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Auto-, and alloantibodies against factor XIII: laboratory diagnosis and clinical consequences

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Summary. Acquired FXIII deficiencies caused by autoantibodies against FXIII subunits represent rare but very severe bleeding diatheses. Alloantibodies in FXIII deficient patients also cause life-threatening bleeding complication, but they develop extremely rarely. In this review we provide an

overview on the diagnosis and classification of anti-FXIII antibodies and analyze 48 patients with autoimmune FXIII deficiency and 4 additional FXIII deficient patients who developed anti-FXIII alloantibody. The patients were collected from peer-reviewed publications from which relevant data could be extracted. With the exception of two cases the antibodies were directed against FXIII-A. The difficulties in the diagnosis of FXIII deficiency in the presence of anti-FXIII antibodies are discussed and a scheme for the functional classification of the anti-FXIII antibodies is recommended. The three main categories are neutralizing and non-neutralizing antibodies and antibodies with combined effect. The methods being used for detecting and quantifying the inhibitory effect on FXIII activation and on the transglutaminase activity of activated FXIII are summarized and techniques for the classification of neutralizing anti-FXIII antibodies are outlined. The importance of clearance studies in these cases is emphasized. Binding assays, useful for the identification of non-neutralizing and combined type antibodies, were collected from literature and their informative power is demonstrated by examples. The most frequently occurring bleeding symptoms in patients with anti-FXIII antibodies were soft tissue bleeding, intracranial bleedings also occurred, but less frequently than in inherited FXIII deficiency. Treatment of such patients is extremely challenging, the main aim should be eradication of the antibody.

Introduction

The plasmatic form of blood coagulation factor XIII (FXIII) is of tetrameric structure (FXIII-A_2B_2); it consists of two catalytic A (FXIII-A) and two carrier/inhibitory B FXIII-B) subunits. Its cellular form, a dimer of FXIII-A is present in platelets, monocytes, macrophages, osteoblasts and chondrocytes. FXIII-A is a pro-transglutaminase, in the plasma it becomes activated by the concerted action of thrombin and Ca^{2+}. Thrombin cleaves off the N-terminal activation peptide (AP-FXIII) from FXIII-A, then in the presence of Ca^{2+} FXIII-B subunits dissociate and the free FXIII-A becomes transformed into an active transglutaminase (FXIIIa; FXIII-A_2*) (Fig. 1). Transglutaminases catalyze an acyl transfer reaction in which a peptide bound glutamine residue is the acyl donor and a primary amine is the acyl acceptor. During the reaction ammonia is released and a substrate primary amine becomes

covalently bound to the glutamine residue. If the acyl acceptor primary amine is the ε-amino group of a peptide-bound lysine residue the end result is the covalent cross-linking of two peptide chains. Cross-linking of peptide chains is the main function of FXIIIa. It promptly crosslinks fibrin γ-chains into dimers and, more slowly, fibrin α-chains into high molecular weight polymers. It also cross-links the main fibrinolysis inhibitor, α_2-plasmin inhibitor to fibrin α-chain. This way FXIII protects newly formed fibrin from the shear stress of circulating blood and from degradation by the powerful fibrinolytic system. In the plasma FXIII-B is in two-fold excess over FXIII-A. FXIII-B is responsible for the long half-life of FXIII in the circulation (8-14 days) and it prevents the spontaneous non-proteolytic activation of FXIII-A in plasmatic conditions (for details see references [1-3]).

FXIII is essential for maintaining hemostasis. Inherited FXIII-A deficiency is one of the most severe inherited bleeding disorders. The overwhelming majority of FXIII-A deficiencies are quantitative (type I) disorders, the qualitative deficiency (type II), where A and B antigen levels are normal, is extremely rare. The general prevalence of FXIII deficiency is 1 in 2 million. However, in populations with high rate of consanguinity and with founder mutation the frequency is much higher. It is the most common inherited bleeding disorder in Iran [4, 5] and in India [6]. Delayed umbilical stump bleeding is a characteristic symptom of FXIII-A deficient newborns. Later in life subcutaneous and intramuscular hematomas and intracranial hemorrhage are the most frequent, many times life-threatening bleeding complications. The high frequency (17-30%) of intracranial bleeding in FXIII-A deficient patients who have not received FXIII concentrate or fresh frozen plasma as prophylactic treatment is particularly noteworthy. In FXIII deficient women menorrhagia is a rather frequent complaint and in women without FXIII prophylaxis 91% of pregnancies resulted in miscarriages [7]. A non-hemostatic consequence of FXIII-A deficiency is impaired wound healing although, very likely due to the complexity of the wound healing process, this is manifested only in some of the patients. The poor healing of excisional wounds in FXIII-A knock out mice strongly supports the involvement of FXIII in the wound healing process [8]. Only a few cases of inherited FXIII-B deficiency have been reported [9-13]. In these patients FXIII activity and FXIII-A antigen level were also decreased and the bleeding phenotype corresponded to mild/moderate FXIII-A deficiency.

