# Laboratory Diagnosis of Factor XIII Deficiency in Developing Countries: An Iranian Experience

Akbar Dorgalaleh, PhD,[1]* Shadi Tabibian, PhD,[1] Mahmood Shams, PhD,[1,2]
Behnaz Tavasoli, PhD,[1] Maryam Gheidishahran, MSc,[1] Morteza Shamsizadeh, MSc[3]

## ABSTRACT

Factor XIII (FXIII) deficiency is an extremely rare bleeding disorder with an approximately 12-times higher than the rest of the world. The International Society for Thrombosis and Hemostasis (ISTH) suggested a standard algorithm for precise diagnosis and classification of FXIII deficiency (FXIIID). However, due to lack of investment in proper equipment and procedures in Iran, almost no part of this algorithm can be used to diagnose Iranian patients. Thus, this study proposes

a guideline for accurate molecular and laboratory diagnosis of FXIIID based on the available tools. Because this study suggests a simple and reliable algorithm for early diagnosis, it can therefore, reduce the rates of morbidity and mortality of FXIIID patients with this condition.

**Keywords:** factor XIII deficiency, laboratory diagnosis, Iran, ISTH, developing countries, rare bleeding disorders

Factor XIII (FXIII) deficiency is a rare bleeding disorder (RBD) with an estimated global incidence of 1 per 2 million. With a high rate of consanguinity, Iran has the highest global incidence of FXIII deficiency (FXIIID) with 483 patients in 2016.[1] This RBD is associated with a high rate of life threatening bleeding episodes, including recurrent pregnancy loss (RPL), umbilical cord bleeding, and intracranial hemorrhage. Central nervous system (CNS) bleeding is a common bleeding diathesis, observed in 32% of Iranian patients with severe congenital

FXIIID, which leads to a high rate of morbidity and mortality.[2,3] Life-threatening bleeding episodes can be reduced significantly with timely diagnosis of FXIIID and appropriate prophylaxis utilizing blood-derived components such as fresh frozen plasma (FFP) and FXIII concentrate, or recombinant FXIII (rFXIII).[3] Clinical presentations offer suitable clues for diagnosis of this disorder.[4]

# Diagnosis of Factor XIII Deficiency

Because FXIII (as a coagulation factor) is not involved in the formation of an early unstable clot, all routine coagulation tests, including bleeding time (BT), prothrombin time (PT), and activated partial thromboplastin time (APTT), show normal results in FXIIID, which complicates the diagnosis. Clot formation, which forms the basis of the aforementioned tests, is normal in FXIIID.[5,6] At the end of the coagulation cascade, when an early clot is formed, FXIII covalently cross-links the unstable fibrin and forms a firm, stable clot.[7] Therefore, stability assessment of the formed clot can be used as a screening test for FXIIID.[8] The clot-solubility test has been used since 1960 to detect FXIIID.[9] In brief, an anticoagulated blood specimen is centrifuged, and the resulting plasma is clotted by calcium chloride ($CaCl_2$). Then, the formed clot is suspended in 5 M urea or

## Abbreviations

FXIII, Factor XIII; FXIIID, Factor XIII deficiency; RBD, rare bleeding disorder; RPL, recurrent pregnancy loss; CNS, central nervous system; FFP, fresh frozen plasma; rFXIII, recombinant FXIII; BT, bleeding time; PT, prothrombin time; APTT, activated partial thromboplastin time; $CaCl_2$, calcium chloride; MCA, monochloroacetic; ISTH, International Society for Thrombosis and Hemostasis; IgG, immunoglobulin G; ELISA, enzyme-linked immunosorbent assay; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PND, prenatal diagnosis; PPP, platelet-poor plasma; FXIIIa, activated FXIII; NADPH, nicotinamide adenine dinucleotide phosphate hydrogen; NADP, nicotinamide adenine dinucleotide phosphate

