NKFIH K 113097
Final report

The main topic of the project concerned the B subunit of coagulation factor XIII (FXIII). The tetrameric FXIII consists of two catalytic A and two inhibitory/protective B subunits (FXIII-A_2B_2). As we have published earlier (Katona et al. Blood 2014;123:1757-63), 99% of FXIII-A is part of the complex, while approximately 50% of FXIII-B exists in free non-complexed form. While the structure, function and clinical importance of FXIII-A has been widely investigated, the role of FXIII-B has been explored to a much lesser extent. The aim of the project was to shed light on certain structural, functional characteristic of FXIII-B, its interaction with FXIII-A2 and fibrinogen. Clinical implications of FXIII-B levels and polymormisms were also explored.

A/ Structural and functional biochemical investigations.

Investigations on the structure of FXIII-B.
Our earlier investigations and a report from Japanese scientists (Souri et al. Biochemistry 2008;47:8656-64, Katona et al. Blood 2014;123:1757-63) suggested that the interaction site in FXIII-B is located to the first two sushi domains. For this reason we concentrated on the structure of these sushi domains. Using homology modeling and molecular dynamic data three-dimensional models for sushi domains 1 and 2 were constructed (Fig. 1A, B). Then, the model was further developed into a structure containing both the first and the second domains (Fig. 1 C).

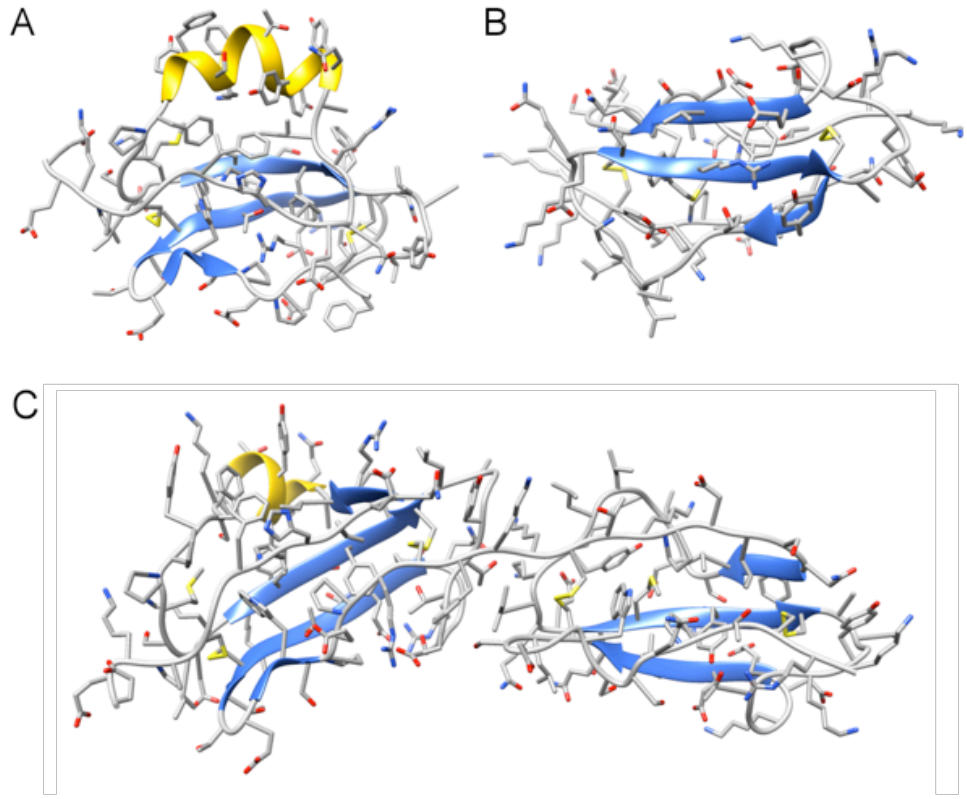

Figure 1. A) The homology model of the first sushi domain of FXIII-B. B) The homology model of the second domain. C) Refined structure of the domain "pair"

Due to the limited experimental data available, we have performed "blind" docking of the sushi domains to FXIII-A_2. Although multiple structures were obtained in all three docking runs with at least three binding sites for both domains (Figure 2), we have failed to identify a "definitive" binding site for any of them. Unfortunately, our excellent molecular modeling and dynamic expert (dr. István Komáromi) passed away during the last year due to melanoma and could not completely close this part of the project. However, even at that stage the in silico experiments could provide a lead to the possible targets protein chemistry investigations, which in turn could further improve our docking strategy.

