

SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD)

Diagnosis and clinical consequences of allo- and autoantibodies against factor XIII-A based on three patient's data

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The Examination takes place at the Library of the Division of Clinical Laboratory Science, Department of Laboratory Medicine, Faculty of Medicine, University of Debrecen, at 11:00 am, 6nd of March, 2018

Head of the **Defense Committee**: Prof. Csongor Kiss, MD, PhD, DSc
Reviewers: Prof. Zoltán Boda, MD, PhD, DSc
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The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen, at 1:00 pm, 6nd of March, 2018

1. Introduction and Literature Review

1.1. The structure and function of factor XIII

Blood coagulation factor XIII (FXIII) consists of two catalytic FXIII-A subunits and two inhibitory/protective FXIII-B subunits, with a heterotetrameric structure (FXIII-A₂B₂) and a molecular mass of 326 kDa.

FXIII-A has a molecular mass of 83 kDa, and consists of four structural domains: β -sandwich domain, catalytic core domain, β -barrel 1 and β -barrel 2 domains. The N terminal activation peptide (AP-FXIII) consists of 37 amino acids. FXIII-A is expressed mainly in cells of bone marrow origin. In plasma 99% is complexed with FXIII-B. Intracellular FXIII-A is a homodimer (FXIII-A₂, cFXIII), found in platelets, monocytes and macrophages.

FXIII-B is a glycoprotein (molecular mass 75 kDa) synthesized by hepatocytes. In plasma the amount of FXIII-B is approximately twice the amount of FXIII-A, with 50% circulating free. It prolongs the half-life of FXIII-A by inhibiting its spontaneous activation and possibly its degradation as well.

FXIII-A₂B₂ becomes activated in the last phase of coagulation by the concerted action of thrombin and Ca²⁺.

First, thrombin removes an activation peptide from the N-terminus of FXIII-A (FXIII-A₂'B₂). Then, in the presence of Ca²⁺ FXIII-B dissociates and the remaining FXIII-A₂ assumes an active configuration (FXIII-A₂^{*}; FXIIIa). FXIIIa is a transglutaminase; its main function is to crosslink fibrin γ and α chains and α_2 -plasmin inhibitor to fibrin through $\epsilon(\gamma\text{-glutaminy})\text{lysyl}$ bonds. This way it protects the newly formed fibrin clot from the shear stress in circulating blood and from fibrinolytic degradation.

1.2. FXIII deficiency

Inherited FXIII-A deficiency is a rare, but severe bleeding diathesis. In the general population its prevalence is approximately 1 in 2 millions, however in populations with the tradition of consanguineous marriages its frequency is 10-fold

higher. According to a classification published in 2012 below 0.05 IU/mL activity is FXIII deficiency with severe bleeding risk, FXIII activity between 0.05 and 0.3 IU/mL corresponds to moderate deficiency and FXIII activity between 0.3 and 0.5 IU/mL correlates with mild hemorrhagic disease. Usually the first sign is delayed type umbilical stump bleeding, other characteristic symptoms in non-supplemented patients include intracranial hemorrhage, subcutaneous and intramuscular bleeding.

FXIII-B deficiency is usually less severe than FXIII-A deficiency, with a FXIII activity of 5-10% and mild bleeding symptoms. FXIII-A type I deficiency is quantitative, while FXIII-A type II deficiency is a qualitative disorder. The database of FXIII-A deficiency mutations (as of November 2017) contains 69 pathological mutations in the FXIII-A gene.

FXIII deficiency is one of the most underdiagnosed bleeding diathesis in the world, partially because routine hemostasis tests are ineffective in establishing the diagnosis. Due to the high risk of hemorrhagic stroke, lifelong substitution therapy, preferably with heat inactivated plasma derived FXIII concentrate: Fibrogammin P (Corifact, CLS Behring, Marburg, Germany) or recombinant FXIII-A₂ (rFXIII-A₂, Novothirteen, Tretten, Cartidecacog, Novo Nordisk, Bagsvaerd, Denmark) is recommended.

The presence of FXIII antibodies poses a great therapeutic challenge, due to the high risk of uncontrollable bleeding. Both allo- and autoantibodies can develop. Anti-FXIII antibody may develop in patients with inherited FXIII deficiency under replacement therapy, but are very rare, previous to our publications only three cases were documented in the literature.

Autoantibodies formed against either of the FXIII subunits are frequently associated with autoimmune or malignant diseases, but their presence could also be idiopathic, particularly in elderly individuals. The presence of autoantibodies results in life-threatening bleeding disorders dominated by intramuscular and subcutaneous bleedings. In most cases the antibodies are of IgG type. Antibodies can develop against both subunits, but antibodies against FXIII-A are more common. This is also a rare clinical condition, although more recently, due to concentrated diagnostic efforts, a number of patients were diagnosed in Japan.

The lack of guidelines means that the treatment is mostly empirical, with a focus on controlling the bleeding, and the quick eradication of the antibody. The therapeutic options include immunosuppression with steroids and cyclophosphamide,

immunoabsorption for the elimination of the antibodies, Rituximab (anti-CD20 monoclonal antibody) therapy and in some cases administration of intravenous immunoglobulins (IVIG).

The diagnosis, classification and management of FXIII deficiency caused by neutralizing or non-neutralizing autoantibodies represent a difficult task. In most cases, only tests detecting neutralizing antibodies are carried out by measuring FXIII activity in the mixture of normal plasma and plasma from suspected individuals. For a semi-quantitative evaluation of this mixing test an adaptation of the Bethesda-Nijmegen assay has been recommended. This test however does not provide information on several important characteristics of the autoantibody. For instance it fails to estimate its binding affinity and its neutralizing capacity. For proper classification it is also important to reveal if the thrombin and/or Ca^{2+} induced activation of FXIII or the activity of FXIIIa are inhibited. In the diagnosis of non-neutralizing autoantibodies demonstration of its binding to either of the FXIII subunits and the demonstration of accelerated clearance of FXIII are essential. Neutralizing and non-neutralizing features may also occur in combination.

