**SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD)**

**LABORATORY DIAGNOSIS, MOLECULAR GENETIC AND PROTEIN BIOCHEMICAL ANALYSIS OF RARE HEMOSTASIS DISORDERS**

by Kitti Bernadett Kovács, MD

Supervisor: Zsuzsanna Bereczky, MD, PhD

UNIVERSITY OF DEBRECEN

KÁLMÁN LAKI DOCTORAL SCHOOL

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Supervisor: Zsuzsanna Bereczky, MD, PhD

Kálmán Laki Doctoral School, University of Debrecen


Head of the **Examination Committee**:  Csongor Kiss, MD, PhD, DSc

Members of the Examination Committee:  Zoltán Boda, MD, PhD, DSc

Hajnalka Andrikovics, MD, PhD


The Examination takes place at the Division of Clinical Laboratory Science, Department of Laboratory Medicine, Faculty of Medicine, University of Debrecen on May 26, 2015, at 11 a.m.


Head of the **Defense Committee**:  Csongor Kiss, MD, PhD, DSc

Reviewers:  Barna Vásárhelyi, MD, PhD, DSc

Zsolt Oláh, MD, PhD

Members of the Defense Committee:  Zoltán Boda, MD, PhD, DSc

Hajnalka Andrikovics, MD, PhD


The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen on May 26, 2015, at 1 p.m.

# 1. Introduction

The term „rare disease" was first introduced in the United States strongly connected to the term „orphan" drug. The basis for the development of the mentioned conceptions was the unprofitable pharmaceutical production of medications used by only a small number of patients besides the increasing production costs. First the products with higher production costs than selling-price were considered „orphan", a year later the definition changed to drugs with less than 200,000 consumers (USA). The current description covers both approaches. The European definition of „orphan" drugs and „rare diseases" came later (1295/1999/EC), accordingly the disease is considered rare if it is life-threatening for the person or significantly disabling with low prevalence (<2-5:10,000 persons); the diagnosis/treatment requires remarkable efforts both financially and mentally. Hemostasis disorders, mainly hemorrhagic diatheses form a specific group among rare diseases. In 2004, the International Society on Thrombosis and Haemostasis (ISTH) formed the Rare Bleeding Disorders (RBD) task force that mediates the research related to these diseases, helps the professionals with reviewing new diagnostic and therapeutic guidelines (www.rbdd.org and www.rbdd.eu). According to the definition of the RBD task force, all coagulopathies and platelet function disorders except for hemophilia A and B and von Willebrand disease is considered as rare. The prevalence of the homozygote or compound heterozygote (i.e. severe) forms of these bleeding disorders is between 1:500,000 to 1:3,000,000. The ISTH „Plasma Coagulation Inhibitors" subcommittee undertook the coordination of basic and clinical research related to rare thrombophilia. The research regarding these diseases are promising in many respects; on the one hand studies on the effect of the newly described causative mutations on the structure and function of the affected protein helps to understand the physiological and pathophysiological processes and new mechanisms can be discovered. On the other hand, the knowledge obtained by the research promotes the development of new diagnostic opportunities and more efficient therapeutic methods. Besides the personal diagnosis and scientific approach the demand of prenatal diagnosis emerges in certain cases requiring special methodology from the part of the laboratory.

For the adequate site, time and intensity of blood coagulation in the body, sensitive balance of pro-and anticoagulant hemostatic factors is required. The intact hemostatic system is based on three pillars: intact vessel wall - the vascular component, proper platelet count and function - the cellular component and adequate quantity and quality of coagulation factors and their inhibitors – the humoral component. The lack or decreased level of coagulation factors

lead to hemorrhagic diatheses, i. e. bleeding disorders, with inherited, acquired or iatrogenic coagulopathy in the background. The first point in the routine diagnostic procedure is the performance of the hemostasis screening tests, which are the prothrombin time (PT), activated thromboplastin time (APTT) and thrombin time (TT) regarding the humoral component. Depending on the affected factor isolated clotting time prolongation or the prolongation of PT and APTT or all three clotting times can be seen at the same time. Negative hemostasis screening tests do not exclude coagulopathy, factor XIII (FXIII) or $\alpha$-2-plasmin inhibitor deficiency should also be considered.

In case of inhibitor system disorders, thrombophilia might develop. Venous thromboembolism (VTE, deep venous thrombosis (DVT), pulmonary embolism (PE)) is one of the most common morbidity and mortality affecting populations in western societies. VTE is still a major cause of pregnancy related morbidity and mortality and stillbirth and is common among young women taking oral anticoncipient drugs. VTE might be recurrent and chronic leading to the painful post-thrombotic syndrome often with ulceration. In the background both genetic and environmental factors play a role. Loss of function mutations of the natural anticoagulants can lead to antithrombin (AT), protein C (PC) and protein S (PS) deficiencies, while abnormal clotting factors or conditions with inherited elevated factor levels (presence of factor V (FV) Leiden mutation and prothrombin (FII) 20210A allele, elevated factor VIII (FVIII) level) are responsible for the wast majority of inherited thrombophilia.

In case of rare coagulopathies and thrombophilia – right because of the low prevalence – large clinical and population studies cannot be carried out, case reports and descriptive studies are important in this field. The genetic alterations responsible for these diseases are heterogeneous, as there are no mutational hot spots in the genes of clotting factors and their inhibitors (inactivators), novel pathological mutations might be discovered upon the investigation of the deficient families. Detailed characterization of these mutations enables exploration of genotype-clinical phenotype, genotype-laboratory phenotype relations. As rare coagulopathies FV and FXIII deficiencies, as rare thrombophilia PC deficiency are presented in this thesis, summarizing the results of our investigations on the characterization of novel mutations.

## 2. Review of the literature
## 2.1. Description of the clotting factors and their deficiencies mentioned in the thesis

## 2.1.1. Characteristics of coagulation factor V

Blood coagulation factor V (FV) is a single-chain glycoprotein (Mr 330 kDa) synthesized in the liver and megakaryocytes. Its plasma concentration is 20 nM (7 μg/mL) and approximately 20% of total FV is found in platelet α-granules. FV has a mosaic-like structure, it has three similar A domains, two C and a highly glycosylated B domain. FV, an essential cofactor of activated factor X, is activated by thrombin. Thrombin removes the B-domain and the remaining heavy chain and light chain are associated via a calcium ion to form the active heterodimer. Activated FV (FVa) enhances the rate of prothrombin activation by several orders of magnitude. The inactivation of FVa is carried out by activated protein C (APC) by sequential cleaving next to three arginine amino acids (Arg506, Arg306 and Arg679). If APC cleaves the negatively charged phospholipid membrane bound native FV next to Arg506, the protein acquires anticoagulant function and serves as a cofactor of APC in the inactivation of membrane bound active FVIII (FVIIIa). In case this anticoagulant protein is cleaved by thrombin next to the Arg709, Arg1018 and Arg1545, the protein gains weak activity in prothrombin activation. Inactivation occurs by the APC mediated cleavage at the Arg306 in this case as well.

The gene for FV (F5) has been localized to chromosome 1q21–25; it spans approximately 80 kilobases and consists of 25 exons and 24 introns. The cDNA encodes a 2224 amino acid long protein. The authentic numbering of the nucleotides and amino acids was carried out according to the publication of Jenny et al. in 1987. The numbering according to the guidelines of the Human Genome Variation Society (HGVS) considers the first methionine as the first amino acid independent of probable cleavage of propeptid of the protein during posttranslational modification. During the maturation of FV, 28 amino acids are cleaved from the original protein, therefore 28 should be added to the amino acid number received according to the authentic numbering (considering only the amino acids of the mature protein) to get the currently accepted HGVS number (www.hgvs.org/mutnomen). As the authentic numbering – mainly because of the widespreadness of the Leiden mutation, Arg506Gln – is still more popular, we follow it in this thesis.

## 2.1.2. Factor V deficiency

Congenital FV deficiency – parahemophilia, Owren's disease (Online Mendelian Inheritance in Man, OMIM +227400) is a rare autosomal recessive bleeding disorder with a prevalence of 1:1,000,000 in the general population. Patients with homozygous FV deficiency usually have

FV activity below 10% (often below 1%) and show moderate to heavy bleeding symptoms. Most heterozygotes are asymptomatic. The vast majority of the cases are type I FV deficient, however one patient with type II deficiency has also been published. Over 50 different mutations in F5 have been described so far in the literature. These mutations are listed in different databases. The majority of missense mutations are located in the A2 and C2 domains. Considering the prevalence of the homozygotes, the heterozygous state should appear with a frequency of 1:1000, however most of these cases remain undiscovered as neither clotting time prolongation, nor bleeding symptoms are prominent.

### 2.1.3. Characteristics of coagulation factor XIII

Blood coagulation factor XIII (FXIII) is a pro-transglutaminase in the circulation. Coagulation factor XIII (FXIII) exists both in plasmatic and cellular forms. Plasma FXIII (pFXIII) is a heterotetramer consisting of two catalytic A subunits (FXIII-A) and two inhibitory/carrier B subunits (FXIII-B). The B subunit is in excess, 50% of it is in a free form in the plasma. The cellular form (cFXIII), a dimer of FXIII-A, is exempt of FXIII-B. A huge amount of cFXIII is present in platelets and in many other cell types. The FXIII-A subunit consists of 732 amino acids (Mr 83kDa). The initiator methionine is followed by a serine, which, after the posttranslational cleavage of the methionine, is N-acetylating and becomes the first amino acid of the protein. The XIII-A subunit does not contain leading sequence, therefore the authentic and the new (HGVS) nomenclature differs only in one amino acid, one should be added to the authentic numbering to get the new one. As the majority of the publications on FXIII deficiencies follow the authentic nomenclature, here we do so as well.

