Manifestations and investigation of functional vascular diseases in children

PhD Thesis

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ABBREVIATIONS

ACA: Anti Centromer Antibody

ANA: AntiNuclear Antibody

APL: AntiPhosphoLipid antibody

AUC: Area Under the Curve

BMI: Body Mass Index

CAS: Cold Agglutinin Syndrome

CTD: Connective Tissue Disease

ENA: Extractable Nuclear Antigen

HR-CT: High-Resolution Computed Tomography

LDF: Laser Doppler Flowmetry

LASCA: LAser Speckle Contrast Analysis

NDC: Non Differentiated Collagenosis

NFC: Nailfold capillaroscopy

PACAP: Pituitary Adenylate Cyclase Activating Polypeptide

RP: Raynaud Phenomenon

ROIs: Regions Of Interests

RS: Raynaud Syndrome

SOM: SOMatostatin

SSc: Systemic Sclerosis

TSH: Thyroid Stimulating Hormone

INTRODUCTION

Raynaud's phenomenon, acrocyanosis and erythromelalgia are functional vascular diseases that differ with respect to clinical signs, prevalence, pathogenesis, therapy and prognosis. The most common form is Raynaud phenomenon.

I. Raynaud phenomenon

Raynaud phenomenon is a transient vasospasm of peripheral arteries and arterioles that classically results in triphasic discoloration of the affected region. The symmetrical triphasic colour pattern is not typical in children. The fingers are the most commonly affected region. It may be associated with sensation of numbness and insensitivity (80%), pain (50%) and prolonged wound healing. The phenomenon is typically triggered by cold exposure or stress. In the primary form the age at onset is the adolescent period, the secondary form can manifest later. It is 3-4 times more common in women. It is classified into primary and secondary RP. Primary Raynaud is more common in which patients are seronegative for immunserological test and have normal nailfold capillaroscopy. Secondary RP refers to the presence of the disorder in association with a related illness, mostly connective tissue disorders. Although, the prognosis of Raynaud phenomenon is good, 5-20 % of patients might develop connective tissue disease. The pathophysiology of Raynaud phenomenon is not completely understood. The mechanism behind the vasospasm might include both central and peripheral neuronal mechanisms, endothelial damage, abnormal immune reactivity and intravascular causes. In primary Raynaud phenomenon only a functional abnormality can be detected, in secondary Raynaud phenomenon structural changes are involved. The diagnosis is based on medical history, physical examination, laboratory tests and nailfold capillaroscopy.

II. Acrocyanosis

The disorder is characterized by symmetrical persistent blue discoloration, most commonly affecting the hands, but rarely feet and face are involved. It can occur often in adolescents and in newborns, too. It is aggravated by cold exposure. The disease is classified into primary form (without related disorders) and secondary form. Secondary acrocyanosis is a manifestation of other disorders, such as systemic autoimmune diseases, hematologic disorders (cold agglutinin syndrome, essential thrombocythemia, paroxysmal nocturnal hemoglobinuria), drug exposures. The diagnosis is based on clinical signs. Specific laboratory abnormalities can only be observed in the secondary forms.

III. Erythromelalgia

The disease is presented in the 5th decade, it is rare in children. It is characterized by warm, red, painful discoloration of the palmar and plantar regions. It is triggered by warm temperature, physical activity, alcohol and caffeine abuse. The painful discoloration is improved with cold exposure and raising of the limbs. It is classified into two forms. There is no other disorder underlying the primary form. The secondary form is associated with myeloproliferative disorders (polcythemia vera, essential thrombocytosis, myelofibrosis), drug exposure (bromocriptine, calcium channel blockers), systemic autoimmune disorders (SLE, rheumatoid arthritis), infection. The diagnosis is based on physical examination.

Methods

Skin microcirculation can be examined by both non-invasive and invasive techniques, such as biopsies or intradermal delivery of drugs.

From 1970s laser Doppler flowmetry has been used as a sensitive, non-invasive method for the measurement of tissue perfusion. As functional tests, warm and cold stimulation test, occlusion test are the most applied procedures. Warm-induced blood flow measurement is more sensitive and specific to determine the severity of the disease and the prognosis. The disadvantage of the sensitive method is the difficult reproducibility and other factors, such as skin thickness, movement artefacts that influence the result.

