ONLINE SUPPLEMENT

MATERIALS AND METHODS

Detection and Titration of FVIII-binding antibodies by ELISA

A detailed description of the FVIII-binding antibody platform and the validation procedure was provided by Whelan et al.\textsuperscript{1}

In short, all anti-FVIII antibody analyses were performed using Ig isotype (IgG, IgA, IgM)- respectively IgG subclass (IgG1-4)-specific ELISAs established in compliance with current regulatory guidelines.\textsuperscript{2,3} PolySorp® microtiter plates (Nunc) were coated with 1µg/mL recombinant human full-length FVIII (rhFLFVIII; Baxalta Innovations GmbH, a Takeda company) diluted in Carbonate-bicarbonate buffer (Sigma-Aldrich) overnight at 4°C. Washing steps were performed with phosphate-buffered saline (PBS; pH 7.4, Thermo Scientific) containing Tween (Merck). To prevent unspecific binding, the assay plate was blocked for 1 hour at room temperature (RT) with bovine serum albumin (BSA, Cytiva). Specific experiments to investigate unspecific binding between anti-FVIII antibodies in plasma samples and BSA were conducted (data not shown). Afterwards, plasma samples and Ig isotype-/IgG subclass-specific positive and negative controls were incubated for 2 hours at RT.

In each Ig isotype- respectively IgG subclass-specific ELISA a FVIII-binding, human monoclonal antibody of the appropriate Ig isotype/IgG subclass was implemented as positive control. All of these control antibodies were raised against the same FVIII epitope (BioRad for Baxalta Innovations GmbH, a Takeda company). Positive control antibodies were spiked into negatively screened plasma pools from healthy donors and were used for the determination of assay sensitivities. The negative control was a plasma pool derived from negatively screened healthy donors.

After plasma sample and Ig isotype-/IgG subclass-specific control incubation, enzyme-conjugated secondary antibodies were added and assay plates were incubated for 1 hour at RT. All detection antibodies were preliminarily tested and their specificity to the appropriate human Ig isotype respectively IgG subclass was demonstrated (data not shown). Conjugate-matched substrates were added prior to incubating the assay plate at RT in the dark. The delta optical density (ΔOD) for each sample was determined by using a Microplate Reader (Synergy HR BioTek Instruments) in dual endpoint analysis.
mode at 405nm (for alkaline phosphatase [AP]), 450nm (for 3,3′,5,5′-Tetramethylbenzidine [TMB]) or 492nm (for o-Phenylenediamine [OPD]) measuring and 630nm reference wavelength. The ΔOD of each sample was blank corrected.

For each assay, predetermined cutoffs were established following a statistical approach based on the background signal of 160 healthy plasma donors according to Jaki T and colleagues.4 For IgM antibody investigation, a “floating cut point” approach was chosen in order to account for daily assay variation.5

In a first step, plasma samples were screened for total IgG, IgA and IgM antibodies binding to rhfFVIII at a minimum dilution of 1:20, in order to prevent unspecific matrix effects. Negative screening results below the assay cutoff were confirmed in a second screening run. Positive samples, for which the ΔOD was greater or equal to the cutoff, were independently titrated at least twice to semi-quantify titers of IgG1-4, IgA and IgM antibodies. The titer of a sample was defined as the highest dilution that still resulted in a positive signal (ΔOD ≥ cutoff). Assay-specific positive controls and samples were geometrically diluted by a factor of 2 starting at a 1:20 dilution. In case, two adjoining titration results (e.g. 1:20 and 1:40) were received for a sample, the higher titer was reported and referred to during FVIII-binding specificity and apparent affinity characterization. In case, two non-adjointing titer results (e.g. 1:80 and 1:320) were determined, the sample was titrated for a third time.