FXIII-A consists of an N-terminal activation peptide of 37 amino acids, followed by a β-sandwich domain, a catalytic core domain and two β-barrel domains. The gene encoding FXIII-A (F13A1) is located on chromosome 6 at 6p25.3–p24.3 position and contains 15 exons and 14 introns.

FXIII-B is a mosaic protein containing 10 sushi domains; its coding gene is located at the 1q31–32.1 position. FXIII-A and B subunits form the FXIII$A_2B_2$ heterotetramer in the circulation, the concentration of which is 14-28 mg/L.

The pFXIII is activated by thrombin and $Ca^{2+}$; thrombin cleaves off the activation peptide from FXIII-A then, in the presence of $Ca^{2+}$ FXIII-B dissociates and the remaining FXIII-A dimer reaches an active configuration. Activated FXIII (FXIIIa) is a transglutaminase, which cross-links glutamine and lysine side-chains by peptide bonds (so-called isopeptide bonds). Its main task in hemostasis is to crosslink fibrin chains and α2-

plasmin inhibitor to fibrin. Apart from its key role in hemostasis, FXIII is needed for wound healing and maintaining pregnancy; it might also has a role in angiogenesis and in cartilage and bone development.

### 2.1.4. Factor XIII deficiency

FXIII activity below 1% of average normal causes severe bleeding diathesis. Such low FXIII activity occurs in inherited FXIII-A deficiency, while in patients with inherited FXIII-B deficiency FXIII activity is higher (usually 5−10%) and the bleeding diathesis is mild to moderate. FXIII deficiency is a rare inherited bleeding disorder. The frequency of diagnosed cases in the general population is approximately 1:2,000,000; however, in countries where consanguineous marriage is a more general practice or in closed communities the frequency might be significantly higher. Due to unawareness at the clinical side and incorrect diagnostic practices in laboratories, FXIII deficiency frequently remains unrecognized. The mutation database for FXIII-A deficiency (http://www.f13-database.de) includes 65 causative mutations in the FXIII-A gene (34 missense mutations, 21 insertions/deletions, nine splice–site mutations and five nonsense mutations). The distribution of the mutations along the gene clearly shows that there is no mutation hot spot. Although the field of inherited FXIII deficiency has been intensively reviewed lately, several unresolved questions remained. The relationship between clinical symptoms, laboratory phenotype and the genetic defect has not been elucidated, partly because of methodological problems or insufficient data on the laboratory phenotype. For this reason, studies in which the description of molecular genetic defect is supplemented with detailed laboratory classification and clinical history are required.

### 2.2. The role of the protein C-protein S system in the regulation of blood coagulation

PC and PS play important roles in the regulation of blood coagulation as natural anticoagulants. PC is activated by thrombin in the presence of thrombomodulin (TM). TM is an endothelial cell surface protein, and upon binding, thrombin becomes a potent activator of PC. Endothelial PC receptor (EPCR) is also highly important in the activation process; EPCR binds PC through its Gla-domain and presents it to the thrombin-TM complex. Thrombin cleaves the activation peptide domain of PC at Arg169 resulting in a 12-amino acid long activation peptide being released from the N-terminal end of its heavy chain. APC inactivates membrane-bound FVa and FVIIIa by cleaving these factors at specific arginine residues. The free form of PS is an important cofactor of APC, enhancing its affinity to negative charged

phospholipid surfaces. Unactivated forms of FV and FVIII are weak substrates for APC. APC can cleave intact FV at the Arg506 site, which results in a FV that serves as a cofactor for APC in FVIIIa inactivation. The main inhibitor of APC is the protein C inhibitor, a single chain serine protease inhibitor glycoprotein synthesized in the liver. Besides its anticoagulant role, PC is important in cell protection.

### 2.2.1. Protein and coding gene structure of protein C

PC is a vitamin K-dependent glycoprotein synthesized by the liver as a single chain protein. It exists in the plasma as a precursor of a serine protease at a concentration of 3–5 mg/L. Its half-life is short, approximately 8 h in the circulation. The mature 62 kDa protein is composed of a heavy (41 kDa) and a light (21 kDa) chain; these chains are held together by a single disulfide bond. The domain structure of PC shows high similarity to other vitamin K-dependent proteins. It has a pre-pro leader sequence (numbered as –42 to –1 by the traditional numbering system, where the first methionine corresponds to –42), which is required for γ-carboxylation of glutamic acid residues in the Gla-domain and for secretion. The mature protein contains a Gla-domain with the nine glutamic acid residues that are carboxylated during posttranslational maturation, a short amphipathic helix, two epidermal growth factor (EGF) domains, an activation peptide and a catalytic domain. To get the new HGVS number, 42 should be added to the amino acid number determined by the traditional numbering. In the thesis we will follow the new nomenclature as it has been followed in our publications in this field. The gene for human PC (PROC) is located at the 2q13–q14 position and contains nine exons encoding for a 1.7-kb messenger RNA (mRNA) and eight introns.

### 2.2.2. Protein C and S deficiencies: epidemiological aspects and clinical symptoms

The first patient with PC deficiency was described by Griffin et al. in 1981; it was associated with recurrent venous thromboembolism. Since then, a large number of deficient patients have been identified and the underlying genetic defects have been clarified in a number of cases. The degree of the risk of thrombosis and the prevalence of PC deficiency among patients with thrombosis and in the general population have been examined in several population studies, with conflicting results. According to the results of the Leiden Thrombophilia Study, one of the first case-control studies on this topic, the risk of first VTE in PC deficiency was 3-fold. In other studies, a 3-11-fold risk of VTE was demonstrated in PC deficiency. In the background of these heterogeneous epidemiological results, methodological variability, gene-gene or gene-environment interactions - many of which have not yet been

discovered may be present. Symptoms of PC deficiency are deep venous thrombosis and/or pulmonary embolism in early adulthood, which is often recurrent. Thrombosis might also develop at unusual sites, such as the proximal extremities and in mesenterial and cerebral veins. In severe PC deficiency when plasma PC or PS concentrations are extremely low, severe thrombosis develops in newborns, frequently in disseminated form, named purpura fulminans. Warfarin-induced skin necrosis is a severe complication of PC deficient patients receiving vitamin K antagonist treatment. In addition to venous thrombosis, patients with PC deficiency can also suffer from thrombotic complications of arterial origin.

### 2.2.3. Molecular genetic background of protein C deficiency, genotype-phenotype correlations

PC deficiency is classified as type I (quantitative) and type II (qualitative) deficiency. In type I deficiency, PC activity and the antigen concentration are decreased equally, suggesting defective synthesis or secretion of the protein, while in type II deficiency, the activity is decreased without a significant decrease in antigen concentrations. The latter type could be due to abnormalities in substrate, calcium-ion or receptor binding. The majority of PC deficient patients are heterozygous for the defect, with typical PC activity values between 30% and 65%. Homozygous or compound heterozygous patients often have undetectable PC concentration and/or activity and exhibit life-threatening thrombosis very early in life. The molecular genetic background of PC deficiency is heterogeneous. Most of the mutations cause type I deficiency, type II deficiency is diagnosed in approximately 10%–15% of cases, though in certain populations type II deficiency is more common due to founder effect. Summary reports of mutations leading to decreased PC concentrations were first described in 1995. To date, approximately 250 causative mutations have been published, which are collected in different databases (http:// www.hgmd.cf.ac.uk and http://www.isth.org).

Most of the mutations causing type I deficiency are single nucleotide substitutions within the coding region of PROC. In vitro expression studies were performed in approximately one third of the cases only. Almost all missense mutations resulted in an absolute block in secretion, however detailed examinations have not been carried out.

Diagnosis of type II deficiency is based on the discrepancy between the results of functional testing and antigen measurements. Missense mutations are the most frequently reported types; the resulting amino acid change involving the Gla-domain or the pro-peptide result in defective calcium and phospholipid binding. Mutations in the serine protease domain result in defective protease activity or decreased substrate binding.

### 2.2.4. Laboratory tests of protein C deficiency

In the diagnosis of PC deficiency functional tests are used as screening tests. These tests can be based on clotting time measurement or can be amidolytic assays using chromogenic substrate. In both assays, PC present in patient plasma is activated by the venom of Agkistrodon contortrix, now commercially available under the trade name Protac. In chromogenic assays, paranitroaniline (pNA) is cleaved-off from a small synthetic peptide by APC. Peptide bound pNA does not absorb light at 405 nm, while the liberated chromogenic compound has an intense color at this wavelength. In clotting time based assays we detect the APC-dependent prolongation of the clotting time in the presence of an activating reagent (APTT, PT, RVVT). Both tests have advantages and disadvantages, the details are not topic of this thesis, but we briefly mention that FV Leiden mutation may influence the clotting test causing underestimation, while this cannot be observed in case of the chromogenic assay. However, the latter are insensitive for certain type II mutations, mainly those affecting phospholipid and/or calcium binding.

### Aims

**1.** FV deficiency was diagnosed in case of a 34-year-old female patient with moderate bleeding symptoms. Our aim was to detect the exact molecular genetic disorder in the background and to investigate its possible consequences by molecular modeling.

**2.** In cases of a male newborn and a 13-year-old girl with severe bleeding symptoms FXIII deficiency was suggested. We aimed to perform detailed laboratory testing and molecular genetic analysis.

**3.** A newborn with neonatal purpura fulminans was compound heterozygous for the p.Asp77Gly and the novel p.Ala163Glu mutations; a female patient who had deep venous thrombosis at the age of 27 carried the novel p.Ala163Val mutation in heterozygous form. We carried out in vitro experiments to examine the fate of the mutant proteins and molecular modeling and molecular dynamic simulations to investigate the structural consequences of the mutations.

