Significance of F8 missense mutations with respect to inhibitor formation

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Summary
We have identified 1,135 haemophilia A patients with missense mutations associated with mild (46%), moderate (22%), severe (16%), and mixed haemophilia phenotypes (11%). Altogether, we detected 374 different missense mutations of which 195 are not listed in the HAMSTeRS database. While missense mutations are strongly under-represented within the factor VIII (FVIII) B-domain, they are evenly distributed throughout the entire F8 cDNA sequence. Only 36 (5%) of 720 patients with missense mutations and known inhibitor status showed an association with inhibitor formation. Inhibitor prevalence was four-fold higher for severe haemophilia compared to mild/moderate phenotypes. Mutations associated with inhibitor formation were especially clustered within the C1/C2 domain compared to the other domains (8.7% C1/C2 domain vs. 3.6% non-C1/C2-domain; p-value: 0.01). Three different missense mutations (T314A [T295A], S2010P [S1991P], R2169H [R2150H]) were associated twice with inhibitor formation. Importantly, we found that the risk of inhibitor formation in association with FVIII missense mutations is significant higher if the amino acid substitution belongs to another physicochemical class than the original residue (p-value 0.039). For this purpose distinct classes of substitutions were grouped in association with side chains properties: class I, small/hydrophobic; class II, neutral; class III, acidic; class IV, basic. Thus, although missense mutations were associated with an overall lower risk of inhibitor formation compared to other F8 gene mutation types, different missense mutations correlate with specific risks for inhibitor formation. These differences have to be identified in assigning risk profiles to aid in choice of preventative treatments designed to prevent inhibitor formation.

Keywords
Missense mutations, haemophilia A, inhibitor formation, domains, amino acid classes

Introduction
Missense mutations are the most common type of mutation among haemophilia A (HA) patients and occur at many positions of the F8 gene. Depending on the amino acid substitution and the specific location, missense mutations are responsible for mild, moderate, and severe haemophilia. Over 630 different missense mutations have been described in the HAMSTeRS database (HAMSTeRS: http://hadb.org.uk/). Some have been shown to interfere with FVIII biosynthesis and secretion, to interact with FIXa, FX, von Willebrand factor (VWF), phospholipid membrane surfaces, or to affect the stability of FVIII after activation (1).

For missense mutations of the B-domain, it is generally believed that the majority of these represent rather rare non-functional polymorphisms than dysfunctional mutations (2). For missense mutations outside the B-domain, a causative effect can likely be assumed, although confirmation of this requires further expression studies and functional characterisation (3).

Missense mutations confer the lowest risk of inhibitor formation (5%) compared to all other mutation types within the F8 gene. Nevertheless, a four-fold greater risk has been reported for missense mutations located within the C1 and C2 domains (4). Previous data suggested that the inhibitor prevalence for missense mutations depends significantly on their specific localisation within the FVIII protein. One other factor that might influence inhibitor formation is the specific amino acid substitution. According to the different properties of the side chains, amino acids can be grouped into different physicochemical classes (5). Missense mutations that result in a switch of amino acid classes are thought to have more pronounced effects on protein function than substitution of an amino acid of the same class.

Investigation of FVIII missense mutations, in general, has provided insights into structure and function of wild-type FVIII, into the underlying mechanisms of HA and into the risk of inhibitor development during replacement therapy (6-8).

In the present study, we aimed to investigate the missense mutations in 1,135 patients with regard to different phenotypes. Since the inhibitor status was known for 720 of these patients, we examined the relative frequencies of inhibitor formation of missense mutations for different phenotypes and for different FVIII do-
mains. Furthermore, we addressed for each missense mutation, whether a change of an amino acid class influence the risk of inhibitor formation.

**Patients, material and methods**

**Patients**

A total of 1,135 patients with missense mutations were included in the study. The inhibitor status was known for 720 patients comprising 36 different inhibitors. The patients’ samples were obtained from different haemophilia centres in Germany with 367 of these from the Haemophilia Centre of Bonn. Patients treated in our centre are all Caucasians, almost all of German origin. The other centres should have similar distribution, so the results most likely mirror the missense mutations of Caucasian and German population, respectively. Informed patient consent was obtained according to the Declaration of Helsinki.