**Confirmation of FVIII-specificity and determination of apparent affinity constants**

An antigen competition-based ELISA approach, based on the aforementioned FVIII-binding ELISA platform, was used to evaluate IgG subclass (IgG1-4)- respectively IgA-specific apparent affinity constants (Kₘₐₓ) in equilibrium as outlined by Hofbauer et al.6

In short, apparent affinity evaluation of anti-FVIII antibodies in a plasma matrix was based on pre-incubating diluted plasma samples containing anti-FVIII antibodies with pre-defined molarities of rhfFVIII before analyzing the remaining free anti-FVIII antibodies by a direct-binding ELISA. During the preincubation step, FVIII-specific antibodies interact with free rhfFVIII molecules and form immune complexes. Antibodies forming immune complexes with competition antigen in solution are unavailable for binding to coated rhfFVIII in the direct-binding ELISA. Calculation of
the apparent affinity constants ($K_A$s) for FVIII-specific antibodies is dependent on the competition behavior of the plasma sample. The curve fit for $K_A$ determination is a non-linear regression model as described by Bobrovnik SA and colleagues and Stevens FJ and Bobrovnik SA. The underlying theoretical considerations for this model incorporate the law of mass action. For immune complex formation by free antibodies and free antigen molecules in solution, antibodies are assumed to target a single epitope per antigen. In equilibrium, the binding strength (affinity) between the interaction partners can be described by an average $K_A$.

In order to deduce information about apparent affinity, the following prerequisites have to be fulfilled by the experimental setting: (i) antigen has to be in excess during competition, which means that immune complex formation must not markedly decrease the availability of free antigen molecules, (ii) a linear correlation between antibody concentration and absorbance signal is considered and (iii) antibody bivalency has to be accounted for. Therefore, a standard binomial distribution is implemented to model the probabilities that either one or both paratopes are available for antigen binding.

Taking these assumptions as well as the assay design into account, antibody-antigen binding in solution is indirectly determined by measuring the ELISA signals from free antibodies at multiple competition antigen concentrations. These data points are subsequently fitted by non-linear regression. The used non-linear regression model is able to discriminate a homogenous apparent affinity population, containing one affinity population, from a bi-modal affinity population, containing two affinity populations with distinct apparent $K_A$s.

For experimental standardization, plasma samples containing FVIII-binding antibodies were diluted in accordance with their pre-determined antibody titers (see “Detection and Titration of FVIII-binding antibodies by ELISA”). Therefore, the ELISA $\Delta$OD had to be approximately two-fold higher than the respective assay $\Delta$OD cutoff. While FVIII-binding IgG1, IgG3, and IgA-positive samples with titers higher or equal to 1:40 qualified for affinity and specificity analyses, samples positive for FVIII-binding IgG2 and IgG4 were included, if their titers were higher or equal to 1:80. Diluted plasma sample duplicates were pre-incubated with eight different concentrations of rhfFVIII in solution for 22 hours at RT. Afterwards, these plasma samples were incubated on PolySorp® microtiter plates (Nunc), which were coated with 1µg/mL rhfFVIII overnight at 4°C, for 45 min at room temperature and shaken at approximately 500 rotations per
minute. Then, enzyme-conjugated Ig isotype-/IgG subclass-specific secondary antibodies (see “Detection and Titration of FVIII-binding antibodies by ELISA”) were added and the assay plate was incubated for 1 hour at RT. Subsequently, conjugate-matched substrates were added prior to incubating the assay plate at RT in the dark. The delta optical density (ΔOD) for each sample was determined by using a Microplate Reader (Synergy HR BioTek Instruments) in dual endpoint analysis mode at 405nm (for alkaline phosphatase [AP]), 450nm (for 3,3′,5,5′-Tetramethylbenzidine [TMB]) or 492nm (for o-Phenylenediamine [OPD]) measuring and 630nm reference wavelength. The ΔODs of each sample were blank corrected and used to calculate non-linear regressions with GraphPad Prism 8.4.3 (GraphPad) according to Bobrovnik SA and colleagues and Stevens FJ and Bobrovnik SA.7,8
For each sample, two models were calculated: Model 1, which assumed homogenous apparent $K_A$ distribution, and Model 2, which was specific for bi-modal apparent $K_A$ distribution. In case both models delivered valid results according to predefined statistical acceptance criteria (R²≥0.7, lower limit of 95% CI > 0, 95% CI detached), an additional extra sum-of-squares F-test was applied supporting model selection. If Model 2 did not deliver highly significant (p<0.001) improvement of data description, Model 1 was chosen. In addition, the non-linear regression model enabled for the identification of the dominant antibody affinity population upon bi-modal apparent $K_A$ distribution by considering for which of the two distinct antibody affinity populations the non-linear regression function fitted more accurately (≥50%).
In case curve fitting was not possible for a sample, the sample was re-analyzed. If nonlinear regression modelling failed twice due to insufficient competition, no $K_A$ values were reported and FVIII specificity was re-evaluated with elevated FVIII competition concentrations (100µg/mL) as outlined in Whelan et al.1 FVIII specificity of IgM positive samples was directly investigated with elevated FVIII competition concentrations, as the non-linear regression model fitting is not possible for multivalent antibody types.
Validation experiments for the apparent affinity ELISA platform were described by Hofbauer et al.6
CLINICAL INFORMATION ON PATIENT NSHA 37, A NON-SEVERE HEMOPHILIA A PATIENT

