Developments in modern hemophilia care

Hassan, S.

Chapter 6

Factor VIII epitope mapping using a random peptide phage-display library approach

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Abstract

Background
Inhibitor development is the most severe complication of hemophilia A care, and is associated with increased morbidity and mortality.

Aims
The aim of this study was to use a novel IgG epitope mapping method to explore the factor VIII (FVIII)-specific epitope profile in the SIPPET cohort population.

Methods
The population consisted of 122 previously untreated patients with severe hemophilia A that were followed-up for 50 days of exposure to FVIII. Sampling was performed before FVIII treatment and at the end of the follow-up. The outcome was inhibitor development. The FVIII-specific IgG epitope repertoire was assessed by means of a novel high-throughput epitope mapping technique using a random peptide phage-display library and the resulting peptide sequences were clustered on the basis of sequence similarity. For each cluster, a consensus motif was generated which was then aligned to the linear sequence of FVIII. The degree to which these clusters of peptide sequences could be used to discriminate between patients with and without an inhibitor was assessed by ROC analysis.

Results
The FVIII-specific antibody response is polyclonal with several clusters. The most predominant clusters in inhibitor-positive patients were mapped to the heavy chain of the FVIII molecule. Using plasma samples taken before exposure to FVIII, three clusters (with the consensus motifs “pxyNw”, “PSLxWK” and “sWphxxxxk”) were identified that predicted inhibitor development (with a C-statistic of 0.73, 0.80 and 0.76 respectively).

Conclusion
Information on immunodominant epitopes could be used to generate novel, less immunogenic FVIII proteins and set up diagnostic tests that predict the risk of inhibitor development before starting treatment with FVIII.

Introduction

Recent advances in the treatment of patients with hemophilia A (HA) have greatly improved clinical outcomes and quality of life. Nevertheless, one of the greatest treatment complications in severe hemophilia A is still the development of anti-factor VIII (FVIII) alloantibodies that neutralize FVIII (also called inhibitors). At least one third of patients treated with FVIII replacement therapy develop an inhibitor during the first 20-30 days of exposure to FVIII (EDs)[1], making treatment with FVIII ineffective. This in turn leads to increased morbidity and mortality among these patients.[1]

This complication is the result of a multi-causal immune response involving both patient- and treatment-related factors.[1] The type of FVIII product is one of the most important risk factors for inhibitor development, with the SIPPET randomized clinical trial showing that patients treated with recombinant FVIII (rFVIII) have an almost twofold higher risk of developing an inhibitor than those treated with plasma-derived FVIII (pdFVIII) products.[2] The pathophysiological mechanisms behind this increased immunogenicity remains unknown. Some plausible biological explanations have been postulated, such as the different post-translational modifications caused by the use of different cell lines during the manufacturing process of rFVIII products and the protective role played by Von Willebrand factor (VWF) in pdFVIII products.[3]

Mature FVIII consists of six major domains (A1, A2, B, A3, C1 and C2) and three acidic linking regions (a1, a2, a3); A1-a1-A2-a2-B-a3-A3-C1-C2. The VWF-FVIII complex forms through a high-affinity interaction between the FVIII light chain and the VWF D-D3 domains.[4] FVIII is activated by limited proteolysis through thrombin cleavage of three peptide bonds at Arg391 (a1-A2 junction), Arg759 (a2-B junction) and Arg1708 (a3-A3 junction).[5] After thrombin cleavage, activated factor VIII (sans B-domain) is released from VWF and binds to phosphatidylserine PS on the extracellular surface of activated platelets.[6,7]

The anti-FVIII humoral immune response is highly polyclonal and consists primarily of IgG antibodies, with variable multiple epitopes among patients and even in the same patient over time.[8] Several studies have examined the immunogenicity of FVIII and the mechanisms underlying inhibitor development during treatment with FVIII.[3, 9, 10] The role of FVIII epitopes in inhibitor development has been previously investigated using different techniques. Specific regions in the A2 (region encompassing Arg484-Ile508)[11], A3 (Gln1778-Asp1840)[12], C1 and C2 (residues Glu2181-Val2243) FVIII domains[13] were shown to be target domains for FVIII alloantibody interaction by

several methods including low resolution immunoprecipitation, western blotting and antibody neutralization assays[8,14], as well as high resolution methods such as the phage display technique[15-18].

In recent years, quantitative immunoproteomics has developed rapidly, offering high throughput analyses at relatively low cost. The aim of this study was to use a novel high-throughput epitope mapping technique based on a random peptide phage-display method in order to explore the overall antibody response before and after exposure to either plasma-derived or recombinant FVIII products and to identify specific immunoprofiles that could be predictive for inhibitor development.

Methods

Patient population

Study samples were obtained from patients enrolled in the SIPPET trial, which was designed to investigate the immunogenicity of different FVIII products in patients with severe hemophilia A who were previously untreated with any FVIII concentrates (PUPs) or minimally treated with blood components.[2] Samples from 122 patients were used for this study. These patients were treated with 8 different FVIII products (4 pdFVIII products and 4 rFVIII products). Inhibitor development was measured using the Bethesda assay with Nijmegen modification.[19] Thirty-nine out of 122 individuals developed an inhibitor.

One sample of citrated plasma was collected at baseline (T0) and two samples at the end of the study (EOS). As previously described[2], in inhibitor-positive patients the end of the study was the time of inhibitor development. In inhibitor-negative patients the study ended when the patient reached 50 EDs or after three years of follow-up (whichever came first).

