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

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Table of Contents

ABBREVIATIONS

α2PI	α2 plasmin inhibitor
BMI	Body mass index
CAD	Coronary artery disease
CAS	Coronary atherosclerosis
FXIII	Blood coagulation factor XIII
FXIII-A	FXIII A subunit
FXIII-A_2	FXIII A subunit homodimer
FXIII-B	FXIII B subunit
FXIII-A_2B_2	Heterotetrameric structure of FXIII
FXIIIa	Activated FXIII
pFXIII	Plasma FXIII
cFXIII	Cellular FXIII
FXIII-A2*	FXIII-A activated by thrombin and Ca^{2+}
FXIII-A'	FXIII-A proteolytically cleaved by trombin
FXIII-A2º	Non-proteolytically activated FXIII-A_2
F13A1	FXIII-A gene
F13B	FXIII-B gene
MI	Myocardial infarction
PCR	Polymerase chain reaction
rFXIII-A_2	Recombinant FXIII-A_2
tFXIII-B	Total FXIII-B
VTE	Venous thromboembolism

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) [1-3]. pFXIII is a heterotetramer (FXIII-A_2B_2) 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 [4-7]. Practically all pFXIII molecules are bound to fibrinogen (K_d 10^{-8} M). This association is independent of the presence of Ca^{2+} [8].

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 (Figure 1). 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 [1].

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-A_2) (Figure 2). In platelets FXIII-A amounts to 3% of total cellular protein [9-11]. 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 [12-15]. FXIII-A has also been detected in chondrocytes, osteoblasts, and osteocytes [16]. 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 [17, 18]. 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 [19]. In recent years recombinant FXIII-A_2 (rFXIII-A_2) was introduced in the therapy of FXIII-A deficient patients [20]. rFXIII-A_2 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 [21-23]. 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 (Figure 3). Each sushi domain is held together by a pair of internal disulfide bonds. Although there were some contradictory results [24], most of the literature suggest that in the plasma free FXIII-B circulates as homodimer (FXIII-B_2) [4-7]. In a recent study on the interaction of FXIII and fibrinogen Byrnes et al. demonstrated that "free" FXIII-B_2 is also bound to fibrinogen [25]. FXIII-B is synthesized by hepatocytes [4, 26], just like fibrinogen, and the two proteins might associate during or immediately after secretion [25].

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 [27, 28]. 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: HNF1α and HNF4α [27, 29].

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-B_2 to FXIII-A_2 [6]. 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 [7]. 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 [30].

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-A'$_2$) is transformed into an active transglutaminase (FXIII-A*$_2$; FXIIIa). The latter step also requires the presence of Ca^{2+} (Figure 1**)**. 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 [1, 2, 31-33]. FXIIIa remains associated with fibrin, in serum no thrombin-cleaved FXIII could be detected [34].

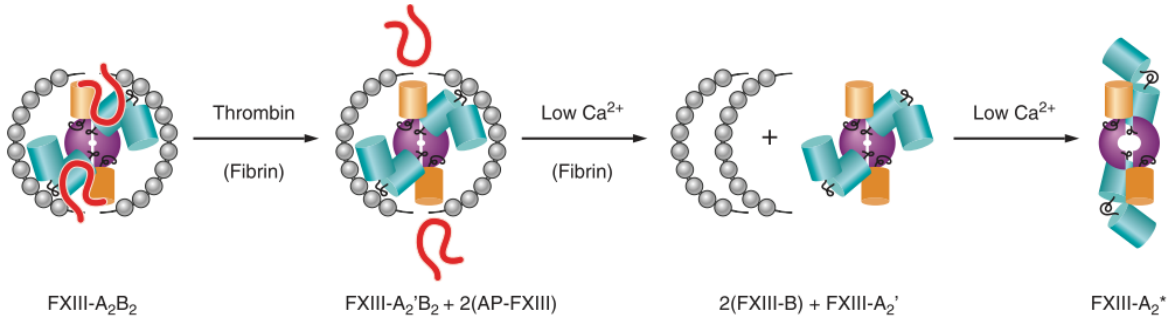

Figure 1. Proteolytic activation of pFXIII in the presence of thrombin and Ca^{2+}. In the first step of proteolytic activation thrombin cleaves off the AP-FXIII resulting in FXIII-A'$_2$B$_2$. Next in the presence of Ca^{2+} FXIII-B dissociates from FXIII-A'$_2$. In the final step FXIII-A'$_2$ is transformed into an active transglutaminase (FXIII-A*$_2$) in the presence of Ca^{2+}. Green and orange cylinders represent β-barrel and β-sandwich domains of FXIII-A, respectively. The central core domains in FXIII-A are depicted as horseshoes in magenta. The activation peptides are shown as red loops. The curved elongated structure consisting of 10 pearls surrounding FXIII-A$_2$ corresponds to FXIII-B. The figure represents a part of figure 7 from reference [1].

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-A_2 assumes the active configuration (FXIII-A°_2), (Figure 2).

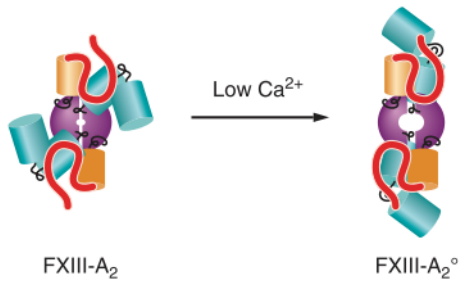

Figure 2. Non-proteolytic activation of cellular FXIII in the presence of Ca^{2+}. In intracellular conditions cFXIII doesn't require proteolytic cleavage for activation. Elevation in intracellular Ca^{2+} concentration causes FXIII to assume an active configuration (FXIII-A°_2). Green and orange cylinders represent β-barrel and β-sandwich domains of FXIII-A, respectively. The central core domains in FXIII-A are depicted as horseshoes in magenta. The activation peptides are shown as red loops. The figure represents a part of figure 7 from reference [1].

1.3. Biochemical and physiological function of FXIII

FXIIIa, like all active transglutaminases, catalyzes an acyl-transfer reaction in two major steps [35]. 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 (ε(γ-glutamyl)lysyl is formed).

The primary physiological substrates of FXIIIa are the α- and γ- chains of fibrin and α2 plasmin inhibitor (α2PI) [35-37]. 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 [38, 39]. The formation of γ-chain dimers is a quick process, and a minimal amount of FXIIIa is sufficient to catalyze it [40]. 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 [41, 42]. α2PI is also cross-linked to fibrin α-chain by FXIIIa [37]. Covalent attachment of α2PI to fibrin is highly important in the regulation of fibrinolysis, it prevents newly formed clot from breakdown by plasmin [37, 43, 44]. Beside α2PI, plasminogen activator inhibitor 2, an inhibitor of urokinase-type plasminogen activator is cross-linked to fibrin by FXIIIa [45, 46]. Thrombin activatable fibrinolysis inhibitor is another substrate for FXIIIa [47]. 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 [48, 49], 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 [23, 50-54] 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-A_2 is present in the plasma. Women with congenital FXIII deficiency cannot maintain pregnancy, the pregnancy is usually lost in the first trimester [55-59]. 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 [60]. 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 [61, 62]. The proangiogenic effect of FXIII has been clearly demonstrated Dardik et al. [63, 64]. In chondrocytes and osteoblasts both FXIII and tissue transglutaminase 2 are present, with possible roles in extracellular matrix formation, assembly, and stabilization [65, 66].