Patient nsHA 37 was part of the nsHA cohort (patients with non-severe hemophilia A). In contrast to all other nsHA patients, Patient nsHA 37 presented with high titer, high affinity FVIII-specific IgG1 and IgG4 as well as low titer, low affinity IgA (see Figures 1A, 2A, 3A in the main manuscript [marked in red]):

Clinical data and FVIII-specific antibody characteristics of Patient nsHA 37 are summarized in Online supplement - Table 3. Patient nsHA 37 received his first FVIII replacement therapy by blood transfusion at 7 years of age after a tooth extraction procedure. Since then, he has been treated with plasma-derived or recombinant FVIII and/or 1-Desamino-8-D-Arginin-Vasopressin (DDAVP; Desmopressin) on demand. Particularly, prolonged coagulation events, gastrointestinal bleeds and operative dentistry procedures required hemostatic therapies. The patient presented once with a hematoma and underwent surgical procedures (leiomyoma resection and left knee arthroplasty), which demanded FVIII supplementation. Until study completion, Patient nsHA 37 had a negative history of FVIII inhibitors. In addition, the patient suffered from heart failure (atrial fibrillation and hypertonia) requiring medication. Associated periodic heart screening procedures could be carried out without the use of FVIII replacement therapies. In 2006, the patient’s HCV infection was successfully treated with Peginterferon alfa-2a (Pegasys®) and Ribavirin (Copegus®).
**TABLES**

**Online Supplement - Table 1:** Additional clinical information relating to patients within the non-severe hemophilia A without FVIII inhibitors- cohort

