

SHORT THESIS FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY (Ph.D.)

FACTOR XIII AND ITS VAL34LEU POLYMORPHISM IN
ATHEROTHROMBOTIC DISEASES

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of
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The Examination takes place at The Lecture Hall of the 1st Department of Medicine, Institute for Internal Medicine, Medical and Health Science Center, University of Debrecen, February 28, 2011.

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BACKGROUND

The plasmatic form of blood coagulation factor XIII (FXIII) is a zymogen (protransglutaminase) of tetrameric structure (A₂B₂). It contains two potentially active A subunits (FXIII-A) and two inhibitory/carrier B subunits (FXIII-B). Cellular FXIII (cFXIII) is a homodimer of FXIII-A, and exists in vast amounts in platelets and megakaryocytes. FXIII-A is synthesized by cells of bone marrow origin, while FXIII-B is synthesized by hepatocytes and the two subunits form a tetrameric complex in the circulation. The reference interval for plasma FXIII-A₂B₂ concentration is 14-28 mg/L. FXIII-A consists of an activation peptide (1-37 amino acids), one β-sandwich (38-184 amino acids), one catalytic (“core” domain, 185-515 amino acids) and two β-barrels (516-628 and 629-730 amino acids). FXIII-B is a mosaic protein, it consists of 10 “sushi” domains, each held together by a pair of disulphide bridges.

Plasma FXIII is transformed into an active transglutaminase (FXIIIa) by the proteolytic action of thrombin in the presence of Ca²⁺. Thrombin removes the activation peptide from FXIII-A, then in the presence of Ca²⁺ FXIII-B dissociates and FXIII-A assumes an enzymatically active configuration (FXIII-A'). The presence of fibrin greatly accelerates the activation process.

Factor XIII is essential for maintaining haemostasis, its deficiency causes severe bleeding diathesis, in non-substituted patients frequently with fatal brain hemorrhage. The primary physiological substrates of FXIIIa are fibrin and α₂-plasmin inhibitor (α₂PI). Cross-linking by FXIIIa improves the mechanical strength, rigidity and elasticity of the clot and increases its resistance to fibrinolysis. FXIIIa cross-links fibrin γ- and α-chains and the major cross-linked products are γ-chain dimers and high molecular weight α-chain polymers. γ-chain dimer formation is an extremely quick process, requires only minute amount of FXIIIa and immediately follows the removal of fibrinopeptide A from fibrin. The multiple cross-linking of

α -chains among several acyl donor and acyl acceptor sites proceeds more slowly than γ -chain dimer formation. Besides γ -chain dimers and α -chain polymers γ - α chain heterodimers and γ -chain trimers/tetramers are also formed. α_2 PI is an excellent acyl donor substrate for FXIIIa and it can be cross-linked to the α -chain of fibrin and fibrinogen rapidly. Its covalent attachment to fibrin plays a primary role in preventing the prompt elimination of newly formed fibrin by the powerful fibrinolytic system. The physiological role of FXIII is unusually diverse for a clotting factor. In addition to hemostasis, FXIII has important functions on at least three major closely related, yet distinct areas. It promotes angiogenesis; it is important for wound healing and plays an essential role in maintaining pregnancy.

The gene coding for human FXIII-A (F13A1) is located at chromosome 6p24-25, it contains 15 exons and 14 introns and spans over 160 kb. Exon I consists of the 5' noncoding region; exon II encodes AP-FXIII. The β -sandwich domain, the catalytic core domain and the two β -barrel domains are encoded by exons II-IV, exons IV-XII, exons XII-XIII and exons XIII-XV, respectively. It is transcribed into a 3.9-kb mRNA, with an 84-base pair 5' untranslated region, a 2.2-kb open reading frame and a 1.6-kb 3' untranslated region.

The gene of FXIII-B is located at 1q31-32. The gene of FXIII-B subunit (F13B) is located at position 1q31-32.1, it is composed of 12 exons producing a 2.2 kb mRNA. Exon I encodes a 20 amino acid leader sequence, exon II-XI encode for the sushi domains, each sushi domain is encoded by a single exon. The last exon codes for a COOH-terminal region of FXIII-B, for the 3'-untranslated region and for the polyA tail. The expression of F13B is directly regulated by transcription factors HNF1 α and HNF4 α .

The following common polymorphisms with amino acid exchange have been described in FXIII-A: Val34Leu, Tyr204Phe, Leu564Pro, Val650Ile and Glu651Gln, among which the

Val34Leu polymorphism, has stirred considerable interest, because of its suspected thrombo-protective effect. The frequency of Leu34 allele among Caucasians is around 25%, in Africans its frequency is significantly lower and in Asians it is extremely rare. The concentration of plasma FXIII in subgroups with different Val34Leu genotypes does not differ significantly indicating that the rate of secretion of FXIII-A variants and their lifespan in the plasma are similar. Considering that the location of this polymorphism is just 3 amino acids up-stream from the thrombin cleavage site, one would expect its influence on thrombin-induced FXIII activation. Indeed, it was demonstrated with both cFXIII and plasmatic FXIII (pFXIII) that the thrombin induced release of AP-FXIII from the Leu34 FXIII-A variant, as well as the consequent activation of FXIII proceed at a 2.5-folds higher rate than in the case of Val34 variant. This finding was also supported by NMR studies testing the binding of the synthetic AP-FXIII segment to thrombin. Faster activation of FXIII results in accelerated fibrin cross-linking and in a higher rate of α_2 PI incorporation into fibrin. These experiments were carried out with purified proteins and it was not known if such a relationship between FXIII-A Val34Leu polymorphism and FXIII activation also exists in the more complex environment of human plasma.

