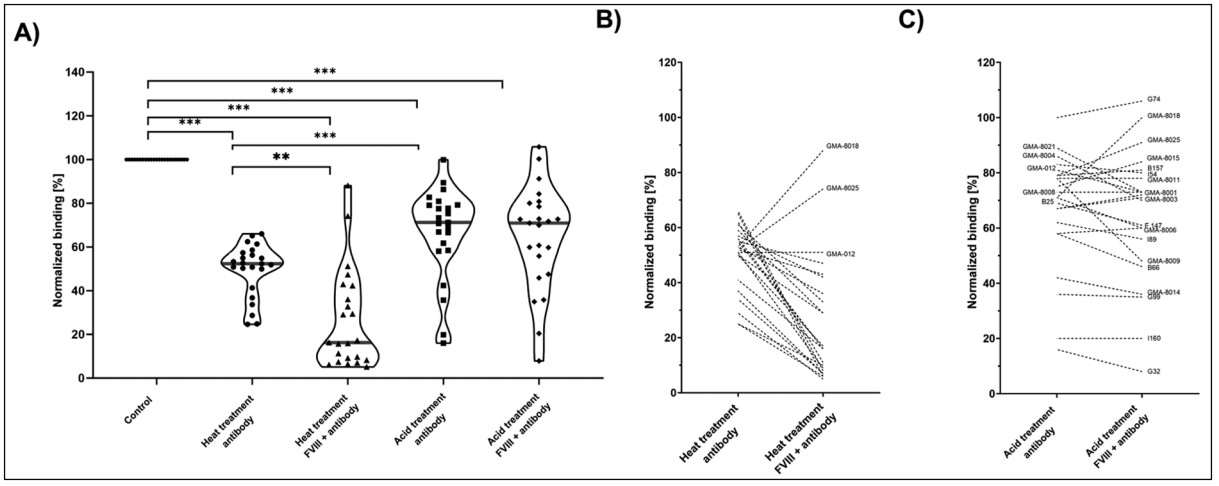

Fig. 3 Normalized binding of monoclonal FVIII-specific antibodies under native and denaturing conditions. (**A**) A total of 23 antibodies were analyzed for binding to FVIII-coated microtiter plates. FVIII and antibodies were either left untreated or treated with acid or heat prior to the ELISA. Statistical analysis was performed with statistical software R. (**B**) Individual view on heat-treatment conditions. (**C**) Individual view on acid-treatment conditions.