2. Objectives

The aim of PhD studies were:

1/ Description of two cases of FXIII-A deficiency caused by FXIII-A anti-autoantibodies, and the presentation of a combined case of inherited FXIII-A deficiency with allo-antibody.

2/ Presentation of the laboratory algorithm leading to the diagnosis.

3/ Adapting the 50% Inhibition Method (IC50) to measure antibody-induced inhibition.

4/ Exploring the biochemical effect of antibodies.

5/ Introducing a new method of surface plasmon resonance (SPR) to determine the affinity of antibodies to FXIII subunits.

2. Materials and Methods

2.1. Protein preparations

The purification of FXIII-A₂B₂ and FXIII-B from human plasma of healthy blood donors was carried out in our institute based on the methods developed by Lóránd et al. and Chung et al. In a repeat freeze-thaw cycle FXIII-A is removed from the complex and then FXIII-B is purified by chromatography, which is done by FPLC (fast protein liquid chromatography). rFXIII-A₂ produced in yeast cells was obtained from Dr. Éva Olsen (Novo Nordisk, Måløv, Denmark).

The patient's plasma IgG preparation was performed on a HiTrap Protein G HP column. First the columns was washed with binding buffer (20 mM Na phosphate buffer, pH 7.0). Next plasma sample diluted 5-fold with binding buffer and passed through a 0.22 µm pore size membrane filter were injected on the column (flow rate 0.5 mL/min). Elution was performed with 0.1 M (pH 2.7), glycine-HCl. Thirteen drops of sample was collected in tubes containing 50-50 µL 1M Tris/HCl (pH 9.0). The whole process was repeated four times. Fractions containing protein (OD > 0.1 as measured at 280 nm) were dialyzed in PBS overnight at 4°C.

2.2. Determination of FXIII activity

Blood samples were collected in anticoagulated vacutainer tubes (1/10 volumes of 0.109 M Na-citrate, Beckton Dickinson, Franklin Lakes, NJ, USA). Plasma was separated by centrifugation (4 ° C, 1400 g, 20 mins) and stored at -80 ° C until use. FXIII activity measurements were also performed from washed platelet as well.

Plasma factor XIII activity was determined spectrophotometrically using the REA-chrom FXIII (REANAL-ker, Budapest, Hungary) in vitro diagnostic reagent kit. Measurements were performed on an EVO P800 Modular (Hitachi, Roche, Switzerland, Basel) analyzer and on a Tecan Infinite M200 (Tecan Group Ltd., Switzerland, Mannedorf) photometer. For calibration the International Standard Factor XIII Plasma, Human calibrator (National Institute for Biological Standards and Control, Potters Bar, United Kingdom) of the World Health Organization (WHO) was used.

Our Montreal collaborative partners used the Berichrom® FXIII test (Siemens, Marburg, Germany) to measure FXIII activity.

2.3. FXIII antigen determinations

Plasma FXIII-A₂B₂, FXIII-A, total and free FXIII-B determinations were carried out by ELISAs. FXIII-A₂B₂ measurement was done by an ELISA method developed in our institute. A biotinylated monoclonal capture-antibody against the B-subunit and a horseradish peroxidase-labelled monoclonal tag-antibody against the A-subunit were added to the diluted plasma samples in the well of a streptavidin coated microplate. The amount of the complex attached to streptavidin-coated microplate was quantitated by measuring peroxidase activity. FXIII-A antigen measurement was similarly carried out on a streptavidin coated platelet, but the antibodies reacted with two different FXIII-A epitopes. Plasma FXIII-B antigen was determined by a sandwich ELISA using 2 monoclonal antibodies against different FXIII-B epitopes. For the measurement of non-complexed “free” FXIII-B the capture antibody in the total FXIII-B assay was replaced by a monoclonal antibody that reacted exclusively with non-complexed FXIII-B. The biotinylated capture antibody, diluted plasma, and the peroxidase labeled detection antibody were mixed and incubated for 1 hour in streptavidin-coated microtiter plates. After extensive washing the peroxidase activity was determined, by measuring absorbance at 450 nm on an iEMS Reader MF microplate analyzer. For the measurement of FXIII-A₂B₂ complex and the A subunit, the WHO calibrator was used as the standard. For the total FXIII-B measurement, a plasma mixture was collected from 40 healthy individuals and used as calibrator and a purified FXIII-B subunit of known concentration was used for free FXIII-B determination.

2.4. Determine the strength of FXIII activation/activity inhibitory activity

1 / Determination of inhibitor strength by Bethesda Nijmegen method.

In the Bethesda-Nijmegen method, several different dilutions of the patient plasma were mixed with a 0.1 M imidazole-buffered (pH: 7.4) control FXIII activity in a 1:1 ratio (tube 1). In the control tube (tube 2), FXIII-deficient plasma was used instead of the patient's plasma. The mixtures were incubated at 37 °C for two hours and then FXIII activity was determined. If the plasma FXIII activity of the patient is not 0, correction should be made. Subsequently, residual activity was calculated from the obtained values according to the following formula:

$$\text{Residual FXIII activity} = (\text{FXIII activity tube 1} / \text{FXIII activity tube 2}) \times 100$$

The result is read from a graph and expressed in Bethesda units (BU). 1 Bethesda unit equals 50% residual factor activity. For calculating BU, the plasma dilution with a residual activity between 25-75% is used. The result from the graph is multiplied by the degree of dilution.

