

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

**REGULATION OF PLASMA FACTOR XIII LEVELS IN
HEALTHY INDIVIDUALS;
FACTOR XIII AND THE RISK OF CORONARY ARTERY
DISEASE**

By Zoltán András Mezei, MD

Supervisor:

Prof. László Muszbek, MD, PhD, DSc, member of HAS



**University of Debrecen
Kálmán Laki Doctoral School**

Debrecen, 2017

**REGULATION OF PLASMA FACTOR XIII LEVELS IN HEALTHY INDIVIDUALS;
FACTOR XIII AND THE RISK OF CORONARY ARTERY DISEASE**

By Zoltán András Mezei, MD

Supervisor: Prof. László Muszbek, MD, PhD, DSc, MHASc

Kálmán Laki Doctoral School
University of Debrecen

Head of the **Examination Committee:** Prof. József Balla, MD, PhD, DSc

Members of the Examination Committee: Prof. Hajna Losonczy MD, PhD

Mariann Harangi, MD, PhD

The Examination takes place at the Library of the Division of Nephrology, Department of Internal Medicine, Faculty of Medicine, University of Debrecen, at 11:00 am, 22nd of September, 2017

Head of the **Defense Committee:** Prof. József Balla, MD, PhD, DSc

Reviewers: Prof. Riitta Lassila, MD, PhD

György Pfliegler, MD, PhD

Members of the Defense Committee: Prof. Hajna Losonczy MD, PhD

Mariann Harangi, MD, PhD

The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen at 13:00, 22nd of September, 2017

1. INTRODUCTION AND REVIEW OF LITERATURE

1.1. The structure of factor XIII

Blood coagulation factor XIII (FXIII) is a pro-transglutaminase that is present in the plasma (pFXIII) and in certain cells (cFXIII). pFXIII is a heterotetramer (FXIII-A₂B₂) that consists of two potentially active A subunits (FXIII-A) and two carrier/inhibitor B subunits (FXIII-B). pFXIII has a molecular mass of 326 kDa, its plasma concentration is 14-28 mg/L. In plasma 99% of FXIII-A circulates in complexed form, while 50% of total FXIII-B (tFXIII-B) circulates as free homodimer, the rest is bound to FXIII-A. Practically all pFXIII molecules are bound to fibrinogen (K_d 10^{-8} M). This association is independent of the presence of Ca^{2+} .

FXIII-A consists of 732 amino acids (molecular mass: 83 kDa), starting with an initiator methionine, followed by a serine. In the mature molecule after the removal of methionine the N-terminal serine undergoes N-acetylation. Amino acid numbering starting with this serine will be used in the followings. FXIII-A has four main structural domains and an N-terminal activation peptide (AP-FXIII, amino acids 1-37). The main structural domains are: a β -sandwich domain (amino acid 38–184), the catalytic core domain (185–515), β -barrel 1 (516–628), and β -barrel 2 (629–731) domains. Beside the active site cysteine (Cys314) there are eight cysteine residues in the molecule, none of which form disulfide bond. The amino acid sequence near the active site is typical for transglutaminases: Gly-Gln-Cys-Trp.

FXIII-A is expressed mainly in cells of bone marrow origin. Megakaryocytes and platelets contain large amount of cFXIII, which is a homodimer of FXIII-A (FXIII-A₂). In platelets FXIII-A amounts to 3% of total cellular protein. Platelet FXIII concentration is 100-150 fold higher than plasma FXIII concentration. cFXIII is also present in monocytes, in their bone-marrow precursor cells, also in monocyte derived macrophages and in tissue macrophages. FXIII-A has also been detected in chondrocytes, osteoblasts, and osteocytes. FXIII-A synthesized by these cells is responsible for the production of FXIII-A component of pFXIII, however the exact cellular site of synthesis and the liberation of cFXIII into the circulation is not known.

The gene encoding human FXIII-A (F13A1) is located on chromosome 6p24–25 and spans over 160 kb. The transcribed mRNA is 3.9-kb in length, consisting of an 84-bp 5'-

untranslated region, a 2.2-kb open reading frame, and a 1.6-kb 3'-untranslated region. F13A1 contains 15 exons and 14 introns. Exon I consists of the 5' noncoding region, and exon II encodes AP-FXIII. The β -sandwich domain is encoded by exons II-IV, the catalytic core domain by exons IV-XII, the two β -barrel domains are encoded by exons XII-XIII, and exons XIII-XV, respectively.

FXIII-B significantly prolongs the half-life of FXIII-A in the circulation. In recent years recombinant FXIII-A₂ (rFXIII-A₂) was introduced in the therapy of FXIII-A deficient patients. rFXIII-A₂ forms complex with the patient's FXIII-B and its half-life in the circulation (usually 9-14 days) is similar to that of FXIII complex. FXIII-B consists of 641 amino acids (molecular mass: 80 kDa), it contains 8,5% carbohydrate. FXIII-B is a mosaic protein, made up of ten so called sushi-domains. Each sushi domain is held together by a pair of internal disulfide bonds. Although there were some contradictory results, most of the literature suggest that in the plasma free FXIII-B circulates as homodimer (FXIII-B₂). In a recent study on the interaction of FXIII and fibrinogen Byrnes et al. demonstrated that "free" FXIII-B₂ is also bound to fibrinogen. FXIII-B is synthesized by hepatocytes, just like fibrinogen, and the two proteins might associate during or immediately after secretion.

The gene of FXIII-B subunit (F13B) is located at position 1q31–32.1. It is of 28 kb length and is composed of 12 exons producing a 2.2 kb mRNA. The exons are interrupted by 11 introns. Exon I encodes a 20-amino acid leader sequence. The sushi domains show a high degree of homology. They are encoded by exon II-XI, each sushi domain being encoded by a single exon. The homology of the sushi domains suggest gene duplication and exon shuffling during evolution. The last exon encodes the C-terminal region of FXIII-B, the 3'-untranslated region, and the polyA tail. F13B is directly regulated by two transcription factors: HNF1 α and HNF4 α .

The exact mechanism of FXIII-A to FXIII-B association is not fully elucidated. Using sequentially recombinant truncated B subunits Souri et al. concluded that sushi domain 1 is responsible for the binding of FXIII-B₂ to FXIII-A₂. Most recently, Katona et al. demonstrated that a monoclonal antibody against a peptide on sushi domain 2 selectively reacted with free FXIII-B and successfully prevented its complex formation with FXIII-A. Based on these results, the N-terminal part of FXIII-B seems to be involved in complex

formation. For FXIII-A the contact site is probably located in β -barrel 1 and β -barrel 2 domains. Deletions in FXIII-A located in these domains result in a lack of complex formation.

1.2. The activation of FXIII

During the final phase of the coagulation cascade FXIII is activated by the concerted effect of thrombin and Ca^{2+} . Initially thrombin cleaves off the AP-FXIII from the N-terminus of FXIII-A. Next in the presence of Ca^{2+} FXIII-B dissociates, and the truncated FXIII-A dimer (FXIII-A₂) is transformed into an active transglutaminase (FXIII-A*₂; FXIIIa). The latter step also requires the presence of Ca^{2+} . The activation process is greatly enhanced by the presence of fibrin. By binding to fibrin, the orientation of thrombin and pFXIII will favor the proteolysis of FXIII-A at Arg37-Gly38. FXIIIa remains associated with fibrin, in serum no thrombin-cleaved FXIII could be detected.

Activation of cFXIII in the intracellular environment is a non-proteolytic process. In the absence of FXIII-B the rise of intracellular Ca^{2+} concentration is sufficient to bring about structural changes of cFXIII due to which FXIII-A₂ assumes the active configuration (FXIII-A^o₂).

