INTRODUCTION

Hemophilia A (HA) is the most common inherited recessive X-linked disorder of blood coagulation caused by deficiency of factor VIII (OMIM, 300841) in the coagulation cascade with an estimated incidence about 1 in 5,000 male live births (OMIM, 2020). Hemophilia A is more common than hemophilia B, representing 80%–85% of the total hemophilia population (Srivastava et al., 2013). In 2019, the World Federation of Hemophilia (WFH), in its global survey in Egyptian population (98,423,595) estimated the number of affected patients with hemophilia as 6,028 (World Federation of Hemophilia report on the Annual Global Survey, 2018).
Hemophilia A is associated with marked increased risk of bleeding. According to the coagulation activity of FVIII, hemophiliacs are categorized into mild (>5%), moderate (1%–5%), and severe (<1%) phenotypes. In severe HA, bleeding might be spontaneous and with no obvious etiology (Santagostino et al., 2010). Heterozygous females with F8 clotting activity level lower than 40% are at risk for bleeding that is usually comparable to that seen in males with mild hemophilia. Also, subtle, abnormal bleeding may occur with a baseline F8 clotting activity between 35% and 60% or higher (Paroskie et al., 2015; Plug et al., 2006).

Human F8 gene has 26 exons and maps to the long arm of the X chromosome at the Xq28 region. The protein product of F8 possesses no enzyme activity. A 19 amino acid signal peptide at the N-terminus leads to the passage of F8 protein through hepatocytes to the blood vessels (Saenko et al., 2002). The matured F8 protein is composed of 2332 amino acids and comprises three homologous A-domains, two homologous C-domains, and a single B-domain. These domains, arranged as A1A2BA3C1-C2 from the N- to the C-terminus. Upon activation, the inactive F8 is proteolyzed via thrombin where the B-domain is cleaved leaving the heterotrimeric active F8 composed of heavy and light chains linked together by a calcium ion (Lenting et al., 1998). The spectrum of F8 gene mutations is heterogeneous as reported at the CDC HA Mutation Project (http://www.cdc.gov/ncbd dd/hemophilia/champs.html, 2016; Payne et al., 2012). Inversion mutations involving intron 22 (Inv22) and intron 1 (Inv1), account for about 45% and 2%–5% of severe phenotype, respectively (Bagnall et al., 2006; Cumming, 2004; Gouw et al., 2012). Inverse shifting PCR (IS-PCR) is used in a rapid and robust manner to detect Inv22 and Inv1 as well as other related DNA rearrangements (Rossetti et al., 2005, 2008). Point mutations are the most prevalent defect in 90%–95% of patients who tested negative for inversion typing, followed by deletions in about 5%–10% (Graw et al., 2005).

Numerous types of previously reported HA gene mutations have been identified in exon 14. Being the largest exon in F8 gene (3,106 bp), exon 14 provides a mutational hotspot and codes partially for two domains (A2 and A3) as well as the entire B-domain of F8 protein (Repesse et al., 2007).

Recently, targeted next-generation sequencing (NGS) is utilized to sequence the whole coding region of F8 (Atik et al., 2020; Bastida et al., 2017; Inaba et al., 2017). HA pedigrees’ linkage analysis can also identify HA carriers providing reliable method used in genetic counseling and prenatal diagnosis (Hussein et al., 2008).

In the current study, Egyptian HA patients with variable clinical phenotypes were studied for the identification of F8 gene mutation spectrum for this serious coagulation disorder, with an ultimate goal of providing proper genetic counseling and prenatal diagnosis.

2  |  PATIENTS AND METHODS

2.1  |  Patients

Sixty HA patients from 43 families were recruited from the Hereditary Blood Disorders Clinic, National Research Centre (NRC), and Hematology clinic, Pediatric hospital, Cairo University. Informed written consents were obtained from the patients or their guardians according to the Helsinki Declaration of 1975, as revised in 1983. Following approval of the National Research Centre Review Board, the following variables were obtained; detailed family history, pedigree analysis up to three generations, thorough clinical examination, coagulation, and immunologic assays. Subsequently, patients were classified according to the F8 activity into mild (>5%), moderate (1%–5%), and severe (<1%) phenotypes.