[1]Department of Hematology and Blood Transfusion, School of Allied Medical Science, Iran University of Medical Sciences, Tehran, [2]Department of Laboratory Sciences, Paramedical Faculty, Babol University of Medical Sciences, Babol, and [3]School of Nursing and Midwifery, Shahroud University of Medical Sciences, Shahroud, Iran

*To whom correspondence should be addressed.
dorgalaleha@gmail.com

monochloroacetic (MCA) acid solutions and incubated at 37°C or at room temperature. Then clot is evaluated at regular intervals for dissolution.[5,9,10]

The clot solubility test is not sensitive and is no longer recommended for screening of FXIIID; quantitative assays should be used to determine the exact plasma level of the FXIII.[6] However, the clot solubility assay is still used in a considerable number of laboratories because of its simplicity and due to the lack of availability of the quantitative screening assay in most routine laboratories.[5,6,9] The International Society for Thrombosis and Hemostasis (ISTH) proposed the following algorithm for proper diagnosis and classification of FXIIID (**Figure 1**).[11]

After measurement of FXIII activity and antigen assay to determine the presence of FXIII inhibitor, a mixing study usually is helpful. In cases with abnormal clot solubility test results, it may be necessary to determine factor deficiency or presence of an inhibitor in a mixing study; the clot solubility assay is performed on a mixture of patient plasma and a similar volume of normal plasma (1:1 proportion). If the result of the assay is corrected, it may be suggestive of a congenital deficiency; if not, an inhibitor to FXIII may be present. Clot-based inhibitor assays are only able to detect neutralizing antibodies. If non-neutralizing antibodies are present, a binding assay is recommended. In this method, immunoglobulin G (IgG) antibodies in the patient plasma are bound to purified FXIII-A2B2, FXIII-A2, and FXIII-B coated to a microtiter plate via enzyme-linked immunosorbent assay (ELISA) or bound to sodium dodecyl sulfate (SDS) via the polyacrylamide gel electrophoresis (PAGE) method. In the PAGE method, the plasma specimen is mixed with SDS that is used as a negative charge for the bound polypeptide proportionate to its mass. The specimen then undergoes electrophoresis on a polyacrylamide gel; proteins will be separated based on their size.[11,12]

This algorithm presents a reliable diagnostic approach. However, in almost all clinical laboratories in Iran and a considerable number of laboratories in the developed world (approximately 20%), the primary available screening test is the clot solubility test, which is not of value in this algorithm.[5,6] Until the required equipment and facilities are available throughout Iran, using a regional diagnostic algorithm seems to be necessary.[6]

## Diagnosis of Factor XIII Deficiency in Iran

Proper diagnosis and classification of FXIIID using a set of laboratory assessments according to international standards is largely unavailable in Iran due to high costs, coupled with a low number of patients (N = 483).[6] Thus, most of these standard assessments are not available. The diagnosis of FXIIID is based on clinical presentations; family history; and laboratory assessment via clot solubility test, FXIII activity assay and, most recently, inhibitor assay.[13-16] Standardization and troubleshooting of available tests are crucial.[6,8,11] In Iran, a patient suspected of having FXIIID is assessed by routine coagulation tests, including PT, PTT, BT, and platelet count. When a normal result is obtained in these tests, the clot solubility test is performed next.[6] Because this test has low sensitivity and specificity and is affected by a number of factors, such as fibrinogen level, clotting agents, and solubilizing factors, it cannot be used as a confirmatory test.[9] Molecular diagnosis of FXIIID is not widely used in Iranian patients; however, a number of hemophilia and research centers use molecular tests to confirm diagnosis of severe congenital FXIIID, for carrier detection and prenatal diagnosis (PND).[1,4,13]