# 3. Methods

## 3.1. Factor V deficiency

### 3.1.1. Patients

A 34-year-old female had surgery-related remarkable blood loss on several occasions. At the age of nine, heavy bleeding developed following tonsillectomy. During her first labour in 1992, severe vaginal bleeding occurred and she required fresh frozen plasma substitution. In 2005 and 2006 she had plastic surgery on her breasts and a cervical conisation, respectively, when again heavy delayed-onset bleeding occurred from the site of operation. Previously, she have never had spontaneous bleeding. The family history was negative for bleeding. The proband's two daughters have not shown bleeding symptoms so far, however they have not gone through any surgical intervention, yet. Blood samples from the proband and her family members were obtained with informed consent and approval of the University Ethical Committee.

### 3.1.2. Materials and methods

### 3.1.2.1. Hemostasis laboratory examinations

Routine hemostasis tests: Upon the first examination hemostasis screening tests were performed on a BCS coagulometer (Siemens/Dade- Behring, Marburg, Germany). Fibrinogen level was determined by the Clauss method (Fibrinogen reagent, Reanal-Ker), the levels of other clotting factors were determined in one-stage clotting tests. FXIII activity was determined in a modified optimized kinetic spectrophotometric assay (REA-chrom FXIII, Reanal-Ker). Activity of α2-plasmin inhibitor was measured in chromogenic test (Stachrom Antiplasmin, Diagnostica Stago). The presence of an inhibitor in the background of decreased FV activity was excluded by 1:1 mixing study of patient: normal plasma carried out both in room temperature and 37°C. Whole blood count was checked on a Sysmex automated hematology analyzer (Sysmex, Kobe, Japan). To check platelet function, bleeding time was determined using Surgicutt template (Edison, NJ). PFA-100 closure time was determined using both collagen/ADP and collagen/epinephrine cartridges (Siemens/Dade-Behring). Platelet aggregation and secretion was checked in a Chrono-log lumiaggregometer (Chrono-log, Havertown, PA). To exclude von Willebrand disease, von Willebrand factor (vWF) antigen level was measured by immunturbidimetric method, ristocetin cofactor activity was checked by aggregometric method, the reagents required were obtained from Siemens/Dade-Behring.

Special tests: FV antigen was determined by a homemade ELISA system using two polyclonal anti-human FV antibodies produced in sheep (Affinity Biologicals, Ancaster, Canada). In the first step the ELISA plate (Thermo Labsystems, Waltham, MA) was coated with the catching antibody (100 µL, 10 µg/mL) and incubated during the night in 4°C. After blocking (with PBS buffer containing BSA), the diluted (1:200) samples were pipetted on the plate. The second, detecting antibody conjugated with peroxidase (100 µL, 4 µg/mL) was added and the detection occurred with o-phenylenediamine substrate. The reaction was stopped by 50 µL 2 M sulphuric acid and the absorbance values were red on 490 nm on an ELISA plate reader (Thermo Labsystems iEMS plate reader). For the calibration, normal and pathological controls Siemens/Dade-Behring Standard Human Plasma and Siemens/Dade-Behring control plasmas were used. Interpretation of the results and setting the reference curve was done using four parametric logistic curve fit of the absorbance values of the dilutions of the standard human plasma.

Platelets were isolated according to Muszbek et al. The whole procedure was carried out at 37°C. Platelet rich plasma (PRP) was obtained form acid-citrate-dextrose anticoagulated blood containing 0,18 µM prostaglandin E1 (PGE1) by centrifugation for 15 minutes at 120×g. The separation of platelets from the PRP from plasma was carried out by centrifugation for 15 minutes at 1300×g. The platelets were then resuspended in solution „A" (140 mM NaCl, 2.5 mM KCl, 0.1 mM MgCl$_2$, 10mM NaHCO$_3$, 0.5 mM NaH$_2$PO$_4$, 1 mg/ml glucose, 10 mM HEPES, pH 7.4) containing 3.6 mg/ml BSA-t, 1 U/ml apirase and 0.3 µM PGE1-t. Platelets were isolated again by centrifugation at 1100×g for 15 minutes and resuspended in the solution „A". Platelets were centrifuged again at 1100×g for 15 minutes and resuspended in solution „A" containing this time only 1 U/ml apirase. The platelet count in the final suspension was set to 1000 G/L in all samples, the lysis of the platelets was done by addition of Triton X-100 (1% final concentration). FV antigen determination from platelets was performed like in case of plasma samples. For the interpretation of the results and setting the reference curve, platelet lysates of healthy persons were used. Four parametric logistic curve fit of the absorbance values of the diluted samples was used.

Routine laboratory examinations (renal and liver function, CRP) from the patient's serum were carried out in the Department of Laboratory Medicine using Roche Integra 800 analyzer (Roche, Mannheim, Germany).

### 3.1.2.2. Molecular genetic examinations

Following DNA isolation from peripheral white blood cells (QIAamp DNA Blood Mini Kit, Qiagen, Hilden, Germany) the 25 exons of F5 along with the exon-intron boundaries were amplified according to van Wijk and Ajzner and then sequenced. Capillary electrophoresis was carried out in an ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems), for the interpretation Sequencing Analysis 5.1.1 softver was used.

### 3.1.2.3. Molecular modeling

To reveal the consequences of introducing a bulky, strongly polar, positively charged group into this region, constant particle number, constant pressure and temperature molecular dynamics simulation was carried out based on the geometry deposited in the protein data bank pdb ID:1y61. The simulations were carried out by Gromacs 3.3. molecular dynamics package using 2 fs time step, OPLS-AA/L force field, TIP3P explicit solvent model, periodic boundary condition and particle mesh Ewald method for long range electrostatics. For visualization of the results, VMD (Visual Molecular Dynamics) program package was used.

## 3.2. Factor XIII deficiency

### 3.2.1. Patients

Two patients with severe bleeding symptoms were included in the study: a neonate boy from Breda, the Netherlands (proband1) and a 13 year-old girl from Ghent, Belgium (proband2).

**Proband 1**

A week old term neonate was admitted to the neonatology ward of Amphia Hospital, Breda, the Netherlands because of jaundice and cephalhaematoma. He was born after uncomplicated home delivery supervised by a midwife. There was no history of clotting disorders in the family. The otherwise healthy term neonate of 3450 grams had prominent jaundice and a large temporo-occipital cephalhaematoma. The patient was treated by phototherapy for 3–4 days and was discharged on day 10 in a healthy condition. Two days later he was readmitted because of bleeding from the umbilical stump proximal to the clamp. Despite local pressure and haemostatic gelatin sponge, the umbilical stump continued bleeding. Routine haemostasis tests including APTT, PT, fibrinogen and platelet count were in the reference interval. FVIII and FIX levels were 211% and 67% respectively. The administration of high dose vitamin K (5 mg) and tranexamic acid (100 mg) were ineffective; however, a single dose of fresh frozen plasma (10 ml/kg body weight) stopped the bleeding instantly. The combination of significant

cephalhaematoma without traumatic delivery and umbilical stump bleeding was highly suspicious for inherited FXIII deficiency. After this bleeding episode, the child demonstrated mild bleeding tendency and he was put on on-demand replacement therapy using FXIII concentrate.

**Proband 2**

A girl, the first child of non-related Caucasian parents without bleeding history, was diagnosed with inherited FXIII deficiency at the age of 13 in the Ghent University Hospital, Belgium. She was born after 39 weeks of pregnancy. There was no report of umbilical cord bleeding after birth. At the age of 3, she was admitted to the hospital with extradural hematoma due to minor head trauma, after which trepanation was performed. Coagulation screening tests (PT, APTT) and platelet count were in the reference interval. Von Willebrand (VW) factor antigen and activity and platelet function tests were normal. Later on, there were several reports of extensive, but superficial bruising over the whole body, which even lead to the suspicion of child abuse. When she was 12 years old, after the removal of a pyogenic granuloma from her right flank seriously impaired wound healing was observed. During wound care follow-up she presented nausea and abdominal pain. Ultrasound and MRI showed an abdominal mass near the left ovary. Explorative laparotomy revealed a massive bleeding (probably due to rupture of the follicle during first ovulation) and 1150 mL blood was removed.

Basic laboratory tests were carried out at the respective hospitals; more detailed laboratory evaluation and molecular genetic analyses were performed in our laboratory. The children's parents and family members gave informed consent and the study fully complied with the Declaration of Helsinki. FXIII activity in plasma and platelet lysate was measured with slight modification in the method described by Kárpáti et al. using TECHNOCHROM® FXIII kit (Technoclone, Vienna, Austria). Washed platelet suspension was prepared as described earlier. Plasma samples anticoagulated by 0.109 mol/L trisodium citrate and platelet pellets were frozen and transported to Debrecen Hungary on dry ice by courier mail. Frozen platelet pellets were solubilized in 1% Triton X-100. Protein concentration of platelet lysate was determined by the BCA Protein Assay Reagent (Pierce, oud-Beijerland, the Netherlands). TECHNOZYM® FXIII Ag, FXIII-A SUB and FXIII-B SUB ELISAs (Technoclone) were used for the determination of FXIII-$A_2B_2$ complex antigen in plasma, FXIII-A antigen in plasma and platelet lysate and FXIII-B antigen in plasma respectively.

