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SEED Haemostasis

Haemophilia

Haemostasis is a complex process that helps to keep the blood in a fluid state and prevent blood loss at the site of injury. While the intact endothelium of blood vessels has an antithrombogenic function that prevents blood coagulation, in the case of vessel wall damage, the exposed sub-endothelial components initiate the formation of a clot that will stop blood loss. Under healthy conditions, the mechanism of clot formation (pro-coagulant) and clot destruction (anti-coagulant) are well balanced. An abnormal increase of the pro-coagulation and/or decrease of the anti-coagulation mechanisms results in thrombotic disorders. These are medical conditions characterised by the formation of an unwanted clot, mostly in veins, but also in the arteries. On the other hand, an abnormal decrease of the pro-coagulation and/or increase of the anti-coagulation mechanisms results in bleeding disorders. In both disorders, we distinguish between acquired and congenital disorders.

Bleeding disorders

Bleeding disorders are a group of medical conditions that are characterised by the inability to form a proper blood clot. These conditions commonly present with prolonged or spontaneous bleeding. People with congenital bleeding disorders may bleed longer or start bleeding spontaneously. The most common bleeding disorder is von Willebrand Disease (VWD), a quantitative or qualitative deficiency in von Willebrand Factor (vWF) [1]. The second largest group of bleeding disorders is haemophilia.

What is haemophilia and which types are common?

Haemophilia is a deficiency in coagulation factors causing long bleeding episodes due to a prolongation of the clot formation process [2, 3, 4]. The most common types of haemophilia are

1. **Haemophilia A**
   a deficiency in coagulation factor VIII (FVIII),
2. **Haemophilia B**
   a deficiency in coagulation factor IX (FIX),
3. **Haemophilia C**
   a deficiency in coagulation factor XI (FXI).

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Who is affected?

Both haemophilia A and B demonstrate X-linked recessive inheritance, hence affecting mainly males (Fig. 1). The genes encoding FVIII and FIX are localised on the X chromosome. Men carry one X and one Y chromosome, whereas women carry two X chromosomes. This means that women can compensate the defective X chromosome, while men cannot. However, in cases of mutations in factor genes, also women might show bleeding tendencies. With a prevalence of 1 : 5,000 neonates, haemophilia A is more frequent than haemophilia B, which appears in 1 : 25,000 neonates [4]. Haemophilia C is characterised by autosomal recessive inheritance, and both genders are equally affected. It is far less prevalent than haemophilia A or B because predominantly Jewish people of Ashkenazi descent are affected [5]. By comparison, VWD has a prevalence of 1 percent of the overall population [6].

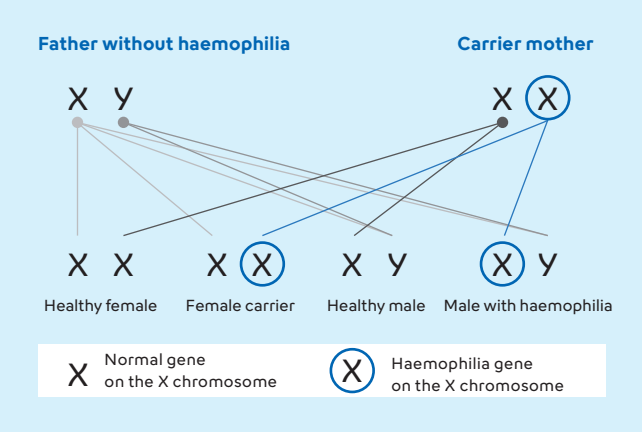

Fig. 1 X-linked recessive inheritance

How is haemophilia classified?

Haemophilia is classified into three classes, based on the frequency and severity of bleeding events as well as factor activity: severe, moderate and mild haemophilia (Table 1) [7]. Some publications also mention a fourth form, the subhaemophilia, but due to its minor clinical difference, subhaemophilia is often treated as equivalent to mild haemophilia. Unfortunately, in most cases, the condition is severe.

What are the clinical symptoms?

