Factor VIII genotype and the risk of developing high-responding or low-responding inhibitors in severe hemophilia A: data from the PedNet Hemophilia Cohort of 1,202 children

The *F8* genotype is an important risk factor for the development of inhibitors against FVIII, but its significance for whether the inhibitor becomes a high-responding (HR) or low-responding (LR) inhibitor has not been studied before. In this large PedNet cohort study (N=1,202) we can highlight the risk to develop inhibitor against FVIII by genotype - stratified as high, intermediate, and low -, but also whether the inhibitor becomes a HR or LR inhibitor, which is clinically important.

Inhibitor development in severe hemophilia A (HA; FVIII<1%) is a feared complication, occurring in around 30% of patients.[1] Inhibitors can be classified as LR or HR, based on whether the historical peak inhibitory titer was <5 BU (Bethesda Units) or >5 BU, respectively. While the genetic *F8* variant is known to be an important risk factor,[2,3] it is not known how the *F8* genetic variant affects the development of a HR or LR inhibitor, which may be important when choosing initial therapy or immune tolerance therapy (ITI) for a patient. Based on the large PedNet Registry cohort, we aimed to study how the *F8* genotype affects the risk (high-, intermediate- or low-risk) of developing inhibitors against FVIII, and, in addition, if the genotype affects if the type of inhibitor becomes a HR or LR inhibitor (*clinicaltrials gov. Identifier: NCT02979119*).

All children aged <18 years with severe HA, registered in the PedNet registry by January 1, 2021, who had undergone at least 50 exposure days (ED) to FVIII concentrate, or who had developed an inhibitor, were included in our study (N=1,202). The well-characterized study group with a population-based inclusion per center, is followed up annually in 33 hemophilia centers in 18 countries and information on FVIII treatments and measurements of inhibitor titers are available, as well as accurate classification into having the LR or HR type inhibitor.[4] Contributors are listed in *Online Supplementary Table S1*.

All genetic reports were reviewed at the coordinating center (Malmö, Sweden) and the variants were revised regarding the nomenclature according to the recommendations of the Human Genome Variation Society (HGVS) and classified according to the American College of Medical Genetics and Genomics criteria and terminology.[5] In this study, only the reported likely pathogenic/pathogenic variants causing HA were included. In line with established *F8* gene databases, the variant effect was classified as missense, nonsense, frameshift, large deletion (>50 base pairs), large duplication (>50 base pairs), small deletion/insertion/duplication (<50 base pairs), silent variant, splice site variant, promoter variant, intron variant, and inversion (inv) which was subdivided into inv22 and inv1.

