Diagnosis of factor XIII deficiency

Akbar Dorgalaleh1, Shadi Tabibian2, Maryam Sadat Hosseini2, Yadolla Farshi2, Fateme Roshanzamir2, Majid Naderi3, Ahmad Kazemi1, Farhad Zaker4, Ali Noroozi Aghideh2, Morteza Shamsizadeh5

1Department of Hematology and Blood Transfusion, School of Allied Medical Sciences, Iran University of Medical Sciences, Tehran, Iran, 2Department of Hematology and Blood Transfusion, School of Allied Medical Sciences, Tehran University of Medical Sciences, Tehran, Iran, 3Department of Pediatrics Hematology & Oncology, Ali Ebn-e Abitaleb Hospital Research Center for Children and Adolescents Health [RCCAH], Zahedan University of Medical Sciences, Zahedan, Iran, 4Cellular and Molecular Research Center, Iran University of Medical Sciences, Tehran, Iran, 5School of Nursing and Midwifery, Shahroud University of Medical Sciences, Shahroud, Iran

Background: Factor XIII (FXIII) deficiency is an extremely rare bleeding disorder with estimated incidence of one per two million. All routine coagulation tests are normal in FXIII deficiency (FXIII-D), which complicates the diagnosis of this disorder. Precise diagnosis of FXIII-D requires more specific tests, including qualitative tests as well as quantitative tests such as FXIII activity, antigen assays, and finally molecular studies to confirm FXIII-D.

Objective: This study was conducted to present different quantitative and qualitative methods as well as molecular approaches for screening and diagnosis of FXIII-D with advantages and disadvantages of each method.

Methods: All relevant English-language publications were searched in Medline (until 2015).

Results and discussion: Clot solubility assay is the most widely used method for detection of FXIII-D but it is not standardized. The sensitivity of this method is dependent upon different factors mainly clotting factors and the solubilizing agents; therefore, FXIII activity assay is recommended for screening of FXIII-D. Among FXIII activity assays, photometric assay is more common but FXIII activity is overestimated in this assay due to lack of sample blank in commercial assay, which can have fatal consequences in severe FXIII-D, for which fluorometric assay is an appropriate alternative preventing the overestimation observed in photometric assay. There are different methods for measurement of FXIII-A2, FXIII-B2, and FXIII-A2B2 as well as detection and quantification of FXIII inhibitor, which are mentioned in detail in this review. There are no mutational hotspots in FXIII-A and FXIII-B genes with a few recurrent mutations in some populations; therefore, full sequencing of FXIII genes has remained a main molecular approach for confirmation of FXIII-D.

Conclusion: Familiarity with different methods for diagnosis of FXIII-D and their advantages and disadvantages can help in appropriate and timely diagnosis of this disorder to prevent misdiagnosis of FXIII-D and its fatal consequences.

Keywords: Factor XIII deficiency, Rare bleeding disorder, Laboratory diagnosis
cell functions, including platelet spreading, monocyte-derived redeployment of cells, phagocytosis, and gene expression.\textsuperscript{2,10}

Activation of both tetrameric pFXIII and dimeric cFXIII occurs through concerted action of thrombin and calcium ions. After thrombin hydrolysis of Arg37–Gly38 peptide bonds of FXIII-A subunit, FXIII activation peptide (AP-FXIII) is detached from N-terminal part of the molecule and appears in plasma. In the presence of Ca\textsuperscript{2+}, FXIII-B subunits dissociate from the complex due to attenuation of the interaction between FXIII-A and FXIII-B subunits because of proteolytic cleavage of FXIII-A by thrombin. Dissociation of FXIII-B is essential for Ca\textsuperscript{2+}-induced transformation of FXIII-A, which results in truncated FXIII-A. Through conformational change of FXIII-A dimer structure, the cysteine of protein active site, which is located within the Tyr–Gly–Gln–Cys–Trp sequence and is covered in the catalytic core domain in parent molecule, becomes unburied and available to react with its substrate. Therefore, FXIII is converted to FXIIIa. Interestingly, full transglutaminase activity of FXIII-A can result from AP-FXIII detachment from one of the two A-subunits of FXIII-A dimer.\textsuperscript{2,9,11,12} As a result of a FXIII-B dissociation during activation, final fibrin clot has no B-subunit, whereas there is still over 90% FXIII-A protein in fibrin network.\textsuperscript{8}

The first fibrin cross-linking by FXIIIa occurs in γ-chain dimers formed between γ406 lysine of one γ-chain and γ398 or γ399 glutamine residue of the other γ-chain of two neighboring fibrin molecules in longitudinal orientation. This dimerization rapidly occurs within 5–10 minutes, resulting in longitudinally antiparallel connection between D-regions of two fibrinogen molecules.\textsuperscript{1,4}

However, cross-linking of α-chains is slower than γ-chains, but the former are dominant structures increasing the rigidity of fibrin clot. A number of residues are involved in α-chain cross-linking such as lysine (Lys 208, Lys219, Lys229, etc.) and glutamine (Glu221, 237, 328, and 366) residues that lead to formation of a high molecular weight polymer. The orientation of α-chain cross-linking is vertical to the longitudinal strands. α-Chain cross-links form a rigid network to protect fibrin clot in pulsatory blood flow to interfere with the degradation effect of plasmin upon the clot. In general, all these FXIIia-induced cross-linking changes drastically affect the viscoelastic properties of fibrin clot, such that it is more resistant than non-cross-linked fibrin in the circulation.\textsuperscript{2–10}

On the other hand, stability of fibrin clot against fibrinolytic system may be enhanced by FXIII. On the one hand, α2-antiplasmin (a physiological inhibitor of plasmin) is bound to α-chain of fibrin by FXIIIa immediately after fibrin polymerization. Moreover, cross-linking of fibrin reduces plasminogen binding to fibrin, decreasing the activation of plasminogen by tissue plasminogen activator. The role of platelet FXIII is not clear; however, cFXIII may enhance the rigidity of fibrin clot after platelet activation. Fibrin polymerization is also accelerated on the surface of activated platelets. On the other hand, bleeding manifestations are more severe in patients with FXIII-A deficiency in comparison with FXIII-B-deficient patients. Therefore, this may indicate the important role of cFXIII in hemostasis.\textsuperscript{8,13}

