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GENETIC STUDY OF HEMOPHILIA A IN IRAQI TEENAGER

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ABSTRACT

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Master of Science in Chemistry

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Hemophilia A (HA) is caused by a deficiency in FVIII, an essential cofactor in the activation of the FX complex. In DNA, SNPs have a major role in gene alterations that disturb gene product sequencing. In this thesis, blood samples were taken to tube with EDTA from 60 patients ranging in age (6-24) years with severe, moderate, and mild deficiency of factor VIII. The samples were stored at -20°C until using. All samples were obtained from the Medical City of Baghdad between the 13th of Jun 2021 to 28th of September of 2021. This research aims to study the clinical and biological factors and the extent of their impact on the F8 gene that causes HA. All were examined by conventional PCR and then sequencing for intron 18 specifically at SNP (rs4898352) located at chrX:154903815 (GRCh38.p13). The results showed severe HA 75% (45/60), moderate 15% (9/60), and mild 10% (6/60). The ages between (20-29) years (n=25) (41.6%) showed the major age range of severe HA. PCR sequencing confirmed the causative mutation for the hemophilic patients showed A>T Nucleotide Location (123909) transversion mutation. There is a strong relationship between family history and HA that caused traits to be passed to children, especially mothers. Our results indicated a big correlation among young ages with HA. Furthermore, early clinical examinations should be performed for early treatment.

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Keywords: Hemophilia A, Conventional PCR, FV III, F8 gene, SNP, rs4898352, FVIII inhibitors, Genetic diagnosis

ÖZET

IRAKLI GENÇLERDE HEMOFİLİ A ÜZERİNE GENETİK ÇALIŞMA

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Hemofili A (HA), FX kompleksinin aktivasyonunda önemli bir kofaktör olan FVIII'deki bir eksiklikten kaynaklanır. DNA'da, SNP'ler, gen ürün dizilişini bozan gen değişikliklerinde önemli bir role sahiptir. Bu araştırma, klinik ve biyolojik faktörleri ve bunların HA'ya neden olan F8 geni üzerindeki etkilerinin derecesini araştırmayı amaçlamaktadır. Bu tezde, yaşları (6-24) arasında değişen şiddetli, orta ve hafif faktör VIII eksikliği olan 60 hastadan EDTA'lı tüpe kan örnekleri alındı. Numuneler kullanılıncaya kadar -20°C'de saklandı. Tüm numuneler 13 Haziran 2021 ile 28 Eylül 2021 tarihleri arasında Bağdat Tıp Şehri'nden alınmıştır. Hepsi konvansiyonel PCR ile incelendi ve ardından intron 18 için spesifik olarak chrX:154903815 (GRCh38.p13) konumunda bulunan SNP'de (rs4898352) sekanslama yapıldı. Sonuçlar şiddetli HA %75 (45/60), orta %15 (9/60) ve hafif %10 (6/60) gösterdi. PCR dizilimi, AT Nucleotid Location (123909) transversiyon mutasyonu gösteren hemofilik hastalar için nedensel mutasyonu doğruladı. PCR dizilimi, hemofilik hastalar için nedensel mutasyonun A>T Nükleotid Yerleşimi (123909) transversiyon mutasyonu gösterdiğini doğruladı. Aile öyküsü ile HA arasında, özelliklerin çocuklara, özellikle de annelere geçmesine neden olan güçlü bir ilişki vardır. Sonuçlarımız, HA ile genç yaşlar arasında büyük bir korelasyon gösterdi. Ayrıca erken tedavi için erken klinik muayene yapılmalıdır.

2022, 46 sayfa

Anahtar Kelimeler: Hemofili A, Konvansiyonel PCR, FVIII, F8 geni, SNP, rs4898352, FVIII inhibitörleri, Genetik teşhis

PREFACE AND ACKNOWLEDGEMENTS

I would to give a big thanks to my father Dr. Adil MORTADHA who always was by my back and draw my future, big thanks to him, and thanks to all my family members that they share and carry a lot of things with me. Thanks to all my friends who support me, and a special thanks to my advisor, Assoc. Prof. Dr. Şevki ADEM and co-advisor, Dr. Mohammed Mezher HUSSEIN for their patience, guidance, and understanding. Finally, I would to thank my friends Dr. Karam MOLBIO, Mr. Mahmood ALI, and Mr. Muthana MAIZAR for their help.

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LIST OF SYMBOLS

%	Percentage
˚C	Celsius
>	Greater-than
<	Less-than
cm	Centimeter
Kb	Kilobyte
mL	Milliliter
mmHg	Millimeter of mercury
ng	Nanogram
pg	Picogram
µL	Microliter

LIST OF ABBREVIATIONS

A	Adenine
AAV	Adeno-associated viral vector
Ag	Antigen
Bp	Base per
C	Cytosine
ChrX	Chromosome X
DNA	Deoxyribonucleic acid
CBD	Congenital bleeding disease
F8	Factor 8
FIIa	Thrombin
FIXa	Activated factor 9
FVIII	Factor 8
FX	Factor X
Fxa	Factor 10
G	Guanine
HA	Hemophilia a
hrRNA	Heterogeneous nuclear ribonucleic acid
HLA	Human leukocyte antigens
HSC	Hematopoietic stem cells
IgG	Immunoglobulin g
IL	Interleukin
INR	International normalized ratio
MHC	Major histocompatibility complex
MAb	Monoclonal antibody
PCR	Polymerase chain reaction
PEG-INF	Pegylated interferon
PFA	Platelet function assay
PGD	Preimplantation genetic diagnosis
PT	Prothrombin time
PTT	Partial thromboplastin time
RNA	Messenger ribonucleic acid
SNP	Single nucleotide polymorphism
snRNPs	Small nucleolar ribonucleoproteins
T	Thymine
TAE	Tris-acetate buffer
TNF-alpha	Tumour necrosis factor-alpha
UV	Ultra violet
VWF	Von willbrand factor

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1. INTRODUCTION

Hemophilia A (HA), is the most prevalent hereditary recessive X-linked blood coagulation disorder resulting from a deficiency in factor VIII (FVIII), with a rate of 1:5,000 to 1:10,000 in men (Ng and Lee 2009). Along with type hemophilia, there are two other forms. Type B is caused by a lack of factor IX (also called Christmas disease) and the third one is very rare Hemophilia type C that is caused by a deficiency in factor XI (also called Rosenthal syndrome).

In this condition, heterogeneous mutations play a significant impact. Many mutations, such as inversion, rearrangement, and deletion frameshift, distort the F8 gene locations on the X chromosome. The FVIII protein is required for the proliferation of the intrinsic coagulation pathway (Hedner *et al.* 2000).

HA is more prevalent than HB, accounting for 80–85% of the hemophilic community. However, both the F8 and F9 genes are prone to novel mutations, with up to one-third of all instances being the result of unexpected spontaneous mutation with unknown family history.

Patients with Hemophilia should be monitored closely to prevent and cure bleeding caused by the insufficient clotting factor. Whenever possible, a deficit in a specific factor should be treated with a measured concentration of that factor. Acute bleeding should be managed expeditiously, preferably within two hours. Even before the physical indicators appear, patients can usually recognize early signs of bleeding. A tingling sensation or "aura" is commonly mentioned.

SNP or A single nucleotide polymorphism is a difference in the sequence of a single nucleotide in DNA. A, C, G, and T are the four nucleotide bases that make up a DNA sequence. SNPs are described as variances in which more than 1% of a populace lacks the same nucleotide at a certain position in the DNA sequence. Once an SNP is found inside a gene, the gene has several alleles. In these situations, SNPs can alter the amino

acid sequence in a variety of ways. They aren't only found in genes; They might be present in non-coding sections of DNA. Since an SNP does not directly cause a problem, it is associated with other disorders. SNPs are utilized to determine which gene or genes are responsible for a particular trait (Sherry *et al.* 2001).

A Variety of mutations in the FVIII gene leading to HA that cause qualitative and quantitative abnormalities in the function of FVIII, an important factor in the activation of the FX complex (Sukarova *et al.* 2008). More than 900 mutations have been discovered in the FVIII coding and untranslated regions (Elmahmoudi *et al.* 2012). Almost 50% of all severe HA cases are due to splice site, missense, nonsense, frameshift mutations, and deletion variations. Most common molecular abnormalities observed in severe HA patients are intron 22 and intron 1 inversions, which occur at a rate of 45–50% and 0.5–5 %, respectively. Missense substitutions commonly result in moderate and mild (Tokoro *et al.* 2020). Numerous techniques have been utilized to find mutations in HA, ranging from linkage analysis to mutation screening methods that identify specific sections of the gene for sequencing to direct sequencing of the entire gene. Direct sequencing, is still not commonly used, because it is highly expensive costs (Al-Allaf *et al.* 2016). Currently, Iraq lacks molecular genotyping services for HA. Screening for molecular abnormalities has become a significant tool in hemophilia care, both for predicting the clinical course and for giving safe genetic counseling to families. Therefore, the objective of this study was to screen and detect the genetic defect that occurred in an intron variant in the SNP ID (rs4898352) of the F8 gene that located at the long arm of the X chromosome (chrX:154903815 (GRCh38.p13)) of Iraqis teenager patients by using conventional Polymerase chain reaction (PCR) and sequencing, then the results compared with world resources NCBI nucleotide blasts.