Inhibitors were reported in 396 of the 1,202 patients (32.9%),

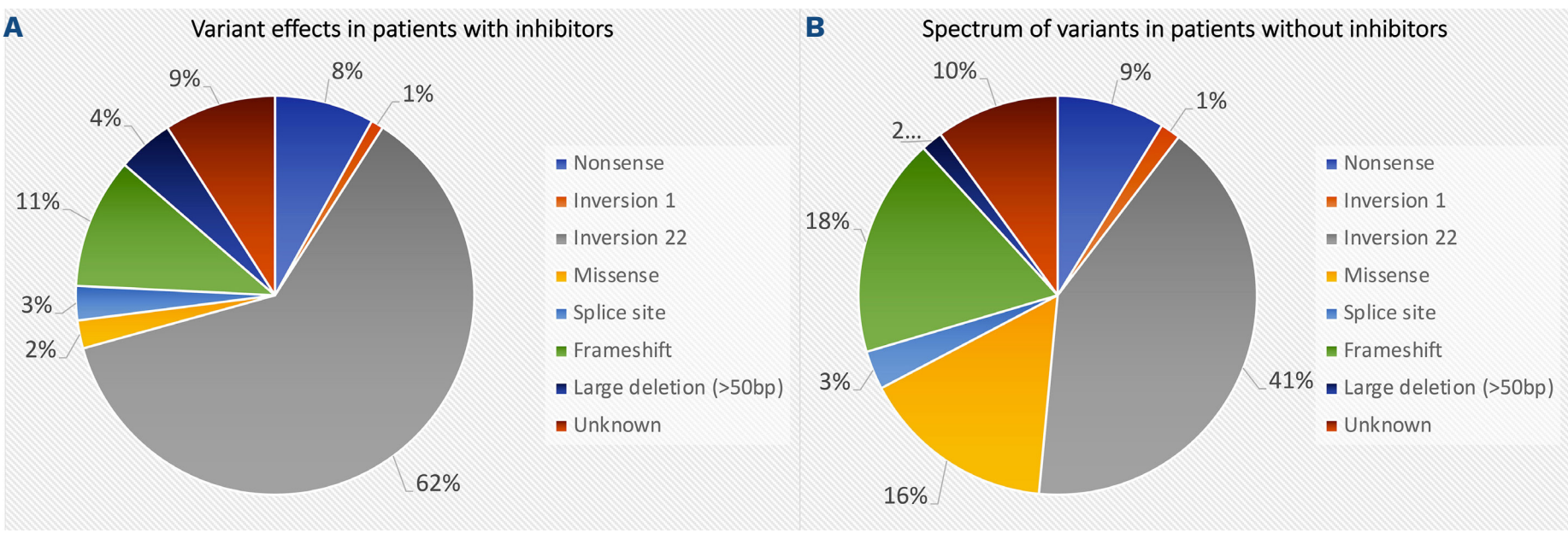

Figure 1. Spectrum of variant effects in patients from the PedNet Registry included in this study. Panel (A) shows the spectrum of variant effects in patients with inhibitors (N=396); panel (B) shows the spectrum of variant effects in patients without inhibitors (N=806). Variant effects below 1% of the cohort are not depicted: promotor, small structural changes in-frame and duplications. No inhibitor was detected in patients with these variant effects.

with 10.6% being LR and 22.3% HR. In 1,086 patients, a genetic report was available (90.3%). The most prevalent variant effects were: inv22 in 47.6% (N=573), frameshift in 15.3% (N=184), missense variants in 11.2% (N=135), nonsense variants in 8.5% (N=102), large deletions >50 bp in 3% (N=32), splice site in 3% (N=36), inv1 in 1% (N=17). When the spectrum of variant effects in patients with inhibitors *versus* patients without inhibitors was analyzed, the inv22 variant was found more often in patients with inhibitors, 62% (244/396) compared to 41% of patients without inhibitors (328/806; *P*<0.00001). Similarly, large deletions (>50 bp) were more prevalent in patients with inhibitors 4.5% (18/396) compared to patients without inhibitors 2% (14/806; *P*=0.0045). Patients without inhibitors showed significantly more frameshift variants (18% *vs.* 11%; *P*=0.0015) and missense variants (16% *vs.* 2%; *P*<0.00001) compared to inhibitor patients (Figure 1).

The highest incidence of inhibitors was seen in patients with large deletions, 56.2% (18/32), followed by inv22 (42.7%; 244/573), nonsense variants (31.4%; 32/102), splice site variants (30.6%; 11/36), inv1 variant (23.5%; 4/17), frameshift variants (22.8%; 42/184) and missense variants (6.7%; 9/135), with the overall lowest inhibitor incidence (Figure 2A; *Online Supplementary Table S2*).

When the effect of the *F8* variant on whether the inhibitor became HR or LR was analyzed (Figure 2B; *Online Supplementary Table S2*), the ratio HR (28.5%; 163/572) to LR inhibitors (14.2%; 81/572) in patients with inv22 was 2.01 (95% confidence interval [CI]: 1.71-2.39). Patients with large deletions (N=32) were more likely to develop a HR (46.9%; 15/32) inhibitor compared to LR (8.3%; 3/32) with a ratio of 5.6 (95% CI: 1.6-16.9). In addition, the HR inhibitor incidence was significantly higher at 73.3% (11/15) for patients with multiple exons, compared to 21.4% (3/14) for the patients with single-exon deletions (*P*=0.036). For LR, no statistical difference was seen between patients with multiple exons *versus* single exon. HR inhibitors were more likely to be present in patients with nonsense variants, occurring in 26.5% (27/102), compared with LR inhibitors in 4.9% (5/102), with a ratio of 5.4 (95% CI: 2.17-13.5). In splice site variants (N=36), HR developed in 19.4% and LR in 11.1% (ratio 1.75; 95% CI: 0.5-5.4). Missense variants had the overall lowest inhibitor incidence (6.7%; 9/135), LR inhibitors developed in 4.4% (6/135) and HR inhibitors in 2.2% (3/135) with a ratio of 0.5 (95% CI: 0.13-1.95). Frameshift variants had no difference in risk of developing HR or LR inhibitors. In the group of 17 patients with the inv1 variant, 23.5% of patients (4/17) developed a HR inhibitor.