Clinical manifestation starts very early and is identical in individuals with haemophilia A and haemophilia B. The most striking sign is an increased appearance of bruises as well as bleeding events after iatrogenic procedures such as injections or minor surgery, nosebleeds and macroscopic haematuria. Very often, haemophilia is already diagnosed in neonates and infants, because bleeding events into joints, usually the lower extremities, are associated with increasing mobility of the child [8]. People with a factor activity of 3 % of the norm or below present an increased bleeding tendency [9]. The clinical symptoms in individuals with haemophilia C are basically the same as with haemophilia A or B; however, the frequency of spontaneous bleeding events is not as high, even in cases of severe FXI deficiency [5].

Which complications may occur?

Severe complications may arise. These complications are joint damages because of re-occurring heavy bleeding periods, or late or inefficient treatment of joint bleeding events. This may lead to heavy joint arthropathies, a reactive inflammatory long-term swelling of the inner joint and increased tissue perfusion (synovitis) causing further bleeding events [10, 11].

Further complications result from internal bleeding. Gastrointestinal bleeding may be severe and consequently cause anaemia. Deep muscle bleeding may cause damage of the muscles, which in turn can cause pain, scarring and may lead to a life-threatening situation if the bleeding appears at the tongue or muscles of the oral cavity, causing for example a respiratory tract blockage. Complications caused by bleeding of organs are rare. Renal haemorrhage is very often painless. It appears as haematuria, posing only a minor threat to life compared with intracranial bleeding or other complications of haemophilia. If not being fatal, they very often can cause serious disabilities such as altered consciousness, seizures and paralysis of facial or limb muscles [12]. Therapy-related issues such as transfusion-transmitted infections (hepatitis or HIV) are rare nowadays due to improved drug safety, although adverse reactions to the applied drug are still likely [13, 14, 15].

Table 1 Challenges in the use of RDTs.

Classification	Factor activity	Bleeding tendency
Severe	< 1 % of the norm (< 0.01 IU/mL)	Spontaneous bleeding in joints, muscles or gastrointestinal tract
Moderate	≥ 1 to 5 % of the norm (≥ 0.01 to 0.05 IU/mL)	Rare spontaneous bleeding in joints, mostly bleeding after trauma
Mild	≥ 5 to 40 % of the norm (≥ 0.05 to 0.40 IU/mL)	Bleeding after injury or surgery, rare bleeding tendency in daily routine

How to treat haemophilia?

The aim of haemophilia treatment is to promote physical and psychological health of the affected individuals by [16]

1. preventing bleeding events and their complications,
2. fast and effective bleeding management,
3. fast and effective complication management,
4. psychological and physiological support.

The primary approach of haemophilia treatment is the prophylactic, regular substitution of the deficient coagulation factor. Treatment should be conducted in accordance with local and/or international standards. Prophylactic medication is usually recommended under the following conditions [17, 18]:

- Infants after first bleeding events or showing other first symptoms
- Relapse into bleedings until relapse-free periods
- At the time of surgery or invasive events
- In case of outstanding physical or psychological stress
- During rehabilitation

Individuals with moderate haemophilia may not require prophylactic medication, and on-demand treatment might be appropriate if their conditions are suitable, because their bleeding tendency is not significantly elevated [9, 16]. The factor substitution therapy usually starts between 7 and 14 months after birth and depends – besides the factor concentration and clinical presentation – on the level of mobility. It is preferred to continue the therapy until the end of the child's growth period, and then may convert into an on-demand treatment if the overall conditions are suitable [18, 19, 20]. However, reverting to a constant prophylactic therapy might become necessary if significant bleeding episodes return. Table 2 summarises the different treatments. [7].

People with haemophilia C usually do not need to undergo a prophylactic therapy, and on-demand therapy is only needed for treatment after bleeding events or as a preventive measure prior to severe medical invasive interventions [5]. Prophylactic medication requires regular monitoring to verify the success of the treatment and to identify the appearance of adverse drug effects.

