Thesis of doctoral (PhD) dissertation

ANGIOLOGICAL ALTERATIONS AND IMMUNO-INFLAMMATORY MECHANISMS IN PRIMARY ANTIPHOPHOLIPID SYNDROME

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Introduction

Antiphospholipid syndrome (APS) is a prothrombotic autoimmun mediated syndrome characterized by recurrent arterial and venous thrombotic events and fetal loss due to circulating antiphospholipid antibodies (APA) including antibodies to cardiolipin (CL) or β2 glycoprotein I (β2GPI), as well as lupus anticoagulant. Autoimmune phenomena have recently been shown to play a role in the initiation and promotion of atherosclerosis; the most important autoantigens are oxidized lipids, heat shock proteins and B2-glycoprotein I. B2GPI is a major target for antiphospholipid antibodies, which are present in patients with primary and secondary antiphospholipid syndrome. Endothelium activation seems to represent one of the pathogenic mechanisms resulting in prothrombotic state of the APS. Anti-B2GPI antibodies bind to endothelial surfaces through adhered β2GPI inducing the appearance of pro-coagulant and pro-inflammatory phenotype. It has also been suggested that the presence of antiphospholipid antibodies is associated with early signs of atherosclerosis. Based on these findings we hypothesized that there is relationship between antiphospholipid antibodies, endothel dysfunction and atherosclerosis. Different laboratory and imaging methods, novel diagnostic procedures became very valuable for the detection of early atherosclerotic lesions in these patients.

Celermayer et al in 1992 described the method entitled flow-mediated vasodilation of the brachial artery. The method is based on the principle that because of shear forces due to flow increase in the brachial artery, endothelial cells are activated, nitrogen-monoxide-synthase, therefore NO is produced, which is secreted from endothelial cells and eventually leads to vasodilation. In early atherosclerosis primarily this particular endothelial function deteriorates, therefore the reduced dilation capability of the arteries can be observed. In healthy individuals the efficacy of flow-mediated vasodilation is almost 10%. A clear dysfunction can be established, if vasodilation does not reach 5%. Another version of this method is the endothel-independent (nitrate-mediated) form.

A newly introduced non-invasive measuring method is the evaluation of augmentation index that is calculated from the data obtained from oscillometric measurements of the brachial artery and the pulse wave velocity. We measured FMD in the brachial artery of 44 patients with primary APS, as well as the von Willebrand factor antigen level in their plasma as markers of endothelial dysfunction, moreover the thickness of the carotid artery, as a sign

for early atherosclerosis and we investigated the relationship between the endothlial function-, and stiffness parameters.

Besides the pathogenic role of aPL antibodies, pro-inflammatory cytokines and also chemokines have been described to play a significant role in the pathogenesis of APS. IL-4 (B-cell stimulation/differentiation/growth factor) stimulates both activated B- and T-cell proliferation and the differentiation of CD4+ T-cells into Th2 cells. IL-4 induces B-cell activation and class switching, and promotes the proliferation and differentiation of activated B-lymphocytes IL-4 plays a pivotal role in the perpetuation of endothelial dysfunction, exaggerated atherosclerosis, and subsequently the development of arterial and venous thromboses. Another important cytokine, IL-6 is capable of inducing the final maturation of B-cells into immunoglobulin-secreting plasma cells upon previous activation by IL-4. By plasma cell activation it significantly stimulates the secretion of antibodies, and therefore may contribute to the humoral pro-inflammatory processes in APS. On the other hand, serum IL-10 levels were decreased in patients with APS. IL-10 during the initial activation delivers negative signals that promote the apoptosis of B cells, and therefore the reduced serum level of the cytokine may down-modulate this important counter-regulatory, immune-suppressive process. Moreover, IL-10 inhibits the production of several cytokines, such as IL-4, IL-5 and IFN-γ, and also reduces the secretion of growth factors and chemokines, and therefore acts as a key counter-regulator of autoimmune processes. Decreased IL-10 levels can therefore be associated with the impaired negative regulation of pro-inflammatory cytokines and lymphocyte activation, which leads to the perpetuation of autoimmune processes in APS.

Since we have evaluated the angiological parameters and endothelial dysfunction in patients with APS, we subsequently assessed whether the endothelial dysfunction showed association with peripheral immunological parameters. In our work we described a broad spectrum of T- and B-cell cytokines, to identify lymphocyte subpopulations and activated T cells and to evaluate growth factors and chemokines in patients with APS. We measured the circulating levels of IL-1, IL-4, IL-6, IL-10 and IFN-γ in patients with primary APS than compared the immunological parameters with the evaluated endothelial function parameters in these patients. In our longitudinal study we followed the effect of certain drugs on endothelial function and followed the changes of endothelial function and vascular stiffness parameters and its relationship with antiphopholipid antibody titer in case of two pregnant women with primary APS during their pregnancy.