Sub-analyses were made to study some factors that in previous studies have been shown to be important for the incidence *per se* of inhibitors. No statistical significance was found when comparing variants in the light chain *versus* heavy chain or variants in the C1/C2-junction *versus* non-C1/C2-junction. In splice site variants, no difference was found between canonical and non-canonical (i.e., +/- 2 bp from splice site) or between poly-A *versus* non-poly-A runs. Notably, 31 of 43 inhibitors (72.1%) in patients with frameshift variants occurred in exon 14, which is the largest exon. No difference in inhibitor incidence could be seen between exon 14, 24.8% (31/125) *versus* outside exon 14, 20.3% (12/59).

In the publication by Oldenburg *et al.* in 2002, a stratification into 'low-risk and high-risk mutations' was made, with low-risk variants defined as <10% and high-risk variants defined as >30% for developing an inhibitor.[6] In the original study, 364 single center patients with all severities of HA were included. High-risk variants included large deletions, nonsense, and inv22 and were also described as 'null-mutations'. Since inv22 is the most common variant, it has also been used as a reference to determine inhibitor

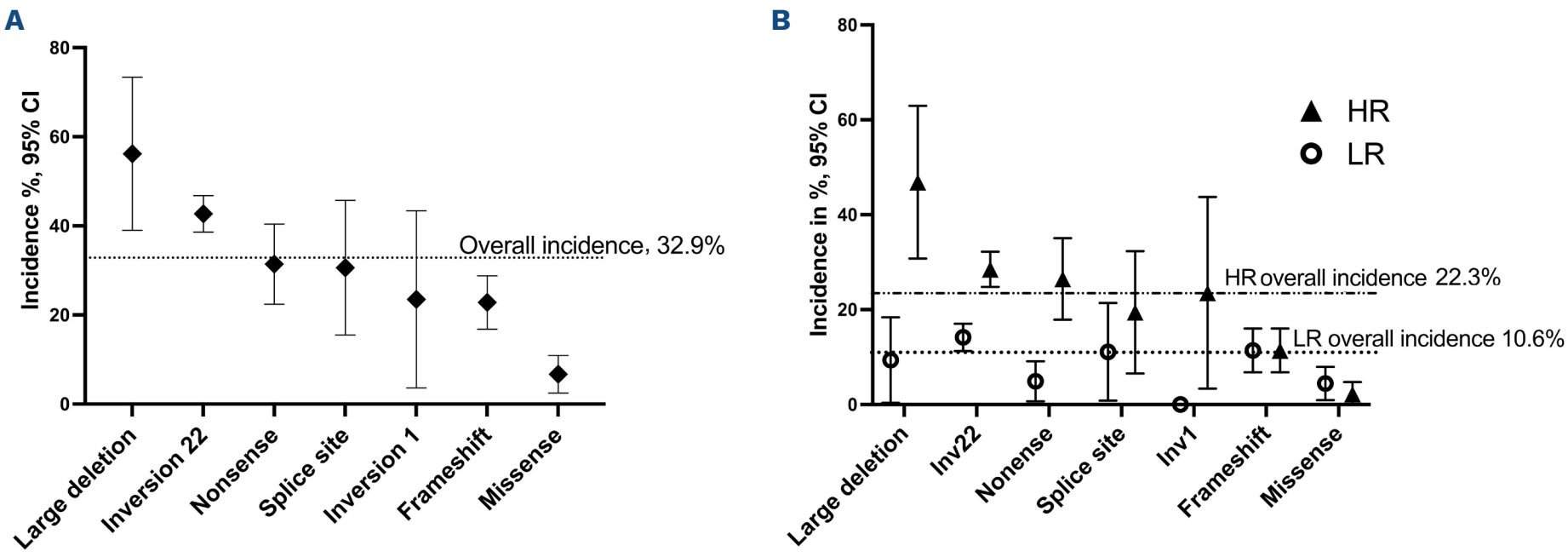

Figure 2. Inhibitor incidence in percentage and 95% confidence interval per variant effect. Panel (A) shows the total inhibitor incidence per genotype and the overall incidence of inhibitors; panel (B) shows the incidence of high-responding (HR) and low-responding (LR) inhibitors per genotype and the overall incidence of HR and LR inhibitors, respectively. CI: confidence interval.