*3.2.2. Genetic analysis*

### 3.2.2.1. F13A1 gene mutation analysis

Following isolation of genomic DNA from peripheral blood leucocytes using QIAGEN Blood kit (Hilden, Germany), coding exons with exon-intron boundaries and promoter region of F13A1 gene were amplified in PCR reactions according to the protocol set in our laboratory. PCR products were cleaned and fluorescent direct sequencing was carried out using Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems) and ABI PRISM 3130 DNA sequenator (Applied Biosystems). For the sequencing reaction, the same primers were used as for the PCR reactions. Data were analyzed by Sequencing Analysis 5.3.1 software and compared to F13A1 gene sequence reported in the National Center for Biotechnology Information GenBank database (accession No. NG_008107). (The traditional nucleotide and amino acid numbering was used throughout the manuscript, which does not include the initiator methionine.)

### 3.2.2.2. FXIII-A mRNA analysis

Blood samples were collected in Tempus Blood RNA tubes (Applied Biosystems) from patient 1, his parents and two healthy individuals. RNA was isolated by Tempus 12-port RNA Isolation kit (Applied Biosystems). Total RNA was reverse transcribed using First Strand cDNA Synthesis Kit for RT-PCR in 20 µL final volume containing 1.6 µg oligo-p[dT]$_{15}$ and 3.2 µg random primers. Real-time PCR reactions using SYBR Green I Master (Roche, Mannheim, Germany) were performed in LightCycler 480 (Roche) in duplicates. FXIII-A gene expressions were normalized to the expression level of Phosphoglycerate Kinase 1 (PGK-1). PCR reaction consisted of 10 µL Master Mix (29 concentration), 300 nM primers for the reference gene (PGK-1) and 600 nM primers for F13A1. F:5′-tcacgagcgttcacctgttc-3′, R:5′-ctgcacatagaaagactgccc-3′ primers and F:5′-cctggatggtcctggagtaa-3′, R:5′-aggga gtcactgctcatgct-3′ primers were used for the amplification of F13A1 exon 3 and exon 14–15, respectively. The amplification programme included heating for 10 min at 95°C, then 40 cycles of PCR reaction each consisting of the following steps: denaturation for 10 s at 95°C followed by 60°C for 30 s and 72°C for 1 s. $2^{-\Delta\Delta Cq}$ method was used for calculation of relative genomic expression.

## 3.3. Protein C deficiency

### 3.3.1. Patients

## 1. patient

Purpura fulminans neonatorum was diagnosed in a newborn. Detailed description and clinical aspects of the case will be presented by another thesis in the future, so now we do not discuss it in more details.

## 2. patient

A 50-year-old woman was sent by her general practitioner to the Outpatient Service of our University for thrombophilia screening, because of her positive history of thrombosis and suspected familiar occurrence. She had left femoral vein thrombosis during pregnancy at the age of 27. Thrombophilia screening at the age of 42 revealed FV Leiden heterozygous mutation, low level of PC activity and antigen (please see details below) elevated Lp(a) and transient positivity for anticardiolipin (ACA) and anti beta2-glycoprotein I (B2GPI) antibodies. At the age of 44 years ulcerative colitis was diagnosed. She has been smoking since the age of 16 (10–15 cigarettes/day). The patient has been on vitamin K antagonist (acenocumarol) treatment since the diagnosis of PC deficiency. The patient's mother had calf vein thrombosis at the age of 38, her brother had left femoropopliteal vein thrombosis at the age of 35 and a right femoral vein thrombosis at the age of 44, the latter was complicated by pulmonary embolism. Her two daughters (30 and 33) are symptom free.

### 3.3.2. Materials and methods

### 3.3.2.1. Routine laboratory methods

Nine volume blood was collected into evacuated tubes containing 1 volume 3.2% Na-citrate and was centrifuged at 2500 rpm, 25 °C for 20 minutes. DNA was extracted from peripheral white blood cells using QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). Plasma and DNA were stored at −80 °C until further use. PT, APTT, TT were performed by commercial reagents on Siemens BCS-XP coagulometer (Siemens, Marburg, Germany). Fibrinogen concentration was determined by the Clauss assay. FVIII activity was measured by APTT-based clotting assay. Thrombophilia testing was performed on the same instrument using Siemens reagents for AT activity (Berichrom ATIII), for PS activity (Protein S Ac) and for PC activity (Protein C clotting reagent). Free PS antigen was determined by Liatest PS (Diagnostica Stago, Asnieres, France). PC antigen was determined by commercial ELISA (Asserachrom PC, Diagnostica Stago). Lupus anticoagulant (LA) screening was executed by the APTT-LA reagent (Diagnostica Stago), ACAs and anti-beta2-glycoprotein I antibodies were detected by Quanta Lite reagents (Innova Diagnostics Inc., USA), FV Leiden and

Prothrombin 20210A polymorphisms were detected by real-time PCR and melting curve analysis on a Light Cycler 2.0 (Roche Molecular Diagnostics, Pleasanton, CA). Plasma homocysteine concentration, lipid levels, liver and kidney function tests were performed by routine laboratory methods.

### 3.3.2.2. Polymerase chain reaction (PCR) and sequencing of the PROC gene

All the 9 exons, exon-intron borders and 5'-flanking part of the PROC were amplified using primer sets designed at our laboratory (available on request along with the detailed protocol). Fluorescent direct sequencing was carried out in ABI PRISM 3130-Avant Genetic Analyzer (Applied Biosystems), for the evaluation Sequencing Analysis 5.4 software was used. According to the recent guidelines amino acid numbering of PC starts at the first initiator methionine, however, traditional numbering started at an alanine residue, which is the first amino acid at the N-terminus of the mature protein. The pre-pro leader sequence, which is cleaved off upon maturation used to be considered as −42 to −1. Here we follow the numbering recommended by the Scientific and Standardization Committee of International Society on Thrombosis and Haemostasis throughout the manuscript and 42 should be subtracted to convert to the traditional amino acid numbering form.

### 3.3.2.3. Transient expression of wild type, p.Asp77Gly, p.Ala163Glu, and p.Ala163Val mutant PC proteins in HEK293 cells

HEK cells were grown in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen) supplemented with 10% fetal calf serum (FCS), 1% L-glutamine (Sigma-Aldrich) and gentamicin antibiotics (Chinoin, Budapest, Hungary). The cDNA clones ORF-NM_000312-pcDNA3.1(+) wt PC and variants c.A230G, c.C488A, and c.C488T, were purchased from ImaGenes GmbH (Berlin, Germany) and proliferated in One Shot® TOP10 Chemically Competent E. coli cells, then purified by the QIAprep Spin Miniprep Kit (Qiagen). HEK cells were transiently transfected in a parallel manner with wt and mutant plasmids. Before transfection, culture media were supplemented with vitamin K (Roche). Transfection was performed using 6 µl of FuGENE HD (Roche) and 2 µg of plasmid DNA in a six-well culture plate. After 50 hours incubation, conditioned media were collected, part of the cells were used for immunostaining and confocal laser scanning microscopy (CLSM), another part of the cells was lysed in a buffer containing 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P40, 0.5% sodium deoxycholate and a protease inhibitor cocktail (Roche). Aliquots of the media and cell lysates were used for PC antigen determination by ELISA (Asserachrom PC:Ag,

Diagnostica Stago) and for Western blotting. The efficiency of the transfection was determined by FluoReporterR LacZ/Galactosidase Quantification Kit (Invitrogen) and the results of the PC ELISA were adjusted accordingly. The activity of wt and mutant PC was determined from the conditioned media by amidolytic assay (Berichrom PC, Siemens) in a microtiter plate and by clotting test (Staclot PC, Diagnostica Stago) in a KC4 coagulometer.

### 3.3.2.4. Western Blotting

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDSPAGE, 5-20% gradient) was executed in non-reducing condition and the gels were electroblotted onto polyvinylidene difluoride (PVDF) membrane, and then blocked by 3% gelatin in 20 mM Tris, 50 mM NaCl, pH 7.5. PC was detected by sheep anti-human PC antibody (Haematologic Technologies, Essex Junction, VT) in a 2000-fold dilution. The immuno-reaction was developed by Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) containing anti-sheep immunoglobulin in a 1000-fold dilution and was visualized by 3,3′-diaminobenzidine (DAB) (Invitrogen).

### 3.3.2.5. Detecting of polyubiquitinated protein C

For detecting the ratio of polyubiquitinated PC in the cell lysates of transfected cells an ELISA method was established in which PC of the cell lysates were captured by polyclonal anti-PC antibody (Diagnostica Stago) coated to the microtiter plate and polyubiquitin was catched by a horseradish peroxidase conjugated anti-polyubiquitin monoclonal antibody (CycLex Co. Ltd., Ina, Nagano, Japan). The reaction was developed by adding tetra-methylbenzidine (TMB) substrate and the optical density was red at 450 nm in a microplate reader. Poly-ubiquitination of mutant proteins was calculated after normalizing the OD values for PC antigen concentration in each cell lysates and expressed as a ratio of mutant to wt polyubiqutinated PC.