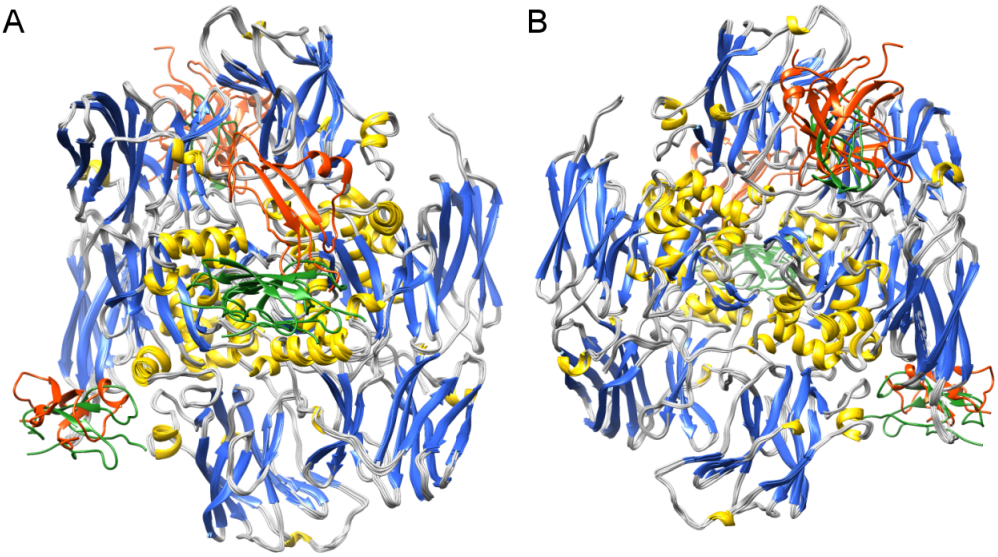

Figure 2. Three possible binding sites of sushi domain 1 (orange) and sushi domain 2 (green), predicted by molecular docking. (Structures with the highest score from the three top-rated clusters created by HADDOCK.)

A further study on the structure of FXIII-B was to explore the monomeric or dimeric state of free FXIII-B. Using fluorescent energy transfer experiments we were able to confirm the results of Souri et al. (Biochemistry 2008;47:8656-64) on the dimeric structure of free FXIII-B in plasmatic conditions. In addition it was also shown that by dilution the dimers dissociate and the subunits assume monomeric structure.

<u>The interaction of FXIII-B with FXIII-A_2.</u>

To study the role of the first two FXIII-B sushi domains in the binding of to FXIII-A_2 we decided to construct individual sushi domains. We were able to express recombinant FXIII-B sushi domains 1+2 in insect cells; however, the production of individual recombinant sushi domains 1 and 2 in these cells remained unsuccessful. In collaboration with the Department of Medical Chemistry, University of Szeged first the chemical synthesis of sushi domain 2 and then that of sushi domain 1 was achieved. It was proven by mass spectrometry that the two disulfide-bonds in the synthetic sushi domains were properly formed and these individual sushi domains can be used in binding experiments. In surface plasmon resonance (SPR) studies FXIII-B sushi domain 2 did not bind to FXIII-A_2; experiments with sushi domain 1 are in progress.

The interaction of FXIII-B with FXIII-A_2 might be influenced by FXIII-B polymorphisms. A common polymorphism in the Asian and European population is intron K nt29756 C>G exchange, which results in the replacement of 10 C-terminal amino acids by 25 novel amino acids. It was suspected that the polymorphism might influence the binding of FXIII-B to FXIII-A_2 and this way it might influence the plasma level of FXIII complex. The hypothesis was addressed by SPR technique. The Kd for the interaction of wild type FXIII-B has been established earlier as 3.14×10^{-10} M. We prepared FXIII-B intron K mutant proteins from the plasma of individuals homozygous for the intron K polymorphism. The Kd with the mutant protein was $8{,}43 \times 10^{-9}$ M, i.e., the mutant protein bound to FXIII-A_2 with less affinity than the wild type. The effect of intron K nt29756 C>G polymorphism on FXIII levels was investigated on 268 healthy individuals. The presence of FXIII-B intron K G allele drastically decreased FXIII activity and FXIII-A_2B_2 antigen levels (Mezei et al. *Thromb Res* **2016**;*148*:101-6). It is suggested that less tight binding somewhat increases the level of free FXIII-A_2 (which is only 1% in the presence of wild type FXIII-B), which is quickly eliminated from the circulation. These findings prompted us to investigate the relationship of FXIII polymorphisms and the risk of thrombotic diseases (see later).

