

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

INVESTIGATION ON THE ROLE OF PROTHROMBOTIC FACTORS IN  
PRIMARY RAYNAUD'S SYNDROME

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ALLERGOLOGY

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The Examination takes place at the Professor Room of Building C, Department of  
Internal Medicine, Faculty of Medicine, University of Debrecen  
12 January, 2017, 11:00 AM

**Head of the Defense Committee: Prof. Margit Zeher MD, PhD, DSc**  
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The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal  
Medicine, Faculty of Medicine, University of Debrecen  
12 January, 2017, 1:00 PM

## **1. THE THESIS BACKGROUND AND OBJECTIVES**

In the last decades, our knowledge of normal and abnormal vascular functions has increased explosively. Nowadays it is well known that the complex system of human vasculature encompasses the endothelial and blood cells as well as the interaction and dynamic change of neurogenic and non-neurogenic regulatory mechanism. Raynaud's syndrome is an ideal for investigating functional disorders of circulatory system, due to its high prevalence and spectacular clinical picture.

The present dissertation summarizes my findings on vascular abnormalities and prothrombotic characteristics in primary Raynaud's syndrome.

### **1.1. Raynaud's phenomenon**

Raynaud's phenomenon is an excessively reduced blood flow in response to cold or emotional stress, causing discoloration of the fingers, toes, and occasionally other areas. The disorder behind this symptom is referred as Raynaud's syndrome (RS). Three color changes are observed in classic Raynaud's. (1) When exposed to cold temperatures, the blood supply to the fingers or toes, and in some cases the nose or earlobes, is markedly reduced; the skin turns pale or white (called pallor) and becomes cold and numb. (2) When the oxygen supply is depleted, the skin color turns blue (called cyanosis). These events are episodic, and (3) when the episode subsides or the area is warmed, the blood flow returns, and the skin color first turns red (rubor), and then back to normal, often accompanied by swelling, tingling, and a painful "pins and needles" sensation.

However, not all patients see all of the aforementioned color changes in all episodes, especially in milder cases of the condition. The attack usually lasts a few minutes, but in some cases there may be up to several hours, the mean duration of 20-25 minutes. Initially, usually only few fingers are affected, but later, along with the disease progression, RS may affect all fingers of both hand. Symptoms of toes, tongue, nose, earlobes, or nipples are less common.

## **1.2. The clinical characteristics of Raynaud's syndrome**

When the disorder's cause is idiopathic, it is referred to as primary Raynaud's syndrome; if the syndrome is secondary to another disease such as systemic sclerosis (SSc), or other connective tissue disorders, it is referred to as secondary Raynaud's syndrome. In primary form, the fingers are usually affected symmetrically, with pure vasospastic attack in the background, without any structural damage in digital arteries. Primary RS patients are more likely to have migraines and angina.

Secondary RS with the organic digital artery lesions can be associated with immunological diseases [eg. systemic sclerosis, systemic lupus erythematosus (SLE)], haematological malignant diseases, diabetes, carpal tunnel syndrome or even thoracic outlet syndrome. Furthermore, various drugs (beta-blockers, amphetamines, ergotamine, bleomycin, vinblastine, cyclosporin) may also trigger RS. In older patients, obstructive vascular disease is the most common reason. In addition to the aforementioned factors, occupational hazards (vibrations, polyvinyl chloride) can cause Raynaud's syndrome, as well.

The true incidence of Raynaud's syndrome is unknown; the prevalence is estimated at 5-20% of the European adult population. The Raynaud's syndrome is four times more common in women than in men. The role of hormonal factors is supported by several observations, and after menopause, symptoms of primary RS often disappear.

Secondary Raynaud's syndrome may occur at any age depending on the basic disease, although the beginning is more common in older age. The incidence of the associated diseases also increases with the age. Raynaud's phenomenon is usually one of the initial symptoms of systemic sclerosis, which may be preceded by years of other signs of the disease (90-95% prevalence). In SLE and Sjögren's syndrome, 35-39% of the patients suffer from Raynaud's syndrome.

## **1.3. The pathophysiology of Raynaud's syndrome**

In Raynaud's syndrome, cold and stress lead to excessive vascular response resulting in decreased perfusion in the affected areas. The proper microcirculation is critically

dependent on the integrity of the endothelium, the appropriate regulation of vascular tone and the characteristics of plasma and cellular components of blood. The etiology of primary Raynaud's phenomenon (PRP) is not entirely known. Abnormalities in the blood vessel wall, as well as in the neural control of vascular tone, and in some circulating mediators were found as part of the pathophysiology of this phenomenon.

