Project K 129287: Molecular and cellular interactions of coagulation factor XIII B-subunit

Scientific report

Coagulation factor XIII exists in two major forms. In the plasma it forms a hetero-tetramer of two potentially active FXIII-A (a pro-transglutaminase) subunits and two protective/inhibitory FXIII-B subunits (FXIII-A2B2). Before starting the project our knowledge on the structure and function of FXIII-B was rather limited. The other form of FXIII was FXIII-A that lacked FXIII-B and, in addition to the plasma, it existed in the intracellular compartment of A few cell types (e.g., platelets, monocytes/macrophages). The aim of our study targeted two major interrelated topics: 1/ To obtain more information on the structure and function of FXIII-B, 2/ How the lack of FXIII-B influences the cellular behavior of FXIII-A.

1/ The structure and function of
Novel structural/functional aspects of coagulation factor XIII

As the first step in the planned research project, we collected and evaluated the data, available on the structure of coagulation factor XIII (FXIII), including our former results. In a commentary review paper (Bagoly and Muszbek J Thromb Haemost 17: 714-6, 2019), based on the most up-to-date results we depicted the structure of the tetrameric plasma FXIII and its changes during the process of activation by thrombin and Ca2+. FIGURE 1 below shows that, as a results of FXIII activation, the non-active (or rather only potentially active) FXIII-A2 subunits become transformed into active monomeric transglutaminase enzyme, while the protective/inhibitory B subunits (FXIII-B) become separated from FXIII-A and remain associated as dimers.

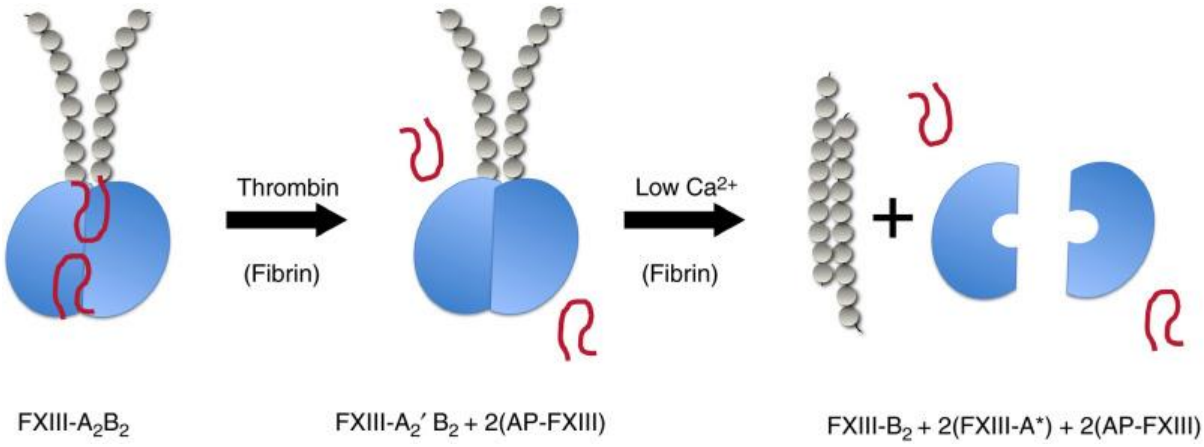

FIGURE 1. Schematic representation of proteolytic FXIII activation (Bagoly and Muszbek J Thromb Haemost 17: 714-6, 2019). *The A subunits of (FXIII-A) are depicted in blue. FXIII B subunits (FXIII-B) are shown as elongated strands that consist of gray pearls representing individual sushi domains. Activation peptides (AP-FXIII) proteolytically removed from FXIII-A by thrombin are shown as red loops. FXIII-A´: FXIII-A cleaved by thrombin; FXIII-A*: FXIII activated by thrombin and Ca2+ (active transglutaminase).*

The glycan structure of FXIII-B and its physiological significance

FXIII-B is a glycoprotein, but the composition and the function of the glycan structure have not been explored earlier. In our study first the glycans bound to FXIII-B were analyzed. They were enzymatically sequentially removed from the protein and the individual sugar moieties were identified by capillary electrophoresis. PNGase F in the traditionally used concentration completely removed N-glycans from the denatured protein. Complete de-glycosylation of the native protein was achieved by repeated digestion at elevated PNGase F concentration.

TABLE 1 The clearance of non-deglycosylated (nD) and deglycosylated (D) FXIII-B from the plasma of FXIII-B knock out (KO) mice.