Which medication is available for treatment?

In the past decades, there has been a variety of treatment options for haemophilia. The most common treatment products are summarised in Table 3 (next page).

What other treatments are there?

In addition to therapy with coagulation factors, adequate first aid treatment is essential. This includes

Protection of the bleeding area
Rest of the affected body part
Ice to cool the affected part
Compression to reduce the blood loss
Elevation of the affected body part [16]

Parts of the treatment include also orthopaedic and physiotherapeutic measures as well as an adjusted lifestyle to maintain the functionality of the joints and limbs and allow for recovery after muscle bleeding events to prevent the person from arthropathy.

Can haemophilia be cured?

Gene therapy aiming to repair the defective FVIII gene has become the hope haemophilia patients for a treatment-free future. Currently several gene therapy products mostly based on vectors employing serotypes of the adeno-associated virus (AAV) designed to deliver FVIII or FIX cDNA into the hepatocytes are in clinical validation studies [35]. First results from those clinical trials are promising with bleedings being largely reduced in patients treated although the expression of FVIII appears to decline with time [35].

Table 2 Summary of factor treatment

Treatment	Intervals and methods
Treatment on demand	In the event of clinically significant bleeding
Primary prophylaxis	Continuous treatment initiated in the absence of documented osteochondral joint disease, determined by physical examination and/or imaging studies, and started before the second clinically evident large joint bleed and an age of three years
Secondary prophylaxis	Continuous treatment started after two or more bleeds into large joints and before the onset of joint disease documented by physical examination and imaging studies
Tertiary prophylaxis	Continuous treatment started after the onset of joint disease documented by physical examination and plain radiographs of the affected joints
Intermittent prophylaxis	Treatment given to prevent bleeding for periods not exceeding 45 weeks in a year

Table 3 Haemophilia treatment

Factor concentrates (plasma-derived or recombinant)	
Description	■ Drugs with high concentrates of coagulation factors, either made from human blood or recombinant (manufactured from genetically engineered cells carrying human factor genes) ■ Recommended by the WHO in preference to cryoprecipitate or fresh frozen plasma (FFP)
Advantages	■ Treatment of choice for haemophilia A and B ■ Well known and monitored by local and international organisations ■ Good product safety with respect to lipid-coated viruses (HIV and HCV) ■ Effective, to-the-point treatment ■ Well-known dose and mode of action
Disadvantages	■ Existing risk of prion-mediated disease through plasma-derived products ■ Possible development of antibodies against the administered factor ■ Severe haemophiliacs need several applications per week
Extended half-life factor concentrates	
Description	■ Drugs with high concentrates of chemically modified coagulation factors (PEGylation, fusion with other proteins) resulting an extended half-life following administration ■ Recommended by the WHO in preference to cryoprecipitate or fresh frozen plasma (FFP)
Advantages	■ Treatment of choice for haemophilia A and B ■ Extended half life reduce frequency of administration and improves patients' quality of life. ■ Wide range of different products available ■ Effective, to-the-point treatment ■ Well-known dose and mode of action
Disadvantages	■ Existing risk of prion-mediated disease through plasma-derived products ■ Possible development of antibodies against the administered factor still apparent
Cryoprecipitates	
Description	■ Prepared from fresh frozen plasma (FFP) ■ Appear as insoluble precipitates and are separated by centrifugation
Advantages	■ The alternative if coagulation factor concentrates are not available ■ Preferable to FFP for haemophilia A treatment
Disadvantages	■ Not for treatment of haemophilia B and C ■ May not be subject to viral inactivation procedures ■ Only low levels of factors available in a single dose ■ Needs several applications per week
Fresh frozen plasma (FFP)	
Description	■ Produced from healthy donors in blood donation centres
Advantages	■ Contains all coagulation factors ■ Usable for treatment of haemophilia A, B and C ■ Locally producible
Disadvantages	■ Needs high volumes due to low FVIII concentrations ■ Sufficient levels of FVIII and FIX are hard to achieve ■ Due to concerns about the safety and quality of FFP, its use is not recommended, if avoidable
Desmopressin (DDAVP)	
Description	■ Synthetic analogue to vasopressin boosting FVIII and vWF levels
Advantages	■ Treatment of choice for mild or moderate haemophilia A forms ■ Less cost-intensive compared with other products ■ Very low risk of transmission of viral infections
Disadvantages	■ No effects on FIX (haemophilia B) and FXI (haemophilia C) ■ Differences in individual patient response
Tranexamic acid	
Description	■ Antifibrinolytic agent promoting clot stability
Advantages	■ Effective in skin and mucosal bleeding management ■ Effective in dental surgery and oral bleeding management
Disadvantages	■ Complementary treatment only
Factor VIII mimetics	
Description	■ Antibodies acting like a FVIII bridging the FIX and FX ■ Latest generation of haemophilia treatment
Advantages	■ No adverse events such as transmission of viral infections, prion-mediated diseases or development of antibodies against FVIII ■ No inactivation by human anti-FVIII antibodies ■ May only need a single weekly application
Disadvantages	■ For haemophilia A treatment only ■ Long-term studies not yet available ■ Monitoring of treatment limited to certain assay types ■ Development of an antibody against emicizumab might be possible