### ***Structural and functional changes of the vascular wall***

The endothelial cells, beside their physical barrier function, play an outstanding role in the regulation of circulation by producing and releasing various cytokines and other factors. Alteration in endothelium-dependent processes, such as overexpression of vasoconstrictor mediators (eg. Endothelin-1, Thromboxane A2) and decrease in release of vasodilator factors (NO, prostacyclin) can play a key role in the development of Raynaud's phenomenon.

In contrast with the primary form with purely functional differences, in secondary RS associated with autoimmune disease, elevated endothelial vasoconstrictor factors along with the activation of immune cells and pro-inflammatory cytokine milieu result in the structural damage of blood vessels. One of the first steps in the disease process is the development of endothelial lesions, such as vacuolar degeneration of endothelial cells and damage of basal membrane. Activation of immune system and derailed inflammatory responses lead to structural damage of vessel wall, hypertrophy of tunica intima and media and fibrosis in adventitia. The worsening circulatory disturbance may cause hypoxic condition in the affected tissues, which can lead to the formation of reactive oxygen species, activation of fibroblasts and further release of growth factors. These processes lead to progressive narrowing of vessel lumen, later occlusion and subsequent tissue and organ damage may occur.

### ***Neurogenic disorders***

The autonomic nervous system, which is a key element of the body's thermal regulation, is believed to play a role in the development of Raynaud's phenomenon. The vascular smooth muscle may overreact to normal sympathetic impulses. Additionally, increased release of noradrenaline from sympathetic nerves, impaired catecholamine degradation, increased density or affinity of alpha adrenoceptors, decreased density or

affinity of beta-2 adrenoceptors, as well as a shift of second messengers such as cAMP, cGMP calcium ions may be important. Freedman et al. reported that peripheral alpha-adrenoceptor sensitivity or density is increased in primary Raynaud syndrome. The alpha-2 adrenergic play a key role in vascular tone regulation of the fingers. Previous studies demonstrated that enhanced reactivity of alpha-2 adrenoceptor can be detected in arteriolar smooth muscle in systemic sclerosis associated secondary RS. Changes in adrenoceptor activity was also reported in chronic vibration caused RS.

Vasodilator effect of autonomic nervous system can be also insufficient. Calcitonin gene related peptide (CGRP) is an endogenous vasodilator. A significant decrease in CGRP-responsive neurons was revealed in skin biopsy of both primary and secondary RS patients. The emotional loading induced vasospasm may be due to the excessive central activation of sympathetic system.

#### ***Intravascular factors - platelets, prothrombotic factors***

A number of intravascular factors play role in the development of Raynaud's phenomenon, such as leukocyte activation, altered cytokine milieu and release of reactive oxygen radicals.

Enhanced platelet activation was reported in both primary and secondary RS. Platelets release a surprisingly large array of mediators, many of which have important roles in inflammation, tissue repair and vascular function. The platelet-derived mediators potentially contribute to chemotaxis, cellular adhesion, altered vascular resistance, altered vascular permeability, angiogenesis, matrix degradation and fibrosis.

Platelets are a rich source of chemokine (C-C motif) ligand 5 (CCL5), platelet factor (PF)-4, beta- thromboglobulin (TG) and epithelial neutrophil activating protein (ENA)-78. Each has important leukocyte-chemoattractant properties attracting neutrophils, eosinophils, monocytes and fibroblasts to the sites of inflammation. Platelet granules also release several important pro-inflammatory mediators, such as interleukin (IL)-1beta, CD40 ligand, platelet-derived growth factors (PDGF). P-selectin (also known as CD62P) is a single-chain transmembrane glycoprotein from a family of important cell adhesion molecules. P-selectin translocates to the platelet membrane following activation, facilitating adhesion, aggregation and leukocyte recruitment. P-selectin is the primary receptor for the interaction between platelets and monocytes,

neutrophils, memory T-cells and natural killer cells, modulating chemokine and cytokine expression in these cells. Additionally, platelets are a rich source of pro-angiogenic and pro-fibrotic growth factors, including transforming growth factor-beta (TGF-beta) and platelet-derived growth factors (PDGF), as well.