To reduce the above mentioned factors, from 1990s laser speckle contrast analysis (LASCA) method was introduced. The technique is based on speckle contrast analysis. It allows real-time cutaneous perfusion imaging of larger body parts in several regions of interests simultaneously and objective measurement of reactive changes. Comparing with other non-invasive blood flow measurements, it allows better temporal and spatial measurement of the perfusion.

The pathophysiology of Raynaud phenomenon is not completely understood, there is an imbalance between vasoconstrictor and vasodilatative process. In these thesis, two sensory neuropeptides, somatostatin and PACAP-38 were analysed. One aim of our investigation was to examine the relationship between these neuropeptide and Raynaud syndrome.

Statistical analysis

Data management of our patient with Raynaud phenomenon and analysis were performed with descriptive statistic methods (median, minimum, maximum). For higher level of analysis χ^2-test, Z-test, Bonferroni correction and Mann-Whitney U test were used with SPSS 24.0 statistical program.

Microcirculation data management and analysis were performed using GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA). Values were expressed as means ± SEM in all the 3 groups of subjects. The normal distribution of investigated parameters was confirmed by D'Agostino-Pearson and Shapiro-Wilk normality tests. In case of normal distribution, values for each group were compared by one-way analysis of variance (ANOVA) followed by Newman-Keuls multiple comparison test. Otherwise, the Kruskal-Wallis test followed by Dunn's multiple comparison test was used for statistical analysis $p<0.05$ was considered as statistically significant. Correlation coefficients between selected clinical and microcirculation parameters were also analysed.

AIM OF THE STUDY

Since there are few comprehensive Raynaud studies in the pediatric population, our aims were the following:

I. Retrospective analysis of the clinical characteristics, laboratory parameters and nailfold capillaroscopy results of the patients investigated in the Department of Paediatrics of the Clinical Centre of the University of Pécs.
II. To compare the vascular responsiveness of healthy controls and adolescents with primary Raynaud phenomenon with a non-invasive functional method and to investigate the role of sensory neuropeptides, such as PACAP and somatostatin.
III. The measurement of microcirculation in a patient diagnosed with acrocyanosis with LAser Speckle Contrast Analysis (LASCA) method to demonstrate the severely impaired perfusion and to monitor the efficacy of the treatment.

RESULTS

I. study: Retrospective analysis of patients with Raynaud phenomenon in the Department of Paediatrics of the Clinical Centre of the University of Pécs.

We retrospective analysed the collected data of 151 children who visited our immunological outpatient consultation with Raynaud Phenomenon from January 2010 to January 2017.

Demographical and clinical signs

76.2 % (115/151) of the children were diagnosed with primary RP, 23.8 % (36/151) carried the diagnosis of secondary RP. 73.5 % were female. The gender was not significantly different between primary and secondary cases ($p>0.05$). The mean age at onset of phenomenon was 11.97 years (range 9-15), the duration until the diagnosis was 2.16 years (0.3-10). In 32 families (21.2%), there were autoimmune diseases or Raynaud phenomenon among the first–degree relatives of the adolescents. The above mentioned parameters were not significantly different between the primary and the secondary forms ($p>0.05$). The pattern of colour changes was based on the medical history and the physical examination. Biphasic (white→red, blue→red) colour change was observed in 56.3 % cases (85/151), 39.7 % (60/151) of the patients had triphasic colour change, while monophasic colour change appeared in 4 % of children. The pattern of colour change was not significantly different between the primary and the secondary group ($p>0.05$). Regarding the triggering factors, exposure to cold was the most common (138/151, 91.4%), while stress was the second most common reason (13/151, 8.6%). The symptoms localized in 80.8 % (122/151) to the upper extremities, both extremities were affected in 19.2 % (29/151) of RP patients.