2. LITERATURE REVIEW

2.1 Hemophilia A

Hemophilia is a term that refers to the love (philia) for blood (hemo), which is an X-linked manifests phenotype marked by excessive and prolonged bleeding neither spontaneously nor after insignificant trauma. A heterogeneous mutation in the F8 gene is cause HA. The disorder affects around 400 000 people worldwide, with just 25% receiving adequate treatment. Since the development of clotting medicines, hemophilia patients' life expectancy has increased. In industrialized countries, patients with hemophilia have a longer life expectancy than in underdeveloped countries, although it is 10 to 15 years less than that of persons without the disorder (Franchini and Mannucci 2013).

2.1.1 Prevalence of hemophilia

Hemophilia A has been identified in people from all walks of life and all ethnic groups. Its prevalence is estimated to be 1 in 5000 male live births or 1 in 10,000 live births. HB is less prevalent, occurring in about 1 in every 30 000 male live births. Hemophilia C, on the other hand, affects roughly 1 in every 100,000 people. In 2015, With 18,383 patients and a frequency of 1.4/100,000 persons, India led the list of nations with the most hemophilia A, B, and C patients, followed by the United States and China (Angelis *et al.* 2015).

Around 10% of females with one F8 disease-causing mutation and one healthy allele have a mild bleeding issue. It is assumed that for every male with hemophilia, there are five possible female carriers (Street *et al*. 2008).

According to the 2016 annual report of the World Federation of Hemophilia (WFH), the total number of Iraqi patients with all types of hemophilia was estimated to be 1346, resulting in a prevalence of 3.7/100,000. Severe hemophilia accounted for 63.4 % of all

hemophiliacs. Hepatitis C virus (HCV), hepatitis B virus (HBV), and HIV infections were found to be prevalent in 22.9%, 0.9%, and 0.2% of the population, respectively. In 45.1% of patients, target joints were identified, whereas clotting factor inhibitors were found in 11.6%. Prophylactic treatment was used by about 27% of the participants. In 2016, there was only one death. In Baghdad, hemophilia's prevalence and occurrence more than doubled over ten years. The rates of various problems were almost similar to those in neighboring nations (Kadhim *et al.* 2019).

2.1.2 The severity of FVIII deficiency with signs and symptoms

The level of clotting factor is often inversely proportional to the severity of bleeding; as a severe phenotype, the clotting factor is (< 1%) of normal; Patients in this category suffered spontaneous bleeding into joints or muscles in the absence of a discernible hemostatic challenge. Range from 1 to 5% categorized as moderate, these patients are suffering from prolonged bleeding due to minor trauma or surgery. Mild patients (>5%) and their bleeding episodes are associated with surgery or serious trauma, but spontaneous bleeding is uncommon (White *et al.* 2001).

The majority of children with severe hemophilia have experienced bleeding throughout their lives, sometimes children with severe hemophilia may not exhibit bleeding symptoms before they start running or walking. Patients with mild severity may not bleed profusely until they are injured or undergo surgery. Some bleedings are life-threatening and must be treated right away. Internal bleeding, such as into the joints or muscles, is the most often occurring sort of bleeding.

The serious bleeding includes joints (hemarthrosis), muscles, particularly in the deep compartments (forearm, iliopsoas, and calf) with estimated frequency 70-80% and 10-20% respectively. Other areas of significant bleeding include the mucosal membranes (in the mouth, gums, nose, and urogenital system), and the CNS, which occur at a rate of 5-10% and <5%, respectively. Life-threatening bleeding can also happen in the intracranial, neck/throat, and gastrointestinal system (Aronstam *et al.* 1979).

If the location of bleeding is not clinically visible, an examination should be undertaken and adequate clotting factors provided during an episode of acute bleeding. Treatment with factors should begin immediately. even before the doctor's diagnosis is completed, severe bleeding episodes, especially in the gastrointestinal tract, neck, head, and chest can be life-threatening.

2.1.3 Monitoring and management

Patients should carry identification that includes their diagnosis, bleeding severity disorder, inhibitor status, type of treatment product, initial dosage, as well as contact information for the treating doctor to facilitate appropriate management in emergencies (Singleton *et al.* 2010). FVIII level can raise adequately by administration of desmopressin injection (Peisker *et al.* 2014). The injection of desmopressin causes a brief increase in the number of clotting factors in the circulation. This implies it can be used as a safety precaution before any dentistry, minor surgical, or invasive procedures, can assist in controlling excessive bleeding. Individual patients should be tested for desmopressin responsiveness, The test will reveal whether the amount of factor VIII has increased.

After any significant bleeding event, they should be seen by a comprehensive care team. patients can be assessed longitudinally with regular standardized examinations, at least once every 12 months, and the early detection of new or emerging problems, allowing treatment regimens to be changed (Hermans *et al*. 2012).

The following notes should be assessed, as well as educated, and reviewed.

- Problems with venous access.
- Hemostasis issues (bleed record).
- The usage of replacement treatment items and the responses to them.
- Musculoskeletal status: determined by clinical examination of muscles and joints, as well as radiographic examination once a year or as needed.

- Transfusion-transmitted infections include HIV, HCV, and HBV, as well as additional infections.
- Inhibitor developments.
- Dental/oral health and psychosocial status.

Clinical bleeding symptoms or plasma procoagulant levels have been used to classify the severity of hemophilia; the latter is the most generally used criteria.

2.1.4 Surgery and invasive procedures

When it comes to oral surgery, patients with hemophilia are at particular risk of bleeding for both intraoperative and postoperative. As a result, oral surgeons and a complete Hemophilia treatment center must work together to treat patients with inherited bleeding disorders. Clotting factor replacement treatment is essential for all surgical procedures requiring an incision. For hemophilia-related issues or unrelated disorders, surgery may be necessary. Those having hemophilia will necessitate more planning and communication with the healthcare team before surgery than those without the condition. A comprehensive hemophilia treatment clinic is the ideal place to take care of a hemophilia patient who needs surgery (Hermans *et al*. 2009).

Patients with bleeding issues should be treated by an anesthesiologist with previous experience. Reliable clotting factor level and inhibitor testing monitoring require appropriate laboratory services. Pre-operative testing should include checking for inhibitors and testing for inhibitors, specifically if the replacement factor recovers much less than expected (Teitel *et al.* 2009). A weekday morning surgery is best since this allows for adequate laboratory and blood bank backup, should it be required. It's important to have enough clotting factor concentrates on hand for both the surgery itself and post-surgery to ensure enough coverage for healing and recovery. Support for plasma components is required if clotting factor concentrates are unavailable. The type of surgery dictates how much clotting factor concentrate to use and how long it should be applied for.

2.1.5 Dental extraction

To help induce the release of VWF from the endothelium cells, individuals with mild types of hemophilia A typically receive desmopressin acetate after consulting with a hematologist before dental extraction. This results in a threefold to fivefold increase in VWF (as well as coagulant factor VIII) levels. Desmopressin acetate can be given intravenously, subcutaneously, or intranasally one hour before the dental treatments. The cardiovascular side effects of intravenous desmopressin acetate, such as a slightly raised pulse rate, hypotension, and nausea, may occur when administered intravenously. Desmopressin acetate is not suggested for patients under the age of 18 and those with coronary artery disease (Anderson *et al*. 2013).

Replacement therapy with factor concentrate or recombinant factor VIII is required before dental extractions can be performed on individuals with moderate to severe hemophilia A. Factor concentrate, on the other hand, is costly and can lead to the formation of autoantibodies or inhibitors that affect clotting, therefore avoidance of unnecessary dental treatments is advised. As a result, there will be less need for replacement medication as well as a lower risk of inhibitor development. As a preventative measure, replacement therapy can be delivered by a healthcare provider; as an emergency treatment, it can be provided by the patient or their caregiver. Within 30-60 minutes of taking factor concentrate, it is advisable to begin the dental procedure. A recombinant factor VIII can be administered to patients with moderate to severe hemophilia A to prevent transfusion of infected blood, which can cause blood-borne infection. Before undergoing any type of dental procedure, patients with hemophilia A should have their factor VIII levels checked. Before basic oral and periodontal surgery, hematologists recommend that a patient's factor VIII concentration be between 50% and 75% of the normal value. However, before maxillofacial surgery, the factor VIII level must be around 75% and 100% (Rafique *et al*. 2013).

2.1.6 Factor VIII

Coagulation and hemostasis come from two important proteins of human blood plasma. FVIII: C (anti-hemophilic factor or pro-coagulant protein) and VWF (Von Willebrand factor) are genetically distinct proteins with diverse biochemical and immunologic properties, as well as distinct and critical physiologic activities (Terraube *et al*. 2010). Reports indicating proteins isolated from human plasma have factor VIII procoagulant (FVIII: C) activity and the ability to react with platelets that could represent an important role in primary hemostasis in vivo. Strengthened the case that factor VIII is a bifunctional molecule. Right now, widely believed the plasma FVIII complex comprising of two ingredients with separate activities, biochemical and immunologic features, and genetic regulation, (Table 2.1) summarizes the features of these components.

Table 2.1 The FVIII-complex components (Mannucci *et al*. 2014)

VIII: C	The FVIII pro-coagulant protein (the anti-hemophilic factor). Categorized as: - Factor VIII pro-coagulant (VIII:C) Measured of the normal plasma pro-coagulant property done by standard coagulation assays. - Factor VIII pro-coagulant antigen (VIII: Cg) Anti-genic determinants that are closely linked to VIII: C. Immunoassays using human antibodies are used to measure this.
VIIIR	The FVIII-related protein (VWF), is a protein with a large polymer that is essential for adhesion and BT of normal platelet in vivo. Categorized as: - FVIII-related antigen (VIIIR: Ag) VIIIR antigenic determinants are recognized by heterologous antibodies. - Ristocetin co-factor (VIIIR:RC) The purpose of normal plasma VIIIR is to promote the agglutination of washed normal platelets by ristocetin.