Based on these observations, increased platelet activation and their altered function may play a potential role in the pathogenesis of Raynaud's syndrome. Studies have shown increased platelet aggregation ability *ex vivo* in primary Raynaud's syndrome as well as systemic sclerosis associated secondary form. Mean platelet volume (MPV) and platelet P-selectin are considered as two sensitive markers of elevated platelet activation. Increased MPV and CD62P levels have been formerly described in inflammation, hyperlipidemia, myocardial infarction, diabetes mellitus, hypertension, and autoimmune diseases such as rheumatoid arthritis. However, the clinical value of MPV in primary RS is still unclear.

The von Willebrand factor (vWF) is a large multimeric glycoprotein produced by many cells including endothelial cells and platelets, facilitating platelet adhesion and aggregation. Several studies have reported increased circulating levels of vWF in SSc, variously reporting their pathological significance in terms of either platelet or endothelial cell activation. Several other studies examined the association between plasma levels of vWF and fibrinogen levels in patients with primary RS, however their results were controversial.

Thrombophilia may develop due to acquired homeostasis changes and/or inherited genetic factors. Factor V substitution of G to A at position 1691 (FVLeiden), is the most common risk factor for venous thrombosis with frequency of 1–11% in Europeans and associated with thrombotic angiopathy. The prothrombin G20210A polymorphism (FIIIG20210A) the substitution of G for A at position 20210 has 0.7–4.0 % prevalence in normal population while it is responsible for 6.2 % of all cases of thrombosis and associated with elevated prothrombin plasma activity. Gene variant in the methylenetetrahydrofolate reductase gene (MTHFR 677C>T polymorphism) is a risk factor for venous and arterial thrombosis which is more prevalent in patients with cardiovascular disease (17 %) than in the normal population (5 %). Carriers for MTHFR T677T mutation have increased total homocysteine (Hcy) levels. Besides, increased levels of homocysteine have been found in patients with primary RS. Higher Hcy level

increases the risk of endothelial injury in these patients, consequently, may affect the threshold of migraine headache. A common polymorphism in the FXIII-A gene-V34L is an important functional polymorphism described in the hemostatic system. This polymorphism affects a valine residue, 3 amino acids upstream to the thrombin cleavage site. It has been clearly demonstrated that the higher rate of proteolytic truncation of L34 variant resulted in earlier activation of FXIII and, consequently, accelerated the cross-linking of fibrin. Moreover, this polymorphism also has a significant effect on fibrin clot structure, probably through the alteration of fibrin cross-linking kinetic. Its prevalence is about 25 % among Caucasians, and in blacks and Asian Indians the allele frequency is lower. The higher prevalence of vascular injury besides genetic predisposition to thrombosis could partly explain the presence of migraine in this group of patients.

## 1.4. Objectives

### *Platelet studies of primary Raynaud's syndrome*

In our work, we investigated the role of platelets in the pathogenesis of primary Raynaud's syndrome. The results obtained from patients were compared to healthy control subjects' data. As the role of platelets in primary RS is controversial, the objectives of our investigations were determined as follows:

- 1) Comparison of the number and mean volume of circulating peripheral platelets in patients with primary RS with the corresponding values of healthy controls.
- 2) Comparison of the platelet activation and CD62P expression measured in patients and control individuals.
- 3) Examination of the relationships between the observed laboratory results and the demographic and clinical characteristics of patients.

### *Von Willebrand factor and fibrinogen tests*

In our study, we examined the potential role of fibrinogen and von Willebrand factor in blood clotting process in the pathogenesis of primary Raynaud's syndrome. Previous studies did not find any association between PRP and vWF and fibrinogen levels. However, they were not only based on small number of subjects, but also their studies did not investigate this association between men and women separately. Since the possible relationship between these factors and primary RS is still not fully elucidated. Our objectives were as follows:

- 1) Determination of von Willebrand factor and fibrinogen levels in a large number of primary RS patients.
- 2) Comparison of the measured values between the male and female populations.
- 3) Examination the risk role of higher von Willebrand factor and fibrinogen levels in the development of primary RS.