Approval for this study was obtained from the medical ethics committee at each study center and informed consent was obtained from all parents/guardians of patients.

Mimotope-variation analysis

Assay set-up

The total IgG epitope repertoire was assessed using mimotope-variation analysis (MVA), a next generation phage display method. (Protobios, Tallinn).[20] MVA was conducted as previously described. Briefly, 2 µl of plasma was incubated

with 5 µl of phage library (~5 × 10^{10} phage particles, derivative of Ph.D.-12, NEB, UK) overnight at +4 °C. The human immunoglobulin G (IgG)-captured phages were pulled down by protein G-coated magnetic beads (NEB, S1506S). Phage DNA was extracted, enriched and samples were barcoded by PCR amplification. Pooled samples were analyzed by Illumina sequencing (50-bp single end read, Genohub, USA). The resulting DNA sequences were *in silico* translated to 12 amino acid (aa) long peptide sequences. To correct for differences in sequencing depth among the samples, the total count of each unique peptide sequence per sample was normalized in its counts per three million. The resulting output consisted of a database of 12-mer peptide sequences with varying degrees of affinity for IgG antibodies. These peptide sequences are often referred to in the literature as "mimotopes", due to the fact that they may mimic the true epitope of an antibody.

Two versions of the assay were performed, the standard MVA assay (described above) and a competition assay. In the MVA competition assay, different factor VIII products (Alphanate (Grifols), Fanhdi (Grifols), Emoclot (Kedrion Biopharma), Factane (LFB), Advate (Baxalta), Kogenate FS (Bayer AG), ReFacto AF (Pfizer), Recombinate (Baxalta)) were used to precondition study samples before competition analyses. In detail, respective FVIII products (final concentration: 3 uM) were incubated with 2 µl of plasma for 2 hours at room temperature before proceeding with the MVA assay as described above.

Removal of target unrelated peptides (TUPs)

One issue in conducting phage display experiments is the presence of so-called target-unrelated peptides (TUPs). These are false-positive results caused by selection-related TUPs which are peptide sequences binding to materials and reagents used in the assay (for example, plastic surfaces, albumin), or propagation-related TUPs caused by faster propagation of some phage clones, resulting in a higher peptide count for some peptide sequences. To minimize the effect of these TUPs, we removed all peptide sequences that were predicted to be TUPs using the SAROTUP software.[21] Briefly, known TUPs were filtered out exploiting the TUPscan and the mimosearch algorithms. Peptides with a high likelihood ($P > 0.8$) to bind to polystyrene, as assessed by the PSBinder algorithm, were also filtered out.

Quality control using intra- and inter-assay replicates

To increase assay reliability, all peptide sequences with a count lower than a certain threshold were removed from the dataset. To establish the level of the threshold, a healthy control sample was compared with all its intra- or inter-assay replicates,

and the percentage of unreplicated peptide sequences according to each possible count threshold was calculated. (Figure S1) Below a peptide count threshold of 250, a strong increase in the percentage of unreplicated sequences was seen (Figure S1) For the following analyses, we only kept sequences retrieved at least 250 times in at least one patient.

Identification of peptide sequences with high affinity for FVIII

FVIII-specific peptide sequences were defined as present in the EOS sample in which the standard MVA assay was performed but not in the EOS sample in which the MVA competition assay was performed (which was depleted of FVIII-specific antibodies). Thus, the count of each peptide sequence in the two EOS samples (standard MVA assay vs. MVA competition assay) was compared using the Fisher's exact test. We corrected for multiple testing using the Bonferroni method. Only peptide sequences significantly underrepresented in the MVA competition assay samples when compared to the standard MVA assay samples were considered to be FVIII-specific peptide sequences and used for further analyses.

Clustering workflow

Each FVIII epitope can be conceptualized as being represented by multiple peptide sequences, each containing the epitope binding motif. Therefore, the Hammock algorithm, a hierarchical clustering algorithm, was used to cluster peptides sequences based on sequence similarity before further analyses.[22] Applying the algorithm resulted in clusters of highly similar peptide sequences. For each cluster, a consensus motif was generated based on the multiple sequence alignment of the sequences. Highly conserved residues (› 60%) were denoted with an uppercase symbol while moderately conserved residues (30%-60%) were denoted with a lower case symbol. Columns in the multiple sequence alignment where no single residue had a prevalence of › 30% were denoted with "x". The total peptide count of each cluster was calculated as the sum of the count of each peptide sequence included in a cluster. The clustering algorithm was performed firstly on the whole dataset and then separately for data from patients using pdFVIII and patients using rFVIII.

Alignment of consensus motifs to FVIII

The consensus motif derived from each cluster of peptide sequences was then aligned to the linear sequence of FVIII.

Statistical analyses

For the descriptive analyses, a PCA plot of all the clusters identified after the clustering step were generated. To find clusters with a significantly higher count among inhibitor-positive patients compared to inhibitor-negative patients, a Wilcoxon Rank Sum test was performed. Correction for multiple testing was done using the Bonferroni method[23], and an adjusted p-value ‹ 0.05 was considered statistically significant.

To find biomarkers that were able to predict inhibitor development before the start of FVIII therapy, clusters showing a significant association with inhibitor development in the samples taken at the end of the study (the EOS samples) were also evaluated in samples taken before FVIII treatment (the T0 samples). Correction for multiple testing was done using the Bonferroni method[23] and an adjusted p-value ‹ 0.05 was considered statistically significant.