	Glycosylated FXIII-B (µg/mL)			
	interval after FXIII-B injection			
FXIII-B KO mice	1 hour	48 hours	120 hours	Weight of mice (g)
1nD	10.97	0.570	0.019	20.3
2nD	10.22	0.677	0.045	23.8
3nD	12.48	0.762	0.049	22.0
4nD	12.68	0.609	0.040	24.9
5nD	9.78	0.585	0.032	25.9
6nD	12.17	0.673	0.040	19.8
7nD	11.42	0.635	0.049	19.3
mean	**11.39**	**0.644**	**0.039**	**22.3**
SD	1.13	0.070	0.010	2.6
	Deglycosylated FXIII-B (µg/mL)			
	interval after FXIII-B injection			
FXIII-B KO mice	1 hour	48 hours	120 hours	Weight of mice (g)
1D	5.18	0.0064	<0.001	22.0
2D	4.17	0.0059	<0.001	22.4
3D	3.68	0.0060	<0.001	23.8
4D	4.11	0.0078	<0.001	25.6
5D	4.05	0.0055	<0.001	19.9
6D	4.89	0.0066	<0.001	19.4
mean	**4.35**	**0.0064**	**<0.001**	**22.2**
SD	0.57	0.0010		2.3

The total N-glycan profile of FXIII-B featured 9 individual structures. The core structure contained two N-acetyl glucose amines, three of which were fucosylated. Each structure contained at least one sialic acid. This is the first report on the comprehensive characterization of the glycan moieties attached to FXIII-B. Introducing a de-glycosylation workflow for the native protein was important to design downstream experiments aiming to understand the biological relevance of FXIII-B glycosylation. In the next series of experiments, we explored the functional/structural role of the glycan moiety. To achieve this goal two major technical problems were solved: 1/ we succeeded in the complete de-glycosylation of native FXIII-B, 2/ we were able to produce FXIII-B knock out mice using the CRISPR/Cas9 technology.

We showed that de-glycosylation did not influence the dimeric structure of FXIII-B. Clearance studies in FXIII-B knock out mice revealed a robust difference in the elimination rate of native and de-glycosylated human FXIII-B2 suggesting that the glycan moiety considerably prolongs the lifespan of FXIII-B in the circulation (TABLE 1). Highlights of the study: 1. Characterization of the glycan moieties attached to FXIII-B was reported for the first time. 2. Complete de-glycosylation of the native protein was achieved by a de-glycosylation workflow. 3. It was shown that the associated glycan structure is not required for FXIII-B dimer formation. 4. The presence of protein-linked glycan prolongs the half-life of FXIII-B and FXIII-A2B2 in the plasma. The results were published in the J Thromb Haemost (Hurják et al. 18:1302-1309, 2020).

FXIII in the human cornea

In our former studies it was shown that human tear contains both FXIII-A and FXIII-B and their concentration becomes highly elevated following penetrating keratoplasty. We extended our study to the presence of FXIII subunits in ocular tissues. FXIII-A but not FXIII-B was detected in corneal keratocytes. 68±13% of CD34+ keratocytes expressed FXIII-A (FIGURE 2). Their distribution in the corneal stroma was unequal; they were most abundant in the subepithelial tertile. Cellular FXIII (cFXIII; FXIII-A2) was of cytoplasmic localization. In the stroma 3.64 ng cFXIII/mg protein was measured. The synthesis of cFXIII by keratocytes was confirmed by RT-qPCR. Isopeptide cross-links the products of transglutaminase action were detected above, but not within the corneal stroma. Slight abnormality of the cornea was detected in about half of FXIII-A deficient patients. The presence and the synthesis of cFXIII in the human keratocytes was established for the first time. The results indicate that cFXIII might be involved in maintaining the stability of the cornea and in the corneal wound healing process. Neither keratocytes nor corneal epithelial cells expressed FXIII-B. However, it was detected in the surrounding vasculature, and it can access corneal FXIII-A in the case of injury or inflammation. We published the results in the International Journal of Molecular Sciences (Orosz et al. 2019; 20:5963).