### ***Prothrombotic polymorphism analysis***

Many previous studies reported on the links between the alterations occurring in the blood coagulation system and the vascular dysfunction, which may lead to the development of various vasospastic disease conditions including Raynaud's syndrome, Prinzmetal's angina and migraine. In our study, we investigated the role of genetic factors predisposing to thrombosis in the development of migraine in patients with primary Raynaud's syndrome. Our objectives were as follows:

- 1) Detection of the factor V Leiden mutation (G and A exchange at position 1691) in primary RS patients.
- 2) Detection of the G20210A prothrombin polymorphism (FII G20210A) in the patients.
- 3) Examination of the methylenetetrahydrofolate reductase (MTHFR) gene C677T mutation in the patient population.
- 4) Determining factor VIII (Laki-Lóránd factor) V34L mutations among patients.
- 5) Comparison of the laboratory results with a special emphasis on the association of migraine.

## 2. PATIENTS AND METHODS

### 2.1. Patients

Patients were recruited from the Autoimmune Outpatient Clinic of the Division of Clinical Immunology, Institute of Medicine, Clinical Center, University of Debrecen, where they received regular follow-up and treatment. Two hundred patients (158 female, 42 male, mean age of  $42.4 \pm 13.7$  years) with the diagnosis of PRP were included in the study. The diagnosis of RS was based according to the criteria by Wigley et al. The diagnosis included a detailed history taking and the observation of bilateral, cold-induced blanching and/or cyanosis of the fingers, or in some cases that of toes as well. We performed a complete serologic examination to detect and exclude the development of any primary connective tissue disease causing secondary RS. 116 age-matched clinical controls without any chronic disease were enrolled (77 female, 39 male, mean age of  $41.6 \pm 14.0$  years). Patients and controls were the subjects of the same geographical and ethnic origin.

In this study, we did not include patients with anamnestic occurrence of angina, myocardial infarction, diabetes mellitus, thrombosis, or connective tissue disease.

Patients were followed up for a mean  $\pm$  S.D. of  $4 \pm 3.5$  years. In our study group, 29% of the patients had a history of migraine. Hyperlipidemia was defined when serum cholesterol level was more than 5.2 mmol/l and/or triglyceride level was more than 1.7 mmol/l and/or regular administration of lipid-lowering agents. Hyperlipidemia ratio was similar (55% vs. 47%) in patients and controls. Notably, there was no significant difference in term of regular statin treatment among patients and controls, and they were not under any medication that influenced the function of bone marrow or platelets for at least three months prior to the sampling. No patients, or controls enrolled in this study were administered immunosuppressive or immune-modulating medications and no one had ongoing infections, either viral, or bacterial. During the study, serum CRP levels of the enrolled subjects were within the reference range ( $<10$  mg / L).

Informed written consent was obtained from the subjects, and the study has been approved by the Ethics Committee of University of Debrecen. All experiments carried out were in compliance with the Helsinki Declaration.

## **2.2. General laboratory tests**

EDTA anticoagulated whole blood samples were analyzed by the Sysmex SF-3000 analyzer (Sysmex Corporation, Kobe, Japan). The samples were processed within two hours after venipuncture. Fasting serum samples were taken for the determination of lipid parameters and C-reactive protein (CRP) and measured on COBAS Integra Analyzer (Roche, Mannheim, Germany) in the Department of Laboratory Medicine of the University of Debrecen.

## **2.3. Determination of autoantibodies**

The determination of autoantibodies was carried out by indirect immunofluorescence technique on Hep2 cells with IFA ANA-Hep2-IgG immunofluorescence kit (Viro-Immun Labor-Diagnostika GmbH, Oberursel, Germany) at the Regional Immunology Laboratory of the Division of Clinical Immunology of the University of Debrecen. To identify fluorescence patterns, Eurostar Plus II fluorescence microscope (EUROIMMUN AG, Lübeck, Germany) was used.

Enzyme-linked immunosorbent assay (ELISA) screening tests for extractable nuclear antigen (ENA) antibodies were also carried out, and in case of positivity, specific determination of antibodies against Sm, Sm/RNP, SS-A, SS-B, Scl-70, Jo-1 was performed by ELISA technique with AUTOSTAT II kits (Hycor Biomedical, Indianapolis, IN, USA), according to the manufacturer's instructions. ETI-MAX 300 device (Diasorin, Saluggia, Italy) was used for the measurements and data evaluation.

#### **2.4. Flow cytometry assay**

Platelets were identified by a fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody to CD42a. Platelet activation was detected by phycoerythrin (PE)-labeled anti-CD62P antibody (Becton Dickinson, San Jose, CA, USA). 40 µl of all anticoagulated peripheral blood samples were fixed in 1 ml 1 % paraformaldehyde. The samples were centrifuged and the pellet was washed in 1 ml phosphate-buffered saline (PBS). Fixed platelets were incubated with monoclonal antibodies for 20 min in the dark at room temperature. Ten thousand dual-color labelled platelet events were acquired on FACS Calibur flow cytometer by using the CellQuest 3.2 software (Becton Dickinson). Results were expressed as percentage of circulating positive platelets.