**Coagulation assays**

Blood was collected by peripheral venous puncture as a 1:10 volume ratio in 3.8% trisodium citrate. At the Bonn centre factor VIII activity (FVIII:C) was determined by an in-house one-stage activated partial thrombin time (aPTT)-based assay, processed on a KC10A coagulation analyser (Trinity Biotech, Lemgo, Germany) with FVIII-deficient plasma from Helena (UK). At the other centers FVIII:C was determined according to local standards. FVIII inhibitor activity was determined using the modified Nijmegen Bethesda method (9). An inhibitor was defined as a current or previous inhibitor with a titer of ≥0.6 Bethesda unit (BU). A value ≥0.6 Bethesda unit (BU) has to be verified at least twice.

**F8 gene analyses**

High-molecular-weight genomic DNA was isolated from whole blood by a salting out procedure (10). Various techniques for F8 gene analyses were used. The positions of mutations were correlated with inhibitor formation are represented by arrows. Positions of mutations T314A [T295A, exon 7], S2010P [S1991P, exon 19], R2169H [R2150H, exon 23] which were associated twice with inhibitor formation are only labelled by one arrow. The different colours of arrows represent different severities of haemophilia (red: severe; orange: moderate; yellow: mild). The exons are also coloured differently for each of the different factor VIII domains (A1/A2/B/A3/C1/C2) without indication of the small acidic regions.

**Figure 1:** Missense mutation distribution according to phenotype for all 1,135 patients.

**Figure 2:** Positions of 326 different missense mutations within the F8 gene and their corresponding phenotypes. The positions of mutations correlated with inhibitor formation are represented by arrows. Positions of mutations T314A [T295A, exon 7], S2010P [S1991P, exon 19], R2169H [R2150H, exon 23] which were associated twice with inhibitor formation are only labelled by one arrow. The different colours of arrows represent different severities of haemophilia (red: severe; orange: moderate; yellow: mild). The exons are also coloured differently for each of the different factor VIII domains (A1/A2/B/A3/C1/C2) without indication of the small acidic regions.
point mutation detection were applied including Denaturing Gradient Gel Electrophoresis (DGGE), Chemical Mismatch Cleavage (CMC), and Denaturing High Performance Liquid Chromatography (DHPLC) (11-13). All identified mutations were verified by direct sequencing. Since 2005, the F8 genetic diagnostic was achieved through first line direct sequencing of all 26 exons and flanking intronic regions of the F8 gene (primers and conditions available on request).

Amino acid classes

For the differentiation of intra- and inter-amino acid class switches we grouped amino acids into four different classes according to the physicochemical properties of their side chains (5, 14). These classes comprised: I. small/hydrophobic amino acids [A, V, F, P, M, I, L, W], II. neutral amino acids [S, Y, N, Q, C, T, H, G], III. acidic amino acids [D, E], and IV. basic amino acids [K, R].

Statistical analysis

A conventional Fisher’s exact test was applied to compare the inhibitor frequencies of missense mutations for different domains and with respect to intra- and inter-amino acid class switches. A p-value <0.05 was considered to indicate statistical significance.

Results

Missense mutations were identified in 1,135 patients. Most missense mutations were associated with mild haemophilia (46%) followed by moderate (22%) and severe (16%) phenotypes (Figure 1). We defined mixed phenotypes (11%) as identical missense mutations observed in several patients, but with different phenotypes (mild/moderate or moderate/severe). No information about phenotype severity was reported for 5% of patients.

Table 2: Number of missense mutations together with associated number of inhibitor formation (patients within brackets) sorted for intra- (dark grey fields) and inter-amino acid (grey fields) changes. The left side of the table gives the number of intra- and inter-amino acid changes and inhibitor formation (in brackets) for each class of amino acid individually; the right side of the table summarises results of intra- and inter-amino acid class substitutions. While statistical evaluation of each single class switch results in different convincing p-values, summarised outcome (total intra-amino acid substitution vs. total inter-amino acid substitution) results in a significant p-value of 0.039 (Fisher’s exact test). According to the properties of their side chains, amino acids are grouped in four different classes: class 1 = small or hydrophobic: A, V, L, I, M, P, F and W; class 2 = uncharged/polar: S, Y, N, Q, C, T, H, G; class 3 = acidic: D, E; class 4 = basic: K, R (5, 14). Three missense mutations have been excluded from this analysis since they represent changes in the original F8 stop codon (TGA). All are non-stop mutations with two being substitutions to Cys and one to Gly. One of the two stop->Cys substitutions was associated with inhibitor formation. Aa: amino acid; wt: wild type.