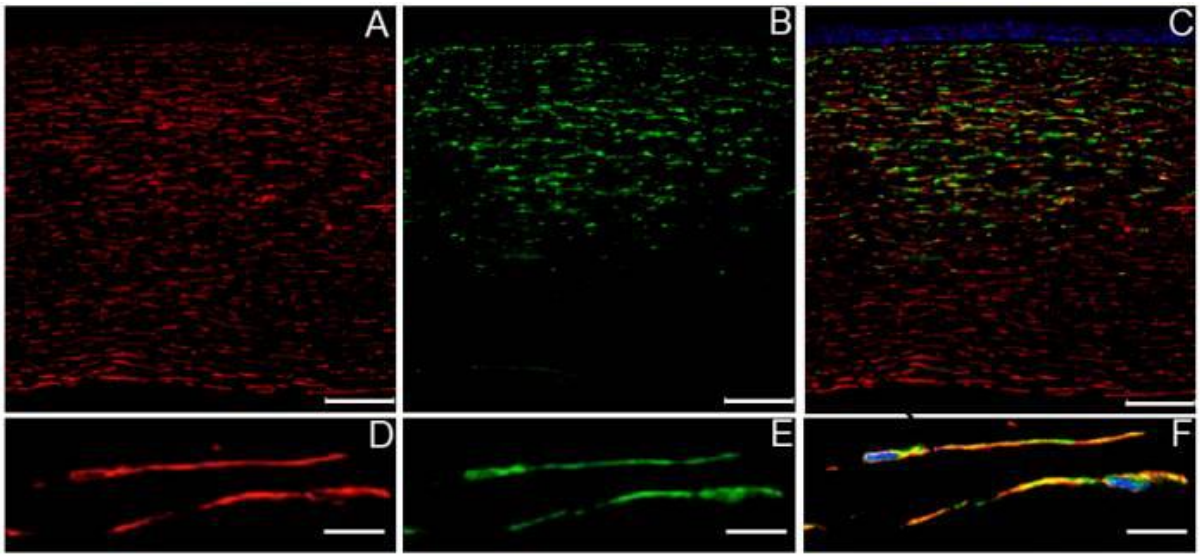

*FIGURE 2 The distribution of CD34 positive keratocytes (**A**) and FXIII-A-positive cells (**B**) and their appearance in overlay picture (**C**) in a cornea section. In the lower part of the figure, two individual keratocytes were selected from the cross-section and shown at higher magnification. CD34 and FXIII-A positivity appear in red and green (**D**,**E**), respectively. The overlay picture (**F**) indicates their different distribution within the cell (DAPI labeled nuclei appear in blue). The scale bars represent 100 µm in A–C and 10 µm in D–F.*

Cell surface exposure of FXIII on activated platelets depends on the type of platelet activation

A further topic we started to investigate was the surface exposure of cFXIII (FXIII-A2) during platelet activation. I addition it was also intended to reveal if FXIII-B can combine with surface exposed FXIII-A. We demonstrated that robust receptor mediated platelet activation by the combination of collagen receptor activating agonists, convulxin (CVX) and the PAR 1 receptor activator thrombin (Thr), results in the trans-location of cFXIII to the cell surface. Similar translocation of the pro-coagulant phospholipid, phosphatidyl serine (PS) was also induced by the combined agonist stimulation. Continuing this line of experiments using quantitative flow cytometry we demonstrated that activation by CVX+Thr exposed cFXIII to the surface of over 60% of platelets and platelet derived microparticles. This finding was confirmed by immune fluorescent labeling for cFXIII (FIGURE 3).

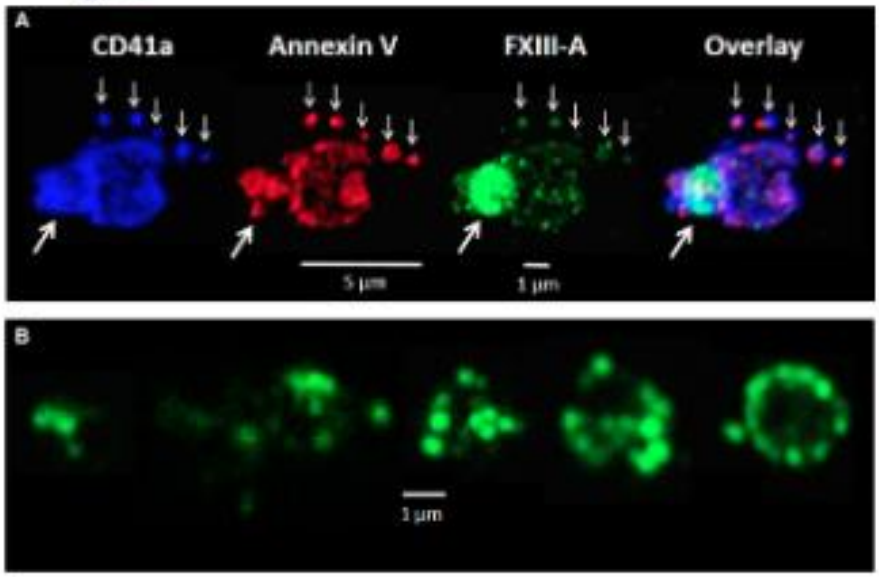

FIGURE 3 Surface labeling of platelets activated by receptor mediated mechanism and of the formed microparticles using immunofluorescent markers. A, Platelet activated by convulxin+thrombin stained for CD41a (blue), annexin V binding (phosphatidylserine labeling, red) and factor XIII A subunit (FXIII-A; green). The small white arrows point to microparticles, while the thick white arrows point to the cap-like structure on an activated platelet. B, FXIII-A positive clumped microparticles formed from platelets activated by convulxin+thrombin. The images are representative of experiments performed on platelets from three different donors

Immune electron microscopy revealed microparticles with preserved membrane structure and microparticles that lacked labeling for membrane glycoprotein CD41a (FIGURE 4). Surface localized cFXIII was observed on both types of microparticles but was more abundant in microparticles (cytoplasmic fragments) not labeled for the membrane marker CD41a. Non-receptor mediated activation of platelets by the Ca2+-ionophore, calcimycin also induced the translocation of PS to surface of platelets, however calcimycin induced activation failed to expose cFXIII. Results measuring the intracellular elevation of Ca2+ level by the two types of platelet activation suggested that the intracellular elevation of Ca2+ concentration is sufficient for the translocation of PS from the internal layer of the membrane, while the translocation of cFXIII from the platelet cytoplasm requires additional receptor mediated mechanism(s) preceding the liberation of Ca2+ from its intracellular storage sites. The surface exposed cFXIII, which consists only FXIII-A subunits could not bind FXIII-B and the active site inhibitor T101 inhibited the surface exposure of cFXIII. The findings indicate that elevation of intracellular Ca2+ concentration is sufficient for the translocation of PS from the internal layer to the membrane surface, while the translocation of cFXIII from the platelet cytoplasm requires additional receptor mediated mechanism(s).

