Thesis for the Degree of Doctor of Philosophy (Ph.D.)

FXIII ACTIVITY, ANTIGEN LEVEL AND FXIII-A VAL34LEU POLYMORPHISM IN CORONARY ARTERY DISEASE

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INTRODUCTION

Blood coagulation factor XIII (FXIII) is a zymogen (protransglutaminase) of tetrameric structure (A_2B_2). It contains two potentially active A subunits (FXIII-A) and two inhibitory/carrier B subunits (FXIII-B). 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 plasma. In the plasma FXIII-A is present only in complex, while FXIII-B can be found also in a free form, this is approximately 50% of its total amount. Plasma concentration of FXIII complex (FXIII) is 21 mg/L. Cellular FXIII lacks FXIII-B and exists as a A_2 dimer (in platelets, monocytes and macrophages). 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). The gene of FXIII-A is located at 6p24-25, it contains 15 exons and 14 introns. The activation peptide is coded by exon 2. FXIII-B is a mosaic protein, it consists of 10 "sushi" domains, two disulphide bridges ensure the forming of the appropriate structure in all of these domains. The gene of FXIII-B is located at 1q31-32.

Plasma FXIII is transformed into an active transglutaminase (FXIIIa) by the proteolytic action of thrombin in the presence of Ca^{2+}. Thrombin removes an activation peptide of 37 amino acids from FXIII-A, 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. The active transglutaminase catalyses an acyl-transfer reaction. In the first step a peptide-bound glutamine residue forms a thioacyl intermediate with the active site Cys314 and ammonia is released. In the absence of an amine substrate hydrolysis of the thioacyl intermediate occurs and the peptide bound glutamine becomes deaminated. If a substrate primary amine is present the acyl group is transferred to the acyl acceptor primary amine and the amine becomes linked to the glutamyl residue through an isopeptide bound. If the substrate amine is provided by the ε-amino group of a peptide bound lysine residue an ε(γ-glutamyl)lysyl is formed and peptide chains become covalently cross-linked. FXIII plays an important role in the last phase of coagulation, the main task of FXIII is to cross-link fibrin chains and to covalently attach proteins important in the regulation of fibrinolysis to the newly formed fibrin network. Cross-linking of fibrin by FXIII improves the mechanical stability 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-plasmin inhibitor is an excellent acyl donor substrate for FXIIIa and it can be cross-linked to the α-chain of fibrin and fibrinogen. Both fibrin cross-linking and the cross-linking of α_2-plasmin inhibitor to fibrin have been suggested to play a major role in protecting fibrin from fibrinolysis. If the activity of FXIIIa is inhibited the degradation of fibrin network by plasmin is highly elevated. The cross-linking of α_2-plasmin inhibitor to fibrin occurs at an early stage of fibrin formation and provides protection of newly formed fibrin against the activated fibrinolytic system, while the increased resistance to thrombolysis of maturated thrombi could be the consequence of extensive cross-linking of fibrin α-chains. In addition to α_2-plasmin inhibitor FXIIIa also cross-links further components of the fibrinolytic system. The physiological significance of the cross-linking of plasminogen and thrombin activatable fibrinolysis inhibitor (TAFI) to fibrin remains elucidated. After cross-linking of type 2 plasminogen activator inhibitor (PAI-2) to fibrin PAI-2 remains fully active. With the exception of pregnancy PAI-2 doesn't appear in the plasma, but activated monocyte incorporated in the thrombus might secrete it. PAI-2 targeted to fibrin by cross-linking might be involved in the protection against urokinase-type plasminogen activator.

The FXIII-A gene is rather polymorphic, in the coding region there are five sequence changes. Among the FXIII-A polymorphisms the Val34Leu is the most well characterized because of its suspected thrombo-protective effect (see later). The polymorphism is located in the 2. exon, in the activation peptide of the FXIII-A protein, only 3 amino acid distance from the thrombin cleavage site. The Leu allele frequency is within a narrow range among the Caucasians (24,5-28,8%). With the exception of the investigation of a smaller group performed in our institute the investigation of the general Hungarian population has not been performed previously. The biochemical consequences of the polymorphism were examined by several studies. As the polymorphic amino acid residue is located in the immediate vicinity of thrombin cleavage site of FXIII-A one would expect that the effect of thrombin is influenced by the Val34Leu substitution. In the case of Leu34 variant FXIII the

release of the activation peptide is 2.5-fold faster than in the case of Val34 FXIII and this effect is independent of fibrinogen level although fibrinogen itself increased the catalytic efficiency. Earlier activation accelerated the cross-linking of fibrin γ-and α-chains and the cross-linking of α_2-plasmin inhibitor to fibrin. It was also demonstrated that FXIII-A Val34Leu polymorphism influences the structure of fibrin. Fibrin fully cross-linked by Leu34 FXIII showed a finer structure with thinner fibers and smaller pores than fibrin cross-linked by FXIII Val34. Although the rate of activation of FXIII by thrombin shows differences according to the Val34Leu genotype using adequate method (see later) and appropriate incubation time there is no difference in the transglutaminase activity by genotype in normal individuals.