Mild to moderate acquired FXIII deficiency due to impaired synthesis of FXIII subunits or their consumption may accompany a number of diseases. However, severe acquired FXIII deficiencies are due to autoantibodies against FXIII subunits. Anti-FXIII antibodies (alloantibodies) may also develop and complicate the clinical course of FXIII deficient patients. The aim of the present review is to give an up-to-date overview on anti-FXIII antibodies including their diagnosis, classification, the clinical consequences and the experience with treatment modalities.

Diagnosis and classification of anti-FXIII antibodies

In the following subchapters a number of specialized and less specialized tests useful in the diagnosis and classification of anti-FXIII antibodies are described. It is to be noted that only FXIII activity and antigen tests are commercially available and are offered by certain clinical laboratories. Other tests described in this review are currently available only in specialized research laboratories.

The first step in the diagnosis of anti-FXIII antibodies

Obviously, the first step in the diagnosis is establishing FXIII deficiency by appropriate screening test(s). To cover almost all subtypes of inherited and acquired FXIII deficiencies the screening test should be an activity assay that is able to measure plasma FXIII activity accurately down to 0.01 IU/mL (1%). Nowadays most laboratories use a commercial FXIII activity assay based on the measurement of ammonia released by FXIIIa from appropriate peptide-bound glutamine and amine substrate. To obtain accurate results in the low activity range correction of the results for plasma blank is essential. The incorporation of amines labeled with fluorescent dye, biotin or radioactivity into a protein substrate could also be used as screening tests [14]. However, these more time-consuming assays are rarely used in routine environments. Monitoring the cross-linking of fibrin chains and the cross-linking of α_2-plasmin inhibitor to fibrin by SDS PAGE or Western blotting could provide further information on the functional activity of FXIII [15-18]. These tests are particularly

important in detecting type III inhibitors (see later). Measurement of FXIII activity in platelet lysate is a useful additional test. In inherited FXIII-A deficiency platelets are also FXIII deficient, while in FXIII-B deficiencies and in deficiencies caused by autoantibodies against FXIII platelet FXIII activity is normal. A more detailed elaboration of FXIII activity measurements is beyond the scope of this review; interested readers should consult the International Society on Thrombosis and Haemostasis, Scientific and Standardization Committee guideline [19] and also recent reviews on the subject [14, 20, 21].

Subtypes of anti-FXIII antibodies and their effect

In the following subchapters allo-, and autoantibodies will be discussed together, because from the point of view of their diagnosis and classification they do not differ. Subtypes of anti-FXIII antibodies are summarized in Table 1. Antibodies may form against either of the FXIII subunits. They may inhibit FXIII activation/activity (neutralizing antibody) or may simply form immune complexes with FXIII-A or FXIII-B and accelerate the elimination of FXIII from the circulation (non-neutralizing antibodies). Anti-FXIII-A antibodies with a combined neutralizing and non-neutralizing effect also exist. Obviously, anti-FXIII-B antibodies are of non-neutralizing subtype. Fig. 1 demonstrates the possible targets of neutralizing anti-FXIII-A antibodies. They may interfere with the thrombin cleavage of FXIII-A (type Ia), or with the Ca^{2+} induced activation of truncated FXIII-A (type Ib). They may inhibit the activity of FXIIIa (type II) or the interaction of FXIII/FXIIIa with fibrin(ogen) (type III). They may also exert multiple inhibitory effects (type IV).