The importance of Ca2+-independent part in the receptor mediated activation pathway was demonstrated by the inhibitory effect of rhosin, an effective RhoA inhibitor. It was also shown that surface exposed FXIII only consists of A subunits in activated form unable to combine with external FXIII-B subunit. Results of the study was published in the Journal of Thrombosis and Haemostasis (Somodi et al, 20: 1223-35, 2022).

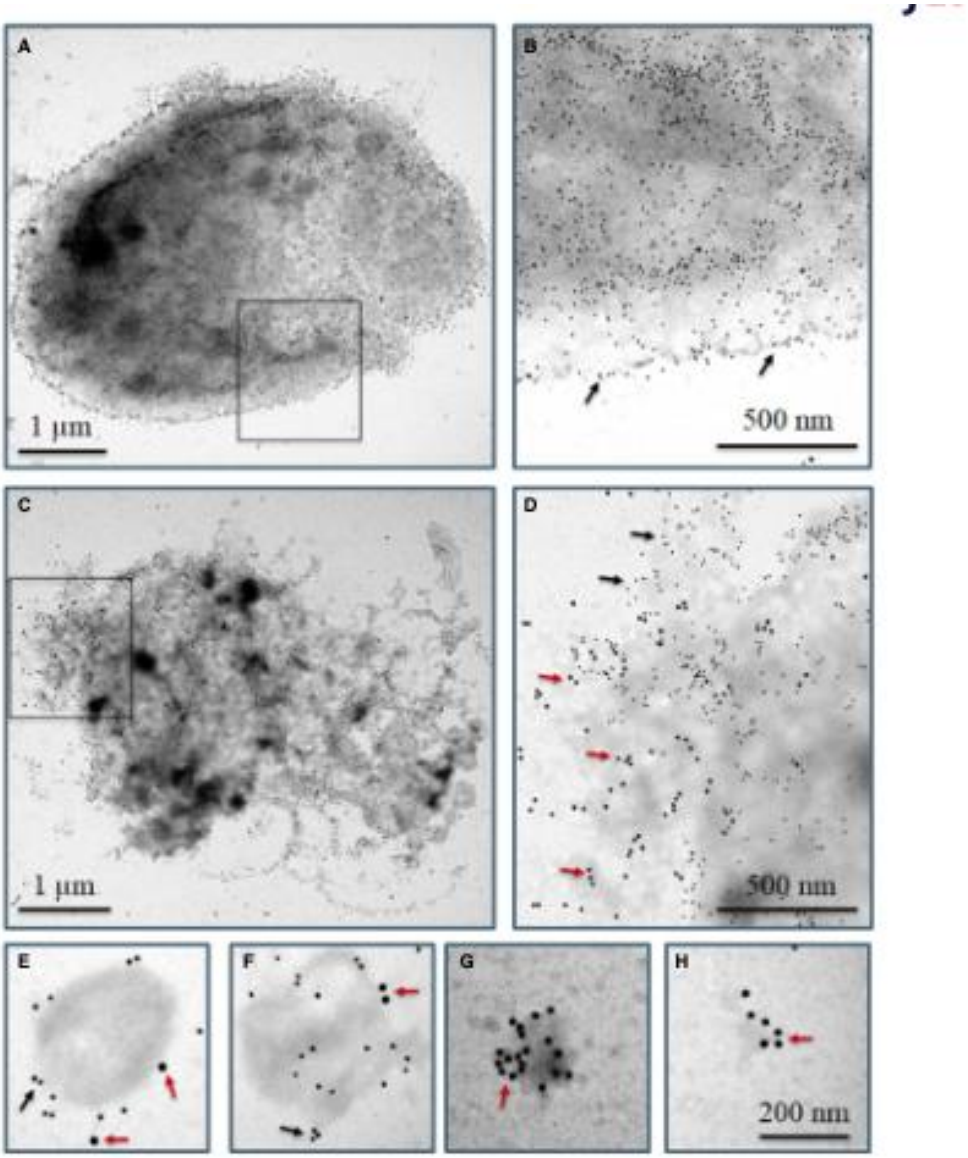

FIGURE 4 Surface labeling of resting and convulxin+thrombin (CVX+Thr) activated platelets using immunogold technique. CD41a antigen and factor XIII A subunit (FXIII-A) are represented by 10 nm and 15 nm gold particles, respectively. A few 15 nm gold particles are depicted by small red arrows; for comparison a few 10 nm gold particles are indicated by small black arrows. A, Non-stimulated whole platelet, (B) higher magnification of the selected area shown in (A), (C) a CVX+Thr stimulated whole platelet, (D) higher magnification of the selected area shown in (C). E–H, Microparticles formed from CVX+Thr activated platelets, (E, F) microparticles labeled for CD41a and cFXIII demonstrate at least partially intact membrane, (G, H) cFXIII positive microvesicles without CD41a membrane labeling. The images are representative of experiments performed with platelets from three different donors