The specific activity of fully activated pFXIII, cFXIII and recombinant FXIII-A₂ of different FXIII-A Val34Leu genotypes are identical. In a few studies, measurement of FXIII activity was performed with FXIII only partially activated by thrombin. In these cases the measured activity reflected the rate of FXIII activation rather than the catalytic concentration of FXIII and higher values were obtained for the Leu34 variant than for the Val34 variant. In conclusion, neither plasma FXIII concentration nor the transglutaminase activity of FXIIIa is influenced by FXIII-A Val34Leu genotype. The rate of activation is, however significantly faster in the case of the Leu34 variant.

Further interesting findings were, that the structure of fibrin clots is influenced by FXIII-A Val34Leu polymorphism, and this effect is modulated by the concentration of fibrinogen. At high fibrinogen concentrations, plasma samples from homozygotes for the Leu34 allele form clots having looser structure, thicker fibers and increased permeability, while at low fibrinogen concentrations fibrin meshwork had thinner, more tightly packed fibers and lower permeability. Practically no fibrinogen concentration dependent changes were observed in the plasma samples of wild type individuals.

The polymorphic nature of FXIII-B has been revealed by isoelectric focusing experiments a long time ago. Based on these experiments FXIII-B was classified into three major population-associated genotypes (European, African, Asian), plus several rare alleles. Two major FXIII-B polymorphisms have been described, His95Arg substitution in the second sushi domain, and C-to-G change at position 29756 in intron K29756 resulting in a novel splice acceptor site. The frequency of FXIII-B Arg95 carriers among healthy Caucasians is about 15%; it is more frequent among Africans and is missing from the Asian population. The polymorphism did not influence FXIII-A, FXIII-B and pFXIII antigen levels. In the plasma of individuals carrying the Arg95 allele, FXIII-A₂B₂ antigen levels were significantly decreased following thrombin activation that might be due to increased dissociation of the subunits. However, these results could not be confirmed by steady-state binding experiments using purified FXIII subunits and therefore, further investigations are needed to draw a final conclusion.

The association of FXIII with the risk of thrombosis includes both major group of thrombotic diseases, venous thromboembolism (VTE) and atherothrombotic diseases and our interest concerned the latter group.

The wide range of data published in the literature and the results of meta-analysis suggest that gene-gene and gene-environmental interactions might significantly influence the effect of FXIII-A Val34Leu polymorphism. The results of several recent studies support such a hypothesis. In young women with obesity the presence of Leu34 allele provided significant protection against MI (OR: 0.33, 95%CI: 0.13, 0.83) while in non-obese subject it did not (OR: 1.68, 95%CI: 0.8, 3.51). The interaction among plasminogen activator inhibitor 1 (PAI-1), insulin resistance and FXIII-A Val34Leu polymorphism represents another example. In subjects possessing the Leu allele insulin resistance and elevated PAI-1 level, related to 4G/5G polymorphism in the PAI-1 gene, led to the loss of cardioprotection. The association between FXIII levels and FXIII polymorphism with the risk of peripheral artery disease (PAD) and atherothrombotic ischemic stroke (AIS), is much less investigated than the association with the risk of coronary artery disease (CAD). As part of our studies deal with this topic the data available in the literature will be reviewed and compared with our data in the Discussion.

The aims of my PhD studies were the followings:

- 1/ To develop a method for the detection of FXIII-A Val34Leu polymorphism, which is applicable for the quick, reliable analysis of a large number of DNA samples, including samples isolated from postmortem material.
- 2/ To establish if the effect of FXIII-A Val34Leu polymorphism on FXIII activation, which has been investigated on purified proteins, also prevails in the more complex environment of human plasma.
- 3/ To reveal the association of FXIII levels and FXIII-A Val34Leu polymorphism with the risk of PAD.

4/ To reveal the association between FXIII-A Val34Leu polymorphism and the risk of AIS with non-fatal or fatal outcome.

MATERIALS, METHODS AND PATIENTS

Development of a melting point analysis method, using fluorescence resonance energy transfer detection (FRET), for the identification of FXIII-A Val34Leu polymorphism

DNA was isolated from citrated whole blood of 113 unrelated subjects [68 wild-type (V/V), 36 heterozygote (V/L), 9 homozygote (L/L)] using MagNA Pure LC Instrument (Roche Diagnostics). Primers and probes were synthesized by TIB MOLBIOL. The detection probe (sensor) labeled with fluorescein at the 3' end covers the polymorphic site. A C→A mismatch was introduced in the sensor to decrease the melting temperature (T_m) for both alleles. The adjacent anchor probe was 5' labeled with the LC-Red640 dye and the 3' end was phosphorylated to prevent probe elongation by Taq polymerase. The gap between the two probes is a single nucleotide. If the probes lie adjacent to each other on a DNA strand, fluorescence resonance energy transfer occurs and the fluorescence of LC Red640 is detected by the fluorimeter component of LightCycler.

PCR reactions were performed in the LightCycler glass capillaries in a final volume of 21 μ l containing 150 ng of genomic DNA, 0.19 [μ mol/l] of each primer, 0.57 [μ mol/l] of anchor probe, 0.38 [μ mol/l] of sensor probe, 2 U Taq DNA polymerase (Roche), 2 μ l of 10 x concentrated PCR reaction buffer (Roche), 42.9 ml/l dimethyl sulfoxide (Sigma), 0.48 [mmol/l] of each dNTP (Roche), plus an extra 2.86 [mmol/l] of $MgCl_2$ (Roche). One sample without DNA, as a negative control, was included in all assay series. Cycling conditions were as follows:

initial denaturation at 95°C for 30 s, 60 cycles of denaturation at 94°C for 0 s, annealing at 50°C for 5 s and extension at 72°C for 10 s. Melting curve analysis consisted of one cycle at 94°C for 15 s and at 35°C for 15 s, followed by increasing the temperature to 70°C at a 0.1°C/s ramping rate. The fluorescent signal of LC Red640 was monitored continuously during the temperature ramp and the results were transformed to derivative melting curves.