### 3.3.2.6. Immunostaining, confocal laser scanning microscopy and qualitative colocalization analysis

Cells fixed in 96% ethanol, 1% acetic acid were incubated with 5% normal human serum in phosphate buffered saline for 15 min to prevent non-specific IgG binding. PC was incubated with 1:100 dilution of rabbit anti-human PC antibody (Sigma-Aldrich) for 60 min. For visualization a secondary antibody (anti-rabbit IgG, produced in goat, Vector, Burlingame, CA) labeled with DyLight 488 (1:100 dilution, 45min incubation) was used. Staining for PC

was combined with an endoplasmic reticulum (ER), or cis-Golgi, or trans-Golgi or 26S proteasome markers in double immunostaining reactions to localize wt and mutant PC. Calnexin, an ER marker was detected by mouse monoclonal antibody (Abcam Ltd, Cambridge, UK, 1:10 dilution, 45 minutes). Cis-Golgi was labeled by mouse anti-Golgi 58 K protein (Sigma-Aldrich, 1:200, 60 min). Mouse anti-mannose 6-phosphate receptor primary antibody (Abcam; 1:10 dilution, 60 min incubation) was used to label trans-Golgi. Finally, proteasome was visualized bymousemonoclonal antibody to 26S Proteasome (Abcam, 1:200 dilution, 60 min). The secondary antibody used for visualization of the mouse monoclonal antibodies was an anti-mouse IgG conjugated with DyLight 549 produced in horse (Vector) (1:200 dilution, 45 min incubation). All reactions were carried out at room temperature; phosphate buffered saline (PBS) was used for the dilution of antibodies and in washing steps. For negative controls identical dilutions of non-immune antisera and idiotypic mouse immunoglobulin-preparations were substituted for primary antibodies. Slides were investigated by confocal laser scanning microscope (LSM700, Zeiss Oberkochen, Germany) equipped with Plan-Apochromat 63X/1.40 oil objective and solid-state lasers. Separation of the fluorescence signals were performed by selective laser excitation (405 nm, 488 nm, 555 nm laser lines) coupled to efficient splitting of the emission using variable secondary dichroic (VSD) beamsplitter. Colocalization of fluorescence signals in image pairs was analyzed using ZEN 2010 imaging software (Carl Zeiss Microscopy) and Protein Proximity Analyser (PPA) software (www.anes.ucla.edu/~wuyong/). In PPA after background reduction by median filter technique, colocalization was characterized by protein proximity index (PPI1, PPI2), which numerical value is equal to the fraction of colocalized molecules in each channel for ideal images free from background and noises. According to our settings PPI1 describes the percentage of PC colocalizing with the different cell organelles and PPI2 describes the percentage of cell organelle colocalizing with PC. The PPA method, as compared to other existing approaches, has the advantage of minimizing the influence of image heterogeneity and broadly distributed background and using the median filter method it provides a universal and stable approach for background reduction. Pearson's correlation coefficient was also used to describe colocalization. Co-localization after manual threshold reduction was also characterized according to Manders and colocalization coefficientsM1 andM2 by using ZEN 2010 software.

### 3.3.2.7. Molecular modeling

Since there was no suitable experimental template structure comprising the activation peptide available for the full length PC, the full length activated PC (APC) structure was built instead. It can be assumed, however, that it had only a marginal structural effect at the sites (residue 163 and especially residue 77 and their proximities) investigated in this study. The residue 77 is very far from the position of activation peptide while the residue 163 and the activation peptide are separated by the EGF2-SP interdomain disulfide bridge. The theoretical full length APC model was derived based on the full-length activated factor IX (FIXa) (PDB ID: 1PFX), the des-Gla APC (PDB ID: 3F6U) and the available PC Gla domain fragment (PDB ID: 1LQV) templates. The latter one was completed using the whole Gla domain geometry from the factor VII (FVII) (1DAN) X-ray structure. The newly build loop structure between the Gla and the EGF1 domain has been refined by means of the Prime module of the Schrodinger suite of software. The unresolved residues in X-ray structures were built by the YASARA program package (Krieger DE. YASARA. www.yasara.org, 2009.). The quality of the model was checked by the procheck software. The effect of p.Ala163Val and p.Ala163Glu mutations on the stability of the structure was studied by molecular dynamics simulations. Since both mutations are on the surface of EGF2 domain and close to the SP domain, only these domains were included in the study. For comparison, the same simulation on the wt protein was also carried out. The mutations were created by the YASARA program package. The energies of the structures were minimized by the GROMACS software package. $Ca2^+$ and $Na^+$ ion as well as structural water molecules were kept according to the original X-ray file. The models were then solvated in a dodecahedral box. The distance between the box wall and the closest protein atom was set to 12 Å. The systems were neutralized and additional $Na^+$ and $Cl^-$ ions were added to set the ionic strength to 0.15M. A short simulated annealing period was applied to heat up the system to 310 K then a 200 ns simulation at constant particle number (N), constant pressure (P = 105 Pa) and constant temperature (T = 310 K) with periodic boundary condition was carried out. OPLS-AA protein force field and TIP3P explicit water model were applied. For the short range electrostatic and van der Waals energy terms 10 Å cut-off distances were used. The long-range electrostatic energy corrections were calculated by means of the particle mesh Ewald (PME) method. In order to apply a longer time step (4 fs) during simulations virtual sites protocol was used. The GROMACS software suites were used for all molecular dynamics calculations and the trajectory evaluations. Protein structure visualization was done by the VMD and the Chimera 1.7 software.

**4. Results**

## 4.1. Factor V deficiency

### 4.1.1. Laboratory results

Prothrombin time (11.3 sec) was at the upper limit of reference interval (8.7–11.3 sec), activated partial thromboplastin time was prolonged (45.9 sec; reference interval 28.3–41.0 sec) while thrombin time and fibrinogen were normal. The presence of an inhibitor was excluded by mixing study. FV activity of the proband was measured at two different occasions. Her plasma FV antigen and activity levels were proportionally reduced and her platelet FV antigen was also reduced. These suggested mild type I FV deficiency. The activity of all other clotting factors (including FXIII and α2-plasmin inhibitor) were normal. Platelet count was within the reference interval. Bleeding time, PFA-100 closure times, platelet aggregation and secretion studies showed no abnormalities. Von Willebrand factor antigen and ristocetin cofactor activity were normal. Liver and kidney function tests were also normal. The patient's younger daughter had similar laboratory phenotype.

### 4.1.2. Results of molecular genetic investigations

Four missense mutations were found in F5 of the proband. The previously published, normal sequence variations, c.2863 A>G (p.Lys897Glu) and c.5380A>G (p.Met1736Val) were found in heterozygous form and the silent c.327A>G (p.Gln51) mutation was present in homozygous form. A novel mutation c.1651G>A (p.Gly493Arg) was detected in heterozygous form. The same mutation was also found in one of the two daughters of the proband, who also showed considerably decreased FV activity and antigen levels. The father and the older daughter of the patient did not carry this mutation.

To prove that this mutation is not a common polymorphism, its presence was checked in 100 healthy Hungarian individuals. The p.Gly493Arg mutation was absent in all cases. According to the hemostasis laboratory results and the pattern of inheritance, this mutation is responsible for the FV deficiency.

### 4.1.3. Investigation of the possible consequences of the mutation by molecular modeling

The Gly493 is located in a loop of 27 amino acids connected by a disulphide bridge between Cys472 and Cys498. In this structure, 17 aminoacids out of 27, including Gly493, were conserved in the corresponding regions of human FVIII and ceruloplasmin and in FV molecules of different species included in the SwissProt database. The site of the mutation is

in a highly hydrophobic, tightly packed environment (491LIG(R)LLLIC498). To reveal the consequences of introducing a bulky, strongly polar, positively charged group into this region, constant particle number, constant pressure and temperature molecular dynamics simulation was carried out based on the geometry deposited in the protein data bank pdb ID:1y61. The simulation revealed that the side chain of Arg does not fit into the (hydrophobic) cavity reserved for the small αH "side chain" of Gly. Its polar guanidinium tail deformed the adjacent, also highly conservative 390ILGPIIRAQVR400 β-sheet and opened a channel to the (polar) solvent environment. Although an expression study that directly links the mutation to FV deficiency was not performed, molecular modeling strongly suggested that the local conformational change resulted in the instability of that region and, finally, to a mis-folding of the A2 domain.

## 4.2. Factor XIII deficiency

### 4.2.1. Proband1
#### 4.2.1.1. Laboratory results

Similarly to the results from the investigations in the Netherlands, we failed to detect plasma FXIII-A2B2 and FXIII-A antigen, while FXIII-B concentration was at the lower limit of reference interval. In platelets, just like in the plasma, both FXIII activity and FXIII-A antigen were below the limit of detection. In case of proband1's mother, the values were within the reference interval: plasma FXIII activity 85.5% (reference range 69-143%); FXIII-$A_2B_2$ antigen concentration 95.3% (20,0 mg/L) (reference range 67-133% (14-28 mg/L)) and FXIII-A antigen concentration 74.8% (8.01 mg/L) (reference range 67-133% (7,1-14,3 mg/L)). The father had decreased FXIII activity (63.3%) and FXIII-$A_2B_2$ antigen concentration (61.7%, 12.9 mg/L) and the FXIII-A antigen concentration was low normal (66.7%, 7.14 mg/L). The level of B subunit was normal in the parents.

FXIII activity and antigen concentration of FXIII-A subunit was below the detection limit in platelet samples as well in case of proband1, while the values were 50% of that of the control (average of FXIII activity and antigen concentration measured from the platelets of two healthy persons) in case of the parents.

#### 4.2.1.2. Molecular genetic investigations
DNA sequencing revealed two causative mutations in the F13A1 gene. The one in exon 8, a c.980G>A replacement resulting in Arg to Gln substitution at position 326, has been reported

by Mikkola et al. In heterozygous form it was also present in his mother, uncle and grandmother. The other causative mutation is a single nucleotide exchange in intron 8 (c.1112+2T>C) that leads to deficient splicing of exon 8. This mutation comes from the father side and is also present in the grandmother. It has not been published and cannot be detected in the FXIII mutation database (http://www.f13-database.de) and in the Human Gene Mutation Database (HGMD; http://www.hgmd.cf.ac.uk).

The FXIII-A mRNA content of whole blood obtained from the proband and his parents was also determined and compared to that of controls. In the patient's blood the expression of FXIII-A mRNA was close to normal, interestingly in her mother's blood the amount of specific mRNA was somewhat increased.