Factor XIII in human macrophage-derived foam cells

Macrophages are major cellular constituents of the atherosclerotic plaque; they may stabilize the plaque by cross-linking structural proteins and they may become transformed into foam cells by accumulating oxidized LDL (oxLDL). The combination of oxLDL staining by Oil Red O and immunofluorescent staining for FXIII-A demonstrated that FXIII-A is retained during the transformation of cultured human macrophages into foam cells. ELISA and Western blotting techniques revealed that the transformation of macrophages into foam cells elevated the intracellular FXIII-A content. This phenomenon seems specific for macrophage-derived foam cells; the transformation of vascular smooth muscle cells into foam cells fails to induce a similar effect. FXIII-A containing macrophages are abundant in the atherosclerotic plaque and FXIII-A is also present in the extracellular compartment. The protein cross-linking activity of FXIII-A in the plaque was demonstrated using an antibody labeling the iso-peptide bonds. Cells showing combined staining for FXIII-A and oxLDL in tissue sections demonstrated that FXIII-A-containing macrophages within the atherosclerotic plaque are also transformed into foam cells. Such cells may contribute to the formation of lipid core and the plaque structurization.

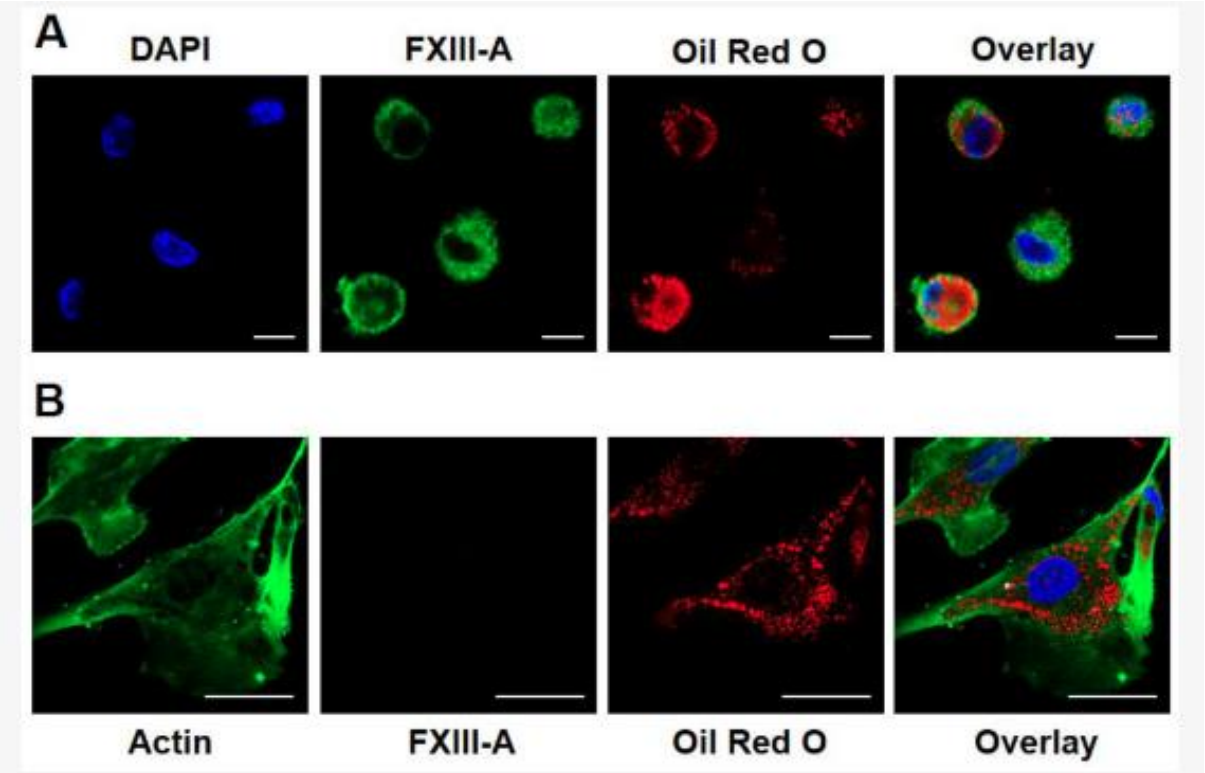

*FIGURE 5. FXIII-A expression in foam cells of different origin. (**A**) FXIII-A in macrophages transformed into foam cells by oxidized LDL ingestion. FXIII-A appears in green, while oxidized LDL is shown in red color. Scale bars correspond to 10 µm. (**B**) The lack of FXIII-A in vascular smooth muscle cells transformed into foam cells by ingestion of enzyme-modified non-oxidized LDL. Smooth muscle actin is depicted in green, while modified LDL is represented by red color. Scale bars correspond to 50 µm.*

The study was published most lately in the International Journal of Molecular Science (Somodi et al. 24: 4802, 2023).