Activation of FXIII in plasma and the recovery of fibrin

9 ml blood was collected in 1 ml 0.105 M trisodium citrate and platelet poor plasma was collected by centrifugation. Among the 23 selected healthy individuals (9 males, 14 females, age 22-45) 7 were wild type, 8 heterozygous and 8 homozygous for Val34Leu polymorphism. Val34Leu genotype was determined by the real-time polymerase chain reaction method described above. Highly purified human FXIII were prepared from the plasma of healthy volunteers with wild-type and homozygous Leu34 FXIII-A genotype. Human thrombin (1690 U/mg) was from Sigma (St.Louis, MO, USA). FXIII deficient plasma was purchased from Sigma and Trinity Biotech (Bray, Ireland).

100 µl of normal plasma of different FXIII-A Val34Leu genotypes were incubated with 0.1-0.3 U/ml thrombin and 18 mM CaCl₂ at 37°C. After various intervals the reaction was stopped by an equal volume of inhibitor cocktail containing 50 mM ethylenediaminetetraacetic acid, 20 mM benzamidine, 50 mM ε-aminocaproic acid, 2 mM iodoacetamide, 0.1 mM d-phenylalanyl-1-prolyl-1-arginine chloromethyl ketone, 100 mM NaCl and 50 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, pH 7.5 to block thrombin and FXIIIa, and to prevent fibrinolysis. The samples were centrifuged, the supernatants were removed and added to 9 volumes of sodium dodecyl sulfate-polyacrilamide gel electrophoresis (SDS-PAGE) sample

buffer. The remaining fibrin clot, if present, was washed with physiological NaCl solution. After dissolving the clot in 1 ml SDS-PAGE sample buffer its protein content was determined and $T_{1/2}$ for fibrin formation (the time between the addition of thrombin and the transformation of 50% of fibrinogen into fibrin polymer) was calculated. Similar experiments were also performed on two different batches of FXIII deficient plasma substituted with 20 $\mu\text{g/ml}$ highly purified FXIII of Val34Val or Leu34Leu genotype. For the experiments 0.17 U/ml thrombin was used. Varying thrombin concentrations in the range of 0.1-0.3 U/ml did not change the tendency of the results. At thrombin concentrations higher than 0.3 U/ml the clotting of plasma and the activation of FXIII was so quick that no meaningful evaluation of the time course of activation process could be carried out.

SDS-PAGE and Western blotting

Aliquots of denatured plasma/serum and fibrin samples were analyzed by SDS-PAGE. After SDS-PAGE part of the samples were electroblotted to Immobilon P membrane (Millipore, Bedford, MA, USA). FXIII-A and FXIII-A' were detected by rabbit anti-FXIII-A antibody (Calbiochem, San Diego, CA, UAS). The immuno-reaction was developed by Vectastain Elite ABC kit (Vector, Burlingame, CA, USA) and visualized by chemiluminescence (ECL Plus; Amersham, Little Chalfont, UK) according to the manufacturers' instructions. The relative amounts of FXIII-A and FXIII-A' were determined by quantitative densitometry using GS-800 Calibrated Densitometer (Bio-Rad, Hercules, CA, USA) and expressed as a percentage of the total FXIII present on the blot at full activation.

The time required for the activation of 50% of FXIII present in the plasma ($T_{1/2}$ FXIII-A') was calculated. The reproducibility of the assay was better than 5%. The amount of γ -chain

dimers was determined by quantitative densitometry of the Coomassie-stained gels and the results were expressed as a percentage of fully cross-linked γ -chains.

Cases and controls

Study on peripheral artery disease (PAD)

302 consecutive PAD patients were recruited over a 2-year period from the 3rd Department of Medicine, University of Debrecen. Patients with history of myocardial infarction, stroke, symptoms of angina and renal insufficiency were excluded. The patients were exempt of acute inflammation during the last two months before blood drawing and did not have any chronic inflammatory state, other than PAD. Finally, 278 patients (173 males and 105 females) with ankle brachial pressure index (ABPI) ≤ 0.9 remained in the study group (173 with Fontaine classification II and 105 with Fontaine classification III). Patients were compared to sex-matched clinical controls (n=278) who also presented at the hospital, but no significant health problem, other than diabetes mellitus in some of the patients, was diagnosed and ABPI was in the range of 0.91-1.3. The mean age \pm SD was 59.0 \pm 9.5 in the control and 64.3 \pm 12.2 in the patient group. The occurrence of diabetes mellitus was 20% and 25% among controls and patients respectively. Thirty percent of controls and 36% of patients were smokers. The differences were not statistically significant. Two hundred and seventeen PAD patients received Aspirin and 33 patients received statins; since in the FXIII levels there was only a non-significant difference (<1%) between PAD patients on treatment and the rest of the patients, these groups were not analyzed separately. Eighty-eight percent of women were menopausal; none of them on hormonal replacement therapy. Ethical approval for the studies was obtained from the Ethics

Committee of the Medical and Health Science Center, University of Debrecen, Hungary.

Individuals in the control groups and patients gave informed consent.

