ONLINE SUPPLEMENT

1. MATERIALS AND METHODS

1.1. Detection of FVIII- and FIX-binding antibodies by ELISAs:

Plasma samples were screened for total IgG, IgA and IgM binding to recombinant, human full-length FVIII (FVIII; Baxalta Innovations GmbH, a Takeda company) as outlined by Whelan et al.[1] In addition, samples were screened for total Ig binding to recombinant human FIX (FIX; Baxalta Innovations GmbH, a Takeda company) with an analogously established ELISA method. In a second run, negative screening results were confirmed. Positive samples were twice independently titrated to semi-quantify anti-FVIII IgG1-4, IgA and IgM respectively anti-FIX total Ig titers. In case, two adjoining titers were received for a sample, the higher titer was reported and referred to for FVIII-specificity and apparent affinity determination or FIX-specificity. For a sample, for which FVIII-specificity and apparent affinity or FIX-specificity could not be demonstrated, the mean titer was reported, if two adjoining titers were received. If two non-adjoining titers were measured, the sample was titrated for confirmation for a third time. Ig isotype-/IgG subclass FVIII-binding and total Ig FIX-binding ELISAs were fully validated according to regulatory guidelines issued by the European Medicines Agency (EMA)[2] and the Food and Drug Administration (FDA)[3]. During validation, an assay precision of ± 1 titer step was determined for Ig isotype-/IgG subclass FVIII-binding and total Ig FIX-binding ELISAs.[1]

1.2. Confirmation of antigen-specificity and determination of apparent affinity constants:

After titer determination, a competition-based ELISA approach enabled for apparent affinity constant (K_A) and FVIII specificity evaluation of FVIII-binding IgG1-4 and IgA antibodies in equilibrium, as outlined by Hofbauer et al.[4] FVIII-binding IgG1, IgG3, and IgA-positive samples, for which a titer of 1:40 was determined at least once, as well as FVIII-binding IgG2- and IgG4-positive samples, for which a titer of 1:80 was determined at least once, were included in affinity and specificity analyses. According to the assay principle, free anti-FVIII antibodies in diluted human plasma samples interact with immobilized FVIII subsequently to preincubation with defined molar concentrations of FVIII in solution (IgG1-3, IgA: 42.11nM to 0.92nM; IgG4: 1.00nM to 0.02nM). Following the assumption of specific equimolar interaction between FVIII-

specific antibodies and FVIII, which causes a decrease in absorbance directly proportional to the concentration of the competition antigen, K_A determination is possible in low volume samples without preliminary antibody purification. The K_As for up to two distinct anti-FVIII antibody affinity clusters per sample and IgG subclass/IgA isotype can be determined by nonlinear regression modeling of delta optical densities received in the competition ELISAs according to Stevens and Bobrovnik and Bobrovnik et al.[5,6] The possibility of fitting a FVIII concentration-dependent, non-linear regression function demonstrates FVIII-specificity of the investigated FVIII-binding Ig isotype/IgG subclass. In case curve fitting could not be achieved, the sample was re-analyzed. If regression modelling failed twice due to insufficient competition, no K_A values were reported and FVIII specificity was separately evaluated with increased FVIII competition concentrations as outlined by Whelan et al.[1] FVIII specificity of FVIII-binding IgM positive samples and FIX specificity of FIX-binding total Ig positive samples were directly investigated with increased competition antigen (FVIII or FIX) concentrations (351nmol). FVIII or FIX specificity was confirmed with a 351nM concentration of competition antigen, if the FVIII- or FIX-binding antibody titer could be reduced by three or more titer steps.

2. ADDITIONAL HIPS-ITI PATIENT CHARACTERISTICS

The following table provides additional information on HIPS-ITI patients:

Online Supplement - Table 1: Additional HIPS-ITI patient characteristics

HIPS-ITI Patient ID	HIPS Patient ID[1]	Family history of hemophilia A	*F8* mutation	Ethnic background[2]	First FVIII inhibitor detection	Family history of FVIII inhibitors
1	21	Yes	Intron 22 inversion	White	ED10	Unknown
2	20	Yes	Duplication	Hispanic	ED10	Yes
3	22	Yes	Intron 22 inversion	Asian or Pacific	ED10	No
4	19	Yes	Intron 22 inversion	White	ED10	Unknown
5	23	Yes	Intron 22 inversion	White	ED11	Yes

HIPS: Hemophilia Inhibitor in Previously Untreated Patients Study; ITI: Immune tolerance induction; ID: Identification number; *F8*: Factor VIII gene; FVIII: Factor VIII; ED: Exposure day