**FXIII deficiency**

FXIII deficiency (FXIIID) is a rare autosomal recessive bleeding disorder with an estimated incidence of one in two million in the general population.\textsuperscript{14} Deficiency of this factor leads to defective cross-linking of fibrin and formation of a weak, unstable clot. In these patients, clots may form normally but begin to break down 24–48 hours later because of weak cross-linking of fibrin, leading to subsequent episodes of bleeding. FXIIID can result from mutations of subunit A or B genes but disorders of subunit A are more common.\textsuperscript{7,15}

Patients with severe FXIIID usually present with less than 1% of pFXIII level, and therefore have a potentially severe bleeding tendency. The clinical manifestations of the disease include delayed wound healing, recurrent spontaneous miscarriage, severe bleeding, and spontaneous intracranial hemorrhage, the main cause of death in these patients.\textsuperscript{16,17}

**Diagnosis of FXIIID**

Evaluation of clinical presentations is helpful in diagnosis of FXIIID. Umbilical cord bleeding is the most common clinical manifestation among patients with FXIIID, which is strongly suggestive of FXIIID. Moreover, CNS bleeding is more common in patients with FXIIID in comparison with other rare bleeding disorders or hemophilia. Delayed wound healing and delayed bleedings are other common presentations among these patients. Recurrent miscarriage in women in childbearing age is another presentation suggestive of FXIIID.\textsuperscript{18} Therefore, the first step in timely diagnosis of FXIIID is taking a proper patient history, including family history. A number of studies revealed that even heterozygotes of FXIIID had an abnormal bleeding pattern.\textsuperscript{19,20} Thus, taking a precise history of patient’s parents or their sibling is another helpful diagnostic factor. After proper assessment of clinical presentations and family history, patients can undergo a full laboratory assessment.

The routine laboratory clotting tests or first-line screening tests such as bleeding time, prothrombin time, activated partial thromboplastin time, and
platelet count are normal in FXIIID. Although clot solubility test is no longer recommended for screening of FXIIID, it is the first screening test nearly in all clinical laboratories in developing world as well as a considerable number of laboratories in developed countries (about 20%). Therefore, clot solubility test cannot be omitted as a screening test but can be improved to detect several forms of FXIIID. Different methods for improvement of clot solubility test will be presented later in this manuscript. Therefore, screening of the disease is based on clot solubility test in 5 M urea or 1% monochloroacetic (MCA) acid. Factor deficiency can be confirmed via estimation of FXIII activity, antigen assay, or molecular analysis as well as detection of causative mutation in FXIII-A or FXIII-B genes. A variety of methods have been introduced for clot solubility test, FXIII activity, and antigen assays as well as inhibitor assessment in patients with FXIIID. Thus, the present review aimed to describe different qualitative and quantitative assays with their advantages and disadvantages as well as molecular approaches for diagnosis of FXIIID, which can lead to proper diagnosis of FXIIID.

Qualitative tests for diagnosis of FXIIID
Blood collection for FXIII screening tests
With regard to prolonged half-life of FXIII, the samples of subjects receiving FXIII prophylaxis should been taken at least 3 weeks after the last treatment. The appropriate anticoagulant for clot solubility test in FXIII assay is 3.2% (105–109 mM) trisodium citrate. This anticoagulant is the most common one for coagulation tests. Although 3.2% (105–109 mM) and 3.8% (129 mM) dehydrated trisodium citrate can be used, the former, which has similar osmolarity with plasma, is preferred and is recommended by scientific and standardization committee of International Society for Thrombosis and Hemostasis (ISTH) and the National Committee for Clinical Laboratory Standards (NCCLS). Another advantage is that prolonged clotting times have been observed in samples collected in 3.8% sodium citrate due to neutralization of calcium ions in the reagent, which are necessary for clotting; however, this prolongation was not observed in 3.2% sodium citrate.

Clot solubility assay
Traditionally, FXIIID is assessed by qualitative assay following normal results in routine coagulation tests. As the most widely used method for detection of FXIIID, clot solubility assay was the first step for diagnosis of this disorder and the first case was diagnosed by this method in 1960. Fibrin clot solubility in urea, acetic acid, or MCA acid solutions has been used for screening of FXIIID. Urea and MCA acid solubility tests are routinely used to detect FXIIID. There is no standard procedure for clot solubility test and different procedures have been introduced for performing this test. Initially, volumes of whole blood is mixed with one volume of 3.2% sodium citrate anticoagulant. Then, calcium chloride and buffer are added with or without thrombin, and the solution is incubated for 1 hour at RT or 37°C for clot formation. After clot formation, it is suspended in 5 M urea or MCA acid solution and incubated at 37°C or RT. Then, clot solubility test is regularly evaluated at 15-minute, 1-hour, and 24-hour intervals. In FXIII-deficient samples, the clot is mostly dissolved within a few minutes to 1 hour, but the clot is stable for 1 day in a normal sample. In acetic acid-based method, formed clot is suspended into acetic acid and then incubated for 6 hours. In the semi-quantitative assay for diagnosis of FXIIID, plasma sample is clotted by adding thrombin, and the clot is then suspended in diluted urea or MCA acid solutions.