2.1.7 Factor VIII with coagulation pathway

Coagulation is a process of clotting factors that results from sequential activation, both co-factors, and zymogens, resulting in the formation of thrombin in (Figure 2.1). The FVIII is a co-enzyme that aids in the formation of activated X and then thrombin. As a result, FVIII is critical for the spread of a hemostatic response. To promote platelet adherence at damaged locations the FVIII in the circulation attached to VWF, that stabilizes FVIII and connects with the subendothelial matrix. Thus, localizing FVIII to these locations. The next activation by activated FX or activated FII-mediated proteolysis through feed-back loops and amplification, activated FVIII (who becomes disengaged from VWF) composes the Case-complex with activated FIX on the platelets surface to amplify the formation of activated factor X (Terraube *et al*. 2010).

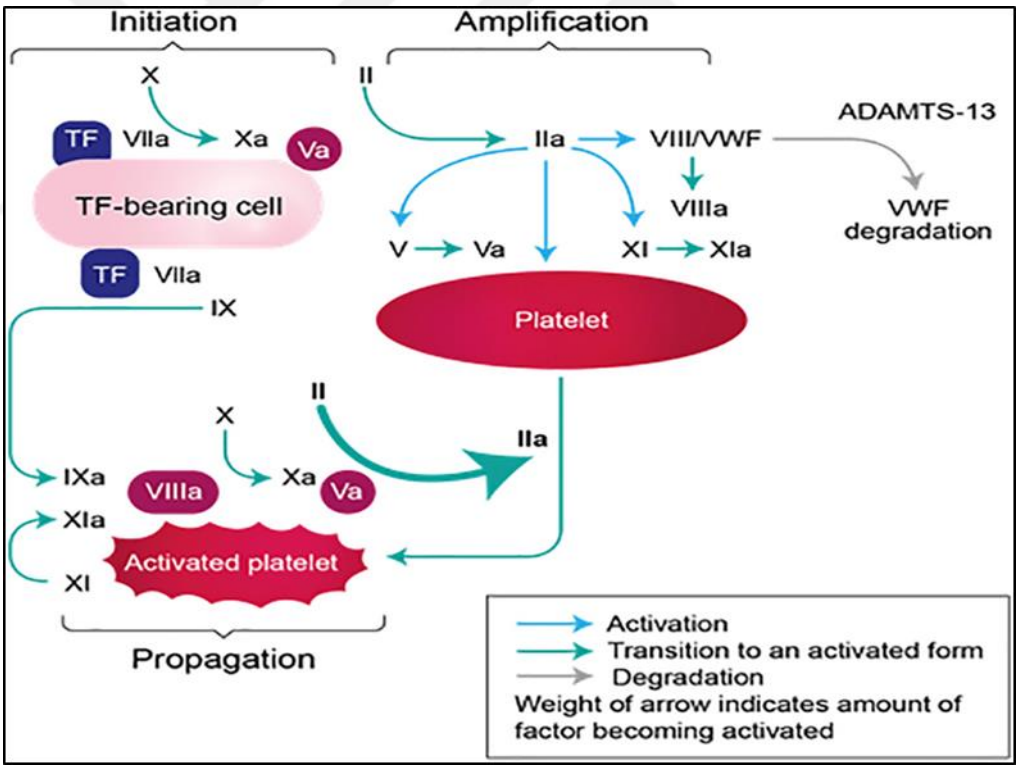

Figure 2.1 Phases of coagulation (Bannow *et al.* 2019)

2.2 Inheritance

Because the disorder is hereditary the risk factor of Hemophilia is a wide range. Generally, X-linked recessive affects males exclusively. A hemizygous male carrying a mutant allele on his single X-linked chromosome is incapable of transmitting the condition to his sons, but all of the daughters will be committing carriers. Heterozygous carrier woman transfers the disorder onto their male sons who are affected. As a result, it can be passed from affected males to male grandchildren via carrier daughters (Dobyns *et al.* 2004). Each male offspring of a carrier woman who has children with a healthy male has a 50% risk of acquiring the disorder, while each female offspring has a 50% risk to be carrier (Figure 2.2).

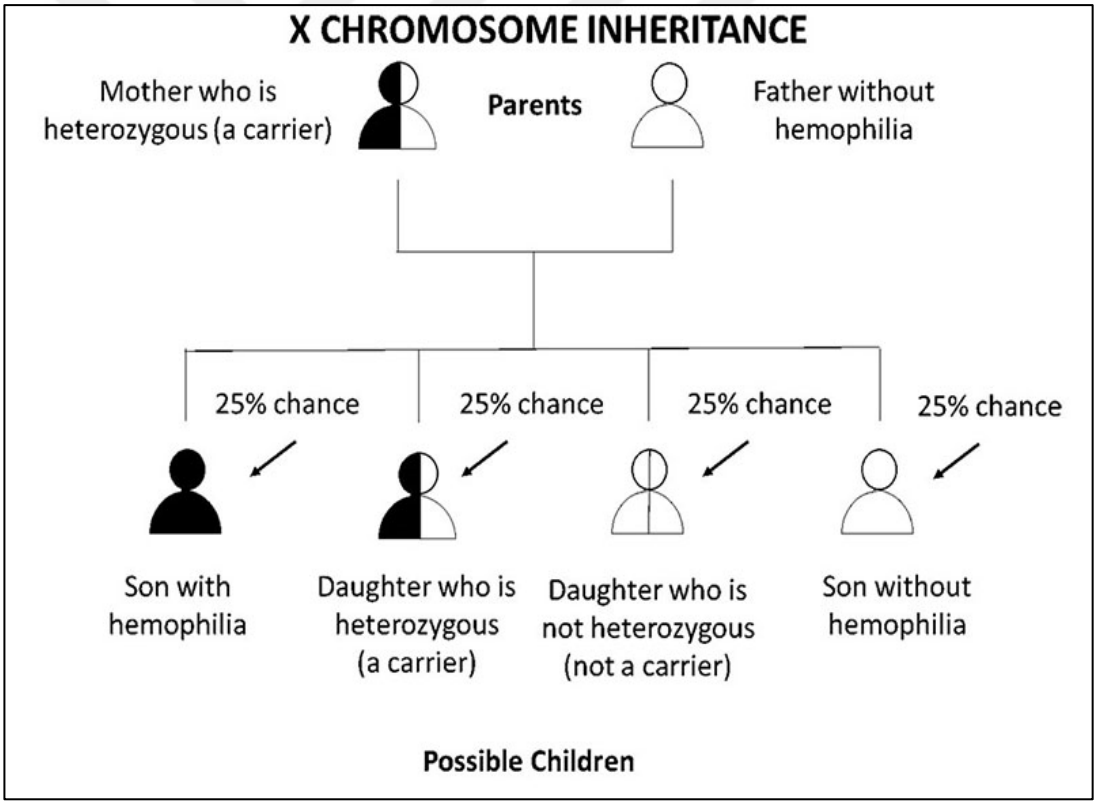

Figure 2.2 The possibilities of affected and non-affected offspring (Migeon 2020)

Females with X-linked recessive diseases can be explained by different circumstances such as a random phenomenon in heterozygous females that can entail a normal allele X and a mutant allele containing X in equal proportion. The majority of a heterozygous

female's cells may have a mutant allele on the active X chromosome. (Skewed X inactivation), resulting in disorder expression (Yang *et al.* 2018). Homozygosity, when a mutant gene is found on the two X chromosomes of females, as in hemophilia A. Females may be harmed by translocations involving an autosome and one of the X chromosomes if the translocation causes damage to a gene on one of the X chromosomes. Additionally, if a female is born with only one X chromosome (Turner syndrome) and has a mutant allele (Shahriari *et al.* 2016).

2.2.1 FVIII in carriers

Carrier females are predicted to have an FVIII plasma concentration half that of healthy women, which is usually enough for adequate hemostasis. Carrier females will benefit from learning their genetics (whether or not they have a mutation) as well as their plasma factor activity status. Hemophilia A carriers with clotting factor levels < 60% are at an increased risk of bleeding. If a carrier's FVIII level is < 60%, they should be classified and treated as a (moderate) HA patient. Carrier females with clotting factor levels < 30% should visit a hemophilia treatment facility regularly (Kadir and James 2009). For at least two reasons, variability in FVIII levels is particularly relevant for the pregnant carrier: First, hemophilia carriers have been linked to an increased risk of both primary and secondary postpartum hemorrhage. Second, a hemophiliac infant born to the carrier is at risk for hemorrhage, particularly in the case of the scalp and intracranial bleeds (Hooper *et al*. 2010).

2.2.2 Gene mutation of F8

F8 is the only gene that has been associated with HA. F8 is a 186-kb segment of genomic DNA found near the distal end of the X-chromosome's arm (Xq28). It has 26 exons, each of which codes for a precursor polypeptide of 2351 amino acids. From the amino terminus to the carboxyl terminus, the mature state of FVIII protein is composed of three homologous A domains, two homologous C domains, and one unique B domain structured as follows: A1A2BA3C1C2. The different domains are critical for FVIII function because each domain has specific binding sites for particular clotting cascade

components. HA may be caused by genetic abnormalities that alter these interaction sites (Keeney *et al.* 2005).