The association between FXIII activity, FXIII antigen concentration and coronary sclerosis (CS)/myocardial infarction (MI) was investigated by a couple of studies. Due to methodological differences, there is some confusion in the literature concerning plasma FXIII activity in individuals of different FXIII-A Val34Leu genotypes. Before discussion the results of the previous studies it is useful to give a brief overview on the assays used for the determination of FXIII activity and antigen concentration. Originally, the solubility of fibrin clot in concentrated urea or monochloroacetic acid solution was used for the determination of FXIII activity. This method is not standardized, not quantitative and detects only very severe (below 1%) FXIII deficiencies. Therefore this method is now considered obsolete and its use as a screening test is to be abandoned. It is a common feature of all functional FXIII assays that first, FXIII is activated by thrombin and Ca^{2+} and then, the transglutaminase activity of FXIIIa is quantitatively determined. Methods for the measurement of transglutaminase activity can be based on two different principles: 1. the measurement of ammonia released in the firs step of transglutaminase reaction, or 2. the determination of small molecular weight amines covalently cross-linked to a protein substrate.

1. The detection of ammonia is performed using a NADP(H) dependent glutamate dehydrogenase (GlDH) indicator reaction. In the kinetic spectrophotometric assay developed by our laboratory one NADPH molecule transformed into NADP corresponds to one molecule of ammonia released, this way the release of ammonia can be continuously monitored at 340 nm. Fibrin polymerization is inhibited by a tetra peptide in the assay. Glycine ethyl ester is used as an amine substrate, and a synthetic dodecapeptide that corresponds to the N-terminal sequence of α_2-plasmin inhibitor as the donor substrate. The introduction of serum blank eliminated the systemic over-estimation – especially at low FXIII activities - observed with the previously used

assays. A further advantage of this assay the use of significantly higher thrombin concentrations for activating FXIII. The high thrombin concentration is sufficient to activate FXIII completely even in situation when fibrinogen cannot support the efficiency of thrombin-induced FXIII activation. FXIII-A Val34Leu polymorphism doesn't influence the results obtained by this assay (see later). The advantages of the assays based on monitoring the released ammonia are the following: they are true kinetic enzymatic methods, one-step, quick tests, easy automatable, a reference interval established according to adequate methods is available.

2. In the amine incorporation assays fluorescent dansylcadaverine, radio-labeled (^{14}C, ^{3}H) putrescine, histamine, or glycine ethyl ester, dinitro-phenyl cadaverin, or biotinylated cadaverine serve as amine substrates which are covalently linked to an acyl donor glutamine residue in a protein substrate. Protein-bound and free amines are separated and the protein-linked fraction is quantitatively measured. A common advantage of these assays is the high sensitiveness, however they are rather time-consuming. In some assays the protein substrate is bound to the surface of a microtiter plate that makes the removal of non-bound amine easier, thus the test requires less time (microtiter plate method). A common problem with the so-called microtiter plate assays using surface-linked substrate proteins that the amount of protein substrate is insufficient to achieve optimal substrate concentration. In these assays the kinetic is far from being zero order and the measured activity is not a linear function of the enzyme concentration. Besides the previously mentioned problems in the commercially available microtiter plate assay too low thrombin concentration (1 U/mL) is used for the activation of FXIII and as a consequence FXIII present in the plasma becomes only partially activated. The rate of thrombin-induced activation of FXIII is influenced by the Val34Leu polymorphism. Leu34 variant FXIII is activated significantly faster than the Val34 protein, and the rate of activation of heterozygous FXIII is in-between. At 1 U/mL thrombin concentration the quantity of thrombin activated fraction strongly depends on FXIII-A Val34Leu genotypes, by increasing the thrombin concentration the differences disappear. Although the specific activity and catalytic efficiency of FXIII of different Val34Leu genotypes are identical, at insufficient thrombin activation the same amount of Val/Val, Val/Leu and Leu/Leu FXIII results in different transglutaminase activities, leading to significant under- (Val/Val) or over-estimation (Leu/Leu) of the real FXIII activity. Due to this problem in the absence of genetic analysis, it is not possible to decide by the assay if a higher FXIII level is true or due to the presence of Leu/Leu FXIII.

ELISA method is used for the determination of plasma FXIII antigen concentration. Both the tetrameric FXIII complex (FXIII A_2B_2) and the individual FXIII subunits can be measured. It is advisable to determine the concentration of the FXIII A_2B_2 as a first tool. A one-step sandwich ELISA using biotinylated anti-FXIII-B and peroxidase-labeled anti-FXIII-A monoclonal antibodies was developed in our laboratory. In a streptavidin-coated microplate the reaction can be carried out quickly. Free FXIII subunits and fibrinogen do not interfere with the assay. The results obtained by the ELISA – with the exception of a rare case when abnormal, not activatable FXIII-A is synthesized – correlated well with the results obtained with the kinetic spectrophotometric assay.

The relationship between CS/MI and plasma FXIII activity and antigen concentration has been addressed in a few studies. Activity measurements in these studies were carried out with the microplate incorporation assay and the results are strongly influenced by the FXIII-A Val34Leu polymorphism. Besides this the results are rather contradictory. To solve this problem it is necessary to explore the association of CS/MI with the FXIII levels determined by a functional test in which the different rate of FXIII activation caused by the FXIII-A Val34Leu polymorphism doesn't influence the detected transglutaminase activity. It is also interesting to investigate if the effect of different Val34Leu genotypes is observed in patients with coronary artery disease using adequate method for determining FXIII levels.