Blood samples were collected from affected individuals, their mothers, siblings and available family members whenever possible for molecular analyses.

3  |  METHODS

3.1  |  Molecular genetic analysis

3.1.1  |  Genomic DNA extraction

DNA was extracted from blood samples of 60 patients, their mothers, and available family members using standard salting out method (Miller et al., 1988).

3.1.2  |  Detection of F8 int22 and int1 rearrangements using IS-PCR

IS-PCR was performed as directed by Rossetti et al. (2008). Briefly, genomic DNA digested with Fast Digest BcII kit according to the manufacturer (Thermo Fisher Scientific). Digested DNA was isolated via phenol chloroform extraction followed by precipitation with ethanol. Ligation of the digested DNA was carried out using Rapid DNA Ligation kit (Thermo Fisher Scientific). Ligated samples undergo PCR using previously published primers.

3.1.3  |  Sequencing of exon 14 of F8 gene

Samples negative for F8 int22 and int1 rearrangements were subjected to sequencing of exon 14. Polymerase Chain Reaction (PCR) was performed on the DNA samples of the patients using primers pairs of six fragments in exon 14 (14A, 14B, 14C, 14D, 14 E, and 14F) of the F8 gene according to
Steve Keeney, 2011 (Keeney, 2010). PCR reaction was carried out in 25 µl reaction mixture containing 1.5 mM MgCl₂, 0.2 mM dNTP, 10 pmoles of each primer, 0.5 U of Taq DNA polymerase, and 200 ng of genomic DNA. PCR conditioning was as follow: initial denaturation for 5 min at 95°C, 30 cycles of 30 s at 94°C, annealing for 30 s at 57°C, extension for 30 s at 72°C, and a final extension for 5 min at 72°C. Amplified fragments were subjected to direct sequencing in both directions using Big Dye Terminator v3.1 Kit and injected to ABI 3500 Genetic Analyzer (Applied Biosystems).

3.1.4 | Targeted NGS of F8

Four patients (two of mild and two of moderate HA patients) who were negative for both int22 rearrangements and any known pathogenic variants in exon 14 were selected for sequencing of the whole coding region of F8 and its flanking intronic regions using targeted NGS (Illumina).

3.1.5 | Data analysis

F8 nucleotide numbering is designated according to coding bases from A (nucleotide+1) from the initiation codon for methionine (ATG) at position −171 (F8: ref. NM_000132.3).

While the protein numbering (p.) follows the amino acid sequences that assign the first residue methionine as +1 in factor VIII sequences (FVIII: NP_000123.1) according to the Human Genome Variation Society guidelines.

Sequence variants were aligned with the corresponding wild-type sequences using BLAST (NCBI) and compared to the HA mutation databases (Human Gene Mutation Database and CDC Haemophilia A Mutation Project database 29).

4 | RESULTS

4.1 | HA phenotyping

Fifty-two HA patients’ age ranged from 2 to 12 years (mean age ~6.7) and eight patients were between 30 and 50 years (mean age ~38). Pedigree analyses revealed positive consanguinity within 50% of studied pedigrees and other affected family members were detected in 37.5%. According to F8 activity, 35% of HA cases were classified as severe HA, 40% as moderate, and 25% as mild phenotype.

4.2 | Inverse-shifting PCR

IS-PCR revealed the presence of three different int22 rearrangements in 9/60 (15%) of all patients and 9/21 (42.8%) of severe HA patients. Inv22 type 1 was found in six patients, del22 type 1 in one patient, and del22 type 2 in two patients (Figure 1). All nine patients were severe HA patients (P1–P9). None of the studied patients had Inv1 (Table 1).

4.3 | Sequencing analysis of exon 14

Sequencing of amplified fragments of exon 14 among 51 HA patients negative for int22 rearrangements and Inv1 could detect molecular alterations in 17/51 as follows:

1. Patient 10 harbored a novel deletion mutation of 2 bp (AA) (NM_000132.3:c.2734_2735delAA, p.(N912Ffs*6)) which creates a frame shift starting at codon N912 and the new reading frame ends in a stop codon 5 positions downstream (Figure 2a). This mutation was submitted in ClinVar with accession number SCV000328623.