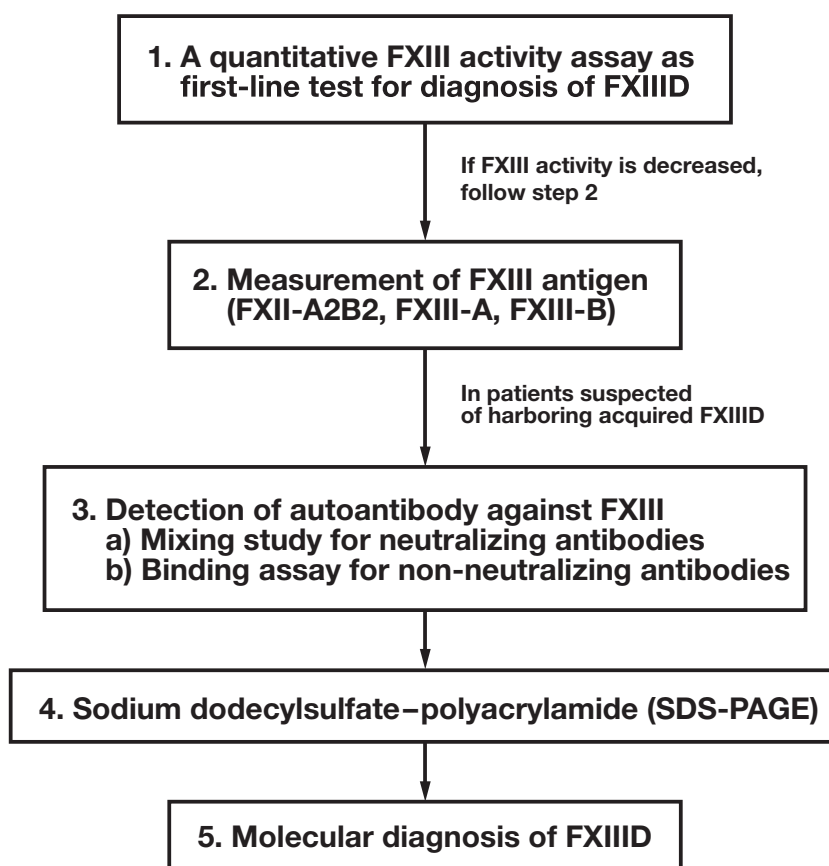## Laboratory Investigation of Patients With Factor XIII Deficiency

### Clot Solubility Test

The only screening assay available for FXIIID in Iran is the clot solubility test; this test is affected by several interfering factors and has low sensitivity and specificity.[6,15] To date, a standard procedure has not been developed; however, several methods and changes have improved it. At baseline, blood is collected with a ratio of 1:9 in 3.2% sodium citrate anticoagulant. The whole blood specimen then is centrifuged to obtain platelet-poor plasma (PPP). After PPP preparation, a clotting agent is added to PPP, and the integrity of the

### Table 1. Detected Mutations in Iranian Patients With Factor XIII Deficiency and Suggested Primers

| Mutation | Exon | Patients, No. (%) | Detection Technique | Primer | Annealing | Digestion Enzyme | Reference |
|---|---|---|---|---|---|---|---|
| Trp187Arg | 4 | 346 (97.1) | PCR-RFLP | F: 5′-TTGCAGACTTGCCTGATTTG-3′<br>R: 5′-CAAGCGATCCTCCCATCTTG-3′ | 58° | Eco130I | [22] |
| Arg77His | 3 | 5 (1.4) | PCR-RFLP | F: 5′-TGCTACCTGCCTTCTTCAGG-3′<br>R: 5′-CCTGCCACTGTTGACATATG-3′ | 56° | Aci I | [22] |
| Arg260Cys & Arg260His | 6 | 2 (0.6) | PCR sequencing | F: 5′-GCTTGCAGAGTGAACACTAGTTT-3′<br>R: 5′-TGACAGGTGTTAACAGATTTTAGG-3′ | 54° | NA | [1] |
| c.689delA | 5 | 1 (0.3) | PCR sequencing | F: 5′-AAGTGTTTGGAAACAGTCTGG-3′<br>R: 5′-ATGAAGTAAAAATGTCCTTGAC-3′ | 54° | NA | [1] |
| Arg382Ser | 9 | 1 (0.3) | PCR-RFLP | F: 5′-CACTTCTTGATCTCTTGGAGCA-3′<br>R: 5′-AGATCAGCAATGAAGCAAGTTC-3′ | 56° | Hae III | [1] |
| c.1981delC | 14 | 1 (0.3) | PCR sequencing | F: 5′-AGAGCAGAACGAGGTTTT-3′<br>R: 5′-GCTTCCCACAGCTCTGCAC-3′ | 52° | NA | [1] |