72.8 % (110/151) of adolescents suffered from pain, 59.6 % (90/151) of the patient detected insensitivity of the fingers. Pain was 4.5 times (OR=4.5 95%, CI:1.5-13.5), insensitivity was 2.6 times (OR=2.6 95%,CI:1.2-5.9) more often observed in patient with secondary RP (OR=4.5 95%, CI:1.5-13.5) compared with the primary group. Delayed wound healing was detected in 13.2 % (20/151) of the patients, it was not significantly different between the primary and the secondary cases ($p>0.05$). Migraine was the most frequent (29.1%, 44/151) accompanying disease of RP patients. Migraine was 3 times (OR=2.916 95%, CI:1.332-6.385) more often observed in patients with secondary RP, but there was no significant difference between the primary and the secondary groups ($p>0.05$). Livedo reticularis was detected in 9.9% (15/151) of the children, more often in the secondary group (16.7 vs 7.8 %), it was not significantly different between the primary and the secondary cases. Sclerodactyly could be observed in 4% (6/151) of the patients. 11.9 % of the children had asthenic habitus, it was more common in secondary Raynaud group.

Diagnostic tests

Routine laboratory tests were all in reference ranges, slightly elevated sedimentation rate was noted transiently in six patients (4%). Thyroid stimulating hormone (TSH) were abnormal in six patients (4%) among which autoimmune thyreoiditis developed later in four cases. The most often positive immune serological test (9.4 % , 14/151) was ANA. 4% of the patients were positive for antiphospholipid antibodies. ENA test were positive in 2.6% (4/180) of the children. ACA was positive in just one patient (0.7 %) without any other clinical sign.

Normal nailfold capillaroscopic pattern was detected in 6 % (6/151) of the adolescents. Non-specific abnormalities were observed in 78.1% (118/151) of patients, it was significantly more common in

primary RP group (94% vs 29.7%, p< 0.01). Pre-scleroderma pattern was noted in 17.9% (27/151) of the adolescents.

Respiratory system involvement was manifested as reduced DLco in 6.6 % (10/151) of the children without pulmonary symptoms. Lung fibrosis or alveolitis were not revealed by HRCT. Cardiac and kidney involvement were not observed. 2.7 % of the patients have gastroesophageal reflux which was not significantly different between primary and secondary cases (p>0.05). The most common associated disorder was allergic rhinitis (14.6%, 22/151). Because of anxiety, 8.6 % (13/151) of the adolescents were attending psychological therapy, mostly patients with secondary RP (13,9% vs75), but there was no significant difference between the primary and the secondary group. The most common associated autoimmune disorders were autoimmune thyreoiditis (6/151) and coeliac disease (3/151), one patient was treated for IBD and and one for psoriasis.

After the age of 18, the patient were followed up in the Department of Rheumatology and Immunology, Medical School of Pécs. In our study 83 adolescents could be followed up. The follow-up period was 28.86 month (0.2-113). No switch occurred from primary Raynaud to secondary form. Two patients were diagnosed with Non-differentiated collagenosis. The colour changes were detected in both extremities, and livedo reticularis was observed. Raynaud colour changes were associated with pronounced pain and numbness. Gastroesophageal reflux was detected. DLco was reduced, but pulmonary structural abnormality was not revealed by HR-CT. There was no sign of any other organ involvement. Systemic autoimmune disease was not developed.

Beyond the lifestyle advice, 64 children got medical treatment. Pentoxyphylline oral medication was used in 10 (6.6%), intravenous in 54 (35.8%) cases. Adolescents with secondary Raynaud had received medical treatment more often (52.8% vs 30.4%), but it was not significantly different between the two groups.

II. study: Functional vascular responsiveness in adolescents with primary Raynaud phenomenon

The aim of the present study was to investigate cutaneous microcirculatory alterations in primary RP adolescents and analyse the heat-induced microvascular responsiveness in relation to the clinical symptoms. Fifty-two adolescents were enrolled in this study at the Department of Paediatrics of the Clinical Centre of the University of Pécs, Hungary in March and April of 2015.

Measurement protocol

After a thorough physical examination, body composition analysis was done with Tanita BC 420 MA Body Composition Analyser. Five minutes later, heart rate and blood pressure were measured.

Venous blood samples were taken for routine laboratory tests and immunoserological tests and samples were also collected for measuring vasoactive sensory neuropeptides.

Microcirculation on the left index finger was measured with the Periflux 5000 system (Perimed AB, Stockholm, Sweden) laser Doppler technology. A thermostatic Laser Doppler probe (Probe 457) was placed on the distal phalanx of the second finger of the left hand. Data from the laser Doppler flowmeter were interfaced to a computer, the perfusion measured in arbitrary perfusion unit (PU) and the kinetics of the response was determined by the time. Baseline mean temperature was maintained at 32 °C and blood flow recorded over 5 minutes. The laser probe was heated to 42 °C for 10 minutes and then 44 °C for a period of maximum 5 minutes.