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 [67, 68]. Platelet FXIII-$A°_2$ cross-links cytoskeletal elements [69] and is involved in certain phases of the platelet spreading [70]. cFXIII has also been implicated in the motility of monocyte-

derived dendritic cells [71] and in the phagocytosis of peripheral blood monocytes [72]. A more detailed description on the diverse role of FXIII can be found in references [1, 3, 73].

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. [74], 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 (1000 Genomes Project; www.internationalgenome.org [75]).

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 [76] and pFXIII [77, 78] 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 [79-81]. Faster activation of FXIII results in rapid fibrin cross-linking and in a larger amount of α2PI incorporation into fibrin [76-78]. 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 [34].

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 [77, 78], cFXIII [76], and rFXIII-A_2 [82] of different FXIII-A p.Val34Leu genotypes are identical. The structure of fibrin clots is influenced by FXIII-A p.Val34Leu polymorphism [77], and this effect is modulated by fibrinogen concentration [83]. 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 [28, 84]. 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 (Figure 3) [85]. The minor allele (Arg95) is relatively rare (7%) in the European population, but it represents the major allele (68%) among black Africans [75].

The polymorphism did not influence FXIII-A, tFXIII-B, or pFXIII antigen levels in a combined group of controls and patients with vascular disease [85]. 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 [85], 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 [86, 87]. 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 (Figure 3). 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 [75]. 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.

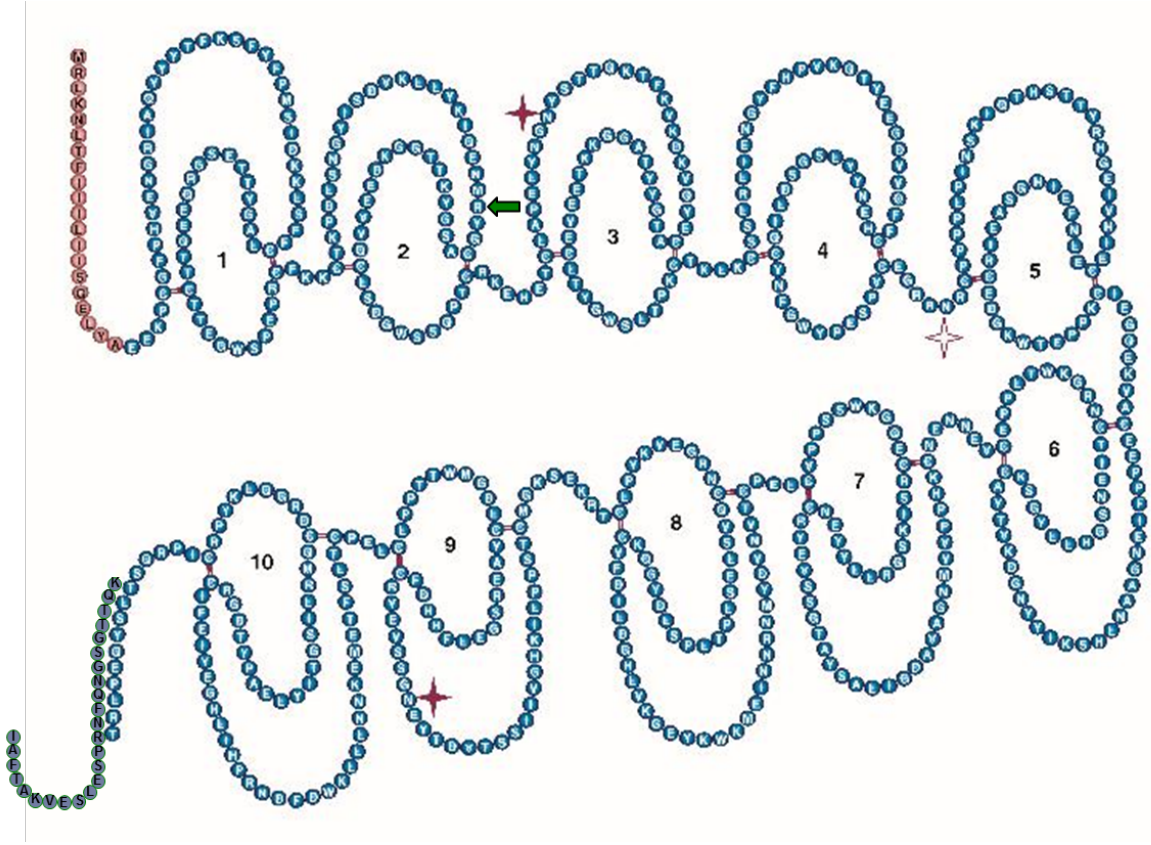

Figure 3. Schematic structure of FXIII-B consisting of 10 sushi domains each held together by two pairs of disulfide bonds. Amino acids in the leader sequence are depicted in purple, and those in the mature protein are shown in cyan. The solid stars point to the site of N-glycosylation, and the empty star points to a potential glycosylation site to which no carbohydrate is attached. Green arrow: FXIII-B p.Arg95His, green tail: the amino acid sequence introduced by FXIII-B intron K c.1952+144 C>G polymorphisms. Modified figure from reference [1].

1.5. Determinants of FXIII level in the plasma

Both genetic and non-genetic factors are involved in the regulation of plasma FXIII levels [88-90]. 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) [90]. pFXIII concentration in individuals with the different FXIII-A p.Val34Leu genotypes did not differ significantly [78] suggesting that their secretion and plasma half-life are similar. Using likelihood analysis with adjustment for age and gender 47% hereditability was established for the plasma level of complex FXIII (FXIII-A_2B_2) in healthy families [88]. FXIII-A p.Val34Leu polymorphism accounted only for a small fraction of this hereditability. 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 [91, 92]. 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 [88], 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 (reviewed in references [93, 94]). In early studies mostly negative results were obtained on the association of levels and coronary atherosclerosis (CAS) or myocardial infarction (MI) [95-98]. 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-A_2B_2 antigen levels in the upper tertile increased the risk of MI 3-fold in females but not in males [99]. Similarly, elevated FXIII level increased the risk of peripheral artery disease in women, but not in men [100]. 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 (reviewed in [93]). Most recently it was reported that genetic markers associated with low tFXIII-B levels increased the risk of ischemic stroke of cardioembolic subtype [101].

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 [102]. Both confirmatory and contradictory results were reported in follow-up studies (reviewed in [93]). 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 [103]. The overall protective effect of Leu34 allele against CAD was confirmed by a meta-analysis of reported findings [104].

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 [105]. The Arg95 allele was associated with an increased mortality (1.7 fold increase after cerebral ischemia of arterial origin in young women [106]). 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) [107]. 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 [101].

Elevated FXIII activity was associated with a small decrease in deep venous thrombotic risk [108]. 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 [109]. 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. [110]. Since then a number of contradictory and confirmatory studies were published, which were summarized in two meta-analyses [111, 112]. 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 [113]. The effect of the polymorphism on the risk of thrombosis (arterial and venous combined) was investigated in patients with antiphospholipid syndrome, as well [114, 115]. 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) [115]. 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 [85].

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-A_2B_2 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. The characteristic features of the study population are shown in Table 2. 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 ≥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 [116]. 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. Results with these patients are shown in Table 5, but 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 [117] 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) [118]. 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 $CaCl_2$, 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 µL sample or blank reagent was added to 25 µL 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 5^{th} and 10^{th} minutes, the ΔA/min of sample and blank reaction were calculated. From the ΔA/min measured with the sample the ΔA/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 µmol/L and triglyceride concentration below 7.5 mmol/L do not interfere with the measurement.