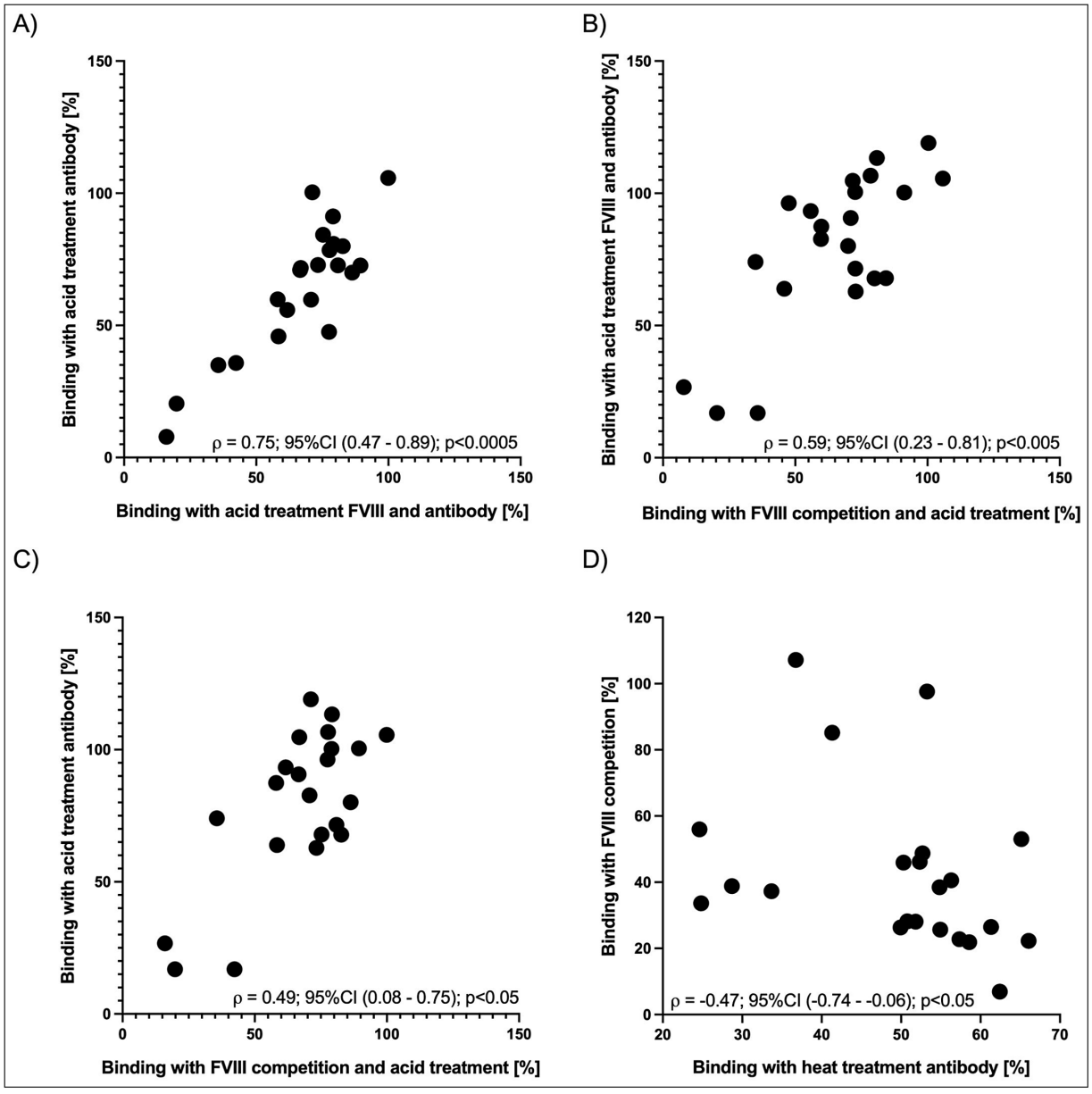

Fig. 4 Linear correlation between binding patterns after different treatments of antibodies and FVIII. Normalized binding percentages were plotted for 22 antibodies. Graphs display (**A**) binding of antibodies after acid treatment versus binding after acid treatment of antibodies and FVIII, (**B**) binding of antibodies after acid treatment of antibodies and FVIII versus binding after FVIII competition and acid treatment, (**C**) binding after acid treatment of antibodies versus binding after FVIII competition and acid treatment, and (**D**) binding of antibodies after heat treatment versus binding after FVIII competition. (**A–D**) Linear Spearman correlation coefficients ρ are given with their 95% confidence intervals (CIs) and p-values.

treatment (linear correlation after Spearman: 0.49, $p < 0.05$, ►**Fig. 4C**) might be explained in a comparable way. Binding of antibodies after FVIII competition and acid treatment is mostly less compared with binding after acid treatment, probably due to the binding of antibodies to renatured FVIII in the sample following neutralization, but a higher proportion of antibodies is rebuilding the complex with FVIII only after the sample is already applied to the FVIII-coated microtiter plate so that binding can also occur to coated FVIII.

Interestingly, we also found an inverse correlation for binding after heat treatment of the antibodies and binding after competition with FVIII (linear correlation after Spearman: −0.47, $p < 0.05$; ►**Fig. 4D**). Thus, antibodies still showing a rather high amount of binding after heat treatment showed less binding after FVIII competition. The correlation was also significant after analysis of partial correlations with other treatment conditions. However, data obtained within this study could not yet help explain this potential spurious correlation.

To summarize, heat treatment of FVIII-containing plasma samples does not only result in a high background in ELISA measurements but also leads to a profound destruction of antibodies and FVIII for the conditions applied. On the other hand, acid treatment seems to have a moderate effect on antibody and FVIII binding that is also more reversible than the effect of heat treatment and can therefore help unmask a

variety of monoclonal FVIII-specific antibodies in plasma samples containing FVIII.

Discussion

With this project, we were aiming to better understand the effects of acid and heat treatment on the interaction of FVIII and FVIII-specific antibodies in the ELISA format. Better knowledge about this topic might help establish more accurate methods for the detection of FVIII-specific antibodies in FVIII-containing plasma samples. This might be of high importance for previously untreated patients with a starting immune response, mild and moderate HA patients who get FVIII on demand, as well as for patients receiving ITI or non-replacement therapy with occasional FVIII administration. With the availability of non-replacement therapies for HA patients, the early detection of FVIII-specific immune responses can help identify the optimal treatment for the individual patient and reliable ELISA methods can help detect FVIII-specific antibodies in the presence of emicizumab, as it influences one-stage assays including the Bethesda assay.