2 / Measurement of inhibitor strength by IC₅₀ determination.

For the determination of 50% inhibitory concentration (IC₅₀) healthy human plasma was used as control, 50 mM HEPES and 100 mM NaCl (pH 7.4) as buffer and the patient's IgG. Purified FXIII-A₂B₂ (1. and 2. patients: 4.2 µg/mL, 3. third patient: 8.3 µg/mL) was incubated with various concentrations of the IgG prepared from the patient's plasma or from normal pooled plasma for 60 min at 37 °C. Then, FXIII was activated by human 20 U/mL thrombin and 10 mM CaCl₂ for 5 min and FXIIIa activity was measured by the ammonia release assay on a Tecan Infinite M200 (Tecan Group Ltd., Mannedorf, Switzerland) photometer. Absorbance was measured for 10 minutes, at 20 seconds intervals. The change in absorbance (ΔA/min) was recorded from the 6th minute and FXIII activity was calculated. FXIII activity was expressed as percent of FXIII activity measured in the presence of normal IgG (100%). IC₅₀ was calculated from the results of two parallel experiments using the software from www.ic50.tk.

2.5. Classification of FXIII activation/activity inhibitory effect

1 / Influence of IgG on the proteolytic activation of FXIII by thrombin

The effect of patient's IgG on the proteolytic cleavage of FXIII-A by thrombin was evaluated by Western blotting. FXIII-A₂B₂ (1. patient: 4.16 µg/mL, 2. and 3. patients: 14.3 µg/mL) was incubated with patient's or normal IgG (1. patient: 2.9 mg/mL, 2. patient: 400 µg/mL, 3. patient: 1 mg/mL) in HEPES buffered saline (pH 7.4) for 60 min. After activation by human thrombin (1. patient: 20 U/mL, 2. patient: 2.5 U/mL, 3. patient: 5 U/mL) and 10 mM CaCl₂ aliquots at 2.5, 5.0, 10.0, 15.0 and 20.0 min were removed for analysis by Western blotting. Sheep polyclonal anti-FXIII-A IgG antibody (Affinity Biologicals, Ancaster, Canada) in combination with Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA) was used for detection and visualization.

2 / Combination of the IgG of patients with thrombin-cleaved FXIII Ca²⁺ induced activation and active FXIII

In the next experiment 4.2 µg/mL FXIII-A₂B₂ was truncated by 20 U/mL thrombin in 50 mM HEPES-t and 100 mM NaCl buffer (37 °C, 10 min) and incubated with normal or patients' IgG (1. patient: 2.92 mg/mL, 2. patient: 0.4 mg/mL, 3. patient: 1 mg/mL) for 60 min at 37 °C, then 10 mM CaCl₂ was added and the transglutaminase activity was measured. This experimental set-up evaluated the combined inhibitory effect of the patient's IgG on the Ca²⁺ induced activation of FXIII-A₂B₂ and on the activity FXIIIa. Measurements were carried out on a Tecan Infinite M200 analyzer.

3 / Effect of patients IgG on thrombin and Ca²⁺ -activated FXIII (FXIIIa) activity

Finally, FXIII was activated by 20 U/mL thrombin and 10 mM CaCl₂ and then, 4.2 µg/mL of the formed FXIIIa was incubated with the previously mentioned concentration of the patient's or normal IgG. After 60 min incubation at 37 °C FXIIIa activity was measured.

2.6. Binding studies

In the case of the third patient in Canada an ELISA using rFXIII-A₂ as coating antigen was developed to determine the antibody titer at various times in the patient's plasma. Serial dilutions of patient's plasma were applied to the coated well and after incubation peroxidase-labeled goat anti-human IgG (Sigma-Aldrich, St. Louis, Missouri, USA) was used for detection. Peroxidase activity was measured by o-phenylenediamine dihydrochloride substrate. The starting plasma dilution was 1:50 and the extent of further dilutions was used for titer calculation.

The binding of patient's IgG to FXIII-A₂B₂, FXIII-B and FXIII-A₂ was monitored by surface plasmon resonance (SPR), using highly purified proteins as ligands and the alloantibody as analyte. SPR measurements were performed on a Biacore X (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and Biacore 3000 instrument (GE Healthcare, Little Chalfont, UK). The patient's IgG was immobilized to one cell of CM5 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) or CMD (XantTec, Düsseldorf, Germany) sensor chip. There was no difference between the two sensor chips. The second cell was the control flow cell, it was covered by either bovine serum albumin (the first two patients) or normal IgG (third patient). Various concentrations of FXIII-A₂B₂, rFXIII-A₂ or FXIII-B in 10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.005% (v/v) surfactant (pH 7.4) were used as analytes and the autoantibodies were used as ligands. From the sensorgrams the association rate constant (k_a), the dissociation rate constant (k_d) and the affinity constant (K_a) were calculated by BIAevaluation software (GE Healthcare, Bio-Sciences AB, Uppsala, Sweden). The response unit (RU) values measured on the control cells was subtracted from the RU values of the main cells with the use of the BIAevaluation software.

2.7. Molecular genetic analysis

In the first patient, from the DNA sample the gene coding for FXIII-A (F13A1) was subjected to a direct fluorescence sequencing of the entire gene, followed by gene sequencing of the patient's parents DNA samples. Duplication of the encoding regions and exon-intron boundaries was accomplished using the GeneAmp2700 PCR (Applied Biosystems, Foster City, USA). The primers used were identical to the oligonucleotides indicated in the FACTOR XIII Registry Database for reference sequence.