1 Reipert et al. Blood Adv. 2020
2 Self-reported by the patient's legal guardian. Genetic analysis of ethnic identity not performed

3. APPARENT OLIGOREACTIVE FVIII-BINDING ANTIBODIES DO NOT BIND TO UNCOATED ELISA PLATES BLOCKED WITH BOVINE SERUM ALBUMIN

In order to demonstrate that FIX-binding of apparent oligoreactive FVIII-binding antibodies in plasma samples of HIPS-ITI Patients 2 (Manuscript - Figure 5B) and 5 (Manuscript - Figure 6B) do not simply represent antibodies that bind to ELISA plates blocked with BSA, appropriate control experiments were conducted. In a first step, uncoated ELISA plates and FIX-coated ELISA plates were blocked with a 2% BSA-PBS solution. In a side-per-side testing approach, serial dilutions of plasma samples obtained from HIPS-ITI Patients 2 and 5 were assessed on uncoated ELISA plates blocked with BSA (HIPS-ITI Patient 2: Online Supplement - Table 2; HIPS-ITI Patient 5: Online Supplement - Table 3) and on FIX-coated ELISA plates blocked with BSA (HIPS-ITI Patient 2: Online Supplement - Table 4; HIPS-ITI Patient 5: Online Supplement - Table 5). While incubation on uncoated ELISA plates blocked with BSA resulted in negative ELISA signals below the cut-off value (OD 0.118), incubation on FIX-coated and BSA-blocked plates resulted in positive ELISA signals indicating the presence of FIX-binding antibodies.

Online Supplement - Table 2: Delta OD values for serial dilutions of plasma samples obtained from HIPS-ITI Patient 2 incubated on uncoated ELISA plates blocked with BSA (A: ELISA plate 1; B: ELISA plate 2)

A

Dilution Factor		1	2	3	4	5	6	7	8	9	10	11	12
		Negative Control (A1-D1) Blank (E1-H1)	Positive Control (FIX Ig) HIGH	Positive Control (FIX Ig) LOW	HIPS-ITI Patient 2 **ED6**	HIPS-ITI Patient 2 **ED10**	HIPS-ITI Patient 2 **ED27**	HIPS-ITI Patient 2 **ED44**	HIPS-ITI Patient 2 **ED53**	HIPS-ITI Patient 2 **ED70**	HIPS-ITI Patient 2 **ED97**	HIPS-ITI Patient 2 **ED120**	HIPS-ITI Patient 2 **ED146**
1:20	**A**	0.007	0.008	0.006	0.013	0.014	0.028	0.022	0.025	0.019	0.020	0.023	0.017
1:40	**B**	0.006	0.002	0.002	0.007	0.007	0.014	0.010	0.011	0.008	0.009	0.015	0.009
1:80	**C**	0.005	0.001	0.000	0.002	0.003	0.007	0.005	0.005	0.006	0.005	0.004	0.004
1:160	**D**	0.008	0.001	0.001	0.001	0.003	0.004	0.002	0.004	0.004	0.003	0.002	0.004
1:320	**E**	0.000	0.001	0.001	0.001	0.001	0.003	0.002	0.002	0.003	0.001	0.002	0.002
1:640	**F**	0.000	-0.001	0.000	-0.001	0.000	-0.001	0.000	0.001	0.001	0.000	0.000	0.002
1:1280	**G**	-0.001	0.000	0.000	0.000	-0.001	0.001	0.001	-0.001	0.001	0.002	0.001	0.001
1:2560	**H**	-0.001	0.000	-0.001	-0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.002
	Titer	**<1:20**	**<1:20**	**<1:20**	**<1:20**	**<1:20**	**<1:20**	**<1:20**	**<1:20**	**<1:20**	**<1:20**	**<1:20**	**<1:20**