Since 1984, when the F8 gene sequence was revealed, a large number of HA mutations have been discovered. The most typical F8 gene inversions are intron 22 and intron 1, which arise in 40–50% and 5–7% of people with severe HA, respectively (Margaglione *et al.* 2008). Large gene deletions can be seen in 5% of patients with severe HA alleles. The remaining severe instances, as well as all moderate and mild cases, are caused by a variety of small insertions-deletions and point mutations, which is one-third of cases, are novel mutations (Xue *et al*. 2010). All severe cases linked with novel stop codons which most resulted from point mutations and also frameshift mutations. Over 1209 mutations have been found in the F8 coding and non-translated regions and displayed in the HAMSTeRS genetic mutation database, which is a global worldwide database.

Splice site mutations are generally severe, although counting on the precise location and change, they can be mild (Margaglione *et al.* 2008). Only about 20% of those with severe hemophilia A have missense mutations, although practically everyone with mild or moderately severe bleeding tendencies has them (Riccardi *et al*. 2010). A transversion mutation also can happen in patients with hemophilia at the F8 gene. A transversion is a type of point mutation that occurs when a single or (two rings) of purine (A or G) is substituted for a single pyrimidine (T or C) ring, or conversely.

While mild - to - moderate hemophilia is not always diagnosed during childhood, severe hemophilia is usually detected at a young age (2-6 years). Even though the amount of activity of the factor correlates with bleeding symptoms, it has been noted that not all hemophilia patients with FVIII levels less than 1% bleed with the same severity and it is rare for children with severe HA to suffer from thrombotic events (Ettingshausen *et al*. 2001).

2.2.3 Intron

A gene's intergenic region is a long segment of noncoding DNA located in the region between its exons (or coding sections). The coding regions of genes with introns are referred to as "split" or "discontinuous" genes because they are not continuous. Eukaryotic creatures are the only ones to have introns. DNA sequencing made it possible to identify introns for the first time in 1977. For a long time, it was assumed that the transcripts of mature eukaryotic mRNA molecules were simply cut off at the terminals. To further understand how introns were removed from transcripts and what they could be doing, scientists did a lot of research (Koonin 2006).

Introns have been proven to have a crucial function in gene expression and regulation. Gene length is increased when introns are inserted, which enhances the possibility of chromosome cross-over and recombination. This can lead to novel gene variants through exon shuffling, deletions, and duplications, which increases genetic variation. Alternate splicing is also possible because of introns. As the exons can be put together in many ways, a single gene can encode different proteins (Keren *et al*. 2010).

Introns and exons of the complete gene are copied by RNA polymerase during transcription into the initial mRNA transcript known as heterogeneous nuclear RNA (hrRNA) or pre-mRNA. Because introns are not transcribed, the translation process cannot proceed until they are deleted from the source text. Splicing refers to the process of removing introns and joining exons in the nucleus to form a fully functional mRNA molecule (Cech and Steitz 2014).

2.2.4 Splicing mechanism

Spliceosome recognition sites and other splicing-related sequences can be found in introns. Using these sites, the spliceosome can distinguish between intron and exon. Sites recognize themselves by small nucleolar ribonucleoproteins (snRNPs).

Spliceosomes are made up of several snRNPs that work together to splice mRNA (Papasaikas and Valcárcel 2016). It is done in three steps (Figure 2.3):

1. At the intron's 5′ terminus, cleavage of the phosphodiester bond occurs between the exon and the GU. Splicing is started by binding to the 5′ splice site of one snRNP (U1), which is complementary to the splice site sequence.
2. Lariat or loop structural synthesis. The intron's free 5′ end is connected to a branch site, a conserved sequence near the intron's 3′ end. To begin the lariat, U2 binds to the branch site and attracts U1. A phosphodiester bond between the free 5′ G and an A at the branch site then forms the lariat.
3. The phosphodiester bond between the second exon and the intron's 3′ AG is cleaved.

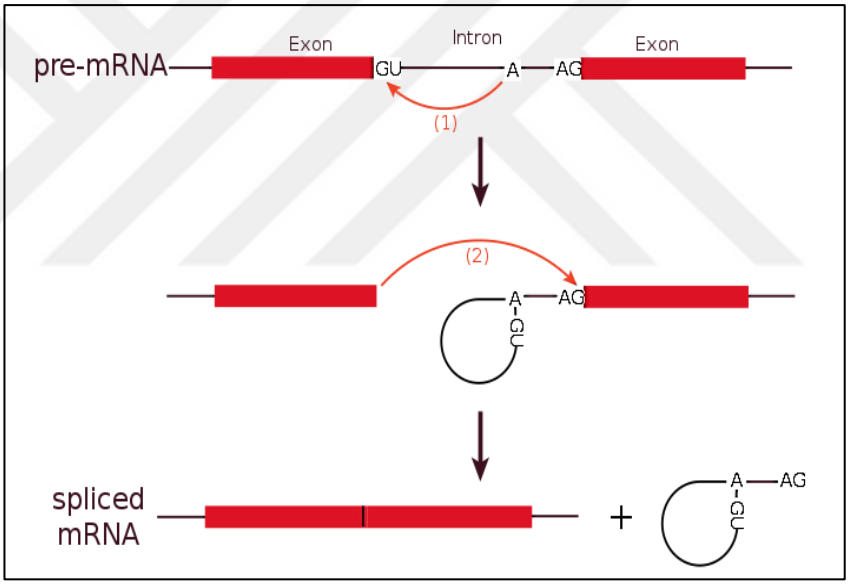

Figure 2.3 The splicing mechanism of introns (Wong *et al.* 2016)

Additionally, introns and the splicing mechanism enable the production of alternative gene products, a process referred to as alternative splicing. Each discontinuous gene is composed of two or more exons, which allows for the assembly of the exons in a variety of ways. Alternative splicing can result in a range of between two and hundreds of distinct messenger RNAs. Alternative splicing could appear in a variety of ways includes, one (or more) exons are ignored from the final mRNA, a portion of the intron

is improperly spliced and remains in the final mRNA, or an alternative splice site is created as a result of the spliceosome removing a portion of one or more exon and an intron (Chen and Manley 2009).

2.3 Other Complications of HA

2.3.1 FVIII inhibitors

In hemophilia, "inhibitors" are IgG antibodies that deactivate the clotting factor. A family history of inhibitors, the FVIII genotype, ethnicity, and specific polymorphisms in immune-modulatory genes are only a few of the genetic factors that have been associated with inhibitor production (Garagiola *et al*. 2018). F8 mutations appear to be the most important genetic predisposition factor for the development of inhibitors, according to research comparing genetic abnormalities to clinical outcomes.

Italian investigation revealed nonsense mutations, large deletions, and inversions were linked to an elevated inhibitor formation, explaining the greater risk in patients with inhibitor family history (Coppola *et al.* 2009).

Specific alloantibodies may come as a result of FVIII concentrate treatment that inhibits factor VIII's function in some patients (inhibitor). The inhibitor has a substantial impact on the patients' subsequent treatment. During the first 25 days of medication, the inhibitor appears most frequently in children. Inhibitors have been found in 10–35 % of severe hemophiliac patients, depending on whether only clinically significant inhibitors with an influence on continued therapy are considered or also temporary detection of antibodies (Boekhorst *et al.* 2008).

Splicing errors were the most major inhibitors found in cases of severe HA, followed by int22 and int1 inversions, massive deletions, and nonsense mutations. The lowest inhibitor prevalence was seen in cases having a missense mutation or small deletion/insertion in severe HA. Their findings imply that the FVIII absence due to null

mutations, as an example, the lack of a second transcript, or splice-site mutations, increases the chance of inhibitor formation. Concordance family investigations, on the other hand, have shown that additional factors play a role in F8 mutations. TNF-alpha and IL-10 may raise by immune regulatory genes polymorphisms or may lower the (CTLA-4) inhibitor risk, and may correlate the high inhibitor risk whose heterogeneous ethnical distribution in non-Caucasian patients (Chaves *et al.* 2010). FVIII haplotypes have lately been shown to play a role in hemophilia, particularly in black hemophiliacs. Anti-factor VIII alloantibodies can form in the black population if FVIII replacement therapy is mismatched (Viel *et al.* 2009).

There is a weak association between the development of inhibitor antibodies and the HLA situated in the MHC class II genotype against FVIII, which is substantially more obvious in patients with int22 inversion when it comes to patients with inhibitor antibody production against factor VIII. In hemophilia, it has been postulated that the interplay between the FVIII genotype and the HLA haplotype may be a determining factor in the formation of inhibitors (Wieland *et al.* 2008).

Mutations that cause hemophilia are important. Mutations in the F8 in which a gene product is missing, such as nonsense or deletion mutations, have a high chance of producing inhibitors; mutations that result in the presence of a gene product (even very little quantities of the protein) have a low risk of producing inhibitors (Coppola *et al.* 2009). In reality, though, the issue is more complicated. Some patients with the same mutations generate inhibitors, whereas others do not. There are other elements at play (Astermark 2010). According to (Margaglione *et al.* 2008), reported that HA patients with high severity and mutations that predicted a null allele developed inhibitors at a higher rate (22-67 %) than cases that have a missense mutation (5 %).

The treatment for this condition is immune tolerance induction. (Street *et al.* 2008). To begin, a tiny dosage of factor VIII is administered. After that, the dose is gradually raised. As a result, the immune system learns to tolerate factor VIII and stops producing inhibitors against it.

2.3.2 Transfusion-transmitted infections

HIV, HBV, and HCV were transmitted by clotting factor products in the 1980s and 1990s resulted in a high death rate among hemophiliacs. In Iraq, the most common transmission infection of hemophilic patients is hepatitis C, this is the result of multiple risk-reduction measures, including careful donor selection and plasma screening, effective viral processes in the production process, and developments in improved diagnostic technology for pathogen identification (Ludlam *et al.* 2005).

