INTRODUCTION

Hemophilia A (HA) is an X-linked recessive bleeding disorder (OMIM 306700) with an occurrence of 1 in 5000 males and is caused by lack or reduced activity of the coagulant factor VIII (FVIII:C). Depending on FVIII:C activity levels, HA is classified as severe (FVIII:C <1 IU/dl), moderate (FVIII:C 1–5 IU/dl), or mild (FVIII:C>5–40 IU/dl). HA is a single-gene disease with more than 3000 unique variants in the F8 gene reported in the EAHAD F8 Variant Database (https://f8-db.eahad.org/index.php)—missense, nonsense, frameshift, splice-site mutations, and large structural changes (large deletions and duplications). The most common variant is the intron 22 inversion causing more than 40% of cases of severe HA.

One of the challenges of genetic testing in patients with HA is the interpretation of sequence variants. Despite standards and guidelines for the classification of variants (Richards et al., 2015) there are still concerns regarding the classification of, for example, silent variants that may cause splicing defects. Substitutions, including silent variants, can affect splicing by directly inactivating or creating a splice site or by interfering with splicing regulatory elements, such as exonic splicing enhancers (ESEs), intronic splicing enhancers (ISEs), exonic splicing silencers (ESSs), or intronic splicing silencers (ISEs; Jian...
et al., 2014). A disruption of an ESE site can result in partial or complete exon skipping. In silico tools can be very helpful in predicting the splicing defects, but analysis of the patient’s RNA is the most reliable method to evaluate the functional effects of a variant.

Here we report a silent variant found in exon 2 in the F8 gene in a patient with a previous von Willebrand disease (VWD) diagnosis. We used in silico tools, American College of Medical Genetics and Genomics (ACMG) classification and mRNA analysis to determine if this variant could affect the patient’s mRNA transcript.

2 | MATERIALS AND METHODS

2.1 | Patient

The patient is a 47-year-old man diagnosed previously with VWD type 1 based on an investigation in Greece because of a clinical family history of VWD. No family members were investigated genetically. Clinically, he had mild bleeding symptoms restricted to prolonged bleeding from minor wounds. He had not undergone any surgery or tooth extraction. After moving to Sweden, renewed blood tests in 2020 showed a prolonged activated partial thromboplastin time (aPTT; 34 s) but close to normal von Willebrand factor (VWF) levels (P−VWAg 0.44 kIE/L, P−VWF GPIbA 0.45 kIE/L) and blood group O. Further investigation showed unproportionally low levels of FVIII:C (0.21 kIE/L). A blood sample was sent to our DNA hemophilia diagnostic laboratory to establish if this low FVIII:C was caused by a variant in the F8 gene.

2.2 | DNA analysis

DNA was extracted from EDTA blood using QIAamp DNA Blood Mini Kit (QIAGEN). Sanger sequencing of promoter, all exons and exon/intron boundaries in the F8 gene and VWF gene (exons associated with VWD type 2N) was performed on the ABI3500 Genetic analyzer (Life technologies) and data were analyzed using SeqScape software v3.0 (Life Technologies). MLPA (Multiplex Ligation-dependent Probe Amplification, MRC Holland) analysis was performed according to the manufacturer’s protocol, using a P178-F8 probe mix.

2.3 | RNA analysis

RNA was extracted from blood collected in PAXgene blood RNA tubes (PreAnalytiX, Hombrechtikon, Switzerland) using the PAXgene Blood RNA kit according to the manufacturer’s procedure (PreAnalytiX). RNA analysis by reverse transcription-polymerase chain reaction (RT-PCR) was performed using Omniscrypt reverse transcriptase (Qiagen, Hilden, Germany) and primers described as above (El-Maarri et al., 2005). An internal control PBGD (human porphobilinogen deaminase gene, Gen-Bank accession number NM_000190.4) was used in each RT-PCR to ensure that the RT-PCR did not fail. Human Liver Total RNA (Life Technologies) was used as a normal control. The PCR products (F8 cDNA) were separated by gel electrophoresis and visualized on a 1% agarose gel. The cDNA bands were extracted from the agarose gel using QIAEXII (Qiagen, Hilden, Germany). The cDNA was then analyzed by Sanger sequencing direct after the RT PCR and after separation on agarose gel.

2.4 | ACMG classification and in silico prediction

The variant classification was performed according to ACMG guidelines (Richards et al., 2015), using VarSome software (Kopanos et al., 2019) and in silico prediction software Alamut Visual version 2.15 (SOPHiA GENETICS), which includes Splice Site Finder-like, MaxEntScan, NNSsplice, GeneSplicer, and ESEfinder for identifying putative ESEs (Cartegni et al., 2003). For information on allele frequency, gnomAD v.2.1.1 was used (Karczewski et al., 2020). The results were discussed and curated in a multidisciplinary team conference (MDT).

3 | RESULTS

3.1 | DNA analysis

Sanger sequencing of F8 gene (NG_011403.2, NM_000132.3) using genomic DNA showed a hemizygous silent variant in exon 2: c.222G>T, p.Thr74Thr. Sanger sequencing of the VWF gene (exons 11, 12, 14–21, 24–28, 52) showed no variants known to cause VWD type N (Normandy). MLPA analysis did not show any deletions or duplications.

3.2 | In silico prediction

The in silico analysis by ALAMUT predicted no effect on the splice sites but a possible disruption of exonic splicing enhancer (ESE) in exon 2 as an effect of the variant F8:c.222G>T.
3.3 | ACMG classification

As a first step, VarSome software was used, showing the conclusion “likely benign” since the variant according to the software fulfilled PM2 (variant not found in gnomAD population database v2.11 and v3.11), BP4 (benign computational verdict based on one benign prediction from DANN vs. no pathogenic predictions and position not conserved strongly) and BP7 (synonymous variant, not predicted splicing). However, as ESE Finder software flagged for a possible disruption of ESE in exon 2, we excluded criteria BP4 and BP7 which gave the final classification of VUS (variant of unknown significance).