MCA acid solubility test was previously performed for detection of FXIII activity. In this method, diluted plasma is mixed with XIII-free fibrinogen, the plasma is clotted by adding thrombin as well as CaCl₂, and FXIII clot solubility is surveyed by adding 1% MCA acid. Clot solubility test is a qualitative assay and can only diagnose the severe form of FXIIID. Urea and MCA acid solubility assays are positive if the level of FXIII is roughly less than 5%. Sensitivity of acetic acid-based assay is nearly twice that of urea and MCA acid solubility assays. However, the acetic acid-based assay is more sensitive and rapid than urea solubility test but is less specific. Normal plasma is dissolved in acetic acid after 4 days, which never occurs in urea-based assay. In routine assay of clot solubility, if the level of FXIII is higher than 5%, patients with FXIIID are falsely considered as normal. The ISTH recommends the following algorithm for diagnosis and classification of FXIIID:

1. A quantitative functional FXIII activity test is requested as a first-line test for diagnosis of FXIIID. This test is able to detect all forms of FXIIID. As a result, if FXIII activity is declined, further investigations are recommended for detection and classification of FXIIID.
2. The concentration of FXIII-A₂B₂ antigen is measured in the plasma. If it is decreased, further analysis should be performed to determine the subtype of deficiency. First, FXIII-A and FXIII-B antigens are measured. For detection of FXIIID in platelets, measurement of FXIII activity and FXIII-A antigen in platelet lysate should be performed.
3. For detection of autoantibody against subunits of FXIII, mixing study and binding assay should be
performed. Mixing study and binding assay should be performed for detection of neutralized antibody against A-subunit and non-neutralized antibody against B-subunit, respectively.

4. If all the above tests are normal, additional tests like evaluation of fibrin cross-linking test by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) are performed.

5. Finally, detection of molecular genetic defect of FXIII is recommended.

Limitation of clot solubility test
The clot solubility test is not standardized, and careful consideration is mandatory. The sensitivity of this method is dependent upon fibrinogen level, the reagent used for clotting (Ca\(^{2+}\), thrombin, etc.), and the solubilizing agents (MCA acid, acetic acid, or 5 M urea). Combination of acetic acid/thrombin, acetic acid/calcium, urea/calcium, and urea/thrombin can modify the relative sensitivity of clot solubility assays. There is no consensus about the sensitivity of traditional clot solubility assay (urea/calcium method) in the literature. For instance, it was reported 3–5% by Jakobsen and Godal in 1974,34 less than 0.5% by Francis in 1980,6,35 1–5% by Jennings et al. in 200321 as well as Lawrie et al.,10 and <0.5–5% by Bolton-Maggs et al.32 and Kohler et al.11 in 2012 and 2011, respectively. Jennings et al.21 also reported that thrombin/acetic acid is sensitive to at least 10% FXIII, whereas the sensitivity of calcium/acetic acid and thrombin/urea is intermediate. In 1980, Francis reported the limited 0–3% detected level of FXIII in plasma by calcium/acetic acid method.6 Reported data by Hsu et al.6 in 2014 have shown that the traditional clot solubility tests only detects 16% of patients with pFXIII level less than 2%, whereas the use of thrombin methods could improve the detection. In this study, the sample with 1% FXIII level was more accurately detected by the traditional method than the sample with 2% FXIII.10,31 Patients with hyperfibrinogenemia must be excluded because this situation prevents clot stabilization. In general, thrombin-based solubility tests, especially thrombin/acetic acid combination methods, are more sensitive.10,31 This method is not though able to differentiate severe form of disease from moderate or mild state, heterozygous from homozygous, and inherited from acquired form.7,31 Since low level of FXIII inhibitor does not decrease FXIII activity to less than 1%, such cases are underestimated in this assay. In addition, administration of FXIII within a few weeks before test gives normal results. Therefore, due to the high number of undiagnosed or delayed diagnosed cases, this method is not recommended as a routine screening test.7,11,31

Quantitative tests in laboratory diagnosis of FXIIID (activity and antigen assay)
If the diagnosis of FXIIID is suspected through qualitative methods, quantitative activity assay followed by antigen assays should be used for confirmation and classification of deficiency.11

Quantitative methods are preferred over the qualitative assays because they are able to diagnose inherited or acquired form of disease and can quantitate FXIII activity as low as 1%. Quantitative assays are also used for monitoring of patients subject to treatment, and are less influenced with heparin or fibrinogen abnormalities.21

Blood collection for FXIII activity and antigen assay
For FXIII assays, just like any other clotting test, the samples should be collected early in the morning (7.00–9.00 AM) in a relaxed gesture. The tubes should be adequately filled to observe 1:9 proportion (by mixing one volume of trisodium citrate and nine volumes of blood specimen). A mark may be provided on the tubes, indicating the exact required volume of blood, and at least 90% of this total volume must be filled. Underfilling might lead to significant sample dilution and subsequent underestimation of quantitative test results, including FXIII level. The volume of anticoagulant should be adjusted in cases with a hematocrit level higher than 55%.

For long-term storage, samples can be frozen at −24°C or lower for up to 3 months or at −74°C for up to 18 months without significant alteration in factor assay results. Frozen samples should be thawed at 37°C for 3–5 minutes immediately prior to testing, and lower temperature is not acceptable due to the likelihood of cryoprecipitation. Frequent thawing and freezing has to be strictly avoided, as it highly affects the activity of coagulation factors.36,37 FXIII activity is relatively equal in different types of plasma, including fresh plasma, fresh frozen plasma, and even outdated blood bank plasma, but FXIII activity is reduced when the plasma is stored at RT for 3 days.38 Plasma can be frozen at −20°C in 0.05 M sodium citrate at pH = 7.1 for 10–12 weeks and longer without loss of FXIII activity. When dithiothreitol is added to the plasma, FXIII activity is increased two- to eightfold during storage.39

FXIII activity assay
Functional quantitative activity assays are capable of detecting all forms of disease, including severe, moderate, and mild deficiencies. In addition, this method can differentiate acquired form of disease from the inherited form. Therefore, these assays are used for exact diagnosis of disease.1,13,37 The following methods are used for measuring FXIII activity.
Photometric assay
This method is based on ammonia release in the first step of transglutaminase reaction of FXIII. In this assay, FXIIIa cross-links a small molecular weight substrate into oligonucleotide containing glutamine. Then, ammonia releases within this reaction and is photometrically measured via an NAD(P)H-dependent reaction. Decreased absorbance is indicative of FXIIIID. The most common kits available for this method are Berichrom FXIII (Dade Behring, Marburg, Germany), REA-chrom FXIII (Reanal, Budapest, Hungry), and Technochrom FXIII (Technocloe, Vienna, Austria).7,10,11

Advantage and disadvantage of photometric assay
As the most common method in clinical laboratories, this method is capable of monitoring the reaction at 340 nm and is a one-step true kinetic assay, which is quickly performed and easily automated on coagulation analyzers. The main disadvantage of this method is relatively low sensitivity that causes a detection limit of 3–5%.6,11 Furthermore, lack of sample blank in commercial assay decreases its precision. A plasma-blank sample is essential for amendment of some ammonia-producing and NADH-consuming reactions independent of FXIIIa in the plasma. Otherwise, without using the sample blank, FXIII activity is overestimated.9,10 In the study of Lim et al.40 in a patient with acquired FXIIIID without detectable level of FXIII-A antigen and positive clot solubility test in urea environment, Berichrom assay detected FXIII activity in a range of 8–14% (0.08–0.14 U/ml). It was concluded that Berichrom assay might not be accurate in low levels of FXIII.