### 4.2.2. Proband2

### 4.2.2.1. Laboratory results

As routine coagulation, and platelet function tests, screening for VW disease and FVIII and FIX were normal, clot solubility test was performed and turned out positive, indicating severe FXIII deficiency. Plasma FXIII-A antigen level of the patient was below 4% by HexamateTM latex immuno-assay (MBL, Nagoya, Japan), while her mother had 72% FXIII-A level. 2.5% FXIII activity was measured in the proband's plasma using BERICHROM FXIII assay (Siemens, Erlangen, Germany) without blank correction. Then, plasma and platelet samples were sent to Debrecen for exploring the full laboratory phenotype and for molecular genetic analysis. Plasma FXIII activity, FXIII-A2B2 and FXIII-A antigens were below the limit of detection, just like FXIII activity and FXIII-A antigen in the platelet lysate. These parameters confirmed severe FXIII-A deficiency. FXIII-B antigen level was 37.8% in the plasma.

### 4.2.2.2 Molecular genetic analysis

Molecular genetic analysis revealed a homozygous single nucleotide deletion in exon 3 (c.215 delA) that led to early stop codon (in codon 74), which explains the severe deficiency and the lack of FXIII-A in plasma and in platelets. Her mother was heterozygous for the same mutation; the father was not available for investigation.

### 4.3. Protein C deficiency

### 4.3.1. Laboratory Phenotype and Genotype of Protein C Deficient Patients

*Patient 1*

The PC activity was below 1 % (normal value for full-term newborns at the age of 5 days is 42±11 %) and the PC antigen concentration was 5%. AT and PS activities were within the age-related reference intervals (60 and 60 %, respectively). The patient was not a carrier of FV Leiden and prothrombin 20210A mutations. She proved to be a compound heterozygote for the c.230A>G, p.Asp77Gly and the c.488C>A, p.Ala163Glu mutations.

*Patient 2*

PC deficiency was diagnosed at the age of 24 upon pregnancy related deep venous thrombosis (DVT). PC activity at the diagnosis was 49 %; the PC antigen concentration was 50%. She also carried FV Leiden mutation in heterozygous form and had elevated Lp(a) level (606 mg/L). No additional risk factor of VTEwas present. Sequencing of PROC revealed a novel, c.488C>T, p.Ala163Val mutation in heterozygous form. Daughters of the patient are exempt of PROC mutation, however they are carriers of FV Leiden mutation. Other family members were not available for detailed laboratory and genetic investigations.

### *4.3.2. Detection and Determination of Wild Type and Mutant Protein C Expressed in HEK293 Cells*

The concentration of the wt PC and the 77Gly, 163Val and 163Glu mutants were determined in the culture media and cell lysates in three separate experiments. The efficiency of the transfection was determined by FluoReporterR lacZ/Galactosidase Quantification Kit and the results of the PC ELISA were adjusted accordingly. The antigen concentration of the wt protein was $0.142 \pm 0.007$ μg/mL and $0.034 \pm 0.003$ μg/mg protein in the media and cell lysates, respectively. The antigen concentration of the wt and mutant PC in the cell lysates did not show marked difference ($0.033 \pm 0.002$; $0.019 \pm 0.002$ and $0.021 \pm 0.002$ μg/mg protein for 77Gly, 163Val and 163Glu, respectively). In the media, the concentration of the 77Gly mutant was measurable, however it was somewhat lower than the concentration of wt protein, ($0.059 \pm 0.001$ μg/mL). The 163Val and 163Glu mutants were undetectable. Both wt and 77Gly PC were clearly visible as discrete bands in the culture media of the transfected cells by Western blotting. In contrast, in case of 163Glu or 163Val mutants very faint bands, if any were present on the Western blots of the media. In accordance with the ELISA results, wt and all mutant PC proteins were detectable in the cell lysates. No PC was detected in mock samples. PC activity of 77Gly in the medium by performing the amidolytic assay was comparable with that of wt PC in three separate experiments (77.5%±15.1%, when PC

activity of the wt PC was considered as 100%). Moreover the specific activity (ie. the activity related to one mg PC protein) of 77Gly was the same as wt PC (104.2%±28.4%, considered wt PC as 100%). No activity could be detected in the media of the Mock, the 163Val and the 163Glu mutants. By performing the clotting assay, the PC activity of 77Gly was 80% ± 9.4% of wt PC. Again, no activity was measured in the mock, the 163Val and 163Glu samples. To detect if there is any difference in the speed of activation between wt and 77Gly PC, medium samples have been pre-incubated with Protac for various times from 1 to 10 minutes and clotting times have been measured by adding APTT reagent and calcium-chloride to the samples. No difference was shown between wt and 77Gly mutant PC in this aspect.

### 4.3.3. Intracellular localization of wild type and mutant protein C

The intracellular localization of the wt, 163Val, 163Glu and 77Gly mutant proteins was visualized by immunofluorescent staining followed by confocal microscopy and quantified by the calculation of colocalization coefficients. Despite spreading intention of expressing the colocalization of two molecules numerically by different computerized techniques and mathematical formulas these methods still suffer from bias. Qualitative analysis of double immunofluorescent staining has still an important role in data interpretation. As it was expected, wt PC was present in the ER, in the cis-Golgi and the trans-Golgi apparatus to a similar extent. The colocalization values with these cell organelles calculated by the PPA software were similar. Similarly to the wt protein, the 163Val, the 163Glu and 77Gly mutants were also detectable in the ER, cis-and trans-Golgi apparatus. 77Gly PC did not show marked colocalization with either cell organelles. The situation was different in the case of the 163Glu and 163Val mutants; in their case the yellow color on the merged pictures suggested accumulation in the 26S proteasome.

The Pearson's colocalization coefficients for 26S proteasome were higher in case of the 163Glu and 163Val PC than in the case of wt and 77Gly PC assuming that these mutants may retain in this cell organelle. The values of the Manders colocalization parameters calculated by ZEN software are more difficult to interpret due to the known problem of this method caused by the high sensitivity to spatial pattern of intercellular structures and the applied controversial thresholding technique for background reduction. By using this method the marker for 26S proteasome showed less than 50% overlap with wt and 77Gly PC (33% and 43%, respectively) and over 50% overlap with 163Glu and 163Val PC (81% and 61%, respectively) indicating that these latter two mutants occupied a relatively large part of 26S proteasome.

### 4.3.4. Investigation of polyubiquitination of wild type and mutant protein C

The ratio of polyubiquitinated mutant PC to wt was more than two in the case of 163Val and 163Glu mutants (2.25 ± 0.49 and 2.95 ± 0.51 for 163Val and 163Glu, respectively), while the 77Gly mutant was not polyubiquitinated (ratio of 77Gly to wt was 0.96 ± 0.10).

### 4.3.5. Structural consequences of p.Asp77Gly, p.Ala163Val and p.Ala163Glu mutations

The p.Asp77Gly mutation is situated on the C-terminal helical section of the Gla domain. The amino acid replaced by the mutation has no direct interaction with any other non-neighboring residues. Therefore, disruption of essential intramolecular H-bonds (or salt bridges) by this mutation is highly unlikely. The situation is different with the other two mutations, which are close to the inter-chain disulphide bond connecting the EGF2 and SP domains. Using molecular dynamics simulations we attempted to reveal how the latter mutations influence the strength of the interaction between the EGF2 and SP domains and/or the relative positions of these domains. To clarify this question the structure snapshots from each trajectory were fitted to the corresponding first snapshots using the respective SP domains for fitting. This way the motion of the EGF2 domain relative to the SP can be easily followed. At the endpoint of simulation carried out for the wt protein the relative position of the two domains are approximately the same as at the beginning of simulation. Remarkably different situation was seen in cases of the mutant proteins, as by fixing the two domains by a disulphide bridge during the whole simulation, the relative positions of the EGF2 and SP domains differ significantly in the first and last frames of simulation trajectories. For the fitted trajectory snapshot geometries the root mean square deviation (RMSD) of the EGF2 domain from the corresponding first snapshot structure were calculated in the case of wt, the 163Glu and 163Val proteins as well. In the case of wt PC the small, nearly constant RMSD values indicate that the relative position of EGF2 domain changed only slightly during the 200 ns simulation period. Contrary, the relative position of EGF2 domain changed significantly at both the 163Glu and 163Valmutations as shown by the respective RMSD values. The deformation of the 163Val mutant protein was more remarkable, although it developed later, than in the 163Glu mutant protein. Summarizing of the simulation results detailed above the positions of the mutant 163Glu and 163Val residues changed significantly during the simulation period as opposed to the wt 163Ala suggesting the possibility of altered folding. A possible consequence of this wrong folding can be the prevention of the disulfide bridge formation.

## 5. Discussion

### 5.1. Factor V deficiency

Our results revealed a p.Gly493Arg mutation of FV in the proband and in one of her daughters. Expression of the mutant allele at mRNA level was not investigated; the low FV antigen level suggests the absence of the mutant protein. Although we did not perform in vitro expression in case of FV deficiency, based on the results of the molecular modeling we can conclude that the local conformational change caused by the mutant arginine has an impact on the stability of the region and results in misfolding of the A2 domain resulting in abnormal structure and degradation of the whole protein. Causative nature of the p.Gly493Arg mutation is supported by several facts. The younger daughter of the patient who inherited only the p.Gly493Arg mutation besides the silent (p.Gln51) mutation not the two other genetic alteration had decreased FV activity and antigen concentration similarly to the patient. We excluded that the p.Gly493Arg mutation would be a common polymorphism by genetic examination of 100 healthy person and the mutation was not present in any case. The mutation affects a highly conserved region of FV protein; the current amino acid is the same in all examined species.