Study on patients surviving or not surviving atherothrombotic ischemic stroke (AIS)

508 consecutive patients who survived IS were included in the study, no one refused. The diagnosis of IS was based on clear, unambiguous clinical symptoms persisting for more than 24 hours, and was confirmed by computed tomography or nuclear magnetic resonance imaging. In 12 patients cardioembolic IS was diagnosed; they were excluded from the study and only patients with AIS remained. It is to be noted that these patients were enrolled at a military hospital and they had a relatively younger age and male dominance. The non-survivor group consisted of 316 patients with AIS, who died in the hospital within 4 weeks after admittance. In this group the diagnosis and the cause of death were confirmed by autopsy and formalin-fixed paraffin-embedded (FFPE) tissue specimens of the patients were stored in the Pathology Department of Diósgyőri Vasgyári Hospital, Miskolc, Hungary. Women involved in the study were not on hormonal replacement therapy.

The population control group that represented the general Hungarian population consisted of 1146 Hungarian individual. We selected age and/or sex matched controls to the patient groups from this population control group. As the age and sex distribution in the groups of patients surviving and not surviving AIS differed significantly, separate matched control groups had to be selected for the two patient groups.

Ethical approval for the studies was obtained from the Ethics Committee of the Medical and Health Science Center, University of Debrecen, Hungary. Individuals in the control groups and patients gave informed consent.

Laboratory methods used in clinical studies

Plasma FXIII activity and antigen were measured by commercially available reagent kits (REACHrom FXIII assay and R-ELISA FXIII, Reanal-ker, Budapest, Hungary) based on methods developed in our laboratory. In the measuring range, the CV for both assays was below 3%. Serum total cholesterol, LDL-cholesterol, HDL-cholesterol, triglyceride, apoAI, apo B, Lp(a) and high sensitivity C-reactive protein (hsCRP), plasma fibrinogen, homocysteine, folic acid and vitamin B₁₂ were determined by routine laboratory methods and used for the determination of parameters independently associated with FXIII levels. Plasma fibrinogen level was measured by a modified Clauss method. Plasma homocysteine, folic acid and vitamin B₁₂ were measured by immunoassays using AxSYM immunanalyser (Abbott Laboratories, Abbott Park, IL).

DNA from controls and patients was isolated from the buffy coat of citrated blood samples by QIAamp DNA Blood Mini Kit (Qiagen). DNA was also extracted from archival FFPE human brain tissues of patients died of fatal AIS. Five 10- μ m sections were prepared from FFPE blocks; de-paraffinization and DNA extraction were performed by EZ1 DNA Tissue Kit using EZ1 Advanced equipment (Qiagen) according to the manufacturer's protocol. Val34Leu polymorphism was determined by real time PCR using FRET detection and melting curve analysis on LightCycler equipment (Roche Diagnostics) using the method described above.

Statistical analysis

The Kolmogorov-Smirnov test was performed to examine the normality of the distribution of different parameters. Fibrinogen, triglyceride, HDL cholesterol, apo AI, hsCRP, homocysteine, folic acid and vitamin B₁₂ were log-transformed to normalize the distribution. An independent

Student's t test was used to assess differences in continuous data. A P value <0.05 was considered to indicate statistical significance. Correlation between ABPI and FXIII levels was determined by Pearson's method. A multiple linear regression analysis was performed for FXIII activity and antigen to determine the parameters independently associated with FXIII levels. The significance of differences in mean FXIII values between the clinical control and PAD patient were tested by analysis of variance (ANOVA). When one-way ANOVA indicated a significant difference, post-hoc pair-wise comparisons were made using the least significant difference test. The effect of elevated FXIII levels on the risk of PAD was also analyzed. The risk represented by FXIII activity, antigen levels being in the upper tertile, as compared to the rest of the patients, and the effect of FXIII-A genotype were expressed as the odds ratio (OR) and 95 percent confidence interval(CI), which were computed from the corresponding regression coefficient in the logistic regression model. χ^2 test was used for differences in category frequencies. Adjusted ORs were obtained by the use of a model that included FXIII-A genotype and age. All statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS).

RESULTS

Evaluation of the newly developed melting point analysis method, using FRET detection, for the identification of FXIII-A Val34Leu polymorphism

In the Materials and Methods section we described a DNA-based method using hybridization probes, melting point analysis and FRET detection on LightCycler (Roche) for rapid identification of the FXIII Val34Leu polymorphism. Melting curve analysis easily discriminated the three different FXIII-A Val34Leu genotypes: T_m for the wild-type (Val/Val) was at 56.4°C,

while T_m for homozygote mutant (Leu/Leu) was at 47.5°C; heterozygotes demonstrated both peaks.

113 individual DNA preparations from a sample pool were tested by the method described above and by PCR-restriction fragment length polymorphism (PCR-RFLP). The results were in 100% concordance. The frequency of the Leu34 allele was 24.2% (95% confidence interval: 16.3-32.1%) which well agrees with the allele frequency obtained by PCR-RFLP on a different group of the same ethnic Hungarian population (25.9%; 95% confidence interval: 22.3-29.5%), and with the data reported for other Caucasian populations. 50 randomly selected DNA samples (26 wild type, 19 heterozygous and 5 homozygous for FXIII-A Val34Leu polymorphism) were also evaluated by fluorescent DNA sequencing using BigDye Terminator Reaction kit on an ABI 310 Genetic Analyzer (Applied Biosystems), again 100% concordance was observed. To test the reproducibility of the assay, DNA samples of all three genotypes were retested on at least 20 different occasions. All results were consistent.