In a comprehensive study by UK NEQAS for blood coagulation, similar results were observed. In a recent study by Ichinose et al.41 on 32 patients with acquired FXIIIID, no correlation was observed between FXIII activity and bleeding severity and even patients with >10% of normal F13 activity had spontaneous bleeding. They stated that this finding may merely reflect the fact that measurement of residual FXIII activities has been performed by ammonia release assay that usually gives high background/blank values. In the normal range of FXIII, overestimation cannot cause a problem but it is highly considerable in severe deficiency.7,10 Since a small level of FXIII (5–10%) is adequate to keep hemostasis, this overestimation may have clinical consequences. In Lim et al. study, FXIII activity levels in severely deficient patients (<2%) were in 8–14% range by Berichrom assay. In the study of Lawrie et al.10 in 2010, Berichrom FXIII assay showed more reproducible results and a level of inter- and intra-assay variation for both normal and pathological controls used in their study compared with other time-consuming methods.

In the study of Levente Karpati et al.42 in 2000, an overestimation of 8% occurred by Berichrom assay in which replacement of NADPH for NADH improved the results but did not omit the problem.43 In fact, Ajzner et al. stated that this overestimation is caused by NADH as a cofactor in Berichrom assay by lactate dehydrogenase in the plasma. Substitution of NADH with NADPH resolved this problem but non-specific NAD(P)H consumption remained as a source of error.44

If the sample blank is not provided commercially, the overestimation problem can be worked out by preparing a blank with added iodoacetamide by user.6,11 Nishida et al.44 reported that when the concentration of hemoglobin is about 0.5–1 μg/μl (50–100 mg/dl), amine incorporation assay does not affect FXIII activity. They reported that when bilirubin is mixed with normal plasma to prepare up to 0.2 μg/μl (20 mg/dl) of plasma bilirubin, activity of FXIII is increased nearly 6% by the mentioned assay, but the activity is not increased by fluorescence assay even in high concentration of bilirubin.37

Putrescine incorporation assay
This assay is based on measuring fluorescence, radio-labeled, or biotinylated amino acid (glycine-ethylester) covalently cross-linked to a glutamine residue in a protein substrate and removing the unbound amino acid. Pefakit FXIIIID was the available kit for this purpose (Pentapharm, Basel, Switzerland).7,10,11

Advantage and disadvantage of putrescine incorporation assay
Several studies reported that this assay is a high sensitive but time-consuming method that is difficult to standardize, and due to separation step, is hard to design a true kinetic assay. In addition its reproducibility is poor and cannot be also adapted to a chemistry clinical analyzer.5,11,44,45 Katona et al.46 in 2000 also reported that this assay is sufficient to determine low range of FXIII but it is cumbersome and not well standardized though. Noticeably, Kohler et al.11 in 2011 implied that amine incorporation test is sensitive to FXIII-A Val34Leu polymorphism and the activity of FXIII highly increased in this situation.

Fluorometric assay
It is a relatively new method based on isopeptidase activity of FXIIIa described by Parameswarn et al.27 Subsequently, this method was improved and became more sensitive by Oertel et al.45 in 2007. N-zyme BioTec is the available kit for this method (Darmstadt, Germany). In comparison with photometric assay, it is direct, simple, and independent of subsequent enzymatic steps.10,11,43
Advantage and disadvantage of fluorometric assay

According to Oertel et al. in 2007, the fluorometric assay is related to FXIII antigen assay results. This study showed that this method is a useful and excellent alternative in conditions such as severe FXIIID in which overestimation occurs in photometric assay. In this study, the determined range for FXIII activity was 0.07–0.14%.7,43,48 (Table 1).

Limitation of FXIII activity assays

Quantitative methods are unable to differentiate low factor activity due to factor deficiency or autoantibodies. Lipemic plasma and elevated ammonia cause falsely increased FXIII activity. For minimizing these interferences, parallel plasma-blank samples are recommended for samples with <10% FXIII activity to correct FXIII-independent NADPH consumption in plasma and avoid overestimation of FXIII activity, which may be clinically significant. In addition, icteric plasma leads to underestimation of FXIII activity.11,21,22

FXIII antigen assay

Several FXIII antigen assays are available, including immunoassays to measure FXIII-A, FXIII-B, and FXIII-A2B2 complex. According to Hsu et al. study in 2014, 80% of laboratories measuring FXIII antigen use HemosIl as an automated or latex-enhanced method for specific FXIII-A assay. Several ELISA methods have also been developed using anti-FXIII-A and anti-FXIII-B monoclonal antibodies for diagnosis of FXIII-A2B2.6 Although electroimmunoassay has been frequently used, it is not adequately sensitive. Radioimmunoassays for A- and B-subunits are difficult and may introduce artifacts. Yorifuji et al. in 1988 used two sandwich ELISAs to differentiate free B-subunits from those in complex with A-subunit in plasma and also measured the zymogen form of FXIII-A2B2. Katona et al. in 2000 stated that the previous assays used to determine FXIII antigen were not calibrated against purified FXIII, so they could not express results as mass concentration. Moreover, all but one of them measured the concentration of A- and B-subunits not the concentration. Moreover, all but one of them measured the concentration of A- and B-subunits not the concentration. Moreover, all but one of them measured the concentration of A- and B-subunits not the concentration. Moreover, all but one of them measured the concentration of A- and B-subunits not the concentration. Moreover, all but one of them measured the concentration of A- and B-subunits not the concentration. Moreover, all but one of them measured the concentration of A- and B-subunits not the concentration. Moreover, all but one of them measured the concentration of A- and B-subunits not the concentration. Moreover, all but one of them measured the concentration of A- and B-subunits not the concentration. Moreover, all but one of them measured the concentration of A- and B-subunits not the concentration.