3.4 | mRNA analysis

F8 cDNA region A (exons 1–8) was divided into two parts: the nested PCR A1 (exons 1–5) and A2 (exon 4–8). Sanger sequencing of the patient’s cDNA after nested PCR showed that the patient had both a normal transcript containing exons 1–4 and a defect transcript lacking exon 2 (Figure 1). The products after nested PCR were visualized on an agarose gel, Figure 2. Exons 1–5 in the normal control (N) is an ~600 bp long band. The patient has two bands: 600 and 500 bp. Exons 4–8 in the region A2 were found to be ~600 bp long fragments in both the patient and the normal control. The patient’s cDNA fragments were extracted from the agarose gel and sequenced. Sanger sequencing of those fragments showed that the 600 bp band, the same size as in the normal control, had a normal sequence containing exons 1–4. The smaller band (500 bp) which was not found in the normal control, had a sequence corresponding to exons 1, 3, and 4, lacking exon 2, Figure 3.

4 | DISCUSSION

The aim of the investigation was to find an explanation for the patient’s low FVIII:C levels causing clinically mild bleeding symptoms. With our standard genetic diagnostic methods—Sanger sequencing of F8 gene, MLPA and additional analysis of the VWF gene—we only found a silent variant in exon 2 in the F8 gene, c.222G>T; p.Thr74Thr. This variant has not been reported in the International Hemophilia Databases (EAHAD, CHAMPS, HGMD), and is absent in the population database gnomAD. When applying ACMG criteria, the variant was predicted to be “likely benign” in the analyzing software or VUS after curating in the MDT as it does not alter the protein sequence and is not situated near a splice site. However, the ALAMUT splice prediction using ESE finder indicated a possible impact by this variant on an ESE site in exon 2.

Our functional study analyzing the patient’s mRNA as described above, revealed that the patient with variant c.222G>T had two mRNA transcripts: one normal containing all the exons, and one shorter, missing exon 2 resulting in a truncated protein. These findings explain the patient’s low FVIII:C and led to the diagnosis of mild HA instead of VWD. Since mild HA and VWD can cause similar bleeding patterns and both present with low FVIII levels, it can be difficult to distinguish between these diseases without DNA analysis, which has clinical importance with regard to treatment and genetic counseling.

A similar variant in the same codon (c.222G>A; p.Thr74Thr), in a patient with mild HA (FVIII 48%), also caused by exon 2 skipping was described by Martorell

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**FIGURE 1** Sanger sequencing of cDNA after the RT-PCR and nested PCR: (a) reference sequence F8 NM_000132.3, parts of exons 1 and 2. (b) The patient’s cDNA sequence read with forward primer. The double peaks contain both the exon 2 and 3 sequences. (c) The patient’s cDNA sequence read with reverse primer. The double peaks contain both the exon 1 and 2 sequences (followed by the exon 3 sequence).

**FIGURE 2** cDNA fragments after amplification by nested PCR. Region A1 (exons 1–5) on the left side presents as two bands in the patient (Pat) and as one band in the normal control (N). Region A2 (exons 4–8) on the right side presents as one compact band both in the patient and in the normal control.
et al. (2015). However, the same variant (c.222G>A) was also investigated by Zimmermann et al. (2013) using ALAMUT and mRNA analysis with no demonstrable effect on splicing. This discrepancy in the results may be explained by different amounts of the normal and aberrant transcripts being synthesized in different patients and/or by not fully reliable splice predictions for silent and other variants more than 2 bp from splice sites. Peripheral blood lymphocytes are not constitutive expressers of F8 such as the hepatic endothelium where FVIII is produced, which is a limitation in this type of studies (Wion et al., 1985).

There is increasing evidence that many human disease genes harbor exonic variants that may affect pre-mRNA splicing (Anna & Monika, 2018). However, mRNA analysis, which should be the most biological and functional method to identify splicing defects and which can increase the clinical diagnostic rate (Wai et al., 2020), has limitations in routine clinical practice. It is time-consuming and often requires a new blood sample from the patient and can only be performed in specialized laboratories. An easier useful way to discover potential splice affecting variants would be more reliable in silico tools with clear disease-specific guidelines helping to interpret and predict splicing defects, but these are still limited. It is likely that silent variants are misinterpreted easily when using classification software and prediction programs only and, in variants of unknown significance, a more extensive genetic DNA- and mRNA work-up is needed to clarify the patient’s phenotype–genotype.

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CONFLICTS OF INTEREST
The authors have declared no conflict of interest.

AUTHOR’S CONTRIBUTION
All authors attest that they meet the current ICMJE criteria for Authorship.

ETHICAL COMPLIANCE
This case report was approved by the general decision on case reports from the Swedish Ethical Review Authority 2020 and performed according to the Declaration of Helsinki. According to Swedish Law and regulations regarding the publication of case reports, the patient was informed of the planned publication, an informed consent was obtained, and a note was made in his Medical Record.

DATA AVAILABILITY STATEMENT
The variant presented in this paper was submitted to the EAHAD database (https://dbs.eahad.org) and will be publicly available (McVey et al., 2020).

ORCID
Anna Olsson https://orcid.org/0000-0002-9974-4880
Nadine G. Andersson https://orcid.org/0000-0001-6058-8350

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FIGURE 3 Schematic presentation of the molecular findings of partial exon skipping (NM_000132.3)
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