2.3.3 Osteoporosis

Bone mineral density (BMD) is reduced in people with hemophilia. An increased number of arthropathic joints decreased joint mobility, and muscle atrophy is all associated with a decreased BMD. Weight-bearing activities (suitable sports) that promote bone density growth and maintenance should be encouraged if joint health permits. The use of bisphosphonate medicine may be recommended, as well as calcium and vitamin D supplementation (Kovacs 2008).

2.3.4 Obesity

In the developing world, obesity and overweight are epidemics, and hemophiliacs tend to be overweight and obese at the same rate as the general population. This is unsurprising, given that patients with hemophilia (PWH) are generally healthy and may not have some of the complications associated with more serious chronic diseases in which the metabolic rate may be raised. Additionally, some parents are overprotective of their hemophilic children and deny them physical activity, putting them in danger of becoming overweight. While overweight and obese hemophiliacs face the same chronic health risks as the general population, PWH faces additional risks. For instance, it has been proven that hemophilic arthropathy occurs more frequently among overweight and obese hemophiliacs, with a concomitant reduction in joint range of motion associated with an elevated body mass index (Wong *et al.* 2011). Another unresolved issue

regarding overweight and obese PWH is the dose of clotting factor concentrate. Factor doses are weight-based, which means that heavier patients receive bigger doses. Fat tissue has less blood flow than muscular and lean tissues, thus it's not obvious whether this is necessary. Though appropriate body weight-based dosing can have a significant economic impact (Majumdar *et al.* 2011), as it stands, this isn't standard practice, and it's unclear if it ever should be. Further study must be done to determine the proper dosage for overweight and obese people because the results could have a large economic impact.

2.3.5 Hypertension

Blood pressure is greater in people with hemophilia than in the general population, and they take more anti-hypertensive medications. Blood pressure should be monitored periodically in people with hemophilia who are hypertensive because of the increased risk of bleeding. A systolic blood pressure of less than 140 mmHg and a diastolic pressure of less than 90 mmHg should be maintained in the absence of additional cardiovascular risk factors (Biere *et al.* 2011).

2.3.6 Hypercholesterolemia

Hemophiliacs have lower average cholesterol levels than the general population, according to research. Patients with hemophilia who are at risk of cardiovascular disease should have their cholesterol levels (total cholesterol, HDL, and LDL) tested. If your cholesterol readings are too high, you should seek medical attention. As a general guideline, the total cholesterol/HDL ratio should not be more than 8 (Biere *et al*. 2011).

2.3.7 Cardiovascular disease

Cardiovascular disease (CVD) risk factors are well known. People who smoke, suffer from high blood pressure, are obese, have high cholesterol levels, or have diabetes are all at risk for cardiovascular disease (CVD) complications. CVD can potentially be

exacerbated by a condition known as a prothrombotic state (Kannel 2005). Three factors are associated with arterial thrombosis: fibrinogen, VWF, and FVIII. Platelet adherence and aggregation are dependent on VWF's role in this process. Furthermore, FVIII is transported by VWF. Thrombus production in stenotic vessels is aided by FVIII, which is involved in the formation of fibrin-rich clots and occluding thrombus. Hemophilia A sufferers, who have a hereditary deficit of clotting factor VIII, may be more susceptible to arterial thrombosis than the general population, according to several observational studies (Darby *et al.* 2007). Thrombosis may be prevented as a result of hypercoagulability, which reduces thrombin production and inhibits the formation of thrombus.

Atherosclerotic plaques have recently been revealed to be prevalent in HA patients, just like in the general population. Hypertension and other well-known cardiovascular risk factors are more common in hemophilia patients. A growing percentage of hemophilia patients are being diagnosed with CVD in clinical practice. Antithrombotic treatment of patients who have a lifetime hypercoagulability and thus a higher risk of bleeding is a significant clinical problem (S-Borensztajn *et al.* 2011).

2.3.8 COVID-19 and hemophilia

Congenital bleeding disease (CBD) is a major issue for the hemophilia community in the pandemic era, but the specific impact of COVID-19 on CBD patients is still largely unknown. SARS-CoV-2 pneumonia has been confirmed in a patient with severe hemophilia A in Wuhan, China, but the patient's symptoms were mild. There are ongoing national and international initiatives to collect data on CBD patients with confirmed or clinically suspected COVID-19. SARS-CoV-2 infection susceptibility or clinical course in CBD patients is not different from the general population at this time (Hermans *et al.* 2020). The severity of the condition is greater in men and hypertensive patients COVID-19. Some studies have shown that PWH is more likely to suffer from hypertension than their age-matched counterparts, and these clinical features could harm these individuals.

The potential impact of a coagulation defect on COVID-19 clinical outcomes in CBD patients is an intriguing question. Severe COVID-19 is linked to abnormalities in coagulation tests; including a mildly prolonged prothrombin time (PT) and decreased platelet count in most patients, and a highly elevated D-dimer, which is associated with the severity of the disease and the risk of death. Endothelial cells and macrophages of the monocyte/macrophage lineage are activated by acute lung injury and hypoxia, resulting in the "cytokine storm" that characterizes stage two of inflammatory disease (Siddiqi and Mehra 2020). Microvascular thrombosis and venous thromboembolism (VTE) in the systemic circulation may be the result of an inflammatory-coagulation cycle, as evidenced by the increasing number of cases reported in COVID-19 (Klok *et al.* 2020).

2.4 Detection of HA and Therapy

2.4.1 Lab diagnosis

One of the main goals of laboratory diagnostics is to figure out what kind of hemophilia someone has and whether or not their clinical diagnosis is correct. Making ensuring that a patient gets the right treatment starts with making sure that they get the right diagnosis. Symptoms of several bleeding disorders might be nearly identical. Screening tests are used to determine the cause of bleeding, ex; bleeding time, platelet count, activated partial thromboplastin time (APTT), and prothrombin time (PT). Recently, approaches for evaluating overall clotting performance such as the thrombin generation test, thromboelastogram, and clot waveform analysis have all been suggested. FVIII assay test to see the amounts of the factor VIII in percentage. When a genetic disorder mutation is found in a member of the family, preimplantation genetic diagnosis (PGD) is now an option for those families (El-Toukhy *et al.* 2010).

2.4.2 Gene therapy

Furthermore, because hemophilia is a monogenic disorder, gene therapy is a good target with a severe to a moderate frequency even with low expression (Batorova *et al.* 2010). As a chronic disease, hemophilia required a fairly accurate factor infusion, which can be fatal, and it is also highly expensive. Numerous gene therapy techniques for hemophilia have been proposed. Both techniques in vivo and ex vivo are used in these strategies. In vivo delivery research employing both vectors (Viral and non-viral), like retroviral and adeno-associated viral vector (AAV), have demonstrated extremely favorable preclinical results, and initial clinical studies have been determined to be safe (Liras and Olmedillas 2009). In terms of efficacy and safety, there are still hurdles ahead, such as possible side effects from vector-mediated cytotoxicity, unexpected immunological reactions, and the danger of insertional alterations. Because viral vectors are not dispersed throughout the body, therapeutic transgene delivery via ex vivo is safer (Montgomery *et al.* 2010). The larger increase from time to time of a severely hemophilic patient was transplanted with autologous skin fibroblasts genetically engineered with the FVIII transgene. It was a safe and well-tolerated medication for patients in a clinical study. However, because the increase in FVIII levels was minimal and only lasted a short time, in this strategy, it was hypothesized that the survival of the transplanted cells, and the FVIII plasma levels, were the biggest barriers. Hematopoietic stem cells (HSC) and endothelial progenitor cells from the patient's blood can be used to deliver the therapeutic coagulation factor (Ide *et al.* 2010). Platelet-based gene therapy, which aims to transfer clotting factors to vascular injury sites through platelets, has recently been proposed as a new approach to gene therapy in hemophilia (Shi and Montgomery 2010). as well as intraarticular gene therapy for hemophilic joints that targets the production of certain proteins (Montgomery *et al.* 2010). To treat severe hemophilia, the infusion of recombinant human activated factor VII (FVIIa) was found to be an effective method of establishing hemostasis, leading to the novel idea of continuously expressing activated FVII from a donor gene (Obergfell *et al*. 2010). A possible transgenic of FVIIa, unlike FVIII, is unlikely to cause an immune response because it is completely tolerated by all hemophilia patients and regulates hemostasis regardless of F8 inhibitors. Shortly, it is suggested that the transgene FVIIa and the

delivery method of gene therapy be used (Margaritis and High 2010). Aside from the fact that gene therapy for hemophiliacs with inhibitor development has only recently been investigated, animal research is still in the early stages (Scott 2010). Finally, in the recent few decades, Hemophilia genetic identification has advanced significantly in recent years, allowing clinicians and patients to better estimate the severity of the condition and its potential long-term effects. This offers better options for genetic counseling, disease prevention, planning of patient therapy, and better detection rate and care of carriers and their offspring.

2.4.3 Treatment

Hemophilia A can be treated using several recombinant FVIII medicines that have received regulatory approval in the United States and Europe (Peyvandi *et al.* .2019). The first FVIII without a B-domain is Antihemophilic Factor (Recombinant). Because it's a newer type of antihaemophilia medication, human serum albumin (HSA) is not used as a stabilizer in the formulation of this factor product (Kelley *et al.* 2010).