*PCR-RFLP, polymerase chain reaction–restriction fragment length polymorphism; PCR, polymerase chain reaction.*

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**1. A quantitative FXIII activity assay as first-line test for diagnosis of FXIIID**

*If FXIII activity is decreased, follow step 2*

**2. Measurement of FXIII antigen (FXII-A2B2, FXIII-A, FXIII-B)**

*In patients suspected of harboring acquired FXIIID*

**3. Detection of autoantibody against FXIII**
   **a) Mixing study for neutralizing antibodies**
   **b) Binding assay for non-neutralizing antibodies**

**4. Sodium dodecylsulfate–polyacrylamide (SDS-PAGE)**

**5. Molecular diagnosis of FXIIID**

**Figure 1**

Suggested algorithm by International Society for Thrombosis and Hemostasis (ISTH) for proper diagnosis and classification of factor XIII deficiency. *FXIII indicates Factor XIII; FXIIID, FXIII disorder*.
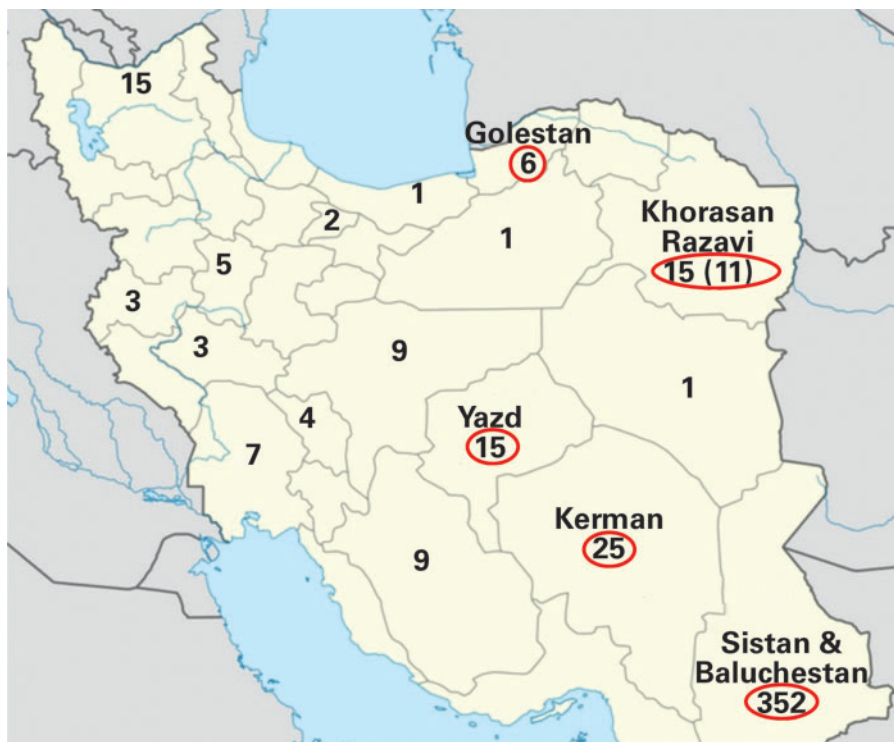
**Image 1**

Iranian provinces in which the greatest number of cases of Factor XIII deficiency are found.

formed clot is assessed at regular intervals.[9,12] The most common solvents include urea or MCA solution and acetic acid. Different clotting agents, including thrombin, calcium, and calcium in combination with thrombin, have been introduced. Several studies[9,12] have determined the specificity and sensitivity of various combinations of clotting and solubilizing factors. In Iran, the most common method of clot solubility test is a combination of $CaCl_2$ as a clotting agent and MCA as a solvent reagent. This method is less sensitive than others and shows abnormal results only in severe FXIIID cases.[12]