Only three patients with heterozygous FV mutation in association with hemorrhagic complications have been reported. The first patient with FV activity of 45% had microscopic hematuria and mild mucosal bleedings. She carried the nt524delG mutation in one allele of F5, however she was also heterozygous for a mutation in the FVII gene. The second patient, who was heterozygous for the p.Glu1608Lys mutation, suffered from spontaneous hematomas and metrorrhagia. Her FV activity and antigen levels were 38% and 50%, respectively. The third patient had hemorrhagic complications following tonsillectomy, but no spontaneous bleeding. His FV activity was 30% and he carried the p.Tyr1702Cys mutation, which is a common cause of FV deficiency in the Italian population. In the latter two cases no other causes of bleeding disorder were reported.

Our patient had no spontaneous bleeding, however her case suggests that upon surgical interventions, significant bleeding may occur in heterozygous FV deficient patients. It is to be noted that plasma FV activity of the proband and her daughter with the p.Gly493Arg mutation was consistently lower than 50%, which might contribute to the bleeding tendency. Investigation of p.Gly493Arg mutation in expression studies would help

us with the understanding of the exact mechanism of FV deficiency. Although the bleeding risks associated with mild factor V deficiency are uncertain, our findings suggest that heterozygosity for some factor V mutations increases bleeding risks and in case of clotting time prolongation, detailed examinations should be carried out before surgical intervention. There are cases, when despite the low level of FV bleeding does not occur or it is mild. One possible explanation is the FV pool of platelets, which is formed in part by the FV synthesis of the megacaryocytes in another part from endocytosis of plasmatic FV to the platelets.

## 5.2. Factor XIII deficiency

In the diagnostic process, the algorithm based on ISTH SSC recommendations was followed. Undetectable FXIII activity in the plasma established the diagnosis of FXIII deficiency in both cases, while undetectable FXIII-A2B2 and FXIII-A antigen together with FXIII-B antigen being over 30% suggested the diagnosis of type I FXIII-A deficiency, which was confirmed by molecular genetic analysis. Undetectable FXIII activity and FXIII-A antigen in platelets excluded the unlikely possibility of an autoantibody. It is to be noted that without the determination of FXIII in platelets a mixing study is satisfactory to exclude the presence of a neutralizing autoantibody, while the exclusion of nonneutralizing autoantibody needs more complicated binding assays. The bleeding symptoms were severe in both cases. In case 1, the delayed umbilical stump bleeding together with the cephalhaematoma led to an early suspicion of FXIII deficiency, and eventually to an early diagnosis. Delayed umbilical stump bleeding has been reported in approximately 80% of new-borns with FXIII-A deficiency. However, its lack does not exclude the diagnosis and one always has to be suspicious when intracranial bleeding occurs at an early age. Intracranial bleeding occurs in 30% of FXIII-A deficient patients not being on prophylactic substitution therapy. In case of the Belgian proband, the clinical history clearly shows a severe bleeding diathesis. Characteristic umbilical bleeding did not occur or it was not prominent enough to be recognized. She was also exempt of intracerebral bleeding. The case emphasizes that when severe bleeding diathesis is recognized in the absence of positive hemostasis screening tests, performing FXIII (and α2-plasmin inhibitor) activity assay is mandatory. Positive clot solubility test, 2.5% FXIII activity by the BERICHROM assay and <4% FXIII-A antigen by the latex immunoassay ensured the diagnosis of FXIII deficiency, but these tests did not indicate the severity of the disease. FXIII activity between 1% and 5% usually causes moderate bleeding diathesis. It is known that without blank subtraction the BERICHROM FXIII assay

overestimates FXIII activity in the low activity range, this explains the discrepancy between the result obtained by the BERICHROM assay and the undetectable FXIII-A antigen plus <1% FXIII activity measured by the TECHNOCHROM FXIII assay.

Proband1 turned out to be compound heterozygote by genetic analysis. A missense mutation in exon 8 resulted in Arg326Gln replacement. Molecular modeling revealed that Arg326 is located near the carboxyl end of the helix containing the active site Cys314 at the amino terminus. The hydrogen bonds involving the Arg326 side-chain atoms serve to tie the active-site helix to other parts of the core domain. Replacing Arg by a neutral residue upsets the electrostatic balance in this region, besides the smaller glutamine side-chain creates a void within the molecule. After transient transfection in COS cells the mutant cDNA expressed mRNA at an equal level to the wild type FXIII-A. Pulse chase experiments with metabolically labeled cells demonstrated remarkable instability and intracellular degradation of Gln326 FXIII-A. The above findings demonstrate the causative nature of this mutation and are compatible with the lack of FXIII-A protein in the plasma and platelets. The other mutation IVS8: c.1112+2T>C clearly predicts a splice–site defect. FXIII-A mRNA content in the patient's blood, that is, in platelets and in monocytes, was nearly normal using primers amplifying exon 3 located before or exons 14–15 located after the splice–site defect. No attempt was made to characterize the transcribed mRNA. The mother of proband1 had higher FXIII activity, FXIII-$A_2B_2$ and FXIII-A antigen in the plasma than would have been expected from a heterozygote. She had mRNA levels somewhat higher than normal what might be related to the relatively elevated FXIII level. However, FXIII activity and FXIII-A antigen levels were approximately 50% in her platelets. The explanation of this is yet unknown, it might be related to the change in the connection of A and B subunits, the genetic cause should be interesting to investigate in the future.

In proband2, there was a homozygous deletion of one adenine from the row of four adenines between nucleotide positions 212–215. Thus, the gaa-aac-aac-aag-ctg nucleotide sequence encoding for amino acids E-N-N-K-L in codons 70–74 changed to gaa-aca-aca-agc-tga encoding for E-T-T-S-STOP, that is, after three new amino acids starting at position 71 the protein synthesis was terminated. This occurred in the β-sandwich very close to the N-terminus, which explains why the patient lacked both FXIII activity and FXIII-A antigen.

Two cases with severe FXIII-A deficiency are reported. A complete evaluation of laboratory phenotypes with the establishment of causative mutations and in one case a three-generation family tree is presented. One of the patients was a compound heterozygote possessing the known mutation c.980G>A, p.Arg326Gln and the novel splice–site defect

c.1112+2T>C. In the other case, a novel single nucleotide deletion in exon 3 (c.212delA) led to an early stop codon allowing the translation of only a small N-terminal part of the molecule. The severe clinical symptoms, the non-detectable FXIII activity and FXIII-A antigen in plasma and platelets harmonize with the molecular defect. From the literature on FXIII deficiency no clear association between clinical severity, laboratory phenotype and genotype could be revealed. This is mainly due to the use of qualitative clot solubility test or quantitative ammonia release assay without plasma blank correction for the determination of FXIII activity. Studies including genetic analysis and complete laboratory work-up based on adequate methodology are required for establishing such association. This study also demonstrates that early diagnosis is essential to avoid severe clinical consequences; awareness of the disease and adequate laboratory support are important in achieving this goal.


## 5.3. Protein C deficiency

PC deficiency was diagnosed in an adult patient with severe venous thrombosis and in a newborn presenting purpura fulminans. The underlying causative mutations have been identified. Two novel mutations (p.Ala163Glu and p.Ala163Val) affect the same position in the EGF2 domain of PC. The third one (p.Asp77Gly) is an already communicated genetic alteration, which has not been characterized, as yet. None of these mutations were detected in 100 healthy individuals having normal PC levels and all the heterozygous carriers of the mutations had decreased PC activity and antigen levels. The p.Ala163Glu and p.Ala163Valmutations affect the same position in the EGF2 domain. Position 163 is conserved among species available in the database and there is also an alanine in the same position of human FX. Although separated by more than twenty residues in the sequence, in the 3D structure Ala163 is in the vicinity of interchain disulphide bond connecting the EGF2 and SP domains.

Altogether 11 different missense mutations have been described in the EGF2 domain (between amino acids 134–178). Surprisingly, no in vitro expression studies have been carried out concerning this domain; even molecular modeling was performed only in the case of the p.Cys140Arg mutation. Based on the results of the few in vitro experiments in PC deficiency almost all missense mutations resulted in an absolute block in secretion due to impaired folding of the mutant protein and only a few mutations allowed the protein to be secreted. It is getting more and more obvious that impairment of protein folding is the major molecule disease mechanism of a great proportion of missense mutations as it results in prolonged

binding of an unfolded conformation of the mutant protein to molecular chaperones, which, in turn, leads to the formation of insoluble aggregates or much more frequently to the degradation of mutant protein by the intracellular proteolytic machinery. Misfolded PC protein accumulation in the ER and Golgi complex was already published.

Altogether 20 PC Gla-domain mutations are listed in the HGMD database; among them 18 are missense. The structural and/or functional consequences of Gla-domain mutations have been investigated in a few cases with heterogeneous conclusions. Gandrille reported the p.Asp77Gly mutation found in one of our patients as a mutation causing type I deficiency. Dávid et al. published this mutation in heterozygous form in two patients with type I deficiencies suffering from severe, repeated VTE. In vitro expression experiments concerning the p.Asp77Gly mutation have not been reported, yet. Harmon et al. investigated the molecular consequence of an Asp to Thr amino acid exchange at this position. They found that PS dependent anticoagulant activity of APC variants (p.Asp77Thr, p.Asp78Ala, and p.Ala81Val) was only slightly decreased, and the 77, 78, 81 Gla residues have little influence on PS interaction. Wildhagen et al. confirmed these results and concluded that 77Asp was not important in PS binding.