FXIII-A_2B_2 and tFXIII-B antigen concentrations were determined by one-step sandwich ELISAs [119, 120]. For the measurement of FXIII-A_2B_2 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-A_2B_2 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 KH_2PO_4, 12 mmol/L Na_2HPO_4, 0.05% Polysorbate 20, 5 g/L bovine serum albumin). 70 µL biotinylated monoclonal anti-FXIII-B antibody was added to the wells of a streptavidin-coated microplate, followed by the addition of 70 µL diluted plasma sample and 70 µL 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 µL/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 µL 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 µL 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-A_2B_2 concentration. FXIII-A_2B_2 antigen concentration was expressed as percentage of normal (pooled plasma) FXIII-A_2B_2 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) [121]. 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 [122]. 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. [123]. 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) (Table 1).

Table 1. Primers and probes used for the determination of FXIII-B p.His95Arg and FXIII-B intron K c.1952+144 C>G polymorphisms.

Primers/Probes	Sequence	Length (bp)
FXIII-B p.His95Arg		
Forward primer	5'-gtaaaagacaagcttagtttcatc-3'	24
Reverse primer	5'-ctacaggttggttgagaagac-3'	21
Sensor	5'-ataacgacatgttctcttgaattttataca-3'-FL	29
Anchor	5'-LC610-actttacatcagagatgtaaccattactcaggtc-3'PH	34
FXIII-B intron K c.1952+144 C>G		
Forward primer	5'-ttccaagacaaaggtaagaag-3'	21
Reverse primer	5'-aacgttgctttcacttcag-3'	19
Sensor	5'-gtttgtttggtgtaaaaaaaatgaagaaaatatt-3'-FL	34
Anchor	5'-LC670-ttttttctttgcaattgccataaagtatgagtgg-3'-PH	34

FL – fluorescein, PH – phosphorylated

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 (the complementer nucleotide is shown in red), 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 complementer nucleotide is shown in red). 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 $MgCl_2$ (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 derivates 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 ± 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 $\chi 2$ 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. [124].

4. RESULTS

4.1. FXIII levels and polymorphisms in healthy individuals

4.1.1. Characterization of study population

Table 2 demonstrates the gender specific differences in the investigated parameters. 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-A_2B_2 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 [75]. As expected, the minor allele frequencies did not show gender specific differences in the study population (Table 2).

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 (Table 3). The relatively weak significant correlation between BMI and FXIII activity and FXIII-A_2B_2 antigen disappeared after adjustment, but remained in the case of tFXIII-B antigen (Table 3). 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 [8, 125, 126]. A highly signifcant correlation was between tFXIII-B antigen level and FXIII-A_2B_2 antigen level or FXIII activity (Table 3).

Table 2. Characteristics of study population; comparison of females and males.

	Total (n=268)	Female (n=160)	Male (n=108)	Significance (p)
Age	38.0 (26.0-48.0)	39.0 (24.3-48.0)	36.5 (27.0-46.0)	0.726
BMI	24.9 (21.5-28.4)	22.7 (20.3-26)	26.7 (24.2-29.2)	<0.001
Smoking (-/+)	200/68	123/37	77/31	0.319
Fibrinogen (g L-1)	3.4±0.6	3.5±0.6	3.2±0.6	0.015
FXIII activity (%)				
non-adjusted	108.4±25.0	110.1±23.7	105.9±26.6	0.175
adjusted		109.1±23.7	107.3±26.6	0.470
FXIII-A_2B_2 antigen (%)				
non-adjusted	105.9±23.8	107.2±22.2	103.9±26.0	0.262
adjusted		106.4±22.2	105.2±26.0	0.646
tFXIII-B (%)				
non-adjusted	108.5±18.0	105.8±18.2	112.4±16.9	0.003
adjusted		106.0±18.2	112.1±16.9	0.003
FXIII-A p.Val34Leu				
wild type	133	82	51	
heterozygote	106	60	46	
homozygote	29	18	11	
Leu34 carrier	50.4%	48.8%	52.8%	0.536
Leu34 allele frequency	30.6%	30.0%	31.5%	0.878
FXIII-B p.His95Arg				
wild type	216	128	88	
heterozygote	47	31	16	
homozygote	5	1	4	
Arg95 carrier	19.4%	20.0%	18.5%	0.875
Arg95 allele frequency	10.6%	10.3%	11.1%	0.817
FXIII-B intron K C>G				
wild type	203	115	88	
heterozygote	60	42	18	
homozygote	5	3	2	
G carrier	24.3%	28.1%	18.5%	0.082
G allele frequency	13.1%	15.0%	10.2%	0.285

Parameters showing non-parametric distribution (age and BMI) are represented by median and interquartile range, while in the case of other parameters with parametric distribution mean±SD are shown. Smoking - and + represent non-smokers and current smokers, respectively. Significance concerns differences between females and males. FXIII activity and FXIII-A_2B_2 antigen were adjusted to age and fibrinogen concentration. tFXIII-B antigen was adjusted to age, BMI and fibrinogen concentration.

Table 3. The effect of various factors on FXIII activity and antigen levels.

		FXIII activity		FXIII-A_2B_2 antigen		tFXIII-B antigen	
		cd (r^2)	significance (p)	cd (r^2)	significance (p)	cd (r^2)	significance (p)
Age	total	0.190	<0.001	0.127	<0.001	0.119	<0.001
	female	0.167	<0.001	0.104	<0.001	0.144	<0.001
	male	0.234	<0.001	0.166	<0.001	0.087	0.002
BMI	total	0.027	0.007	0.019	0.026	0.148	<0.001
	female	0.040	0.011	0.020	0.073	0.136	<0.001
	male	0.044	0.029	0.043	0.031	0.104	0.001
BMI adjusted	total	0.000	0.776	0.000	0.786	0.092	<0.001
	female	0.000	0.869	0.000	0.913	0.055	0.003
	male	0.021	0.135	0.023	0.118	0.083	0.003
Smoking	total	0.010	0.102	0.005	0.240	0.005	0.268
	female	0.036	0.017	0.020	0.080	0.008	0.275
	male	0.000	0.949	0.000	0.954	0.000	0.919
Smoking adjusted	total	0.004	0.276	0.002	0.492	0.002	0.526
	female	0.018	0.096	0.008	0.263	0.001	0.747
	male	0.000	0.853	0.000	0.878	0.000	0.868
Fibrinogen	total	0.207	<0.001	0.166	<0.001	0.181	<0.001
	female	0.184	<0.001	0.159	<0.001	0.271	<0.001
	male	0.227	<0.001	0.168	<0.001	0.140	<0.001
Fibrinogen adjusted	total	0.189	<0.001	0.145	<0.001	0.133	<0.001
	female	0.187	<0.001	0.161	<0.001	0.231	<0.001
	male	0.165	<0.001	0.109	0.001	0.079	0.004
tFXIII-B	total	0.414	<0.001	0.417	<0.001	n.a.	n.a.
	female	0.496	<0.001	0.484	<0.001	n.a.	n.a.
	male	0.401	<0.001	0.417	<0.001	n.a.	n.a.
tFXIII-B adjusted	total	0.286	<0.001	0.312	<0.001	n.a.	n.a.
	female	0.335	<0.001	0.362	<0.001	n.a.	n.a.
	male	0.276	<0.001	0.301	<0.001	n.a.	n.a.

BMI and smoking were adjusted to age, fibrinogen to age and BMI, tFXIII-B to age, BMI and fibrinogen. cd: coefficient of determination.

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. [78]. tFXIII-B level was not affected, either (Table 4). A slight decrease of FXIII-A_2B_2 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 (Figure 4A-C).

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 (Table 4).Only in the case of complex FXIII-A_2B_2 antigen was the increase statistically significant. On the other hand, after adjustment, the increase in all FXIII parameters became statistically significant (Table 4). 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 (Figure 4D-F).