What lab tests may I perform?

The presence of haemophilia is primarily detected by a prolongation of the activated partial thromboplastin time (APTT) assay, a screening test to detect anomalies in the intrinsic and common pathway of the coagulation cascade. The prolongation of the APTT in seconds correlates with the factor sensitivity of the reagent in use, and it is recommended to use reagents with high factor sensitivity when screening for potential factor deficiencies. Other screening assays such as prothrombin time (PT), thrombin time (TT), fibrinogen (Fbg) and the platelet count show normal results regardless of the severity of the haemophilia, while the bleeding time lacks sensitivity and specificity [21]. Thus, these tests are not recommended to screen for haemophiliacs.

Table 4 Expected findings in screening assays

Assay	Result
Prothrombin Time (PT)	Normal
Activated Partial Thromboplastin Time (APTT)	Prolonged
Thrombin Time (TT)	Normal
Fibrinogen (Fbg)	Normal
Platelet count (PLT)	Normal

Prolonged findings in the APTT assay should be confirmed by a repeated measurement of the patient sample mixed with pooled normal plasma in a 50:50 mixture, to separate a real factor deficiency from a deficiency caused by an inhibitor such as Lupus Anticoagulant (LA).

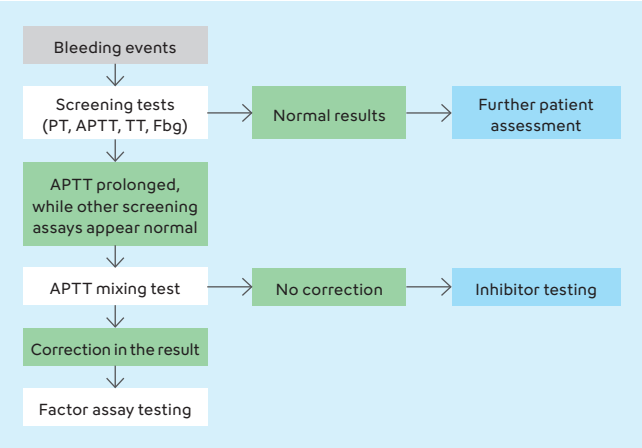

Fig. 2 Flow chart of routine assays for the determination of haemophilia

The subsequent factor activity examination is carried out by specific factor assays. Two types of factor assays to assess the activity of FVIII and FIX are available:

a) one-stage APTT-based factor assays,
b) chromogenic factor assays and two-stage assays.