Table 1. Classification of variant effects in high-, intermediate- and low-risk to develop inhibitors, low-responding inhibitors, and high-responding inhibitors.

Risk to develop inhibitor	Variant effect
All inhibitors	
High risk	inv22, large deletions
Intermediate risk	nonsense, splice site, inv1
Low risk	missense, frameshift
LR inhibitors	
High risk	inv22
Intermediate risk	frameshift, splice site, large deletions
Low risk	missense, nonsense
HR inhibitors	
High risk	inv22, large deletions
Intermediate risk	nonsense, splice site, inv1
Low risk	missense, frameshift

LR: low-responding; HR: high-responding; inv: inversion.

development in other variants, e.g., in the meta-analysis of Gouw *et al.*[3] We chose not to compare the other variant effects with inv22 since the incidence of inhibitors in inv22 varies hugely between studies: e.g., in the meta-analysis of Gouw *et al.*, the incidence varied between 0-77% in 30 studies with different population sizes. Garagiola *et al.* (2018) suggested a stratification into low-, intermediate- and high-risk variants after a review of the literature: large insertion/deletion (multiple exons) and nonsense mutations on the light chain were classified as high-risk; large insertion/deletion (single exon), nonsense mutations on the heavy chain, inv22 and inv1, as intermediate risk, and frameshift, missense mutations and splice-site mutations as low risk.[7] In our study, the risk of inhibitor development was evaluated for each genotype by calculating the incidence per variant effect *versus* the incidence for all other variant effects combined. Based on these results, we propose a division into high-, intermediate and low-risk for the development of inhibitors of all types but also with new information about the risk of HR or LR type of inhibitor (Table 1). We can confirm in our study that patients with large deletions, but also inv22, could be classified as high-risk variants whereas, on the other hand, patients with frameshift and missense could be classified as low-risk variants; nonsense, splice site, inv1 as intermediate-risk variants. The same calculation was not only done for inhibitor development, but also for development of HR and LR inhibitors (Table 1).

The strengths of this study are the large well-characterized study group with a population-based inclusion per center prospectively collected with very detailed information on the first 50 ED and the genotype characterized and being curated following current American College of Medical Genetics and Genomics guidelines in 90.3% of the patients. The spectrum of variants in our cohort have been described previously and are comparable with those cited in other locus-specific databases, such as EAHAD and CHAMPS.[8] Despite the large cohort there were relatively limited numbers of patients with splice site, inv1 and large deletion variants as well as for analyses of heavy chain *versus* light chain, or canonical to non-canonical variants in splice sites.

A LR inhibitor is a minor clinical problem and ITI may not be advisable in those patients.[9] Therefore, our results, which can not only be used to assess the risk of each type of inhibitor when the *F8* genotype is known in the patient, may also have a valuable clinical relevance when it comes to weighing in the risk of different types of inhibitors into the choice of therapy.

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Disclosures

NGA has served as a speaker and/or on advisory boards for CSL Behring, Octapharma and Sobi. VL has been as a speaker and/or advisor for Bayer, Novartis, NovoNordisk, Octapharma, Roche, Sobi, and Takeda. MK-K has been a speaker and/or advisor for Bayer, Sobi, Takeda and Roche. MK-K has received grants from Roche and financial support for travel, accommodation, and expenses from Bayer, NovoNordisk, Sobi, Takeda and Jazz. FP has received financial support for travel and accommodation from Roche. TSM has no conflicts of interest to disclose. RL has received compensation for consultancy work (DMC, advisory board) or

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Contributions

All authors have participated in the concept and design; analysis and interpretation of data; drafting and/or revising of the manuscript. Each author listed on the title page of the manuscript has approved the submission of this version of the manuscript and takes full responsibility for the manuscript. Contributors belonging to the PedNet Study Group are listed as the collaborative group "PedNet Group" in *Online Supplementary Table S1* of the *Online Supplementary Appendix*.

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Data-sharing statement

All data used in this study are from the PedNet Registry, which is governed by the non-profit-making organization PedNet Hemophilia Research Foundation. The data that support the findings of this study are available from the Registry of the PedNet Hemophilia Research Foundation. Restrictions apply to the availability of these data, which were used under license for this study. Data are available from the authors with the permission of the PedNet Registry Foundation (www.pednet.eu).

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