1.3. Biochemical and physiological function of FXIII

FXIIIa, like all active transglutaminases, catalyzes an acyl-transfer reaction in two major steps. First a peptide bound glutamine substrate forms a binary complex with the enzyme. A thioacyl intermediate is formed between the carboxamide group of the glutamine side chain and the active site Cys314 during which ammonia is released. In the next step, if a substrate primary amine group is present, the acyl group is transferred to the acyl acceptor primary amine and the amine becomes attached to the glutamyl residue via an isopeptide bond. In the absence of an amine substrate the thioacyl intermediate is hydrolyzed, and the glutamine residue is deamidated. If the primary acceptor amine is an ϵ -amino group of a peptide-bound lysine residue, the peptide chains become covalently cross-linked ($\epsilon(\gamma$ -glutamyl)lysyl is formed).

The primary physiological substrates of FXIIIa are the α - and γ - chains of fibrin and α 2 plasmin inhibitor (α 2PI) . FXIIIa cross-links fibrin γ -chains into dimers and α -chains into high molecular weight polymers that improves the mechanical strength of the newly formed fibrin clot and protects it from the shear stress of circulating blood. The formation of γ -chain dimers is a quick process, and a minimal amount of FXIIIa is sufficient to catalyze it. The formation of the high molecular weight α -chain polymers is a slower process, and requires multiple cross-linking of α -chains through acyl donor and acyl acceptor sites. In small amounts γ - α chain heterodimers and γ -chain trimers and tetramers are also formed. α 2PI is also cross-linked to fibrin α -chain by FXIIIa. Covalent attachment of α 2PI to fibrin is highly important in the regulation of fibrinolysis, it prevents newly formed clot from breakdown by plasmin. Beside α 2PI, plasminogen activator inhibitor 2, an inhibitor of urokinase-type plasminogen activator is cross-linked to fibrin by FXIIIa. Thrombin activatable fibrinolysis inhibitor is another substrate for FXIIIa. The physiological significance of the latter two cross-linking processes is questionable. In addition to proteins involved in hemostasis a broad range of FXIIIa substrates have been described, however their cross-linking in in vivo conditions and, if it occurs, the physiological implications remain to be proven.

The main functions of FXIII can be deduced from symptoms of FXIII deficient patients. The severe bleeding diathesis in non-supplemented patients with inherited FXIII-A deficiency clearly shows that factor XIII is essential for effective hemostasis. Umbilical stump bleeding occurs in a high percentage (80%) of FXIII-A deficient infants. Deficient patients frequently suffer of ecchymoses, subcutaneous and intramuscular bleedings. Life-threatening intracranial bleeding is reported in about 30% of cases. Inherited FXIII-B deficiency is less severe; in this case 5-10% non-complexed FXIII-A₂ is present in the plasma. Women with congenital FXIII deficiency cannot maintain pregnancy, the pregnancy is usually lost in the first trimester. Impaired wound healing is a relatively frequent finding in FXIII deficient patients, and the role of FXIII in wound healing was proven in FXIII deficient mice. It has been also suggested that FXIII in tears is involved in corneal wound healing, whereas high FXIII levels in tear may represent a risk factor for neovascularization by promoting angiogenesis. The proangiogenic effect of FXIII has been clearly demonstrated Dardik et al. In chondrocytes and osteoblasts both FXIII and tissue transglutaminase 2 are present, with possible roles in extracellular matrix formation, assembly, and stabilization.

A few publications also demonstrate the intracellular role of cFXIII. During platelet activation only a part of cFXIII becomes activated by a non-proteolytic mechanism. Platelet FXIII-A₂ cross-links cytoskeletal elements and is involved in certain phases of the platelet spreading. cFXIII has also been implicated in the motility of monocyte-derived dendritic cells and in the phagocytosis of peripheral blood monocytes.

1.4. FXIII subunit polymorphisms

In F13A1 a number of common polymorphisms with amino acid exchange were described: FXIII-A p.Val34Leu (c.103G>T, rs5985), FXIII-A p.Tyr204Phe (c.614A>T, rs3024477), FXIII-A p.Leu564Pro (c.1694C>T, rs5982), FXIII-A p.Val650Ile (c.1951G>A, rs5987) and FXIII-A p.Glu651Gln (c.1954G>C, rs5988). Among the FXIII-A polymorphisms the Val to Leu exchange at position 34, first described by Mikkola et al., was intensively studied. The frequency of the rare Leu34 allele is 23.3% in Caucasians, 11.7% in Africans and is extremely rare in Asians.

Considering that the location of FXIII-A p.Val34Leu polymorphism is just 3 amino acids upstream from the thrombin cleavage site, it's not surprising it has an influence on thrombin-induced FXIII activation. It was demonstrated with both cFXIII and pFXIII, that the thrombin-induced release of AP-FXIII from the Leu34 FXIII-A variant and the consequent activation of FXIII proceed at a 2.5-fold higher rate than in the case of the Val34 variant. Further studies on the binding of synthetic AP-FXIII to thrombin also supported this information. Faster activation of FXIII results in rapid fibrin cross-linking and in a larger amount of α 2PI incorporation into fibrin. The results of these initial experiments were verified in the more complex environment of human plasma and led to a similar conclusion regarding the effect of the FXIII-A p.Val34Leu genotype on thrombin-induced FXIII activation.

Initially the effect of the FXIII-A p.Val34Leu polymorphism on the specific activity of FXIIIa was controversial. In early studies FXIII was only partially activated by thrombin. In these experiments FXIII activity measurements expressed the rate of FXIII activation rather than the full catalytic activity, resulting in higher FXIII activity values for the Leu34 allele. It is now clear that the specific activity of fully activated pFXIII, cFXIII, and rFXIII-A₂ of different FXIII-A p.Val34Leu genotypes are identical. The structure of fibrin clots is

influenced by FXIII-A p.Val34Leu polymorphism, and this effect is modulated by fibrinogen concentration. At high fibrinogen levels, plasma samples from homozygotes for the Leu34 allele form clots with a looser structure, thicker fibers, and increased permeability, while at low fibrinogen concentrations the fibrin meshwork had thinner, more tightly packed fibers, and lower permeability. In the plasma samples of wild-type individuals no fibrinogen concentration-dependent changes were observed. The biochemistry of other common FXIII-A polymorphisms has not been investigated in details.

The polymorphic nature of FXIII-B was demonstrated a long time ago using isoelectric focusing techniques. On the basis of isoelectric focusing experiments, three major population-associated phenotypes were described: FXIII-B*1, FXIII-B*2, and FXIII-B*3, characteristic of European, African, and Asian populations, respectively. Molecular genetic and biochemical techniques revealed two major polymorphisms in the F13B gene. An A to G transversion within exon 3 (rs6003) leads to a His to Arg amino acid exchange at position 95 in the mature protein. The minor allele (Arg95) is relatively rare (7%) in the European population, but it represents the major allele (68%) among black Africans.

The polymorphism did not influence FXIII-A, tFXIII-B, or pFXIII antigen levels in a combined group of controls and patients with vascular disease. Increased subunit dissociation was found in plasma from subjects possessing the Arg95 allele. However, when the variants were purified to homogeneity and binding was analyzed by steady-state kinetics, no difference was observed, therefore further experiments are needed to clarify its exact physiological properties.

In 2009 a C-to-G change at nucleotide position 29756 in intron K (c.1952+144 C>G, rs12134960) leading to a novel splice acceptor site was described in the F13B gene. This polymorphism results in an allele-specific splicing product, in which the last 10 amino acids are exchanged by an alternative sequence consisting of 25 amino acids. The variant sequence includes two additional lysine and one glutamic acid residues. These charged amino acids change the isoelectric point of the protein. The polymorphism that corresponds to FXIII-B*3 characteristically occurs in Asians (67% in East Asians and 32% in South Asians). In African and European populations the allele frequency was found to be 3%, and 16%, respectively. Although such a profound structural change introduced by the polymorphism would be

expected to alter some of the biochemical features of the molecule and may have an effect on disease susceptibility, these possibilities have not been explored.