During the investigation the above mentioned parameters were measured:

- heat-induced hyperaemia expressed as the area under the response curve (AUC)

- time to peak response (the time to attain maximal cutaneous perfusion in seconds)
- peak perfusion value
- heat-induced percentage increase.

The cooling test could not be performed because of the symptoms of RP patients.

Results

Clinical parameters

Patients with Raynaud symptoms were divided into two groups according to the symptoms existing on the day of the study or in the past 2 months. Four of the participants included in the control group were diagnosed with primary RP and were therefore moved to the group of Raynaud patients without current symptoms.

Most patients except one in each group among the Raynaud patients were girls. The mean duration of primary RP was 23.2±4.9 months in the group of Raynaud without symptoms and 38.4±6.9 months in the group of Raynaud with symptoms, the difference, however, was not statistically significant.

Compared to healthy controls body fat percentage values were significantly higher in Raynaud children without symptoms and significantly lower in symptomatic patients compared to controls. BMI was also significantly lower in symptomatic patients compared to either healthy controls or patients with symptoms. In the group of RP with symptoms 1 person was found severely underweight, and 3 were underweight but none of the patients were anorectic. At the same time in the group of RP without symptoms only one patient was underweight, however four of them were obese.

Microcirculatory impairment in adolescents with Raynaud phenomenon

Baseline perfusion at 32 °C was 97.6±22.4 perfusion units (PU) in symptomatic Raynaud patients which was significantly lower compared with either healthy volunteers (248.3±23.5 PU, $p<0.001$) or patients without symptoms (187.4±27.9 PU, $p<0.05$). The difference between healthy controls and asymptomatic participants was not statistically significant. Heating to 42 °C and 44 °C induced a gradual increase in cutaneous blood flow. The maximum blood flow at 42 °C was significantly reduced in RP adolescents with current symptoms (358.6±43.9 PU), compared with healthy subjects (555.9±8.2 PU, $p<0.05$). However, there was no significant difference in this response parameter between healthy controls and patients without symptoms (482.3±28.7 PU) or between the two Raynaud groups.). Analysing the percentage changes from baseline to maximal flow during heating to 42 °C, significantly greater increase was detected in symptomatic RP adolescents (452.9±93.4 %) compared with either the controls (185.1±42.5 %, $p<0.01$), or the asymptomatic RP patients (241.7±61.5 %, $p<0.05$). This parameter did not differ in the disease group without symptoms in comparison with healthy subjects, similarly to the absolute perfusion values. Heating to 44°C induced perfusion changes similar to the 42 °C stimulus and statistical comparisons revealed the same differences between groups. Additional analysis of the perfusion change revealed that the kinetics of the heat-induced response was also altered in patients with RP in comparison with healthy controls. The AUC of the 42°C heat-induced perfusion response was significantly greater in both Raynaud disease groups compared with the control group. No significant difference in AUC was found between symptomatic and asymptomatic patients. On the other hand, latency to reach the maximum perfusion at 42 °C was significantly longer in both patient groups (symptomatic: 236.4±17.4 s, asymptomatic 164.6±7.4 s) compared with healthy controls (101.9±4.7 s, $p<0.001$ for both). Moreover, the latency was also significantly different between the two RP patient

groups. Correlations between microcirculatory parameters and mean blood pressure or body fat percentage or disease duration were analysed, but no significant relationships were detected.

Vasoactive sensory neuropeptide results

Plasma concentrations of vasoactive sensory neuropeptides were not altered in primary Raynaud adolescents. PACAP-38 and somatostatin-LI were reliably measurable in the systemic circulation, however there were no significant differences between their concentrations in the three groups.

III. study: Severe acrocyanosis precipitated by cold agglutinin secondary to infection with Mycoplasma pneumoniae in a pediatric patient

In our investigation we examined the severely impaired perfusion and the efficacy of medical treatment a girl diagnosed with acrocyanosis.