| Patient ID | Race      | Type of F8 mutation          | Relatedness | Age [y] | FVIII treatment | FVIII product type | anti-HCV antibodies [Yes/No] | HCV qRT-PCR [Pos/Neg] |
|------------|-----------|------------------------------|-------------|---------|----------------|-------------------|------------------------------|---------------------|
| nsHA 1     | Caucasian | Point mutation               | none        | 53      | On demand      | n.a.              | No                           | -                   |
| nsHA 2     | Caucasian | Point mutation               | none        | 41      | On demand      | n.a.              | Yes                          | Neg                 |
| nsHA 3     | Caucasian | No mutation identified       | none        | 45      | On demand      | Plasma-derived    | No                           | -                   |
| nsHA 4     | Caucasian | Splice site mutation         | none        | 35      | On demand      | n.a.              | No                           | -                   |
| nsHA 5     | Caucasian | Point mutation               | none        | 73      | On demand      | n.a.              | Yes                          | Neg                 |
| nsHA 6     | Caucasian | Point mutation               | none        | 76      | On demand      | Recombinant       | Yes                          | Pos                 |
| nsHA 7     | Caucasian | Point mutation               | none        | 20      | On demand      | Plasma-derived    | No                           | -                   |
| nsHA 8     | Caucasian | Point mutation               | none        | 76      | On demand      | Plasma-derived    | No                           | -                   |
| nsHA 9     | Caucasian | Point mutation               | none        | 67      | On demand      | Recombinant       | No                           | -                   |
| nsHA 10    | Caucasian | Point mutation               | none        | 63      | On demand      | n.a.              | No                           | -                   |
| nsHA 11    | Caucasian | Point mutation               | none        | 54      | On demand      | n.a.              | n.a.                         | -                   |
| nsHA 12    | Caucasian | Point mutation               | none        | 67      | On demand      | Recombinant       | No                           | -                   |
| nsHA 13    | Caucasian | Splice site mutation         | none        | 62      | On demand      | n.a.              | No                           | -                   |
| nsHA 14    | Caucasian | Point mutation               | none        | 45      | On demand      | Recombinant       | No                           | -                   |
| nsHA 15    | Caucasian | Point mutation               | none        | 42      | On demand      | n.a.              | No                           | -                   |
| nsHA 16    | Caucasian | No mutation identified       | none        | 37      | On demand      | Recombinant       | No                           | -                   |
| nsHA 17    | Caucasian | Point mutation               | none        | 62      | On demand      | Recombinant       | Yes                          | Neg                 |
| nsHA 18    | Caucasian | Point mutation               | none        | 50      | On demand      | Recombinant       | n.a.                         | -                   |