To assess the discriminative performance of the clusters that were also significantly associated with inhibitor development in the T0 samples, we calculated the C-statistic and plotted a receiver operating characteristic (ROC) curve. In addition, a cut-off was selected using Youden's index for each cluster, and based on this cut-off we calculated the sensitivity and specificity of each cluster for inhibitor development.

Results

The MVA assay methodology was applied to 124 previously untreated patients with hemophilia. Of this group, thirty-nine patients were inhibitor-positive. The mean number of unique peptide sequences generated for each patient was 356,365. After removing potential target-unrelated peptides, the mean number of unique peptides generated for each patient decreased to 313,340. From this dataset, we kept only the peptide sequences with a count of at least 250 in at least one patient and used this dataset to identify FVIII-specific peptide sequences as described in the Methods section. This yielded 286 unique peptide sequences per patient.

FVIII-specific epitope profile of patients that developed an inhibitor after replacement therapy with pdFVIII or rFVIII

As shown in Table 1, we found 17 clusters with a significantly higher count in patients who developed an inhibitor as compared with patients who did not. The PCA plot showed a clear difference between patients with or without inhibitors (Figure 1). Clusters that were more common in inhibitor-positive patients were predominantly mapped to the heavy chain of the FVIII molecule (Table 2).

Clusters associated with an inhibitory response against rFVIII

The clustering workflow was then applied to FVIII-specific peptide sequences retrieved in the rFVIII group. Eleven clusters had a significantly higher count among inhibitor-positive patients when compared to inhibitor-negative patients at the end of the study (Table 3). Of these 11 clusters, one cluster (with the consensus motif "pxyNw") was also significantly associated with inhibitor development in the baseline (T0) samples (Figure 2). This cluster was mapped to the A2 domain. The C-statistic of this cluster was 0.73 (95%CI: 0.60-0.86), sensitivity was 86% and specificity was 59%. (Figure 3A)

Clusters associated with an inhibitory response against pdFVIII

Next, the clustering workflow was then applied to the FVIII-specific peptide sequences retrieved in the pdFVIII group. In this group, we found 14 clusters with a significantly higher count among inhibitor-positive patients when compared to inhibitor-negative patients at the end of the study (Table 4). Of these 14 clusters, two were also significantly associated with inhibitor development in the baseline (T0) samples. (Figure 2) The C-statistic of the first cluster (with consensus motif "PSLxWK") was 0.80 (95%CI: 0.66 – 0.93), sensitivity was 76% and specificity was 77%. (Figure 3B) This cluster was mapped to the B domain. The C-statistic of the second cluster (with consensus motif "sWphxxxxk") was 0.76 (95%CI: 0.63 – 0.89), sensitivity was 88% and specificity was 59%. (Figure 3B) This cluster was mapped to the C2 domain.

Discussion

Summary

We assessed the FVIII-specific epitope profile of 122 previously untreated patients with hemophilia A, using a novel random peptide phage-display assay. Our results show that the FVIII-specific antibody response is

highly polyclonal, as our analysis generated many different clusters. In our cohort, we saw an overall slightly stronger response against the A1-A2-B domains than the C1-C2 domains. Using samples obtained before exposure to FVIII, we identified three clusters of peptide sequences (with the consensus motifs "pxyNw", "PSLxWK" and "sWphxxxxk"), that were predictive for inhibitor development (with an C-statistic of 0.73, 0.80 and 0.76 respectively).

Table 1. Consensus motifs of clusters of peptide sequences with a significantly higher count in either inhibitor-negative (INH-) patients or inhibitor-positive (INH+) patients in all patients.

Consensus motif	Mean peptide count* in IHN- Group	Mean peptide count* in IHN+ Group	Adjusted P-value	FVIII Domain(s)	Number of unique peptide sequences in cluster (%)	Peptide count of cluster (%)
kxPxstw	6.10	8.50	2.4e-05	A2	31 (0.11%)	62423 (0.18%)
Yvntxxxt	5.70	8.20	8.0e-04	A1	41 (0.15%)	48730 (0.14%)
pxxWxKp	6.40	8.80	8.2e-04	C1	66 (0.24%)	93414 (0.26%)
kxxTgpq	5.60	7.60	2.2e-03	A2	35 (0.13%)	37907 (0.11%)
KnxHxxxxp	5.50	7.90	2.5e-03	A3	73 (0.26%)	154368 (0.44%)
QxxlPf	4.80	7.20	3.5e-03	A2	73 (0.26%)	89871 (0.25%)
WDrxxxxt	4.60	6.90	8.4e-03	A1	16 (0.06%)	35821 (0.1%)
lsxpK	6.40	8.40	2.3e-02	A1	46 (0.17%)	85081 (0.24%)
QPxxPf	7.60	9.40	4.0e-02	A1	275 (0.99%)	385819 (1.09%)
snHk	6.70	3.90	1.4e-03	B	38 (0.14%)	42742 (0.12%)
pxPtxn	7.30	5.30	3.0e-03	B	49 (0.18%)	43449 (0.12%)
kxtPxnIS	6.70	4.20	3.3e-03	A2	37 (0.13%)	48498 (0.14%)
pskT	8.10	6.50	5.1e-03	B	47 (0.17%)	64470 (0.18%)
kxRPtxxt	8.20	6.40	1.4e-02	A1	86 (0.31%)	122694 (0.35%)
YxDxxLN	9.20	7.50	1.6e-02	A2	224 (0.81%)	334062 (0.94%)
rxxDTxxs	10.40	9.30	2.0e-02	B	421 (1.52%)	532983 (1.5%)
pqNtk	9.20	7.50	3.1e-02	B	130 (0.47%)	216865 (0.61%)

* Mean peptide count is reported as the mean 2log.Total number of unique peptide sequences: 27775. Total peptide count: 35452858.