Medical history, clinical symptoms

The 9 year-old girl became subfebrile and developed unproductive cough two weeks before admission to the hospital. She was treated with amoxicillin-clavulanate antibiotic and fluticasone proprionate and salbutamol inhalation treatments. One week later painful, bluish discoloration of fingers occurred which was worsening in cold temperature. She was referred to our emergency unit. On physical examination, her fingers and toes were cold, blue and tender, with a small cutaneous necrosis of the digital phalanx of the fourth finger. Sclerodactyly was not observed. Radial and axillary pulses on both sides were equal and regular, blood pressure and heart rate were in the normal range. The elevated arm test was not indicative for thoracic outlet syndrome. Crepitation was observed above the middle lobe of the right pulmonary tract. No further abnormalities were seen on physical and neurological examination.

Diagnostic results

Routine laboratory tests were all in reference ranges, erythrocyte sedimentation rate (ESR) was slightly elevated. Direct antiglobulin test (Coombs) detected the presence of both IgM and C3d. IgM exhibited a peak auto-agglutination at 4 °C, its reactivity gradually vanished above room temperature and was

inactive at 37 °C. Immunoserological investigations revealed elevated anti-nucleosoma antibody (76.9 UI/ml, normal < 20 UI/ml), while other autoantibodies were in the normal range. Infectious serology results indicated recent Mycoplasma pneumoniae infection. Chest radiography revealed pneumonia in the right lobe.

On the basis of clinical examination and the result of laboratory tests, we reasonably suspected that the girl had acrocyanosis precipitated by cold agglutinin secondary to infection with Mycoplasma pneumoniae. Autoimmune disorders and drug-induced acrocyanosis were ruled out based on the medical history and the laboratory tests.

The patient was treated with 2 week-long oral clarithromycin treatment and intravenous pentoxyphyllin infusions (200 mg/dosi) three times a week in the beginning. No adverse events, such as hypotension, occurred during pentoxyphyllin treatments. After the first 3 months of therapy, the frequency of infusions was set to every 2-3 weeks according to the improving clinical signs and continued for 12 months in every three weeks. No plasma exchange therapy was necessary. The patient was advised to avoid cold temperatures and keep her peripheries warm, gloves should be worn. There were no problems with adherence to medical advice.

Functional investigations

Since the symptoms persisted for over two months after the first admission, we decided to use two non-invasive methods to determine the viability of tissues, the degree of tissue perfusion impairment and the responsiveness of the microvasculature. Periflux 5000 system (Perimed AB, Stockholm, Sweden) applies the Laser Doppler Flowmetry (LDF) technology for perfusion measurement. A thermostatic Laser Doppler probe was placed on the distal phalanx of the second finger of the right hand to measure the effect of local heating on skin microcirculation (42 °C for 15 min then to 44 °C for 5 min). Severely

reduced basal blood flow was detected on the affected fingers with delayed vasodilatation after local heating.

The Laser Speckle Contrast Analysis (LASCA) technology used by the PeriCam PSI equipment (Perimed AB, Stockholm, Sweden) allows real-time cutaneous perfusion imaging of larger body parts in several regions of interests (ROIs) simultaneously. This measurement also showed reduced perfusion in the acral regions and a significant perfusion increase in response to an acute 2-h-long pentoxyfillin infusion (200 mg) 1 hour after finishing the treatment.

Summary of our results

1. Based on our first study, the following conclusions could be emphasized:

- Medical history: Detailed family medical history is necessary for the etiology and background (inherited disorders- familial accumulation). The accurate physical examination is essential, but the phenomenon cannot be classified based on the clinical signs.
- ANA test and nailfold capillaroscopy are necessary for the classification and determination of the prognosis of Raynaud phenomenon.
- Recognition of harmless Raynaud phenomenon is necessary, because it could be the first sign of connective tissue disease. Additional tests (cardiology, pulmonology, gastroenterology, psychology) are essential to ensure early detection of other organ involvement.

2. To our knowledge, our second study is the first one in paediatric population to show altered heat-induced cutaneous hyperaemia responses in relation with the clinical severity and symptomatology using a sensitive, non-invasive method.

3. Microcirculatory changes develop before morphological abnormalities are seen with nail-fold capillaroscopy, so altered microvascular response to thermal stimuli could be an early marker, but a follow-up study is needed to determine whether this parameter could be an objective severity and prognosis indicator.

4. The main conclusion of our third study is that if a child present with acrocyanosis, early diagnosis and etiology is essential for successful treatment.

LIST OF PUBLICATIONS:

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