Titerendpoint (cut-off): ΔOD - Blank >0.118

B

Dilution Factor		1	2	3	4	5	6	7	8	9	10	11	12
		Negative Control (A1-D1) Blank (E1-H1)	Positive Control (FIX Ig) HIGH	Positive Control (FIX Ig) LOW	HIPS-ITI Patient 2 **ED161**	HIPS-ITI Patient 2 **ED184**	HIPS-ITI Patient 2 **ED203**	HIPS-ITI Patient 2 **ED236**	HIPS-ITI Patient 2 **ED263**	HIPS-ITI Patient 2 **ED294**	HIPS-ITI Patient 2 **ED326**	HIPS-ITI Patient 2 **ED377**	HIPS-ITI Patient 2 **ED388**
1:20	**A**	0.005	0.004	0.005	0.015	0.015	0.014	0.013	0.015	0.019	0.019	0.017	0.016
1:40	**B**	0.005	0.002	0.002	0.009	0.008	0.007	0.008	0.006	0.011	0.012	0.008	0.008
1:80	**C**	0.004	0.001	0.000	0.005	0.003	0.004	0.005	0.003	0.004	0.005	0.004	0.006
1:160	**D**	0.006	0.001	0.002	0.002	0.003	0.002	0.002	0.003	0.003	0.004	0.003	0.004
1:320	**E**	0.001	-0.001	0.001	0.003	0.001	0.002	0.001	0.001	0.002	0.000	0.002	0.003
1:640	**F**	-0.001	-0.001	0.001	0.000	0.001	0.000	-0.001	0.001	-0.001	0.001	0.005	0.000
1:1280	**G**	0.000	-0.001	-0.001	0.002	-0.001	0.000	0.000	0.000	0.001	-0.002	-0.005	0.001
1:2560	**H**	0.000	-0.001	-0.001	-0.001	0.000	0.000	0.000	0.000	0.000	-0.014	-0.001	0.000
	Titer	**<1:20**	**<1:20**	**<1:20**	**<1:20**	**<1:20**	**<1:20**	**<1:20**	**<1:20**	**<1:20**	**<1:20**	**<1:20**	**<1:20**

Titerendpoint (cut-off): ΔOD - Blank >0.118

HIPS: Hemophilia Inhibitor in Previously Untreated Patients Study; ITI: Immune tolerance induction; ED: Exposure day; OD: Optical density; ΔOD: Difference between optical density at 492nm and 650nm

Online Supplement - Table 3: Delta OD values for serial dilutions of plasma samples obtained from HIPS-ITI Patient 5 incubated on uncoated ELISA plates blocked with BSA

Dilution Factor		1	2	3	4	5	6	7	8	9	10	11	12
		Negative Control (A1-D1) Blank (E1-H1)	Positive Control (FIX Ig) HIGH	Positive Control (FIX Ig) LOW	HIPS-ITI Patient 5 **ED285**	HIPS-ITI Patient 5 **ED336**	HIPS-ITI Patient 5 **ED356**	HIPS-ITI Patient 5 **ED360**					
1:20	**A**	0.006	0.004	0.005	0.005	0.007	0.006	0.008					
1:40	**B**	0.006	0.004	0.003	0.004	0.004	0.004	0.006					
1:80	**C**	0.005	0.000	0.000	0.000	0.002	0.002	0.002					
1:160	**D**	0.005	0.000	0.000	0.003	0.002	0.001	0.001					
1:320	**E**	0.002	0.000	0.000	0.000	0.002	0.002	0.002					
1:640	**F**	-0.002	-0.001	-0.001	0.001	0.000	0.001	-0.001					
1:1280	**G**	0.001	-0.001	-0.002	-0.001	0.001	0.001	0.000					
1:2560	**H**	0.000	0.000	0.000	0.001	0.001	0.000	0.000					
	Titer	**<1:20**	**<1:20**	**<1:20**	**<1:20**	**<1:20**	**<1:20**	**<1:20**					

Titerendpoint (cut-off): ΔOD - Blank >0.118

HIPS: Hemophilia Inhibitor in Previously Untreated Patients Study; ITI: Immune tolerance induction; ED: Exposure day; OD: Optical density; ΔOD: Difference between optical density at 492nm and 650nm

Online Supplement - Table 4: Delta OD values for serial dilutions of plasma samples obtained from HIPS-ITI Patient 2 incubated on FIX-coated ELISA plates blocked with BSA (A: ELISA plate 1; B: ELISA plate 2)