The control of the PCR product was performed with electrophoresis on a 3% agarose gel GelRed (Izinta Trading Ltd., Budapest, Hungary). PCR product purification was done with a Big Dye Terminator Cycle Sequencing Kit (Applied Biosystem, Foster City, USA), and direct sequencing was carried out on an ABI PRISM 3130 DNA Sequencer (Applied Biosystem, Foster City, USA). Capillary electrophoresis and fluorescence detection of sequenced products were performed using an Avant Genetic Analyzer (Applied Biosystem, Foster City, USA), Sequencing Analysis 5.3.1 software was used to evaluate electrophoresis. The traditional nucleotide and amino acid numbering was used, which does not include the initiator methionine.

3. Results

3.1. First patient

3.1.1. Case history

The patient was a French Canadian girl diagnosed with FXIII deficiency in March 1994, at the age of 3. She initially presented with a history of easy bruising and prolonged bleeding from minor cuts. The FXIII activity was < 0.01 IU/mL. The FXIII-A level was < 0.07 IU/mL with normal FXIII-B. Parents were consanguineous; FXIII activity was of 0.54 IU/mL for the father and 0.77 IU/mL for the mother. At time of diagnosis, parents expressed concerns about the safety of plasma-derived FXIII concentrates and the father and the paternal grandmother donated plasma for prophylaxis. Just before the initiation of prophylactic treatment, the patient received Fibrogammin[®] (CSL Behring) together with paternal plasma for a significant hematoma. From June 1994 until July 1995, she received prophylactic monthly transfusion of either paternal or grandmother plasma and did not experience any bleeds. The FXIII level after 30 minutes of 15 mL/kg of paternal plasma was at 0.26 IU/mL. The development of anti-FXIII-A alloantibody and the subsequent clinical course are described in the Results. This patient was briefly mentioned in an earlier publication.

In July 1995, one year after having started the prophylaxis, the patient reported spontaneous hematomas despite plasma transfusion. A plasma-derived FXIII concentrate (Fibrogammin[®]) was tried but was also unable to prevent bleeding. 24 hours after the injection of 40 IU/kg Fibrogammin[®] the FXIII level was only at 0.025 IU/mL. An anti-FXIII activity of 6.6 BU was measured. All prophylactic treatments were stopped. Eradication of the inhibitor was first attempted by two courses of IVIG in November 1995. Anti-FXIII titer was 4.5 BU before the first IVIG infusion and 0.76 BU 5 weeks after the second one.

Two months later, she presented with an intracranial hemorrhage after a minor fall. She was treated with dexamethasone and 90 U/kg Fibrogammin[®]. She responded to the treatment with a rise in FXIII level up to 1.1 IU/mL. An immune tolerance induction inspired by the Malmö protocol (cyclophosphamide, IVIG, high dose FXIII) was attempted. Following this protocol, she was maintained on 33 IU/kg Fibrogammin[®] every 7 to 10 day. During this period the FXIII level pre Fibrogammin[®] injection exceeded 0.1 IU/mL and the level 30 minutes after the injection was around 0.45 IU/mL.

After 1 year she started having lower biological response to FXIII concentrate. FXIII levels before and after weekly injections of 40 IU/kg of Fibrogammin[®] were around 0.05 IU/mL and 0.1 IU/mL, respectively. She was otherwise clinically well without spontaneous bleeding. With this evidence of inhibitor relapse, a second Malmö protocol was introduced. Again, she showed a good initial response but for short duration (6 months). Because she was clinically well, she was kept on high doses Fibrogammin[®] (60 IU/kg twice a week) for the next 3 years. Dosages were increased to 100 IU/kg twice a week for four months to try to induce immune tolerance, which was not successful.

In 2000 she was having multiple spontaneous bleedings and another attempt of inducing immune tolerance was carried out. Two-volume exchange plasmapheresis along with immunoadsorption daily for 5 days was performed, which was followed by IVIG (1 g/kg/day) for two days. Continuous perfusion of FXIII at a rate of 5 IU/kg/hour was given throughout the treatment and continued thereafter. There was an initial good response with a rise of FXIII level from 0.03 IU/mL to 1.06 IU/mL. However, three days post immunoadsorption, while she was still on the continuous FXIII perfusion, the FXIII level fell back to 0.04 IU/mL. Another 2 cycles of 3 days of plasmapheresis and immunoadsorption followed by IVIG were done at an interval

of 7 days. Prednisone 1.5mg/kg and cyclophosphamide 2 mg/kg were started on day 4. Again, the initial response was good (FXIII level 1.55 IU/mL) but brief (FXIII 0.13 IU/mL on her last day of plasmapheresis). She was discharged on continuous infusion of FXIII at a rate of 5 IU/kg/h, which was changed for bolus 2 months later. For the subsequent years, she was maintained on a daily dose of 30 IU/kg of FXIII. She initially had many spontaneous bleeds that became less frequent over time. During this period, there was clinical and laboratory evidence on the persistence of inhibitor, which was also shown by ELISA technique.

In 2006 she fell and bumped her head on the ground. She did not lose consciousness but was amnesic of the event. She was immediately treated with Fibrogammin[®] 25 IU/kg and brought to the hospital. On admission she was completely alert with a Glasgow score of 15. The initial brain CT scan showed a subdural hematoma and other discrete signs of bleeding. She was treated with Fibrogammin[®] 25 IU/kg every 6 hour along with dexamethasone. Less than 24 hours later, she became more and more somnolent. The repeated CT scan showed multiple intracerebral haemorrhages. She rapidly became comatose and died a few hours later.