| nsHA 19    | Caucasian | Point mutation               | none        | 44      | On demand      | Recombinant       | n.a.                         | Pos                 |
| nsHA 20    | Caucasian | Point mutation               | Brother nsHA 21 | 27  | On demand      | Recombinant       | No                           | -                   |
| nsHA 21    | Caucasian | Point mutation               | Brother nsHA 20 | 32  | On demand      | Recombinant       | No                           | -                   |
| nsHA 22    | Caucasian | Point mutation               | none        | 51      | On demand      | Recombinant       | No                           | -                   |
| nsHA 23    | Caucasian | Point mutation               | none        | 24      | On demand      | Recombinant       | No                           | -                   |
| nsHA 24    | Caucasian | Point mutation               | none        | 50      | On demand      | n.a.              | No                           | -                   |
| nsHA 25    | Caucasian | Splice site mutation         | none        | 69      | On demand      | Recombinant       | Yes                          | Neg                 |
| nsHA 26    | Caucasian | Splice site mutation         | none        | 34      | On demand      | Recombinant       | No                           | -                   |
| nsHA  | Ethnicity | Phenotype | Mutation | Age | Administration | Source | Result  | Status |
|-------|-----------|-----------|----------|-----|----------------|--------|---------|--------|
| 27    | Caucasian | Point mutation | none | 55  | On demand     | n.a.   | Yes     | Neg    |
| 28    | Caucasian | Point mutation | none | 44  | On demand     | n.a.   | Yes     | Neg    |
| 29    | Caucasian | Point mutation | none | 19  | On demand     | Plasma-derived | No | -     |
| 30    | Caucasian | Point mutation | none | 69  | On demand     | Recombinant | Yes | Neg   |
| 31    | Caucasian | Point mutation | none | 59  | On demand     | Recombinant | Yes | Neg   |
| 32    | Caucasian | Splice site mutation | none | 49  | On demand     | n.a.   | Yes     | Neg    |
| 33    | Caucasian | Splice site mutation | none | 61  | On demand     | Plasma-derived | No | -     |
| 34    | Caucasian | Point mutation | none | 44  | On demand     | n.a.   | Yes     | Neg    |
| 35    | Caucasian | Point mutation | none | 30  | On demand     | Recombinant | No | -     |
| 36    | Caucasian | Point mutation | none | 36  | On demand     | Recombinant | No | -     |
| 37    | Caucasian | Point mutation | none | 64  | On demand     | Recombinant | Yes | Neg   |
| 38    | Caucasian | Point mutation | none | 54  | On demand     | n.a.   | No      | -      |
| 39    | Caucasian | Splice site mutation | none | 21  | On demand     | Recombinant | No | -     |
| 40    | Caucasian | Point mutation | none | 61  | On demand     | Recombinant | No | -     |
| 41    | Caucasian | Point mutation | none | 51  | On demand     | Recombinant | n.a. | -     |
| 42    | Caucasian | Point mutation | Brother nsHA 43 | 51  | On demand     | Recombinant | Yes | Neg   |
| 43    | Caucasian | Point mutation | Brother nsHA 42 | 49  | On demand     | Plasma-derived | Yes | Neg   |
| 44    | Caucasian | Point mutation | none | 55  | On demand     | Recombinant | No | -     |
| 45    | Caucasian | Point mutation | none | 52  | On demand     | Recombinant | n.a. | -     |
| 46    | Caucasian | Point mutation | Brother nsHA 47, nsHA 48 | 52  | On demand     | Plasma-derived | No | -     |