Inhibitor assay in FXIIID

Although development of FXIII inhibitors is an extremely rare phenomenon,51 it must be considered to differentiate between acquired and congenital FXIIID in cases with bleeding events presenting with normal results of screening tests. In congenital FXIIID, inhibitor development is defined as an immune response to exogenous FXIII due to receiving replacement therapies. This event is suspected when a patient no longer responds to replacement therapies and continues to bleed.52 Acquired FXIII inhibitor is an immunoglobulin G from subclass 1 (IgG1) in which the light chain is exclusively λ-chain.53

FXIII inhibitors may be directed against FXIII-A, including neutralizing and non-neutralizing antibodies, or may be non-neutralizing inhibitors specific for FXIII-B. Neutralizing anti-FXIII-A antibodies result in a high reduction in pFXIII activity (usually below 3%), while pFXIII-A2B2 antigen and FXIII-A antigen are normal or slightly reduced (usually 20–70%). However, all these parameters will be highly reduced in presence of non-neutralizing antibodies. Both neutralizing and non-neutralizing anti-FXIII-A antibodies lead to higher than 30% level of pFXIII-B antigen. If an anti-FXIII-B antibody exists, the results are rather similar to non-neutralizing antibodies against FXIII-A but pFXIII-B antigen is considerably decreased.11
FXIII antibodies mainly occur spontaneously or in patients who are under long-term treatment with specific drugs, including isoniazid, penicillin, phenytoin, practolol, and amiodarone. The presence of these antibodies has also been reported in a few cases with an underlying disease such as monoclonal gammopathy of undetermined significance, rheumatoid arthritis, and systemic lupus erythematosus. As these inhibitors are usually associated with severe bleeding diathesis, early diagnosis and management of patients is necessary. Patients developing FXIII inhibitors need to be managed by plasmapheresis or immunosuppressive agents such as corticosteroids and cyclophosphamide as well as replacement therapies, which may result in a reduction of inhibitor titer and improvement of clinical phenotype. Rituximab is another choice, which may be administered alone or in combination with other immunosuppressive drugs. It is an anti-CD20 monoclonal antibody that can decrease antibody production by CD20+ B cells. Choosing the best therapeutic protocol should be based on inhibitor level, type of inhibitor (including low responding and high responding), and the patient’s response to a specific agent.

**Screening and quantitative assays of FXIII inhibitor**
Mixing study is usually helpful to determine the presence of FXIII inhibitor. In cases with abnormal clot solubility test results, it may be necessary to determine factor deficiency or presence of an inhibitor. In this method, clot solubility assay is performed on a mixture of patient plasma and a similar volume of normal plasma (1:1 proportion) with 100% FXIII activity. Therefore, in cases with FXIIIID, it leads to about 50% activity in the mixture. If the result of the assay is corrected, it may be suggestive of a congenital deficiency but if not corrected, an inhibitor to FXIII may be present.

Clot-based inhibitor assays are only able to detect neutralizing antibodies, and binding assay is recommended if non-neutralizing antibodies are present either against FXIII-A or -B subunits. In this method, IgG antibodies in the patient plasma are bound to purified FXIII-A2B2, FXIII-A2, and FXIII-B coated to a microtiter plate of ELISA or bound to SDS in PAGE method. The former is a sandwich method in which addition of a second enzyme-linked antibody against human IgG followed by an appropriate substrate leads to quantitative assessment of inhibitor. In the PAGE method, plasma sample is mixed with SDS as an anionic detergent and provides a negative charge for the bound polypeptide proportionate to its mass. The sample then undergoes electrophoresis on a polyacrylamide gel through which the proteins will be separated based on their size. Separated proteins are further investigated through western blotting in which the samples are transferred to a nitrocellulose membrane and are then detectable by immunochemical techniques.

Further investigation on FXIII inhibitor through antibody titration is commonly performed by Bethesda assay. For this assay, the patient plasma is mixed with the same volume of pooled normal plasma, and control sample is a 50:50 mixture of normal plasma and buffer. This traditional method is performed by measuring residual FXIII activity after incubation of patient plasma and control in different dilutions for 2 hours in 37°C. A semi-log plot of the residual FXIII activity (%) against inhibitor unit (Bethesda unit per milliliter) is then used to convert factor activity into inhibitor unit. The Bethesda units (BUs) are then detectable by immunochromatographic techniques.

**Table 1: Available methods for measuring FXIII activity**

| Type of activity assay | Name of available kits | Detection limit | Reference range | Advantage | Disadvantage |
|-----------------------|------------------------|-----------------|-----------------|-----------|-------------|
| Photometric assay     | Berichrom FXIII       | <5%             | 70–140 U/dl     | Easily automated | Low sensitivity |
|                       | REA-chrom FXIII       | <3%             | 69–143 U/dl     | one-step true kinetic assay | |
|                       | Technochrom FXIII     | <5%             | 70–140 U/dl     |           | |
| Putrescine incorporation assay | Pefakil FXIIIID kit | —               | 46–200 U/dl (wild type for Val34Leu) | High sensitivity | Time-consuming |
|                       |                        |                 | 97–251 U/dl (hetero for Val34Leu) |           | |
|                       |                        |                 | 151–483 U/dl (homo for Val34Leu) |           | |
| Fluorometric assay    | N-zyme BioTec         | <5%             | 70–140 U/dl     | Direct | |