In our studies we have found colocalization of 163Val and 163Glu mutants with the 26S proteasome, which is a known potential site of intracellular degradation of misfolded proteins after polyubiquitination. These results are supported by the proved higher rate of polyubiquitination of 163Val and 163Gly PC indirectly showing intracellular degradation. In summary, our results suggest that p.Ala163Glu and p.Ala163Val mutations lead to structural and secretion defect.

In our study individuals carrying the p.Asp77Gly mutation in heterozygous form had proportionally decreased plasma PC activity and antigen level suggesting type I deficiency. Since a high amount of the 77Gly PC was detected in the medium of the transfected cells no severe secretion defect was confirmed. Moreover both the amidolytic and clotting functional PC assays showed normal activity of the secreted 77Gly protein. It was also demonstrated that the rate of activation of 77Gly by Protac did not differ from the wt PC. Double immunofluorescent staining showed no colocalization with either cell organelles and no increased polyubiquitination was detected. This part of the molecule seems to be conserved in mammalian species and in other vitamin K dependent coagulation proteins. At position 77, aspartic acid is present in human PC and in FX, and either aspartic acid or the characteristically similar glutamic acid is present in FVII and FIX as well as in PC of other species. However, molecular modeling studies could not reveal marked intramolecular

structural abnormality. The mutation might affect the interaction with the outer part of cell membrane, its receptors, might influence the stability of protein complexes in which PC participates. Based on the presence and normal activity of 77Gly PC in the medium and as no marked secretion defect could be confirmed by immunofluorescent studies one would expect at least 70% PC concentration in the plasma of heterozygous patients. Still, heterozygous patients with this mutation had PC antigen level in plasma only around 50%. However, the conversion of in vitro secretion by HEK cells to the in vivo secretion by hepatocytes is too mechanical. Putting all our findings together, p.Asp77Gly mutation leads to a PC with normal FVa and FVIIIa inactivation effect, and as no marked secretion defect could be seen, it might have an impact on the intermolecular interactions in which PC is involved and also on the clearance of PC. These hypotheses could be an interesting subject of further research.

In summary, we have investigated the consequences of three mutations within PROC gene, detected in two patients with thrombosis. While the mutations at position 163 (p.Ala163Glu and p.Ala163Val) lead to abnormal folding and as a consequence impaired secretion, mutation at position 77 (p.Asp77Gly) showed no marked difference from wt PC either concerning secretion or activation/activity and no structural abnormality was suggested by molecular modeling. As this mutation appears as type I in all carriers we can surmise that either the intermolecular interactions and/or the increased elimination of this protein may be responsible for the deficiency.

**Novel results of the PhD thesis**

By performing detailed investigations of laboratory phenotype and genotype of patients suffering from rare coagulopathies (FV and FXIII deficiencies) and in thrombophilia (PC deficiency), novel, yet uncharacterized mutations have been found. To explain the pathogenic nature of these mutations protein biochemistry investigations and molecular modeing studies were carried out. In details:

**Factor V deficiency**

- Type I FV deficiency was diagnosed in a female patient with moderate bleeding based on prolonged APTT, decreased FV activity and proportionally decreased FV antigen. The novel p.Gly493Arg mutation in the F5 gene is responsible for the moderate bleeding symptoms of our young female patient who is heterozygous for this mutation.

- The detected mutation affects the highly conserved part of the FV protein; the amino acid itself is the same in all examined species. As a consequence of the mutation –

based on the „in silico" molecular modeling – abnormally folded FV protein is formed that is intracellularly degraded.

- Our case calls the attention to the probable bleeding complications of symptomless heterozygote FV deficient patients with FV levels below 50% and above 10% upon surgical interventions. Clinical phenotype may be more severe in FV deficiency than it is expected from laboratory results, thus complex laboratory investigations are highly important.

**Factor XIII deficiency**

- Two patients suffered from severe FXIII deficiency was investigated by complex methodology established by our study group to characterize their deficiencies based on functional and immunological determination of FXIII in the plasma and in platelets.

- The causative mutations were determined by molecular genetic investigations. One of the patients was compound heterozygote for the c.980G>A, p.Arg326Gln and the novel c.1112+2T>C mutation causing splice-site defect. In case of the latter we have also performed mRNA quantitation and exclude the possibility of nonsense mediated decay. We have detected a novel single nucleotide deletion in the other patient (c.212delA) that led to early stop codon allowing the translation of the N-terminal part of the molecule only.

- Having performed the correct laboratory diagnosis of two patients with severe factor FXIII deficiency we called the attention to the importance of early diagnosis and that genotype-phenotype associations can be examined only by adequate laboratory methods giving exact results in low FXIII activity range as well. This is also important for the prophylaxis and laboratory follow-up of the patients.

**Protein C deficiency**

- We have identified the mutations in the PROC gene causing type I protein C deficiency in two patients with severe deep venous thrombosis. The p.Ala164Glu and p.Ala163Val novel mutations affected the same position of PC protein in the EGF2 domain, while the p.Asp77Gly mutation is an already published but yet not characterized mutation in the Gla domain.

- The consequences were examined by in vitro expression studies and molecular modeling in all cases: the mutations at position 163 caused altered protein folding and secretion disorder and we were the first to detect the accumulation of the mutant PC in the 26S proteasome and the increased polyubiquitination of the abnormal protein.

- We have shown that the p.Asp77Gly mutation leads to type I deficiency but neither abnormal folding nor secretion defect were seen. The antigen concentration, activation and activity of the 77Gly PC do not differ from those of the wild type, in vitro expressed PC, however, its activity in the circulation is about 50%.

- The role of novel, not yet examined mechanisms (altered intermolecular interactions and/or enhanced protein clearance) were suggested in case of the p.Asp77Gly mutation.

## 6. Summary

Inherited hemostatic disorders are rare, they can be life-threatening to the affected person or might be disabling; their prevalence is <2-5:10 000. Depending whether the pro-or the anticoagulant part of the hemostatic system is affected, hemorrhagic diatheses or thrombophilia might develop. In the present thesis we focus on factor V (FV) and factor XIII (FXIII) deficiencies regarding coagulopathies, and protein C deficiency concerning thrombophilia. The above-mentioned diseases are presented via case reports and in vitro studies. The causative mutations are described in all cases and if possible, the molecular genetic testing of the family members has been also carried out. The consequences of the novel or previously not characterized mutations is investigated in protein biochemistry experiments and/ or molecular modeling.

FV deficiency:

- the p.Gly493Arg mutation was detected in heterozygous form in a 34-year-old female with moderate bleeding symptoms and in one of her asymptomatic daughters;
- based on molecular modeling, the mutation leads to local conformational changes, instability of the affected region and improper folding of the A2 domain.

FXIII deficiency:

- We presented two cases of severe FXIII-A deficiency. The newborn proband1 was compound heterozygote for the c.980G>A, p.Arg326Gln and the novel c.1112+2T>C mutations, the latter leads to splice site defect. The 13-year-old proband2 carried the novel c.212delA single nucleotide deletion causing early stop codon enabling the translation of only small portion of the N-terminal part of the molecule.

Protein C deficiency:

- we examined the molecular consequences of three PROC mutations found in two patients suffering from venous thromboembolism. A neonate was a compound heterozygote and had purpura fulminans, a 50-year-old female had recidive severe deep venous thrombotic events;
- the mutations affecting the 163 position (p.Ala163Glu and p.Ala163Val) cause abnormal folding and as a consequence impaired secretion;
- the mutation affecting position 77 (p.Asp77Gly) probably influences the intermolecular interactions in which PC takes part and/or leads to increased elimination of the mutant protein.

Candidate: Kitti Bernadett Kovács
Neptun ID: JBEG2D
Doctoral School: Kálmán Laki Doctoral School
MTMT ID: 10038836

## List of publications related to the dissertation

1. **Kovács, K.B.**, Pataki, I., Bárdos, H., Fekete, A., Pfliegler, G., Haramura, G., Gindele, R., Komáromi, I., Balla, G., Ádány, R., Muszbek, L., Bereczky, Z.: Molecular characterization of p.Asp77Gly and the novel p.Ala163Val and p.Ala163Glu mutations causing protein C deficiency.
   *Thromb. Res. Epub ahead of print (2015)*
   DOI: http://dx.doi.org/10.1016/j.thromres.2015.01.011
   IF:2.427 (2013)

2. Katona, É., Muszbek, L., Devreese, K., **Kovács, K.B.**, Bereczky, Z., Jonkers, M., Shemirani, A., Mondelaers, V., Ermens, A.A.M.: Factor XIII deficiency: Complete phenotypic characterization of two cases with novel causative mutations.
   *Haemophilia. 20* (1), 114-120, 2014.
   DOI: http://dx.doi.org/10.1111/hae.12267
   IF:2.468 (2013)

3. Bereczky, Z., **Kovács, K.B.**, Muszbek, L.: Protein C and protein S deficiencies: Similarities and differences between two brothers playing in the same game.
   *Clin. Chem. Lab. Med. 48* (Suppl.1), S53-S66, 2010.
   DOI: http://dx.doi.org/10.1515/CCLM.2010.369
   IF:2.069

4. **Kovács, K.B.**, Tisza, B., Komáromi, I., Muszbek, L., Bereczky, Z.: Inherited factor V deficiency associated with a novel heterozygous missense mutation (p.G493R) in a patient with excessive surgical bleeding.
*Thromb. Haemost. 102* (4), 787-789, 2009.
DOI: http://dx.doi.org/10.1160/TH-09-04-0272
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Address: 1 Egyetem tér, Debrecen 4032, Hungary Postal address: Pf. 39. Debrecen 4010, Hungary
Tel.: +36 52 410 443 Fax: +36 52 512 900/63847 E-mail: publikaciok@lib.unideb.hu, ¤ Web: www.lib.unideb.hu

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