The most commonly used assay for haemophilia lab testing is the one-stage APTT-based factor assay. A plasma deficient in any of the factors comprised in the intrinsic pathway will result in a prolonged partial thromboplastin time (APTT). A mixture of the respective factor-deficient plasma and the patient plasma is tested in the APTT assay, and the result is interpreted using a reference curve obtained with dilutions of standard plasma or a normal plasma pool mixed with the deficient plasma. A patient plasma deficient in a specific factor will not be able to compensate for the absence of the factor in the corresponding coagulation factor-deficient plasma and therefore result in a prolonged APTT [23]. Samples determined with one-stage APTT-based FVIII and FIX assays must be tested in at least three different dilutions and compared with a reference plasma. A single dilution of the plasma in question is not recommended, due to higher imprecision and potential inaccuracy in the presence of an inhibitor [16, 24].

However, there are limitations, e.g. due to interference with LA, and one-stage APTT-based factor assays might fail to detect individuals with mild haemophilia A and certain mutations in factor VIII. These individuals have a bleeding history but appear normal in APTT and one-stage APTT-based factor assays, while showing abnormal results in chromogenic assays. Nevertheless, the reverse can also occur in some clinical cases [25, 26]. Therefore, more than one type of FVIII assay must be used for a sufficient primary diagnosis of haemophilia A.

Patients with mild haemophilia B may have a normal or near normal APTT. Thus, in undiagnosed mild bleeding disorders, a factor IX assay should be performed, even if the APTT is normal [22]. Complementary assays to one-stage APTT-based factor assays are the FVIII chromogenic assay (e.g. Sysmex Factor VIII chromogenic assay, BIOPHEN™ FVIII:C) and FIX chromogenic assay (e.g. BIOPHEN™ FIX). Chromogenic assays to determine FXI activities are currently not available.

The monitoring of extend half-life factor concentrate pose a challenge to the laboratory as many products cause false high or false low results depending on the reagent and method used (OSA or chromogenic). But suitable assays for the determination of factor activities in treated patients have been identified for each extended half-life product [36, 37].

Ideally, inhibitor testing for people with severe haemophilia A should be performed frequently. Referring to local recommendations, inhibitor testing should be part of the regular assessment and linked to the number of drug administrations. It should also be increased with advanced age [27, 28].

What else do I have to consider during laboratory examination?

A positive first diagnosis of haemophilia A should always be challenged in differential diagnosis against VWD, a genetic disorder caused by an absent or defective vWF. Because vWF appears as the carrier of FVIII in blood and prevents its early dissociation, deficiencies in vWF are also associated with decreased concentrations of FVIII. It is therefore very important to differentiate haemophilia A from VWD type 2N and the more severe form, VWD type 3. Preferred vWF assays are vWF:Ag, vWF:RiCo, vWF:FVIII and vWF:CB assays [29].

The differential diagnosis should always start with vWF:Ag, followed by activity assays if vWF:Ag appears normal. Figure 3 presents the flowchart to diagnose haemophilia including the exclusion of VWD. However, VWD may already have been excluded prior to haemophilia testing, because of its higher prevalence or other reasons (for example a female patient). In such a case, the test procedure might stop after determining abnormal results in factor assays.

How to detect an inhibitor to FVIII?

A major adverse drug effect is the development of an antibody against the FVIII, so-called 'acquired haemophilia A' (AHA). Factor concentrates made from human donors, but also recombinant factor compounds, may be recognised by the receiver's immune system as a foreign object, leading to the development of antibodies. This makes the therapy less effective and the person may have long-term bleeding events even though being under factor substitution treatment. In contrast to this, many individuals with an inhibitor may not show any clinical signs, but in laboratory testing, lower concentrations of the coagulation factor are detected than would be expected after drug administration. In this case, further laboratory investigation is essential to prevent a deterioration of the person's condition. The most common test to do so is the Nijmegen-Bethesda assay (NBA). The original Bethesda method was developed to standardise the measurement of inhibitors in a FVIII neutralisation assay. In the Nijmegen modification, the pH and the protein concentration of the test mixture were further standardised. The plasma in question is mixed with a pooled normal plasma in different dilutions. The remaining factor concentration is determined after two hours of incubation and compared with a control plasma. In the NBA, the FVIII in the test mixture is less prone to artefactual deterioration, and the test has improved specificity compared with the Bethesda assay [32].