1.5. Determinants of FXIII level in the plasma

Both genetic and non-genetic factors are involved in the regulation of plasma FXIII levels. A single study evaluated the effect of age, gender, smoking and hypertension on FXIII subunit and activity levels in elderly healthy individuals (age: 63.8 ± 16.8 years). pFXIII concentration in individuals with the different FXIII-A p.Val34Leu genotypes did not differ significantly suggesting that their secretion and plasma half-life are similar. Using likelihood analysis with adjustment for age and gender 47% heritability was established for the plasma level of complex FXIII (FXIII-A₂B₂) in healthy families. FXIII-A p.Val34Leu polymorphism accounted only for a small fraction of this heritability. The Phe204 allele of the FXIII-A p.Tyr204Phe polymorphism was reported to be associated with decreased pFXIII level and activity, whereas the Leu564 allele of the p.Pro564Leu variant resulted in lower FXIII plasma level with increased FXIII activity. However, these results obtained with non-validated methods needs to be confirmed. As the common FXIII-B polymorphisms were discovered later than the analysis reported in reference, evidently they were not included in the study. To date no study has investigated the effect of FXIII-B polymorphisms on plasma FXIII levels in healthy individuals.

1.6. Factor XIII in thrombotic diseases

Clinical studies suggest that FXIII might contribute to the risk of thrombotic diseases. In early studies mostly negative results were obtained on the association of levels and coronary atherosclerosis (CAS) or myocardial infarction (MI). However, the results of these studies, were not analyzed according to gender and FXIII activity was determined by an assay strongly influenced by FXIII-A p.Val34Leu polymorphism. In our laboratory it was shown that FXIII activity and FXIII-A₂B₂ antigen levels in the upper tertile increased the risk of MI 3-fold in females but not in males. Similarly, elevated FXIII level increased the risk of peripheral artery disease in women, but not in men. No clear picture emerged from the few studies on the association of FXIII level and the risk of ischemic stroke which partly due to

the lack of distinction between atherothrombotic and cardioembolic stroke. Most recently it was reported that genetic markers associated with low tFXIII-B levels increased the risk of ischemic stroke of cardioembolic subtype.

In the first report on the association of FXIII-A p.Val34Leu polymorphism and coronary artery disease (CAD) Kohler et al., demonstrated the protective effect of Leu34 allele against MI. Both confirmatory and contradictory results were reported in follow-up studies. It was presumed that gene-gene and gene-environment interactions might be responsible, at least in part, for the variability of the findings obtained by different laboratories. Indeed, we have demonstrated that the Leu34 allele decreased the risk of CAD only in patients with an elevated fibrinogen concentration. The overall protective effect of Leu34 allele against CAD was confirmed by a meta-analysis of reported findings.

Only a few studies investigated the effect of FXIII-B polymorphism and the risk of atherothrombotic disease. In a study by Reiner et al., the homozygous presence of the FXIII-B Arg95 allele lowered the risk of nonfatal MI in postmenopausal women. The Arg95 allele was associated with an increased mortality (1.7 fold increase after cerebral ischemia of arterial origin) in young women. Macrae et al. investigated the effect of FXIII-B p.His95Arg, FXIII-B intron K c.1952+144 C>G and FXIII-A p.Val34Leu polymorphisms in patients with abdominal aortic aneurysm. Of the three polymorphisms only the Arg95 allele was found to be associated with a moderately increased risk of aortic aneurysm (relative risk: 1.240, CI 1.093–1.407, $p = 0.006$). A genome-wide association study indicated that FXIII-B polymorphisms might be important factors in assessing the risk of ischemic stroke, however individual polymorphisms were not pinpointed.

Elevated FXIII activity was associated with a small decrease in deep venous thrombotic risk. However, the FXIII activity assay used in this study was influenced by the presence of the FXIII-A p.Val34Leu polymorphism, and the measured elevated FXIII activity and protective effect could be the result of relatively high proportion of individuals carrying the Leu34 allele in non-thrombotic controls. In this study FXIII-A and tFXIII-B antigen levels in individuals with and without the history of venous thromboembolism (VTE) did not differ, and FXIII subunit levels were not associated with the risk of thrombosis. Similarly, in a more recent longitudinal study elevated FXIII-A levels did not influence the risk of VTE. However,

in this study mean FXIII-A level both in individuals with and without VTE was above the upper limit of reference interval (142% and 141%, respectively).

The protective effect of FXIII-A p.Val34Leu polymorphism against deep vein thrombosis was first reported by Catto et al. Since then a number of contradictory and confirmatory studies were published, which were summarized in two meta-analyses. Both meta-analyses demonstrated a moderate protective effect of the Leu34 allele against VTE. The effect of Leu34 allele might be modulated by other factors. In an earlier publication the presence of the Leu34 allele was protective against deep venous thrombosis at fibrinogen concentration in the >90% percentile, but only in men. The effect of the polymorphism on the risk of thrombosis (arterial and venous combined) was investigated in patients with antiphospholipid syndrome, as well. FXIII-A p.Val34Leu polymorphism in neither study provided protection against thrombosis. However, in the study by de la Red et al. the Leu34 allele was protective against thrombosis in patients with fibrinogen level in the upper tertile (>3.40 g/L). There has been only a single study on the association of of FXIII-B polymorphisms and VTE. In this study FXIII-B p.His95Arg polymorphism was shown to be a risk factor of VTE.

2. OBJECTIVES

The aim of the PhD studies were:

1. To explore the effect of age, body mass index (BMI), smoking and fibrinogen level on FXIII activity, FXIII-A₂B₂ and FXIII-B antigen levels in males and females in a non-elderly healthy population.
2. To determine how the three FXIII polymorphisms, FXIII-A p.Val34Leu, FXIII-B p.His95Arg, FXIII-B intron K C>G and their combinations influence FXIII levels in healthy individuals.
3. To investigate the effect of FXIII-B p.His95Arg and FXIII-B intron K c.1952+144 C>G polymorphisms on the risk of CAD.

4. To determine how the three FXIII polymorphisms, FXIII-A p.Val34Leu, FXIII-B p.His95Arg, FXIII-B intron K c.1952+144 C>G influence FXIII levels in CAD patients.
5. To investigate the possible interaction between FXIII-B polymorphisms and FXIII-A p.Val34Leu polymorphism concerning FXIII levels and the risk of CAD.

3. MATERIALS AND METHODS

3.1. Cases and Controls

3.1.1. Study of FXIII levels and polymorphisms in healthy individuals

Two hundred and sixty-eight apparently healthy young and middle-aged adults from Eastern Hungary were enrolled in the study. Fifty-three individuals had moderate hypertension (between 145/90 and 165/95 Hgmm) and were on antihypertensive therapy, which was not considered as exclusion criteria. Among the 160 women 19 were taking oral contraceptive and 36 were in menopause. The BMI were calculated and smoking habit was recorded.

3.1.2. Study on FXIII levels and polymorphisms in CAD patients and clinical controls

Six hundred and eighty-seven consecutive patients admitted for coronary angiography to investigate suspected coronary artery disease were recruited for the study from a single center (Institute of Cardiology, University of Debrecen, Debrecen, Hungary) over a one and a half year period. Patients with $\geq 50\%$ stenosis in a major coronary artery or in one of its branches were graded as coronary atherosclerosis positive (CAS+), while patients with no or less significant stenosis were graded as CAS-. Patients with a positive or negative history of MI were classified as MI+ or MI-, respectively. MI was defined according to the Joint ESC/ACCF/AHA/WHF Task Force for the Universal Definition of Myocardial Infarction. Patients without significant coronary stenosis and with the lack of a history of MI were considered as the clinical control group (CAS-MI-) to which subgroups of patients with CAS and/or MI (CAS-MI+, CAS+MI-, CAS+MI+) were compared. Patients in the small CAS-MI+ group suffered MI in the absence of significant coronary stenosis. In this subgroup, the rupture of plaques that did not cause significant stenosis and/or coronary vasospasm must

have been responsible for the previous MI. The small number excluded any kind of meaningful statistical evaluation. A large number of individuals (n = 994) representing the general Hungarian population were recruited in the framework of the Hungarian General Practitioners' Morbidity Sentinel Stations Program and served as population controls for the study.