3.1.2. FXIII determinations and molecular genetic testing

FXIII activity, FXIII-A₂B₂ antigen and FXIII-A antigen levels were all below the limit of detection. As expected, FXIII-B antigen level was only moderately decreased. Molecular genetic analysis revealed a novel nonsense mutation, c.127C>T, p.Gln42STOP in Exon 2, in homozygous form. As the mutation stops protein synthesis at amino acid position 42, i.e., just after the activation peptide, it is to be considered a causative mutation. Three known polymorphisms were also detected: c.103G>T, p.Val34Leu, c.1704A>G, p.Glu567Glu and c.1954G>C, p.Glu651Gln, all in homozygous forms. Exon 2 in the parents' DNA samples was also sequenced and both the father and the mother were heterozygotes for the causative mutation.

3.1.3. Binding of the alloantibody to the factor XIII and its subunits

The binding of alloantibody to FXIII to plasma FXIII (FXIII-A₂B₂) and to rFXIII-A₂ was assessed by SPR technique using highly purified proteins as ligands in

different concentration (15.625 nM, 31.25 nM, 62.5 nM, 93.75 nM, 125 nM and 156.25 nM). Based on the results an equilibrium dissociation constant $K_d=1.44 \times 10^{-8}$ M ($K_a=8,24 \times 10^7$ M⁻¹) was calculated, which is in the range of antigen-antibody association. The association and dissociation rate constants were 4.82×10^4 M⁻¹ sec⁻¹ (k_a) and 5.68 sec⁻¹ (k_d). Similar results were obtained when FXIII-A₂B₂ was used as ligand; in the latter case the K_d was 1.31×10^{-8} M ($K_a=1.05 \times 10^8$ M⁻¹, $k_a=8.17 \times 10^3$ M⁻¹s⁻¹, $k_d=9.07 \times 10^{-5}$ s⁻¹).

3.1.4. Determination of the inhibitory capacity of alloantibody

The inhibitor titer was determined by the Bethesda-Nijmegen method in the Canadian laboratory. In our laboratory the patient's IgG was pre-incubated with plasma FXIII, then FXIII was activated by thrombin and Ca²⁺ and the transglutaminase activity was measured. The results were expressed as percentage of activity measured in the presence of the same concentration of IgG prepared from normal pooled human plasma. Normal IgG even in the highest concentration was without effect on FXIII activity, while the patient's IgG inhibited FXIII activity and above 2 mg/mL completely blocked it. The IC₅₀ for the inhibition was 0.34 mg/mL.

3.1.5. Classification of the alloantibody

The previously set-up did not distinguished between the effect of the antibody on the activation steps and on the inhibition of activated FXIII. For this reason, experiments were designed in which the effect of antibody on the cleavage of FXIII-A by thrombin, on the Ca²⁺ induced activation of thrombin cleaved FXIII and on activated FXIII could be separately investigated. As it was demonstrated by Western blotting, the removal of activation peptide from FXIII-A was hindered by the antibody, but was not completely prevented. In the presence of normal IgG FXIII-A was transformed into its proteolytically cleaved form (FXIII-A') by thrombin within 2.5 min. Quantitative densitometric analysis of the Western blot showed that at the same time in the presence of patient's IgG only 59% of FXIII-A was cleaved by thrombin, and after 5 min 31% of FXIII-A still remained intact. This situation did not change up-to 20 min of incubation.

To explore if Ca^{2+} induced FXIII activation were influenced by the alloantibody, first the activation peptide was cleaved off from FXIII-A by thrombin, then the resulted FXIII-A₂B₂ was incubated with the patient's (and normal) IgG and, after the addition of Ca^{2+} , the transglutaminase activity was measured. This way the combined effect of alloantibody on the Ca^{2+} induced dissociation of the B subunits, on the transformation of FXIII-A' dimers into active transglutaminase and on FXIIIa activity was investigated. At the patient's IgG concentration, that exerted maximal inhibitory effect, only 9% of the transglutaminase activity measured after incubation with normal IgG could be recovered.

Finally, it was tested if the alloantibody inhibits the transglutaminase activity of fully activated FXIII (FXIII-A*₂). In this case 57% of the transglutaminase activity was inhibited at saturating IgG concentration.

3.2. Second patient

3.2.1. Case description and diagnosis

Due to intramuscular bleeding in the thighs a 75 years old patient was transferred from a county hospital to the University Clinic. He had no previous history of bleeding. The usual hemostasis screening tests (prothrombin time, activated partial thromboplastin time, thrombin time, and PFA-100 closure times) and α_2 -plasmin inhibitor activity were in the reference intervals. Factor VIII activity, von Willebrand factor ristocetin cofactor and antigen were 196%, 232% and 221%, respectively. The patient was positive for IgM type antiphospholipid antibody. At admittance FXIII activity measured by the modified sensitive ammonia release assay in the patient's plasma was 2%. FXIII-A₂B₂ and FXIII-A antigens in the plasma were below the limit of detection (<0.1%), FXIII-B antigen was 30%. In platelet lysate FXIII activity and FXIII-A antigen were 117% and 128%, respectively. The presence of neutralizing autoantibody was verified by a mixing study. FXIII activity of 1:1 mixture of normal control plasma (106% FXIII activity) with FXIII-A deficient plasma was 56%, while 1.2% FXIII activity was measured in the mixture of patient plasma with normal plasma. An adaptation of the Bethesda type assay with Nijmegen modification showed high (63.2 BU) inhibitor titer. At admittance no acute bleeding

was observed and to eradicate the antibody, treatment with steroids in combination with cyclophosphamide (daily dose: 200 mg intravenously for 5 days and subsequently 100 mg orally for 10 days) was initiated. The patient also received a weekly dose of 700 mg rituximab for four weeks. A minor bleeding (hematoma at the right buttock induced by a minor trauma) was successfully stopped by administration of fresh frozen plasma and NovoSeven (60 µg/kg every 3 hours for 4 days) in combination with tranexamic acid. After one month the patient was released symptom free with 3% FXIII activity and with an antibody titer of 23 BU. Two weeks later he was readmitted with hemorrhagic shock (Hgb: 48 g/L) due to severe retroperitoneal bleeding as verified by CT. In spite of NovoSeven plus tranexamic acid administration and continuous transfusions the bleeding could not be controlled and after one week the patient passed away.