A

										Titerendpoint (cut-off): ΔOD - Blank >0.118			
		1	2	3	4	5	6	7	8	9	10	11	12
Dilution Factor		Negative Control (A1-D1) Blank (E1-H1)	Positive Control (FIX Ig) HIGH expected titer: 1:160 (+/- 1 titer step)	Positive Control (FIX Ig) LOW expected titer: 1:40 (+/- 1 titer step)	HIPS-ITI Patient 2 **ED6**	HIPS-ITI Patient 2 **ED10**	HIPS-ITI Patient 2 **ED27**	HIPS-ITI Patient 2 **ED44**	HIPS-ITI Patient 2 **ED53**	HIPS-ITI Patient 2 **ED70**	HIPS-ITI Patient 2 **ED97**	HIPS-ITI Patient 2 **ED120**	HIPS-ITI Patient 2 **ED146**
1:20	**A**	0.021	0.425	0.205	0.085	0.124	0.556	0.565	0.560	0.536	0.533	0.533	0.521
1:40	**B**	0.017	0.281	0.121	0.046	0.079	0.545	0.536	0.536	0.509	0.529	0.495	0.507
1:80	**C**	0.017	0.176	0.067	0.022	0.039	0.491	0.487	0.504	0.492	0.460	0.455	0.458
1:160	**D**	0.002	0.114	0.039	0.012	0.022	0.488	0.453	0.465	0.453	0.447	0.420	0.415
1:320	**E**	0.002	0.062	0.021	0.005	0.012	0.457	0.412	0.410	0.417	0.399	0.350	0.349
1:640	**F**	0.000	0.033	0.014	0.002	0.005	0.397	0.340	0.350	0.341	0.313	0.274	0.270
1:1280	**G**	0.001	0.017	0.006	-0.001	0.003	0.333	0.275	0.265	0.269	0.245	0.196	0.192
1:2560	**H**	-0.004	0.010	0.003	0.001	0.003	0.264	0.202	0.208	0.199	0.170	0.132	0.127
	Titer	**<1:20**	**1:80**	**1:40**	**<1:20**	**1:20**	**1:5120***	**1:5120***	**1:5120***	**1:2560**	**1:2560**	**1:1920****	**1:1920****

*** 1:5120 titer confirmed in additional analysis runs**
**** Once a titer of 1:1280 and once a titer of 1:2560 was determined**

B

										Titerendpoint (cut-off): ΔOD - Blank >0.118			
		1	2	3	4	5	6	7	8	9	10	11	12
Dilution Factor		Negative Control (A1-D1) Blank (E1-H1)	Positive Control (FIX Ig) HIGH expected titer: 1:160 (+/- 1 titer step)	Positive Control (FIX Ig) LOW expected titer: 1:40 (+/- 1 titer step)	HIPS-ITI Patient 2 **ED161**	HIPS-ITI Patient 2 **ED184**	HIPS-ITI Patient 2 **ED203**	HIPS-ITI Patient 2 **ED236**	HIPS-ITI Patient 2 **ED263**	HIPS-ITI Patient 2 **ED294**	HIPS-ITI Patient 2 **ED326**	HIPS-ITI Patient 2 **ED377**	HIPS-ITI Patient 2 **ED388**
1:20	**A**	0.024	0.441	0.208	0.528	0.548	0.547	0.569	0.555	0.547	0.557	0.499	0.513
1:40	**B**	0.017	0.286	0.114	0.505	0.520	0.522	0.516	0.491	0.480	0.475	0.472	0.464
1:80	**C**	0.018	0.185	0.065	0.475	0.451	0.441	0.426	0.466	0.463	0.445	0.426	0.423
1:160	**D**	0.020	0.110	0.035	0.419	0.414	0.408	0.398	0.382	0.384	0.356	0.353	0.347
1:320	**E**	0.001	0.065	0.019	0.347	0.340	0.334	0.307	0.319	0.313	0.288	0.269	0.257
1:640	**F**	0.000	0.034	0.009	0.276	0.251	0.252	0.222	0.238	0.233	0.208	0.189	0.174
1:1280	**G**	0.000	0.019	0.006	0.191	0.177	0.172	0.154	0.162	0.154	0.134	0.105	0.100
1:2560	**H**	0.000	0.010	0.001	0.124	0.119	0.117	0.097	0.106	0.102	0.082	0.060	0.055
	Titer	**<1:20**	**1:80**	**1:20**	**1:2560**	**1:2560**	**1:1920***	**1:1280**	**1:1280**	**1:1280**	**1:1280**	**1:960****	**1:960****

*** Once a titer of 1:1280 and once a titer of 1:2560 was determined**
**** Once a titer of 1:640 and once a titer of 1:1280 was determined**

HIPS: Hemophilia Inhibitor in Previously Untreated Patients Study; ITI: Immune tolerance induction; ED: Exposure day; OD: Optical density; ΔOD: Difference between optical density at 492nm and 650nm

<u>Online Supplement - Table 5:</u> Delta OD values for serial dilutions of plasma samples obtained from HIPS-ITI Patient 5 incubated on FIX-coated ELISA plates blocked with BSA