<u>The binding of FXIII-B to fibrinogen.</u>

In the plasma FXIII is bound to fibrinogen, but the structural basis of the interaction was not known. The γ' fibrinogen chain, which contributes 8% to the total fibrinogen chains, is 20 amino acids longer than the γA chain. The extra 20 amino acids C-terminal peptide in the γ' is highly acidic and contains two sulfated tyrosine residue. γA/γA (peak 1) and γA/γ' (peak 2) fibrinogen were separated by ion exchange chromatography. There are contradictory results on the binding of FXIII to γA/γA and γA/γ' fibrinogen. Mosesson (J Thromb Haemost 2005;3:1894-904) published that FXIII binds to the γ'-chain, while Byrnes et al. reported that FXIII binds to the γ-chain 390-396 residues through the B subunit. The 390-396 sequence is present in both the γA and the γ' chains. Using ELISA techniques significant amount of plasma FXIII-B was measured in the γA/γ' fibrinogen (the fibrinogen:FXIII-B molar ratio was 17.4). FXIII (FXIII-A_2B_2) was also bound to the γA/γ' variant, in this case the molar ratio was 749. γA/γA fibrinogen contained much less (1000-fold less) FXIII-B and FXIII-A_2B_2. The results were also confirmed by Western blotting technique. Binding experiments using surface plasmon resonance (SPR) technique showed similar binding to both fibrinogen variants, which is not surprising because peak 2 fibrinogen already contained FXIII. To further explore the problem we synthesized two peptides, the sequence of one peptide corresponded to 390-396 residues of the γ-chain (γA peptide), the other one corresponded to the extra C-terminal peptide present on the γ'-chain with sulfated tyrosine residues (γ' peptide). A Kd 6.13×10^{-8} was estimated by SPR for the interaction of FXIII-B and the γ' peptide. For the short γA peptide SPR could not be used, in this case a Kd of 2.4×10^{-8} was measured by isothermal titration calorimetry (ITC). The results suggest that FXIII-B binds to the γA sequence present in both the γA and γ' chains and the γ' peptide represent another binding site for FXIII-B in the γ' chain. This finding might explain the different FXIII content of peak 1 and peak 2 fibrinogen. FXIII-B bound to the single binding site on γA/γA fibrinogen is lost during ion exchange chromatography, while the double binding sites on γ'-chain results in much stronger binding that resists the conditions of ion exchange chromatography. This latter part of the study is ready for publication.

<u>Analyzing the carbohydrate side chain of FXIII-B.</u>

FXIII-B is N-glycosylated at Asn142 and Asn525. We intended to study the functional significance of the carbohydrate side-chains, i.e. its role in extending the life-span of FXIII-A_2B_2 complex in the circulation. However, we had to realize that the composition of these side chains has not been fully revealed. FXIII-B prepared from human plasma was denatured and digested by PNGase-F endoglycosidase. The released florescent-labeled glycans were analyzed by capillary electrophoresis. Six of the nine well separable peaks were identified and sialidase digestion was used for the identification the remining three peaks. Finally the following structures were revealed: A3G(4)3S(6)3, A2G(4)2S(6)2, A2BG(4)2S(6)2, F(6)A2G(4)2S(6)2, F(6)A2BG(4)2S(6)2, A2[3]BG(4)1S(6)1, A2G(4)2S(6)1, A2BG(4)2S(6)1, F(6)A2[3]G(4)2S(6)1. We attempted to release the carbohydrate from non-denatured FXIII-B, but so far only partially succeeded.

B) Development of an immunoassay for the determination of free FXIII-B in the plasma

Earlier we developed an ELISA type immunoassay that measured total FXIII-B antigen level (Ajzner et al Blood 2009;113:723-5). First we tested a slightly modified version of this assay in clinical conditions to establish total FXIII-B concentration in the plasma of FXIII-A deficient patients. In a patient with anti-FXIII-A alloantibody the total FXIII-B level was 30% of normal average (Pénzes et al. *Haemophilia* **2016**;*22*:268-75), in two patients with acquired FXIII-A deficiency due to anti-FXIII-A autoantibody the total FXIII-B level was 46% and 50-60% respectively (Pénzes et al. *J Thromb Haemost* **2016**;*14*:1517-20, Kun et al. *Haemophilia* **2017**;*23*:590-7). These findings demonstrated that in addition to inherited FXIII-A deficiency the total FXIII-B level also decreased in acquired deficiencies due to anti-FXIII-A antibodies.