Coronary artery disease (CAD) is a major health issue in both women and men, however the time of its onset, the course of the disease, the presentation of clinical symptoms and the response to therapy show gender specific features. Rupture or erosion of atherosclerotic plaque leads to the activation of blood coagulation and platelets and eventually, to intra-coronary thrombus formation causing acute ischemic events. Although the role of clotting factors in the cardiovascular risk has not yet been fully explored, it is generally appreciated that the prothrombotic state resulting from the elevation of certain clotting factors and suppression of fibrinolysis increase the risk of MI. Besides the so-called classical risk factors, recently the role of some hemostasis factors also suggested. Among these fibrinogen and fibrinolytic inhibitor PAI-1 are the most well established hemostatic risk factors for CAD and gender-related differences could also be demonstrated concerning the effects of these risk factors. It is interesting that fibrinogen levels of women were generally higher than those of men and significant positive correlations of fibrinogen with high blood pressure was observed only in women. In the Framingham Study the relationship of fibrinogen with CAD was only significant in diabetic women. In subjects with CAD

higher PAI-1 levels were found in females than in males. Since blood coagulation FXIII is intimately related to fibrinogen and is a key factor in the regulation of fibrinolysis it is suggested that FXIII can play a role in the development of CS/MI and based on the results of the above mentioned studies there can be also a difference between males and females.

The association between FXIII-A Val34Leu polymorphism and MI was investigated by several studies, however the results are rather contradictory. In the first papers it was demonstrated that the presence of the Leu34 allele provided a protective effect against MI. Although, contradictory studies have also been published. It has already been suggested that gene-environmental and gene-gene interactions might be responsible for the conflicting results on the effect of FXIII-A Val34Leu polymorphism. As the rate of change in fibrin clot permeability with increasing fibrinogen concentrations decreased stepwise with increasing number of Leu34 alleles it has been proposed that the protection by Val34Leu polymorphism becomes effective only at higher fibrinogen concentrations. However, this hypothesis has not been tested on patients with CAD. Because of the contradictory results concerning the protective effect of Leu34 allele, performing a meta-analysis based on the published literature is also necessary.

AIMS

1. Investigation of FXIII-A Val34Leu polymorphism in the general Hungarian population and in patients with cardiovascular disease and determination the frequency of Leu34 allele in the general Hungarian population. To explore if the presence of FXIII-A Leu34 allele is protective against CS/MI and if this protective effect is dependent on fibrinogen level.
2. Meta-analysis, based on our results and the previously published data, to evaluate the contradictory results concerning the association between FXIII-A Val34Leu polymorphism and CS/MI.
3. Investigation of association between FXIII activity/antigen concentration and CS/MI using adequate laboratory methods, to establish if elevated FXIII levels represent a risk for these diseases in males and females.
4. Determination of FXIII activity and antigen concentration in different FXIII-A Val34Leu genotypes in controls and in CS/MI patients using adequate laboratory methods for measuring FXIII levels.

PATIENTS, MATERIALS AND METHODS

Patients and control individuals:

One thousand and ten consecutive Hungarian patients admitted for investigation of suspected CAD were recruited over an 18-month period from the Department of Cardiology, University of Debrecen, Medical and Health Science Center. Coronarography was performed for demonstrating the presence, localization and severity of coronary sclerosis. Patients having single, double or triple vessel CS based on the presence of ≥50% stenosis in a major coronary artery or in one of its branches were graded as CS+, patients without or with less significant coronary stenosis were graded as CS-. The diagnosis of MI was established at the time of its onset according to the criteria of the American College of Cardiology (ACC) and the European Society of Cardiology (ESC). At least three months were allowed to elapse between the onset of MI and the time of obtaining blood samples for laboratory investigations. Because of the uncertainty of the diagnosis of MI or the lack of one or more laboratory parameters 55 patients were excluded from the study. Patients without significant coronary stenosis and with a lack of a history of MI were considered as the clinical control group (CS-MI-). All the other patients were subdivided according to the presence or absence of CS and/or MI leading to the following subgroups: CS+MI-, CS-MI+ and CS+MI+. Besides the presence or absence of CS and MI the presence of diabetes mellitus, hypertension and smoking were also registered. Continuous smoking was considered if at least 10 cigarettes were consumed a day. Ethical approval was obtained from the Ethics Committee of the Medical and Health Science Center, University of Debrecen, Hungary and the subjects gave informed consent.

The population control group consisted of 1146 Hungarian individuals. The sampling frame for the reference group included all those registered with the participating practices in the Hungarian General Practitioners' Morbidity Sentinel Stations Program coordinated by the Institute of Preventive Medicine/School of Public Health. 22 practitioners were selected from four counties in a way to represent the distribution of settlement size of each county and thereafter were asked to invite individuals randomly according to a previously specified algorithm from their practices.

Laboratory methods:

The blood sampling was carried out in a fasting state. The clinical chemistry and immunochemistry investigations were performed using a native serum on the same day. EDTA anticoagulated blood was used for the determination of homocysteine, the plasma was separated within two hours and stored at -20°C before measurement. Fibrinogen, FXIII activity and antigen were determined using Na-citrated plasma, the samples were stored at -20°C. Serum cholesterol, LDL-cholesterol, HDL-cholesterol, triglyceride, apo AI, apo B and lipoprotein (a) (Lp(a)) were determined by routine laboratory methods using Integra 700 laboratory analyzer (Roche Diagnostics, Mannheim, Germany). C-reactive protein (CRP) was determined using Roche Integra 400 laboratory analyzer. Plasma fibrinogen level was measured by a modified Clauss method (Fibrinogen assay kit, Reanal-Ker Ltd, Budapest, Hungary). Plasma homocysteine concentration was determined by fluorescence polarization immunoassay on AxSYM immune-analyzer (Abbott Laboratories, Abbott Park, IL). Plasma FXIII activity was determined by a modified optimized kinetic spectrophotometric assay developed in our institute (REA-chrom FXIII assay kit, Reanal-Ker Ltd.). The determination of FXIII A_2B_2 complex concentration in the plasma was carried out by a one-step sandwich enzyme-linked immunosorbent assay (ELISA) developed also in our institute (R-ELISA, Reanal-Ker Ltd.). DNA was isolated from the buffy coat of citrated blood samples by QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). FXIII-A Val34Leu polymorphism was determined by real time PCR with fluorescence resonance energy transfer detection and melting curve analysis on LightCycler equipment (Roche Diagnostics) according to a protocol developed in our laboratory.

Statistical methods:

The Kolmogorov-Smirnov test was performed to examine the normality of the distribution of different parameters. Parameters significantly deviated from normal (Gaussian) distribution were log-transformed to normalize the distribution. Results were expressed as mean (95 percent confidence interval), log-transformed results were expressed as geometric mean and anti-logged 95 percent confidence interval. An independent Student's t test was used to assess differences in continuous data. χ^2 test was used for differences in category frequencies. Spearman bivariate correlation coefficients were calculated to identify significant associations of FXIII antigen or

activity with other variables. 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 different patient groups were tested by analysis of variance (ANOVA). When one-way ANOVA indicated a significant difference, post-hoc pair-wise comparisons were made using the LSD (least significant difference) test. The effect of elevated FXIII levels on the risk of CS and MI was also analyzed. The risk represented by FXIII activity and antigen and fibrinogen levels being in the upper tertile as compared to the rest of the patients was expressed as the odds ratio (OR) and 95 percent confidence interval, which were computed from the corresponding regression coefficient in the logistic regression model. Age, gender, diabetes mellitus, smoking and different laboratory parameters were introduced into the logistic regression model to calculate adjusted odds ratios. Association between FXIII-A Val34Leu polymorphism and plasma FXIII levels were investigated in an ANOVA model. A P value of 0.05 or less was considered to indicate statistical significance. All statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS 11.5).

We used the routine of "meta" of the statistical package STATA for the meta-analysis of FXIII-A Val34Leu polymorphism. We used the "metabias" routine of the software package STATA to assess the possibility of publication bias (see later) by making funnel plot and performing the Egger's test.

RESULTS

Main characteristics of the different patients' groups:

Among the 955 recruited patients undergone coronarography investigations 302 had neither significant coronary stenosis nor MI, they represented the clinical control group (CS-MI-). 312 patients had significant CS without the history of MI (CS+MI-). 307 patients had both CS and MI (CS+MI+), while 34 patients had no significant CS, but positive history of MI in the anamnesis (CS-MI+). In this subgroup of patients coronary plaque with non-significant stenosis and/or coronary vasospasm must have been responsible for the previous MI. As expected, in the subgroups of patients with CS and/or MI there was a male dominance, as opposed to the clinical control group. The mean age of CS-MI- group was 55,5±10,2 years, the mean age of CS-MI+ was practically identical (55,4±10,3 years). Patients in the CS+ groups were significantly

older than patients without CS (CS+MI-: 60,8±10,1 years, p<0,001; CS+MI+ 58,6±10,7 years p<0,001).
Diabetes mellitus was more frequent among patients with CS and/or MI than among clinical controls. Smoking habits did not differ in the different patients groups.
Fibrinogen, triglyceride, HDL-cholesterol, homocysteine and Lp(a) did not show Gaussian distribution, logarithmic transformation was performed to normalize the distribution before further analysis. As compared to clinical controls fasting triglyceride and homocysteine levels were significantly elevated, while HDL-C and apo A levels were decreased in the groups of patients with CS (CS+MI-) and CS plus MI (CS+MI+). The increase of fibrinogen and Lp(a) levels was significant only in the CS+MI+ group. Serum cholesterol, LDL-cholesterol and apoB showed no differences among the patient groups.

Characteristics of the population control group:

The population control group (1146 individuals) consisted of 46.2% male and 53.5% female, the geomean of age was 46.2 years (95% CI: 45.2-47.1). When the patient groups were compared to the population control group the ORs were adjusted for these two (gender, age) parameters.

Determination of the frequency of FXIII-A Val34Leu polymorphism in the general Hungarian population and the different patient groups, investigating the effect of Leu34 allele on the risk of developing CS and MI, investigating the effect on the risk according to the fibrinogen level:

The distribution of FXIII-A Val34Leu genotypes, the Leu34 carrier and allele frequencies in the population control and clinical control groups were practically identical. Leu34 allele frequency in the general population was 25.9%, in the clinical control group it was 25.8%. Leu34 carrier frequency was 45.1% in the general population and 44.7% in the clinical control group. In the CS-MI+, CS+MI- and CS+MI+ groups the Leu34 allele frequency were 32.4%, 26.6% and 23.9% respectively, the Leu34 carrier frequency were 55.9%, 45.9% and 42.7% respectively. The differences from the general population and the clinical control group were not significant in the χ^2 test. The CS-MI+, CS+MI- and CS+MI+ groups were compared to the population control group (OR adjusted for gender and age) and to the clinical controls (OR adjusted for gender, age, cholesterol, fibrinogen, diabetes mellitus, Lp(a)

and homocysteine). Comparison of CS-MI+, CS+MI- and CS+MI+ patient groups with the population control or clinical control group revealed that neither Leu34 carriership nor Leu34 homozygosity exerted a statistically significant effect on the risk of CS and/or MI in the whole patient population. The effect of Leu34 allele and Leu34 homozygous genotype on the risk of CS and MI was also calculated in the combined group including all CS+ patients (CS+MI- and CS+MI+ groups) and in the combined MI+ group (CS-MI+ and CS+MI+). Significant effects on CS and MI were not detectable.