Detection and measurement of neutralizing anti-FXIII-A antibodies

The first step in the diagnosis of anti-FXIII antibodies is to test their inhibitory effect by a mixing study. By the Bethesda-Nijmegen inhibitor test [22] an approximate quantification of the inhibitory antibody could also be achieved. This is a two tube assay in which case FXIII activity of the mixture

of normal and FXIII-A deficient plasmas (tube 1) is compared to the activity measured with the mixture of normal and patient's plasmas (tube 2). Neutralizing antibody is suspected if FXIII activity in the mixture of normal and patient's plasma is at least 10% less than the FXIII activity of normal and FXIII-A deficient plasma. In that case the determination of antibody titer is warranted using various dilutions of the patient's plasma for mixing. Residual FXIII activity (RA) in the mixture of normal plasma and patient's plasma dilution is calculated by dividing the activity measured in tube 1 (T1) with the activity in tube 2 (T2):

$$RA\ (\%) = (T1\ \text{activity} / T2\ \text{activity}) \times 100$$

The results are expressed as Bethesda units (BU); one BU corresponds to 50% RA. BU should be read from a graph (Fig. 2A) using the RA value of a plasma dilution that falls into the range of 25-75%. The result is multiplied with the dilution factor. Residual activity above 75% measured with undiluted plasma does not support the presence of an inhibitor. Step-by-step execution of the Bethesda-Nijmegen inhibitor assay is described in reference [21]. It is to be noted that in early studies the inhibitor titer was defined as the highest plasma dilution still showing detectable inhibition in mixing study, but this definition is no longer used.

Most recently we adapted a pharmacological inhibitor assay for the measurement of the inhibitory capacity of neutralizing anti-FXIII-A antibody by determining the antibody concentration required for 50% inhibition of FXIII (IC50). In this assay the combined inhibition of FXIII activation and FXIIIa activity is measured. Various concentrations of the patient's IgG or normal IgG is pre-incubated with plasma FXIII, then FXIII is activated by thrombin and Ca^{2+} and FXIII activity is measured. Normal IgG had no effect on FXIII activity, and the IC50 value for the patient's IgG could be calculated (an IC50 tool kit is useful for the calculation; www.ic50.tk). A typical IC50 curve from reference [23] is shown on Fig. 2B. This assay was used in two further studies for the quantification of neutralizing anti-FXIII-A antibodies [24, 25]. The advantage of IC50 determination over the Bethesda-Nijmegen assay is the exact information on the inhibitory capacity of the antibody. Its disadvantage is the requirement of purified FXIII and IgG.

In rare cases in the anti-FXIII antibody did not inhibit FXIII activity measured by an amine incorporation assay, but it caused impaired cross-linking of fibrin chains. These type III antibodies are detected by monitoring the crosslinking of fibrin γ-, and α-chains using SDS PAGE analysis of the fibrin clot [17, 18, 26]. The inhibition of fibrin chains cross-linking might also be due to an antibody directed against fibrin(ogen) and not against FXIII [18].

Characterization of neutralizing anti-FXIII-A antibodies

Once the presence of a neutralizing anti-FXIII-A antibody was revealed, the next step is to explore the mechanism by which it exerts the inhibitory effect. A type Ia antibody, which interfered with the liberation of AP-FXIII from FXIII-A by thrombin, was described in an early study from Lorand et al. [27]. In this case the antibody inhibited FXIII activity only if it was pre-incubated with the zymogen. The inhibition of AP-FXIII release by thrombin could also be part of a type IV multiple inhibitory effects. Such effect could be monitored by Western Blotting or by the determination of AP-FXIII using immunoassays or HPLC technique. To our knowledge, the latter techniques have not been used for such a purpose. We investigated the effect of anti-FXIII-A antibodies on the truncation of FXIII-A by thrombin in three independent studies by Western Blotting. Fig. 3A demonstrates the marked inhibitory effect of an alloantibody [24]. In another study an anti-FXIII-A autoantibody only slightly affected the truncation of FXIII-A [23], while in a third case the autoantibody failed to influence the release of AP-FXIII by thrombin [25].

To separate the inhibitory effect on Ca^{2+} induced activation of thrombin cleaved FXIII (FXIII-A_2'B_2) and on FXIIIa two assays are needed. In the first set-up, plasma FXIII truncated by thrombin is incubated with the patient's antibody at saturating concentration, then FXIII-A_2'B_2 is activated by Ca^{2+} and the transglutaminase activity of the resulted FXIIIa is measured. The result reflects the combined inhibitory effect on Ca^{2+} induced activation and on FXIIIa activity. In a second set-up, FXIII activated by thrombin and Ca^{2+} is incubated with the antibody and the inhibition of FXIIIa is tested. Inhibition only in the first assay suggests the presence of type Ib antibody inhibiting

Ca^{2+} induced activation. Equal inhibition in both set-ups indicates exclusively anti-FXIIIa effect, i.e. the presence of type II antibody. More considerable inhibition in the first assay than in the second one suggests a double inhibitory effect, i.e. the inhibition of both Ca^{2+} induced FXIII-A_2'B_2 activation and FXIIIa (multiple type IV inhibition). Fig. 3B demonstrates the effect of anti-FXIII-A antibodies in three separate cases. In case 1 [24] both Ca^{2+} induced FXIII-A_2'B_2 activation and FXIIIa were inhibited to a considerable extent by an alloantibody (multiple inhibition). In case 2 [23] the full inhibitory effect of an autoantibody was due to the inhibition of FXIIIa (type II antibody), while in case 3 [25] a moderate inhibition of Ca^{2+} induced FXIII-A_2'B_2 activation also contributed to the full inhibitory effect.