For the determination of free, non-complexed FXIII-B in the plasma two monoclonal anti-FXIII-B antibodies were produced, one of which bound only to free FXIII-B and inhibited complex formation with FXIII-A_2, the other one bound to both free and complexed FXIII-B. The combination of these two antibodies in a sandwich ELISA resulted in a sensitive immunoassay. The clinical usefulness of the assay was tested on plasma samples of a patients receiving FXIII-A concentrate. It was shown that before the administration of FXIII total and free FXIII-B was the same, i.e. only free FXIII-B was present in the plasma, while following plasma FXIII (FXIII-A_2B_2) administration the two values deviated, the total FXIII-B level well exceeded that of free FXIII-B (Kun et al. *Haemophilia* **2017**;*23*:590-7). The above three publications were included in the submitted PhD thesis of Mária Kun.

C/ Clinical investigations

<u>The effect of FXIII-B p.His95Arg and intron K polymorphisms on the risk of myocardial infarction and on FXIII level</u>

In a nested case-control study we explored the effect of coagulation factor XIII-B subunit polymorphisms on the risk of coronary artery disease, and on FXIII levels. The following two polymorphisms were investigated: p.His95Arg and intron K nt29756 C>G polymorphisms. In the study, 687 patients admitted for coronary angiography to investigate suspected coronary artery disease and 994 individuals representing the general Hungarian population were enrolled. The patients were classified according to the presence or absence of significant coronary atherosclerosis (CAS) and the history of myocardial infarction (MI). The p.His95Arg polymorphism did not influence the risk of CAS or MI. The FXIII-B intron K nt29756 G allele provided significant protection against CAS and MI in patients with a fibrinogen level in the upper tertile. However, this effect prevailed only in the presence of the

FXIII-A Leu34 allele, and a synergism between the two polymorphisms was revealed. Carriers of the intron K nt29756 G allele had significantly lower FXIII levels, and FXIII levels in the lower tertile provided significant protection against MI. It was suggested that the protective effect of the combined polymorphisms is related to decreased FXIII levels. The results were published in *Int J Mol Sci* Mezei et al. **2015**;*16*:1143-59 and it was part of the dr. Zoltán Mezei's PhD thesis (2017).

FXIII levels and frequency of FXIII-B polymorphisms in patients who suffered myocardial infarction at a young age; a case control study

In this study we evaluated the association of FXIII levels with MI in young patients. The effect of FXIII-A p.Val34Leu, FXIII-B p.His95Arg and F13B IVS11, c.1952+144 C>G (Intron K) polymorphisms on FXIII levels and on the risk of MI were also assessed. Patients with ST elevation MI below 40 (MI, n=119), age-matched clinical controls (CC, n=101) without MI and coronary artery disease and healthy controls (HC, n=120) were enrolled in the study. FXIII activity and FXIII-A_2B_2 antigen were significantly elevated in patients with the history of MI. FXIII activity and antigen were somewhat elevated in Arg95 and significantly decreased in Intron K "G" carriers. Smoking had an independent increasing effect on FXIII activity and FXIII-A_2B_2 antigen. Intron K C>G polymorphism significantly decreased the risk of MI in patients with elevated fibrinogen. Among the investigated factors Intron K C>G polymorphism and smoking have the most powerful effect on FXIII levels and on the risk of MI in the young. The increased FXIII level of smokers may contribute to their increased risk of MI. The manuscript summarizing the results has been submitted to the journal of *Mol Cell Biochem* and has been accepted with minor revision.

The effect of FXIII-B polymorphisms and FXIII levels on the risk of venous thromboembolism.

The aims of the study were 1/The determination of FXIII activity and antigen levels in patients with a history of VTE and to explore how they are influenced by sex and FXIII-B polymorphisms. 2/ To reveal the association of FXIII levels and FXIII-B polymorphisms with the risk of VTE. 218 VTE patients and equal number of age and sex matched controls were enrolled in the study. Adjusted FXIII activity and FXIII-A_2B_2 antigen levels were significantly higher in females with a history of VTE than in the respective controls. FXIII-B levels were significantly lower in male VTE patients than in controls. FXIII-A_2B_2 antigen levels in the upper tertile increased the risk of VTE in females (adjusted OR:2.52; CI:1.18-5.38). Elevated FXIII-B antigen level had a protective effect only in males (adjusted OR:0.19; CI:0.08-0.46). FXIII-B Intron K c.1952+144 C>G polymorphism significantly lowered FXIII activity, FXIII-A_2B_2 and FXIII-B antigen levels in both groups. FXIII-B polymorphisms did not influence the risk of VTE. The results were published in the journal *Thromb Res* Mezei et al. **2017**;*158*:93-7.