Table 1: Number of patients by phenotype and number of inhibitor patients within each phenotype.

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Patients</th>
<th>Inhibitors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mild</td>
<td>336</td>
<td>10</td>
</tr>
<tr>
<td>Moderate</td>
<td>195</td>
<td>7</td>
</tr>
<tr>
<td>Severe</td>
<td>116</td>
<td>15</td>
</tr>
<tr>
<td>Mixed</td>
<td>73</td>
<td>4</td>
</tr>
<tr>
<td>All patients</td>
<td>720</td>
<td>36</td>
</tr>
</tbody>
</table>

We found 374 different missense mutations. Of these, 195 are not among those reported in the HAMSteRS database. Positions of the various missense mutations (326 with known phenotype) are labelled on the F8 gene sequence (Figure 2). The missense mutations are distributed throughout the F8 gene, with the greatest representation in exons 4, 7 and 23. Notably, missense mutations are strongly underrepresented within the B domain.

Missense mutations are typically associated with low inhibitor risk. Within our patient cohort, inhibitor status was available for 720 patients. Of these, 5.0% (36/720) developed inhibitors. Inhibitor formation was observed for pairs of patients at position 314 [295] (T314A [T295A]; both patients exhibit mild haemophilia), at position 315 [296] (S2150P [S1991P]; both patients exhibit severe haemophilia), and at position 2169 [2150] (R2169H [R2150H]; both patients exhibit moderate haemophilia). The first mentioned amino acid position gives numbering according to Human Genome Variation Society (HGVS) whereas number in brackets gives the “legacy” amino acid position of the mature protein.

The distribution of mutations of different severities, as well as fraction of inhibitors for each severity, is summarised in Table 1 and Figure 2. The inhibitor frequency is four-fold greater for se-
vere haemophilia than for milder forms of the disease. Of the 36 inhibitor patients, 10 patients had missense mutations within the A1 domain, nine within the C1 domain, and eight within the C2 domain (▶ Figure 2).

Missense mutations restricted to the C1 and C2 domains exhibited a significantly greater inhibitor frequency (8.7%) than mutations outside the C1 and C2 domains (3.6%) (p-value of 0.01). This difference was even more pronounced, when we compared inhibitor/missense mutation ratio of the C2 domain with the inhibitor/missense ratio for all non-C2 FVIII domains combined (14.3% vs. 4.2%; p-value: 0.004). Additionally, we found that inhibitor risk of missense mutations within the C1/C2 domain and non-C1/C2 domains is not constant, but differs between mild/moderate and severe haemophilia (mild/moderate: 7.1% [C1/C2] vs. 1.9% [non-C1/C2]; severe: 18.5% [C1/C2] vs. 11.3% [non-C1/C2]).

In a further analysis, we assigned four different classes of amino acids according to the properties of their side chains (class 1:

Table 3: Correlation between inhibitor frequency of missense mutations and intra- and inter-amino acid class switches. Numbering of codons is according to Human Genome Variation Society (HGVS) as well as to the mature protein (mat. prot.) Intra-amino acid switches are given in bold. Amino acids are presented with the one letter code.