Our data indicate that heat treatment does not only lead to high background in ELISA but also leads to irreversible changes in the binding properties of FVIII-specific antibodies and FVIII. On the other hand, acid treatment mainly results in a temporary denaturation of antibodies and FVIII, which makes the treatment feasible for disruption of interactions of FVIII–antibody complexes without leading to a high reduction in the following detection of most FVIII-specific antibodies in ELISA. Of note, the neutralization of acid-treated plasma samples prior to the addition to the FVIII-coated wells as performed here could result in the re-formation of antibody–FVIII complexes and therefore reduce the efficiency of antibody detection. A different protocol was already described for antibodies against therapeutic proteins, where neutralization of acidic samples was performed in the microplate only.[12] We also tested this procedure but did not see any significant difference to the protocol described in this article but a trend to a lower signal (data not shown). We assume a denaturing effect of the basic TRIS solution on the coated FVIII molecules.

With the applied acid-treatment protocol, we were also able to detect masked FVIII-specific antibodies in plasma samples of a patient undergoing ITI and therefore strongly suggest using acid denaturation as an alternative for the detection of FVIII-specific antibodies in FVIII-containing plasma samples. However, the method is not sufficient for the detection of some FVIII-specific antibody entities. For instance, we were not able to restore the binding of human monoclonal BO2C11 after FVIII competition using acid treatment. As monoclonal human antibodies specific for FVIII are rare, we conducted further analysis with murine monoclonal antibodies. Our data show that, in general, binding of FVIII-specific antibodies to immobilized FVIII can be competed by the addition of FVIII to the antibody samples, although the extent of competition depends on the binding strength of the antibody, represented as B50 value in this study; binding of antibodies with low B50 value could not easily be competed with FVIII. It was already reported that non-inhibitory antibodies in general have rather low affinities.[9] On the contrary, antibodies developed during the first immune response toward FVIII will most often have low affinities. So, competition analysis, which is sometimes used for proving specificity in ELISA, might sometimes result in a misinterpretation of plasma samples from HA patients during the first exposure days.

Antibodies specific for FVIII that cannot easily be competed due to their low affinity might not have a negative impact for the patient. However, they also might reflect a non-tolerant state and negatively influence the FVIII pharmacokinetics. In addition, these antibodies generally are the first ones that are produced and therefore might help detect the beginnings of a FVIII-specific immune response. Luckily, as these antibodies cannot easily be competed by FVIII, they will be most likely detected in a sensitive ELISA even in the presence of FVIII.

Concerning heat and acid treatment, the binding strength of antibodies did not correlate with binding after any treatment applied. We saw a wide range of effects of acid treatment on the binding of monoclonal antibodies with a median signal reduction of 28.79% without finding any correlations that might help more precisely characterize the type of antibody that will not be detected by acid treatment of the sample. As most antibodies analyzed were directed against the A2 or C2 domain of FVIII and other specificities were underrepresented, domain specificities of antibodies were not considered for analysis, as shown in ►**Supplementary Table S1** (available in the online version only). Interestingly, it was shown that the constant region of antibodies is more sensible to acid treatment, while the Fab fragment of antibodies is more easily degraded by heat treatment.[13] As the Fab fragment contains the antigen binding site of an antibody, this might explain why binding to FVIII was more reduced after heat treatment than after acid treatment. On the contrary, this observation helps to explain why the use of acid treatment is favorable when the disruption of the antibody–antigen interaction is planned to be only temporary. Of note, the analysis of FVIII-specific antibodies in a monoclonal setting using PBS for dilution is not fully comparable with plasma samples. For the latter, the reaction of the full set of plasmatic proteins to heat or acid treatment can have an impact on antibody detection, as antibodies might as well be integrated into other protein aggregates that can inhibit antibody binding unrelated to the conformation of the antibodies.

To summarize, for a better understanding of the different impacts of heat and acid treatment on individual FVIII-specific antibodies, further physicochemical analysis would have to be performed. However, for routine diagnosis of plasma samples, acid treatment is already a useful method because of the earlier-reported advantages over heat treatment. Acid treatment allows a more reliable detection of FVIII-specific antibodies in plasma samples containing FVIII to better understand the state of tolerance, especially in previously untreated patients and inhibitor patients on or

after ITI or non-replacement therapy to better guide treatment decisions.

What Is Known about This Topic?

- FVIII-specific antibodies in FVIII-containing plasma samples can be undetectable without pretreatment of the sample.
- Heat treatment is known to produce an unspecific background in ELISA, which can lead to false-positive results.

What Does This Paper Add?

- We are comparing the effect of heat and acid treatment on plasma samples as well as monoclonal FVIII-specific antibodies.
- We can conclude that acid treatment is a superior method for the detection of FVIII-specific antibodies in plasma samples containing FVIII.

Conflict of Interest

None declared.

Acknowledgments

We gratefully acknowledge Pete Lollar (Atlanta, Georgia, United States) for providing monoclonal murine antibodies F147, I89, B66, I54, G74, G99, B25, B167, G32, and I160.

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