Further investigation on FXIII inhibitor through antibody titration is commonly performed by Bethesda assay. For this assay, the patient plasma is mixed with the same volume of pooled normal plasma, and control sample is a 50:50 mixture of normal plasma and buffer. This traditional method is performed by measuring residual FXIII activity after incubation of patient plasma and control in different dilutions for 2 hours in 37°C. A semi-log plot of the residual FXIII activity (%) against inhibitor unit (Bethesda unit per milliliter) is then used to convert factor activity into inhibitor unit. The Bethesda units (BUs) are then detectable by immunochromatographic techniques.
of Bethesda assay is a more sensitive and specific assay with reliable results. The difference of this method is buffering the pooled normal plasma with 0.1 M imidazole buffer at pH = 7.4 and the use of FXIII-deficient plasma in control mixture. In presence of a low inhibitor level (<1 BU), the Bethesda assay may lead to a false positive result. In this condition, the Nijmegen modified assay provides zero level.58,61

In cases with residual FXIII activity less than 25%, the patient plasma must be further diluted and then retested. The results showing ≥0.6 BU/ml must be considered as clinically significant. If the inhibitor level is constantly <5 BU/ml, it will be defined as low responding inhibitor, which may be transient and disappear within a period of 6 months after the first investigation while high responding inhibitors are defined as ≥5 BU/ml levels, which are commonly persistent.58-61

**Molecular diagnosis of FXIIIID**

Inherited FXIIIID is a rare autosomal recessive disorder most often caused by mutation in FXIII-A subunit gene. Molecular defects in this subunit occur with a rate of one per two million.62 The most common molecular defects in FXIII-A subunit are nonsense mutations (>50% of cases). Nonsense mutations and deletions/insertions are other common gene defects in patients with FXIII-A deficiency.63 Several common disease-causing gene defects are observed among different populations. IVS5-1G>A is the most common mutation observed among European patients with different nationalities, including patients from the Netherlands, Poland, UK, etc. Arg661stop mutation in exon 14 of FXIII-A gene is another recurrent gene defect among European patients reported in Finland, Switzerland, Poland, Sweden, etc.29,63-65 This common mutation was also observed in India.66 Arg326Gln FXIII-A gene mutation has been detected in patients from Germany and the Netherlands.58,67 Thus, in patients with European origin, these three mutations can be selected as the first step in molecular diagnosis of FXIIIID. In Iranian patients with FXIIIID, the first selected mutation should be Trp187Arg observed in 348 patients with severe congenital FXIIIID.15,68-70 The second selected mutation in these patients should be Arg77His mutation, a frequent mutation among Iranian patients with FXIIIID.15,70 Among Indian patients, mutation in exon numbers 6 and 10 has been commonly detected.66 IVS1 A246G polymorphism seems to be the most common FXIII polymorphism in Indian patients, which is a suitable diagnostic marker of FXIIIID in this country (FF). Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) or PCR of a special exon can be the first diagnostic marker used to track FXIIIID in these countries. However, in other parts of the world, repeated disease-causing mutations are not observed, and over 120 different mutations are distributed throughout the FXIII gene, full sequencing of which is inevitable.7

A limited number of mutations have been observed in FXIII-B gene. Due to carrier role of this subunit for FXIII-A, mutations in this subunit are not associated with severe FXIIIID. Because of the small size of FXIII-B gene, it is easier to detect mutation in this subunit.7,64

**DNA sequence analysis**

FXIII-A gene with 15 exons and 160 kb is too large to use sequencing as a screening technique for unknown mutations; however, this is inevitable in most patients in general population due to lack of common genetic defects. PCR-RFLP with single PCR product sequencing can be considered as a reliable diagnostic technique in some Iranian, Swiss, and Finnish patients. When mutation screening technique fails in these patients, whole FXIII gene sequencing may be inevitable. With new automated sequencing techniques, only a small quantity of template DNA is required. Once a mutation in a family or close relative is detected or founder effect is confirmed, diagnosis of patients or carriers can be simply done by PCR followed by sequencing of amplified fragment or restriction enzyme digestion (PCR-RFLP) wherever the mutation creates a restriction enzyme site.71-73 Due to lack of a common mutation and the small size of FXIII-B gene, whole gene sequencing of this subunit is more practical in individuals suspected to defects in this subunit.

**Prenatal diagnosis**

Umbilical stump bleeding and intracranial hemorrhage are common life-threatening bleeding diatheses among neonates with severe congenital FXIIIID.16 With regard to these severe bleeding episodes, prenatal diagnosis (PND) has a crucial role in high-risk individuals. PND can be easily performed by PCR-sequencing or RFLP (PCR-RFLP) in chorionic villus sampling (CVS) within the first trimester of gestation with identification of FXIIIID causing mutations in the family members of a fetus.74 Although PND can be performed in fetuses with a positive family history of FXIIIID, most patients with FXIIIID are new cases with regard to inheritance pattern of the disease and therefore PND is not easy to perform in most cases.

CVS sample should be washed by phosphate-buffered saline prior to DNA extraction and amplification by PCR. A standard biopsy can yield more than 50 μg DNA. PCR-sequencing or PCR-RFLP can determine the status of fetus within 1 or 2 days.70,71
Conclusion
Because of normal routine coagulation laboratory tests, diagnosis of FXIIID is a challenge. FXIIID is still often missed all over the world with fatal consequences due to life-threatening bleeding. Despite the progress in laboratory diagnosis of FXIIID, clot solubility assay has remained as the most widely used method for detection of FXIIID. Therefore, familiarity with limitations of this quantitative test and appropriate approaches to improve the specificity and sensitivity of tests may contribute to timely diagnosis of FXIIID. In countries with well-equipped coagulation laboratories, FXIII activity can be used for screening of suspected patients to FXIIID. Although FXIII activity assay is an appropriate method for detection of FXIIID, different methods of FXIII activity have limitations that should be considered for precise diagnosis of FXIIID. All circumstances including patient’s preparation, sampling, as well as limitations of every method should be considered in qualitative and quantitative assays such as FXIII activity, antigen, and inhibitor assays in order to avoid the effect of these potentially interfering factors on test results.

Acknowledgments
This research has been supported by Tehran University of Medical Sciences and Health Services grant 26770.

Disclaimer statements
Contributors All authors had equal role in the work.

Funding None.

Conflicts of interest No conflict of interest.