<table>
<thead>
<tr>
<th>Wildtype Aa</th>
<th>Mutated Aa</th>
<th>Codon No HGVS / [mat. prot.]</th>
<th>Number of inhibitors</th>
<th>Class of Aa Property</th>
<th>Severity</th>
<th>FVIII-domain</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>T</td>
<td>723 / [704]</td>
<td>1</td>
<td>1 &gt; 2</td>
<td>moderate</td>
<td>A2</td>
</tr>
<tr>
<td>D</td>
<td>A</td>
<td>182 / [163]</td>
<td>1</td>
<td>3 &gt; 1</td>
<td>mild</td>
<td>A1</td>
</tr>
<tr>
<td>E</td>
<td>G</td>
<td>2093 / [2074]</td>
<td>1</td>
<td>1 &gt; 2</td>
<td>mild</td>
<td>C1</td>
</tr>
<tr>
<td>F</td>
<td>S</td>
<td>1937 / [1918]</td>
<td>1</td>
<td>1 &gt; 2</td>
<td>severe</td>
<td>A3</td>
</tr>
<tr>
<td>G</td>
<td>E</td>
<td>2266 / [2247]</td>
<td>1</td>
<td>1 &gt; 2</td>
<td>mild</td>
<td>C1</td>
</tr>
<tr>
<td>K</td>
<td>E</td>
<td>185 / [166]</td>
<td>1</td>
<td>4 &gt; 3</td>
<td>severe</td>
<td>A1</td>
</tr>
<tr>
<td>L</td>
<td>Q</td>
<td>216 / [197]</td>
<td>1</td>
<td>1 &gt; 2</td>
<td>severe</td>
<td>A1</td>
</tr>
<tr>
<td>M</td>
<td>R</td>
<td>11 / [-19]**</td>
<td>1</td>
<td>1 &gt; 4</td>
<td>severe</td>
<td>signal-seq.</td>
</tr>
<tr>
<td>N</td>
<td>K</td>
<td>188 / [169]</td>
<td>1</td>
<td>2 &gt; 4</td>
<td>severe</td>
<td>A1</td>
</tr>
<tr>
<td>P</td>
<td>K</td>
<td>2319 / [2300]</td>
<td>1</td>
<td>1 &gt; 1</td>
<td>mild</td>
<td>C2</td>
</tr>
<tr>
<td>Q</td>
<td>T</td>
<td>2126 / [2107]</td>
<td>1</td>
<td>4 &gt; 2</td>
<td>severe</td>
<td>A1</td>
</tr>
<tr>
<td>S</td>
<td>Q</td>
<td>2119 / [2100]</td>
<td>1</td>
<td>2 &gt; 4</td>
<td>severe</td>
<td>C1</td>
</tr>
<tr>
<td>R</td>
<td>C</td>
<td>391 / [372]</td>
<td>1</td>
<td>4 &gt; 2</td>
<td>severe</td>
<td>ar1</td>
</tr>
<tr>
<td>H</td>
<td>1800 / [1781]</td>
<td>1</td>
<td>4 &gt; 2</td>
<td>moderate</td>
<td>A3</td>
<td></td>
</tr>
<tr>
<td>L</td>
<td>2169 / [2150]</td>
<td>2</td>
<td>4 &gt; 2</td>
<td>severe</td>
<td>C2</td>
<td></td>
</tr>
<tr>
<td>Q</td>
<td>2326 / [2307]</td>
<td>1</td>
<td>4 &gt; 2</td>
<td>moderate</td>
<td>C2</td>
<td></td>
</tr>
<tr>
<td>S</td>
<td>C</td>
<td>2213 / [2194]</td>
<td>1</td>
<td>2 &gt; 2</td>
<td>severe</td>
<td>C2</td>
</tr>
<tr>
<td>P</td>
<td>T</td>
<td>314 / [295]</td>
<td>2</td>
<td>2 &gt; 1</td>
<td>mild</td>
<td>A1</td>
</tr>
<tr>
<td>W</td>
<td>C</td>
<td>412 / [393]</td>
<td>1</td>
<td>1 &gt; 2</td>
<td>severe</td>
<td>A2</td>
</tr>
</tbody>
</table>