Objectives

1. The aim of our study was to perform complex angiological examination in primary antiphospholipid syndrome, during in 44 patients with primary APS were performed the brachial artery flow-, and nitrate-mediated vasodilatation ang carotid artery intima-media thickness measured. In addition, the stiffness parameters such as the augmentation index and pulse wave velocity was also occurred. We were looking for a relationship between the stiffness parameters, endothelial function and carotid artery intima-media thickness.

2. After recording the initial angiological status in primary APS we started longitudinal study in order to follow the effect of certain drugs on endothelial function. Dividing our patients according to cardiovascular complications we controlled their angiological parameters per month than every third month. We had opportunity to follow the changes of endothelial function and vascular stiffness parameters and its relationship with antiphopholipid antibody titer in case of two pregnant women with primary APS during their pregnancy.

3. Parallel with angiological parameters immunological tests were performed in patients with APS in order to search for the relationship between the immunological and angiological alterations. We investigated the correlation between the immuno-inflammatory differences in the peripheral blood and endothelial dysfunction.

Patients and methods

I. Angiological measurments in antiphospholipid syndrome

Fourty-four (25 female and 19 male) consecutive patients with the diagnosis of primary APS according to the 2006 Myakis et al criteria were included. The thromboembolic classification was based on the Bick and Baker criteria. The mean age of the patients was: 52 ± 15 year (26-55). Venous or arterial vascular events occured in 25 and 19 patients, respectively and recurrent abortions as was the presenting symptom in one patient.

The gynecological history of 25 female patients with primary APS terms in 10 cases only successful pregnancy, in 7 cases healthy, successful pregnancies and abortions, in 3 cases only abortion, while 5 patients had no pregnancy earlier. Subjects with venous thrombosis (25 patients) received acenocumarol therapy alone (4 patients), aspirine alone (12 patients), or the combination of acenocumarol and aspirine (9 patients). Arterial thrombotic events included acute coronary syndrome (3 patients), stroke (5 patients) and peripherial arterial occlusive disease (11 patients). All of these patients were on statin, ACE inhibitor and aspirin; furthermore three of them were on acenocumarol and two on clopidogrel also. No vascular events occured within the last 3 months. The control group included 36 healthy females and males. The study population was assessed for major cardiovascular risk factors, including family history of coronary artery disease, smoking and diabetes mellitus. Blood glucose, cholesterol and triglyceride levels were determined.

We determined the flow-mediated and nitrate-mediated vasodilatation and carotid artery intima-media thickness in 44 patients with primary APS and von Willebrand factor antigen level determinations were made parallel with measurements. Detectable angiological differences in primary APS were compared with 36 healthy control subjects endothelial function parameters. Beside endothelial function parameters, the stiffness parameters were performed too.

II. Longitudinal studies

25 patients with primary antiphospholipid syndrome (16 women and 9 men) were included in prospective longitudinal study. The patients' mean age was 53 ± 10 years (28-50). They were followed by endothelial dysfunction and stiffness parameters for therapeutic suggestibility.

According to other associated cardiovascular complications they were divided into different treatment groups: statin (10 patients), ACE inhibitors (10 patients), beta-blockers (2 patients) and immunosuppressive (3 patients) receiving treatment. After the first detailed study we controlled their angiological parameters per month than every third month. We had opportunity to follow the changes of endothelial function and vascular stiffness parameters and its relationship with antiphopholipid antibody titer of two pregnant women with primary APS during their pregnancy. The young female patients were taking aspirin or acenokumarol according previous thrombotic manifestations but after the plan of pregnancy they were taking low molecular weight heparin. The dose of LMWH has been set by the level of anti Xa factor. The first patient was 24 year old, who had in her history deep vein thrombosis complicated with massive pulmonary embolism. The present investigation was her first pregnancy. The second, 28-year old gravida had two habitual abortion earlier.

III.Immunoinflammatory mechanisms and angiological alterations

Twenty-eight patients (12 males and 16 females), newly diagnosed with primary APS, were involved. In addition to the angiological investigations the immunological status of patients was also examined, the following parameters have been defined: T-helper (Th1/Th2), cytotoxic T-cell, activated CD4 + and CD8 + cells ratio, IL-4 and IL-10 expression rate, and soluble cytokine levels (IFN-γ, IL-4, IL-10, IL-6, IL-1 és IL-8). The measurements were performed with Coulter EPICS XL flow cytometer. The control group included 36 patients with stable coronary disease. The study population was assessed for major cardiovascular risk factors, including family history of coronary artery diseases, smoking and diabetes mellitus. Blood glucose, cholesterol and triglyceride levels were evaluated in each case. It seemed logical to choose stable coronary patients as control group because they were subjects in whom atherosclerosis is present. The measurments were made in Regional Laboratory of Immunology of 3rd Department of Internal Medicine.