2.4.4 Vaccination

Hemophilia treatment centers and patient organizations must work together to educate people with bleeding disorders about vaccines and contribute to an effective vaccination program. As a result, people with bleeding disorders are not considered a high-priority target population for vaccination against COVID-19 or its severe variants. The vaccine should be injected into the muscle. It is best to use a needle with the smallest gauge possible (between 25 and 27 gauge) (Chirumbolo 2021). To reduce bruising and swelling, apply pressure to the injection site for at least ten minutes after the injection. The injection site should be checked several minutes and 2-4 hours after the procedure to ensure that there are no post-injection hematomas. If you experience pain or swelling in your arm for more than two days after your injection, do not be alarmed. Hematomas and allergic reactions should be reported to a hemophilia treatment center in the event of any complications.

Patients who experience an allergic reaction (fever, warmth, redness, itchy skin rash, shortness of breath, or swelling of the face or tongue) should contact their doctor or go to the nearest hospital emergency room immediately, as it can be life-threatening. Some vaccines contain polyethylene glycol (PEG) as an excipient, which should be discussed with patients who have had allergic reactions to PEG-containing extended half-life clotting factor concentrates. After an FVIII injection for patients with severe/moderate hemophilia, the injection should be given (Chirumbolo 2021). No hemostatic precautions are needed in patients with a basal FVIII or FIX level greater than 10%. Patients with rare bleeding disorders, such as thrombocytopenia and/or platelet function disorders, should all receive vaccinations to prevent the spread of the disease. Before vaccination, anticoagulant patients should have their prothrombin time (PT) and international normalized ratio (INR) tested within 72 hours to ensure that their INR is stable and within the therapeutic range. Because of the complications of hemophilia and their treatments, vaccination is not contraindicated. Conditions such as hepatitis C and HIV therapy do not preclude vaccination (Siemieniuk *et al.* 2020).

3. MATERIALS AND METHODS

3.1 Study Design

This is a genetic study confirmed by conventional PCR and Sanger sequencing, we depended on the samples that taken from HA patients to see if there is a mutation happened in the SNP (rs4898352) in intron 18 of the F8 gene located at Chromosome chrX:154903815 (GRCh38.p13). All patients with cancer, COVID-19, and other diseases that effects on blood viscosity were excluded. The study is performed in Baghdad at Nabu scientific Foundation and the Korean company Macrogen for PCR sequencing diagnostics from 30th of September to 27th of November.

3.1.1 Patients

Sixty Hemophilic Patients Type A for severe, moderate, and mild cases. Their age ranged from (1 - 29 years) all of the patients received treatment, and some of them got a blood transfusion. All samples were taken from the pediatric teaching hospital in the Medical City in Baghdad. Collection period from the 13th of Jun 2021 to the 28th of September 2021. All were taken under permission of ethical committee.

3.1.2 Control group

The study included 60 samples taken from healthy people of the same range of age and the same conditions of storage.

3.2 Materials

Table 3.1, Table 3.2, Table 3.3, and Table 3.4, details all of the materials, chemicals, instruments, and kits that were used in this investigation with their respective manufacturing industry. Data analysis was done by the Geneious Prime software program.

Table 3.1 Materials and their manufacturer companies

NO.	MATERIALS	COMPANY AND ORIGIN
1	Cotton	China
2	Eppendorf ‘s tubes	Eppendorf. Germany
3	Gloves	China
4	Syringes	China
5	EDTA tubes	Vacutainer scientific. UK
6	Tips (Blue 1 mL, yellow 100M)	China
7	Adhesive bandage/tape	
8	Glassware, cylinders	
9	Racks	

Table 3.2 Instruments and their manufacturer companies

NO.	INSTRUMENT	COMPANY. ORİGİN
1	Autoclave	Hirayama. Japan
2	Cooling centrifuge	Eppendrof. Germany
3	Deep Freeze -80	Blider. Germany
4	Electrophoresis System	Cleaver. UK
5	Gel Documentation	Cleaver. UK
6	Gradient Thermocycler	Eppendrof. Germany
7	Micropipettes	Eppendrof. Germany
8	Quibt 4	Invitrogen. USA
9	Refrigerator	Kelon. Korea
10	Sensitive balance	Denver. European Union
11	SimpliAmp Thermal Cycler	Thermo Fisher Scientific. USA
12	Thermomixer	Eppendrof. Germany
13	Vortex	VELP. Germany
14	Water Distillater	GFL. Germany

Table 3.3 Chemicals used in the study

NO.	CHEMİCAL	COMPANY. ORİGİN
1	Absolute ethyl alcohol (99.9%)	Diamound. France
2	Agarose	Cleaver. UK
3	ddH2O	Bariq. Iraq
4	DNA Marker (100-1000) bp	NEB. UK
5	Free Nuclease Water	NEB. UK
6	Normal Saline	Bioneer. Iraq
7	RedSafe dye	Intron. South Korea
8	TAE Buffer (50X)	Carl ROTH

Table 3.4 Kits used in this study

PRODUCT NAME	DESCRİPTİON AND COMPONENTS	COMPANY. ORİGİN
FavorPrep™ Blood / Cultured Cell Genomic DNA Extraction Maxi Kit	For the purification of genomic DNA (gDNA), the Monarch Genomic DNA Purification Kit is an all-in-one solution for lysis, RNA removal, and purification from a wide range of biological materials. Additional steps can be taken to enhance lysis in these difficult-to-lyse samples, such as bacteria and yeast.	FAVORGEN. Taiwan
OneTaq® 2X Master Mix	OneTaq® DNA Polymerase is an optimized blend of Taq and Deep Vent™ DNA polymerases for use with routine and difficult PCR experiments.	NEB. England
Qubit™ dsDNA HS Assay Kit	The Qubit dsDNA HS (High Sensitivity) Assay Kit is developed specifically for use with the Qubit Fluorometer. Double-stranded DNA (dsDNA) is detected with a high degree of specificity, while RNA is not.	ThermoFisher®. USA

3.3 Methods

3.3.1 Sampling

Three milliliters taken from sixty patients by vein puncture using tourniquet were obtained from Pediatric teaching hospital in the Medical City in Baghdad between 13th of Jun 2021 to 28th of September of 2021. Blood samples were placed into EDTA anticoagulant tubes which were labeled and stored in a deep freeze at -20 ℃. Then DNA was isolated from these blood extractions, Intron 18 in the F8 gene was amplified in a thermal cycler by using a specific primer. Then, amplification products for intron 18 of F8 gene were sent to Macrogen Company (Korea) for sequencing.

3.3.2 DNA extraction

Blood samples from elderly and newly diagnosed and treated Hemophilia A patients were used to extract total genomic DNA (gDNA), and from blood samples collected

from healthy volunteers. FAVORGEN (Taiwan) provided the FavorPrep™ Blood gDNA mini kit, which was used to extract DNA according to the given procedure:

1. At room temperature, 200 µL of blood samples were vortexed for 10 minutes, then 20 µL of proteinase K was added and thoroughly mixed.
2. Microcentrifuge tubes (1.5 mL) were allowed to stand for 15 min, and the tubes vortexed for 10 sec then incubated for 15 min in 60˚C.
3. Each tube received 200 µL of cell lysis buffer solution, which was mixed for 10 seconds by vortexing. Then the tubes were incubated at 70°C for 15 minutes.
4. After incubation, each tube was filled with 200 µL of 100% Ethanol (preferred cold) and vortexed for 10 sec to mix.
5. For DNA filtering, contents of microcentrifuge tubes (1.5 mL) were placed in the FABG column and centrifuged for 1 min at 13.500 RPM.
6. We Added 400 µL from the W1 solution in the FABG column. Then centrifugation at 13,500 RPM for 1 minute. after that, the west was poured in bleach.
7. Wash buffer, 600 µL added and centrifuged 1 minute at 13,500 RPM.
8. The west poured, after that centrifuged again for 3 minutes at high speed.
9. FABG columns are discarded and kept the filter and moved the tubes to another microcentrifuge tubes then kept them cooling for 2 minutes.
10. Seventy microliters of Elution buffer (deionized water) were droped into the tubes and left to stand for 10 minutes.
11. Centrifuged at 3000 RPM for 3 minutes to distribute the elution into whole filters. After that centrifuged at 13,500 for 1 minute. The elute indicates the DNA solution that was maintained at -20°C until it was used.

3.3.3 PCR primers

Success in a polymerase chain reaction (PCR) experiment is dependent on the design of the primers. When designing primers for a specific region of DNA to be amplified, it is important to ensure that the primers anneal to the plus strand, which is typically aligned in the direction of 5' to 3' in most cases (also referred to as the non-template or sense strand).

To do this, we gently used particular primers produced by Geneious Prime software to amplify a specific position of intron 18 in the F8 gene product size (123691-124234), which was 544 bp. A list of the primers can be found in Table 3.5.

Table 3.5 Oligonucleotide primers used for the amplification of intron 18

PRİMERS	SEQUENCE (5'- 3')	PRODUCT SİZE (BP)
rs52-F	AAGGATTCGATGGTATCTGCT	544 bp 123691-124234
rs52-R	TAAAGGGACAGTGAAGAACCA	

3.3.4 Quantitation of dsDNA by qubit 4.0

The assay is extremely selective for double-stranded DNA (dsDNA) over RNA and has a detection limit of 10 pg/µL to 100 ng/µL. At room temperature, the assay is performed, and the signal is stable for three hours. The assay is highly tolerated for common impurities such as salts, free nucleotides, solvents, detergents, and protein.

1. Qubit® dsDNA HS Reagent was diluted 1:200 in Qubit® dsDNA HS buffer to create the Qubit® working solution.
2. Each tube designated as a standard was filled with 190 µL of Qubit® working solution, followed by the addition of 10 µL of each specified standard solution.
3. Each tube prepared for sample was filled with 197 µL of the Qubit® working solution and then 3 µL of sample was added individually.
4. All components have been vortexed and incubated for 3 minutes at room temperature.
5. For the purpose of establishing a concentration curve, standards tubes have been put into the Qubit device.