The effect of FXIII-A Val34Leu polymorphism on the activation of FXIII in whole plasma

In collaboration with Dr. Bagoly (Zsuzsa Bagoly doctoral thesis, University of Debrecen 2008) we have shown that at a thrombin concentration that occur in physiological conditions the activation of FXIII takes place exclusively on the surface of fibrin and the truncated form (FXIII-A₂') never appears in the serum. Continuing this work, first I studied the quantitative correlation between fibrin clot formation and FXIII activation. Once fibrin is formed (2 min after the addition of thrombin and Ca²⁺), FXIII becomes attached to its surface and truncated by thrombin. The formation of fibrin is the initiator of FXIII activation and there is a highly significant

correlation between the time required for half maximal fibrin formation ($T_{1/2}$ for fibrin formation) and the time required for half maximal proteolytic activation ($T_{1/2}$) of FXIII.

$T_{1/2}$ values for FXIII activation in the plasma from Val34Val wild-type individuals, from Val34Leu heterozygotes and from Leu34Leu homozygotes were practically identical.

The results indicate that in whole plasma fibrin polymerization and not FXIII-A Val34Leu polymorphism is the primary determinant for the onset of FXIII activation. However, a closer analysis of the time course of fibrin formation and FXIII activation revealed that even in whole plasma FXIII-A Val34Leu polymorphism is not without effect on the proteolytic activation process. Fibrin formation preceded FXIII activation in all three genotypes, but the lag time between fibrin formation and FXIII-A truncation was significantly longer in the plasma of wild-type individuals than in the plasma of Leu/Leu homozygotes. γ -chain cross-linking starts with the activation of FXIII, but then it slightly precedes it, which is not surprising given that a small amount of activated FXIII is sufficient for full dimerization of γ -chains.

To provide further evidence for the influence of Val34Leu polymorphism on the thrombin activation of FXIII in plasma, experiments on two FXIII deficient plasma samples, substituted with highly purified FXIII of Val34Val or Leu34Leu genotype, were performed. In these plasma samples everything but FXIII genotype was the same. Although the two plasma samples demonstrated a different time course of fibrin polymerization, the formation of truncated FXIII-A in samples containing FXIII of Val/Val genotype clearly lagged behind the appearance of FXIII-A' in samples containing FXIII of Leu/Leu genotype.

Factor XIII levels in PAD patients

Characteristics of subject groups

Patients with PAD were 5.3 years older than subjects in the clinical control group. Diabetes mellitus and smoking was somewhat more frequent among PAD patients than among controls; however the difference did not reach the level of statistical significance. With the exception of homocysteine only marginal differences were detected between the patient and control group in the traditional risk factors. The elevation of total plasma homocysteine concentration in patients with PAD was highly significant, and it seemed to be related to decreased B12 vitamin levels. Neither FXIII activity nor FXIII A₂B₂ antigen levels of clinical controls differed significantly from the reference interval established in our laboratory. Non-adjusted plasma FXIII activity and antigen (FXIII A₂B₂) levels were significantly higher in the patient group.

Adjusted factor XIII levels

Mean FXIII activity and antigen levels adjusted for the respective independently associated parameters were moderately, but significantly higher in the patient than in the clinical control group. When the results were analyzed according to gender, in women the presence of PAD was associated with a statistically significant (8-9%) elevation of FXIII activity and antigen levels. In the male PAD subgroup somewhat lower elevations were observed and the difference in FXIII antigen concentrations did not reach the level of statistical significance. No difference in adjusted FXIII levels were found in PAD patients with Fontaine II and Fontaine III stage disease (not shown), and ABPI values did not show significant correlation with FXIII activity or antigen level.

Elevated factor XIII as risk factor of peripheral artery disease

It was also investigated if FXIII levels in the upper tertile (FXIII activity > 120 %, FXIII antigen >25.5 mg/L) represent a risk of PAD as compared to the rest of the patients. In males elevated FXIII activity or antigen level did not increase the risk of PAD. In contrast, elevated FXIII activity conferred a more than two-fold, statistically significant risk of PAD on females. FXIII antigen in the upper tertile also represented a two-fold risk of PAD in women, however in this case the P value was somewhat above the limit of statistical significance.

The effect of FXIII-A Val34Leu genotype on the risk of peripheral artery disease (PAD)

There was no significant difference in genotypes, carrier or allele frequencies between the two groups. Similar negative results were obtained, if the groups were subdivided according to gender and the female and male patient groups were compared with their respective control groups.

The odds ratios were calculated against sex matched control groups. No significant effect of Leu34 carriership or the presence of one or two Leu34 alleles was detected. The situation was the same if female or male patients were compared with their respective controls.

The effect of factor XIII A subunit Val34Leu polymorphism on the risk of atherothrombotic ischemic stroke (AIS)

Gender-, and age-dependent distribution of FXIII-A Val34Leu genotype in the general population

As we intended to investigate gender specific differences in the effect of FXIII-A Val34Leu genotypes on the risk of non-fatal AIS, first we explored the frequency of these genotypes in a population control group that represent the general Hungarian population. There was no gender-

specific difference in the frequency of FXIII-A Val34Leu genotypes, Leu34 carriers and Leu34 allele in the population control group. The effect of age on the distribution of Val34Leu genotype was investigated by dividing the population control group into age tertiles and comparing them to each other. The lack of statistically significant difference among the age groups indicates that in the general population FXIII-A Val34Leu polymorphism does not provide survival advantage or disadvantage.

