# EDITORIAL

## Integrating Next Generation Sequencing (NGS) into Haemophilia A Genetic Diagnosis.

Haemophilia A (HA, OMIM#306700) is a rare X-linked hereditary disorder that manifests as a bleeding disorder in males and affects 1 in 5000 boys. It is caused by a deficiency of coagulant factor 8 (*F8)*, which is a co-enzyme, critical in accelerating the generation of *Factor 10a* (*F10a)* and thrombin. In the blood circulation, *F8* binds to *von Willebrand factor (VWF)* and is activated (*F8a)* by *F10a*. The *F8a* forms the *Xase* complex with *Factor 9* to potentiate *F10a* generation [1]. *F8* gene is on Xq28, spans about 180kb and has 25 introns and 26 exons, ranging in size from 69 to 3,106 bp [2]. Depending on their *F8* levels, people with haemophilia A (PwHA) experience spontaneous bleeding or difficulty in controlling the bleed. PwHA clinical manifestations include haemathrosis, deep muscle haematoma, cerebral bleed with minimal or no antecedent trauma, cephalohaematoma in neonates, prolonged bleeding after tooth extraction or circumcision, delayed bleeding, and poor wound healing after surgery [3]. A coagulation assay showing extended activated partial thromboplastin time (aPTT), normal *VWF* levels, and low factor *F8* clotting activity is used to make the diagnosis: <1% *F8* levels in severe PwHA, 1-5% *F8* levels in moderate PwHA, and >5%-40% *F8* levels in mild PwHA. Genetic testing of PwHA identifies the pathogenic mutation of *F8* gene, and is used to forecast the clinical phenotype, evaluate the likelihood of developing *F8* inhibitor (antibodies), and determine the best treatment plan. Intron 22 inversion is the most common *F8* mutation, seen in 45% of PwHA, while 16% due to deletions or insertions, 15% from missense mutations, 10% with nonsense mutations, 3% with large deletions, 3% with splice-site mutations, 2% with intron 1 inversion, and 4.6% with unknown mutation [4]. Efforts to genotype PwHA around the globe have identified large numbers of unique mutations in the *F8* gene. The *F8* mutation list now contains 3,756 unique mutations that are known to cause HA. The mutation list can be downloaded at the CDC Hemophilia A Mutation Project (CHAMP) Mutation List, accessible at https://www.cdc.gov/ncbddd/hemophilia/champs.html.

Genetic testing starts with screening for intron 22 and intron 1 inversions, followed by sequencing of coding regions and splice sites of the *F8* for PwH with non-inversion mutations. Samples that do not yield any findings will be subjected to Multiplex Ligation-dependent Probe Amplification (MLPA) to detect large deletions. The diagnostic workflow for haemophilia A is depicted in Figure 1. Recent use of next-generation sequencing (NGS) has increased the identification of mutations to the point that genetic variants unrelated to haemophilia A or incidental mutations are being discovered. This may be a problem in terms of how to handle accidental discoveries that may signal a risk of another disease or condition [5].

Basically, there are two types of sequencing, Sanger, a chain terminating or first-generation sequencing and NGS, a second-generation sequencing, that can perform massive parallel sequencing. The difference between the two is in the volume of samples. Sanger sequences one DNA fragment at a time, but can read 500-1000bp length of DNA, whereas NGS sequences several but small fragments (50-500bp length of DNA) in a single run. The advantage of using NGS in haemophilia A is that it has higher sequencing depth, which

leads to more mutation discovery but, it is not cost-effective for sequencing low targets and a small number of samples. Sanger sequencing on the other hand, is suitable for low volume samples, but it is labour intensive and more expensive. The following are two studies that used NGS to detect non-inversion mutations causing haemophilia A. Borràs N et al (2021) investigate the molecular epidemiology of 106 Cuban haemophilia patients using NGS [6]. Long-range PCR (LR-PCR) was used to identify intron 22 inversion, while intron 1 inversion was detected by conventional PCR. NGS-based sequencing of *F8, F9*, and *VWF* genes were performed and analysed in all patients. A customized gene panel was designed to target all exonic and intronic flanking regions of 30 genes. Primer design, amplification, and indexing were performed with the LP 48.48 Access Array integrated fluidic circuit (IFC; Fluidigm, CA, USA), a nanofluidic chip that enables processing of 693 PCRs from 48 samples. All indexed amplicons were pooled in a single tube and then underwent adapter ligation and clean up. The NGS libraries were simultaneously sequenced on a MiSeq system (Illumina, CA, USA). 131 candidate variants along the *F8, F9*, and *VWF* genes were identified which included 72 unique variants and 28 of them (39%) were not previously described. Putative variants were identified in 105/106 patients. The mutations identified by NGS and bioinformatic analyses leads to reclassification of 85% of PwHA in this study [6].