1. Endothelium-dependent (flow-mediated; **FMD**) and endothelium independent (nitrate-mediated; **NMD) vasodilation**

Endothelium-dependent vasodilation was assessed with a 7. 5-MHz linear array transducer (Sonos 5500; Hewlett-Packard, Soma Technology Inc. CT, USA) by scanning the brachial artery in longitudinal section. Endothelial function testing was performed by the same person

(H. D.) and the evaluation was done offline by digital softver technique (AVITA, Gtech Information Systems, IL, USA). All subjects were refraining from smoking and eating for 8 hours, and without any medications for ≥12 hours before the exercise and endothelial function tests. To minimize mental stress, care was taken to make the patients as comfortable as possible, and the procedure was performed in a quiet air-conditioned room (22°C to 25°C). The right arm was stabilized with a cushion, and a sphygmomanometric cuff was placed on the forearm. A baseline image was acquired, and blood flow was estimated by time-averaging the pulsed Doppler velocity signals obtained from a mid-artery sample volume. Then the cuff was inflated to at least 50 Hgmm above systolic blood pressure for 5 minutes and released abruptly. Postocclusion diameters were obtained at 60 seconds after deflation. FMD (flow-mediated vasodilation) was calculated as the percent change in diameter compared to preocclusion values. A mid-artery pulsed Doppler signal was obtained immediately upon cuff release and not later than 15 seconds after cuff deflation to assess hyperemic velocity.

After at least 10 minutes another image was acquired to reflect the reestablished baseline conditions. Diameter measurements were taken at least 3 times at 3- to 4-minute intervals after 0.4 mg sublingual nitroglycerin administration. The maximal FMD and NMD diameters were determined based on the average of 3 consecutive diameter measurements as the percent change in diameter compared with baseline. Blood flow was calculated by multiplying the velocity time integral by the heart rate and the vessel cross-sectional area. The inter-observer analysis found the variability on 20 patients: 8.95 %. The intra-observer analysis was performed on 10 healthy individuals 3 times, with 30 minutes interval between the analyses. The intra-observer variability was: 4.6 %. We performed the variation coefficient for baseline diameter in 20 cases, it was: 0.86 %, so the accuracy of the method is appropriate according to the international recommendation.

2. Carotid Duplex Ultrasound Investigations; Measuring of carotid artery intima-media thickness (IMT)

Ultrasound examinations were performed immediately after blood sampling with a color-coded HP SONOS 5500 (Hewlett Packard Soma Technology Inc. CT, USA) carotid duplex equipment with a 7.5 MHz linear transducer. The investigation included longitudinal and transverse examinations of the carotid arteries. Measurements of (intima-media thickness)

IMT were performed at about 10 mm proximal to the carotid bulb or 20 mm proximal to the flow divider. IMT was measured as the distance between the leading edge of the first echogenic line (lumen-intima interface) and the second echogenic line (upper layer of the adventitia) in the far (deeper) artery wall. All measurements were performed at the end of the heart cycle, with the transducer was in the mediolateral direction. Offline analysis was performed by using digital video images (AVITA, Gtech Information Systems, IL, USA). 5-5 separate measurements were performed on both carotid arteries and we took the average of these measurements each side separately. When we evaluate the left and right IMT values we considered not the mean of the measurements, but the higher value for the assessment.

3. Assessment of AIx and PWV—stiffness parameters

Measurements were carried out by using a TensioClinic device (Tensiomed Corp., Budapest, Hungary). The measurement is based on the fact that the contraction of the heart causes pulse waves in the aorta. The first wave reflects on the aorta at the bifurcation; therefore, during systole the second wave is easily detectable and a late systolic peak appears. The second reflected wave depends on the stiffness of the large artery, the time (RT S_{35}) spent and the peripheral resistance-dependent amplitude. AIx can be calculated by the amplitude of the reflected and the first wave, which is the pressure difference between the late-systolic peak pressure and the early-systolic peak pressure divided by the late-systolic peak pressure. The TensioClinic arteriograph can assess this parameter from the oscillometric data obtained from the 35 mmHg suprasystolic pressure of the brachial artery

$$\text{AIx } (\%) = (P2 - P1)/PP \times 100$$

PWV

PWV is the quotient between the jugular fossa and symphisis distance and the reflection time (RT S_{35} is the reflection time at 35 mmHg suprasystolic pressure). A *jugular fossa–symphysis* distance is anatomically identical with the distance between the aortic trunk and the bifurcation. In order to have reproducible results, the patients had to rest at least for 5 min before the measurement, and also the investigation room was completely quiet without any disturbing effects