									Titerendpoint (cut-off): ΔOD - Blank >0.118			
		1	2	3	4	5	6	7	8	9	10	11	12
Dilution Factor		Negative Control (A1-D1) Blank (E1-H1)	Positive Control (FIX Ig) HIGH expected titer: 1:160 (+/- 1 titer step)	Positive Control (FIX Ig) LOW expected titer: 1:40 (+/- 1 titer step)	HIPS-ITI Patient 5 **ED285**	HIPS-ITI Patient 5 **ED336**	HIPS-ITI Patient 5 **ED356**	HIPS-ITI Patient 5 **ED360**					
1:20	**A**	0.023	0.526	0.252	0.042	0.337	0.269	0.194					
1:40	**B**	0.019	0.360	0.141	0.022	0.240	0.166	0.129					
1:80	**C**	0.019	0.243	0.078	0.011	0.178	0.113	0.085					
1:160	**D**	0.019	0.149	0.042	0.007	0.117	0.068	0.050					
1:320	**E**	0.001	0.084	0.022	0.002	0.067	0.037	0.025					
1:640	**F**	-0.001	0.045	0.010	0.002	0.035	0.016	0.012					
1:1280	**G**	0.001	0.025	0.007	0.000	0.018	0.010	0.003					
1:2560	**H**	-0.002	0.013	0.002	0.001	0.008	0.003	0.002					
	Titer	**<1:20**	**1:160**	**1:40**	**<1:20**	**1:80**	**1:60***	**1:40**					

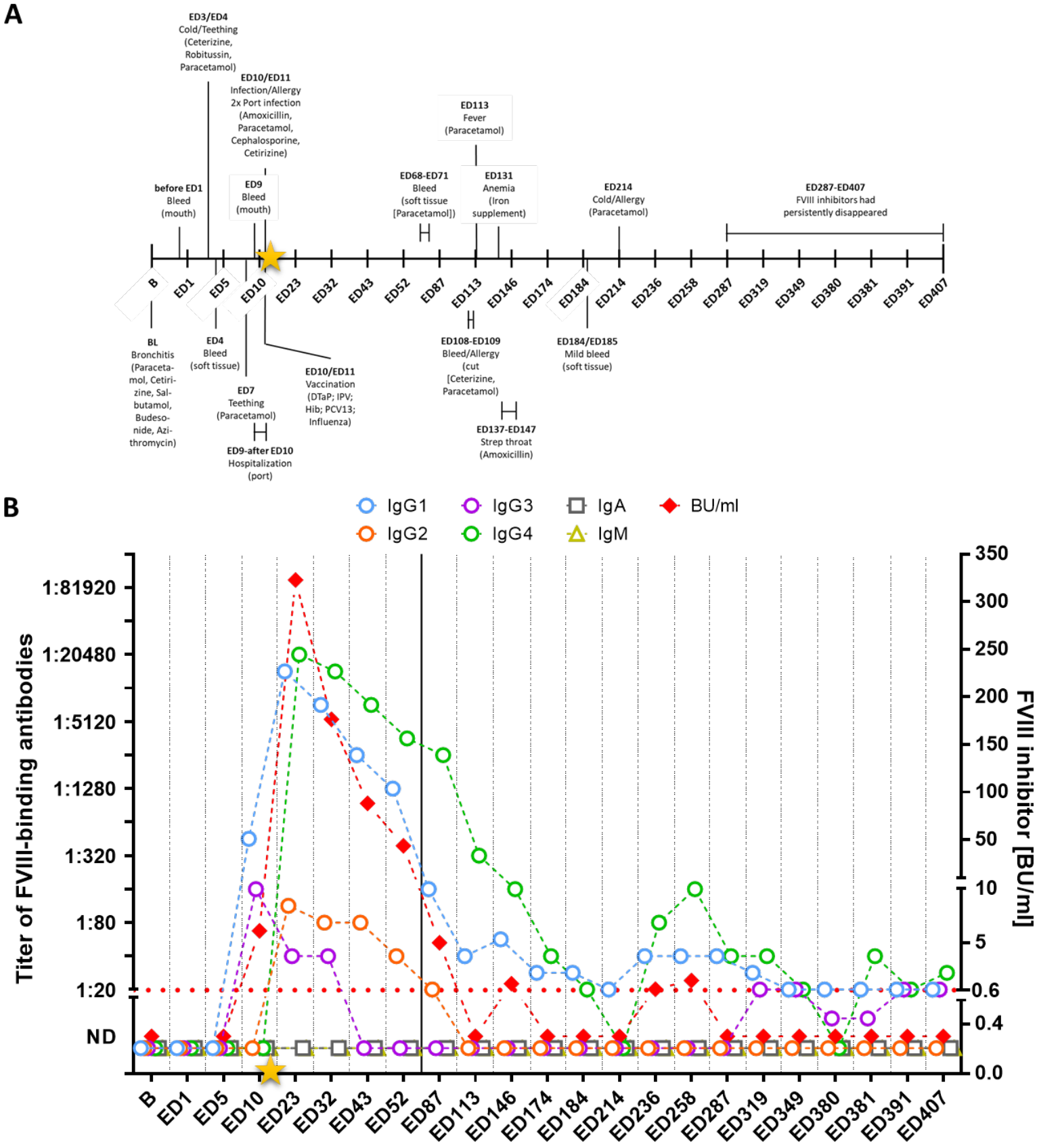
*** Once a titer of 1:40 and once a titer of 1:80 was determined**