Clearance of FXIII concentrate from the plasma

Non-neutralizing anti-FXIII antibodies and combined type anti-FXIII-A antibodies accelerate the clearance of FXIII from the plasma. The time course of FXIII elimination is followed by FXIII activity or antigen assay following the administration of FXIII concentrate and the half-life of FXIII in the plasma is determined. Fig. 4A shows such a clearance study demonstrating the considerably shortened half-life of FXIII due to an anti-FXIII-B autoantibody [28]. As opposed to the normal half-life of FXIII in the circulation (9-12 days [29-31]), the half-life of administered plasma FXIII concentrate was shortened to 17 hours. Such pharmacokinetic monitoring of administered FXIII has also been reported in two Japanese studies [32, 33]. It is to be noted that in case of anti-FXIII-A antibody with combined effect, due to the prompt inhibition of FXIII, activity measurement cannot be used for half-life determination. On the other hand, the allo-, or autoantibody present in the patient's plasma might interfere with the binding of antibody used for antigen determination resulting in severe underestimation of FXIII antigen concentration. In this case parallel detection of FXIII antigen by Western blotting could clarify the situation (see reference [23], Supporting information).

Binding assays for the characterization of anti-FXIII antibodies

Anti-FXIII antibodies may bind to FXIII-A or FXIII-B and the antibodies directed against either of the FXIII subunits may also react with FXIII-A_2B_2. Binding assays are essential for the diagnosis of non-neutralizing antibodies and for the classification of combined type anti-FXIII-A antibodies. Various methods have been utilized for investigating the binding of anti-FXIII antibodies to the FXIII-A_2B_2 complex and to individual FXIII subunits. In the first report on the binding of an alloantibody to purified plasma FXIII, an Ouchterlony gel diffusion test was used. In another early binding study FXIII was immunoabsorbed from the plasma by the patient's IgG [34].

ELISA techniques were also used for the detection of anti-FXIII antibody binding. In indirect ELISAs the plates are coated with FXIII and the binding of IgG isolated from the patient's plasma are detected by enzyme-labeled secondary antibody [35, 36]. Fig. 4B demonstrates the results of such a study, in this case purified FXIII-A_2, FXIII-B_2 and FXIII-A_2B_2 were all used as coating antigens [28]. The patient's IgG reacted with FXIII-B and FXIII-A_2B_2, but not with FXIII-A_2 indicating the presence of an anti-FXIII-B autoantibody in the plasma. In two studies competitive ELISA assay was used to identify the target of the anti-FXIII antibody. In these studies FXIII-A_2B_2, FXIII-A_2, FXIII-A_2'B_2 and FXIII-A_2' were pre-incubated with the patient's IgG [16, 37]. Then the incubation mixture was transferred to an ELISA plate the surface of which was coated with FXIII-A_2B_2. The IgG that remained unbound in the fluid phase but bound to the coated FXIII was detected by enzyme labeled anti-human IgG. Using such a technique in one study the primary target of the autoantibodies was identified as FXIII-A_2' [16]. In another study the antibody reacted with FXIII-A_2B_2 and FXIII-A_2'B_2 equally well [37].

The dot blot assay for the detection of anti-FXIII antibody binding to different FXIII species was first introduced by Lorand et al. [16]. In the assay the respective FXIII species are blotted to nitrocellulose membrane and the reaction with the patient's diluted plasma or purified IgG is detected by labeled secondary antibody. This technique was intensively used by the Japanese group, and contributed to the diagnosis of a relatively high number of patients with anti-FXIII antibodies (see

Table S1 and references [32, 33, 38-45]). In the latter cases rFXIII-A_2 and rFXIII-B_2 were used as antigen. An immuno-chromatographic binding assay for the detection of anti-FXIII-A antibodies with 90% diagnostic reliability has also been published [46]. Western blotting has also been used for the detection of antibody binding to FXIII subunits [28]. In this case in the sample buffer containing high concentration of SDS structural changes may occur in the protein at high temperature. If the SDS PAGE is carried out in reducing condition the reduction of disulfide bonds may induce further structural changes. On Fig. 4C the binding of an autoantibody to FXIII-B, but not to FXIII-A on the Western blot indicates that the autoantibody is directed against FXIII-B. The disappearance of the binding reaction in reducing condition suggests that the autoantibody reacted with a structural epitope. The 10 pairs of disulfide bonds are essential for keeping the structures of the 10 sushi-domains in FXIII-B and breaking them results in drastic structural rearrangements.