#### **2.5. Determination of von Willebrand factor and fibrinogen levels**

Hemostasis tests and determination of plasma fibrinogen levels of sodium citrate anticoagulated peripheral blood were made by using BCS XP device (Siemens Healthcare Diagnostics, Deerfield, IL, USA).

The vWF levels were determined by ELISA according to the manufacturer's instructions in the Department of Laboratory Medicine of the University of Debrecen.

#### **2.6. Genetic polymorphism analysis**

Genomic DNA was isolated by using MagNA Pure LC Instrument (Roche Diagnostics) according to manufacturer's protocol. To detect the G1691A mutation in Factor V gene, G20210A mutation in Factor II, C677T mutation in MTHFR gene, and FXIII-A V34L the real-time fluorescence PCR were performed as described earlier by using LightCycler (Roche Diagnostics).

## **2.7. Statistical analyses**

The SPSS ver. 12.0 and 13.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. To assess the distribution of data Kolmogorov-Smirnov test was used. In cases of normal distribution, we determined mean  $\pm$  standard deviation (SD) values and used two-sample t test for statistical comparison of the experimental data.

In cases of distributions different from normal, median, minimum and maximum values were calculated, and Mann-Whitney test was used.

Comparisons between groups were performed with the Chi<sup>2</sup>-test for binary and categorical data. For analyzing a dataset in which there are one or more independent variables and dichotomous dependent variable, logistic regression [odds ratios (ORs) and 95% CI's] was obtained to calculate adjusted ORs, and to assess the independent associations. Differences were considered statistically significant at  $p < 0.05$ .

### 3. RESULTS

#### 3.1. Results of platelet studies

There was no significant difference between the average years of age in primary RS group ( $42.4 \pm 10.0$  years) vs. control group ( $41.6 \pm 14.0$  years). However, male to female ratio differed significantly ( $p < 0.05$ ) between patients (female/male: 158/42) and normal subjects (female/male: 77/39) and thus it was adjusted, when appropriate, to other parameters during statistical analyses. Serologic results for autoimmune diseases were negative in all PRP individuals. Hyperlipidemia ratio was similar (55 % vs. 47 %) in both groups, and no inflammatory process was detected by the measuring of CRP level and WBC count.

##### *Platelet count and mean volume*

Platelet (PLT) count was significantly lower in our primary RS patients compared with healthy controls ( $250 \pm 4.6 \pm 10^9/l$  vs.  $268 \pm 5.4 \pm 10^9/l$ ,  $p < 0.05$ ).

MPV was significantly increased in patients compared to clinical controls ( $11.0 \pm 0.1$  fl vs.  $8.8 \pm 0.1$  fl,  $p < 0.001$ ). Additionally, there was a significant and negative correlation between MPV and PLT count ( $R = -0.470$ ,  $p < 0.001$ ).

##### *Examination of CD62P expression*

The platelet CD62P expression was significantly increased value in primary RS patients compared to the control group ( $1,3 \pm 1,1$  % vs.  $0,7 \pm 1,1$  %,  $p < 0,001$ ). A significant positive correlation was found between CD62P expression of platelets and their numbers ( $R = 0,137$ ,  $p = 0,032$ ).

##### *Correlations between clinical data and laboratory results*

PLT counts, MPV and CD62P values were associated with neither the age/gender of patients nor the disease duration. Similar statistical significance ( $p < 0.001$ ) was calculated for MPV and CD62P levels when female and male patient subgroups were compared to female and male clinical controls. MPV values in total group were adjusted for PLT count and gender, and for PLT count in the female patient

group. Furthermore, adjustment for confounders did not influence the results. Adjusted ORs by multiple logistic regression analysis showed that both MPV (OR: 15.8, 95 % CI: 8.1–30.6) and CD62P (OR: 11.3, 95 % CI: 4.8–26.1) were significantly ( $p < 0.001$ ) and independently related to primary RS during the analysis of either the whole patient cohort or in different gender subgroups, separately.