The intron K c.1952+144 C>G polymorphism has a robust effect on FXIII levels. In intron K G carriers FXIII activity, FXIII-A_2B_2 antigen and tFXIII-B antigen are considerably lower than in wild type individuals (Table 4). The differences were highly significant both in the non-adjusted and adjusted evaluations. In the latter case FXIII activity and FXIII-A_2B_2 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 (Figure 4A,B). Among the combinations the constellation of the two minor alleles had the most powerful effect on FXIII activity and FXIII-A_2B_2 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 (Figure 4C). 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 (Figure 4D-F). 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.

Table 4. The effect of FXIII subunit polymorphisms on FXIII activity and antigen levels.

FXIII polymorphisms	FXIII activity (%)		FXIII-A_2B_2 antigen (%)		tFXIII-B (%)	
	non-adjusted	adjusted	non-adjusted	adjusted	non-adjusted	adjusted
FXIII-A p.Val34Leu						
wild type (n=133)	110.5±24.2	110.0±24.2	109.2±23.8	108.8±23.8	108.8±16.4	109.1±16.4
Leu34 carrier (n=135)	106.3±25.6	106.8±25.6	102.6±23.4	103.0±23.4	108.2±19.5	109.1±19.5
significance (p)	0.163	0.210	0.022	0.022	0.810	0.997
FXIII-B p.His95Arg						
wild type (n=216)	107.4±25.0	106.8±25.0	104.3±23.3	103.8±23.3	107.8±18.1	107.9±18.1
Arg95 carrier (n=52)	112.5±24.6	114.9±24.6	112.5±25.0	114.5±25.0	111.3±17.4	114.0±17.4
significance (p)	0.191	0.010	0.026	0.001	0.205	0.007
FXIII-B intron K C>G						
wild type (n=203)	112.2±25.2	111.3±25.2	109.5±24.3	108.8±24.3	112.0±17.3	111.6±17.3
G carrier (n=65)	96.3±20.0	99.2±20.0	94.6±18.0	96.9±18.0	97.7±15.8	101.0±15.8
significance (p)	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

FXIII activity and FXIII-A_2B_2 antigen levels were adjusted to age and fibrinogen concentration, tFXIII-B antigen level was adjusted to age, gender, BMI and fibrinogen concentrations.

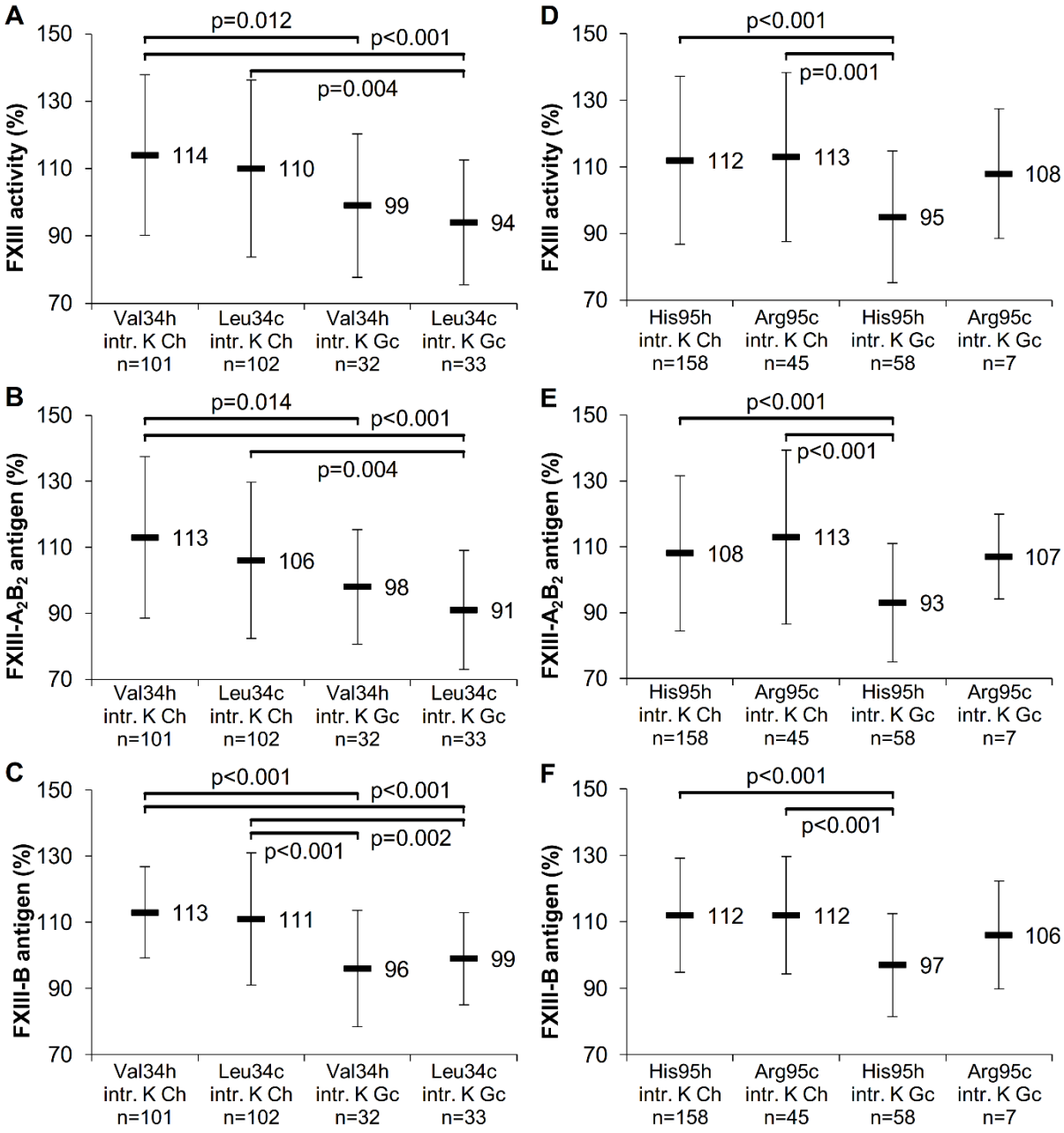

Figure 4. The effect of FXIII-B polymorphisms on FXIII levels. Effect of FXIII-B intron K c.1952+144 C>G polymorphism in combinations with FXIII-A p.Val34Leu (A-C) or FXIII-B p.His95Arg (D-F) polymorphisms on FXIII activity (A,D), FXIII-A_2B_2 antigen (B, E) and tFXIII-B antigen (C, F) levels is shown. Non-adjusted mean±SD values are shown. After adjustment of FXIII activity and FXIII-A_2B_2 antigen to age and fibrinogen concentration and tFXIII-B antigen to age, gender, BMI and fibrinogen concentration essentially the same results were obtained. The numbers beside the thick horizontal lines represent numerical mean values. Val34h, His95h and intr. K Ch: homozygotes for the major alleles in p.Val34Leu, p.His95Arg and intron K C>G polymorphisms, respectively. Leu34c, Arg95c, intr. K Gc: carriers of the respective minor alleles.

4.2. FXIII and coronary artery disease

4.2.1. Characterization of study population

The general characteristics of the study groups are shown in Table 5. Briefly, 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 in Table 5 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.

Table 5. General characteristics of the patient groups.