4. Von Willebrand factor antigen level

Blood samples were collected from 44 patients with primary antiphospholipid syndrome and from 36 healthy individuals into Vacutainer (Becton Dickinson, Rutherford, NJ) tubes containing 0, 105 M sodium citrate as anticoagulant (blood-anticoagulant ratio: 9:1) Samples were centrifuged at 1500 g, 22 °C for 20 min and plasma was used. The von Willebrand factor antigen (VWF: Ag) level was measured by STA Liatest VWF (Diagnostica Stago, Asnieres, France) immunoturbidimetric assay using microlatex particles coated with policlonal rabbit anti-human VWF antibodies. After mixing the reagent with plasma, the degree of agglutination was proportional to the amount of VWF present in the sample. The reference range was 50-160 %. The intraassay CV (coefficient of variability) of the STA diatest vWf assay was 1.9 % and 2.7 % in normal and pathologic range, respectively, and the interassay CV was 2.9 % and 4.5 %.

5. Identification of lymphocyte subpopulations and activated T cells

In order to determine lymphocyte subpopulations (T, Th, T cytotoxic, B, NK and NKT cells) and activated T cells isolated from heparinized blood samples, monoclonal antibodies against cell surface markers CD3, CD4, CD8, CD19 and CD56 (BD Biosciences, San Diego, CA, USA and Immunotech, Marseille, France) were used. The expression of T-lymphocyte activation markers such as HLA-DR and CD69 were also determined on CD3+ cells (BD Biosciences). Samples were processed according to the Coulter Q-PREP protocol and system. Measurements were performed and events were collected on Coulter EPICS XL flow cytometer (Beckman Coulter Inc., Miami, FL, USA). Lymphocytes, monocytes and granulocytes were separated based upon their size and granulation pattern. Lymphocyte subpopulations were quantified as their percentage in the entire population.

6. Detection of intracytoplasmic cytokines

CD4+ and CD8+ cells isolated from heparinized whole blood were used for intracytoplasmic cytokine measurements, as previously described. Since the cytokine content of resting lymphocytes is very difficult to detect, prior to the labelling process, cells were stimulated using 25 ng/ml phorbol myristate acetate (Sigma Aldrich Corp., St Louis, MO, USA) and 1 ng/ml ionomycine (Sigma Aldrich Corp.) for 4 h at 37°C and 5% CO_2. Excretion of *de novo* synthesized cytokines from the Golgi apparatus were inhibited by 10 µg/ml brefeldin-A

(Sigma Aldrich Corp.). Subsequently, cell surface labelling of CD4 and CD8 antigens was performed using quantum-red conjugated specific monoclonal anti-CD4 and anti-CD8 antibodies (Sigma Aldrich Corp.) at room temperature for 30 min. Red blood cells were eliminated by using FACS Lysis Solution (BD Biosciences), then cell membrane of leucocytes were permeabilized with Permeabilizing Solution (BD Biosciences), for 10 min each, at room temperature in dark. This was followed by washing and labelling of intracytoplasmic cytokines with specific monoclonal antibodies: FITC-labelled anti-human-IFN-γ, phycoerythrin-conjugated anti-human-IL-4 (BD Biosciences), PE-conjugated anti-human-IL-10 (Caltag Laboratories, Burlingame, CA, USA) for 30 min at room temperature in dark. The cells were then fixed with 1% paraformaldehyde solution as the last step of the labelling protocol. Samples were immediately evaluated by flow cytometry. Lymphocytes, granulocytes and monocytes were gated and separated based on their morphological properties.

7. Determination of serum soluble cytokines

Serum IL-4, IL-10 and IFN-γ was measured by corresponding BD OptEIA ELISA kits (BD Biosciences) according to the manufacturer's instructions.

8. Statistical Analysis

Statistica for Windows, version 6.0 (StatSoft), was used for data analysis. We presented mean values ± SD (standard deviation) of the angiological parameters. Normality of continuous variables was checked by the Kolmogorov-Smirnov and Liliefors test. In case of normal distribution, T-test and one-way and breakdown ANOVA were used. In case of nonnormal distribution, the Mann-Whitney *U* test and the Kruskal-Wallis ANOVA were used. P values less than 0.05 were considered statistically significant.