3.1.3. Ethical approval

All enrolled individuals were informed about the study according to the study protocol and gave written informed consent. Ethical approval for the study was obtained from the Regional Ethics Committee at the Medical Faculty, University of Debrecen, Hungary.

3.2. Laboratory Methods

Fasting blood samples were collected from the antecubital vein into vacutainer tubes (Beckton Dickinson, Franklin Lakes, NJ, USA) without anticoagulation or with anticoagulant (Ethylenediaminetetraacetic acid or 1/10 volume of 0.109 M citrate). Serum and platelet poor plasma were separated by centrifugation at 2500g for 20 min, and samples were stored at -80 °C until determination. From the buffy coat of citrated blood samples DNA was isolated by QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). DNA samples were stored at -70 °C until determination.

FXIII activity was measured by a one-step ammonia release assay designed in our laboratory and produced by Reanal-ker kft, Budapest, Hungary (REA-chrom FXIII kit). In this assay FXIII is activated by thrombin and Ca^{2+} , and crosslinks the amine substrate glycine ethyl ester to a glutamine residue of a specific oligopeptide substrate. During the reaction ammonia is released, which is continuously monitored by a glutamate dehydrogenase catalysed NADPH indicator reaction. The rate of consumption of NADPH is directly proportional to the FXIII activity, and is measured spectrophotometrically by the decrease of absorbance at 340 nm. The NADPH to NADP^+ conversion is linear between 5 and 10 minutes of the reaction measurement. A plasma blank measurement is done to compensate for FXIII-independent ammonia producing reactions.

For the measurement of plasma FXIII activity lyophilized NADPH was dissolved in 3 mL distilled water. The NADPH concentration in the solution was 0.8 mmol/L. The

lyophilized Activator reagent was dissolved in the NADPH solution. Detection reagent was also dissolved in 3 mL distilled water and equal volume of the two reagents were combined. This mixture was used for the preparation of blank and sample reagent solution. The blank reagent solution had 1/20 volume Inhibitor (23.2 mmol/L 2-iodoacetamide and 5.8 g/L Na-azide) added to 1 volume of reagent mixture. The sample reagent had 1/20 volume of Stabilizer solution (5.8 g/L Na-azide) added to 1 volume of reagent mixture. The final reagent had the following composition: 20 kU/L thrombin, 10 mmol/L CaCl₂, 5 mg/L polybrene, 2 mmol/L fibrin polymerization inhibitory peptide (GPRP), 0.1 mmol/L dithiothreitol, 4.4 mmol/L α_2 -plasmin inhibitor N-terminal (1-12) peptide, 5 mmol/L glycineethyl ester, 0.35 mmol/L NADPH, 20 kU/L GluDH, 0.6 mmol/L ADP, 7 mmol/L α -ketoglutarate and 5.4 g/L bovine serum albumin in 60 mmol/L HEPES buffer, pH 7.7.

In the assay 250 μ L sample or blank reagent was added to 25 μ L plasma sample. During the reaction the decrease in absorbance at 340 nm was monitored for 10 minutes at 37 °C. In the first 5 minutes of the reaction the endogenous ammonia present in the sample is decomposed, and FXIII becomes fully activated. By measuring the absorbance change between the 5th and 10th minutes, the $\Delta A/\text{min}$ of sample and blank reaction were calculated. From the $\Delta A/\text{min}$ measured with the sample the $\Delta A/\text{min}$ value measured in the blank reaction was subtracted. For calibration a plasma pool (plasma samples from 20 healthy individuals) was used and its activity was considered as 100%. FXIII activity was expressed as percentage of normal (pooled plasma) FXIII activity. The method is linear up to a 300% FXIII activity, bilirubin concentration below 200 μ mol/L and triglyceride concentration below 7.5 mmol/L do not interfere with the measurement.

FXIII-A₂B₂ and tFXIII-B antigen concentrations were determined by one-step sandwich ELISAs. For the measurement of FXIII-A₂B₂ antigen diluted plasma sample, peroxidase-labeled monoclonal anti FXIII-A tag antibody and biotinylated monoclonal anti-FXIII-B (capture) antibody were added to the wells of a streptavidin-coated microplate. The amount of the complex attached to streptavidin-coated microplate was quantitated by measuring peroxidase activity. Only FXIII-A₂B₂ reacted in this assay, non-complexed A or B subunits showed no reaction.

Calibrator (normal pooled plasma), control and patients plasma samples were diluted 1:1000 using a dilution buffer (0.5 mol/L NaCl, 3 mmol/L KH₂PO₄, 12 mmol/L Na₂HPO₄,

0.05% Polysorbate 20, 5 g/L bovine serum albumin). 70 μ L biotinylated monoclonal anti-FXIII-B antibody was added to the wells of a streptavidin-coated microplate, followed by the addition of 70 μ L diluted plasma sample and 70 μ L horseradish peroxidase-labeled anti-FXIII-A antibody. The plate was incubated and shaken for 1 hour at room temperature at 300 rpm. The incubation step was followed by washing the plate 4 times with 300 μ L/well washing buffer (0.14 mol/L NaCl, 3 mmol/L KH_2PO_4 , 12 mmol/L Na_2HPO_4 , 0.05% Polysorbate 20). The next step was the addition of 200 μ L substrate (3,3',5,5'-tetramethylbenzidine) followed by incubation at room temperature for 30 minutes. The enzymatic reaction was stopped by the addition of 50 μ L 2 mol/L H_2SO_4 . The absorbance of the formed colored complex was read at 450 nm in an ELISA reader. Measurements were carried out in duplicates, and the mean absorption value was used for the calculation of FXIII-A₂B₂ concentration. FXIII-A₂B₂ antigen concentration was expressed as percentage of normal (pooled plasma) FXIII-A₂B₂ concentration. The measurement of tFXIII-B antigen was carried out by a similar method. In this case the capture and tag antibodies were raised against different FXIII-B epitopes. The antibodies bound to free and complexed FXIII-B equally well.

The rate method described by Clauss was used for the measurement of fibrinogen concentration (Fibrinogén LX kit, produced by Reanal-ker kft, Budapest, Hungary). In this method the time of the clot formation induced by high concentration of thrombin shows a linear correlation with fibrinogen concentrations in a relative broad range (0.8-6,0 g/L). The inclusion of polybrene, a heparin antagonist, into the diluting buffer enabled the measurement of fibrinogen in presence of therapeutic heparin concentration. Lipid parameters, C-reactive protein (CRP), and homocysteine concentrations were measured by routine laboratory methods.

3.3. Determination of FXIII-A and FXIII-B subunit polymorphisms

FXIII-A p.Val34Leu was determined using a melting point analysis method, with fluorescence resonance energy transfer detection (FRET) as described by Shemirani et al. For the determination of FXIII-B p.His95Arg and FXIII-B intron K c.1952+144 C>G polymorphisms a dual color experimental protocol was developed, allowing the determination of both polymorphisms from a single reaction mix. The primers were purchased from

Integrated DNA Technologies (Leuven, Belgium) and Detection probes were synthesized by Kromat Ltd. (Budapest, Hungary).