Former techniques did not allow calculating the kinetic parameters of the binding between the antibody and the respective FXIII species. Modern methods of protein biochemistry, like surface plasmon resonance (SPR), isothermal titration calorimetry and thermophoresis are able to provide such information. To our knowledge isothermal titration calorimetry and thermophoresis have not been used for this purpose. We employed SPR in three cases to determine the kinetic parameters of the antibody-antigen reaction [23-25]. Fig. 4D shows SPR sensorgrams from an experiment in which the autoantibody was the surface-bound ligand and various concentrations of rFXIII-A_2 served as analyte [25]. The Ka was 2.66 x 10^8 M^{-1} for FXIII-A_2 and 1.65 x 10^8 M^{-1} for FXIII-A_2B_2 (the latter experiments are not shown), which demonstrates high affinity binding. Complex formation of FXIII-A_2 with FXIII-B_2 did not influence the affinity of FXIII-A_2 toward the autoantibody. The rate constants (ka and kd) indicated quick binding and slow dissociation of the complex. FXIII-B did not react with the autoantibody.

We found only two reports in which epitope mapping for an anti-FXIII antibody was attempted. Most recently, using linear B-cell epitope mapping Nixon et al. localized the binding epitope of a neutralizing anti-FXIII-A autoantibody to peptides including amino acids 45-54 and 378-387 [47]. In an earlier study, without reporting experimental details, peptide sequences 490-500 and 391-400 were reported as binding epitopes for the anti-FXIII-A autoantibody [48].

Analyses of patients with anti-FXIII autoantibodies

The prevalence of symptomatic anti-FXIII-A autoantibodies is low. In addition to its rarity, unawareness and poor diagnostic practices also contribute to the low number of recognized patients. The majority of individual cases were described in case reports and summarized in reviews. Two systematic reviews on acquired FXIII inhibitors, published in 2013 and 2016, included 30 and 63 cases, respectively [49, 50]. The latter publication included only 36 case reports and the rest of the patients came from 3 case series, in two of which individual patients were not characterized. Three further reviews have been published on acquired autoimmune FXIII deficiency [45, 51, 52]. In two of them detailed characterization of 33 and 35 individual cases was described [51, 52]. Most recently Japanese authors published a comprehensive valuable review that included 59 Japanese and 34 non-Japanese cases [45]. In this review cases published in abstracts only and unpublished cases from the authors were also included. Our strategy was to include only peer-reviewed reports published in English. Reports published only as abstracts or listed in reviews without detailed description were excluded. Similarly, cases with inhibitors that were proven not to be autoantibodies were not enlisted, either. Following such a strategy altogether 48 cases were collected in Table S1 and included in the analysis.

Patients with autoimmune FXIII deficiency

The mean age of the patients at the time of diagnosis was 61.9±22.2 years; the median age was 70 with interquartile range (IQR) 56.0-77.5. Similar mean age (59.7) of patients with anti-FXIII autoantibody was reported by Tone et al. [50]. In our patient cohort (Table S1) there is a female dominance (60.4%) and female patients were 6 years younger than male patients (means: 59.5±24.7 vs. 65.6±17 years). Female predominance was also reported in four other reviews [49-52]. The gender distribution among Japanese patients was considerably different; in this case females contributed only 37.3% to the patients' group [45]. It is interesting that two separate age cohorts exist among the patients (Fig. 5). The one below 40 mainly consists of females (80%), while the one above 50 shows

more even gender distribution (female/male: 21/17). Omitting the two pediatric cases (both with age 9) 6 out of 8 patients in the younger cohort suffered of SLE (75%), while in the older cohort only 18% had underlying autoimmune disease. In the review of Franchini et al. 25% of autoimmune FXIII deficient patients was associated with an autoimmune disease. Much lower prevalence (15.2%) of underlying autoimmune disease was reported for the Japanese patient population [45]. 31.3% of patients enlisted by us had drug-related background, among them 8 patients were on isonicotinylhydrazide (INH) therapy to treat tuberculosis. Only in four cases was the development of autoantibody related to a malignant disease. In 16 cases (33.3%) no related condition was reported.