Results

I. **Angiological measurments in antiphospholipid syndrome**

Regarding cardiovascular risk factors (age, systolic- and diastolic blood pressure, plasma level of total cholesterol, HDL-cholesterol, LDL-cholesterol and triglicerid, smoking habits, body mass index), there was no significant difference between the two groups. To asses endothelial function, brachial artery flow-mediated dilation was measured by high resolution ultrasonography. FMD (flow-mediated vasodilation) in patients with primary APS was significantly lower than that of controls (3.43 ± 2.86% vs. 7.96 ± 3.57%; $p<0.0001$). However, no significant diffrence was found in NMD (nitrate-mediated vasodilation) results between the two groups (13.71 ± 6.98% vs. 12.63 ± 9.88%).To reveal any diffrences in endothelial impairment between the arterial and venous thrombosis group, FMD was calculated separately in patients with arterial or venous thrombotic events. Our results show that FMD in patients either with arterial (19 patients) or venous involvement (25 patients) was significantly lower than in the control group. (3.71 ± 2.77% and 3.21 ± 2.96% vs. 7.96 ± 3.57, respectively). Moreover, FMD data did not differ between the two groups of patients. Carotid artery IMT was significantly higher in primary APS group than in controls (0.74 ± 0.2 mm vs. 0.54 ± 0.085 mm; $p=0.0037$). There were no plaques in the control group. We found plaques in carotid artery of 23 patients. None of them casued significant stenosis. We found between flow-mediated vasodilation and carotid artery intima-media thickness negativ linear correlation ($R= -0.4725$, $p=0.002$) in patients with APS. When analyzing the arterial or venous thrombosis group separately, we found that in the subgroup of primary APS with venous involvement the carotid artery intima-media thickness (IMT) was significantly higher than in controls, similarly to the subgroup of primary APS with arterial involvement (0.7 ± 0.22 mm and 0.72 ± 0.18 mm vs. 0.54 ± 0.085 mm; $p=0.044$, $p=0.0074$). There was no difference between the two subgroups. To asses endothelium activation and/or damage, vWF antigen levels were determined in the plasma of primary APS patients and controls. The von Willebrand factor antigen level was significantly higher in patients with primary APS than in the control group (157.91 ± 52.5% vs. 125.87 ± 32.8%; $p=0.012$) reflecting endothelial injury.

II. Longitudinal studies in primary antiphopholipid syndrome

Examing the effect of drugs on endothelial function, in the therapeutic subgroup, where ACE inhibitors were introduced, FMD from 2.6% to 4.7% , AIx from 3.2% to -18.1% improved, whereas in statin group from 3.8% to 7% over. In this subgroup showed no significant improvement in AIx, but already the starting value was in the normal range.
We had opportunity to follow the changes of endothelial function and vascular stiffness parameters and its relationship with antiphopholipid antibody titer of two pregnant women with primary APS during their pregnancy.

Two pregnant women with primary APS were followed before-, during their pregnancy ang during the first 6 weeks of the maternity period. The first gravida was 24 year old who has in a history of deep vein thrombosis complicated with pulmonary embolism, have taken before her pregnancy aspirin and acenocoumarol, but after the plan of pregnancy was taken LMWH. During pregnancy, she showed aCL and B2GPI positivity (CL: 14.1 U / ml; B2GPI: 8.1 U / ml), while LA negativity. The FMD value was in the normal range (9.24% - 10.77%) but markedly increased the titre of antibodies (CL: 59.61 U / ml; B2GPI: 42.56 U / ml) simultaneously with LA positivity and also vasculitis developed. In parallel with a significant decline in FMD (3.2%) was detectable the symtoms of preeclampsia, which was decided to terminate the pregnancy. In the maternal stage she was taken high doses of steroids, which moderated the clinical symptoms and antibody titers showed a downward trend to. The abnormal value of FMD normalized to 8.7% within a few weeks. Subsequently, the dose of steroids was gradually reducing – then we left. The second, 28-year old gravida had two habitual abortion earliar. She was during pregnancy consistently anti CL positive (9.1 U / ml). The parameters of endothelial function were always in normal range (8.36% - 10.94%). In the last trimester was appearing B2GPI antibody positivity, in addition to aCL titer increasing was observed, whereas in parallel with this laboratory alterations FMD decreased significantly. She had no other clinical symptoms, so we decided to just close up. The uncomplicated birth was on the 39th week.

III. Immunological parameters in primary antiphospholipid syndrome

III/1. T-cells in primary APS

In order to characterize the immune status of our patients, we assessed the following parameters: number and percentage of T, Th (T-helper), Tc (cytotoxic T cell), NKT (natural killer T-cell), B cells and NK (natural killer) cells, on CD3+ cells, markers of activation (HLA-DR and CD69), intracellular IFN-γ, IL-4 and IL-10 expression of *in vitro* activated CD4+ and CD8+ cells, and soluble IL-1, IL-4, IL-6, IL-8, IL-10 and IFN-γ cytokine levels.

Tcells, T-cell subsets, Bcells

Patients with APS vs controls

We found significant differences in peripheral blood double positive CD4+IL10+ (17.63 ± 12.97% *vs* 4.32 ± 5.83%) and double positive CD8+IL10+ (17.9 ± 10.76% *vs* 6.44 ± 17.24%) cell percentages in APS patients when compared with the control group. The phenotyping of the basic cellular composition of APS patients and patients with stable coronary disease failed to show significant differences in peripheral CD3+, CD4+, CD8+, CD19+, CD56+ and CD3+CD56+ cell percentages.