Ethics approval None.

References
1 Komaromi I, Bagoly Z, Muszbek L. Factor XIII: novel structural and functional aspects. J Thromb Haemost. 2011;9(1):9–20.
2 Hoppe B. Fibrinogen and factor XIII at the intersection of coagulation, fibrinolysis and inflammation. Thromb Haemost. 2014;112(6):649–58.
3 Bagoly Z, Koncz Z, Harsfalvi J, Muszbek L. Factor XIII, clot structure, thrombosis. Thromb Res. 2012;129(3):382–7.
4 Lorand L. Factor XIII and the clotting of fibrinogen: from basic research to medicine. J Thromb Haemost. 2005;3(7):1337–48.
5 Kohler HP. Interaction between FXIII and fibrinogen. Blood. 2013;121(11):1931–2.
6 Dorgalaleh A, Tabibian S, Hosseini S, Shamsizadeh M. Guidelines for laboratory diagnosis of factor XIII deficiency. Blood Coagul Fibrinolysis. 2011;22(5):396–401.
7 Jennings I, Kitchen S, Woods T, Preston F. Problems relating to the laboratory diagnosis of factor XIII deficiency: a UK NEQAS study. J Thromb Haemost. 2003;1(12):2603–8.
8 Favaloro EJ, Lippi G, Adcock DM. Preanalytical and postanalytical variables: the leading causes of diagnostic error in hemostasis?. Semin Thromb Hemost. 2008;34(7):612–34.
9 Katona E, Penzes K, Molnar E, Muszbek L. Intracranial hemorrhage pattern in the patients with factor XIII deficiency. Ann Hematol. 2014;93(4):693–7.
10 Naderi M, Zarei T, Haghpanah S, Eshghi P, Miri-Moghaddam E, Karimi M. Intracranial hemorrhage pattern in the patients with factor XIII deficiency. Ann Hematol. 2016;95(6):956–62.
11 Kohler HP, Ichinose A, Seitz R, Ariëns RA, Lai T-S, Weisel JW, Greenberg CS, Grant PJ. Role of factor XIII in fibrin clot formation and effects of genetic polymorphisms. Blood. 2002;100(3):743–54.
12 Dorgalaleh A, Tabibian S, Alizadeh S, Kashani Khatib Z, Tabibian S, Kazemi A, et al. Polymorphism of thrombin-activatable fibrinolysis inhibitor and risk of intracranial haemorrhage in factor XIII deficiency. Haemophilia. 2014;20(1):e89–92.
13 Eshghi P, Mahjour S, Naderi M, Dehborzorgian J, Karimi M. Long-term prophylaxis in patients with factor XIII deficiency complicated by intracranial haemorrhage in Iran. Haemophilia. 2010;16(2):383–5.
14 Levy JL, Greenberg C. Biology of factor XIII and clinical manifestations of factor XIII deficiency. Transfusion. 2013;53(5):1201–31.
15 Ivasekivicius V, Biswas A, Bevans C, Schroeder V, Kohler HP, Rott H, et al. Identification of eight novel coagulation factor XIII subunit A mutations: implied consequences for structure and function. Haematologica. 2010;95(6):956–62.
16 Mahmoudi M, Peyvandi F, Afsarabadi A, Ghaffarpasand F, Karimi M. Bleeding symptoms in heterozygous carriers of inherited coagulation disorders in southern Iran. Blood Coagul Fibrinolysis. 2011;22(5):396–401.
17 Levy JL, Greenberg C. Biology of factor XIII and clinical manifestations of factor XIII deficiency. Transfusion. 2013;53(5):1201–31.
18 Favaloro EJ, Lippi G, Adcock DM. Preanalytical and postanalytical variables: the leading causes of diagnostic error in hemostasis?. Semin Thromb Hemost. 2003;1(12):2603–8.
19 Favaloro EJ, Lippi G, Adcock DM. Preanalytical and postanalytical variables: the leading causes of diagnostic error in hemostasis?. Semin Thromb Hemost. 2003;1(12):2603–8.
20 Mahmoodi M, Peyvandi F, Afsarabadi A, Ghaffarpasand F, Karimi M. Bleeding symptoms in heterozygous carriers of inherited coagulation disorders in southern Iran. Blood Coagul Fibrinolysis. 2011;22(5):396–401.
21 Jennings I, Kitchen S, Woods T, Preston F. Problems relating to the laboratory diagnosis of factor XIII deficiency: a UK NEQAS study. J Thromb Haemost. 2003;1(12):2603–8.
22 Favaloro EJ, Lippi G, Adcock DM. Preanalytical and postanalytical variables: the leading causes of diagnostic error in hemostasis?. Semin Thromb Hemost. 2003;1(12):2603–8.
23 Katona E, Penzes K, Molnar E, Muszbek L. Measurement of factor XIII activity in plasma. Clin Chem Lab Med. 2012;50(7):1191–202.
24 Hayward CP, Moffat KA, Lim W. Prophylactic and perioperative replacement therapy for acquired factor XIII deficiency: reply to a rebuttal. J Thromb Haemost. 2005;3(1):187–8.
25 Favaloro EJ, Lippi G, Adcock DM. Preanalytical and postanalytical variables: the leading causes of diagnostic error in hemostasis?. Semin Thromb Hemost. 2003;1(12):2603–8.
26 Favaloro EJ, Lippi G, Adcock DM. Preanalytical and postanalytical variables: the leading causes of diagnostic error in hemostasis?. Semin Thromb Hemost. 2003;1(12):2603–8.
27 Favaloro EJ, Lippi G, Adcock DM. Preanalytical and postanalytical variables: the leading causes of diagnostic error in hemostasis?. Semin Thromb Hemost. 2003;1(12):2603–8.
28 Favaloro EJ, Lippi G, Adcock DM. Preanalytical and postanalytical variables: the leading causes of diagnostic error in hemostasis?. Semin Thromb Hemost. 2003;1(12):2603–8.
29 Favaloro EJ, Lippi G, Adcock DM. Preanalytical and postanalytical variables: the leading causes of diagnostic error in hemostasis?. Semin Thromb Hemost. 2003;1(12):2603–8.
30 Favaloro EJ, Lippi G, Adcock DM. Preanalytical and postanalytical variables: the leading causes of diagnostic error in hemostasis?. Semin Thromb Hemost. 2003;1(12):2603–8.
31 Favaloro EJ, Lippi G, Adcock DM. Preanalytical and postanalytical variables: the leading causes of diagnostic error in hemostasis?. Semin Thromb Hemost. 2003;1(12):2603–8.
32 Favaloro EJ, Lippi G, Adcock DM. Preanalytical and postanalytical variables: the leading causes of diagnostic error in hemostasis?. Semin Thromb Hemost. 2003;1(12):2603–8.
33 Favaloro EJ, Lippi G, Adcock DM. Preanalytical and postanalytical variables: the leading causes of diagnostic error in hemostasis?. Semin Thromb Hemost. 2003;1(12):2603–8.
34 Favaloro EJ, Lippi G, Adcock DM. Preanalytical and postanalytical variables: the leading causes of diagnostic error in hemostasis?. Semin Thromb Hemost. 2003;1(12):2603–8.
35 Favaloro EJ, Lippi G, Adcock DM. Preanalytical and postanalytical variables: the leading causes of diagnostic error in hemostasis?. Semin Thromb Hemost. 2003;1(12):2603–8.
36 Favaloro EJ, Lippi G, Adcock DM. Preanalytical and postanalytical variables: the leading causes of diagnostic error in hemostasis?. Semin Thromb Hemost. 2003;1(12):2603–8.
Francis J. The detection and measurement of factor XIII activity: a review. Med Lab Sci. 1980;37(2):137–47.