Autoimmune FXIII deficiency is a very severe life-threatening bleeding diathesis. The most frequently reported symptoms were large intramuscular and subcutaneous hematomas (20 and 19 reports, respectively). Additionally, hematomas in soft tissue, which very likely represents muscle and subcutaneous tissue, are also described in 10 reports. Altogether 85.4% of the patients demonstrated soft tissue hematomas indicating that this is the major site of bleeding in autoimmune FXIII deficiency. 17 patients (35.4%) suffered of excessive post-surgical bleedings, there were two report on impaired wound healing. Further relatively frequent bleeding symptoms were retroperitoneal bleeding (16.7%), ecchymosis (16.7%) hematuria (12.5%) and intracranial hemorrhage (8.3%). It is to be noted that in inherited FXIII-A deficiency 30%, 19% and 17% were reported for the occurrence of intracranial hemorrhage [6, 53, 54]. These data suggest that in autoimmune FXIII deficiency intracranial hemorrhage is a somewhat less frequent complication than in inherited FXIII deficiency.

Diagnosis of FXIII deficiency in patients with anti-FXIII autoantibody

There were only 6 studies (12.5%) in which the diagnosis of FXIII deficiency was based exclusively on clot solubility test (Table S1). In 42 studies a quantitative FXIII assay was used to establish/confirm the diagnosis, in 26 of these studies clot solubility was also investigated. Among the quantitative tests in 16 cases an amine incorporation test, in 17 cases an ammonia release assay, in 6 cases both assays were used, while in three reports the FXIII activity assay was not specified. Since

the late nineties when commercial ammonia release FXIII activity kits became available this test became the most popular FXIII activity assay. Impaired fibrin cross-linking was demonstrated by SDS PAGE in the plasma of 17 patients. It is to be noted that in the rare case of type III autoantibodies FXIII activity measured by amine incorporation or ammonia release assay might be only moderately decreased or even normal (see cases 5, 9, 17, 20). In such cases carrying out a cross-linking test could be of diagnostic importance.

There were 22 cases in which a numerical value of FXIII activity measured at the time of the diagnosis was reported. The activity varied between undetectable (n=5) and 18%, most of them were below 10%. As compared to inherited FXIII deficiencies the activities in autoimmune FXIII deficiencies were higher than expected. This might be due to incomplete inhibition of FXIII activation/FXIIIa activity by the autoantibody and/or to the overestimation of FXIII activity by ammonia release FXIII assays without blank subtraction [55]. Although FXIII antigen determination is not essential for establishing the diagnosis, in 17 cases antigen determinations (FXIII-A_2B_2, FXIII-A, FXIII-B antigens) were carried out as additional tests. In the case of antigen assays possible interference of the patient's anti-FXIII-A autoantibody with the assay should be excluded. For such a purpose parallel detection of FXIII-A in the patient's plasma by Western blotting is to be carried out [23].

Detection and characterization of the patients' anti-FXIII autoantibody

The most common test for the detection of neutralizing autoantibodies used to be the mixing study, which was carried out in 39 cases. An inhibitor titer was determined in 22 patients, in most cases (n=16) with the Bethesda-Nijmegen assay (Table S1). The titer varied between 1.1 BU to 63.2 BU, but in the majority of the cases (n=10) it was ≥10 BU. In a few studies changes in the BU titer were also used for monitoring the effectiveness of eradication therapy [25, 47, 52, 56-60]. Residual FXIII activity in a 1:1 mix of the patient's and normal plasma was also used to monitor the therapeutic effectiveness [33, 41, 42]. In our experience the most precise information on the neutralizing capacity

of an autoantibody is provided by IC50 determination. In two studies 50 μg/mL and 170 μg/mL IC50 concentrations were measured [23, 25]. In theory, one can calculate from these values the amount of FXIII that would be needed to overshoot the inhibitory effect of the autoantibody. In the above cases it would have been evidently hopeless to overcome the inhibitory effect of the autoimmune IgG by the administration of even high dose of FXIII concentrate. In the case of type III inhibitor when FXIII activity was normal or close to normal the mixing study might be positive only with the clot solubility assay [17, 18, 61, 62]. In three cases the diagnosis was confirmed by demonstrating the inhibition of fibrin cross-linking in the presence the patients' IgG [17, 18, 26], in one of these studies the epitope of the autoantibody was on fibrinogen/fibrin and not on FXIII [18].