Amongst activated T cells, unlike peripheral CD3+/HLADR+ cells, CD3+/CD69+ cells were represented in increased percentages in APS patients when compared with those found in controls (10.4 ± 7.73% *vs* 12.23 ± 10.93%, $P = 0.63$ and 1.66 ± 1.61% *vs* 1.16 ± 1.01%, $P = 0.27$, respectively).

APS patients with venous vs arterial manifestations

Comparing the cell percentages of CD4+IL10+ and CD8+IL10+ T cells in the peripheral blood of APS patients with venous *vs* arterial manifestations we did not find significant differences between the two subgroups, yet double positive CD4+ (14.61 ± 13.42 and 19.31 ± 12.39 *vs* 4.32 ± 5.83, $P = 0.0014$ and $P < 0.0001$, respectively) and CD8+ (17.82 ± 12.42 and 17.82 ± 10.56 *vs* 6.44 ± 7.24, $P = 0.0014$ and $P < 0.001$, respectively) cell percentages were significantly higher in patients with venous and arterial manifestation compared with patients with stable coronary disease.

III/2. Assessment of Th0/Th1/Th2 cells by intracellular cytokine profiling

Patients with APS vs controls

When analysing the fine functional structure of the immune system of patients with APS, we found that Th 0 (CD4+/IFN𝛾+/IL4+) and T cytotoxic 0 (CD8+/IFN𝛾+/IL4+) cell percentages were significantly decreased in patients with APS compared with patients with stable coronary disease (0.54 ± 0.85% *vs* 1.4 ± 1.68%, $P = 0.008$ and 0.49 ± 0.47% *vs* 1.46 ± 2.08%, $P = 0.01$). At the same time, Th1 and Th2 cell percentages did not differ significantly between these groups (22.78 ± 11.65% *vs* 24.98 ± 9.48% and 0.31 ± 0.35% *vs* 0.45 ± 2.08%).

APS patients with venous vs arterial manifestations

The percentage of Th0, Tc0, Th1, Th2 cells in APS patients with venous *vs* arterial manifestations was found comparable between the two subgroups.

APS patients with venous or arterial manifestation vs patients with stable coronary disease

The comparison of Th0, Tc0, Th1 and Th2 cell proportions in the disease subsets showed that the percentages of Th 0 (CD4+/IFN𝛾+/IL4+) and T cytotoxic 0 (CD8+/IFN𝛾+/IL4) cells were significantly decreased in patients with venous and arterial manifestations compared with patients with stable coronary disease (0.29 ± 0.27% and 0.72 ± 1.05% *vs* 1.4 ± 1.68%, $P = 0.01$) and (0.5 ± 0.58% and 0.46 ± 0.39% *vs* 1.46 ± 2.08%, $P = 0.039$ and 0.031, respectively).

III/3. Circulating cytokines

Patients with APS vs controls

Of serum-soluble cytokines, IFN-𝛾 was present in higher concentrations in the sera of APS patients compared with patients with stable coronary artery disease (99.89 ± 243.98 pg/ml *vs* 31.03 ± 69.05 pg/ml). Serum IL-1 levels also presented in higher concentrations in APS patients (9.18 ± 20.2 pg/ml *vs* 4.89 ± 10.93 pg/ml). Interestingly, IL-4 and IL-6 were found to be present in significantly higher titres in APS patients (31.46 ± 60.69 pg/ml *vs* 1.4 ± 3.23 pg/ml, $P = 0.015$ and 24.76 ± 13.94 pg/ml *vs* 10.23 ± 11.97 pg/ml, $P < 0.05$). Serum IL-10 and IL-8 levels were decreased in patients with APS compared with stable coronary patients (8.18 ± 10.21 pg/ml *vs* 27.03 ± 70.18 pg/ml and 79.28 ± 37.7 pg/ml *vs* 128.65 ± 241.5 pg/ml)

APS patients with venous vs arterial manifestations compared with patients with stable coronary artery disease

The concentration of circulating cytokines in APS patients with venous or arterial manifestations did not differ significantly. Interestingly, serum IL-1 and IL-4 levels were significantly increased in APS patients with arterial manifestations compared with patients with stable coronary artery diseases (18.36 ± 27.73 pg/ml *vs* 4.89 ± 10.93 pg/ml, $P < 0.05$ and 48.71 ± 80.05 pg/ml *vs* 1.4 ± 3.23 pg/ml, $P = 0.031$)

III/4. Association between angiological parameters and the immune status

The evaluation of the relationship between the angiological parameters and immune status of patients with primary APS showed that carotid artery IMT, AIx and PWV had strong positive linear correlation with serum levels of IL-4 ($R = 0.7$ and $P = 0.015$; $R = 0.7$ and $P = 0.015$; $R = 0.899$ and $P < 0.001$, respectively). An interesting connection was detectable between PWV and CD8+IL10+ and CD8+IL10– cell percentages. We found significant negative linear correlation between PWV and CD8+IL10+ cell percentages ($R = –0.395$ and $P = 0.045$) and significant positive linear correlation between PWV and CD8+IL10– cell percentage