FXIII activity in the patient's plasma was always low, but measurable; it varied between 1-4% during the 7-week course. In parallel measurements FXIII-A₂B₂ and FXIII-A antigen were undetectable in the plasma. This discrepancy made us suspect that the autoantibody present in the patient's plasma interfered with the two antigen assays, since both used the same anti-FXIII-A monoclonal antibody. This hypothesis was proven by the detection of approximately normal amount of FXIII-A in the patient's plasma using Western blotting. This finding suggests that in the case of autoimmune FXIII-A deficiency FXIII antigen determination might not be the adequate method for measuring FXIII in the plasma and for monitoring the changes of FXIII during eradication therapy.

3.2.2. SPR measurements, determination of binding constants

Various concentrations of FXIII-A₂B₂, recombinant FXIII-A₂ (rFXIII-A₂) and FXIII-B were used as analytes. From the sensorgrams we calculated the following kinetic parameters: for rFXIII-A₂; $K_a=1.96 \times 10^9 \text{ M}^{-1}$, $K_d=6.72 \times 10^{-10} \text{ M}$, $k_a=5.77 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$, $k_d=3.93 \times 10^{-4} \text{ s}^{-1}$. For FXIII-A₂B₂; $K_a=7.00 \times 10^9 \text{ M}^{-1}$, $K_d=1.04 \times 10^{-8} \text{ M}$, $k_a=4.56 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$, $k_d=1.07 \times 10^{-4} \text{ s}^{-1}$. FXIII-B did not bind to the antibody.

3.2.3. Determination of the inhibitory capacity of autoantibody

The Western blotting experiment suggested that the autoantibody did not accelerate the clearance of FXIII from the circulation significantly. To prove that the antibody was of the neutralizing type, 4.2 µg/mL pFXIII pre-incubated with various concentrations of the patient's IgG for 60 min at 37°C was activated by 20 U/mL human thrombin (Sigma, St. Louis, MO) and 10 mM Ca²⁺. The transglutaminase activity of FXIIIa was measured by the ammonia release assay. In this assay the combined inhibitory effect of the IgG on FXIII activation and the transglutaminase activity of FXIIIa was measured. The IC50 was 50 µg/mL which represents a powerful neutralizing effect. The low IC50 value suggests that the autoantibody present in the patient's plasma was well over the quantity required for the complete blockage of FXIII activation/activity

3.2.4. Classification of the alloantibody

To classify the autoantibody first its effect on the thrombin induced release of activation peptide was investigated. In this case 14.2 µg/mL pFXIII pre-incubated with 400 µg/mL normal or patient's IgG was activated by 2.5 U/mL thrombin and 10 mM Ca²⁺ at 37°C. After various time of incubation, aliquots were removed for analysis by Western blotting using sheep polyclonal anti-FXIII-A IgG antibody (Affinity Biologicals, Ancaster, Canada) for detection. In the presence of patient's IgG the release of activation peptide, i.e. FXIII-A transformation to truncated FXIII-A (FXIII-A') is slightly slower than in the presence of normal IgG.

In the next experiment 4.16 µg/mL FXIII truncated by 20 U/mL thrombin for 10 min was incubated with the antibody (400 µg/mL patients' or normal IgG) for 60 min. The truncated FXIII was then activated by 10 mM Ca²⁺ and FXIII activity was determined. The patient's IgG exerted considerable (86%) inhibitory activity. In these arrangements the combined effect of the autoantibody on the Ca²⁺ induced FXIII-A₂ activation and on the transglutaminase activity of FXIIIa was measured.

Finally, the patient's IgG (400 µg/mL) was incubated with 4.16 µg/mL thrombin and Ca²⁺ activated FXIII for 60 min and then the transglutaminase activity was measured. Again the inhibition of FXIIIa by the patient's IgG was considerable (85%).

3.3. Third patient

3.3.1. Case description and diagnosis

An 82 year-old woman presented to the emergency room with visual symptoms that progressed to complete visual loss in her left eye. A large ecchymosis was noted in her right arm. A left occipital hemorrhagic stroke was diagnosed by head CT scan. Previously, the patient went through several surgical procedures (knee and hip replacements, operation with adenocarcinoma complicated by hepatic infiltration) without abnormal bleeding. At admittance routine laboratory investigations, including blood count and coagulation screening tests, were normal. However, the clot solubility test was grossly abnormal, and FXIII activity was below the limit of detection. A Bethesda-Nijmegen assay revealed the presence of an anti-FXIII inhibitor with a titer >16 BU.

Following the detection of neutralizing anti-FXIII antibody in the Bethesda-Nijmegen assay, an anti-FXIII-A ELISA was developed to follow the antibody titer in parallel with FXIII activity measurements. The initially high antibody titer (1:128) significantly decreased by day 6 of prednisone+cyclophosphamide therapy, but remained between 1:4-1:16 titer until day 13. In parallel, FXIII activity, undetectable during the first days, started to raise somewhat, but fluctuated at a low level (between 1.4% and 4.3%). In the meantime plasmapheresis was initiated on day 9 and weekly administration of Rituximab started on day 12. A significant elevation of FXIII activity was measured on day 15 and 16 while the antibody decreased. (It is to be noted however, that the starting dilution in titer determination was 1:50, and a titer of 2 represented detectable antibody at a dilution of 1:100.) In this situation replacement therapy was started on day 16. 1250 IU human plasma derived FXIII concentrate (Corifact; CSL Behring, King of Prussia, PA) was administered daily for 4 consecutive days. The substitution therapy sharply increased plasma FXIII activity up-to 82%. After the cessation of substitution FXIII activity started to decline with an approximate half-life of 2 days, but did not decrease below 1%. On day 39 FXIII activity was 4.6% and by days 68 and 106 it reached the level (above 30%) required for appropriate hemostasis in non-stress situations. The patient was eventually discharged to a rehabilitation facility. A few months later due to the recurrence of her

adenocarcinoma invading bile duct and intra-hepatic biliary tracts the patient's condition deteriorated and she died of presumed septic shock.