Patients (n)	CAS−MI− (237)	CAS−MI+ (26)	CAS+MI− (214)	CAS+MI+ (210)
Gender (male/female)	97/140	19/7 †	144/70 ‡	164/46 ‡
Age	54 (48–64)	56 (47–65)	61 (54–70) †	59 (51–68) †
Diabetes mellitus (−/+)	218/19	20/6 *	175/39 †	161/49 ‡
Current smoker (−/+)	205/32	22/4	184/30	175/35
Triglyceride (mmol/L)	1.47 (1.03–2.19)	1.46 (1.29–2.40)	1.67 (1.25–2.26) *	1.81 (1.35–2.48) ‡
Cholesterol (mmol/L)	5.60 ± 1.13	5.78 ± 1.17	5.71 ± 1.33	5.56 ± 1.12
HDL-C (mmol/L)	1.23 (1.01–1.49)	1.13 (1.01–1.31)	1.12 (0.96–1.33) †	1.05 (0.90–1.25) ‡
LDL-C (mmol/L)	3.50 ± 0.98	3.70 ± 0.99	3.65 ± 1.15	3.48 ± 0.95
ApoA-I (g/L)	1.44 ± 0.27	1.34 ± 0.21	1.39 ± 0.30	1.32 ± 0.25 ‡
ApoB (g/L)	1.03 (0.90–1.18)	1.15 (0.88–1.26)	1.10 (0.95–1.27) *	1.11 (0.96–1.29) †
Lp(a) (mg/L)	126 (99–368)	99 (99–300)	126 (99–441)	170 (99–642) *
Homocysteine (µmol/L)	11.92 (9.68–14.75)	13.38 (10.39–15.16)‡	13.66 (10.98–16.28)‡	13.62 (11.18–17.19)‡
Fibrinogen (g/L)	3.78 (3.13–4.44)	3.58 (2.95–5.11)	3.88 (3.29–4.62)	4.06 (3.23–5.03) *
FXIII activity (%)	101 ± 20	103 ± 26	100 ± 22	101 ± 22
FXIII-A_2B_2 antigen (%)	108±23	111±28	105±25	107±24

Values for age, triglyceride, HDL-C, apoB, Lp(a), homocysteine and fibrinogen are medians with the interquartile range in parenthesis, all other variables are means ± SD. CAS+ and CAS−, patients with and without coronary atherosclerosis, respectively; MI+ and MI−, patients with and without a history of myocardial infarction, respectively. * $p < 0.05$, † $p < 0.01$, ‡ $p < 0.001$ for comparison with the clinical control group (CAS−MI−).

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 (Table 6) and were similar to the data from the 1000 Genomes Project (www.internationalgenome.org) [75]. 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 (Table 6). 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 (data not shown). In Table 6, the 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 (Table 7).

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 FXIIII-A p.Val34Leu polymorphism decreased the risk of CAS and MI in individuals with elevated fibrinogen levels [12]. 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). Table 8 demonstrates that 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.

Table 6. FXIII-B p.His95Arg and intron K c.1952+144 C>G genotype distribution in clinical control and patient groups: The effect of polymorphisms on the risk of coronary artery disease.

	Population controls n=994	CAS−MI− n=237	CAS+MI− n=214	CAS+MI+ n=210	CAS+ n=424	MI+ n=236
FXIII-B p.His95Arg						
wild type n(%)	831 (83.6%)	202 (85.2%)	189 (88.3%)	180 (85.7%)	369 (87.0%)	203 (86.0%)
heterozygote n(%)	155 (15.6%)	33 (14.0%)	25 (11.7%)	30 (14.3%)	55 (13.0%)	33 (14.0%)
homozygote n(%)	8 (0.8%)	2 (0.8%)	-	-	-	-
R95 carrier frequency	16.4%	14.8%	11.7%	14.3%	13.0%	14.0%
R95 allele frequency	8.6%	7.8%	5.8%	7.1%	6.5%	7.0%
OR for R95 carriers non-adjusted	-	-	0.76 (0.44, 1.32)	0.96 (0.57, 1.63)	0.86 (0.55, 1.36)	0.94 (0.56, 1.57)
OR for R95 carriers adjusted	-	-	0.76 (0.41, 1.39)	1.18 (0.64, 2.17)	0.95 (0.57, 1.59)	1.11 (0.62, 2.01)
FXIII-B intron K c.1952+144 C>G						
wild type n(%)	712 (71.6%)	158 (66.7%)	155 (72.4%)	151 (71.9%)	306 (72.2%)	173 (73.3%)
heterozygote n(%)	259 (26.1%)	74 (31.2%)	52 (24.3%)	55 (26.2%)	107 (25.2%)	59 (25.0%)
homozygote n(%)	23 (2.3%)	5 (2.1%)	7 (3.3%)	4 (1.9%)	11 (2.6%)	4 (1.7%)
G carrier frequency	28.4%	33.3%	27.6%	28.1%	27.8%	26.7%
G allele frequency	15.3%	17.7%	15.4%	15.0%	15.2%	14.2%
OR for G carriers non-adjusted	-	-	0.76 (0.51, 1.14)	0.78 (0.52, 1.17)	0.77 (0.55, 1.09)	0.73 (0.49, 1.08)
OR for G carriers adjusted	-	-	0.82 (0.53, 1.28)	0.87 (0.55, 1.39)	0.82 (0.56, 1.21)	0.80 (0.51, 1.26)

The ORs were calculated by comparing different patient groups with the CAS−MI− (clinical control) group. The respective 95% CIs are shown in parenthesis after the OR values. ORs were adjusted for gender, age, diabetes mellitus, current smoking, total cholesterol, Lp(a), homocysteine and fibrinogen concentrations. CAS+ and CAS−, patients with and without coronary atherosclerosis, respectively; MI+ and MI−, patients with and without a history of myocardial infarction, respectively; OR, odds ratio; *n*, number of individuals in each subgroup.

Table 7. FXIII-B p.His95Arg and intron K c.1952+144 C>G genotype distribution in population control and patient groups; the effect of polymorphisms on the risk of coronary artery disease.

	Population controls n=994	CAS−MI− n=237	CAS+MI− n=214	CAS+MI+ n=210	CAS+ n=424	MI+ n=236
FXIII-B p.His95Arg						
wild type n(%)	831 (83.6%)	202 (85.2%)	189 (88.3%)	180 (85.7%)	369 (87.0%)	203 (86.0%)
heterozygote n(%)	155 (15.6%)	33 (14.0%)	25 (11.7%)	30 (14.3%)	55 (13.0%)	33 (14.0%)
homozygote n(%)	8 (0.8%)	2 (0.8%)	-	-	-	-
R95 carrier frequency	16.4%	14.8%	11.7%	14.3%	13.0%	14.0%
R95 allele frequency	8.6%	7.8%	5.8%	7.1%	6.5%	7.0%
OR for R95 carriers non-adjusted	-	-	0.74 (0.48, 1.14)	0.83 (0.55, 1.25)	0.79 (0.57, 1.08)	0.80 (0.54, 1.19)
OR for R95 carriers adjusted	-	-	0.80 (0.50, 1.38)	0.87 (0.55, 1.38)	0.81 (0.56, 1.18)	0.83 (0.53, 1.29)
FXIII-B intron K c.1952+144 C>G						
wild type n(%)	712 (71.6%)	158 (66.7%)	155 (72.4%)	151 (71.9%)	306 (72.2%)	173 (73.3%)
heterozygote n(%)	259 (26.1%)	74 (31.2%)	52 (24.3%)	55 (26.2%)	107 (25.2%)	59 (25.0%)
homozygote n(%)	23 (2.3%)	5 (2.1%)	7 (3.3%)	4 (1.9%)	11 (2.6%)	4 (1.7%)
G carrier frequency	28.4%	33.3%	27.6%	28.1%	27.8%	26.7%
G allele frequency	15.3%	17.7%	15.4%	15.0%	15.2%	14.2%
OR for G carriers non-adjusted	-	-	0.89 (0.70, 1.36)	0.98 (0.70, 1.36)	0.98 (0.76, 1.26)	0.91 (0.66, 1.26)
OR for G carriers adjusted	-	-	1.07 (0.73, 1.56)	1.08 (0.74, 1.57)	1.04 (0.77, 1.40)	1.01 (0.70, 1.45)

The ORs were calculated by comparing different patient groups with the population control group. The respective 95% CIs are shown in parenthesis below the OR values. ORs were adjusted for gender and age. CAS+ and CAS−, patients with and without coronary atherosclerosis, respectively; MI+ and MI−, patients with and without a history of myocardial infarction, respectively; OR, odds ratio.