During analysis, MPV values were adjusted for PLT and WBC counts in the total cohort and female subgroup, while CD62P was adjusted for PLT count and gender in total group, and for PLT count in female subgroup. Of note, 30.2 % of control group and 98 % of patient group belonged to the upper tertile ( $> 49.3$  fl) of MPV, whilst 33.6 % of controls and only 67 % of PRP subjects were in the upper tertile ( $> 0.99$  %) in terms of CD62P positivity.

### **3.2. Results of von Willebrand factor and fibrinogen studies**

No differences among groups were seen in vWF levels. Fibrinogen level, moderately but significantly, was elevated in female group ( $3.5 \pm 0.8$  g/L vs.  $3.1 \pm 0.8$  g/L,  $p = 0.016$ ).

#### ***Correlations between genders and laboratory results***

We defined vWF and fibrinogen levels according to tertiles and compared them separately. Fibrinogen level in the lowest tertile was significantly elevated in female group ( $p = 0.045$ ) compared with male group. We obtained no significant difference of fibrinogen level among other tertiles between male and female groups. vWF level in the upper tertile [ $> 137.6\%$ , odds ratio (95% confidence interval): 1.022 (0.466–2.239),  $p = 0.958$ ] did not confer a significant risk of primary RS between two groups of men and women. Neither fibrinogen in the upper tertile [ $> 3.8$  g/L, odds ratio (95% confidence interval): 0.931 (0.333–2.608),  $p = 0.892$ ] conferred any significant risk of primary RS between females and males.

### **3.3. Results of prothrombotic polymorphism analysis**

Based on anamnestic data, 29 % of primary RS patients suffered from migraine (n = 58). Duration of RS was significantly longer in patients with migraine than patients without migraine, in the whole group and when the two sexes were considered separately ( $p < 0.001$ ).

#### ***Prevalence of genetic polymorphisms***

Among all patients, 20 (10%) possessed the factor V Leiden allele polymorphism. Eighteen of these patients were heterozygous, and two were homozygous for the FV Leiden allele. Prevalence of the FII20210A allele polymorphism was 5%. No individuals homozygous for the FII20210A allele were detected. Interestingly, there was no MTHFR homozygous mutation among patients with migraine. The frequency of the T allele was significantly different between cases with migraine (50%) and without migraine (32%) (OR 2.1, 95% CI: 1.4–3.3,  $p = 0.001$ ). Seventy-six cases (38%) were heterozygous for the FXIII V34L polymorphism, and 14 cases were homozygous for FXIII 34L (7%). A significantly higher allele frequency of MTHFR 677T in patients with PRP and migraine could be shown in this study. There was no correlation, however, between the occurrences of migraine and FII20210A, FVLeiden, or FXIII V34L mutations.

## **4. DISCUSSION**

### **4.1. Platelet studies in primary Raynaud's syndrome**

Several factors may contribute to the pathogenesis of primary RS, but the complex pathomechanism of this disease is still unknown. Among many conditions, alterations in hemostasis such as elevated levels of tissue plasminogen activator antigen, marked activation of GPIIb/IIIa receptor with increased thromboxane levels in platelets were formerly documented in patients with primary RS. Interestingly, conflicting data were published in terms of higher level of platelet-derived microparticles in the disease. However, the percentage of platelet-monocyte and platelet-neutrophil heterotypic aggregates were significantly elevated in primary RS patients compared to healthy controls. Overall, platelet hyperactivity and arterial thrombosis in the digits might be the part of disease etiology.

MPV is a routinely measured platelet parameter by automated hematology analyzers, and its abnormal level may indicate altered platelet reactivity and potential vascular risk. MPV may increase due to the accelerated platelet turnover with higher level of reticulated platelets from bone marrow. According to previous publications, this marker with elevated levels predicted the development and progress of cardio- and cerebrovascular diseases or peripheral artery disease. MPV was higher than normal in severe dyslipidemia and diabetes mellitus as well. On the other hand, an increase in MPV has been also shown to be associated with hypertension. In other studies, changes in MPV have been linked to alterations in thrombogenesis, and the level of circulating autoantibodies and inflammatory agents.

In the present study, MPV seems to reflect enlarged platelet reactivity as a potential cause or consequence of the development of primary RS, which was also demonstrated by CD62P positivity. Almost all patients (98%) belonged to the upper tertile of MPV, but it was 2/3 of the patient population in case of CD62P. MPV levels remained significantly higher in the total patient group or either gender subgroup compared to controls, even after adjustment for different confounders. Thus, MPV seems to be a more sensitive novel marker for PRP than CD62P. Future studies are

needed to examine the functional role of platelets with high MPV values in PRP with or without thrombotic events.