Table 5 VWD forms associated with haemophilia presentations

VWD form	Clinical presentation	vWF:AG	vWF:FVIII	vWF:RiCo	vWF:CB
VWD type 2N	■ Deficiency in the binding of vWF to FVIII ■ Individuals presenting with bleeding tendency of a mild or moderate haemophilia	Normal	Abnormal	Normal	Normal
VWD type 3	■ Complete absence of vWF ■ Individuals presenting with bleeding comparable with severe haemophilia A	Abnormal	Abnormal	Abnormal	Abnormal

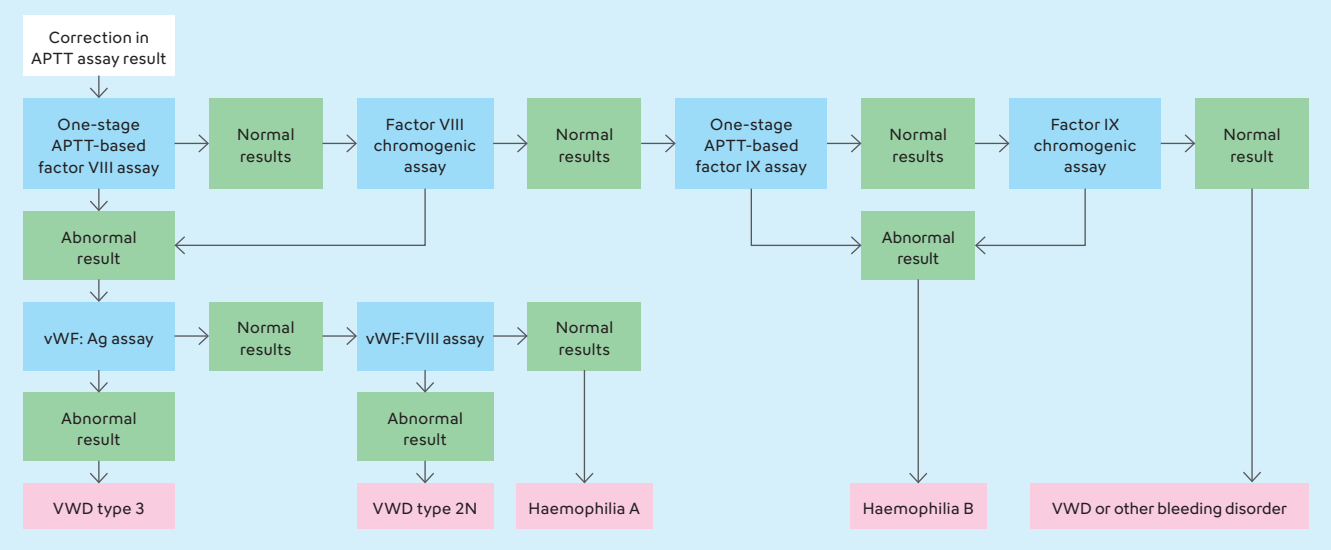

Fig. 3 Flow chart for determination of haemophilia by special assays (without haemophilia C testing)