Table 8. The effect of FXIII-B p.His95Arg and intron K c.1952+144 C>G polymorphisms on the risk of coronary artery disease in patients with an elevated fibrinogen concentration.

Subjects	CAS−MI− n = 63	CAS+MI− n = 75	CAS+MI+ n = 79	CAS+ n = 154	MI+ n = 88
FXIII-B p.His95Arg					
Wild-type n	55 (87.3%)	69 (92.0%)	67 (84.8%)	136 (88.3%)	74 (84.1%)
Heterozygote n	8 (12.7%)	6 (8.0%)	12 (15.2%)	18 (11.7%)	14 (15.9%)
Homozygote n	–	–	–	–	–
Arg95 carrier frequency	12.7%	8.0%	15.2%	11.7%	15.9%
Arg95 allele frequency	6.3%	4.0%	7.6%	5.8%	8.0%
OR for Arg95 carriers non-adjusted	–	0.6 (0.20, 1.83)	1.23 (0.47, 3.23)	0.91 (0.37, 2.22)	1.3 (0.51, 3.32)
OR for Arg95 carriers adjusted	–	0.81 (0.23, 2.92)	1.55 (0.52, 4.65)	1.07 (0.39, 2.96)	1.5 (0.51, 4.38)
FXIII-B intron K c.1952+144 C>G					
Wild-type n	38 (60.3%)	56 (74.7%)	61 (77.2%)	117 (76.0%)	70 (79.5%)
Heterozygote n	24 (38.1%)	17 (22.7%)	16 (20.3%) *	33 (21.4%) *	16 (18.2%) †
Homozygote n	1 (1.6%)	2 (2.6%)	2 (2.5%)	4 (2.6%)	2 (2.3%)
G carrier frequency	39.7%	25.3%	22.8% *	24.0% *	20.5% *
G allele frequency	20.6%	14.0%	12.7%	13.3% *	11.4% *
OR for G carriers non-adjusted	–	0.52 (0.25, 1.07)	0.45 * (0.21–0.93)	0.48 * (0.26, 0.90)	0.39 * (0.19, 0.81)
OR for G carriers adjusted	–	0.35 * (0.15, 0.83)	0.42 * (0.19, 0.96)	0.38 † (0.19, 0.79)	0.37 * (0.17, 0.84)

Elevated fibrinogen concentration represents the upper tertile of fibrinogen concentration (>4.3 g/L) in all study subjects. ORs were adjusted for gender, age, smoking, Lp(a), serum triglyceride and homocysteine concentrations. ORs were calculated by comparing different patient groups with the CAS−MI− (clinical control) group. The respective 95% CIs are shown in parenthesis below the OR values. CAS+ and CAS−, patients with and without coronary atherosclerosis, respectively; MI+ and MI−, patients with and without a history of myocardial infarction, respectively; OR, odds ratio; *n*, number of individuals in each subgroup; * $p < 0.05$, † $p < 0.01$.

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 (data not shown). 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 (Table 9). 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 (Table 9). 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.

Table 9. Effect of combined FXIII-A Leu34 and FXIII-B intron K c.1952+144 G carriership on the risk of CAD in individuals with fibrinogen concentration in the upper tertile.

Subjects	CAS−MI− n = 63	CAS+MI− n = 75	CAS+MI+ n = 79	CAS+ n = 154	MI+ n = 88
Val34 Homozygotes, intron K C Homozygotes (n)	19	30	33	63	37
Leu34 carriers, intron K C Homozygotes (n)	19	26	28	54	33
Unadjusted OR	–	0.87 (0.38, 1.98)	0.85 (0.38, 1.91)	0.86 (0.41, 1.78)	0.89 (0.41, 1.97)
Adjusted OR	–	1.33 (0.51, 3.52)	0.81 (0.31, 2.08)	1.08 (0.48, 2.45)	0.94 (0.38, 2.34)
Val34 homozygotes, intron K G Carriers (n)	10	10	15	25	15
Unadjusted OR	–	0.63 (0.22, 1.81)	0.86 (0.32, 2.30)	0.75 (0.31, 1.85)	0.77 (0.29, 2.04)
Adjusted OR	–	0.59 (0.18,1.96)	0.92 (0.31, 2.76)	0.76 (0.29, 2.02)	0.86 (0.29, 2.52)
Leu34 Carriers, intron K G Carriers (n)	15	9	3	12	3
Unadjusted OR	–	0.38 (0.14, 1.04)	0.12 (0.03, 0.45) †	0.24 (0.10, 0.60) †	0.10 (0.03, 0.40) ‡
Adjusted OR	–	0.30 (0.09, 0.96) *	0.08 (0.02, 0.39) †	0.19 (0.07, 0.55) †	0.08 (0.02, 0.36) ‡
Synergy factor unadjusted	–	0.69 (0.23, 2.98)	0.16 (0.03, 0.85) *	0.37 (0.10, 1.35)	0.15 (0.03, 0.80) *
Synergy factor adjusted	–	0.38 (0.23,1.62)	0.11 (0.02, 0.61) *	0.23 (0.06, 0.85) *	0.10 (0.02, 0.53) †

The wild-type individuals (Val34 and intron K C homozygotes) served as the reference in each study group. ORs were calculated by comparing different patient groups with the CAS−MI− (clinical control) group. The respective 95% CI values are shown in parenthesis after the OR and synergy factor values. ORs were adjusted for gender, age, smoking, Lp(a) and serum HDL-C concentrations. CAS+ and CAS−, patients with and without coronary atherosclerosis, respectively; MI+ and MI−, patients with and without a history of myocardial infarction, respectively; OR, odds ratio; n, number of individuals in each subgroup; * $p < 0.05$, † $p < 0.01$, ‡ $p < 0.001$.

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 (Table 10). 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 (data not shown).

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 (Figure 5A,B), as well as in the CAS+ group (Figure 5E,F). 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 (Figure 5G,H). 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 (data not shown). 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 (Table 11).

Table 10. The effect of FXIII-B subunit polymorphisms on FXIII activity and antigen concentration.

Subjects	Wild Type for the Mutation			Carriers of the Mutation		
	n	FXIII activity (%)	FXIII-A_2B_2 antigen (%)	n	FXIII activity (%)	FXIII-A_2B_2 antigen (%)
FXIII-B p.His95Arg polymorphism						
All	594	103 ± 21	109±24	93	109 ± 23†	114±24*
CAS−MI−	202	103 ± 20	109±22	35	112 ± 23*	116±25
CAS+MI−	189	101 ± 22	108±24	25	107 ± 25	113±26
CAS+MI+	180	106 ± 22	111±25	30	107 ± 20	113±20
CAS+	369	103 ± 22	109±25	55	107 ± 22	113±23
MI+	203	105 ± 23	111±25	33	109 ± 21	114±22
FXIII-B intron K c.1952+144 C>G polymorphism						
All	486	106 ± 21	113±24	201	97 ± 21‡	101±22‡
CAS−MI−	158	106 ± 21	114±24	79	100 ± 20*	104±20†
CAS+MI−	155	106 ± 22	113±24	59	94 ± 21‡	99±23‡
CAS+MI+	151	109 ± 20	115±23	59	98 ± 24‡	103±26‡
CAS+	306	107 ± 21	114±24	118	96 ± 22‡	100±25‡
MI+	173	108 ± 21	115±24	63	99 ± 24†	103±25†

FXIII levels are expressed as mean ± SD. FXIII levels were adjusted to gender, smoking, serum total cholesterol and plasma fibrinogen levels. The levels of significance were calculated for the difference between wild type individuals and carriers of the respective mutation. CAS+ and CAS−, patients with and without coronary atherosclerosis, respectively; MI+ and MI−, patients with and without a history of myocardial infarction, respectively; *n*, number of individuals in each subgroup; * $p < 0.05$, † $p < 0.01$, ‡ $p < 0.001$.