In our study, significantly lower PLT count was observed in primary RS patients when compared to clinical controls suggesting that platelet consumption may be augmented in the disease due to their activation. Furthermore, megakaryocytes may be subsequently triggered to produce young and thus larger platelets. In contrast, elevated PLT count was measured in patients with RA versus non-RA subjects. This discrepancy might be due to the distinct pathophysiological function of activated platelets in the background of PRP and RA. These results support our hypothesis that elevated MPV is independently associated with PRP, but age, gender, and the disease duration do not alter this parameter.

#### **4.2. Studies on von Willebrand factor and fibrinogen**

Endothelium injuries lead to fibrin generation, in parallel, fibrinolytic system prevents excessive fibrin accumulation. Altered plasma fibrinolysis may contribute to the obstruction of free flow of blood, which is an important underlying mechanism of thrombotic vascular diseases. Previous studies on association between RS and vWF and fibrinogen levels were not only based on small number of subjects, but also their studies did not investigate this association between men and women separately, thus the possible relationship between these factors and primary RS remained controversial. Our data indicates that plasma levels of vWF and fibrinogen are not associated with primary RS in women or men.

#### **4.3. Prothrombotic polymorphism analysis**

The frequency of the prothrombotic risk factors FVLeiden mutation, FIII20210A mutation, and FXIII-A V34L polymorphism did not significantly differ in PRP patients with migraine and non-migrainous primary RS patients. Our results add to a series of recent reports on the relationship between FVLeiden and FII20210A genotypes and

migraine. As others, our data suggest that the factor XIII 34L allele does not play a protective role against migraine.

It has been postulated that neurogenic and vascular hypotheses mostly explain the migraine. Neurogenic hypothesis predicts that neural events cause changes in blood flow. Cortical spreading depression which consists of slow dispersion wave of intensive neuronal burst activity and is followed by depression of spontaneous and evoked activity could activate trigeminal nerve. Vascular hypothesis explains the migraine attacks by alterations in the perivascular nerves of the cerebral vessels. It has been demonstrated that direct stimulation of the cerebral and meningeal blood vessels produced ipsilateral headaches.

The enzyme MTHFR is responsible for the reduction of 5,10-methylene-THF to 5-methyl-THF, the required substrate in the remethylation process. A homozygous mutation of the MTHFR gene, due to a point mutation 677 C to T changing alanine to valine at amino acid 222, has been related to thermolabile variant of the enzyme. This mutation causes hyperhomocysteinemia by reducing 5-MTH availability. Thermolabile MTHFR is the most frequent inherited defect of the Hcy pathway. Individuals homozygous for the MTHFR mutation have significantly higher Hcy levels than wild-type or heterozygous ones. Hyperhomocysteinemia is an independent risk factor for cardiovascular diseases and it contributes to vascular damage by several mechanisms. It has direct toxic effect on endothelial cells and may strengthen the injury provoked by oxidative stress. Studies in humans and animals have shown negative effects of mild hyperhomocysteinemia on the blood vessels. Vascular endothelium dysfunction by homocysteine effect may influence migraine vulnerability through the activation of trigeminal nerve. The roles of elevated levels of Hcy on neurons have been reported to include generation of reactive oxygen species. In our patient group, the MTHFR mutation had significantly higher allele frequency in migrainous patients compared with patients without migraine. As the disease duration was significantly prolonged in patients with migraine, higher plasma Hcy levels for a longer period of time could affect blood vessels, consequently, it could lower the threshold of migraine headache in this group of patients. Thus, longer duration of primary RS and the presence of MTHFR 677T allele could increase the risk of migraine headache. However, a former investigation revealed a significantly lower allele frequency of MTHFR 677T in

patients with PRP compared to healthy individuals. On the contrary, a number of studies reported higher allele frequency in patients suffering from migraine. So we cannot exclude that the increased frequency of MTHFR 677T allele is primarily associated with the migraine, regardless of the existing primary RS.

Moreover, there is no rationale for premising that a thrombophilic state caused by the FVLeiden, FIIG20210A, and FXIII V34L mutations should be associated with migraine in primary RS.