General characteristics of the patients surviving AIS

In this patient group the median age of males suffering AIS was somewhat higher than that of females and hyperlipidemia (cholesterol >5.2 mmol/L and/or triglyceride >1.7 mmol/L) was more frequent in male than in female patients. There was no gender difference in the frequency of diabetes mellitus and hypertension.

The effect of FXIII-A Val34Leu genotype on the risk of non-fatal ischemic stroke

There was no significant difference in genotypes, carrier or allele frequencies between the two groups. Similar negative results were obtained, if the groups were subdivided according to gender and the female and male patient groups were compared with their respective age-matched control groups.

The odds ratios were calculated against age and sex matched control groups as well as against the whole population control group. No significant effect of Leu34 carriership or the presence of one or two Leu34 alleles was detected. The situation was the same if female or male patients were compared with their respective controls.

The effect of FXIII-A Val34Leu genotype on the risk of fatal ischemic stroke

The general characteristics of the study group of patients who died of AIS is shown in. Although there was a statistically significant difference in the age of females and male other characteristics of the study group showed no gender difference.

Due to the high age of patients died of AIS, the median age of sex-matched control group that could be selected from control population was 10 years lower than that of the patient group. The frequency of Val/Val, Val/Leu genotypes, Leu34 carriers and Leu34 allele did not differ significantly between the group of patient with fatal AIS and their respective sex-matched controls. However, the frequency of Leu34 homozygotes was significantly higher in the non-divided patient group ($p = 0.007$) and among female patients with fatal AIS ($p = 0.009$) than in the respective control groups. The difference between the male patient group and the respective control group was not significant.

Age adjusted ORs were calculated against sex-matched controls and against the population control group. Neither Leu34 carriership nor hetrozygosity for the Leu34 allele influenced the risk of fatal AIS significantly in either gender. In contrast, homozygous occurrence of the Leu34 allele significantly increased the risk of fatal AIS in the total patient group. However, this effect was restricted to females, to which gender a more than 3-fold increased risk of fatal AIS was conferred by the presence of two Leu34 alleles.

DISCUSSION

FRET based melting point analysis method for genotyping FXIII-A Val34Leu polymorphism

Our FRET based melting point analysis method was used throughout the study for genotyping FXIII-A Val34Leu polymorphism and the method has been adopted by other laboratories. Two

other real-time PCR methods, apyrase-mediated allele-specific primer extension and 5' nuclease assay, has also been described for genotyping FXIII-A Val34Leu polymorphism. The results obtained by the assays were compared to those obtained by DNA sequencing on a relatively small number (17 and 20, respectively) of samples. Both methods seem to represent a promising alternative to earlier assays. However, as discrimination among different genotypes by these methods is based on quantitative measures, comparisons with reference methods on a larger number of samples would have been required to confirm their specificity. The real time PCR method presented in this study uses FRET detection and differentiates among genotypes on the basis of a clear difference in the T_m of wild type and mutant allele. With LightCycler the amplification of the target DNA and determination of the genotype are performed in the same closed capillary, limiting the risk of error and contamination considerably. The LightCycler software allows for obtaining a large amount of information from a single run. The assay is specific, reproducible and the total analysis of 31 samples in the LightCycler takes less than 30 min. Its specificity has been confirmed by comparison with the most frequently used PCR-RFLP method and with DNA sequencing on a large number of samples. Since the introduction of the assay more than 3,000 patients' samples have been tested for Val34Leu polymorphism in our laboratory and in all cases a clear discrimination among the different FXIII-A Val34Leu genotypes could be observed.

Effect of fibrin formation and factor XIII-A subunit Val34Leu polymorphism on the activation of factor XIII

The aim of this study was to determine the relative importance of fibrin formation and FXIII-A Val34Leu polymorphism in controlling the time and the rate of the proteolytic step of FXIII

activation and to reveal their interaction in whole plasma. In accordance with earlier reports factor XIII bound to the newly formed fibrin polymer in its native non-truncated form, but with a short lag phase it soon became cleaved by thrombin. The results suggest that at a thrombin concentration that occur in physiological conditions the activation of FXIII takes place exclusively on the surface of fibrin and after its formation FXIIIa remains associated with its substrate. There was a highly significant correlation between the time required for half maximal activation ($T_{1/2}$) for FXIII activation and $T_{1/2}$ for fibrin formation. These results indicate that in whole plasma fibrin polymerization and not FXIII-A Val34Leu polymorphism is the primary determinant for the onset of FXIII activation. However, a closer analysis of the time course of fibrin formation and FXIII activation revealed that even in whole plasma FXIII-A Val34Leu polymorphism is not without effect on the proteolytic activation of FXIII. The lag time between fibrin formation and FXIII-A truncation was significantly longer in the plasma of wild-type individuals than in the plasma of Leu/Leu homozygotes. Further evidence on the influence of Val34Leu polymorphism on the thrombin activation of FXIII in plasma, was provided by experiments on FXIII deficient plasma samples, substituted with FXIII of Val34Val or Leu34Leu genotype. The proteolytic activation of FXIII-A started at the same time, but proceeded significantly faster in samples containing FXIII of Leu/Leu genotype than in the samples containing FXIII of Val/Val genotype. In conclusion, in whole plasma the onset of FXIII activation is primarily determined by fibrin formation; however, once the activation of FXIII starts, the rate of activation is modulated by Val34Leu polymorphism.