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small/hydrophobic; class 2: neutral; class 3: acidic; class 4: basic). Then we posed the question, whether inhibitors arise more frequently if the wild-type amino acid is replaced by an amino acid of another class (inter-class substitution) or if it is substituted by an amino acid from the same class (intra-class substitution). We hypothesised that the former case would result in a higher risk of inhibitor formation than the latter because of the highly different physicochemical properties between wild-type and substituted amino acids. ► Table 2 shows inhibitor risk of intra- (dark grey fields) and inter-amino acid (grey fields) class switches for each amino acid position separately and in combination. P-values were calculated by correlating the frequencies of inhibitor formation of intra- and inter-amino acid class switches with the respective number of missense mutations. Evaluation of each wild-type amino acid class separately showed that the most significant risk of inhibitor formation (p value 0.048) exists when inter-amino acid class switches occur at class 1 amino acids (small/hydrophobic). Comparison of all intra- and inter-amino acid switches showed that only three of 168 intra-amino acid substitutions (1.8%) were associated with inhibitor formation. In contrast, a significantly greater incidence of inhibitor formation was observed in the case of inter-amino acid substitutions (32/549; 5.8%; p=0.039). All inter- or intra-amino acid substitutions for missense mutations correlated with inhibitor formation are summarised in detail in ► Table 3.

Discussion

Missense mutations can result either in mild, moderate or severe haemophilia phenotypes. It has also been reported that the same missense mutation could result in different phenotypes that we define as mixed phenotypes. One reason for this could be the use of different FVIII:C assays for the determination of the FVIII:C from patient plasma. Accordingly, it has previously been demonstrated that discrepancy between one-stage and chromogenic FVIII:C assays exists for various mutations (15). Poulsen et al. reported FVIII:C assay discrepancies in about one third of patients with non-severe haemophilia (16). Thus, the results of FVIII:C activity assays are also dependent upon which assay is used for routine diagnosis. The use of different APTT reagents and the analytic system add to the inter-laboratory influences on FVIII activity (17). Further factors which could influence the phenotype of patients are prothrombotic mutations and inter-individual variance in the pharmacokinetics of FVIII based on different ABO blood group of patients (18, 19). The different blood groups influence the level of von Willebrand factor, the carrier for FVIII.

In our study, missense mutations conferred a low risk of inhibitor formation (5%), similar to the results of recent publications (4, 20). A possible explanation for this is that patients with missense mutations synthesise some endogenous protein that, although functionally altered, is sufficient to induce immune tolerance. However, depending on the localisation of the mutations, differences were observed in inhibitor risk within our study data. While for mutations within the C1 and C2 domains (codon 2039 [2020] to 2351 [2332]) inhibitor formation was 8.7%, mutations outside the C1 and C2 domains occurred with a frequency of only 3.6% (p-value of 0.01).

The clustering of mutations with greater inhibitor risk within the C1 and C2 domains (17 out of 36) can be seen in ► Table 3 and is in agreement with previously reported data (21), but is not in exact agreement with data from the ongoing INSIGHT study (22). Within the INSIGHT study, correlation between missense mutations and inhibitors was found for sequence stretches from codon 550 [531] to 687 [668] (within the A2 domain), and from 1780 [1761] until 2352 [2333] (last segment of A3/C1/C2). Differences here may stem from sampling bias due to the inclusion of large families (e.g. R612C [R593C]).

Altogether, among the group of inhibitor patients we identified 17 missense mutations within the C1 and C2 domains of which 12 were affected with mild/moderate haemophilia. These data suggest that within all phenotypes amino acid substitutions in the C1 and C2 domains lead to changes in the three-dimensional structure of the FVIII protein which may influence von Willebrand factor binding and FVIII binding to phospholipid membranes (4).

Data from the haemophilia A database (http://hadb.org.uk/) and the present study show that a distinct subset of missense mutations in the F8 gene can be found repeatedly within different patients. This could either be caused by a founder effect, as for Y1699F [Y1680F] within the German population or as for R612C [R593C] within the Dutch population, but could also be caused by independent spontaneous mutational events. The repeated occurrence of the same missense mutation in different patients appears frequently at CpG dinucleotides which are often methylated and therefore sensitive for C→T substitutions on the coding strand as well as for G→A substitutions on the antisense strand. Since codons for Arginine contain the highest incidence of CpGs, they are mostly affected. In our study comprising a cohort of 1135 HA patients, three most common missense-mutations are Y1699F [Y1680F (52x), R546W [R527W] (46x), and R2169H [R2150H] (36x).