To reveal if the effect of Leu34 allele was influenced by plasma fibrinogen concentration, patients with fibrinogen in the upper quartile (>4.6 g/L) were separately investigated. ORs were adjusted for age, gender, Lp(a), homocysteine, triglyceride and current smoking because they were independently associated with CS and MI. In this case a statistically significant protection was conferred by the Leu34 allele against the combined presence of CS and MI (OR 0.40, 95%CI: 0.18-0.89, $p<0.05$). The protective effect of the Leu34 allele against CS and against MI could also be confirmed in the combined groups of patients. OR for the combined CS+ group was 0.46, (95%CI: 0.22-0.98), OR for the combined MI+ group was 0.41, (95%CI: 0.18-0.93). These findings suggest that in the Hungarian population the protective effect of Leu34 allele is restricted to individuals with higher fibrinogen concentration. As fibrinogen is an acute phase reactant it was also investigated if FXIII-A Val34Leu polymorphism exerts its protective effect in patients with elevated level of the inflammation marker CRP. However, in patients with CRP level in the upper quartile no protective effect of Leu34 allele against CS or MI could be demonstrated. This suggests that the protective effect of Leu34 allele is specific for fibrinogen itself and not for acute phase reaction (inflammation).

Evaluation of the contradictory results concerning the association between FXIII-A Val34Leu polymorphism and CS/MI by meta-analysis:

Besides our results we selected studies from MEDLINE where the definition of coronary artery disease was based on the occurrence of myocardial infarction or significant coronary artery stenosis verified by coronary angiography. We finally selected the results of sixteen studies that involved 5346 cases and 7053 control subjects. The vast majority of the enrolled people were Caucasian, 11 studies were published from Europe, 1 from South-America and 4 from North-America. Since the range of the study specific odds ratios was wide and the test for heterogeneity was significant we chose to use empirical random-effects Bayes model to obtain study

specific estimates of the odds ratios for each study and to combine these estimates. This method gives more conservative estimates than conventional fix-effect methods, furthermore it shrinks the study specific estimates towards the combined value reducing the influence of studies with extreme results. Furthermore, besides considering coronary artery disease as outcome, we analyzed the data using only myocardial infarction as outcome.

The overall risk of coronary artery disease was 18% less (OR 0.82, 95% CI: 0.73-0.94) in subjects with Val/Leu genotype than in subjects with Val/Val genotype. For the Leu/Leu genotype the combined OR was 0.89 (95% CI: 0.69-1.13). When Leu allele carriers (Val/Leu and Leu/Leu together) were compared to subjects with Val/Val genotype the estimate of the combined OR was 0.81 (95% CI: 0.70-0.92). Considering only myocardial infarction as outcome did not materially change this result. Carrying the Leu34 allele represented a 16% less risk of MI (OR 0.84, 95%CI: 0.76-0.94).

The result of the Egger' test for publication bias was statistically significant (p=0.002). In accordance with this, the funnel plot indicates that some small studies that did not find beneficial effect in the Leu allele carriers might be missing in the published literature.

Association of FXIII levels with coronary sclerosis/myocardial infarction, investigating the differences between males and females:

In none of the patient groups did non-adjusted FXIII activity or antigen deviate from the FXIII levels measured in clinical controls. Neither FXIII activity nor FXIII A_2B_2 antigen level of clinical controls differed significantly from those measured in the healthy reference population previously determined in our laboratory. Plasma FXIII activity and complex plasma FXIII (FXIII A_2B_2) antigen showed a high level of correlation in all patient groups (correlation coefficients varied between 0.86-0.93, $P<0.001$), demonstrating that the two methods measure different properties of the same plasma component. Multiple linear regression demonstrated that gender, smoking, cholesterol and fibrinogen levels were independently associated with FXIII activity and FXIII antigen levels, and means of FXIII levels adjusted for these parameters were used in further analysis. When the subgroups of patients were not subdivided according to gender FXIII levels, adjusted for independently associated variables, did not differ significantly among the subgroups. However, when the individual patient groups were broken down according to gender, the presence of MI

was associated with statistically significant gender-related differences in FXIII levels. CS and/or MI did not influence FXIII levels in males, similarly CS alone was without effect in females. However, if they had a positive history for MI, women suffering from CS showed elevated FXIII activity and antigen levels compared with CS+MI- females (FXIII activity: 107%, 95%CI: 100-115 versus 98%, 95%CI: 92-105, p=0.02, FXIII antigen: 24.1 mg/L, 95%CI: 22.4-25.8 versus 22.1 mg/L, 95%CI: 20.6-23.5, p=0.02). As compared to clinical controls FXIII antigen was significantly higher in the CS+MI+ female group. The same difference between FXIII activity in the clinical controls and in the CS+MI+ group did not reach the level of statistical significance. The highest adjusted FXIII levels were observed in females of the CS-MI+ groups (FXIII activity: 112%, 95%CI: 97-126; FXIII antigen: 25.1 mg/L, 95%CI: 21.7-28.5), however due to the small number of patients in this group the difference between this group and the respective clinical control group did not reach the level of statistical significance. The combined female MI+ groups (CS-MI+ plus CS+MI+) also showed elevated FXIII levels (108 %, 95% CI: 101-115 %; P=0.04) and antigen (24.2 mg/L, 95% CI: 22.6-25.5 mg/L; P=0.02) levels when compared with clinical controls.