## **5. THE NEW SCIENTIFIC RESULTS**

The results of our study on primary Raynaud's syndrome (RS) indicate the involvement of altered platelet functions in the disease pathogenesis. We found significantly decreased platelet numbers in patients, compared to the results of healthy individuals. Regarding mean platelet volume (MPV), we found significantly elevated values in the disease, moreover, the statistical analysis revealed a clear negative correlation between the aforementioned parameters. Of note, according to our data, CD62P expression was significantly elevated on platelets in primary RS, which indicates the activated state of the platelets. The increased MPV values also seem to reflect enlarged platelet reactivity as a potential cause or consequence of the development of primary RS.

We did not find any alteration in plasma levels of von Willebrand factor or fibrinogen, which indicates that they are not associated with primary RS in women or men.

Our investigations on prothrombotic polymorphisms revealed that the longer duration of primary RS and the presence of MTHFR 677T allele may increase the risk of migraine headache. Moreover, there is no rationale for premising that a thrombophilic state caused by the FV Leiden, FII G20210A and FXIII V34L mutations should be associated with migraine in primary RS.

**LIST OF PUBLICATIONS RELATED TO THE DISSERTATION**



Registry number: DEENK/27/2016.PL  
Subject: Ph.D. List of Publications

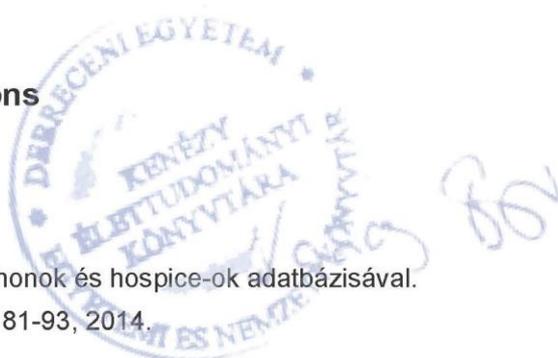
Candidate: Alajos Takáts  
Neptun ID: BRCAN7  
Doctoral School: Doctoral School of Pharmaceutical Sciences

### List of publications related to the dissertation

1. **Takáts, A.T.**, Shemirani, A., Zsóri, K.S., András, C., Csiki, Z.: Association of von Willebrand factor and fibrinogen plasma levels with primary Raynaud's phenomenon in male and female patients.  
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DOI: <http://dx.doi.org/10.1007/s00296-011-2236-9>  
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2. **Takáts, A.T.**, Shemirani, A., Zsóri, K.S., András, C., Csiki, Z.: Prothrombotic polymorphisms in patients with Raynaud's phenomenon and migraine.  
*Acta Physiol. Hung.* 99 (4), 430-435, 2012.  
DOI: <http://dx.doi.org/10.1556/APhysiol.99.2012.4.7>  
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3. Shemirani, A., Nagy, B., **Takáts, A.**, Zsóri, K.S., András, C., Kappelmayer, J., Csiki, Z.: Increased mean platelet volume in primary Raynaud's phenomenon.  
*Platelets.* 23 (4), 312-316, 2012.  
DOI: <http://dx.doi.org/10.3109/09537104.2011.618563>  
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4. **Takáts A.**: Gasztronterológiai betegek kezelése időskorban.  
In: Idősgondozási kézikönyv - A magyarországi idősothonok és hospice-ok adatbázisával.  
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7. Tari K., Lukovich P., Morvay K., **Takáts A.**, Wacha J., Öreg Z., Kupcsulik P.: A colonosopos vizsgálat előkészítése: Létezik-e betegbarát módszer?  
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8. Egri, G., **Takáts, A.**: Monoclonal antibodies in the treatment of lung cancer.  
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9. Lukovich P., Lakatos P.L., Keresztes K., Wacha J., **Takáts A.**, Morvay K., Tari K., Kupcsulik P.:  
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10. Novák J., Csiki Z., Sebesi J., **Takáts A.**, Demeter P., Sipka S.: Helicobacter pylori-ellenes antitestszint-emelkedés Schönlem-Henoch-puipurában.  
*Orv. Hetil.* 144 (6), 263-267, 2003.
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DOI: <http://dx.doi.org/10.1055/s-2001-17195>  
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14. Rákóczy G., Márk G., **Takáts A.**, Szentmihályi A., Joós Á.: Helicobacter előfordulása Helicobacter pylori pozitív szülők gyermekeinél.  
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**Total IF of journals (all publications): 8,477**

**Total IF of journals (publications related to the dissertation): 4,749**

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

10 March, 2016