The primers were designed to ensure that the amplicon length for FXIII-B p.His95Arg (amplified DNA sequence 228 bp) and FXIII-B intron K c.1952+144 C>G (amplified DNA sequence 226 bp) are similar. The similar length enables the amplification of the two sequences in a single PCR reaction. FXIII-B p.His95Arg sensor was designed to be complementary with the DNA sequence containing the mutated nucleotide, while the sensor probe for FXIII-B intron K c.1952+144 C>G was designed to be complementary with the wild type DNA sequence. The sensor were labeled with fluorescein at the 3' end and the anchors were labeled with LC610 for the FXIII-B p.His95Arg probe and with LC670 for the FXIII-B intron K c.1952+144 C>G probe, respectively. The gap between the sensor and anchor is 3 bp and 4 bp respectively. The two dyes in the anchors were chosen so that when the sensor and acceptor probe lie adjacent to each other on a DNA strand fluorescence resonance energy transfer could take place. The overlap of the spectra emitted by the LC610 and LC670 dyes is minimal.

Amplification with polymerase chain reaction (PCR), FRET detection and melting curve analysis were carried out in a LightCycler® 480 real-time PCR instrument (Roche, Mannheim, Germany). PCR reactions were carried out in a 96 well plate. The final volume of the reaction mixture was 20 µL. It contained 5 µL of sample DNA (20-100 ng/µL), 10 µmol/L of each primer, 2 µmol/L of each probe, 4 µL Genotyping Master mix (Roche) and 1 µL of 25 mM MgCl₂ (Roche). In each run positive controls for both polymorphisms (established by DNA sequencing) and negative controls (no DNA) were included. Cycling started with an initial denaturation step at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 10 s, annealing at 51 °C for 10 s and extension at 72 °C for 10 s. Melting curve analysis was one cycle at 95 °C for 1 min and at 45 °C for 1 min, followed by an increase of temperature to 75 °C with a ramp rate of 0.06 °C/s. Fluorescence signal was monitored during melting curve analysis for both LC610 and LC670. The derivatives of melting curves were used for analysis.

3.4. Statistical analysis

The distribution of parameters was examined by the Kolmogorov–Smirnov and Shapiro-Wilk tests. The results of continuous variables were expressed as the mean \pm SD, while the results of non-continuous variables were shown as the median and interquartile range. Between group differences were analyzed by Student's t test when normally distributed and by Mann-Whitney test when the distribution was non-parametric. Differences in category frequencies were evaluated by the χ^2 test. Pearson's correlation coefficient was calculated to characterize the strength of the linear relationship between two variables. Multiple linear regression analysis was performed to adjust for parameters independently associated with FXIII levels. The significance of differences in mean FXIII levels was tested by the analysis of variance (ANOVA) using the Bonferroni correction for multiple comparisons. The effect of each polymorphism was analyzed in logistic regression models and expressed as odds ratio (OR) and 95% confidence interval (CI). Adjusted ORs were obtained by the use of a model that included the polymorphism and all independently-associated parameters. A p-value of less than 0.05 was considered to indicate statistical significance. Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS 22.0, Chicago, IL, USA). The synergy factor was calculated as described by Cortina-Borja et al.

4. RESULTS

4.1. FXIII levels and polymorphisms in healthy individuals

4.1.1. Characterization of study population

Males and females were of similar age; there were more current smokers among males (29%) than among females (22%), but the difference was not statistically significant. Males had considerably higher BMI and somewhat lower fibrinogen level than females. In our population neither FXIII-A₂B₂ antigen level nor FXIII activity showed gender specific differences, while tFXIII-B concentration was significantly higher in males than in females.

In the case of the three investigated polymorphisms the distribution of genotypes followed the Hardy-Weinberg equilibrium in all subgroups and did not deviate significantly from the data in the 1000 Genomes Project (www.internationalgenome.org) for European

populations. As expected, the minor allele frequencies did not show gender specific differences in the study population.

4.1.2. Relation of non-genetic parameters to FXIII levels

Age positively and significantly correlated with all three FXIII parameters both in males and females. The relatively weak significant correlation between BMI and FXIII activity and FXIII-A₂B₂ antigen disappeared after adjustment, but remained in the case of tFXIII-B antigen. After adjustment to age and fibrinogen level no significant effect of current smoking on FXIII activity and FXIII antigen levels could be demonstrated in either sex. Plasma fibrinogen concentration significantly correlated with FXIII activity and FXIII antigen concentrations in a gender independent manner. This is not surprising because in the plasma practically all FXIII, including free FXIII-B is bound to fibrinogen. A highly significant correlation was between tFXIII-B antigen level and FXIII-A₂B₂ antigen level or FXIII activity.

4.1.3. The effect of major FXIII polymorphisms and their combinations on FXIII levels

FXIII-A Leu34 allele carriership did not influence FXIII activity, similarly to result published by Balogh et. al. tFXIII-B level was not affected, either. A slight decrease of FXIII-A₂B₂ antigen, with a low level of statistical significance, was observed in Leu34 carriers. However, when the effect of FXIII-B intron K polymorphism was eliminated, i.e., when the effect of Leu34 allele in intron K C homozygotes or in intron K G carriers was investigated separately even this low level of statistical significance disappeared.

Non-adjusted FXIII activity and tFXIII-B levels did not differ significantly among the groups of different FXIII-B p.His95Arg genotypes although there was a tendency of increase in the presence of the minor allele. Only in the case of complex FXIII-A₂B₂ antigen was the increase statistically significant. On the other hand, after adjustment, the increase in all FXIII parameters became statistically significant. We also investigated the effect of FXIII-B p.His95Arg polymorphism separately in individuals wild type for the intron K C allele and in individuals carrying the G allele. In this case the effect of Arg95 allele prevailed only in intron K G carriers, but due to the low number of individuals in this group the increase was not statistically significant.

The intron K c.1952+144 C>G polymorphism has a robust effect on FXIII levels. In intron K G carriers FXIII activity, FXIII-A₂B₂ antigen and tFXIII-B antigen are considerably lower than in wild type individuals. The differences were highly significant both in the non-adjusted and adjusted evaluations. In the latter case FXIII activity and FXIII-A₂B₂ antigen levels were adjusted to age and fibrinogen concentration, tFXIII-B antigen level was adjusted to age, gender, BMI and fibrinogen concentrations. The FXIII level lowering effect of intron K G allele prevailed independently of the FXIII-A p.Val34Leu polymorphism, i.e., the presence of intron K G allele resulted in decreased FXIII activity and FXIII complex antigen concentration both in Val34 homozygotes and in Leu34 carriers. Among the combinations the constellation of the two minor alleles had the most powerful effect on FXIII activity and FXIII-A₂B₂ levels. The difference between the Val34-intron K C and the Leu34 carrier-intron K G carrier subgroups was the highest, which suggests a kind of synergism between the two minor alleles. As mentioned earlier FXIII-A p.Val34Leu polymorphism was without effect on tFXIII-B antigen levels. The intron K G allele exerted its effect independently of FXIII-B p.His95Arg polymorphism, the FXIII-B p.His95Arg polymorphic variants did not influence the effect of intron K G allele on the measured FXIII parameters. In His95 homozygotes the decrease of FXIII levels in intron K G allele carriers was statistically significant, in Arg95 carriers, due to the low number of individuals, it did not reach the level of significance.

4.2. FXIII and coronary artery disease

4.2.1. Characterization of study population

The ratio of males was significantly higher in patients with CAS and/or with a history of MI. As compared to clinical controls (CAS–MI–), patients in the CAS+MI– and CAS+MI+ groups were 5–7 years older. Diabetes mellitus was more frequent among patients with CAS and/or MI than in the CAS–MI– group. The frequency of current smoking did not differ among the groups. Triglyceride and apoB levels were significantly elevated, and HDL-C was significantly decreased in both the CAS+MI– and CAS+MI+ groups. The decrease of apoA-I level and the increase of Lp(a) and fibrinogen concentrations were significant only in the CAS+MI+ group. Homocysteine levels were significantly higher in patients with CAS and/or MI than in clinical controls. FXIII activity and antigen levels were practically the same in all

study groups. FXIII levels were influenced by gender, smoking, serum total cholesterol and plasma fibrinogen concentrations, as was demonstrated by the multiple linear regression models in our study population. Antihypertensive treatment was uniformly between 59%–68% in all patient groups. The history of treatment length, intensity and efficiency was rather uncertain; thus, these data were not included and were not used in subsequent analyses.