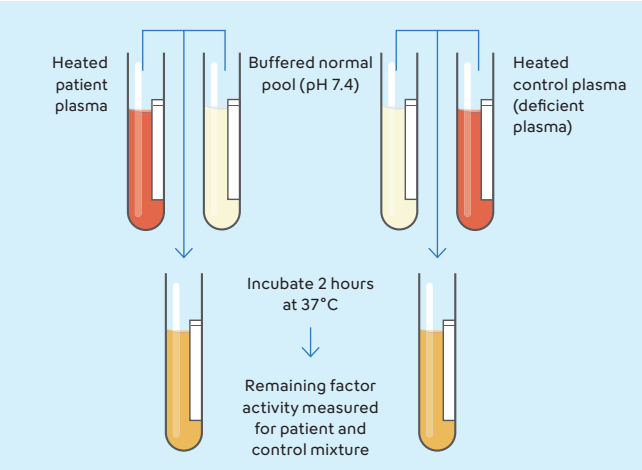

Fig. 4 Nijmegen-Bethesda test

The results are reported in Nijmegen-Bethesda-Unit (NBU). One NBU is defined as the amount of inhibitor which results in 50 % residual FVIII activity of a defined test mixture. Thus, 100 % of residual factor activity is equal to 0 NBU, 50 % of residual factor activity is equal to 1 NBU, and so on. If the residual factor activity of undiluted sample is < 25 %, retesting with diluted sample is necessary. Individuals with > 5 NBU/mL are called high responders, individuals with < 5 NBU/mL low responders.

However, even with a standardised procedure, several factors can affect the performance of the test and it is important for laboratory staff to be aware of their impact on the result outcome.

- Neither calibrator nor quality controls are available
- High imprecision (particularly between operators)
- Potentially false inhibitor titres if LA is present
- Results of NB assay are strongly dependent on deficient plasma used for control and substrate
  - Contains vWF as natural carrier of FVIII, thus vWF concentration important for FVIII stability
  - If vWF concentration is low, FVIII residual activity will be 30–50 % lower
- Buffered normal pooled plasma may contain variable concentrations of FVIII, and high concentrations of FVIII can lead to determining a falsely decreased inhibitor titre in test plasma
- pH has an impact on the inhibitor concentration
- Residual FVIII activity in the patient's plasma (if not or insufficiently heated) can interfere and cause lower inhibitor titres
- An incubation time of two hours does not cover proteolytic inhibitors; thus, a prolonged incubation time is needed

A suitable alternative in detecting FVIII inhibitors in human plasma might be qualitative or quantitative assays of the ELISA sandwich type. ELISA assays such as ZYMUTEST™ Anti-VIII MonoStrip IgG specifically measure human auto- and allo-antibodies to FVIII of the IgG isotype using immobilised (recombinant) FVIII. The advantages of this assay type over NBA are that there is no interference with LA and it can be easily used in laboratories having knowledge in ELISA testing. These standardised assays exhibit higher sensitivity (> 95 %) and specificity, notably when using age- and gender-specific cut-offs.

NBA still appears as the gold standard in AHA testing and is required to detect IgM isotypes, to exclude the diagnosis, or in strongly suspected AHA cases. On the other hand, ELISA-type assays are more accurate and suitable for use, and helpful in cases of borderline NBA results [31, 33].

Accordingly, the presence of antibodies directed against FVIII has an impact on the treatment. Depending on the antibody level, either higher FVIII concentrate levels or, alternatively, treatment with recombinant factor VII (rFVII) are required. In case factor substitution therapy is inefficient, immunoadsorption apheresis should be considered. The goal of the AHA treatment is the elimination of the anti-FVIII antibodies. This can be achieved, for example, by establishing immune tolerance [34].

Before you leave …

Haemophilia is a bleeding disorder making affected individuals bleed longer and, in severe cases, spontaneously. Caused by a genetic defect on the X chromosome, less coagulation factor activity is available. Even though for haemophilia A and B mainly men are affected, women might also show an increased bleeding tendency under certain conditions. With haemophilia C, both genders are affected equally. Prevention of long-term harm resulting from inappropriate treatment is a major aim of patient management, besides the prevention and management of bleeding episodes. Treatment has significantly been improved over the last decades, and latest-generation treatment is more efficient, secure and convenient than ever before. And the future holds a potential cure with gene therapy redeeming haemophilia patients from life-long treatment. Proper lab diagnostics remain essential for diagnosing and therapy monitoring but can be managed using the right factor activity determination by APTT-based one-stage and/or chromogenic assays. However, the diagnostic workflow should include the diagnosis of a condition which signs and/or symptoms are shared by other conditions such as VWD. Adverse drug effects became less severe over the past decades but remain important to be considered to make sure that treatment is effective and ensure the best possible conditions of life for affected individuals.

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