Inaba H et al (2017) performed whole genetic analysis of *F8* gene using NGS on 45 Japanese PwHA, with 31 of them had previous genetic analysis being done. 48 gene locus was amplified in 14 overlapping regions (5-23kb) by LR-PCR. In total, approximately 197 kb (including the upstream and downstream regions of *F8*) were amplified. After purification, the PCR fragments were mixed in equimolar amounts. The DNA library was prepared by fragmentation using a Nextera XT DNA sample preparation kit (Illumina Inc., San Diego, CA, USA). The paired-end adapter-ligated fragments of the pooled libraries were attached to the flow cell and sequenced using the amplicon sequencing application of the MiSeq software program (Illumina Inc.). The obtained nucleotide sequences were aligned to the GRCh37/hg19 coordinates of an *F8* reference sequence (ENSG00000185010) using the Burrows-Wheeler Aligner. The variants were detected using the Genome Analysis Toolkit and were annotated by the VariantStudio software program (Illumina Inc.) NGS sequencing coverage was sufficiently high (>20 reads) and analysis allowed for the identification of genetic variants within 99% of *F8*. Each patient had an average of 140 variants discovered. After analysing these variants and comparing them to previously identified mutations, it was confirmed that NGS could detect single nucleotide variants and tiny deletions with great accuracy and efficiency. However, structural variants (such as inversions and huge duplications) were not recognised correctly. Following bioinformatic filtration, 27 unusual and unique variants were discovered in 16 patients. PredictSNP2 analysis predicted that three of these variants were deleterious. The remaining variants needed more research to determine their functions and impacts on *F8* expression.

## Conclusion

Next-generation sequencing (NGS) is a powerful tool that can examine the entire *F8* gene and detect variants deep within the gene, leading to the discovery of novel and unique variants. Some of these novel variants have been identified as deleterious and is the cause of haemophilia A. Unfortunately, many variants are yet unknown in terms of clinical

significance. Nonetheless, identifying and understanding these variants may in the near future provide a better tool for precise and personalized PwHA treatment.

**Keywords**: *Haemophilia A, long-range PCR; genetics, next generation sequencing.*

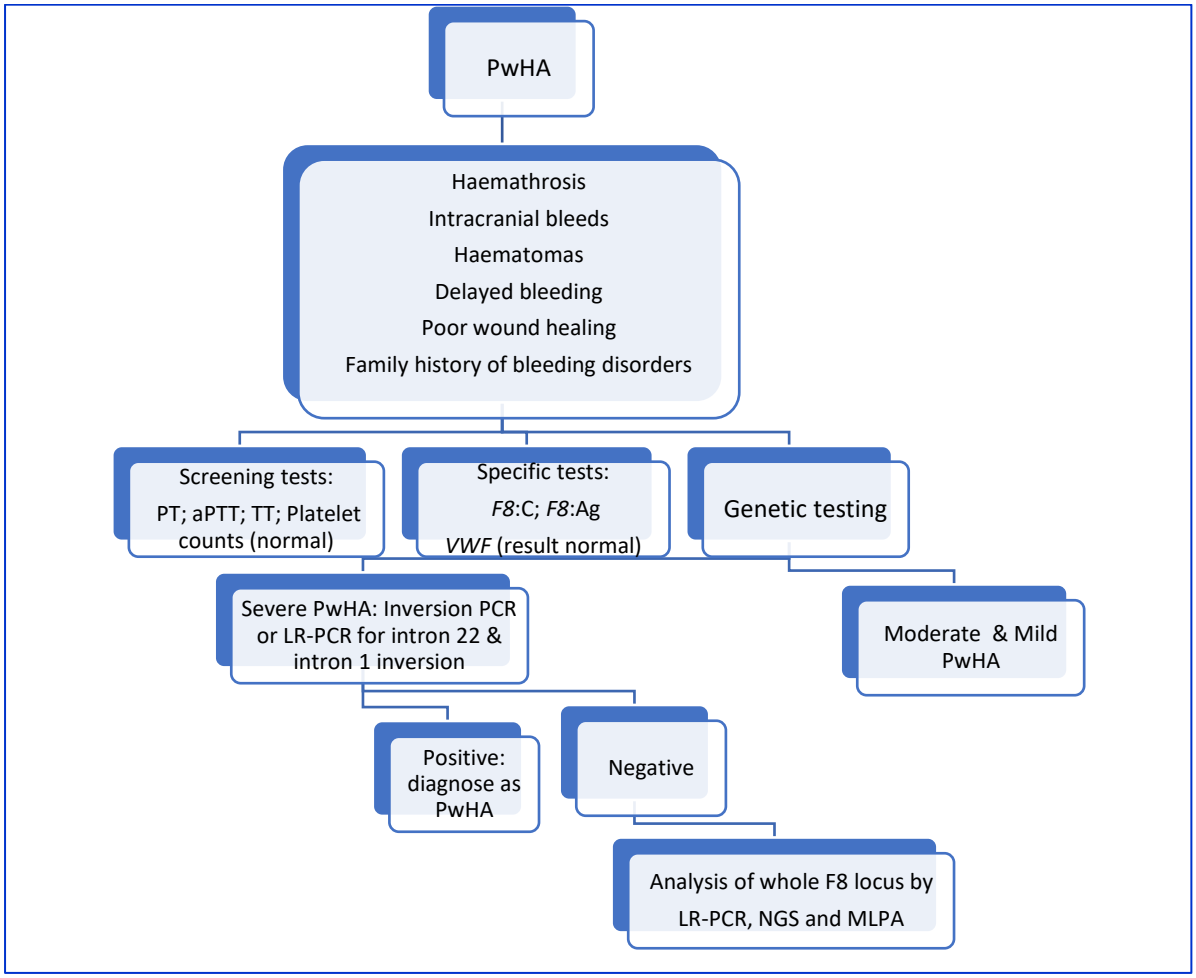


Figure 1. Diagnostic workflow of PwHA. PwHA, person with haemophilia A; PT, prothrombin time; aPTT, activated partial thromboplastin time; TT, thrombin time; *F8*:C, Factor *8* clotting assay; *F8*: Ag, F*8* antigen Elisa assay; LR-PCR, long range polymerase chain reaction; NGS, next generation sequencing; MLPA, Multiplex Ligation-dependent Probe Amplification. Adapted from Pezeshkpoor B et al [5]

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