Schroeder V, Durrer D, Meili E, Schubiger G, Kohler HP. Congenital factor XIII deficiency in Switzerland: from the worldwide first case in 1960 to its molecular characterisation in 2005. Swiss medical weekly. 2007;137(19-20):272–8.

Kamitsui H, Tani K, Yasui M, Taniguchi A, Taira K, Tsukada S, et al. Activity of blood coagulation factor XIII as a prognostic indicator. Haematologica. 2009;94(5):719–23.

Mackie I, Cooper P, Lawrie A, Kitchen S, Gray E, Laflan M. Guidelines on the laboratory aspects of assays used in haemostasis and thrombosis. Int J Lab Hematol. 2013;35(1):1–13.

Shroff J, Coon N, Naderi M, Korimi M. Factor XIII deficiency: a review of literature. JBC. 2012;4(2):85–91.

Lim W, Moffat K, M Hayward C. Prophylactic and perioperative replacement therapy for acquired factor XIII deficiency. J Thromb Haemost. 2004;2(6):1017–9.

Ichinoe A, Okazi T, Souri M. Clinical features of 32 new Japanese cases with autoimmune haemorrhha-philia due to anti-factor XIII antibodies. Haemophilia. 2015 Sep;21(5):653–8.

Karpati L, Penke B, Katona E, Balogh I, Vamosi G, Muszbek L. A modified, optimized kinetic photometric assay for the determination of blood coagulation factor XIII activity in plasma. Clin Chem. 2000;46(12):1946–55.

Aujizer N, Muszbek L. Prophylactic and perioperative replacement therapy for acquired factor XIII deficiency: a rebuttal. J Thromb Haemost. 2006;4(11):2075.

Nishida Y, Ikematsu S, Fukutake K, Fujimaki M, Fukutake K, Ajzner E, Muszbek L. Prophylactic and perioperative replacement therapy for acquired factor XIII deficiency. J Thromb Haemost. 2004;2(6):1017–9.

Michell C, Platt A, Kelly F, Soucie J. Validation of batch tests for determination of blood coagulation factor XIII activity: a systematic review. PLoS ONE. 2014;9(10):e108683–16.

Green AR. Postgraduate haematology. London: John Wiley & Sons; 2010.

Ivaskevicius V, Windyga J, Baran B, Schroeder V, Junen J, Bykowska K, et al. Phenotype-genotype correlation in eight Polish patients with inherited Factor XIII deficiency: identification of three novel mutations. Haemophilia. 2007;13(5):649–57.

Kulkarni BP, Nair SB, Vijapurkar M, Mota L, Shanbhag S, Ali S, et al. Molecular pathology of rare bleeding disorders (RBDs) in India: a systematic review. PLoS ONE. 2014;9(10):e108683–16.

Gomez Garcia E, Poort S, Stibbe J, Sturk A, Schaap M, Kappers M, et al. Two novel and one recurrent missense mutation in the factor XIII A gene in two Dutch patients with factor XIII deficiency. Br J Haematol. 2001;112(2):513–8.

Naderi M, Doragalaei A, Alizadeh S, Tabibian S, Hosseini S, Shamsizadeh M, et al. Clinical manifestations and management of life-threatening bleeding in the largest group of patients with severe factor XIII deficiency. Int J Hematol. 2014;100(5):443–9.

Naderi M, Alizadeh S, Kazemi A, Tabibian S, Zaker F, Bamedi M, et al. Central nervous system bleeding in pediatric patients with factor XIII deficiency: a study on 23 new cases. Hematol 2015;20(2):112–5.

Eshghi P, Cohon N, Lak M, Naderi M, Peyvandi F, Menegatti M, et al. Arg7?His and Trp187Arg are the most common mutations causing FXIII deficiency in Iran. Clin Appl Thromb Hemost. 2012;18(1):100–3.

Goodeve A. Laboratory methods for the genetic diagnosis of bleeding disorders. Clin Lab Haematol. 1998;20(1):3–19.

Dalal A, Pradhan M, Agarwal S. Genetics of bleeding disorders. Int J Hum Genet. 2006;6(1):27–32.

Peyvandi F, Kunicki T, Lillicrap D. Genetic sequence analysis of inherited bleeding diseases. Blood. 2013;122(20):3423–31.

Killick CJ, Barton CJ, Aslam S, Staden G. Prenatal diagnosis in factor XIII-A deficiency. Arch Dis Child Fetal Neonatal Ed. 1999;80(3):F238–9.