The population control (PC) group consisted of 45% males and 55% females. The median age was 48 years (interquartile range: 34–57 years). When the patient groups were compared to the PC group, the ORs were adjusted for these two parameters.

4.2.2. The effect of the FXIII-B polymorphisms on the risk of CAD

The minor allele frequencies of both p.His95Arg and intron K c.1952+144 C>G polymorphisms in the CAS–MI– and PC groups were practically identical and were similar to the data from the 1000 Genomes Project. The distribution of genotypes in both control groups corresponded to the Hardy–Weinberg equilibrium. The allele frequencies in the patient groups did not differ significantly from those in the two control groups.

The Arg95 carriership was without effect on the risk of CAS or MI. In the case of the intron K polymorphism, the ORs were below 1.0 in all patient groups, but the level of protective effect conferred by this polymorphism did not reach statistical significance. Adjustment for independently-associated variables did not change the situation. Similar results were obtained when the ORs were separately calculated for males and females. Patients with CAS and/or MI were compared to the clinical control group, and ORs were calculated accordingly. Comparison to the PC group gave similar results.

4.2.3. The effect of the FXIII-B polymorphisms on the risk of CAD in individuals with elevated fibrinogen concentration

In a previous paper, we demonstrated that the FXIII-A p.Val34Leu polymorphism decreased the risk of CAS and MI in individuals with elevated fibrinogen levels. In this study we investigated the effect of FXIII-B polymorphisms on the risk of CAD in individuals with fibrinogen level in the upper tertile. The combined study group (clinical controls and patients) was used to calculate the lower limit of the upper tertile of fibrinogen concentrations (>4.3 g/L). In individuals with fibrinogen concentration in the upper tertile, the p.His95Arg polymorphism was without effect, while the intron K c.1952+144 C>G polymorphism

conferred a significant protective effect against CAS and MI. In the case of the CAS+MI- group, the protective effect became statistically significant only after adjustment.

4.2.4. The effect of combined FXIII-A p.Val34Leu and FXIII-B polymorphisms on the risk of CAD

Combined FXIII-A Leu34 and FXIII-B Arg95 carriership did not exert any effect on the risk of CAD in patients with elevated fibrinogen level. When FXIII-A Leu34 carriership and FXIII-B intron K G carriership, separately and in combination, were compared to the wild-type (Val34 intron K C) genotype, an interesting relationship was revealed. Separately, neither of these alleles conferred significant protection against CAS and/or MI in patients with an elevated fibrinogen level. However, their combination exerted highly significant protection against MI in these patients, and after adjustment, the protective effect against CAS without MI also became significant. The synergistic effect of the two polymorphisms in the protection against CAD was also demonstrated by synergy factor calculations. In the case of MI+ patients, the synergy factor significantly differed from 1.0, and the low values suggest an efficient interaction, leading to a considerable protective effect in patients possessing both the FXIII-A Leu34 allele and the FXIII-B intron K G allele.

4.2.5. The effect of FXIII-B polymorphisms on FXIII levels

Within the whole study population, carriers of the Arg95 variant had slightly, but significantly higher, FXIII levels than wild-type individuals. In the different subgroups a similar tendency was observed, but with the exception of FXIII activity in clinical controls, the differences did not reach the level of statistical significance, which is likely due to the relatively low number of individuals in the study groups and, consequently, to the lower statistical power. In case of intron K c.1952+144 C>G polymorphism carriers, they had significantly lower FXIII levels than wild-type individuals, and this difference was significant, not only in the whole study population, but also in all subgroups. Comparison of non-adjusted FXIII levels resulted in the same conclusion.

The presence of intron K G allele significantly decreased FXIII levels independently of its combination with FXIII-A Val34 homozygotes or Leu34 carriers in the whole study population, as well as in the CAS+ group. In MI+ patients, there was a similar tendency, but the extent of decrease in the FXIII levels was statistically significant only if intron K G and FXIII-A Leu34 carriership were combined. As compared to patients homozygous for the

FXIII-A Val34 allele and carrying the intron K G allele, FXIII levels of patients carrying both FXIII-A Leu34 and intron K G alleles were decreased, but the differences were not statistically significant.

4.2.6. The effect of low FXIII levels on the risk of CAD

As FXIII-B intron K c.1952+144 C>G polymorphism and its combination with FXIII-A p.Val34Leu polymorphism decreased FXIII levels, it was intriguing to find out if decreased FXIII levels were associated with protection against CAD. To address this question individuals with FXIII levels in the lower tertile were compared to those with FXIII levels in the upper tertile. In the total population, not stratified according to fibrinogen level, the low FXIII activity and antigen levels were without significant effect on the risk of CAS and MI. In patients with fibrinogen concentration in the upper tertile the ORs for CAS were below 1.0 but the protective effect of low FXIII levels was not statistically significant, while low FXIII activity or antigen levels significantly decreased the risk of MI.

5. DISCUSSION

5.1. FXIII levels and polymorphisms in healthy individuals

Ariens et al. reported higher FXIII-A antigen concentration in females and no difference in FXIII activity. We determined FXIII-A₂B₂ antigen level; however, as 99% of FXIII-A in the plasma is complexed with FXIII-B the two parameters are comparable. Our result on FXIII antigen seemingly contradict the data reported earlier. However, there was a considerable difference in the age of women enrolled into the two studies. In our study the median age of women was 39 years and only 22.5% of them were in menopause, while in the study with contradictory results the mean age of women was 65 years. Although no specific data was given, the great majority of women enrolled in that study must have been in menopause. This discrepancy supports the suggestion by Ariens et al., that studies on the effect of menopausal status and estrogen replacement therapy on FXIII levels, particularly on FXIII-B level are warranted.

Age positively and significantly correlated with all three FXIII parameters both in males and females. Similarly, positive correlation was observed by Ariens et al. between age and FXIII subunit antigen levels, but not between age and FXIII activity. The discrepancy is very likely due to methodological differences. In the method they used FXIII is only partially activated by thrombin. The rate of thrombin activation and thereby the measured FXIII activity, is strongly influenced by the common FXIII-A p.Val34Leu polymorphism. Due to the relatively high frequency of this polymorphism in the Caucasian population, FXIII activity measured by this method in the normal population scatters in a wide range and it is poorly related to FXIII-A antigen. Indeed, in their study the correlation between the catalytic FXIII-A subunit level and FXIII activity was weak, the coefficient of determination (cd; r^2) was only 0.02. In the FXIII assay used in our study FXIII was fully activated and the measured activity was independent from the p.Val34Leu polymorphism. Using this assay there was a strong correlation between FXIII-A₂B₂ antigen and FXIII activity (cd: 0.845, $p < 0.001$) and FXIII activity, just like FXIII-A₂B₂ antigen, showed significant age dependence.

The strong correlation of tFXIII-B antigen level with FXIII-A₂B₂ antigen level and FXIII activity is not surprising. Similar strong correlation between the two FXIII subunits has been reported earlier. FXIII-A level is low in FXIII-B deficiency, while the administration of FXIII-A to FXIII-A deficient patients increases plasma tFXIII-B concentration. These findings suggest that the two subunits are involved in the regulation of each other's plasma concentration by influencing synthesis, secretion or lifespan in the plasma.

Non-adjusted FXIII activity and tFXIII-B levels did not differ significantly among the groups of different FXIII-B p.His95Arg genotypes although there was a tendency of increase in the presence of the minor allele. This finding is similar to the results of an earlier study on a combined group of 192 healthy controls and 252 patients with vascular disease. In their study Komanasin et al., found no differences in FXIII activity, subunit antigen levels and FXIII-A₂B₂ levels in relation to different p.His95Arg genotypes. However in our case the minor Arg95 allele significantly increased the complex FXIII-A₂B₂ antigen concentration. Furthermore after adjustment, the increase in all FXIII parameters became statistically significant.