2. Nine patients (P12–P20) exhibited previously reported nonsense mutation (NM_000132.3:c.2440C>T, p.(R814*)) (Figure 2c).

3. Moreover, the previously reported natural variant (NM_000132.3:c.3780C>G, p.(D1260E)) (rs1800291) was detected in six patients (P21–26), which was inherited in a heterozygous state in four of their mothers (Figure 2d).

4.4 | Targeted NGS of F8 gene

Targeted NGS of the whole coding regions of F8 and its flanking introns revealed no known pathogenic mutations in the selected four HA patients who were negative for int22 rearrangements and exon 14 known pathogenic variants. The only detected alteration was (NM_000132.3:c.3780C>G, p.(D1260E)) (rs1800291) in P21 which was identified earlier by direct Sanger Sequencing of exon 14.

5 | DISCUSSION

Based on the clinical presentation of the patients, we initially performed the molecular analysis of the most pathogenic F8 variants in severe HA patients known as inversion which leads to abnormal protein product.

In our cohort of 60 HA patients we found six mutations including intron 22 rearrangements, nonsense mutations, and...
small deletions. Reviewing the HAMSTeRS, HGMD, and CHAMP database one of these six mutations is novel.

In the current study 21 (35%) of our studied patients, presented with severe clinical phenotype. Nine (42.8%) of these patients harbored three different int22-related rearrangements. Abou-Elew et al. (2011) reported intron 22 rearrangements in 70% of severe cases, combining our results with that of Abou-Elew et al. (2011), intron 22 rearrangements would be responsible for about 21% of all reported Egyptian hemophilia molecular pathology and 53% of severe cases, (Table 2). Both studies fall within the range of reported molecular pathology of severe HA in different populations (Abdulqader et al., 2020; Acquila et al., 2004; Astermark et al., 2005; Coppola et al., 2009; Hill et al., 2005; Vinciguerra et al., 2006). Considering these collective studies, targeted molecular diagnosis for severe HA phenotype in Egyptians should target Inv22 together with del22 to start.

By excluding IVS22 and IVS1 mutations, 11 (52.4%) out of 21 severe HA studied patients, mainly harbored pathogenic mutations within exon 14 corresponding to the B-domain of factor VIII. FVIII B-domain has no homology sequence to any other known genes and participates in the intracellular processing and trafficking of factor VIII. (Acquila et al., 2004; Santacroce et al., 2008). Preferential mutational hotspots are usually detected in FVIII B-domain, in most cases creating a premature stop codon within the reading sequence (Kemball-Cook et al., 1998).

Among the studied patients of B-domain affection, patient No 10 harbored a novel frameshift mutation within exon 14 of F8 gene, due to a novel deletion (NM_000132.3:c.2734_2735delAA, p.(N912Ffs*6)). Frameshift mutation (NM_000132.3:c.3091_3094delAGAA, p.(K1031Lfs*9)) detected in Patient No 11, was similarly correlated with severe HA phenotype in American and Italian populations (Coppola et al., 2009). Both detected deletion mutations are speculated to affect the protein product through disturbing the architecture of the B-domain binding and thus alter the function with consequent severe phenotype presentation.

Patients No 12–20 also presenting with severe phenotype, harbored the reported nonsense mutation (NM_000132.3:c.2440C>T, p.(R814*)), that might point to this mutation as a hotspot in Egyptian severe HA patients or to the presence of common ancestors as per previous reports (Abdulqader et al., 2020; Acquila et al., 2004; Atik et al., 2020; http://www.factorviii-db.org/index.php, 2017; http://factorviii-db.org, 2014; Hill et al., 2005; Santacroce et al., 2008). The (NM_000132.3:c.2440C>T, p.(R814*)) nonsense mutation results in altered mRNA decay which would allow for the production of the truncated protein sufficient for dampening the severe clinical outcome.