HIPS: Hemophilia Inhibitor in Previously Untreated Patients Study; ITI: Immune tolerance induction; ED: Exposure day; OD: Optical density; ΔOD: Difference between optical density at 492nm and 650nm

4. ADDITIONAL LONGITUDINAL MONITORING DATA OF CLINICAL EVENTS

The following figures provide longitudinal monitoring data of clinical events for HIPS-ITI Patients 1, 3 and 4:

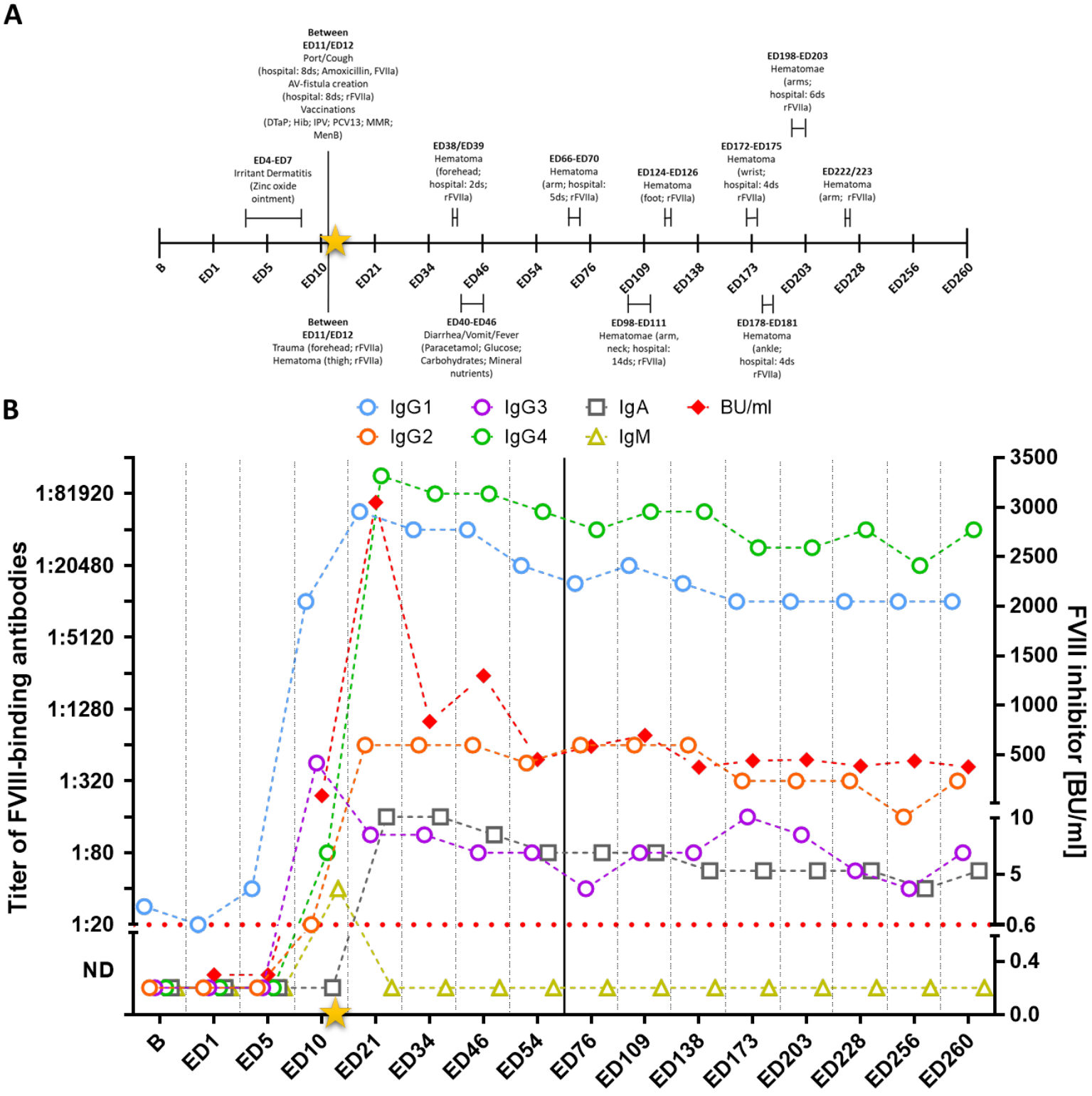
<u>Online Supplement - Figure 1:</u> FVIII-specific antibodies in HIPS-ITI Patient 1 during HIPS and HIPS-ITI and timeline of clinical events