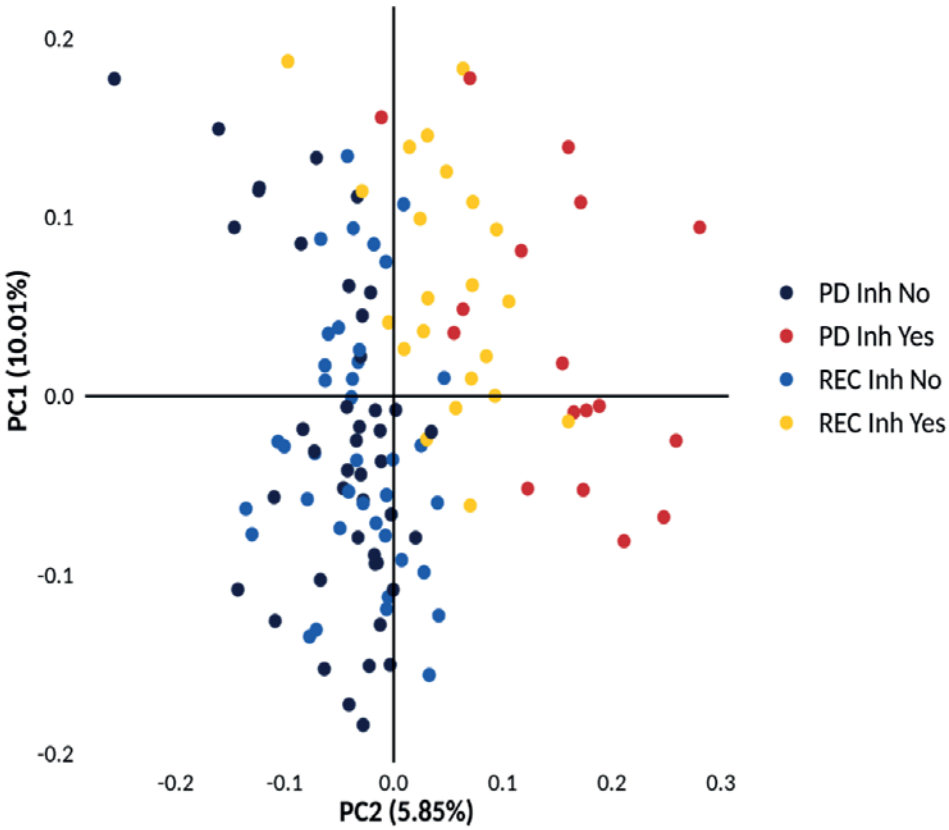
Comparison with the literature

The consensus motif "pxyNw" was mapped to the A2 domain, on residues Phe528 to Trp532 of FVIII. It has been previously reported that this region is part of a binding site for FIXa.[24] To our knowledge, there have been no previous publications of an epitope targeting this region of FVIII. Interestingly, this epitope motif was more common in inhibitor-negative patients than in inhibitor-positive patients.

The consensus motif "sWphxxxxk" was mapped to the C2 domain, on residues Ser2331 to Arg2339, which have been reported to be involved in binding to von Wille-

Figure 1. Principal component analysis performed on clusters of peptide sequences found at the end of the study, for the total cohort.

PD Inh No: inhibitor-negative patient using pdFVIII.
PD Inh Yes: inhibitor-positive patient using pdFVIII. REC Inh No: inhibitor-negative patient using rFVIII. REC Inh No: inhibitor-positive patient using rFVIII.



brand factor and phospholipids. Furthermore, there have been reports describing anti-FVIII antibodies targeting this region.[25]

The consensus motif "PslxWk" was mapped to the B domain, on residues Glu1037 to Phe1042. To our knowledge, there have been no previous publications of an epitope targeting this region of FVIII.

Table 2. Distribution of consensus motifs on domains of FVIII.

FVIII domains	Count (total group)	Count (pdFVIII group)	Count (rFVIII group)
INH+ clusters	9	13	7
A1	4 (44%)	3 (23%)	1 (14%)
A2	3 (33%)	2 (15%)	2 (29%)
B	0 (0%)	6 (46%)	3 (43%)
A3	1 (11%)	1 (8%)	1 (14%)
C1	1 (11%)	0 (0%)	0 (0%)
C2	0 (0%)	1 (8%)	0 (0%)
INH- clusters	8	1	4
A1	1 (12%)	1 (100%)	1 (25%)
A2	2 (25%)	0 (0%)	1 (25%)
B	5 (62%)	0 (0%)	2 (50%)
A3	0 (0%)	0 (0%)	0 (0%)
C1	0 (0%)	0 (0%)	0 (0%)
C2	0 (0%)	0 (0%)	0 (0%)

INH+ clusters: clusters that were more common in inhibitor-positive patients. INH- clusters: clusters that were more common in inhibitor-negative patients

Interestingly, this epitope motif (that was mapped to the B-domain) was more common in inhibitor-positive patients than in inhibitor-negative patients. This is in contrast with previous studies that have suggested that antibodies against the B-domain might be predominantly of the non-neutralizing type[26-28], as the B-domain is not essential for the role of FVIII in blood clotting and is cleaved off after FVIII is activated.