It is not surprising that the primary determinant of the initiation of FXIII activation is the formation and at least partial polymerization of fibrin. It is known that the conversion of fibrinogen into fibrin, i.e. the formation of at least partially polymerized fibrin I and fibrin II

greatly enhances the thrombin-catalyzed activation of FXIII. The cleavage of FXIII-A by thrombin in the fibrin I-FXIII complex ($k_{cat}/K_m = 1.4 \times 10^7 \text{ M}^{-1} \text{ sec}^{-1}$) was 100-fold more efficient than the cleavage of free, uncomplexed FXIII ($k_{cat}/K_m = 1.4 \times 10^5 \text{ M}^{-1} \text{ sec}^{-1}$). At the same time only a 2.5-fold increase in the rate of AP-FXIII release is conferred by the Leu34 allele to the thrombin induced activation process. Thus, the onset of fibrin polymerization well overrules any effect of Val34Leu polymorphism and becomes the initiator of FXIII activation. However, the promoting effect of polymeric fibrin is rapidly lost, when catalytically competent FXIIIa is allowed to form. When the degree of γ -chain dimerization exceeds approximately 40%, for which minor amount of FXIIIa is sufficient, the promoter effect of fibrin is completely abrogated. This means, that although the role of FXIII Val34Leu polymorphism in the initiation of FXIII activation in whole plasma is negligible, after initiation the rate of the activation is modified by the polymorphism, and the release of AP-FXIII proceeds significantly faster in the case of Leu34 allele. It is interesting to speculate if such a difference in the rate of activation has any physiological significance. It has been shown that the higher rate of proteolytic truncation of Leu34 FXIII-A resulted in accelerated cross-linking of α_2 PI to fibrin α -chain. The formation of this heterodimer is a fast process, only slightly lags behind γ -chain dimerization, i.e. Leu34 FXIII-A provides an earlier protection of newly formed fibrin against the prompt elimination by the fibrinolytic system.

The fact that cross-linking of fibrin by FXIIIa down-regulates the adhesion of platelets to fibrin might offer the following hypothesis: earlier activation of Leu34 FXIII-A would result in earlier cross-linking of fibrin in the growing thrombus, that would down-regulate platelet adhesion and inhibit further thrombus growth. The influence of FXIII-A Val34Leu polymorphism on fibrin structure, making it more prone to fibrinolysis, is another alternative

hypothesis. This mechanism seems to be effective only at high fibrinogen concentration. The biochemistry of this effect of Val34Leu polymorphism is still to be revealed.

Factor XIII and the risk of peripheral artery disease

FXIII levels were elevated in patients with PAD, and this was more pronounced in women than in men. The extent of FXIII elevation did not correlate with the severity of PAD. One may conclude from these results that the existence of PAD and not the difference in its severity is associated with the elevation in FXIII levels. In a small (n=50) early study elevation of non-adjusted FXIII levels has also been observed in PAD patients, however in this case the patients were not characterized and differences according to gender and severity of the disease were not analyzed.

The reason for the elevation of FXIII levels in PAD is not known. Plasma FXIII is a tetrameric complex of two potentially active A and two inhibitor/carrier B subunits (FXIII-A and FXIII-B). FXIII-B is in excess to FXIII-A, i.e., the actual amount of FXIII A₂B₂ complex is determined by the amount of FXIII-A. Megakaryocytes are a major source of FXIII-A synthesis and circulating platelets contain a huge amount of FXIII-A. Although platelet FXIII-A is retained during platelet activation, platelets may become damaged when pressed through occluded arteries and release their FXIII-A content into the circulation. Such a mechanism could contribute to the increase of FXIII level in patients with PAD.

Elevated FXIII level conferred a more than two-fold, statistically significant risk of PAD on females. Several pieces of evidence suggest that impaired fibrinolysis caused by elevated PAI-1 level and decreased tPA activity increases the risk of PAD. Factor XIII is a major regulator of fibrinolysis; it strengthens fibrin clot and makes it more resistant to shear forces and

to fibrinolysis. At elevated FXIII levels this mechanism could be more forceful and in PAD, together with the elevation of α_2 PI level, could participate in impairing the fibrinolytic potential. In addition, FXIIIa has also been shown to enhance migration and proliferation of smooth muscle cells and monocytes, two processes, which have also been implicated in the progression of atherosclerosis. It remains to be seen, why the effect of elevated FXIII level is more prominent in women.

It is known that the onset of acute complications of atherothrombotic disease occurs at older ages in women than in men, but even after adjustment for age, women have poorer outcome. Although the mechanism has not been revealed, it seems that in females hemostatic risk factors are more important determinants of atherothrombotic diseases than in males. Our finding suggests that elevated FXIII is a gender-specific risk factor of these two atherothrombotic diseases and supports the suggestion that in atherothrombotic diseases the clotting/fibrinolytic system plays a role more prominent in females than in males.

Only a single report was found on the association of FXIII-A Val34Leu polymorphism and PAD. In this study, like in our study, the polymorphism did not influence the occurrence of PAD, but there was a later onset of PAD in Leu/Leu homozygotes (mean age 67.3 years) than in Val/Val wild type individuals (mean age: 64.1 years). The lack of data on the association of FXIII-A Val34Leu polymorphism and PAD warrants further investigations.