It was also investigated if elevated FXIII levels represent a risk of MI and CS. Patients with FXIII levels in the upper tertile (FXIII activity > 110 %, FXIII antigen > 24.1 mg/L) were compared with patients having lower FXIII levels according to the risk of CS and/or MI in logistic regression model. In males elevated FXIII activity or antigen level did not increase the risk of CS or MI. When the male patient subgroups CS-MI+, CS+MI- and CS+MI+ were compared to the male clinical control subgroup, in none of the cases deviated the odds ratio from 1.0 significantly. Similarly, elevated FXIII levels did not confer a significant risk of CS to women without the history of MI (CS+MI- versus CS-MI-). These findings suggest that elevated FXIII levels do not represent a risk of the development of severe atherosclerosis in either of the sexes. In contrast to the respective male subgroup, when the female subgroup with CS and previous MI (CS+MI+) was compared to female clinical controls (CS-MI-) high odds ratios with high statistical significance (elevated FXIII activity: CS+MI+ versus CS-MI-: 3.091, 95% CI: 1.648-5.798, P<0.001 and elevated FXIII antigen: CS+MI+ versus CS-MI-: 2.346, 95% CI:1.269-4.336, P=0.007) were calculated. To separate the effect of elevated FXIII level on the risk of CS and MI even more clearly, subgroups of female patients with and without the history of MI, but with significant CS were compared (CS+MI+ versus CS+MI-). Elevated FXIII levels represented a significantly increased risk of MI in female patients with CS. OR for elevated FXIII activity was 1.873 (95% CI:1.015-3.458, P=0.04) and OR for elevated FXIII antigen

was 1.999 (95% CI:1.051-3.765, P=0.03). Based on these results it was demonstrated that elevated FXIII levels represented a risk on developing MI in females with CS, but the same effect was not demonstrated in the case of male patients.

Effect of coronary sclerosis/myocardial infarction on plasma FXIII levels in different FXIII-A Val34Leu genotypes:

It was demonstrated in our previous study that FXIII activity and antigen concentration did not show any difference among certain Val34Leu genotypes in healthy individuals. Although there was a slight tendency of decreasing FXIII activity and antigen with increasing number of Leu alleles in the CC group, the differences among FXIII-A genotypes were not significant. In contrast, FXIII activity and antigen level in CS+ patients homozygous for FXIII-A Leu34 allele was significantly (10%) lower than in CS+ patients with Val/Val genotype ($p<0.05$). In the CS+ group (n=619) FXIII activity in Val/Val, Val/Leu and Leu/Leu subgroups were 104% (95%CI: 100-107), 103% (95%CI: 99-107) and 94% (95%CI: 86-103) respectively. FXIII antigen concentration inVal/Val, Val/Leu and Leu/Leu subgroups were 23.2 mg/L (95%CI: 22.4-24.1), 22.7 mg/L (95%CI: 21.8-23.6) and 20.9 (95%CI: 19.0-22.9) respectively. In patients with the history of MI (MI+, n=341) FXIII levels showed a more prominent decrease in Leu/Leu subgroup. In this patient group the difference between Val/Leu heterozygotes and Leu/Leu homozygotes was also statistically significant. FXIII activity in Val/Val, Val/Leu and Leu/Leu subgroups were 107% (95%CI: 102-112), 106% (95%CI: 101-111) and 88% (95%CI: 76-99) respectively. FXIII antigen concentration in Val/Val, Val/Leu and Leu/Leu subgroups were 24.0 mg/L (95%CI: 22.8-25.1), 23.2 mg/L (95%CI: 22.0-24.5) and 20.3 (95%CI: 17.6-23.0) respectively. The CS+ group contained patients with and without the history of MI. To find out if the decrease of FXIII levels in Leu/Leu homozygotes was also evident in CS+ patients without the history of MI this group of patients (CS+MI-) were separately assessed. Although there was a tendency of decrease with increasing number of Leu34 allele the differences were not statistically significant.

Theoretically, the decrease of FXIII activity could be due to the decrease in the concentration of FXIII molecules or to the decreased activity of the variant factor. The parallel decrease of FXIII activity and antigen levels suggested the former possibility. To give a more correct assessment of the problem, specific FXIIIa activities, i.e. activities of a given amount of FXIII protein, were calculated. Practically identical specific FXIIIa activities were obtained in all patient groups with

any FXIII-A genotypes (7.00-7.32 Unit/mg) which demonstrates that the decreased FXIII activity in the Leu/Leu patient groups was due to the decrease of FXIII concentration.