| 47    | Caucasian | Point mutation | Brother nsHA 46, nsHA 48 | 60  | On demand     | Recombinant | Yes | Neg   |
| 48    | Caucasian | Point mutation | Brother nsHA 46, nsHA 47 | 55  | On demand     | n.a.   | No      | -      |
| 49    | Caucasian | Point mutation | none | 40  | Prophylaxis    | Recombinant | n.a. | -     |
| 50    | Caucasian | Point mutation | none | 78  | On demand     | Recombinant | No | -     |
| 51    | Caucasian | Point mutation | none | 72  | On demand     | n.a.   | Yes     | Neg    |
| 52    | Caucasian | Point mutation | none | 54  | On demand     | Recombinant | Yes | Neg   |
| 53    | Caucasian | No mutation identified | none | 71  | On demand     | n.a.   | No      | -      |
| 54    | Caucasian | Point mutation | none | 36  | On demand     | Recombinant | Yes | Neg   |
| 55    | Caucasian | Point mutation | Grandfather nsHA56, nsHA57 | 77  | On demand     | Recombinant | No | -     |
| 56    | Caucasian | Point mutation | Grandson nsHA55, Brother nsHA57 | 23  | On demand     | Recombinant | No | -     |
| 57    | Caucasian | Point mutation | Grandson nsHA55, Brother nsHA56 | 21  | On demand     | Recombinant | No | -     |
| 58    | Caucasian | Point mutation | none | 55  | Prophylaxis    | Plasma-derived | Yes | Neg   |
| 59    | Caucasian | Point mutation | none | 59  | On demand     | Plasma-derived | Yes | Pos   |
| 60    | Caucasian | Point mutation | none | 62  | On demand     | Recombinant | Yes | Neg   |
| 61    | Caucasian | Point mutation | none | 61  | On demand     | n.a.   | No      | -      |
| 62    | Caucasian | Point mutation | none | 34  | On demand     | Recombinant | No | -     |
| ID  | Ethnicity | Point mutation | Treatment Type | Prophylaxis | Recombinant | Heritability | Treatment | Treatment Source |
|-----|-----------|----------------|----------------|-------------|-------------|--------------|-----------|------------------|
| nsHA 63 | Caucasian | Point mutation | none | 21 | Prophylaxis | Recombinant | No | - |
| nsHA 64 | Caucasian | Point mutation | none | 55 | On demand | n.a. | No | - |
| nsHA 65 | Caucasian | Point mutation | none | 43 | On demand | Plasma-derived | Yes | Neg |
| nsHA 66 | Caucasian | Point mutation | none | 68 | On demand | Recombinant | Yes | Neg |
| nsHA 67 | Caucasian | Point mutation | none | 24 | On demand | Recombinant | No | - |
| nsHA 68 | Caucasian | Point mutation | none | 66 | On demand | Recombinant | Yes | Neg |
| nsHA 69 | Caucasian | Point mutation | none | 62 | On demand | Recombinant | Yes | Pos |
| nsHA 70 | Caucasian | Point mutation | none | 52 | On demand | n.a. | Yes | Neg |
| nsHA 71 | Caucasian | Point mutation | none | 45 | On demand | n.a. | No | - |
| nsHA 72 | Caucasian | Point mutation | none | 27 | On demand | Recombinant | No | - |
| nsHA 73 | Caucasian | Point mutation | none | 80 | On demand | n.a. | No | - |
| nsHA 74 | Caucasian | Point mutation | none | 40 | On demand | Recombinant | Yes | Pos |
| nsHA 75 | Caucasian | Point mutation | none | 39 | On demand | Plasma-derived | No | - |
| nsHA 76 | Caucasian | Point mutation | none | 39 | On demand | n.a. | No | - |
| nsHA 77 | Caucasian | n.a. | none | 32 | On demand | Recombinant | n.a. | - |
| nsHA 78 | Caucasian | n.a. | none | 41 | On demand | Recombinant | n.a. | - |
| nsHA 79 | Caucasian | n.a. | none | 56 | On demand | Recombinant | n.a. | - |
| nsHA 80 | Caucasian | n.a. | none | 42 | On demand | Recombinant | n.a. | - |
| nsHA 81 | Caucasian | n.a. | none | 60 | On demand | Recombinant | n.a. | - |