Beside the cluster of missense mutations within the C1 and C2 domains, there are also individual amino acid substitutions associated with a high risk of inhibitor formation. The increased influence of specific missense mutations on inhibitor formation up to 50% was recently reported for R612C [R593C], Y2124C [Y2105C], R2169H [R2150H], W2248C [W2229C], and P2319L [P2300L] (4). In our study covering 720 patients with known inhibitor status, inhibitors arose only twice in 28 patients presenting the R2169H [R2150H] substitution, twice in three patients affected with a S2010P [S1991P] substitution, and twice in 15 patients with the Y314A [Y295A] substitution (► Table 3). The S2010P [S1991P] substitution may be a further amino acid change which presents a high risk of inhibitor development because this mutation has not yet been reported in the Haemophilia A Mutation Database (status: September 30, 2012). Further substitutions with a potential risk of inhibitor formation are the other newly found amino acid changes within this study showing association with inhibitor formation (see ► Table 3; newly identified amino acid changes are labelled by an asterisk). Another amino acid substitu-
tion with a high risk of inhibitor formation is the D2093G [D2074G] change. Combining our data with the Mutation Database, the D2093G [D2074G] change occurred nine times whereas four patients showed inhibitor formation.

One further factor that seems to influence inhibitor formation, and that bears attention in risk calculations of inhibitor formation, is the differentiation between intra- and inter-amino acid class switches (Table 2). Interestingly, the separate evaluation of wild-type amino acid class at each individual position revealed a significantly elevated risk of inhibitor formation for inter-amino acid switches of class 1 amino acids (p-value 0.048). Although the number of intra- and inter-amino acid switches of class 2 amino acids was equally high as for class 1 amino acids, the p-value was not significant. However, a clear tendency for a higher risk of inhibitor formation could be shown for inter-amino acid class switches. In contrast to this, no tendency of higher inhibitor risk could be shown in case of class 3 and 4 switches, probably caused by the small number of intra-amino acid switches. Overall the combination of all intra- and inter-amino acid changes clearly presents a generally higher risk of inhibitor formation in the event of inter-amino acid switches (p < 0.039) (Table 2).

Beside the specific missense mutation, the presence or absence of inhibitors in patients is also influenced by other genetic variables as polymorphisms of genes involved in immune response (HMC class II, interleukin-10, tumour necrosis factor-α, and CTLA-4). (23). Aside from the genetic determinants a number of environmental variables with potential to have an impact on this process have been extensively discussed. These variables include the treatment regime, type of replacement concentrate and additional, concomitant danger signals of the immune system (24).

Based on the detailed breakdown of missense mutations and the substantial statistical analysis, the presented study allows a more precise assignment of the inhibitor risk. Currently it is discussed that, on a given genetic background defined by the type of F8 gene mutations as well as by polymorphisms of immune response genes, modifications of the treatment regimens may allow to move a “bad risk” patient to a “good risk” patient (and vice versa). So far, one preliminary study including previously untreated patients presented a new early prophylaxis regimen that seems to avoid immunological danger signals, thus reducing FVIII inhibitor development considerably (25). In case of mild haemophilia the application of desmopressin (DDAVP) should also be considered as a good alternative treatment for many patients. Future studies will show whether this personalised risk assessment and treatment continues to lower the risk of inhibitor formation.

In conclusion, FVIII missense mutations are not only the most prevalent and manifold mutation type, but may provide further information about structurally and functionally important regions of the FVIII protein with respect to potential immunogenicity. This observation may be important as a first step to establishing individually personalised treatments to prevent inhibitor formation.

Conflicts of interest
None declared.

References
15. Oldenburg J, Pavlova A. Discrepancy between one-stage and chromogenic factor VIII activity assay results can lead to misdiagnosis of haemophilia A phenotype. Hämostaseologie 2010; 4: 207-211.

What is known about this topic?
• Missense mutations confer the lowest risk of inhibitor formation (5%) compared to all other mutation types within the F8 gene. However, dependent on type and position of mutations, inhibitor formation differs notably.

What does this paper add?
• Independent of position, the risk of inhibitor formation in association with F8 missense mutations is significantly higher if the amino acid substitution belongs to another physicochemical class than the original residue (p-value 0.039).