International collaborative study to assign value for total Factor XIII-B subunit antigen to the WHO 1st International Standard for Factor XIII Plasma, (02/206)

Communication from the ISTH SSC Subcommittee on Factor XIII and Fibrinogen. For the ELISA used by participating laboratories we provided the anti-FXIII-B monoclonal antibodies, and the assay was carried out as we designed. Combination of results produced an overall mean of 0.98 units/mL with an inter-laboratory variability (geometric coefficients of variation - GCV%) of 18.3% [95% confidence interval: 0.86-1.11]. Real-time and bench stability studies indicated good stability and preservation of the FXIII-B subunit analyte in the WHO 1st IS FXIII Plasma (02/206). Following agreement by study participants, ISTH/SSC Experts, WHO-ISTH Liaison Group and the SSC Board, the WHO/ECBS established the current WHO 1st IS Factor XIII plasma (NIBSC code 02/206) by additionally assigning it with a Total FXIII-B subunit antigen value of 0.98 IU/ampoule. (Raut et al. J Thromb Haemost 2022; 20: 521-31, 2022). This study was an important step improving the reproducibility and accuracy of FXIII-B measurement in human plasma.

Terminal Phase Components of the Clotting Cascade in Patients with End-Stage Renal Disease

A key component of the last stage of coagulation cascade is FXIII which circulates in association with fibrinogen and in activated form, it works on fibrin by cross-linking fibrin chains and α_2PI to fibrin. Hemostasis disorder in patients with end-stage renal disease (ESRD) is frequently associated with bleeding diathesis but it may also manifest in thrombotic complications. Analysis of individual coagulation and fibrinolytic factors may shed light on the background of such a paradox situation. We explored components essential for fibrin formation/stabilization in ESRD patients being on maintenance hemodiafiltration (HDF) or hemodialysis (HD). Pre-dialysis fibrinogen, factor XIII (FXIII) antigen concentrations and FXIII activity were elevated (FIGURE 6), while α_2-plasmin inhibitor (α_2PI) activity was decreased. The inflammatory status, as characterized by C-reactive protein (CRP) was a key determinant of fibrinogen concentration, but not of FXIII and α_2PI levels. During a 4-h course of HDF or HD, fibrinogen concentration and FXIII levels gradually elevated. When compensated for the change in plasma water, i.e., normalized for plasma albumin concentration, only FXIII elevation remained significant. There was no difference between HDF and HD treatments. Individual HDF treatment did not influence α_2PI activity, however after normalization it decreased significantly. HD treatment had a different effect, α_2PI activities became elevated but the elevation disappeared after normalization. Elevated fibrinogen and FXIII levels in ESRD patients might contribute to the increased thrombosis risk, while decreased α_2PI activity might be associated with elevated fibrinolytic potential. Results were summarized in the following article: Pénzes et al. Int J Mol Sci 21: 8426, 2020.