ID: identification number; F8: factor VIII gene; FVIII: factor VIII (protein); HCV: hepatitis C virus; qRT-PCR: quantitative real-time polymerase chain reaction; nsHA: non-severe hemophilia A; n.a.: not available
**Online Supplement - Table 2:** Additional clinical information relating to patients within the severe hemophilia A without FVIII inhibitors- cohort

| Patient ID | Race     | Type of F8 mutation | Relatedness | Age [y] | FVIII treatment | FVIII product type | anti-HCV antibodies [Yes/No] | HCV qRT-PCR [Pos/Neg] |
|------------|----------|---------------------|-------------|---------|----------------|-------------------|-----------------------------|-----------------------|
| sHA 1      | Caucasian | Nonsense mutation   | Brother sHA 16 | 22      | Prophylaxis     | Plasma-derived     | No                          | -                     |
| sHA 2      | Caucasian | No mutation identified | none        | 33      | Prophylaxis     | Recombinant        | Yes                         | Neg                   |
| sHA 3      | Caucasian | Missense mutation  | none         | 30      | On demand       | Recombinant        | Yes                         | Neg                   |
| sHA 4      | Caucasian | Deletion            | Brother sHA 24 | 19      | Prophylaxis     | Recombinant        | No                          | -                     |
| sHA 5      | Caucasian | Nonsense mutation   | none         | 47      | On demand       | Plasma-derived     | Yes                         | Neg                   |
| sHA 6      | Caucasian | Splice site mutation | none         | 29      | Prophylaxis     | Recombinant        | No                          | -                     |
| sHA 7      | Caucasian | Inversion           | none         | 18      | Prophylaxis     | Plasma-derived     | No                          | -                     |
| sHA 8      | Caucasian | Inversion           | none         | 44      | Prophylaxis     | Recombinant        | n.a.                       | -                     |
| sHA 9      | Caucasian | Inversion           | none         | 21      | Prophylaxis     | Recombinant        | No                          | -                     |
| sHA 10     | Caucasian | Inversion           | Brother sHA 11 | 23      | On demand       | Recombinant        | Yes                         | Pos                   |
| sHA 11     | Caucasian | Inversion           | Brother sHA 10 | 18      | On demand       | Recombinant        | No                          | -                     |
| sHA 12     | Caucasian | Missense mutation  | none         | 44      | On demand       | Recombinant        | No                          | -                     |
| sHA 13     | Caucasian | Deletion            | none         | 46      | Prophylaxis     | Recombinant        | n.a.                       | -                     |
| sHA 14     | Caucasian | Inversion           | none         | 41      | Prophylaxis     | Recombinant        | n.a.                       | -                     |
| sHA 15     | Caucasian | Inversion           | Brother sHA 37 | 28      | Prophylaxis     | Plasma-derived     | Yes                         | Neg                   |
| sHA 16     | Caucasian | Nonsense mutation   | Brother sHA 1 | 18      | Prophylaxis     | Plasma-derived     | No                          | -                     |
| sHA 17     | Caucasian | Missense mutation  | none         | 30      | On demand       | Recombinant        | Yes                         | Neg                   |
| sHA 18     | Caucasian | Inversion           | none         | 28      | Prophylaxis     | Recombinant        | No                          | -                     |
| sHA 19     | Caucasian | Inversion           | none         | 29      | On demand       | Recombinant        | Yes                         | Neg                   |
| sHA 20     | Caucasian | Inversion           | none         | 45      | Prophylaxis     | Plasma-derived     | Yes                         | Neg                   |
| sHA 21     | Caucasian | Missense mutation  | none         | 34      | On demand       | Recombinant        | Yes                         | Pos                   |
| sHA 22     | Caucasian | Inversion           | none         | 28      | Prophylaxis     | Recombinant        | Yes                         | Neg                   |
| sHA 23     | Caucasian | Deletion            | none         | 31      | On demand       | Recombinant        | Yes                         | Pos                   |
| sHA 24     | Caucasian | Deletion            | Brother sHA 4 | 24      | Prophylaxis     | Recombinant        | No                          | -                     |
| sHA 25     | Caucasian | Inversion           | none         | 28      | Prophylaxis     | Plasma-derived     | Yes                         | Pos                   |
| sHA 26     | Caucasian | Inversion           | none         | 44      | On demand       | Recombinant        | Yes                         | Neg                   |
| sHA 27     | Caucasian | Inversion           | none         | 27      | On demand       | Recombinant        | No                          | -                     |
| sHA 28     | Caucasian | Inversion           | none         | 33      | On demand       | Recombinant        | No                          | -                     |
| sHA 29     | Caucasian | Inversion           | none         | 31      | Prophylaxis     | Recombinant        | Yes                         | Neg                   |
| sHA 30 | Caucasian | Inversion | none | 33 | Prophylaxis | Recombinant | Yes | Neg |
|--------|-----------|-----------|------|----|-------------|-------------|-----|-----|
| sHA 31 | Caucasian | Inversion | none | 40 | On demand   | Plasma-derived | Yes | Pos |
| sHA 32 | Caucasian | Inversion | none | 45 | On demand   | Recombinant   | Yes | Pos |
| sHA 33 | Caucasian | Missense mutation | none | 53 | On demand   | Recombinant   | Yes | Pos |
| sHA 34 | Caucasian | Missense mutation | none | 24 | Prophylaxis | Recombinant   | No  | -   |
| sHA 35 | Caucasian | Inversion | none | 31 | Prophylaxis | Recombinant   | Yes | Neg |
| sHA 36 | Caucasian | Missense mutation | none | 42 | Prophylaxis | Recombinant   | Yes | Neg |
| sHA 37 | Caucasian | Inversion | Brother sHA 15 | 24 | Prophylaxis | Plasma-derived | No  | -   |
| sHA 38 | Caucasian | n.a. | Brother sHA 39 | 26 | Prophylaxis | Recombinant | n.a. | -   |
| sHA 39 | Caucasian | n.a. | Brother sHA 38 | 26 | Prophylaxis | Recombinant | n.a. | -   |