In an early study, FXIII-A p.Val34Leu accounted only for a small part of the 47% heritability of FXIII-A₂B₂. In a separate study FXIII activity was not affected by carriership

of the Leu34 allele. Our findings further strengthen these results, the presence of the FXIII-A p.Val34Leu polymorphism didn't influence FXIII activity and tFXIII-B antigen levels. The small decrease of FXIII-A₂B₂ antigen, with a low level of statistical significance in Leu34 carriers, disappeared, when when the effect of FXIII-B intron K polymorphism was eliminated.

To our knowledge, these are the first reports where the effect of the FXIII-B intron K c.1952+144 C>G polymorphism on FXIII levels was investigated in healthy individuals or CAD patients. The intron K c.1952+144 C>G polymorphism had a powerful effect on FXIII levels of healthy individuals. In intron K G carriers FXIII activity, FXIII-A₂B₂ antigen and tFXIII-B antigen were considerably lower than in wild type individuals. The differences were highly significant both in the non-adjusted and adjusted evaluations. This effect was independent of the presence of FXIII-A p.Val34Leu or FXIII-B p.His95Arg polymorphisms.

Interestingly among the FXIII-B intron K c.1952+144 C>G and FXIII-A p.Val34Leu combinations the constellation of the two minor alleles had the most robust effect on FXIII activity and FXIII-A₂B₂ levels. The difference between the Val34-intron K C and the Leu34 carrier-intron K G carrier subgroups was the highest, which suggests a kind of synergism between the two minor alleles.

The reason for the association of the FXIII-B intron K c.1952+144 C>G polymorphism and decreased FXIII levels is not known. FXIII-A₂ and FXIII-B₂ form a tight complex in the plasma, and the *K_d* for their interaction is in the range of 10⁻¹⁰ M. Interaction with FXIII-B is highly important for keeping the catalytic FXIII-A dimer in circulation. In patients with severe FXIII-B deficiency and in FXIII-B knockout mice, the FXIII-A level is considerably decreased. Following the administration of FXIII-A₂ concentrate prepared from human placenta to FXIII-B deficient patients, FXIII-A quickly disappeared from the plasma. When FXIII-B-deficient mice were supplemented with recombinant FXIII-B, FXIII-A levels, fibrin crosslinking and transglutaminase activities increased in their plasma, indicating that FXIII-B assisted the maintenance of FXIII-A levels in the circulation. In the absence of FXIII-B, the short half-life of FXIII-A₂ might be related to its spontaneous non-proteolytic activation in plasmatic condition. One can speculate that the replacement of 10 C-terminal amino acids plus the added extra 15 amino acids to the C-terminus in intron K mutants might influence either the interaction of the two subunits or the clearance of FXIII-A₂B₂ from the circulation.

The former hypothesis is not supported by findings that locate the FXIII-A binding epitope in the first two N-terminal sushi domains of FXIII-B. It has been shown that plasma free FXIII-B₂, just like FXIII-A₂B₂, is also bound to fibrinogen, and that both the 1st and 10th sushi domain of FXIII-B are involved in the binding. Taking the above results into consideration further studies are warranted to explain the influence of FXIII-B splice variant on plasma FXIII levels.

5.2. FXIII and the risk of coronary artery disease

After Kohler et al. demonstrated the protective effect of FXIII-A p.Val34Leu polymorphism against MI, the follow up studies were contradictory. It was presumed that gene-gene and gene-environment interactions might be responsible, at least in part, for the variability of the findings obtained by different laboratories and on different populations. Indeed, a study published from our laboratory demonstrated that the Leu34 allele decreased the risk of CAD only in patients with an elevated fibrinogen concentration. In the end the overall protective effect of Leu34 allele against VTE and CAD was confirmed by meta-analyses of the reported findings. The main aim of the present study was to investigate the effect of FXIII-B polymorphisms on the risk of CAD.

Carriership of the minor allele of either the FXIII-A p.His95Arg or FXIII-B intron K polymorphism did not influence the risk of CAD significantly, although statistically non-significant protection by the intron K polymorphism against CAD (ORs in the range of 0.73–0.78) was revealed. It was shown in our earlier study that the protective effect of FXIII-A p.Val34Leu polymorphism against MI prevailed only in individuals with a high fibrinogen concentration. The protection against CAD by the FXIII-A Leu34 allele at a high fibrinogen concentration might be related to the fibrinogen concentration-dependent effect of this polymorphism on the fibrin clot structure. At a high fibrinogen level, plasma samples from homozygotes for the Leu34 allele form clots having a looser structure, thicker fibers and increased permeability, while at low fibrinogen concentrations, the fibrin meshwork had thinner, more tightly-packed fibers and lower permeability. Similarly to the FXIII-A p.Val34Leu polymorphism, the protection by the intron K G allele against CAD was evident only for patients with elevated fibrinogen concentration; the adjusted OR was reduced by approximately 60% for the CAS+MI⁻, CAS+MI⁺, CAS⁺ and MI⁺ groups. It is to be noted

that smoking is an important determinant of fibrinogen level, and indeed, in our study population, current smokers had a significantly higher median fibrinogen level (4.21 g/L, interquartile range: 3.53, 5.08) than currently non-smoking individuals (3.85 g/L, interquartile range: 3.16, 4.60; $p < 0.001$). For this reason, the results adjusted for current smoking and other confounders were also calculated. Adjusted results demonstrate that the putative protective role of the FXIII-B intron K c.1952+144 C>G polymorphism was independent of the investigated cardiovascular risk factors.

Besides the gene-environment interaction, gene-gene interactions can also modify the risk of CAD. It has been reported that the combined presence of both FXIII-A Leu34 and FXIII-B Arg95 alleles lowered the risk of nonfatal MI in postmenopausal women. No such interaction between these polymorphisms could be demonstrated in our study. In contrast, investigating the interaction of FXIII-A p.Val34Leu and FXIII-B intron K c.1952+144 C>G polymorphisms, a surprising interaction between the two polymorphisms was revealed. When compared to individuals, wild-type for both polymorphisms, the protective effect of the intron K G allele disappeared in the absence of the Leu34 allele. The results suggest that the protective effect of intron K G carriership is due to that portion of patients who also possess the FXIII-A Leu34 allele. Without the concomitant presence of this FXIII-A polymorphism, the FXIII-B intron K G carriership is not protective. The same seems to be the situation with the protective effect of the FXIII-A p.Val34Leu polymorphism. In a previous study involving a higher number of individuals, Leu34 carriers had a significantly decreased risk of MI in patients with a fibrinogen level in the upper quartile (OR: 0.41, 95% CI: 0.18, 0.93). In the present study, there was also a tendency of the decreased risk of MI in Leu34 carriers (OR: 0.61, 95% CI: 0.33, 1.12 unadjusted) with the fibrinogen level in the upper tertile. However, the protective effect of the Leu34 allele prevailed only in the presence of the intron K G allele. The results of the synergy factor calculation proved the synergistic action of the two polymorphisms in the protection against CAD.

The relationship between FXIII level and the risk of arterial or venous thrombosis is a complex issue, which is influenced by a number of confounders. Elevated FXIII levels represent a significant risk of myocardial infarction or peripheral arterial disease in females, but not in males. It has been shown that the homozygous presence of the FXIII-A Leu34 allele decreased the FXIII levels in CAS+ and MI+ patients. Arg95 allele had only minor effect on

FXIII activity and FXIII-A₂B₂ antigen levels. A slight elevation of FXIII activity was observed only in clinical controls, but not in the CAD subgroups. In contrast, FXIII-B intron K c.1952+144 C>G polymorphism exerted a major effect on FXIII levels. The presence of the G allele resulted in significantly lower FXIII activity and FXIII-A₂B₂ antigen level in all study groups. As carriership of FXIII-B intron K c.1952+144 G allele uniformly decreased FXIII activity and antigen levels and the decrease was most prominent when the intron K G and FXIII-A Leu34 alleles were both present, it can be presumed, that their protective effect is related to decreased FXIII levels. The hypothesis that the FXIII-B intron K c.1952+144 C>G polymorphism, in combination with the FXIII-A p.Val34Leu polymorphism, exerts its beneficial effect through the decrease of the FXIII level was supported by the protection against MI of patients with FXIII levels in the lowest tertile.