DISCUSSION

The frequency of FXIII-A Leu34 allele in the Caucasian population varies within 24.5-28.8%, a rather narrow range. The allele frequency observed in our population control group (25.9%) and in clinical controls (25.8%) is well within this narrow range. In blacks and Asian Indians the allele frequency is lower and in the Japanese population this polymorphism is extremely rare. In the initial case-control study on the relationship of FXIII-A Val34Leu polymorphism and the risk of MI a protective effect of Leu34 carriership against MI was suggested. This finding was confirmed by studies from Finland, Brazil, Northern Italy and Turkey. In contrast, smaller studies from Southern France and Spain, as well as a large Italian study recruiting 1210 patients and controls did not reveal any effect of the Val34Leu polymorphism on the risk of MI. These studies suggested that FXIII-A Val34Leu polymorphism was without any effect on the risk of MI. The present study, as well as two studies from North-America demonstrated that, in contrast to an earlier hypothesis, the absence of significant protective effect of Leu34 allele against MI in the general population is not restricted to the Mediterranean area or to populations with relatively low incidence of CAD.

It has been suggested that the deviating results on the effect of FXIII-A Val34Leu polymorphism could be due to gene-environmental and gene-gene interactions that might influence the protective effect of the polymorphism. The cardio-protection exerted by the Leu34 allele was lost in the presence of features associated insulin resistance, particularly in individuals with high plasminogen activator inhibitor 1 (PAI-1) level. Based on biochemistry results the interaction between fibrinogen as a risk factor and FXIII-A Val34Leu polymorphism in the determination of the risk of CAD is an intriguing possibility. The biochemical consequences of FXIII-A Val34Leu polymorphism include the increased rate of proteolytic activation of the Leu34 variant by thrombin and the alteration in the structure of fibrin. Decrease in clot permeability with increasing fibrinogen concentration is diminished by the Leu34 FXIII variant. It was also shown by scanning electron microscopy that at high fibrinogen concentration (5.0 and 7.7 g/L) plasma samples homozygous for the Leu allele form clots with looser structures and

thicker fibers. Fibrin with such a structure is degraded by fibrinolysis at a faster rate than tight fibrin made of thin fibers. Our finding that the protective effect of Leu34 allele prevails only at high fibrinogen concentrations is in complete accordance with these in vitro experimental results and indicates that in such condition the protection provided by the Leu34 allele might be related to an accelerated fibrinolytic degradation of fibrin network. The results suggest that FXIII-A Val34Leu polymorphism and fibrinogen concentration should be evaluated together in the stratification for risk of CAD.

The development of coronary sclerosis and the onset of acute thrombosis in the coronary vessels leading to acute myocardial ischemia are two separate, although, in the majority of cases, causatively interrelated events. It is not easy to separate the effect of Leu34 variant on CS and MI within the high fibrinogen quartile. When CS+MI- group of these patients was compared to the respective clinical controls a somewhat lower OR (0.79) was calculated but the protective effect was not statistically significant. Although the comparison of the combined CS+ group with clinical controls resulted in a statistically significant decrease of OR (0.46), this group included almost all patients with MI. Investigating the MI+ patients separately a significant protective effect (OR: 0.41) was also demonstrated. In conclusion, the Leu34 allele provides protection against coronary thrombosis leading to MI in patients with high fibrinogen level. It remains to be seen if an impaired atherosclerotic process also contributes to the protective effect.

We performed an evaluation of the contradictory results concerning the association between FXIII-A Val34Leu polymorphism and CS/MI by meta-analysis. The individual study results pooled in this analysis showed considerable heterogeneity; the range of the study specific odds ratio without the shrinkage was 0.31-1.11. Based on the published information we could not identify any potential determinants that could explain this heterogeneity.

Although the biological mechanism (see above) in which a genetic factor causes a disease remains unchanged in the different populations, the actual strength of the association may vary considerably as the prevalence of the relevant environmental factors vary. It is to be noted that some of the negative studies came from Mediterranean countries, in which the prevalence of environmental risk factors is lower than in populations from Middle- and Northern Europe, and in these cases presumably no further protection could be provided by a relatively moderate genetic protection factor. On the other hands, our study with negative outcome involved a

population that is at high risk of coronary artery disease, one of the highest in Europe, and in this case the protective effect of Val34Leu polymorphism might have been overcome by strong environmental and other genetic risk factors. However, we have shown the protective effect of Leu34 allele in a well-defined subgroup of the population with high fibrinogen concentration. Because of the considerable heterogeneity, we used empirical Bayes model in our study. Compared to conventional random-effects meta-analysis this reduces the influence of studies with extreme results. Based on the results of our meta-analysis it is demonstrated that the presence of Leu34 allele significantly protective against coronary artery disease in the Caucasian population, however this protective effect is dependent on different environmental factors and gene-gene interactions. The analysis raises the possibility of publication bias. Because of the large sampling error the results of small studies tend to vary around the true value of the parameter of interest to a larger extent than the results of large studies. However, there was only one small study that found less beneficial effect than our pooled estimate. If these findings exist but remained unpublished then our estimate on the beneficial effect of FXIII Val34Leu polymorphism might be somewhat more optimistic than the true effect.