Atherothrombotic Ischemic Stroke (AIS) and FXIII A Val34Leu polymorphism

We were able to collect 13 publications in the literature on the relationship of FXIII-A Val34Leu polymorphism and the risk of IS. Most of the studies as well as an interim mini-metaanalysis, demonstrated the lack of association of FXIII-A Val34Leu genotypes with the risk of IS,

although in a few reports protection against IS or increased risk of IS was associated with the polymorphism. In nine of these publications AIS and cardioembolic IS cases were considered as a common group. However, due to the different pathomechanism of the two types of IS, the same genetic or environmental factors might exert different, occasionally opposing, effects on the risk of AIS and cardioembolic IS. Thus, the results obtained with the common group of patients might obscure results that could have been obtained by separate risk assessment. Only in 4 published smaller studies was AIS separated from cardioembolic stroke in the analysis of the risk associated with FXIII-A Val34Leu polymorphism. In two of these studies (n=31 and n=121) the polymorphism was found neutral. In another study involving 105 AIS patients an OR of 0.63 (CI: 0.37-1.10) suggested a protective effect against AIS, however it did not reach statistical significance. In two cases the effect of FXIII-A Val34Leu polymorphism on the risk of small vessel disease stroke was investigated. No protective effect was found by in one study (n=159), while in another small study (n=66) the Val/Val genotype conferred a 2.1-fold increased risk of stroke (CI: 1.1- 3.9). To eliminate the possibility of false negative result due to small sample size, our study involved 496 patients, and it is far more the largest study in which the effect of FXIII-A Val34Leu polymorphism on the risk of AIS, separately from cardioembolic IS was investigated. Comparison with both a sex and age matched control group and with a general population control group, which reflects the general occurrence of the polymorphism in the population, could not detect any protective effect of Leu34 carriership and homozygous or heterozygous Leu34 variants against the risk of AIS.

The results obtained with CAD and PAD (see above) seemed to justify the gender specific analysis of the effect of FXIII-A Val34Leu polymorphism on the risk of AIS. Two former studies have been published on the association of FXIII-A Val34Leu polymorphism and

IS in females, however, here again, AIS and cardioembolic stroke were not analyzed separately. It has been reported a nearly 4-fold increase of IS in young women carrying two copies of the Leu34 allele. However, this study involved only 36 cases. In a larger study (n = 190) on young women no significant effect of Val34Leu polymorphism on the risk of IS could be revealed. In our study on AIS survivor patients the polymorphism failed to exert a significant effect on the risk of AIS in either gender.

In summary, FXIII Val34Leu polymorphism does not seem to be a significant protective or risk factor of AIS, although genetic and environmental factors might modulate this picture and might be responsible for some of the contradictory findings. As discussed earlier, the modulatory effect of insulin resistance and fibrinogen level on the effect of FXIII-A Val34Leu polymorphism has been revealed in CAD patients. Such interactions still await to be investigated in the case of AIS. The only study in this respect concerned the interactive effect of fibrinogen level and FXIII-A Val34Leu polymorphism on the outcome of thrombolytic therapy of IS. Patients with wild type FXIII-A and low fibrinogen level displayed the best clinical outcome; while carriers of the Leu34 allele having high fibrinogen level showed almost no clinical response.

To our knowledge this is the first study concerning the effect of Val34Leu polymorphism on the risk of fatal AIS. In our study neither Leu34 carriership nor Val34Leu heterozygosity influenced the risk of fatal AIS significantly. In contrast, the presence of double Leu34 alleles conferred an approximately three-fold risk of AIS with fatal outcome on women, but not on men. This interesting gender-specific effect suggests that, although homozygous form of Leu34 allele did not influence the risk of the onset of AIS, it increased the risk of more severe consequences

in women. The fact that the OR in heterozygous women was close to 1.0 excludes a gene dosage effect.

The findings reported in this study raise two questions. 1/ Why homozygous presence of Leu34 allele increases the risk of fatal IS? 2/ Why is the high risk of fatal AIS conferred only on women? The Leu allele that confers protection against MI has been shown to be a risk factor of primary intracerebral hemorrhage. The fatal outcome of AIS in many cases is due to hemorrhagic transformation of AIS. Following this line it might be that homozygous form of FXIII-A Val34Leu polymorphism increases the risk of hemorrhagic complication of acute AIS. As discussed above the increased severity of acute events in females suffering of CAD has been connected to hemostatic risk factors. Our results on the gender-specific association of FXIII-A Val34Leu polymorphism and fatal AIS support such hypothesis. However, the biochemical background of the increased sensitivity of women to hemostatic risk factors remains to be explored.

SUMMARY

A new method based on real time PCR with fluorescence resonance energy transfer (FRET) detection and melting curve analysis was developed for the detection of factor XIII A subunit (FXIII-A) Val34Leu polymorphism. The rapid, simple method is well applicable for large-scale analysis. The results with this method showed 100% coincidence with those obtained by the traditional PCR-RFLP assay and fluorescent DNA sequencing.

In plasma the time of thrombin induced fibrin formation closely correlated with the time of FXIII activation, while there was no significant correlation between the onset of FXIII activation and FXIII-A Val34Leu genotype. However, in the case of Leu34 variant the proteolytic activation of FXIII proceeded significantly faster than in the case of Val34 variant. The results suggest that in whole plasma the onset of FXIII activation is determined by fibrin formation, while the rate of activation is modulated by Val34Leu polymorphism.

FXIII levels were elevated in patients with peripheral artery disease (PAD) and the elevation was more evident in females than in males. The severity of PAD did not show significant correlation with FXIII levels. FXIII levels in the upper tertile conferred a 2-2.3-fold increased risk of PAD to females, but not to males, i.e., elevated FXIII could be considered a gender specific risk factor of PAD. FXIII-A Val34Leu polymorphism did not influence the risk of PAD.

No association was revealed between the risk of non-fatal atherothrombotic ischemic stroke (AIS) and FXIII-A Val34Leu genotype. In contrast, in females homozygous presentation of Leu34 allele represented a more than 3.0-fold increased risk of AIS with fatal outcome. FXIII-

A Val34Leu polymorphism does not influence the occurrence of AIS, but has a gender specific effect on the severity of its outcome.