FXIII antigens were monitored until day 39. As a tendency, FXIII-A₂B₂ antigen level followed FXIII activity. However, FXIII-A₂B₂ antigen was usually higher than FXIII activity. This is very likely due to the combined effect of the antibody. Binding of FXIII to the antibody might have accelerated its elimination from the circulation and decreased the antigen level. As the antibody might also inhibited the activation of FXIII and the activity of FXIIIa, these additional effects could explain the lower FXIII activity compared to FXIII antigen level.

As free FXIII-B concentration, expressed as percentage of average normal, cannot be used in such a comparison, here the mass concentrations of the three FXIII species were used. In the first days, due to the low FXIII-A₂B₂ level in the circulation, total and free FXIII-B antigen levels were practically identical. Free FXIII-B amounted to 50-60% of total FXIII-B level measured in normal plasma and corresponded to the free FXIII-B level present in the plasma of healthy individuals. After starting supplementation the two values deviated. Due to the administration of FXIII-A₂B₂ concentrate the total FXIII-B level well exceeded that of free FXIII-B. As in the later stage considerable amount of FXIII-A₂B₂ was present in the plasma, total FXIII-B level remained somewhat higher than free FXIII-B.

3.3.2. Determination of boundary parameters

The interaction between rFXIII-A₂ and the autoantibody was characterized by the following parameters: k_a : $1.52 \times 10^5 \text{ Ms}^{-1}$, k_d : $5.72 \times 10^{-4} \text{ sec}^{-1}$, K_a : $2.66 \times 10^8 \text{ M}^{-1}$, $K_d = 6.76 \times 10^{-9} \text{ M}$. The binding constants for FXIII-A₂B₂ were: k_a : $1.41 \times 10^5 \text{ Ms}^{-1}$, k_d : $8.17 \times 10^{-4} \text{ sec}^{-1}$, K_a : $1.65 \times 10^8 \text{ M}^{-1}$, $K_d = 1.34 \times 10^{-8} \text{ M}$. FXIII-B did not bind to the patient's IgG. The results clearly demonstrate that the autoantibody binds to both rFXIII-A₂ and FXIII-A₂B₂ with high affinity. The similar K_a values for the two FXIII species indicate that the antibody binds to non-complexed FXIII-A₂ and to FXIII-A₂ present in complex equally well, i.e. the presence of FXIII-B does not interfere with the binding. No binding to non-specific IgG was observed.

3.3.3. Determination of the inhibitory capacity of autoantibody

FXIII-A₂B₂ was incubated with the patient's IgG or non-specific IgG and then FXIII activity was measured. 50% inhibition was achieved at 170 µg/mL IgG concentration and 500 µg/mL IgG would have been sufficient to block FXIII completely. The low IC₅₀ value suggests that the autoantibody present in the patient's plasma was well over the quantity required for the complete blockage of FXIII activation/activity. Non-specific IgG was without effect.

3.3.4. Classification of autoantibody

In the followings the separate effect on these events was investigated at saturating concentration of the patient's IgG. There was hardly any difference between the truncation of FXIII-A by thrombin in the presence of patient's IgG and non-specific IgG. In contrast, the patient's IgG exerted highly significant (83%) combined inhibition of the Ca²⁺ dependent FXIII activation/activity. The latter experimental set-up did not distinguish between effects on the activation of FXIII-A₂B₂ and on FXIIIa activity. In the last series of experiments the inhibitory effect of the patient's IgG on FXIIIa activity was investigated separately from its effect on the Ca²⁺ induced activation. In this case 71% inhibition of the transglutaminase activity was established.

4. Discussion

The colleague of our institute summarized the original reports describing FXIII antibodies and their consequences. So far, there have been reports of 48 patients with bleeding symptoms caused by FXIII autoantibody. Five additional publications, summarizing FXIII autoantibodies were published as well. With the exception of the Japanese cases, the majority of patients were women. The age composition of non-Japanese cases showed that there was a younger age group of below 40 years, consisting mostly of women and a group above 60 years. In the first group, the appearance of autoantibodies was often associated with autoimmune diseases, while in the older age group it was often idiopathic. The two patients with autoimmune

FXIII deficiency we analyzed were from the elderly group (75 year old male and 82 year old female) and in the latter case the autoantibody was associated with malignant disease. In the male patient there was severe musculoskeletal haemorrhage, which is very common in this group of patients. In the case of the elderly lady, the intracranial bleeding was the first severe bleeding symptom.

In the first patient the homozygous novel nonsense mutation, c.127C>T in Exon 2, resulted in an early stop codon p.Gln42STOP, which made it unlikely that even traces of FXIII-A were synthesized. In her case, alloautoantibodies developed during substitution therapy, posing a tremendous therapeutic challenge, and resulting in two severe intracranial bleeding episodes. The incidence of alloantibody production against clotting factors in factor deficiency varies widely. Compared to severe haemophilia A, where the incidence of alloantibodies is approximately 30%, in FXIII deficiency, it is extremely rare. Her case highlights that alloantibodies, although a rarity, may develop in inherited FXIII deficiency and are very difficult to eradicate. Including our case, there are only **four** patients documented in the literature.