The association between cardiovascular diseases and plasma FXIII levels was investigated by only a few studies. In these studies the above mentioned microplate amine incorporation assay was used to determine FXIII activity and the results were highly dependent on FXIII-A Val34Leu polymorphism. Since there was no genotype dependent reference interval used in these studies and the comparison of patients and controls were not performed according to the different genotypes we can compare our results only with the antigen levels of these studies. There were also a low number of patients recruited to these studies. In a study involving 276 white European patients undergoing routine coronary angiography increased FXIII-A antigen was measured only in patients with severe CS. In a setup similar to our study design, 362 patients with significant coronary stenosis were compared with 134 patients having normal angiogram and no association between CS and FXIII activity or antigen levels was found. No difference in FXIII levels between patients with and without MI were reported, with the exception of a small study involving 63 male MI+ patients, in which decreased adjusted FXIII-A antigen level, with unchanged FXIII activity was observed. The results of the above studies on male or predominantly male patients with basically negative outcome well agree with the negative outcome of the present study concerning the undivided predominantly male patient population. The results of

the previous studies, probably due to the relatively small number of subjects, were not analyzed according to gender. We recruited almost 1000 patients this allowed to create subgroups according to gender. In our study female patients with the history of MI had higher FXIII activity and antigen levels than female patients without MI, independently from the presence or absence of CS. This finding indicates a gender-specific association between the elevation of FXIII levels and MI.

In male patients FXIII activity or antigen levels in the upper tertile neither represented a significant risk of MI or CS nor exerted a protective effect against these conditions. In sharp contrast to men, the comparison of CS+MI+ female group with female clinical controls indicated that a 2.5-3.0-fold risk of this condition was associated with FXIII levels in the upper tertile. In women – similarly to men - elevated FXIII level did not increase the risk of CS in MI- patients, while significantly increased risk of MI was observed in females with CS. These findings suggest that the elevation of FXIII level increases the risk of MI by mechanisms other than the development of atherosclerotic plaques. On the basis of the results elevated FXIII can be regarded as a gender (women) specific risk factor of MI, and FXIII determination could be a candidate to be included in the risk stratification in women.

By preventing the prompt degradation of fibrin clot elevated FXIII could play a role in sustaining the thrombus occluding coronary arteries and promoting its growth. It remains to be seen, why such a mechanism operates only in women. In any case, the results support the suggestion that in the development of CAD the clotting system plays a role more prominent in females than in males.

Due to methodological differences, there is some confusion in the literature concerning plasma FXIII activity in individuals of different FXIII-A Val34Leu genotypes. In assays at full activation of FXIII by thrombin the differences in FXIII activity among FXIII-A Val34Leu genotypes disappear and there are no Val34Leu genotype-dependent differences in the specific activities of FXIIIa. In this study we used a FXIII assay which measures the activity of fully activated FXIII, the results obtained with this assay reflect the catalytic concentration and show good correlation with estimated plasma FXIII antigen values.

We demonstrated a statistically significant decrease of both FXIII activity and antigen levels in CS+ and MI+ patients homozygous for the Leu34 allele.

From the parallel decrease of FXIII antigen and activity and from the practically identical FXIIIa specific activities in the different patient and genotype groups it became evident that the Val34Leu mutation did not influence the activity of FXIIIa (in contrast to some previous results), but the concentration of FXIII was

decreased in the Leu34 homozygous CS+ and MI+ groups. The reason for the FXIII-A Val34Leu genotype specific decrease of FXIII level only in the patient groups and not in the healthy individuals is not clear. One can presume that in the patients, particularly in MI+ patients, there is a continuous or occasional small-scale activation of blood coagulation including the activation of FXIII. The Leu34 variant, especially its homozygous form, is activated at a higher rate than the Val34 variant and the quick removal of activated FXIII from the circulation results in decreased FXIII levels.

SUMMARY

Blood coagulation factor XIII (FXIII) is a zymogene (protransglutaminase) of tetrameric structure (A_2B_2). Thrombin removes an activation peptide of 37 amino acid residues from FXIII-A, then in the presence of Ca^{2+} the carrier/inhibitory FXIII-B dissociate and FXIII-A assumes an enzymatically active configuration. The main function of FXIII in normal hemostasis is to cross-link fibrin chains and to attach proteins important in the regulation of fibrinolysis to the fibrin network. Among FXIII-A gene polymorphisms Val34Leu polymorphism is the most well-characterized because of its suspected thrombo-protective effect.

The association between FXIII levels and coronary sclerosis (CS) and myocardial infarction (MI) was investigated using adequate laboratory methods. The effect of elevated FXIII levels and FXIII-A Val34Leu polymorphism on the risk of CS/MI was also examined.

The presence of FXIII-A Leu34 allele or homozygous Leu34 genotype alone did not change the risk of CS/MI in the Hungarian population. However, when patients with elevated fibrinogen level were separately investigated, the Leu34 allele provided a statistically significant protection against MI. Fibrinogen concentration modulates the effect of Leu34 allele on the risk of MI, its protective effect emerges at increasing fibrinogen concentration.

The general protective effect of FXIII-A Leu34 allele against coronary artery disease in the Caucasian population was demonstrated by a meta-analysis of 16 studies. However, it was also indicated that the prevalence of this effect depends on environmental factors and gene-gene interactions.

We first described in the literature that elevated FXIII level was an independent risk factor for MI in females and suggested that FXIII determination is to be included in the gender-specific risk profile.

We demonstrated that in patients with CS and MI FXIII-A Val34Leu polymorphism influences plasma FXIII levels. In MI+ patients homozygous for the Leu34 allele FXIII levels were significantly lower than in heterozygous and wild type patients. The specific activity of FXIII was independent of FXIII-A Val34Leu polymorphism. It is presumed that in MI+ Leu34 homozygous patients faster activation of Leu34 FXIII is combined with a higher extent of low-scale thrombin formation, more FXIIIa is formed which is then eliminated from the circulation resulting in lower FXIII levels.

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