Overall, two out of three consensus motifs that were predictive for inhibitor development were directed against the A2 and C2 domains respectively, which is in line with the results of previous studies that suggest that most antibodies are directed against the A2 and C2 domains.[8, 29-31]

Previous studies have also shown that the peptide presentation profile of monocyte-derived dendritic cells changes when exposed to the FVIII-VWF complex[32,33]. In

our study, the overall epitope profile in the rFVIII group was similar to that of the pdFVIII group in terms of the distribution across FVIII domains of the epitope motifs. However, due to the very small number of consensus motifs, no definitive conclusions can be drawn from these results.

Table 3. Consensus motifs of clusters of peptide sequences with a significantly higher count in either inhibitor-negative (INH-) patients or inhibitor-positive (INH+) patients in the recombinant-derived FVIII treatment group.

Consensus motif	Mean peptide count* in IHN- Group	Mean peptide count* in IHN+ Group	Adjusted P-value	FVIII Domain(s)	Number of unique peptide sequences in cluster (%)	Peptide count of cluster (%)
PTNlxk	7.50	10.00	2.2e-04	B	40 (0.16%)	98681 (0.67%)
sxPxfT	5.00	7.80	3.7e-03	A3	32 (0.13%)	35683 (0.24%)
kyQqlsxxlp	5.20	7.60	1.2e-02	A2	11 (0.04%)	23800 (0.16%)
Qqyxp	7.10	8.90	1.4e-02	A2	39 (0.15%)	60093 (0.41%)
tyvEPxqxxr	5.90	7.80	2.0e-02	A1	8 (0.03%)	32077 (0.22%)
ppxxnxs	5.80	8.30	2.3e-02	B	56 (0.22%)	53923 (0.36%)
pSdsVxs	4.30	7.00	4.3e-02	B	14 (0.06%)	28539 (0.19%)
pWsk	10.40	8.40	3.6e-03	B	147 (0.58%)	276673 (1.87%)
pSNp	6.80	3.80	7.4e-03	A1	42 (0.17%)	31351 (0.21%)
qxixNsK	7.70	4.50	2.8e-02	B	119 (0.47%)	194793 (1.31%)
pxyNw	8.40	5.40	4.7e-02	A2	63 (0.25%)	73568 (0.5%)

* Mean peptide count is reported as the mean 2log. Total number of unique peptide sequences: 25235. Total peptide count: 14820947.

The presence of peptide sequences with high affinity for anti-FVIII antibodies in samples taken before treatment with FVIII might seem unexpected at first glance. However, several studies have reported the presence of non-neutralizing anti-FVIII antibodies in healthy controls.[34] In addition, a previous study using pre-treatment samples of the current cohort reported that roughly 10% of patients had measurable anti-FVIII antibodies.[35] This suggests that natural autoreactivity against endogenous FVIII is relatively common in patients as well as healthy controls. Another hypothesis could be that the detected antibodies were not initially directed against FVIII, but were the result of previous exposure to a pathogen (e.g. a bacteria or virus) that contained a similar epitope. This cross-reactivity of the antibody response has been previously reported in several auto-immune disorders.[36]

Figure 2. Figure showing the location on the FVIII molecule and the mean peptide count in the pre-treatment samples of the three clusters (with motifs "pxyNw", "PslxWk" and "sWphxxxxk") that were able to predict inhibitor development before exposure to FVIII.

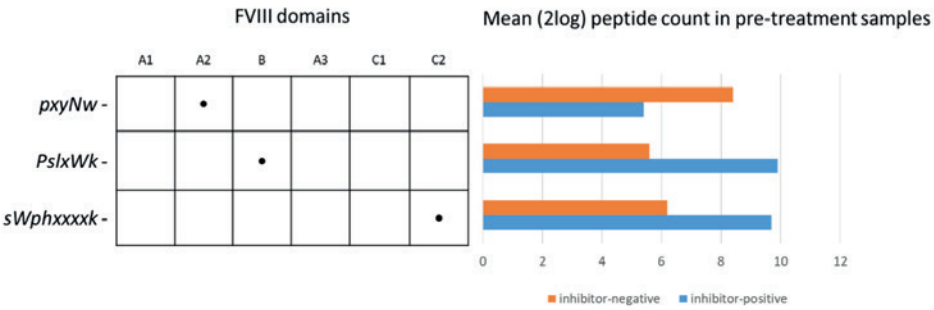
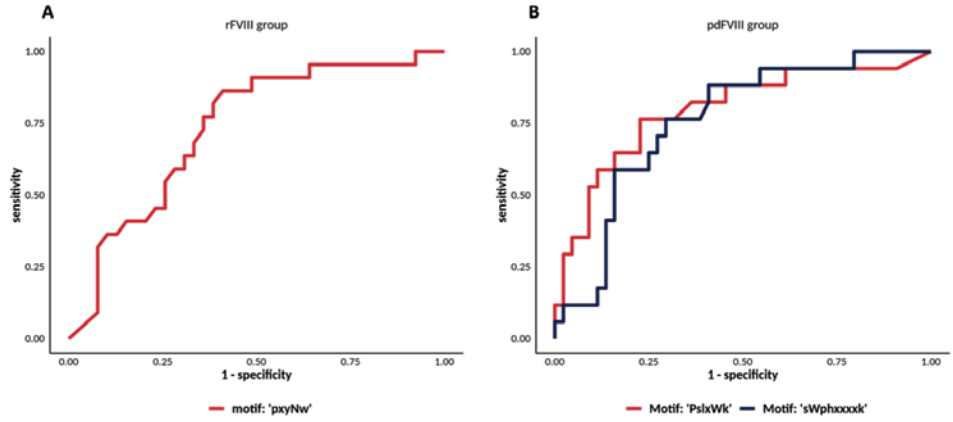


Figure 3. ROC curves showing the degree to which the three clusters (with motifs "pxyNw", "PslxWk" and "sWphxxxxk") were able to predict inhibitor development before exposure to FVIII.