FXIII deficiency in general, autoimmune FXIII deficiency and alloantibodies against FXIII in particular is a challenging and often underdiagnosed condition; many considering that FXIII deficiency is the most underdiagnosed bleeding diathesis. The development and publication of the ISTH SCC guidelines on the diagnosis and classification of FXIII deficiency in recent years greatly improved the diagnostic efficiency of the inherited form of FXIII deficiency. Alas, the same can not be said for the diagnosis of FXIII antibodies. In the case of our three patients we pursued a diagnostic algorithm, which enabled the proper classification of the FXIII antibodies, the establishment of the correct diagnosis, and could be the basis of future diagnostic guidelines. We introduced novel techniques, previously not employed in the diagnostic process of FXIII deficiencies. We endorsed the diagnostic value of the Bethesda-Nijmegen assay in the characterization of neutralizing FXIII antibodies, however, this test does not provide information on several important characteristics of the autoantibodies. For this reason, we introduced IC₅₀ determination, a technique generally used in pharmacology for characterizing an inhibitor. Using this technique, we were able to demonstrate that low concentration of the patient's IgG, well below its plasma concentration, were capable of neutralizing a considerable amount of FXIII, far above the amount of FXIII concentrate to be administered.

A further step in the classification of neutralizing antibodies to determine whether the antibody is involved in activation of FXIII or activity of FXIIIa and what steps affect the inhibitor. The first step in the characterization of the inhibitory effect is monitoring the truncation of FXIII-A by thrombin using Western blot. Then, the effect of Ca^{2+} on thrombin cleaved FXIII is tested in the presence of the antibody. Such set-up tests the combined effect of the antibody on the activation by Ca^{2+} and on the activity of FXIIIa. As the effect on FXIIIa can be measured separately, one can deduce what the effect is on Ca^{2+} induced activation. Using these diagnostic approach, the neutralizing antibodies can be characterized. The IgG isolated from our first patient's plasma exerted a multiple effect by inhibiting both thrombin and Ca^{2+} induced FXIII activation and also interfering with the transglutaminase activity of FXIIIa. The fast elimination of FXIII from the circulation was indicated by the accelerated clearance of substituted FXIII. It was concluded that in her case the anti-FXIII-A alloantibody combined subtype with multiple neutralizing effect. In the second patient's case although the primary target of the antibody was FXIIIa (corresponding to type II inhibition), due to some inhibition of the release of activation peptide by thrombin, the pattern rather corresponded to It was concluded that in her case the anti-FXIII-A alloantibody has to be considered as of combined subtype with multiple neutralizing effect. In the second patient's case although the primary target of the antibody was FXIIIa (corresponding to type II inhibition), due to some inhibition of the release of activation peptide by thrombin, the pattern rather corresponded to type IV inhibition. In our third case, the autoantibody failed to influence the cleavage of FXIII-A, but also exerted a combined effect: it bound to FXIII-A with high affinity accelerating the decay of FXIII in the plasma and inhibited the activity of FXIIIa.

In the characterization of severe acquired FXIII deficiency caused by an autoantibody against either of the FXIII subunits, the first step is the determination of antibody target, and the binding affinity. Among modern techniques available for measuring binding affinity, like microcalorimetry, thermophoresis, SPR, the latter was selected in our study. In all three patient we determined that the antibody didn't bound to FXIII-B, and showed similar binding affinity to both FXIII-A₂ and FXIII-A₂B₂. The presence of FXIII-B in the complex did not influence the binding of the antibody to FXIII-A. In the case of the alloantibody, instead of IgG, FXIII was used as ligand in the measurement, and K_d was published in that article. In the case of the two

autoantibodies IgG was used as ligant, and Ka value was subsequently published. Nonetheless, it can be concluded, that the allo/auto antibodies bound with high affinity to FXIII-A. The SPR technique could be used in the characterization of antibodies against other clotting factors as well.

Due to the lack of a unified guideline, the treatment of these patients is rather eclectic. FXIII substitutions aren't effective in most cases, and even in those patients the effect is temporary. There is a consensus, that the first priority is the eradication of the antibody. This can be done by either eliminating the antibody, or by eradicating the antibody producing cells. The techniques used in the former (plasmapheresis, immunoabsorption) lack effectiveness. After elimination, the antibody quickly reappears, as it's produced anew. In some cases immunosuppression therapy (steroids, cyclophosphamide, rituximab) seems to be effective. Additionally IVIG infusion can be administered. In the case of the patient with the alloantibody, our Canadian colleagues tried to induce immunotolerance according to the Malmö protocol (cyclophosphamide, IVIG, high dose FXIII). Immunotolerance induction was tried three times with transitory improvement in patient health for one year and half year respectively, but in the end hemostasis could be controlled temporarily by administering 30 IU/kg FXIII concentrate daily (the monthly dose of a usual FXIII deficient patient). The life expectancy of all three patients was poor. In the first patient, after a minor head injury, due to the alloantibody subdural and multiple intracranial hematoma developed, leading to the patient's death. The second patient passed away after uncontrollable bleeding from a retroperitoneal hematoma. The third patient showed signs of clinical improvement, but the autoantibody persisted, and he had very low levels of FXIII. After several months he died because of a septic shock and adenocarcinoma, so we don't know what effects the continuous presence of the antibody might have had. The three case history presented above, as well as numerous fatal outcomes published in the literature suggest the importance of a treatment guideline, and the need for new therapeutic approaches.

In summary, we think that the various methods presented proved their usefulness in complete antibody characterization. It is presumed that such characterization may lead to better laboratory evaluation of autoimmune FXIII deficiencies and perhaps also to more appropriate therapeutic approaches.



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List of publications related to the dissertation

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