The study on FXIII levels and the risk of CAD has several limitations, including the general limitations of case-control studies. To overcome the latter problems, a prospective study concerning the effect of FXIII-B polymorphisms on the risk of myocardial infarction has been initiated in our laboratory. Due to the relatively low number of enrolled individuals, results in the groups with fibrinogen levels in the upper tertile should be confirmed on a larger cohort. A larger study population would also allow the exploration of the gene dosage effect. Among MI survivor patients, only those referred to cardiac catheterization were included in the study, which represents a selection bias. The study was conducted only on Hungarian patients; its extension to cohorts from other nations could provide further support to the protective effect associated with the minor allele of FXIII-B intron K c.1952+144 C>G polymorphism.

6. SUMMARY

The following two interrelated topics were studied: 1/ The regulation of FXIII activity and antigen levels by FXIII subunit polymorphisms and non-genetic factors in healthy individuals, 2/ The effect of FXIII levels and FXIII polymorphisms, particularly FXIII-B subunit polymorphisms on the risk of CAD.

The results suggest that in healthy individuals, plasma FXIII levels are subjected to multifactorial regulation with age, fibrinogen level and FXIII-B intron K c.1952+144 C>G

polymorphism being the major determinants. Gender had no influence on FXIII activity or FXIII-A₂B₂ antigen concentration in our study. Surprisingly tFXIII-B antigen concentration was significantly elevated in males compared to females. Carriers of the intron K G allele had significantly lower plasma FXIII activity, FXIII-A₂B₂ and tFXIII-B antigen concentration than wild type individuals. Although FXIII-B intron K c.1952+144 C>G polymorphism exerted its effect on FXIII levels regardless of the presence of FXIII-A p.Val34Leu polymorphisms, it is worth noting that the constellation of these two minor alleles had the most powerful effect on FXIII activity and FXIII-A₂B₂ levels. The difference between the Val34-intron K C homozygotes and the Leu34 carrier-intron K G carrier subgroups was the highest, suggesting a kind of synergism between the two minor alleles. Revealing the regulatory factors that influence FXIII level in healthy individuals might help our understanding of the involvement of FXIII in thrombotic diseases and initiate further research on this topic.

The FXIII-B p.His95Arg polymorphism did not influence the risk of CAS or MI, while the FXIII-B intron K G allele was associated with significant protection against CAS and MI in patients with a fibrinogen level in the upper tertile. Interestingly, the protective effect of the intron K G allele 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 G allele had significantly lower plasma FXIII activity and FXIII-A₂B₂ antigen concentration as found in healthy individuals. Likewise, the concomitant presence of both Leu34 allele and intron K G allele had the lowest FXIII activity and FXIII-A₂B₂ antigen concentration, similarly to healthy individuals, while carriers of both Val34 and intron K C alleles had the highest FXIII activity and FXIII-A₂B₂ antigen concentration. As FXIII levels in the lower tertile were associated with significant protection against MI, one might conclude that the protective effect of combined FXIII-B intron K G and FXIII-A Leu34 carriership is related to decreased FXIII levels.

GRANT SUPPORT

The study was supported by the GINOP-2.3.2-15-2016-00050 project. The project is co-financed by the European Union and the European Regional Development Fund. Additional support was received from the National Research, Development and Innovation Fund, Hungary (K 113097) and from the Hungarian Academy of Sciences (MTA 11003 TKI417).

SZÉCHENYI 2020



MAGYARORSZÁG
KORMÁNYA

Európai Unió
Európai Regionális
Fejlesztési Alap



BEFEKTETÉS A JÖVŐBE

7. LIST OF PUBLICATIONS



UNIVERSITY OF DEBRECEN
UNIVERSITY AND NATIONAL LIBRARY



Registry number: DEENK/311/2016.PL
Subject: PhD Publikációs Lista

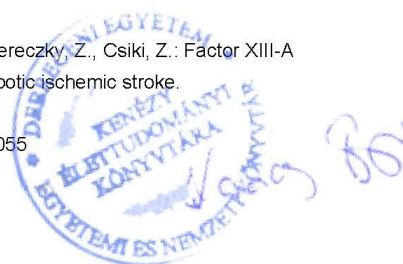
Candidate: Zoltán András Mezei
Neptun ID: FZJBEJ
Doctoral School: Kálmán Laki Doctoral School
MTMT ID: 10037483

List of publications related to the dissertation

1. **Mezei, Z. A.**, Katona, É., Kállai, J., Bereczky, Z., Molnár, É., Kovács, B., Ajzner, É., Bagoly, Z., Miklós, T., Muszbek, L.: Regulation of plasma factor XIII levels in healthy individuals; a major impact by subunit B intron K c.1952+144 C>G polymorphism.
Thromb. Res. 148, 101-106, 2016.
DOI: <http://dx.doi.org/10.1016/j.thromres.2016.10.025>
IF: 2.320 (2015)
2. **Mezei, Z. A.**, Bereczky, Z., Katona, É., Gindele, R., Balogh, E., Fialat, S., Balogh, L., Czuriga, I., Ádány, R., Édes, I., Muszbek, L.: Factor XIII B Subunit Polymorphisms and the Risk of Coronary Artery Disease.
Int. J. Mol. Sci. 16 (1), 1143-1159, 2015.
DOI: <http://dx.doi.org/10.3390/ijms16011143>
IF: 3.257

List of other publications

3. Shemirani, A. H., Antalfi, B., Pongrácz, E., **Mezei, Z. A.**, Bereczky, Z., Csiki, Z.: Factor XIII-A subunit Val34Leu polymorphism in fatal atherothrombotic ischemic stroke.
Blood Coagul. Fibrinolysis. 25 (4), 364-368, 2014.
DOI: <http://dx.doi.org/10.1097/MBC.0000000000000055>
IF: 1.403



Address: 1 Egyetem tér, Debrecen 4032, Hungary Postal address: Pf. 39. Debrecen 4010, Hungary
Tel.: +36 52 410 443 Fax: +36 52 512 900/63847 E-mail: publikaciok@lib.unideb.hu, Web: www.lib.unideb.hu



4. Antalfi, B., Pongrácz, E., Csiki, Z., **Mezei, Z. A.**, Shemirani, A. H.: Factor XIII-A subunit Val34Leu polymorphism in fatal hemorrhagic stroke.
Int. J. Lab. Hematol. 35 (1), 88-91, 2013.
DOI: <http://dx.doi.org/10.1111/j.1751-553X.2012.01465.x>
IF: 2.114
5. Koncz, Z., Bagoly, Z., Haramura, G., **Mezei, Z. A.**, Muszbek, L.: Thrombomodulin-dependent effect of factor V Leiden mutation on the cross-linking of alfa2-plasmin inhibitor to fibrin and its consequences on fibrinolysis.
Thromb. Res. 130 (3), 528-534, 2012.
DOI: <http://dx.doi.org/10.1016/j.thromres.2012.05.019>
IF: 3.133
6. Koncz, Z., Bagoly, Z., Haramura, G., **Mezei, Z. A.**, Muszbek, L.: Thrombomodulin-dependent effect of factor VLeiden mutation on factor XIII activation.
Thromb. Res. 129 (4), 508-513, 2012.
DOI: <http://dx.doi.org/10.1016/j.thromres.2011.06.030>
IF: 3.133

Total IF of journals (all publications): 15,36

Total IF of journals (publications related to the dissertation): 5,57

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

24 November, 2016