As Inv22 and Inv1 in addition to exon 14 sequencing could only unravel the molecular pathology in severe HA
TABLE 1  F8 mutations in Egyptian hemophilia A patients

| Patients | Mutations | Nucleotide changes | Amino acid changes | Novelty | Disease severity |
|----------|-----------|--------------------|--------------------|---------|----------------|
| P1       | Inv22 type1 | -----             | -----             | Reported | Severe         |
| P2       | Inv22 type1 | -----             | -----             | Reported | Severe         |
| P3       | Inv22 type1 | -----             | -----             | Reported | Severe         |
| P4       | Inv22 type1 | -----             | -----             | Reported | Severe         |
| P5       | Inv22 type1 | -----             | -----             | Reported | Severe         |
| P6       | Inv22 type1 | -----             | -----             | Reported | Severe         |
| P7       | Del 22 type1 | -----             | -----             | Reported | Severe         |
| P8       | Del 22 type2 | -----             | -----             | Reported | Severe         |
| P9       | Del 22 type2 | -----             | -----             | Reported | Severe         |
| P10      | Frameshift | c.2734_2735delAA  | N912Ffs*6         | Novel   | Severe         |
| P11      | Frameshift | c.3091_3094delAGAA| K1031Lfs*9        | Reported | Severe         |
| P12–20   | nonsense   | c.2440C>T        | p.Arg814*         | Reported | Severe         |

TABLE 2  Genotype–phenotype findings in two cohorts of Egyptian hemophilia A patients

| Genotypes of intron 22-related re-arrangements | Number of patients studied by Abou-Elew et al. (2011) | Number of patients included in our study | Total reported Egyptian HA | HA phenotype |
|------------------------------------------------|------------------------------------------------------|----------------------------------------|-----------------------------|--------------|
| Wild-type allele                               | 20 (66.7%)                                           | 51 (85%)                               | 71 (78.8%)                  | 22 mild, 33 moderate, and 16 severe |
| Intron 22 inversion type 1 (Inv22-1)           | 6 (20%)                                              | 6 (10%)                                | 12 (13.3%)                  | 11 severe and one moderate         |
| Intron 22 inversion type 2 (Inv22-2)           | 1 (3.3%)                                             | 0 (0%)                                 | 1 (1.1%)                    | All severe                         |
| Intron 22 deletion type 1 (Del22-1)            | 1 (3.3%)                                             | 1 (1.6%)                               | 2 (2.2%)                    | All severe                         |
| Intron 22 deletion type 2 (Del22-2)            | 2 (6.7%)                                             | 2 (3.3%)                               | 4 (4.4%)                    | All severe                         |
| Total                                          | 30 (100%)                                            | 60 (100%)                              | 90 (100%)                   | 22 mild, 34 moderate, and 34 severe |

FIGURE 2  Genotyping patterns of different Inversion 22 (Inv 22) rearrangements using inverse shift PCR (IS-PCR). The figure shows genotyping patterns of different Inv22 rearrangements detected by IS-PCR on 1.5% agarose gel. On the left, Del22 type 1 (one band at 457 bp) and Inv22 type 1 (two bands at 559 & 457 bp) are shown. On the right gel, the Del22 type 2 (one band at 405 bp) is shown versus Del22 type 1 (one band at 457 bp) and wild type (WT; two bands at 457 & 405 bp). The sizes of 100 bp DNA marker (M) bands are shown.
cases within our study, targeted NGS of the whole coding regions of F8 and its flanking introns were performed for two mild and two moderate HA patients.

Failure of detection of any pathogenic mutations through targeted NGS analyses correlates with mild or moderate phenotype. However, suggesting the possibility of either a rare or unique individual variants located deep within the introns of F8 gene (Atik et al., 2020; Bastida et al., 2017; http://www.factorviii-db.org/index.php, 2017; http://factorviii-db.org, 2014). No further analyses could be performed due to budget limitation.

The natural homozygous variant (NM_000132.3:c.3780C>G, p.(D1260E)), was detected in six of mild and moderate studied HA patients where we failed to detect the causative mutation. It was also detected in a heterozygous state in four of patient’s mothers providing valuable linkage analyses. Viel et al. (2007) provided evidence for significant association between this nonsynonymous variant located in B-domain of F8 gene and FVIII coagulation activity. FVIII: C level, the association persisted with each C-allele additively increasing the FVIII: C level by 14.3 IU dl (−1) (p = 0.016). This variant was also previously detected with 23 entries from different populations (Bastida et al., 2017; Casaña et al., 2008; Fernández-López et al., 2005; Reitter et al., 2010; Repesse et al., 2007; Viel et al., 2007).