IV. Comparing arterial stiffness, flow-mediated vasodilation of the brachial artery and the thickness of the carotid artery intima-media in patients with APS

Investigate the connection between endothelial function and stiffness parameters FMD showed strong negative linear correlation wit augmentation index (FMD–AIx: R= -0,594, p<0,001) and with pulse wave velocity (FMD–PWV: R= -0,655, p= 0,0002). Intima-media thickness of carotid artery correlated with stiffness parameters positively (IMT–AIx: R= 0,59, p=0,0012; IMT–PWV: R= 0,6 és p < 0,001).

Discussion

Besides traditional risk factors in pathogenesis of atherosclerosis, non-traditional, immune-inflammatory risk factors come to the front, and the recognition and therapeutic intervention of these factors have important prospectives in the prevention of atherothrombotic events. Patients with certain autoimmune diseases, such as SLE, APS or RA are characterized to have accelerated atherosclerosis, have a high risk for atherosclerotic cardiovascular events. Accelerated atherosclerosis in APS is a clear example of an atherothrombotic event, caused by a non-traditional risk factor. Antiphospholipid antibodies were found to play a role in the pathomechanism of atherosclerosis in several animal models, and these data were in accordance with human findings. Anti-β2GPI antibodies have been shown to activate endothelial cells by inducing prothrombotic and proinflammatory phenotype characterized by the upregulation of adhesion molecules, cytokines and tissue factors. Antiphospholipid antibodies contribute to atherosclerotic processes, and play a pivotal role in the pathogenesis. Antiphospholipid-mediated endothelium perturbation has a crucial role in APS-associated vasculopathy. Cronic endothelial dysfunction predisposes to organic damage of the vascular wall that, in a preclinical stage, before overt disease, can be detectable by ultrasound of the carotid intimal-medial thickness (IMT). Numerous studies provided evidence of increased carotid IMT in APS. Antiphopholipid syndrome is an autoantibody-mediated acquired thrombohilic state. Pathological autoantibodies, mainly anti-B2GPI has anti-endothelial activity. By binding to the endothelial cells it shifts the endotelial cells towards a prothrombotic and proinflammatory state. These functional changes can be detected with sensitive angiological methods, which are already present in the current diagnostic repertoire.

The aim of the study was therefore to model the immune-inflammatory changes mediated by cytokines in primary APS, in parallel with the assessment of angiological parameters, reflecting the endothelial dysfunction. Our results demonstrate endothelial injury and impaired endothelial-dependent vasodilation in correlation with increased IMT in these patients. According to our findings several other investigations showed that carotid artery IMT is increased in patients with primary APS than in controls, supporting an atherogenic role of antiphospholipid autoantibodies in APS. We found elevated vWF levels in patients with primary APS patients, suggesting that endothelial injuries caused by antiphospholipid antibodies. Examing the effect of drugs on endothelial function in patients with primary APS

significant improvement was detectable in endothelial function and in augmentation index in therapeutic subgroup where ACE inhibitors were introduced and following the changes of endothelial function and vascular stiffness parameters and its relationship with antiphopholipid antibody titer of two pregnant women with primary APS during their pregnancy we could detect a significant increase in antibody titer in in both cases parallel with the deterioration of endothelial function.

We gained new information on primary APS, which aids in the better understanding of the pathomechanism, on the other hand helps to identify angiological disturbances behind the clinical symptoms. We could verify T-cell activation signified by a predominant Th2 response. With the aid of the parallel functional and morphological assessment, we found abnormal arterial elasticity, signifying endothelial dysfunction, pathological arterial stiffness, characteristic to early atherosclerosis, and the early sign of atherosclerosis, the increment in carotid IMT. We found a correlation between the immuno-inflammatory and angiological parameters, soluble IL4 and carotid IMT, as well as pulse wave velocity (PWV) and augmentation index. We found a similar association between the percentages of CD8+ cells and PWV; also within CD8+ cells, we could identify regulatory intracellular IL10+ cells. Our results could be important in the development of biological therapies in the future.

New Results

1. We examined the endothelial function parameters in 44 patients with primary antiphospholipid syndrome, and detected as a new result the damage the brachial artery flow-mediated vasodilation and the abnormally increased carotid artery intima-media thickness. The relationship in terms of the angiological parameters we demonstrated negative linear correlation between flow-mediated vasodilatation and carotid IMT.

2. In parallel angiological studies vonWillebrand factor antigen levels were determined, and significantly higher level demonstrated in primary APS.