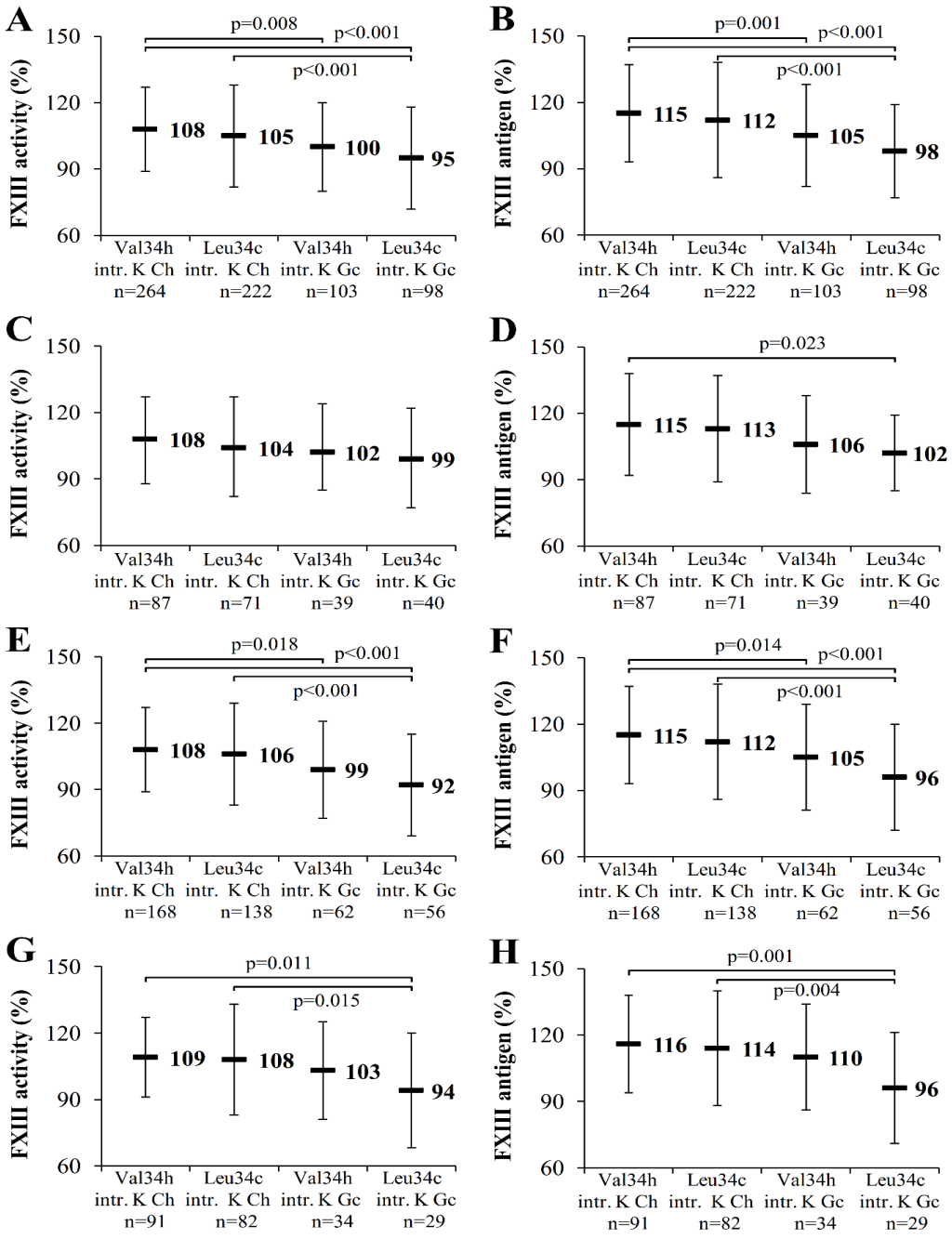

Figure 5. The effect of FXIII-A p.Val34Leu, FXIII-B intron K c.1952+144 C>G polymorphisms and their combination on FXIII activity and antigen levels. FXIII levels adjusted for gender, smoking, serum total cholesterol and plasma fibrinogen levels are expressed as the mean ± SD; the numerical values of the means are also shown beside the thick horizontal lines. Val34h, His95h and intr. K Ch: homozygotes for the major alleles in p.Val34Leu, p.His95Arg and intron K C>G polymorphisms, respectively. Leu34c, Arg95c, intr. K Gc: carriers of the respective minor alleles. Significant differences between genotype combinations are indicated by the p-values associated with the horizontal lines on the upper part of the figure. FXIII activity (A,C,E,G) and antigen (B,D,F,H) levels are demonstrated in the whole study group (A,B), in the CAS−MI− (C,D), in the CAS+ (E,F) and in the MI+ (G,H) patient groups.

Table 11. The effect of FXIII levels in the lower tertile on the risk of CAD in patients with an elevated fibrinogen concentration.

Subjects	CAS−MI−	CAS+MI−	CAS+MI+	CAS+	MI+
FXIII activity upper tertile (n)	21	33	40	73	47
FXIII activity lower tertile (n)	24	24	22	46	24
Adjusted OR	–	0.65 (0.25, 1.69)	0.38 (0.15, 0.98) *	0.52 (0.23, 1.17)	0.39 (0.16, 0.96) *
FXIII antigen upper tertile (n)	19	32	40	72	46
FXIII antigen lower tertile (n)	24	25	24	49	25
Adjusted OR	–	0.57 (0.23, 1.42)	0.36 (0.14, 0.91) *	0.49 (0.22, 1.08)	0.35 (0.14, 0.86) *

Elevated fibrinogen concentration represents the upper tertile of fibrinogen concentration (>4.3 g/L) in all study subjects. ORs were calculated by comparing different patient groups with the CAS−MI− (clinical control) group. The respective 95% CI values are shown in parenthesis after the OR values. ORs were adjusted for gender, age, smoking, Lp(a), serum triglyceride and homocysteine concentrations. CAS+ and CAS−, patients with and without coronary atherosclerosis, respectively; MI+ and MI−, patients with and without a history of myocardial infarction, respectively; OR, odds ratio; *n*, number of individuals in each subgroup; * $p < 0.05$.

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 [90]. We determined FXIII-A_2B_2 antigen level; however, as 99% of FXIII-A in the plasma is complexed with FXIII-B [7] the two parameters are comparable. Our result on FXIII antigen seemingly contradict the data reported in reference [90]. 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 [90] 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 (Table 3). Similarly, positive correlation was observed by Ariens et al. between age and FXIII subunit antigen levels, but not between age and FXIII activity [90]. 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 [76-78]. 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 [90]. 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-A_2B_2 antigen and FXIII activity (cd: 0.845, $p<0.001$) and FXIII activity, just like FXIII-A_2B_2 antigen, showed significant age dependence.