Linkage analysis application utilizing such polymorphic sites, helped in successfully providing genetic counseling services for families with informative mothers (Hussein et al., 2008).

In conclusion, the current study concluded that severe HA is associated with major disruptions of the F8 gene, whereas mild and moderate hemophilia correlates with other alterations that need further molecular analyses. Facing the high management cost, QoL, and limited resources in developing countries, the study is an important step to characterize the molecular pathology underlying HA coupled with linkage analyses, as essential tools for providing patients and their families with proper genetic counseling and prenatal services.

ACKNOWLEDGMENTS
This work was supported financially by the Science and Technology Development Fund (STDF), Egypt. Grant No.33461. The authors would like to acknowledge the support of (STDF).

CONFLICT OF INTEREST
The authors have no conflict of interest relevant to this article.

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REFERENCES
Abdulqader, A., Mohammed, A. I. Rachid, S., Ghoraishizadeh, P., & Mahmoud, S. N. (2020). Identification of the intron 22 and intron 1 inversions of the factor VIII gene in Iraqi Kurdish patients with hemophilia A. Clinical and Applied Thrombosis/Hemostasis, 26, 1076029619888293.
Abou-Eleew, H., Ahmed, H., Raslan, H., Abdelwahab, M., Hammond, R., Mokhtar, D., & Arnaout, H. (2011). Genotyping of intron 22-related rearrangements of F8 by inverse-shifting PCR in Egyptian hemophilia A patients. Annals of Hematology, 90, 579–584.
Acquila, M., Passino, M., Lanza, T., Molinari, A. C., Caprino, D., Bottini, F., & Bicocchi, M. P. (2004). Identification of mutations in exon 14 including five novelities in 13 Italian patients with haemophilia A. Haemophilia, 10, 744–746.
Astermark, J., Oldenburg, J., Escobar, M., White, G. C. 2nd, Bernortp, E., & Malmö International Brother Study group. (2005). The Malmö International Brother Study (MIBS). Genetic defects and inhibitor development in siblings with severe hemophilia A. Haematologica, 90(7), 924–931.
Atik, T., Işık, E., Onay, H., Akgün, B., Shamsali, M., Kavakli, K., Evim, M., Tuysuz, G., Ozbek, N. Y., Şahin, F., Salçoglu, Z., Albayrak, C., Oymak, Y., Unal, E., Belen, F. B., Yılmaz, E., Balkan, C., Baytan, B., Küpesiz, A., … Özkanay, F. (2020). Factor 8 gene mutation spectrum of 270 patients with haemophilia A: Identification of 36 novel mutations. Turkish Journal of Hematology, 37(3), 145–153.
Bagnall, R. D., Giannelli, F., & Green, P. M. (2006). Int22-related inversions causing hemophilia A: A novel insight into their origin and a new more discriminant PCR test for their detection. Journal of Thrombosis and Haemostasis, 4, 591–598.
Bastida, J. M., González-Porras, J. R., Jiménez, C., Benito, R., Ordoñez, G. R., Álvarez-Román, M. T., Fontecha, M. E., Janusz, K., Castillo, D., Fisac, R. M., García-Grade, L. J., Aguilar, C., Martínez, M. P., Bermujez, J., Herrera, S., Balanzategui, A., Martín-Antorán, J. M., Ramos, R., Cebeiro, M. J., … Sarasquete, M. E. (2017). Application of a molecular diagnostic algorithm for haemophilia A and B using next-generation sequencing of entire F8, F9 and VWF genes. Thrombosis and Haemostasis, 117, 66–74.
Casaña, P., Cabrera, N., Cid, A. R., Haya, S., Beneyto, M., Espinós, C., Cortina, V., Dasi, M. A., & Aznar, J. A. (2008). Severe and moderate hemophilia A: Identification of 38 new genetic alterations. Haematologica, 93(7), 1091–1094.
Centers for Disease Control and Prevention (CDC). (2016). Hemophilia A mutation Project (CHAMP). http://www.cdc.gov/ncbddd/he-mo-philica/champs.html. Accessed 29th March 2016.
Coppola, A., Murgaglione, M., Santiagoostino, E., Rocino, A., Grandone, E., Mannucci, P. M., & Di Minno, G.; AICE PROFIT Study Group. (2009). Factor VIII gene (F8) mutations as predictors of outcome in immune tolerance induction of hemophilia A patients with high-responding inhibitors. Journal of Thrombosis and Haemostasis, 7, 1809–1815.
Cumming, A. M. (2004). The factor VIII gene intron 1 inversion mutation: Prevalence in severe hemophilia A patients in the UK. Journal of Thrombosis and Haemostasis, 2, 205–206.
EAHAD Coagulation Factor Variant Databases (2017). [cited 13th March 2017]. http://www.factorviii-db.org/index.php
Factor 8 Variant Database. (2014). http://factorviii-db.org/
Fernández-López, O., García-Lozano, J. R., Núñez-Vázquez, R., Pérez-Garrido, R., & Núñez-Roldán, A. (2005). The spectrum of mutations in Southern Spanish patients with hemophilia A and identification of 28 novel mutations. *Haematologica*, 90(5), 707–710.