Binding assays were used in 17 cases for the detection/classification of non-neutralizing and combined type antibodies. In a few cases the binding assays were also useful in identifying the FXIII subunit and the activation products of FXIII to which the anti-FXIII antibody had considerable affinity. ELISA type [16, 28, 33, 36, 37, 40, 41, 63] and dot blot assays [16, 32, 39-42, 64] were used in 8 and 7 cases. Occasionally the techniques used for the characterization of antibody binding were Western blotting [17, 28], immuno-chromatographic test [33, 63] and surface plasmon resonance (SPR) technique [23, 25]. Affinity constants and other kinetic parameters were determined only by SPR.

Classification of FXIII autoantibodies

In all cases in which the immunoglobulin was specified (n=25) IgG was found to be the effective autoantibody, in a single case IgM also contributed to the effect (Table S1, case no: 27). In 6 cases (no: 4, 8, 12, 17, 18, 22) the type of IgG light-chain was also explored, the κ to λ ratio was 4:2. All patients included in Table S1 had anti-FXIII-A autoantibodies, with the single exception of an anti-FXIII-B IgG (no: 48). It is to be noted that 3 additional Japanese cases with anti-FXIII-B antibodies were reported and in 7 patients autoantibodies against both FXIII subunits were published [45]. However, these cases were not described in detail and for this reason they were not included in our

analysis. The anti-FXIII autoantibody described by Ajzner et al. reacted with both free FXIII-B and FXIII-A_2B_2 and due to their highly increased clearance FXIII activity and all FXIII antigen concentration was below 1%, causing severe clinical symptoms [28]. Two Japanese FXIII-B deficient patients were very likely milder cases with 13% and 41% FXIII activities [44].

We attempted to classify the reported autoantibodies retrospectively according to the principles laid down in Table 1. Among the 48 cases in a single one neither neutralizing nor non-neutralizing effect could be established (no: 10). In two cases, including the anti-FXIII-B autoantibody, the accelerated clearance of FXIII occurred in the absence of significant inhibitory effect (no: 27 and 48). In 9 cases the accelerated clearance was combined with some kind of neutralizing effect (no: 19, 23, 30, 31, 32, 38, 45, 46, 47), while in the rest of the cases FXIII deficiency was exclusively due to the inhibitory action of the autoantibody. In the latter group further classification could be carried out in 23 cases, while in 22 cases the data provided in the respective publications were not sufficient to establish the proper subtype. Among the 23 cases one type Ia and one Ib (no: 3 and 8), five type II (no: 7, 13, 18, 22, 39), four type III (no: 2, 5, 9, 17, 20) and two type IV (no: 12, 42) antibodies could be verified. The above results indicate that accelerated elimination of FXIII is part of the pathogenesis in a significant number of cases (non-neutralizing and combined type autoantibodies). A further point is the occurrence of type III autoantibodies that can be only detected by fibrin polymerization assays.

Treatment of patients with FXIII autoantibodies

There is no guideline for the treatment of patients with bleeding diathesis due to autoantibodies against FXIII. The data in Table S1 demonstrate the therapeutic modalities that have been tried so far. In most of the cases to overshoot the autoantibody present in the plasma even by high dose of FXIII concentrate seemed hopeless. The main aim of the therapy should be to eliminate/decrease the autoantibody. Only temporary relief was achieved by plasmapheresis and plasma exchange, the autoantibody usually rapidly returns after the treatment. The most successful attempts were

eradication of the autoantibody by various combinations of corticosteroids, cyclophosphamide, cyclosporine and anti-CD20 (rituximab). 23% of the patients died of bleeding complications. 50% of the patients fully recovered and in 27% of them the recovery was only partial. However, it is to be noted that the follow-up periods varied significantly.

Anti-FXIII alloantibodies

The development of alloantibodies against FXIII subunits is extremely rare. Alloantibodies against FXIII-A were reported in three cases, while anti-FXIII-B alloantibody was discovered only in a single case, respectively (Table S2). Only in cases 3 and 4 were the inherited genetic defects identified. In all cases the patients suffered of severe bleeding symptoms, which in case 3 led to death at the age of 15. The diagnostic procedures and treatments were similar to those described for anti-FXIII autoantibodies. In all cases of anti-FXIII-A alloantibodies were classified as combined type. In case 3 IC50 was also determined and was found to be 340 μg/mL.

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The authors state that they have no conflict of interest.