A) Timeline of clinical events documented for HIPS-ITI Patient 1 throughout HIPS and HIPS-ITI including (i) adverse events with short descriptions, (ii) infections and administered treatment, (iii) hospitalizations, duration of hospitalizations and administered treatment, (iv) immunizations and administered vaccinations, (v) additionally administered medications and supplements. The yellow star marks the initiation of ITI treatment at ED11.
B: Baseline; ED: Exposure day; DTaP: Diphteria, tetanus, pertussis; IPV: Inactivated polio vaccine; Hib: *Haemophilus influenzae* type b; PCV13: Pneumococcal conjugate vaccine

B) FVIII-specific antibody titers (IgG1, IgG2, IgG3, IgG4, IgA, IgM as indicated) and FVIII inhibitors (BU/mL) for HIPS-ITI Patient 1. The red dotted line represents the limit for positive evaluation of FVIII inhibitors (0.6 BU/mL). The yellow star marks the initiation of ITI treatment at ED11. The vertical line indicates the end of HIPS and the start of HIPS-ITI.
FVIII: Factor VIII; ND: not detectable (below the detection limit of 1:20 for FVIII-binding antibodies); B: Baseline; ED: Exposure day; Ig: Immunoglobulin; BU/mL: Bethesda Units/milliliter

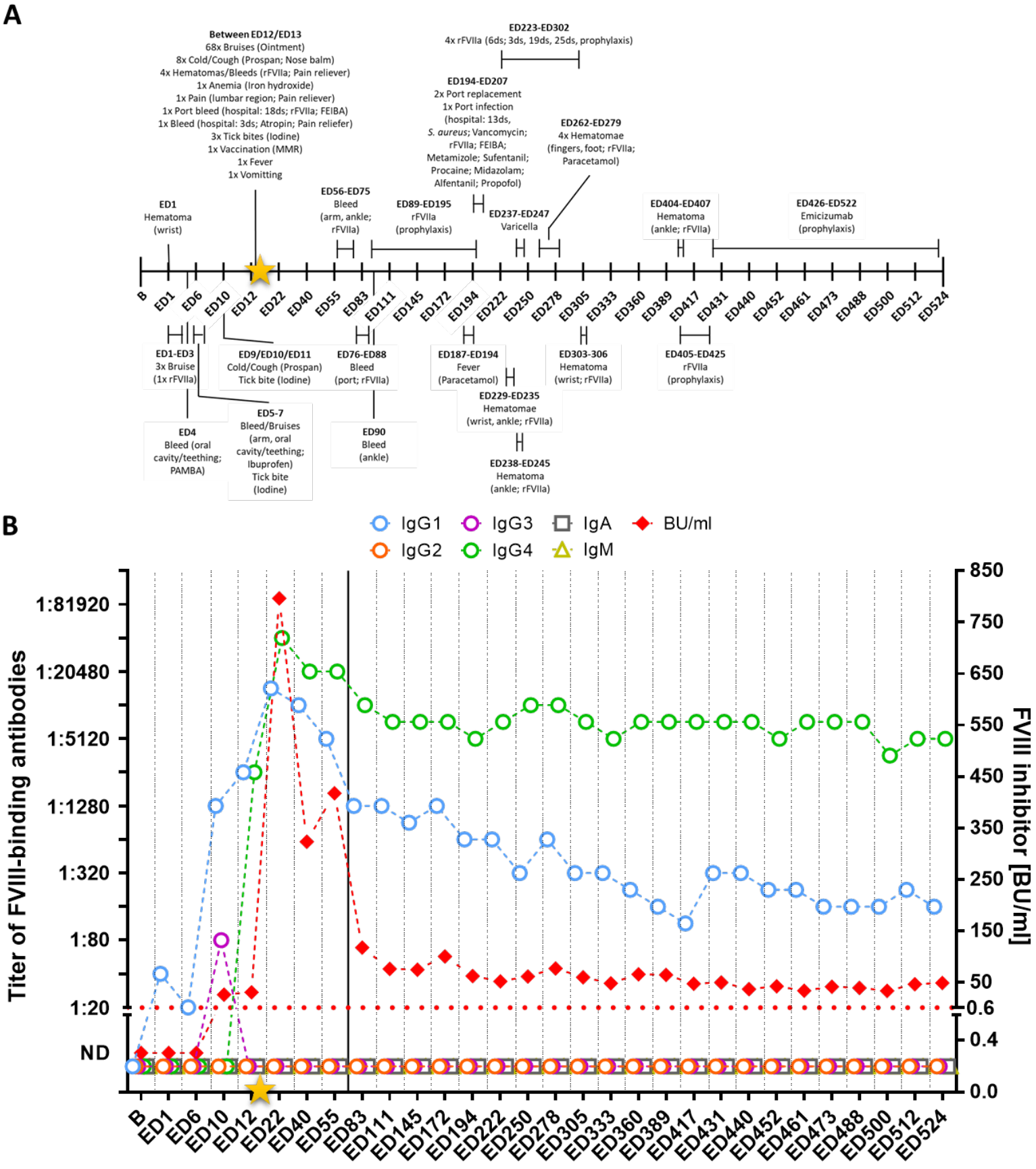
<u>Online Supplement - Figure 2:</u> FVIII-specific antibodies in HIPS-ITI Patient 3 during HIPS and HIPS-ITI and timeline of clinical events