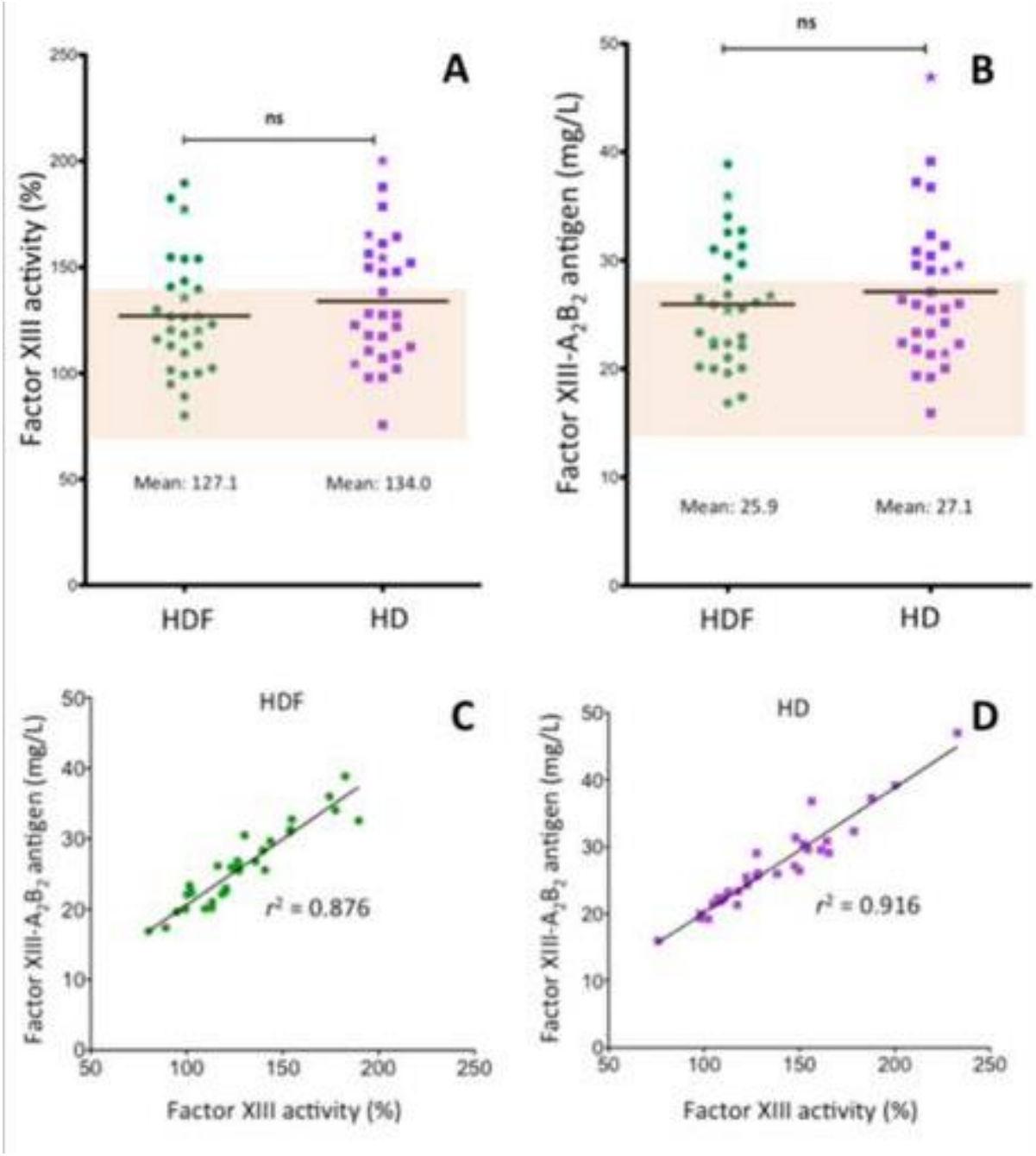

FIGURE 6. FXIII levels in the plasma of ESRD patients. Plasma FXIII activity (A) and antigen concentration (B) of patients being on chronic HDF treatment or after two weeks of HD treatment. (C) and (D): correlation of the results obtained by activity measurement and immuno-assay.

Antibodies against FXIII

Hemorrhagic diathesis due to anti-factor XIII (FXIII) autoantibody is a rare but severe disorder. Challenges of the diagnosis and treatment is demonstrated by the case of a 67-year-old female without previous bleeding history, who suffered huge muscular hematoma. In this interesting clinical case FXIII deficiency was caused by an autoantibody against FXIII-A subunit. It was demonstrated that such an autoantibody, although it decreases FXIII-A level and blocks FXIII activation/activity, is without effect on the plasma level of FXIII-B. In a previous study we demonstrated that FXIII-A level is regulated by FXIII-B, the present finding shows that vice versa this is not the case. Without blank subtraction 18% plasma FXIII activity was measured; however, after correction for blank the activity was below the limit of detection and the lack of fibrin cross-linking in the patient's plasma confirmed the latter result. FXIII-A_2 antigen was not detectable by enzyme-linked immunosorbent assay (ELISA); however, it was well detected by western blotting. The autoantibody showed high affinity toward FXIII-A_2. Its considerable inhibitory activity was demonstrated by high titer in Bethesda units and the low immunoglobulin G concentration required for inhibition. The main biochemical effect was the inhibition of Ca^{2+} -induced activation. Eradication therapy was only partially successful. Interestingly, four months after the last hemorrhagic event the patient suffered deep vein thrombosis complicated by pulmonary embolism. The findings were described in the following publication: Bovet et al. J Thromb Haemost 18: 1330-1334, 2020.

In a previous publication (Blood 2014 123: 1757-63) a monoclonal anti-FXIII-B antibody that prevented the interaction between the FXIII-A and FXIII-B subunits was developed. The antibody reacted with the recombinant combined first and second sushi domains of FXIII-B, and its epitope was localized to the peptide spanning positions 96 to 103 in the second sushi domain. We synthesized the corresponding peptide and checked it inhibitory effect on the subunit's interaction. Our future aim is to prevent the interaction between the two FXIII subunits. It is envisioned that preventing the binding to FXIII-B a limited decrease of FXIII-A with antithrombotic potential could be achieved. The publication concerning these results is under preparation.