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Table 1 Classification of anti-FXIII antibodies

Subunit specificity	Effect	Type
Anti-FXIII-A		
	Neutralizing	Inhibition of FXIII activation (type I)
		Inhibition of the release of activation peptide (type Ia)
		Inhibition of the activation of thrombin cleaved FXIII by Ca^{2+} (type Ib)
		Inhibition of FXIIIa activity (type II)
		Inhibition the binding to fibrin (type III)
		Multiple neutralizing effect (type IV)
	Non-neutralizing	Acceleration of FXIII clearance without the inhibition of activity/activation
	Combined	Inhibition of FXIII activity/activation and acceleration of FXIII clearance
Anti-FXIII-B		
	Non-neutralizing	Acceleration of FXIII clearance

The principles of this classification were laid down by Lorand et al. [16]. The Table represents a slight modification of the version published by Pénzes et al. [24].

Legend to the Figures

Fig. 1. The mechanism of plasma FXIII activation and the possible targets of inhibition by neutralizing anti-FXIII-A antibodies. Red lines represent the N-terminal activation peptides. Orange and green cylinders depict the β-sandwich and the two C-terminal β-barrel domains, respectively. The central core domain, between the β-sandwich and β-barrel 1 domains, is shown in magenta. The 10 sushi domains of FXIII-B are represented by interconnected gray beads. Thrombin cleaves off the activation peptide. Ca^{2+} at plasmatic concentration (low Ca^{2+}) induces the dissociation of FXIII-B from the FXIII-A dimer, the latter then assume enzymatically active configuration. Fibrin (shown in parenthesis) accelerates the first two steps of the activation process. (This cartoon contains part of a figure that has been published in Physiol Rev, see reference [1]). The possible targets of the inhibition by anti-FXIII-A antibodies are encircled in red.

Fig. 2. Quantification of neutralizing anti-FXIII-A antibodies. (A) Diagram for the calculation of Bethesda units from residual FXIII activity. (B) IC50 measurement. The graph demonstrates the IgG concentration-dependent inhibition of FXIII activation/activity. IgG was prepared from the plasma of a patient with of anti-FXIII-A autoantibody. FXIII was pre-incubation with various concentration of the patient's IgG and then it was activated by thrombin and Ca^{2+} and the transglutaminase activity was measured [23].

Fig. 3. Characterization of neutralizing anti-FXIII-A antibodies. (A) Impaired thrombin-induced release of activation peptide in the presence of an anti-FXIII-A alloantibody. Plasma FXIII was activated by thrombin and Ca^{2+} for various times and the release of activation peptide was demonstrated by the accumulation of truncated FXIII-A (FXIII-A') on the Western blot. In the presence of normal IgG complete truncation of FXIII-A occurred within 2.5 min, while in the

presence of the patient's IgG even 10 min was not sufficient for complete truncation. The figure is a slightly modified version of the one originally published in the journal Haemophilia [24]. (B) Three cases demonstrating the effect of neutralizing anti-FXIII-A antibodies on the Ca^{2+} induced activation of FXIII truncated by thrombin and on fully activated FXIII (FXIIIa). Open bars represent experiments in which FXIII truncated by thrombin (FXIII-A_2'B_2) was incubated with the antibody and then FXIII-A_2'B_2 was activated by the addition of Ca^{2+} and the activity of the resulted FXIIIa was measured. Closed bars show the results of experiments in which FXIII was first activated by both thrombin and Ca^{2+} then the formed FXIIIa was incubated with the antibody and the activity of was determined. Measured FXIII activities were expressed as percentage of FXIII activity measured in the absence of the antibody (relative FXIII activity). The data shown on the graph were calculated from the results published in references [23-25].

Fig. 4. Detection of anti-FXIII antibodies by clearance study and by binding assays. (A) Accelerated clearance of FXIII after the addition of plasma FXIII concentrate (Fibrogammin-P, Dade-Behring, Marburg, Germany) to a patent with anti-FXIII-B autoantibody. The half-life was determined as 17 hours. (B) The IgG prepared from the plasma of a patient with anti-FXIII-B autoantibody bound to FXIII-B and to FXIII-A_2B_2 coated to an ELISA plate. (C) Binding of the patient's IgG to non-reduced FXIII-B but not to FXIII-A as demonstrated by Western blotting. After reduction of FXIII-B the reaction disappeared. (D) SPR sensorgrams demonstrating the binding of an anti-FXIII-A autoantibody to rFXIII-A_2. The Ka of the interaction was calculated. Panels A,B,C were originally published in Blood (reference [28]), panel D was published in Haemophilia (reference [25]).

Fig. 5. Age and gender distribution of patients with anti-FXIII autoantibodies.