It has been suggested that the calcium/urea method is sensitive to 1 to 5 U/mL of FXIII, whereas thrombin/acetic acid is sensitive to at least 10 U/mL. Calcium/acetic acid and thrombin/urea methods are reported to provide intermediate-level sensitivity. Although the acetic acid–based assay is more sensitive and rapid than the urea solubility test, it is less specific.[9,12]

## Factor XIII Activity Assay

FXIII activity assay is a specific test for precise detection and confirmation of diagnosis. Until recently, this assay was rarely used for diagnosis of FXIIID in Iran.[6] This test currently is restricted to referral coagulation laboratories. Several methods have been introduced for FXIII functional assays, including photometric, putrescine incorporation, and fluorometric.[5,6,12]

In the commonly used photometric assay, activated FXIII (FXIIIa) crosslinks the amine substrate in the glutamine residue of the $\alpha_2$-plasmin inhibitor. During this reaction, ammonia is released and used for glutamine formation, and nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) is transformed to nicotinamide adenine dinucleotide phosphate hydrogen (NADP). The decrease in NADPH level causes absorbance reduction at 340 nm, which indicates FXIIIa activity.[10,11,16] Among activity assays, the putrescine incorporation assay is a highly sensitive but time-consuming method.[10,11]
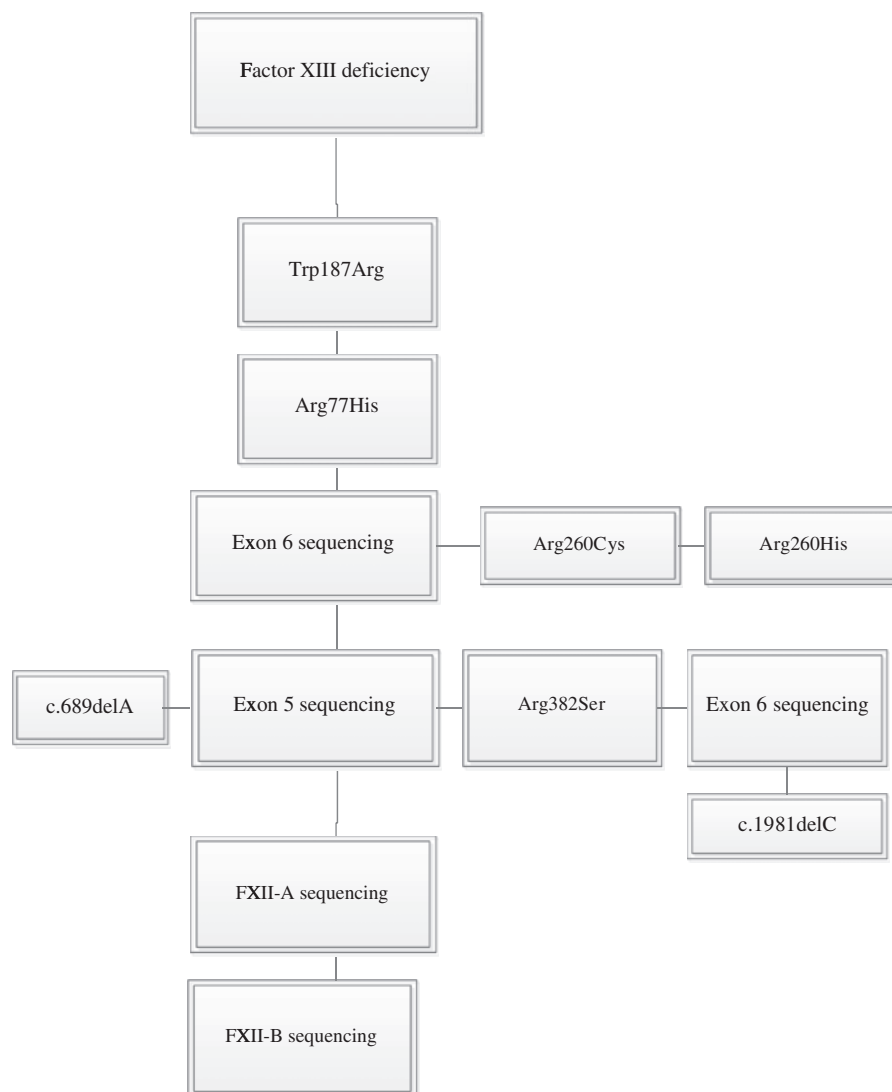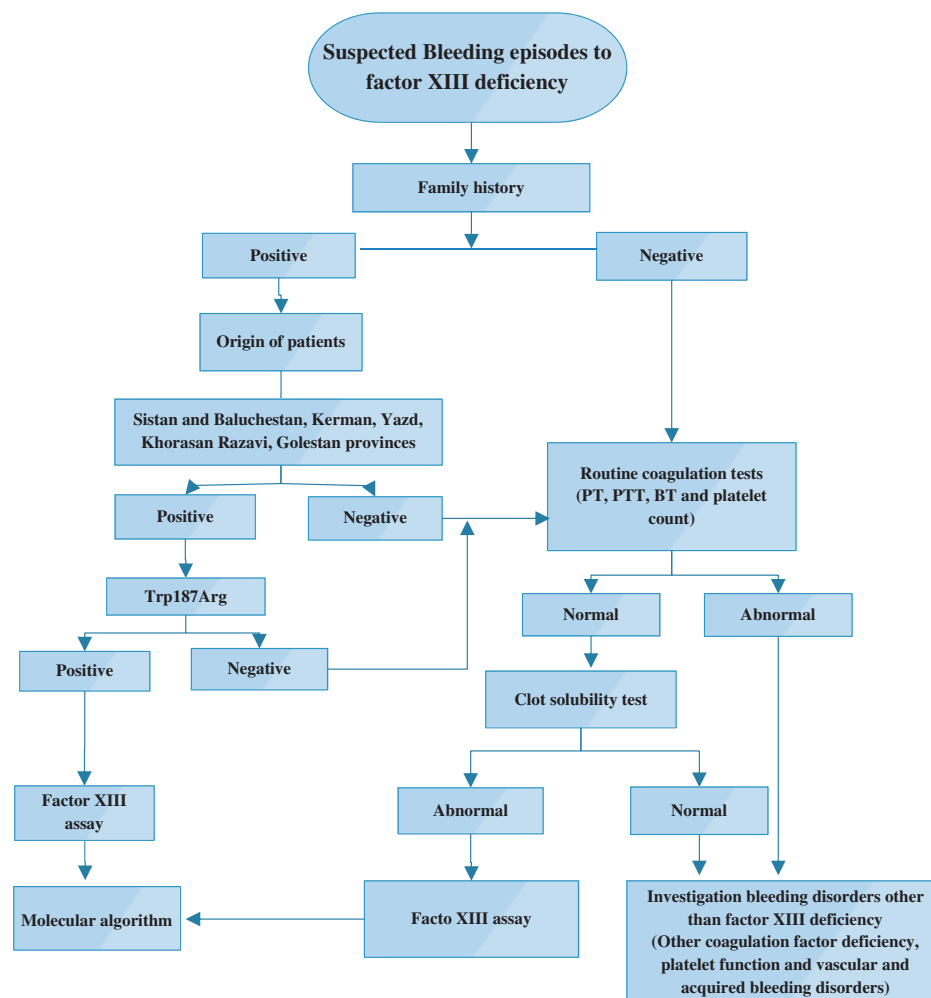
**Figure 2**

Suggested algorithm for molecular diagnosis of factor XIII deficiency in Iran.