Gouw, S. C., van den Berg, H. M., Oldenburg, J., Astermark, J., de Groot, P. G., Margaglione, M., Thompson, A. R., van Heerde, W., Boekhorst, J., Miller, C. H., le Cessie, S., & van der Bom, J. G. (2012). F8 gene mutation type and inhibitor development in patients with severe hemophilia A: Systemic review and meta-analysis. *Blood*, 119, 2922–2934.

Graw, J., Brackmann, H., Oldenburg, J., Schneppenheim, R., Spannag, M., & Schwaab, R. (2005). Haemophilia A: From mutation analysis to new therapies. *Nature Reviews Genetics*, 6, 488–501.

Hill, M., Deam, S., Gordon, B., & Dolan, G. (2005). Mutation analysis in 51 patients with haemophilia A: report of 10 novel mutations and correlations between genotype and clinical phenotype. *Haemophilia, 11*(2), 133–141.

Hussein, I. R., El-beshlawy, A., Salem, A., Mosaad, R., Zaghoul, N., Ragab, L., Fayek, H., Gaber, K., & El-ekiabi, M. (2008). The use of DNA markers for carrier detection and prenatal diagnosis of haemophilia A in Egyptian families. *Haemophilia, 14*, 1082–1087. https://doi.org/10.1111/j.1365-2516.2008.01779.x

Inaba, H., Shinozawa, K., Amano, K., & Fukutake, K. (2017). Identification of deep intronic individual variants in patients with hemophilia A by next-generation sequencing of the whole factor VIII gene. *Research and Practice in Thrombosis and Haemostasis, 1*(2), 264–274.

Keeney, S. (2010). Primers for PCR amplification. HAMSTErS. The HAMSTErS. The

Kembell-Cook, G., Tuddenham, E. G., & Wacey, A. I. (1998). The factor VIII structure and mutation resource site: HAMSTErS version 4. *Nucleic Acids Research, 26*, 216–219.

Lenting, P. J., van Mourik, J. A., & Mertens, K. (1998). The life cycle of coagulation factor VIII in view of its structure and function. *Blood, 92*, 3983–3996.

Miller, S. A., Dykes, D. D., & Polesky, H. F. (1988). A simple salting out procedure for extracting DNA from human nucleotide cells. *Nucleic Acid Research, 16*, 1215.

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OMIM, Online Mendelian Inheritance in Man. (2020). https://www.omim.org. Updated January 3, 2020.

Paroskie, A., Gailani, D., DeBaun, M. R., & Sidonio, R. F. (2015). A cross-sectional study of bleeding phenotype in haemophilia A carriers. *British Journal of Haematology, 170*, 223–228.

Payne, A. B., Miller, C. H., Kelly, F. M., Michael Soucie, J., & Craig Hooper, W. (2012). The CDC Haemophilia A Mutation Project (CHAMP) mutation list: A new online resource. *Human Mutation, 34*(2), E2382–E2391.