Limitations

This approach has some major limitations. Firstly, it has been shown that only a handful of contact residues within an epitope make a significant contribution to antibody binding.[37] In this study, we tried to identify these residues by clustering highly similar peptide sequences and generating a consensus motif. Using alanine walk mutational analysis, the study by Kahle et al.[18] showed that there was reasonable agreement between a given consensus motif and the crucial binding residues of an epitope. Therefore, the consensus motifs derived from the multiple sequence align-

ment of each cluster of peptide sequences can, in theory, be considered to be potential epitope motifs. However, the accuracy of this approach is unknown and further verification is needed to identify the exact residues involved in binding to an antibody.

Table 4. Consensus motifs of clusters of peptide sequences with a significantly higher count in either inhibitor-negative (INH-) patients or inhibitor-positive (INH+) patients in the plasma-derived FVIII treatment group.

Consensus motif	Mean peptide count* in IHN- Group	Mean peptide count* in IHN+ Group	Adjusted P-value	FVIII Domain(s)	Number of unique peptide sequences in cluster (%)	Peptide count of cluster (%)
PslxWk	5.60	9.90	1.3e-04	B	90 (0.34%)	83908 (0.41%)
qxNxStk	4.10	8.20	7.1e-04	B	52 (0.2%)	59042 (0.29%)
SqnK	8.40	11.40	7.9e-04	B	128 (0.48%)	314257 (1.52%)
Wskp	4.20	8.30	1.2e-03	B	39 (0.15%)	41553 (0.2%)
PHtxk	6.10	10.10	1.3e-03	A2	91 (0.34%)	100570 (0.49%)
pwwp	5.20	8.80	2.4e-03	A1	26 (0.1%)	32816 (0.16%)
PxtFxKp	5.20	8.80	4.2e-03	A1	52 (0.2%)	40083 (0.19%)
iKPxl	4.30	8.40	5.0e-03	B	22 (0.08%)	37313 (0.18%)
sWphxxxxk	6.20	9.70	6.1e-03	C2	41 (0.15%)	94409 (0.46%)
txpmMss	3.70	8.10	1.0e-02	A3	26 (0.1%)	37165 (0.18%)
sGPQ	3.60	7.80	1.0e-02	A2	24 (0.09%)	32688 (0.16%)
nqnK	5.80	10.00	1.2e-02	B	92 (0.35%)	195716 (0.95%)
pdxTpwp	5.00	8.80	1.4e-02	A1	45 (0.17%)	51658 (0.25%)
KxxNexY	7.30	3.70	2.5e-02	A1	57 (0.21%)	81886 (0.4%)

* Mean peptide count is reported as the mean 2log. Total number of unique peptide sequences: 26641. Total peptide count: 20631911.

Furthermore, peptide sequences were clustered based on sequence similarity. However, peptide sequences targeted by the same antibody could have similar physicochemical properties despite not being similar in terms of their amino acid sequence. In this case, clustering based on sequence similarity will not yield optimal results and alternative approaches that take the physicochemical properties of peptide sequences into account might prove more useful.

Each cluster of peptide sequences contained only a small proportion (0.03-1.52%) of the total number of unique peptide sequences available for the clustering step.

Ideally, each cluster would have contained a large proportion of the total number of unique peptide sequences as this would have provided us with stronger evidence for a cluster being related to an epitope.

In addition, the final epitope motifs were mapped to FVIII by aligning the motifs to the linear sequence of FVIII. However, it has been reported that the majority of B-cell epitopes are conformational.[38, 39] (although the exact proportion of B-cell epitopes purported to be conformational is unknown) Therefore, the accuracy of this approach is most likely not high. An alternative approach would involve mapping the epitope motifs to the three-dimensional structure of FVIII, using an in-silico approach. However, a recent study that assessed a set of B-cell epitope prediction algorithms against a benchmark dataset reported that all algorithms performed relatively poorly at mapping a potential epitope to the right location on an antigen.[40]

We removed all peptide sequences that were predicted to be target-unrelated (based on software exploiting publicly available repositories[21]) from the final peptide sequence database. However, the residual impact of target-unrelated peptide sequences that were not removed from the database on the results is difficult to quantify. In addition, some peptide sequences have affinity to both elements of assay as well as an IgG antibody. (i.e. they can be classified as both target-unrelated and target-related peptides) By removing these peptide sequences, we might have inadvertently also removed some important peptide sequences from the initial database.

From the output of the assay, only peptide sequences with a count higher than 250 were selected, this resulted in a much smaller dataset. The cut-off was based on the intra- and inter-assay replicability (Figure S1). It is possible that many peptide sequences that were the target of a FVIII-specific antibody were removed in this step.

Lastly, our analysis of the immune response did not include non-peptidic epitopes (such as the glycans present on the surface of FVIII). One difference between rFVIII and pdFVIII is in their respective glycosylation patterns.[41] Unfortunately, our approach does not allow assessment of the impact of differing glycosylation patterns on immunogenicity.

Conclusion

The reported information on immunodominant epitopes may aid the development of novel, less immunogenic FVIII proteins. In addition, we found several clusters of

peptide sequences that were detectable in patients without any exposure to exogenous FVIII. Information on these clusters could be used to set up diagnostic tests that predict the risk of inhibitor development before starting treatment with FVIII.