FXIII and vascular smooth muscle cells

As described earlier macrophages and macrophage-derived foam cells express FXIII and contribute to the structural organization of atherosclerotic plaque. Our aim was to explore if human aortic smooth muscle cells (HAoSMCs), another major cell type in the atherosclerotic plaque, may also express FXIII. However, osteoblastic transformation induced by Pi and Ca2+ failed to elicit the expression of cellular FXIII in HAoSMCs. The negative results prompted us to investigate how active FXIII (FXIIIa) influence these cells. EZ4U, CCK-8 and CytoSelectWound Healing assays were used to investigate the effect of external FXIII on the proliferation and migration of HAoSMCs. The Sircol Collagen Assay Kit was used to monitor collagen secretion. Thrombospondin-1 (TSP-1) levels were measured by ELISA. Cell-associated TSP-1 was detected by the immunofluorescence technique. The TSP-1 mRNA level was estimated by RT-qPCR. Activated recombinant cFXIII (rFXIIIa) increased cell

proliferation (FIGURE 7) and collagen secretion. In parallel, a 67% decrease in TSP-1 concentration in the medium and a 2.5-fold increase in cells were observed. TSP-1 mRNA did not change significantly. These effects of FXIIIa might contribute to the pathogenesis of atherosclerotic plaques. The results were published in the International Journal of Molecular Science (Bogáti et al. 2022; 23:5845).

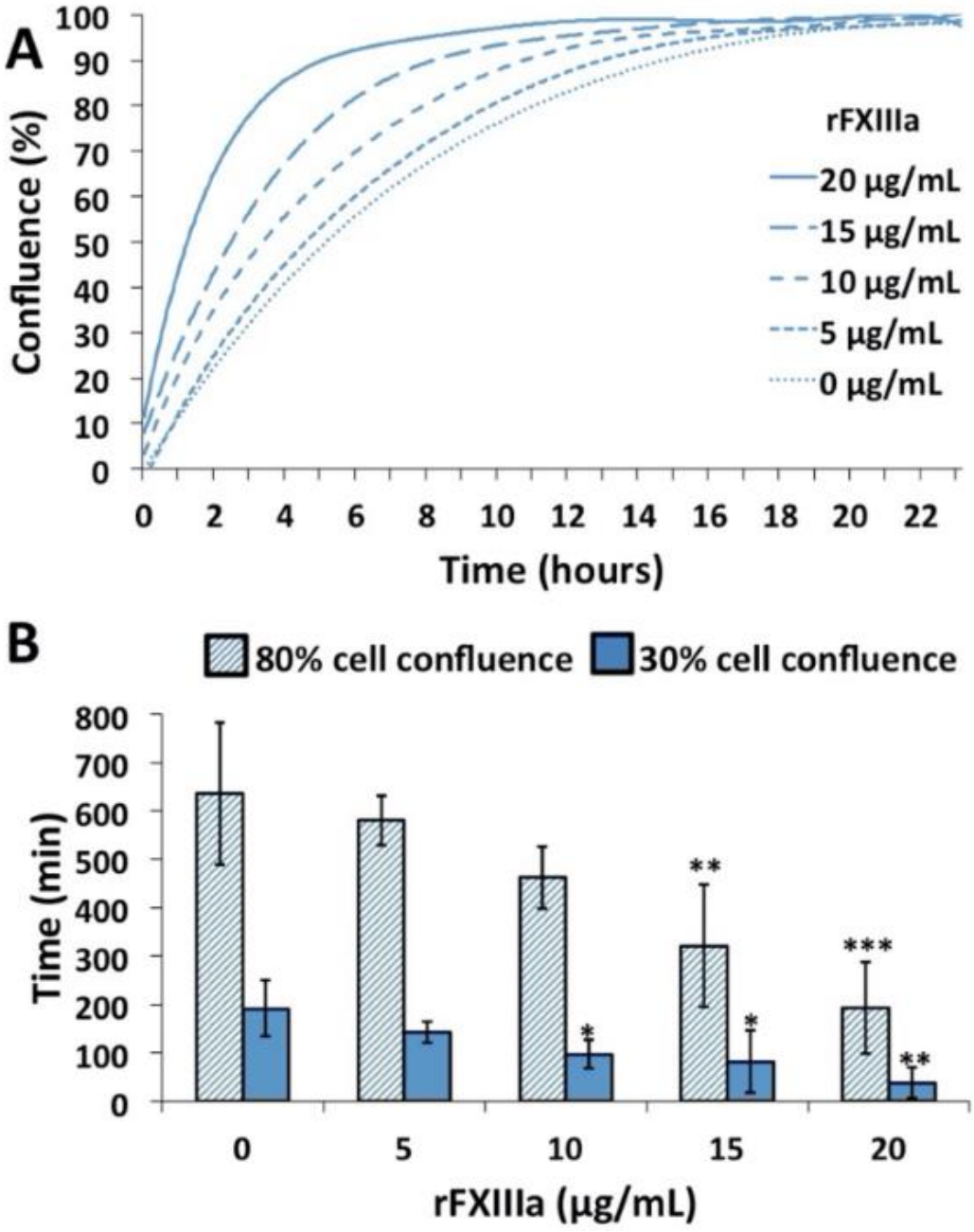

*FIGURE 7. Recombinant active FXIII (rFXIIIa) accelerates the closure of in vitro gap wound in human aortic smooth muscle cell culture. (**A**) After removing the insert from the cell culture, the gradual closure of the cell-free gap by proliferating and migrating cells at various rFXIIIa concentrations was continuously monitored by real-time microscopy. (**B**) The time required for reaching 30 and 80% cell confluence was determined. The statistical comparison is between time 0 and the respective time points; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.*