## Molecular Diagnosis of Factor XIII Deficiency in Iran

Molecular diagnosis of FXIIID is rarely performed in Iran. Most diagnoses of the condition have been performed in southeast Iran, which has the highest incidence of FXIIID worldwide.[1,6] Several studies[1,4,17] indicate that Trp187Arg is the only FXIIID-causing mutation in this area, due to the founder effect (the founder effect is the loss of genetic variation in a new population that is established by small number of people from a larger population). Another study on Iranian patients found His77Arg to be another common disease-causing mutation. Several other mutations were found but not observed recurrently. Regarding these studies, in a patient suspected of having FXIIID, Trp187Arg should be the first selected mutation for diagnosis or confirmation of the condition. The Arg77His mutation on exon 3 should be selected as the second disease-causing mutation.[1,18] Sequencing of exon number 6 of the *FXIII-A* gene should follow because the second most common mutations Arg260Cys and Arg260His have been observed among Iranian patients[1,18] (**Table 1**).

**Figure 3**
Suggested algorithm of diagnosis of factor XIII deficiency in Iran.

Because *FXIII-A* has 15 exons, sequencing of other exons that have established mutations in Iranian patients, including exons number 5, 9, and 14, should be considered before full sequencing of *FXIII-A*. If full sequencing of *FXIII-A* detects no mutation, *FXIII-B* sequencing should be performed.

## An Algorithm for Diagnosis of Factor XIII Deficiency in Iran

Regarding available data about Iranian patients with FXIIID, a specific diagnostic algorithm can be proposed for reliable, early, one-time diagnosis. The majority of Iranian patients with FXIIID displays a wide spectrum of clinical presentations.[1,4,18-]

[20] Their clinical manifestations can provide clues for timely diagnosis of FXIIID. Umbilical cord bleeding is the most common presentation strongly suggestive of FXIIID in Iran, as it is elsewhere.[1,21] Moreover, recurrent miscarriage, intracranial hemorrhage, and soft tissue hematoma have been reported, along with delayed and prolonged wound bleeding.[1] After precise physical examination and assessment of clinical presentations, precise family history is the second-most-helpful diagnostic criterion.[15] A study of 190 Iranian patients indicated that 78% of patients had parents who were close relatives genetically and 12% had parents who were distant relatives genetically, whereas only 10% had parents who were not genetically related.[15] Therefore, a precise, detailed family history is important.

In a patient with a positive family history for FXIIID, the third appropriate step toward proper diagnosis is to address the question of ethnic origin. Of the 473 reported patients with FXIIID in Iran, 352 were residents of the Sistan and Baluchestan province in southeastern Iran and 62 patients were residents of the Kerman, Khorasan Razavi, Yazd, and Golestan provinces, which have a relatively high rate of relocation from Sistan and Baluchistan[1] (**Image 1**). Two studies[1,22] determined that the FXIIID-causing mutation in Sistan and Baluchestan Province is Trp187Arg, which emphasizes a founder effect. In a patient from Sistan and Baluchestan Province with a positive family history for FXIIID, a simple but reliable polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) test can lead to early confirmation of the deficiency.

Because approximately all Iranian provinces with a high number of patients with FXIIID live in the vicinity of Sistan and Baluchestan Province or have a high rate of migration from this province, Trp187Arg should be selected as the first molecular test for FXIIID diagnosis in every Iranian patient. In a patient with a negative family history for FXIIID and without origins from Sistan, Baluchestan, Kerman, Khorasan Razavi, Yazd, or Golestan provinces, all routine coagulation tests should be performed at baseline. Normal results of these tests would rule out most other bleeding disorders (**Figure 2**).

A normal result in routine coagulation tests can be followed by the clot solubility test. In this step, one can obtain a normal or abnormal result in the solubility assay. Due to the low specificity of clot solubility, an abnormal result of this test should be followed by more reliable tests, such as the FXIII activity assay. Finally, with a proper molecular approach, the molecular basis of this disease can be distinguished (**Figure 3**). **LM**

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