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References

1. Peyvandi F, Garagiola I, Young G. The past and future of haemophilia: diagnosis, treatments, and its complications. *Lancet*. 2016;388(10040):187-197. doi:10.1016/s0140-6736(15)01123-x
2. Peyvandi F, Mannucci PM, Garagiola I, et al. A Randomized Trial of Factor VIII and Neutralizing Antibodies in Hemophilia A. *N Engl J Med*. 2016;374(21):2054-2064. doi:10.1056/nejmoa1516437
3. Cormier M, Batty P, Tarrant J, Lillicrap D. Advances in knowledge of inhibitor formation in severe haemophilia A. *Br J Haematol*. 2020;189(1):39-53. doi:10.1111/bjh.16377
4. Yee A, Oleskie AN, Dosey AM, et al. Visualization of an N-terminal fragment of von Willebrand factor in complex with factor VIII. *Blood*. 2015;126(8):939-942. doi:10.1182/blood-2015-04-641696
5. Pezeshkpoor B, Schreck U, Biswas A, et al. An in silico and in vitro approach to elucidate the impact of residues flanking the cleavage scissile bonds of FVIII. *PLoS One*. 2017;12(7). doi:10.1371/journal.pone.0180456
6. Lollar P, Parker CG. Subunit Structure of Thrombin-Activated Porcine Factor VIII. *Biochemistry*. 1989;28(2):666-674. doi:10.1021/bi00428a038
7. Van Dieijen G, Tans G, Rosing J, Hemker HC. The role of phospholipid and factor VIII(a) in the activation of bovine factor X. *J Biol Chem*. 1981;256(7):3433-3442. doi:10.1016/s0021-9258(19)69627-4
8. Lollar P. Pathogenic antibodies to coagulation factors. Part one: Factor VIII and Factor IX. *J Thromb Haemost*. 2004;2(7):1082-1095. doi:10.1111/j.1538-7836.2004.00802.x
9. Lai J, Hough C, Tarrant J, Lillicrap D. Biological considerations of plasma-derived and recombinant factor VIII immunogenicity. *Blood*. 2017;129(24):3147-3154. doi:10.1182/BLOOD-2016-11-750885
10. Lacroix-Desmazes S, Voorberg J, Lillicrap D, Scott DW, Pratt KP. Tolerating Factor VIII: Recent Progress. *Front Immunol*. 2020;10:2991. doi:10.3389/fimmu.2019.02991
11. Healey JF, Lubin IM, Nakai H, et al. Residues 484-508 contain a major determinant of the inhibitory epitope in the A2 domain of human factor VIII. *J Biol Chem*. 1995;270(24):14505-14509. doi:10.1074/jbc.270.24.14505
12. Barrow RT, Healey JF, Gailani D, Scandella D, Lollar P. Reduction of the antigenicity of factor VIII toward complex inhibitory antibody plasmas using multiply-substituted hybrid human/porcine factor VIII molecules. *Blood*. 2000;95(2):564-568. doi:10.1182/blood.v95.2.564

13. Healey JF, Barrow RT, Tamim HM, et al. Residues Glu2181-Val2243 Contain a Major Determinant of the Inhibitory Epitope in the C2 Domain of Human Factor VIII. *Blood*. 1998;92(10):3701-3709. doi:10.1182/blood.v92.10.3701
14. Lavigne-Lissalde G, Rothschild C, Pouplard C, et al. Characteristics, mechanisms of action, and epitope mapping of anti-factor VIII antibodies. *Clin Rev Allergy Immunol*. 2009;37(2):67-79. doi:10.1007/s12016-009-8119-0
15. Villard S, Lacroix-Desmazes S, Kieber-Emmons T, et al. Peptide decoys selected by phage display block in vitro and in vivo activity of a human anti-FVIII inhibitor. *Blood*. 2003;102(3):949-952. doi:10.1182/blood-2002-06-1886
16. Villard S, Piquer D, Raut S, Léonetti JP, Saint-Remy JM, Granier C. Low molecular weight peptides restore the procoagulant activity of factor VIII in the presence of the potent inhibitor antibody ESH8. *J Biol Chem*. 2002;277(30):27232-27239. doi:10.1074/jbc.M203415200
17. Mühle C, Schulz-Drost S, Khrenov A V., Saenko EL, Klinge J, Schneider H. Epitope mapping of polyclonal clotting factor VIII-inhibitory antibodies using phage display. *Thromb Haemost*. 2004;91(3):619-625. doi:10.1160/th03-07-0473
18. Kahle J, Orlowski A, Stichel D, et al. Epitope mapping via selection of anti-FVIII antibody-specific phagepresented peptide ligands that mimic the antibody binding sites. *Thromb Haemost*. 2015;113(2):396-405. doi:10.1160/TH14-01-0101
19. Duncan E, Collecutt M, Street A. Nijmegen-Bethesda assay to measure factor VIII inhibitors. *Methods Mol Biol*. 2013;992:321-333. doi:10.1007/978-1-62703-339-8_24
20. Sadam H, Pihlak A, Jaago M, et al. Identification of two highly antigenic epitope markers predicting multiple sclerosis in optic neuritis patients. *EBioMedicine*. 2021;64. doi:10.1016/j.ebiom.2021.103211
21. He B, Chen H, Li N, Huang J. Sarotup: A suite of tools for finding potential target-unrelated peptides from phage display data. *Int J Biol Sci*. 2019;15(7):1452-1459. doi:10.7150/ijbs.31957
22. Krejci A, Hupp TR, Lexa M, Vojtesek B, Muller P. Hammock: A hidden Markov model-based peptide clustering algorithm to identify protein-interaction consensus motifs in large datasets. *Bioinformatics*. 2016;32(1):9-16. doi:10.1093/bioinformatics/btv522
23. Dunn OJ. Multiple Comparisons among Means. *J Am Stat Assoc*. 1961;56(293):52-64. doi:10.1080/01621459.1961.10482090
24. Celie PHN, Van Stempvoort G, Jorieux S, Mazurier C, Van Mourik JA, Mertens K. Substitution of Arg527 and Arg531 in factor VIII associated with mild haemo-