ID: identification number; F8: factor VIII gene; FVIII: factor VIII (protein); HCV: hepatitis C virus; qRT-PCR: quantitative real-time polymerase chain reaction; sHA: severe hemophilia A; n.a.: not available
**Online Supplement - Table 3:** Clinical data and FVIII-specific antibody characteristics of Patient nsHA 37

| Age at sampling [y] | BMI at sampling [kg/m²] | Hemophilia A family history | F8 mutation status | lowest FVIII:C [%] | Characteristics of FVIII-binding antibodies with confirmed specificity† |
|---------------------|-------------------------|-----------------------------|-------------------|------------------|--------------------------------------------------------------------------------------------------|
| 64                  | 38.61                   | positive                    | 2 missense mutations: Glu132Asp (A1-domain) Arg612Cys (A2-domain) | 10%              | IgG1:  
  Titer - 1:320  
  Kₐ₁ - 1.75•10¹⁰ M⁻¹  
  Kₐ₂ - 4.44•10⁷ M⁻¹  
  IgG4:  
  Titer - 1:480  
  Kₐ₁ - 2.79•10¹⁰ M⁻¹  
  IgA:  
  Titer - 1:40  
  Kₐ₁ - 1.69•10⁹ M⁻¹  
  Kₐ₂ - 1.72•10⁷ M⁻¹ |

BMI: body mass index; F8: factor VIII gene; FVIII: blood coagulation factor VIII protein; FVIII:C: factor VIII clotting activity; AB: antibody; Glu: glutamic acid; Asp: aspartic acid; Arg: arginine; Cys: cysteine; Ig: Immunoglobulin; Kₐ: (apparent) affinity constant

† Apparent Kₐ of dominant antibody affinity population is highlighted in bold.
## Online Supplement - Table 4: Prevalence comparisons of FVIII-binding antibodies with confirmed FVIII specificity in hemophilia A patients differentiated by their anti-HCV antibody status

| FVIII-specific IgG subclass/Ig Isotype | Cohort comparison by anti-HCV AB status [number of patients] | Statistical test | p-value |
|--------------------------------------|---------------------------------------------------------------|------------------|---------|
| pooled Igs                           |                                                               |                  |         |
| nsHA                                 | HCV AB pos [15] vs HCV AB neg [14]                           | X²               | 0.019*  |
| sHA                                  | HCV AB pos [7] vs HCV AB neg [3]                             | FET              | 0.467   |
| nsHA                                 | HCV AB pos [15] vs sHA [7]                                   | X²               | 0.095   |
| sHA                                  | HCV AB neg [14] vs. sHA [3]                                  | FET              | 0.737   |
| IgG1                                 |                                                               |                  |         |
| nsHA                                 | HCV AB pos [11] vs HCV AB neg [11]                           | X²               | 0.091   |
| sHA                                  | HCV AB pos [4] vs HCV AB neg [3]                             | FET              | 1.000   |
| nsHA                                 | HCV AB pos [11] vs. sHA [4]                                  | X²               | 0.090   |
| sHA                                  | HCV AB neg [11] vs. sHA [3]                                  | FET              | 1.000   |
| IgA                                  |                                                               |                  |         |
| nsHA                                 | HCV AB pos [7] vs HCV AB neg [9]                             | X²               | 0.445   |
| sHA                                  | HCV AB pos [4] vs HCV AB neg [0]                             | FET              | 0.126   |
| nsHA                                 | HCV AB pos [7] vs. sHA [4]                                   | FET              | 0.729   |
| sHA                                  | HCV AB neg [9] vs. sHA [0]                                   | FET              | 0.098   |

FVIII: factor VIII; Ig: Immunoglobulin; HCV: Hepatitis-C Virus; AB: antibody; nsHA: non-severe hemophilia A patients; sHA: severe hemophilia A patients; HCV AB pos: anti-Hepatitis-C Virus antibody positive; HCV AB neg: anti-Hepatitis-C Virus antibody negative; X²: Chi-squared test; FET: Fisher’s exact test; p-value: level of significance

Significant p-values are indicated in bold and marked with an asterisk:

* *p≤0.050*
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