Plug, I., Mauser-Bunschoten, E. P., Brocker-Vriends, A. H., van Amstel, H. K., van der Bom, J. G., van Dienen-Homan, J. E., Willemse, J., & Rosendaal, F. R. (2006). Bleeding in carriers of hemophilia. *Blood, 108*, 52–56.

Reitter, S., Sturm, R., Horvath, B., Freitag, R., Male, C., Muntean, W., Streif, W., Pabinger, I., & Mannhalter, C.; Austrian Molecular Haemophilia Study Group. (2010). Spectrum of causative mutations in patients with haemophilia A in Austria. *Thrombosis and Haemostasis, 104*(1), 78–85.

Repesse, Y., Sloua, M., Ferrandiz, D., Gautier, P., Costa, C., Costa, J. M., Lavergne, J. M., & Borel-Derlon, A. (2007). Factor VIII (F8) gene mutations in 120 patients with hemophilia A: Detection of 26 novel mutations and correlation with F8 inhibitor development. *Journal of Thrombosis and Haemostasis, 5*, 1469–1476.

Rossetti, L. C., Radic, C. P., Larripa, I. B., & De Brasi, C. D. (2005). Genotyping the hemophilia inversion hotspot by use of inverse PCR. *Clinical Chemistry, 51*, 1154–1158. https://doi.org/10.1373/clinchem.2004.046490

Rossetti, L. C., Radic, C. P., Larripa, I. B., & De Brasi, C. D. (2008). Developing a new generation of tests for genotyping hemophilia causative rearrangements involving int22h and int11h hotspots in the factor VIII gene. *Journal of Thrombosis and Haemostasis, 6*, 830–836.

Saenko, E. L., Ananyeva, N. M., Tuddenham, E. G., & Kembell-Cook, G. (2002). Factor VIII - Novel insights into form and function. *British Journal of Haematology, 119*, 323–331.

Santacroce, R., Acquila, M., Belvini, D., Castaldo, G., Garagiola, I., Giacomelli, S. H., Lombardi, A. M., Minuti, B., Riccardi, F., Salvato, R., Tagliabue, L., Grandone, E., & Margaglione, M.; The AICE-Genetics Study Group. (2008). Identification of 217 unreported mutations in the F8 gene in a group of 1,410 unselected Italian patients with hemophilia A. *Journal of Human Genetics, 53*, 275–284.

Santagostino, E., Mancuso, M. E., Tripodi, A., Chantarangkul, V., Clerici, M., Garagiola, I., & Mannucci, P. M. (2010). Severe hemophilia with mild bleeding phenotype: molecular characterization and global coagulation profile. *Journal of Thrombosis and Haemostasis, 8*(4), 737–743.

Srivastava, A., Brewer, A. K., Mauser-Bunschoten, E. P., Key, N. S., Kitchen, S., Llinas, A., Ludlam, C. A., Mahliangu, J. N., Mulder, K., Poon, M. C., & Street, A.; Treatment Guidelines Working Group on Behalf of The World Federation Of Hemophilia. (2013). Guidelines for the management of hemophilia. *Haemophilia, 19*(1), e1–e47.

Viel, K. R., Machhia, D. K., Warren, D. M., Khachidze, M., Buil, A., Fernstrom, K., Souto, J. C., Peralta, J. M., Smith, T., Blangero, J., Porter, S., Warren, S. T., Fontcuberta, J., Soria, J. M., Flanders, W. D., Almasy, L., & Howard, T. E. (2007). A sequence variation scan of the coagulation factor VIII (F8) structural gene and associations with plasma F8 activity levels. *Blood, 9*, 3713–3724.

Vinciguerra, C., Zawadzki, C., Dargaud, Y., Pernod, G., Berger, C., Nouger, C., & Négrier, C. (2006). Characterisation of 96 mutations in the factor VIII gene correlating with severe haemophilia A in 120 patients. *Journal of Thrombosis and Haemostasis, 4*(2), E2382–E2391.

World Federation of Hemophilia report on the Annual Global Survey 2014, 2015, 2016, 2017, 2018. http://www1.wfh.org/publications/files/pdf-1627.pdf. Accessed July 7, 2018.