A) Timeline of clinical events documented for HIPS-ITI Patient 3 throughout HIPS and HIPS-ITI including (i) adverse events with short descriptions, (ii) infections and administered treatment, (iii) hospitalizations, duration of hospitalizations and administered treatment, (iv) immunizations and administered vaccinations, (v) additionally administered medications and supplements. The yellow star marks the initiation of ITI treatment at ED12.
B: Baseline; ED: Exposure day; AV: arteriovenous; rFVIIa: activated, recombinant FVII; DTaP: Diphteria, tetanus, pertussis; Hib: *Haemophilus influenzae* type b; IPV: Inactivated polio vaccine; PCV13: Pneumococcal conjugate vaccine; MMR: Measles, mumps, rubella; MenB: Meningococcal bacteria type B

B) FVIII-specific antibody titers (IgG1, IgG2, IgG3, IgG4, IgA, IgM as indicated) and FVIII inhibitors (BU/mL) for HIPS-ITI Patient 3. FVIII-binding antibodies with titers at or close to the detection limit, eg at baseline (B), are included for information. The red dotted line represents the limit for positive evaluation of FVIII inhibitors (0.6 BU/mL). The yellow star marks the initiation of ITI treatment at ED12. The vertical line indicates the end of HIPS and the start of HIPS-ITI.
FVIII: Factor VIII; ND: not detectable (below the detection limit of 1:20 for FVIII-binding antibodies); B: Baseline; ED: Exposure day; Ig: Immunoglobulin; BU/mL: Bethesda Units/milliliter

<u>Online Supplement - Figure 3:</u> FVIII-specific antibodies in HIPS-ITI Patient 4 during HIPS and HIPS-ITI and timeline of clinical events



A) Timeline of clinical events documented for HIPS-ITI Patient 4 throughout HIPS and HIPS-ITI including (i) adverse events with short descriptions, (ii) infections and administered treatment, (iii) hospitalizations, duration of hospitalizations and administered treatment, (iv) immunizations and administered vaccinations, (v) additionally administered medications and supplements. The yellow star marks the initiation of ITI treatment at ED13.
B: Baseline; ED: Exposure day; rFVIIa: activated, recombinant FVII; PAMBA: p-aminomethylbenzoic acid; FEIBA: Factor VIII inhibitor bypassing activity; MMR: Measles, mumps, rubella; *S. aureus: Staphylococcus aureus*

B) FVIII-specific antibody titers (IgG1, IgG2, IgG3, IgG4, IgA, IgM as indicated) and FVIII inhibitors (BU/mL) for HIPS-ITI Patient 4. FVIII-binding antibodies with titers at or close to the detection limit, eg at baseline (B), are included for information. The red dotted line represents the limit for positive evaluation of FVIII inhibitors (0.6 BU/mL). The yellow star marks the initiation of ITI treatment at ED13. The vertical line indicates the end of HIPS and the start of HIPS-ITI.
FVIII: Factor VIII; ND: not detectable (below the detection limit of 1:20 for FVIII-binding antibodies); B: Baseline; ED: Exposure day; Ig: Immunoglobulin; BU/mL: Bethesda Units/milliliter

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