philia A: characterization in terms of subunit interaction and cofactor function. *Br J Haematol*. 1999;106(3):792-800. doi:10.1046/J.1365-2141.1999.01590.X

25. Nogami K, Shima M, Giddings JC, Takeyama M, Tanaka I, Yoshioka A. Relationship between the binding sites for von Willebrand factor, phospholipid, and human factor VIII C2 inhibitor alloantibodies within the factor VIII C2 domain. *Int J Hematol*. 2007;85(4):317-322. doi:10.1532/IJH97.06192
26. Whelan SFJ, Hofbauer CJ, Horling FM, et al. Distinct characteristics of antibody responses against factor VIII in healthy individuals and in different cohorts of hemophili A patients. *Blood*. 2013;121(6):1039-1048. doi:10.1182/blood-2012-07-444877
27. Lebreton A, Lapalud P, Chambost H, et al. Prevalence and epitope specificity of non-neutralising antibodies in a large cohort of haemophilia A patients without inhibitors. *Thromb Haemost*. 2011;105(6):954-961. doi:10.1160/TH10-10-0668
28. Vincent AM, Lillicrap D, Boulanger A, et al. Non-neutralizing anti-FVIII antibodies: different binding specificity to different recombinant FVIII concentrates. *Haemophilia*. 2009;15(1):374-376. doi:10.1111/J.1365-2516.2008.01909.X
29. Prescott R, Nakai H, Saenko EL, et al. The inhibitor antibody response is more complex in hemophilia a patients than in most nonhemophiliacs with factor VIII autoantibodies. *Blood*. 1997;89(10):3663-3671. doi:10.1182/blood.v89.10.3663
30. Astermark J. Basic aspects of inhibitors to factors VIII and IX and the influence of non-genetic risk factors. *Haemophilia*. 2006;12(SUPPL. 6):8-14. doi:10.1111/j.1365-2516.2006.01360.x
31. Scandella DH, Nakai H, Felch M, et al. In hemophilia A and autoantibody inhibitor patients: The factor VIII A2 domain and light chain are most immunogenic. *Thromb Res*. 2001;101(5):377-385. doi:10.1016/S0049-3848(00)00418-7
32. Sorvillo N, Hartholt RB, Bloem E, et al. von Willebrand factor binds to the surface of dendritic cells and modulates peptide presentation of factor VIII. *Haematologica*. 2016;101(3):309-318. doi:10.3324/haematol.2015.137067
33. Jankowski W, Park Y, McGill J, et al. Peptides identified on monocyte-derived dendritic cells: A marker for clinical immunogenicity to FVIII products. *Blood Adv*. 2019;3(9):1429-1440. doi:10.1182/bloodadvances.2018030452
34. Abdi A, Bordbar MR, Hassan S, et al. Prevalence and incidence of non-neutralizing antibodies in congenital hemophilia a—a systematic review and meta-analysis. *Front Immunol*. 2020;11. doi:10.3389/fimmu.2020.00563

35. Cannavò A, Valsecchi C, Garagiola I, et al. Nonneutralizing antibodies against factor VIII and risk of inhibitor development in severe hemophilia A. *Blood*. 2017;129(10):1245-1250. doi:10.1182/BLOOD-2016-06-720086
36. Rojas M, Restrepo-Jiménez P, Monsalve DM, et al. Molecular mimicry and autoimmunity. *J Autoimmun*. 2018;95:100-123. doi:10.1016/j.jaut.2018.10.012
37. Novotny J. Protein antigenicity: A thermodynamic approach. *Mol Immunol*. 1991;28(3):201-207. doi:10.1016/0161-5890(91)90062-O
38. Barlow DJ, Edwards MS, Thornton JM. Continuous and discontinuous protein antigenic determinants. *Nature*. 1986;322(6081):747-748. doi:10.1038/322747a0
39. Haste Andersen P, Nielsen M, Lund O. Prediction of residues in discontinuous B-cell epitopes using protein 3D structures. *Protein Sci*. 2006;15(11):2558-2567. doi:10.1110/ps.062405906
40. Sun P, Chen W, Huang Y, Wang H, Ma Z, Lv Y. Epitope prediction based on random peptide library screening: Benchmark dataset and prediction tools evaluation. *Molecules*. 2011;16(6):4971-4993. doi:10.3390/molecules16064971
41. Peyvandi F, Miri S, Garagiola I. Immune Responses to Plasma-Derived Versus Recombinant FVIII Products. *Front Immunol*. 2021;11. doi:10.3389/fimmu.2020.591878

Figure S1. Data quality control. Experimental reproducibility was assessed by comparing technical replicates. Intra-assay reproducibility **(A)** and inter-assay reproducibility **(B)** by minimum count threshold was assessed by calculating the percentage of sequences not present in both technical replicates. A threshold of 250 was chosen to ensure maximum experimental reproducibility.