The strong correlation of tFXIII-B antigen level with FXIII-A_2B_2 antigen level and FXIII activity is not surprising. Similar strong correlation between the two FXIII subunits has been reported earlier [90]. FXIII-A level is low in FXIII-B deficiency [51], while the administration of FXIII-A to FXIII-A deficient patients increases plasma tFXIII-B concentration [127, 128]. 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 (Table 4). 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-A_2B_2 levels in relation to different p.His95Arg genotypes [85]. However in our case the minor Arg95 allele significantly increased the complex FXIII-A_2B_2 antigen concentration. Furthermore after adjustment, the increase in all FXIII parameters became statistically significant (Table 4).

FXIII-A p.Val34Leu accounts only for a small part of the 47% hereditability of FXIII-A_2B_2 [88]. In a separate study FXIII activity was not affected by carriership of the Leu34 allele [78]. 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-A_2B_2 antigen, with a low level of statistical significance in Leu34 carriers, disappeared, when when the effect of FXIII-B intron K polymorphism was eliminated (Figure 4A–C).

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-A_2B_2 antigen and tFXIII-B antigen were considerably lower than in wild type individuals (Table 4). 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 (Figure 4).

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-A_2B_2 levels (Figure 4A,B). 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-A_2 and FXIII-B_2 form a tight complex in the plasma, and the *K*d for their interaction is in the range of 10^{-10} M [7]. 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 [19, 129-134]. Following the administration of FXIII-A_2 concentrate prepared from human placenta to FXIII-B deficient patients, FXIII-A quickly disappeared from the plasma [19]. 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 [134]. In the absence of FXIII-B, the short half-life of FXIII-A_2 might be related to its spontaneous non-proteolytic activation in plasmatic condition [135]. One can speculate that the replacement of 10 C-terminal amino acids plus the added extra 15 amino acids to the C-terminus in intronK mutants might influence either the interaction of the two subunits or the clearance of FXIII-A_2B_2 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 [6, 7]. It has been shown that plasma free FXIII-B_2, just like FXIII-A_2B_2, is also bound to fibrinogen [25], and that both the 1st and 10th sushi domain of FXIII-B are involved in the binding [136]. 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 [102], 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 [103]. In the end the overall protective effect of Leu34 allele against VTE and CAD was confirmed by meta-analyses of the reported findings [104, 111]. 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 [103]. 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 [83]. 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 [137], 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 presented in Tables 6–11 and Figure 5. 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 [138]. 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 [105]. 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 demonstrated in Table 9 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) [103]. 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 (data not shown). However, the protective effect of the Leu34 allele prevailed only in the presence of the intron K G allele (Table 9). 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, for details see references [93, 94, 139]. Elevated FXIII levels represent a significant risk of myocardial infarction or peripheral arterial disease in females, but not in males [99, 100]. It has been shown that the homozygous presence of the FXIII-A Leu34 allele decreased the FXIII levels in CAS+ and MI+ patients [140]. Arg95 allele had only minor effect on FXIII activity and FXIII-A_2B_2 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-A_2B_2 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 [141]. 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-A_2B_2 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-A_2B_2 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-A_2B_2 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-A_2B_2 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-A_2B_2 antigen concentration, similarly to healthy individuals, while carriers of both Val34 and intron K C alleles had the highest FXIII activity and FXIII-A_2B_2 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.

7. ÖSSZEFOGLALÁS

A dolgozat két összefüggő téma vizsgálatát írja le: 1/A véralvadás XIII-as faktor (FXIII) B alegységének (FXIII-B) polimorfizmusai és egyéb nem genetikus tényezők hatása a FXIII aktivitás és antigén szintjére egészséges egyénekben, 2/A FXIII szintek és a FXIII polimorfizmusok, különösen a FXIII-B alegység polimorfizmusainak a hatása a szívkoszorúér-betegség kockázatára.

Az eredmények azt mutatják, hogy egészséges egyénekben a FXIII plazma szintje multi faktoriális szabályzás alatt áll, melyek közül a kor, a fibrinogén koncentráció és a FXIII-B intron K c.1952+144 C>G polimorfizmus a legfontosabb. A nemnek nem volt hatása a FXIII aktivitás illetve a komplex plazma FXIII (FXIII-A_2B_2) antigén szintekre. Meglepő módon a totál FXIII-B (tFXIII-B) antigén szint férfiakban szignifikánsan magasabb volt mint nőkben. A FXIII-B intron K G allél hordozókban a FXIII aktivitás, FXIII-A_2B_2 és tFXIII-B antigén szintek szignifikánsan alacsonyabbak voltak mint a vad típusú egyének esetében. Habár a FXIII-B intron K c.1952+144 C>G polimorfizmus hatása a FXIII szintekre független a FXIII A alegység (FXIII-A) p.Val34Leu polimorfizmus jelenlététől, a két minor allél együttes jelenléte volt a legnagyobb hatással a FXIII aktivitásra és FXIII-A_2B_2 antigén szintre. A FXIII szintek csökkenése a Leu34-intron K G allél hordozók esetében volt a legnagyobb. A FXIII szintjét befolyásoló faktorok ismerete elősegítheti a FXIII trombotikus betegségekben betöltött szerepének a megértését és további kutatások kiindulópontja lehet.

A FXIII-B p.His95Arg polimorfizmus nem befolyásolta a coronaria ateroszklerózis (CAS) illetve a miokardiális infarktus (MI) rizikóját, ezzel szemben a FXIII-B intron K G allél csökkentette a CAS és MI rizikóját azon egyéneknél, akiknél a fibrinogén szint a felső harmadban volt. Érdekes módon a FXIII-B intron K G allél védő hatása csak a FXIII-A Leu34 allél jelenlétében jelentkezett; a két polimorfizmus hatása közt egy szinergizmus volt kimutatható. Az intron K G allél hordozók szignifikánsan alacsonyabb FXIII aktivitással és FXIII-A_2B_2 antigén szinttel rendelkeztek, hasonlóan az egészséges populáción kapott eredményeinkhez. Ugyanúgy, mint az egészséges populáció esetén a legalacsonyabb FXIII aktivitást és FXIII-A_2B_2 antigén szintet a Leu34 és az intron K G allélek együttes jelenléte esetén mértünk, míg a Val34 és az intron K C allélre egyaránt homozigóták rendelkeztek a legmagasabb FXIII aktivitással és FXIII-A_2B_2 antigén szinttel. Mivel az alsó harmadban levő FXIII szintek szignifikánsan csökkentették a MI rizikóját, kijelenthető, hogy a FXIII-B intron K G és a FXIII-A Leu34 allélok közös védő hatása összefügg az alacsony FXIII szintekkel.

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9. LIST OF PUBLICATIONS

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

Candidate: Zoltán András Mezei
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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., Fiatal, 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

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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.
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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
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10. KEYWORDS

Coronary artery disease

FXIII activity

FXIII antigen

FXIII-A p.Val34Leu polymorphism

FXIII-B p.His95Arg polymorphism

FXIII-B intron K c.1952+144 C>G polymorphism

Fibrinogen

Gender

Healthy individuals

Myocardial Infarction

11. TÁRGYSZAVAK

Koszorúér-betegség

FXIII aktivitás

FXIII antigén

FXIII-A p.Val34Leu polimorfizmus

FXIII-B p.His95Arg polimorfizmus

FXIII-B intron K c.1952+144 C>G polimorfizmus

Fibrinogén

Nem

Egészséges személyek

Myocardialis infarctus

12. ACKNOWLEDGEMENTS

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14. APPENDIX

Two original publications related to the dissertation.