3.3.5 Molecular sequencing of rs4898352

Conventional PCR is a highly effective approach for exponentially amplifying DNA sequences. Amplification by polymerase chain reaction (PCR) requires a pair of primers that are complementary to the sequence of interest. DNA polymerase extends primers.

An entire quantity of 25ul was used for PCR. Table 3.6 lists the components of the reaction. Using specified primers, the Intron 18 of the F8 gene was amplified. The polymerase chain reaction was performed using the amplification protocol provided in Table 3.7, and the amplification products were then run on a 1.5% agarose gel.

Table 3.6 Reaction mixture components for amplification of intron 18

COMPONENT	**VOLUME (µL)**
Master Mix: dNTPs, *Taq* DNA Polymerase, reaction buffer, and MgCl2.	12.5
F-Primer	1.5
R-Primer	1.5
DNA Template	5
D.W.	4.5
Total volume	25

Table 3.7 PCR amplification conditions for the intron 18

STEPS	**STAGES**	**TEMPERATURE (˚C)**	**TIME**	**NO. OF CYCLES**
Initial Denaturation	1	95	3 min.	1
Denaturation	2	95	45 sec.	35
Annealing		51	45 sec.	
Extension		72	1 min.	
Final Extension	3	72	7 min.	1

3.3.6 Agarose gel electrophoresis principle

Separating proteins, DNA, and/or RNA with this technique is commonplace nowadays. Using an electric field, negatively charged nucleic acid molecules migrate toward the anode (positive) pole. Migration is governed exclusively by molecular weight, with smaller molecules moving more quickly than bigger ones. In addition to size separation, agarose gel electrophoresis can be used to fractionate nucleic acids to further purify a target band. Through the use of an ultraviolet transilluminator and a stained gel, the desired "band" can be excised using the technique's extension.

3.3.7 1X TAE buffer preparation:

Ten milliliters of a stock solution of TAE (Tris-acetate-EDTA buffer) was added to 90 mL of deionized water, the 1X TAE Buffer was formed.

3.3.8 Preparation of agarose gel

First, 1.1 Grams of agarose powder was added to prepare 1.5% into a flask that contained 100 of 1X TAE buffer. The flask's contents were boiled in a microwave oven till the particles were dissolved. Finally. The solution was chilled to 55-60˚C, then 5 µL of RedSafe was added to the flask and gently mixed the contents.

3.3.9 Horizontal casting of agarose gel

The Agarose gel solution was poured into the gel tray and allowed to solidify completely at room temperature for 20-30 minutes. Following that, the fixed comb was gently removed and the tray was placed in a tank filled with 1X TAE buffer until it reached a height of 2-3 mm above the top of the gel.

3.3.10 Gel electrophoresis

Firstly, 5 µL of quick-load purple 100 bp DNA ladder was added. Afterward, samples of DNA were loaded into the wells, and electrophoresis was carried out for 1-2 hours at a voltage of 5 volts/cm. The DNA bands were seen with the aid of a UV-light transilluminator as shown in Figure 3.1.

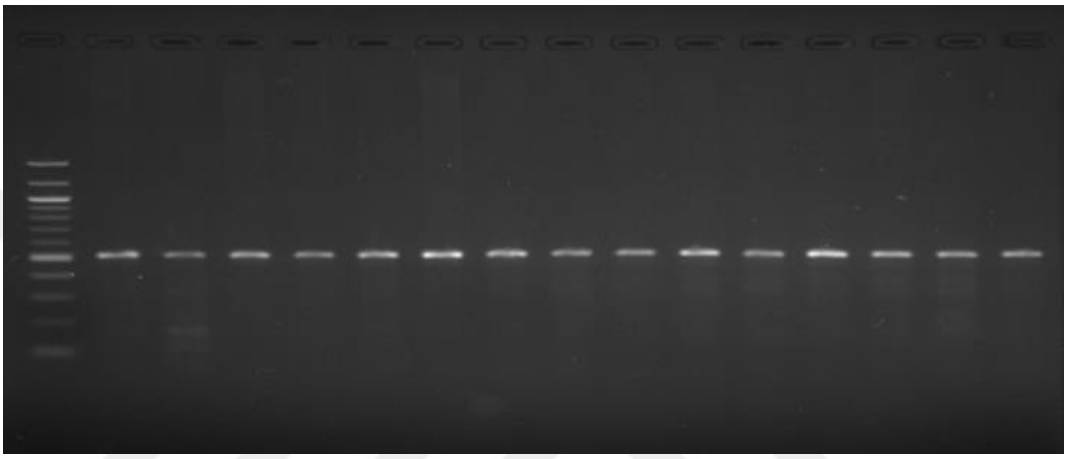

Figure 3.1 Bands of DNA under UV-light

Amplification products of intron 18 of the F8 gene were sent to Macrogen Company (Korea) for sequencing by Sanger method to identify the SNP that occur in rs4898352. The data analysis of the results was done by using Bioinformatics tools Geneious Prime software, then were compared with the information of the (NCBI) gene bank for standard F8 gene.

3.4 Statistical Analysis

Values were presented as mean ±SD and $P \leq 0.05$ was done by using statistical package for the social science (SPSS) version 16.0 and. Student t-test and Pearson's correlation test were used to comparing the mean ± SD and to analyze any relationships. $P \leq 0.05$ was considered statistically significant.

4. RESULTS AND DISCUSSION

This research was carried out on (60) Iraqi participants with hemophilia A their age (6-24) years were matched with the same number of a healthy control group to assess the major causative of the disease based on the mutation in X chromosome in HA patients and clinical information of family history, age, and weight.

The data illustrated in (Table 4.1) showed the distribution of HA patients according to the percentage levels of factor VIII activity, it pointed that the majority of the studied cases were classified as having severe HA (75%) (45/60) followed by moderate (15%) (9/60) and mild (10%) (6/60) this data was on the same line with the previous studies (Franchini *et al.* 2010), (Tonbary *et al*. 2010), (Song *et al*. 2021) those findings emphasize the importance of taking preventative measures as soon as possible.

Table 4.1 Distribution of samples according to the percentage of factor VIII activity levels

FACTOR 8 PERCENTAGE LEVEL %	SEVERITY	N	%
> 5	Mild	6	10
1-5	Moderate	9	15
< 1	Severe	45	75
Total		60	100

As noted in Table 4.2 the age ranged between (20-29) years (25) (41.6%) was the major age range with severe HA followed by the age ranged between (1-9) years (12) (20%), The age difference between patients and the control group is not statistically significant (Larsson 1985). The mean of age and weight of patients and controls shown in Table 4.3 and Figure 4.1 shown the mean of age.

Table 4.2 Characteristics of the severity of factor VIII for samples about age groups, family history and weight

VARIABLES		SEVERITY					
		MILD		MODERATE		SEVERE	
		No.	%	No.	%	No.	%
Age group (Years)	1-9	2	3.3	2	3.3	12	20
	10-19	4	6.6	5	8.3	8	13.3
	20-29			2	3.3	25	41.6
Family history	Father					2	3.3
	Mother			6	10	23	38.3
	Brother			4	6.6	10	16.6
	Uncle			2	3.3	13	21.6
Weight (Kg)	20-39	1	1.6	1	1.6	16	26.6
	40-59			4	6.7	2	3.3
	60-79	3	5	4	6.7	21	35
	80-99	2	3.3			6	10

Table 4.3 Studied characteristics about age and weight for samples in Hemophilia A cases and control groups

VARIABLES	CASE MEAN ± SD N (60)	CONTROL MEAN ± SD N (60)	P. VALUE
Age	16.27±6.57	15.87±5.73	0.15
Weight	55.85±21.76	46.85±21.6	0.00

The data illustrated in Table 4.4 confirmed the causative mutation for patients with hemophilia A that categorized in groups; group (A) include patients with transversion mutation, group (B) without mutation, and group (C) include controls, in the presents study that located deep the intron 18 of gene F8 within SNP ID rs4898352 at the Chromosome chrX:154903815 (GRCh38.p13), by using conventional PCR, the primer sequence was verified by Geneious Prime software, the finding of the research after

sequencing shoed A>T Nucleotide Location (123909) Transversion type of mutation is the most cause of HA disease within Iraqi patients as shown in samples of a group (A).