3. Examing the effect of drugs on endothelial function in 25 patients with primary APS significant improvement was detectable in endothelial function and in augmentation index in therapeutic subgroup where ACE inhibitors were introduced.

4. We followed the changes of endothelial function and vascular stiffness parameters and its relationship with antiphopholipid antibody titer of two pregnant women with primary APS during their pregnancy we could detect a significant increase in antibody titer in in both cases parallel with the deterioration of endothelial function.

5. Examing immunological features in primary antiphospholipid syndrome, significantly higher CD4 + / IL10 + and CD8 + / IL10 + cell ratio were detected. CD4 positive and CD8 positive cell ratio is significantly higher in early, not yet requiring stage too and it indicates increased immuno-inflammatory activity in primary antiphospholipid syndrome. From circulating cytokines IL-4 and IL-6 levels were significantly higher in APS compared stable coronary disease while IL-1 was significantly elevated in arterial APS.

6. We firstly compared in primary APS the observed angiological alterations with immunological variations. We found the strong positive linear correlation of carotid IMT, AIx and PWV with serum IL-4. Remarkable the negative relationship between

CD8 + / IL10 + cell ratio and a positive linear correlation of CD8 + / IL10-cell ratio and pulse wave velocity.

7. Examining the relationship between endothelial function and stiffness parameters in primary APS, we found strong negative linear correlation between FMD and augmentation index and pulse wave velocity. FMD as indicator of endothelial dysfunction ang definitive vascular damage signaling carotid IMT showed a positive linear relationship with stiffness parameters.

LIST OF PUBLICATION

Scientific publications of the thesis:

1. **Dér Henrietta,** Kerekes György, Veres Katalin, Szomják Edit, Soltész Pál.
   Arteria brachialis flow-mediált vasodilatáció, carotis intima-média vastagság és augmentációs index (AIx) összehasonlító vizsgálata.
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3. Henrietta Der*, Pal Soltesz*, Katalin Veres, Renata Laczik, Sandor Sipka, Gyula Szegedi, Peter Szodoray.
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   * egyenrangú elsőszerzők

4. Pal Soltesz, **Henrietta Dér,** Gyorgy Kerekes, Peter Szodoray, Gabriella Szucs, Katalin Danko, Yehuda Shoenfeld, Gyula Szegedi, Zoltan Szekanecz.
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   IF: 1, 459

Other publications:

1. Veres K., Szomják E., Kerekes Gy., **Dér H**., Szerdahelyi Sz., Tumpek J., Soltész P. **Foszfolipid ellenes antitestek alsó végtagi perifériás érbetegekben.** Érbetegségek 2006, 1: 21-26.

2. Szomják Edit, **Dér Henrietta**, Veres Katalin, Kerekes György, Tóth Judit, Ifj. Péter Mózes, Bakó Gyula, Soltész Pál. **Perifériás érbetegeink klinikai adatainak elemzése, különös tekintettel a kor- és nembeli sajátosságokra, valamint az intervenciós radiológiai beavatkozások késői eredményeire** Magyar Belorvosi Archívum 2006, 59: 260-266.

3. P. Szodoray, O.Timar, **H. Der**, E: Szomjak, G. Lakos, M. Aleksza, B. Nakken, G. Szegedi, P. Soltesz. **Th1/Th2 Imbalance, Measured by Circulating and Intracytoplasmic Inflammatory cytokines – Immunological Alterations in Acute Coronary Syndrome and Stable Coronary artery Disease** Scandinavian Journal of Immunology 2006, 64: 336-344. **IF: 1,912**

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5. Soltész Pál, Prohászka Zoltán, Füst György, **Dér Henrietta**, Kerekes György, Szodoray Péter, Zeher Margit, és Szekanecz Zoltán **Vasculopathiák autoimmun vonatkozásai.** Orv. Hetilap 2007, 148: 53-57.

6. Gabriella Szűcs, Orsolya Tímár, Zoltán Szekanecz, **Henriett Dér**, György Kerekes, Szilvia Szamosi, Yehuda Shoenfeld, Gyula Szegedi, Pál Soltész.

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7. Szekanecz Z, Kerekes G, **Dér H**, Sándor Z, Szabó Z, Végvári A, Simkovics E, Soós L, Szentpétery A, Besenyei T, Szücs G, Szántó S, Tamási L, Szegedi G, Shoenfeld Y, Soltész P.

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9. Soltész P, Veres K, Szomják E, Kerekes G**, Dér H,** Sándor Z, Dezso B, Dévényi K, Szekanecz Z.

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10. Kerekes G, Szekanecz Z, **Dér H,** Sándor Z, Lakos G, Muszbek L, Csipö I, Sipka S, Seres I, Paragh G, Kappelmayer J, Szomják E, Veres K, Szegedi G, Shoenfeld Y, Soltész P.

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Scientometry

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