Table 4.4 Genetic data and clinical information of causative mutations in hemophilia A patients for the factor 8 gene

GROUPS (N)	SNP ID	GENE	CONSEQUENCE	NUCLEOTIDE LOCATION	STATUS (A>T)	TYPE	CHROMOSOME
A (31)	rs4898352	F8	Intron Variant	123909	A	Transversion	chrX:154903815 (GRCh38.p13)
B (29)	rs4898352	F8	Intron Variant	123909	T	-	chrX:154903815 (GRCh38.p13)
C (60)	rs4898352	F8	Intron Variant	123909	T (except 1)	-	chrX:154903815 (GRCh38.p13)

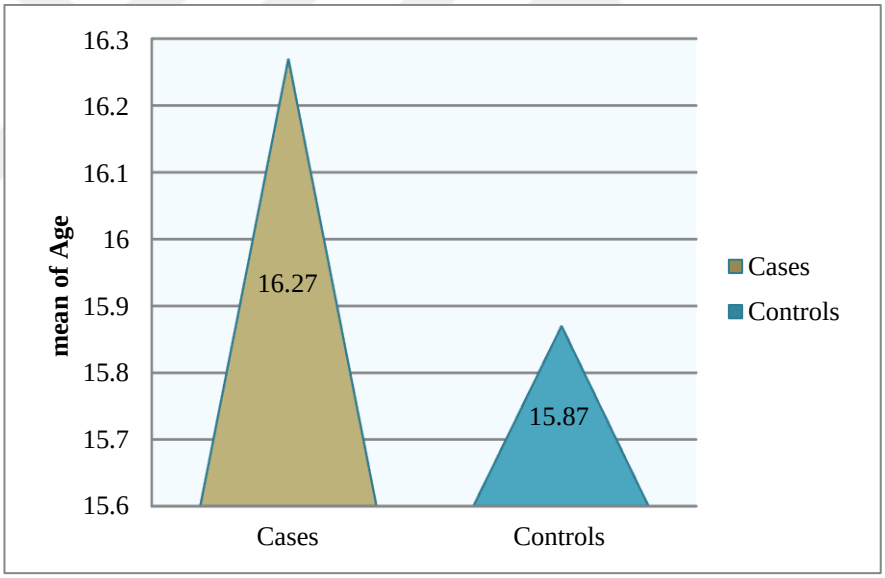

Figure 4.1 The distribution of patients and control according to age

Studying the family history status is critical to investigate the family history of individuals with a HA to establish which members of the family are at risk of developing HA, so there is special considerable attention on infants and children with hemophilia A (Srivastava *et al.* 2013). According to the finding of this research revealed

that patients with family history were more than those without a family history (Bertamino *et al*. 2017).

Moreover, this research found that HA patients were higher with mother family history classified as severe (23) (38.3%) followed by uncle (13) (21.6%) then brother (10) (16.6%) while patients with father family were only (2) (3.3%) that also were with great similarity with that found in (Caesar Mahmoud Abu Arra *et al*. 2020).

Our study showed that there was an increase in weight in patients with HA There were statistically significant differences between the weight of HA patients and the weight of persons of the control group which agrees with (Shapiro and Makris 2019). The mean of weight of patients and control shown in Figure 4.2.

According to the researchers, hemophilia is uncommon to be a direct cause of obesity, patients who have muscle/joint pain, limited range of motion, or fear of bleeding may reduce their exercise levels, which could lead to weight gain or difficulty decreasing weight (El-Safy *et al.* 2021), (Hofstede *et al*. 2008).

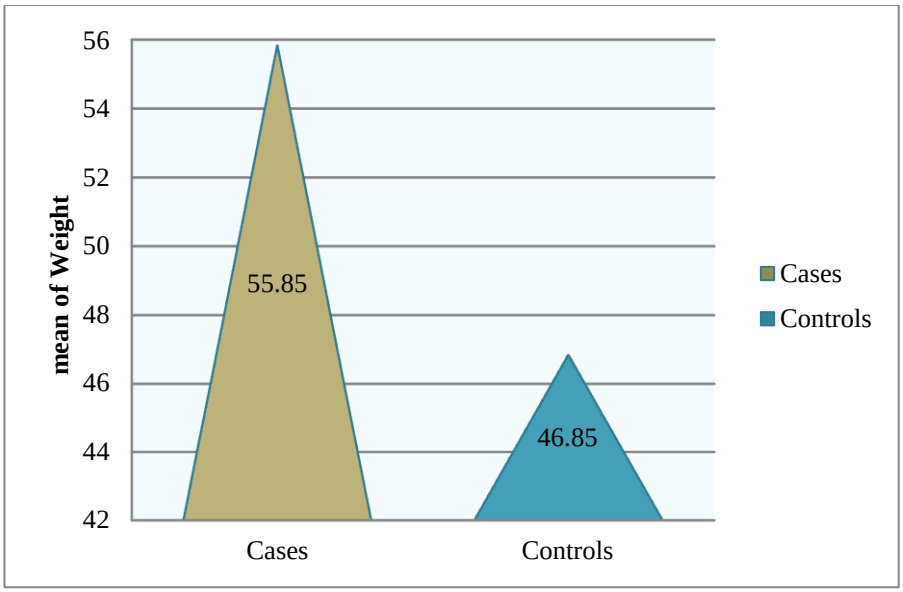

Figure 4.2 The distribution of patients and control according to weight

The effective defect of intron converted by a missense single nucleotide polymorphism SNP is the molecular alteration found in HA patients (Tokoro *et al*. 2020).

The rest samples group (B) was with a normal nucleotide sequence. were with no nucleotide mutation noted as shown in Table 4.4. This is due to the presence of many clinical reasons other than the genetic mutation in intron 18, as well as pathological reasons or other conditions. For all persons in the healthy control group, there is no nucleotide mutation in deep of the intron 18 of gene F8 within SNP ID rs4898352 at the Chromosome chrX:154903815 (GRCh38.p13).

All controls were healthy and have no change in rs4898352 except for sample number (55) was observed with nucleotide mutation A>T deep in intron 18 of gene 8 that may be due to had one of the causatives mutated of HA that made him save and unaffected by the disease. Figure 4.3 shown the data analysis results in Geneious prime software.

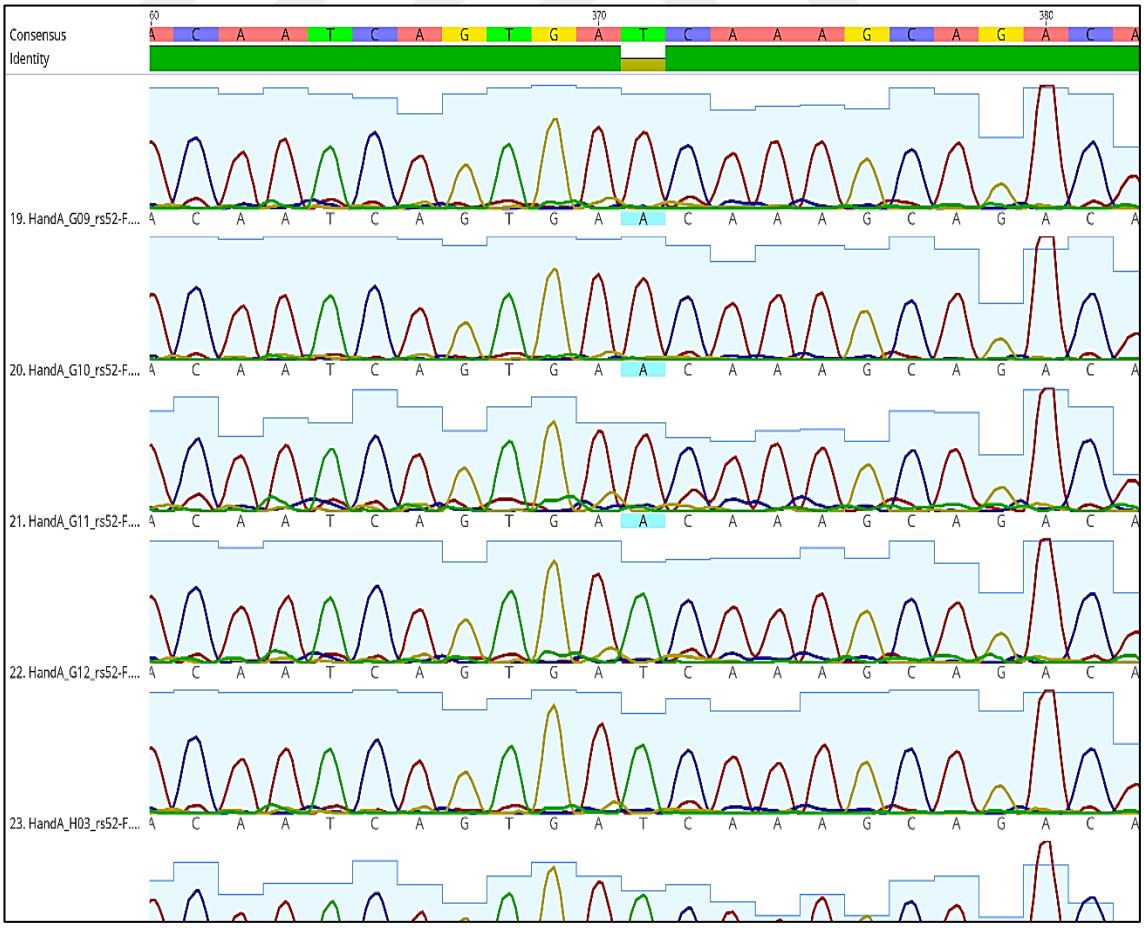

Figure 4.3 Data analysis of patients

5. CONCLUSIONS AND RECOMMENDATION

5.1 Conclusions

Through what was reached in this research, we find that the early ages of males are more susceptible to HA, and of these, the most dangerous are those who have a history of illness from a family member, especially the mother with severity (23) patients (38.3%).

It was also noted from the results that the severity of HA infection among the sample taken for the study is the most severe the percentage of FVIII activity levels (45) cases (75%), and it was also noted that the patients' weights increased were with severity stage at the age ranged (20-29) years (25) cases (41.6%).

When conducting genetic studies on patients with HA, the presence of genetic mutations that cause the disease was noted the causative mutation for patients with HA that located deep the intron 18 of F8 gene within SNP ID rs4898352 at the Chromosome chX:154903815 (GRCh38.p13), the finding of the research shoed A>T Nucleotide location 123909 Transversion type of alternation is one of the major causes of hemophilia A disease within Iraqi patients.

5.2 Recommendation

- Male infants with a family history of hemophilia A should be observed with appropriate laboratory tests for early detection of the disease.
- If HA is present, the infant should be treated directly with FVIII concentrates, also should be given before and after any surgery.
- Medicines and herbal remedies that affect platelet function, including aspirin, should be used only under strong medical supervision.

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