Role of nociceptin/ orphanin FQ and Heat shock protein 70 in ischemic cardiovascular diseases

Doctoral thesis

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1. INTRODUCTION

Ischemic cardiovascular diseases (CVD) of atherosclerotic origin are still leading cause of death in the US and in Europe. CVD causes nearly half of all deaths in Europe and the estimated costs are billions of euros each year.Atherosclerosis is the main cause of most cardiovascular diseases, such as coronary artery disease (CAD), peripheral and carotid artery disease, aortic aneurysms and cerebrovascular diseases. Vascular and cancer-related illnesses are the main cause of death in the developed countries. In addition, WHO expects cardiovascular diseases to undertake cancer and to be the major killer globally within 15 years owing to both its rapidly increasing prevalence and risk factors such as metabolic syndrome throughout the world.These facts underline the need for intensified research and prevention on the cardiovascular field in order to fight this devastating disease.The concept that chronic inflammation takes a causative part in the progression of atherosclerosis, and adaptive immunity is deeply involved in this process has gained widespread acceptance.

Inflammatory stress factors alone - such as smoking, hyperhomocysteinemia or high C-reactive protein levels - do not always explain the extent of calcified vasculature and later cardiovascular risk in peripheral artery disease and CAD.

Measuring the extent of atherosclerosis by surrogate markers such as vascular calcification, not clearly defines plaque vulnerability and plaque remodeling.
A wider range of surrogate startegies, such as novel biomarkers of arterial calcification and atherosclerosis are therefore urgently needed to help risk startification and avoid later major CVD complications.Hsp 70 and nociceptin/orphanin FQ are potent markers of inflammation.Since atherosclerosis is a chronic inflammatory disease, we propose nociceptin/orphanin FQ and heat shock protein 70, as novel markers of the chronic inflammation in severe atherosclerosis.
We also introduce N/OFQ as a possible marker of ischemia/reperfusion injury of acute coronary syndromes.

Nociceptin/orphanin FQ

Identification of the opioid receptor-like ORL1 receptor (recently named as NOP) and its endogenous peptide nociceptin/orphanin FQ (N/OFQ) little over a decade ago opened new perspectives in the research of peptide-based signalling pathways in the nervous sytem. Both the peptide and its receptor are widely distributed in the central as well as in the peripheral nervous system and take an important role in modulating numerous biological functions including pain transmission, anxiety, memory, food intake, locomotor activity and regulate functions of some peripheral systems such as the airways, gastrointestinal and genitourinary systems.The role of N/OFQ in the cardiovascular system was demonstrated in a large number of experimental studies. Based on *in vitro* and *in vivo* results it is generally accepted that N/OFQ induces hypotension, bradycardia and vasodilation by influencing the central nervous system and peripheral tissues both *directly* and *indirectly* by reducing sympathetic and increasing parasympathetic influence on neurons innervating the heart and the vascular system.Clinical studies on patients with different types of CVDs are still very limited. In a human study, N/OFQ was found elevated in patients with acute stroke and transient ischemic attack. It was suggested that elevated plasma N/OFQ level is the consequence of stroke. High plasma N/OFQ levels were found in acute unstable angina pectoris, but not during induced myocardial ischemia. No evidence exists regarding the role of human N/OFQ in chronic ischemic cardiovascular diseases due to atherosclerosis which commonly involve chronic inflammation and recurrent pain.

Heat shock protein 70

The Hsp70 class is the most studied subtype and includes the constitutively expressed Hsp-8 and inducible Hsp70-1, also called HspA1B (Hsp70 in the following refers to the inducible form) members. Hsp70 is traditionally considered as intracellular cytoprotective chaperone, and its level can increase several-fold in response to stress. It has been recognised that Hsp70 are present in the peripheral circulation of normal individuals providing the first evidence that Hsp70 may be released into the extracellular environment not only in response to stress but also under physiologic conditions. Hsp70 has immunoregulatory properties with both pro- and anti-

inflammatory effects in a cross-species manner. The role of Hsp70 is still unclear in the pathology of atherosclerosis. It is not clear, whether a dysregulated soluble Hsp70 levels are indeed a consequence or a cause of atherosclerosis. The extent of atherosclerosis and the presence of cardiovascular risk factors (homocysteine, CRP, smoking, diabetes) might explain the dysregulation of the Hsp70 system. Further analysis is required in order to explain the "chaperokine" properties of Hsp70 in the etiology of atherosclerosis and calcification.

Since both Hsp70 and nociceptin have their distinguished effects on endothelial cells, establishing and studying their role as atherosclerosis markers are questions of mutual interest.

2. AIMS

With a pilot cohort study of atherosclerotic chronic ischemic cardiovascular and acute coronary syndrome patients patients we looked for the answers to the following questions:

2.1. Do nociceptin/orphaninFQ levels correlate with the severity of ischemic heart failure?

A little is known about the role of the N/OFQ system in the human cardiovascular system. Therefore we aimed to investigate the correlations between N/OFQ levels and the severity of ischemic heart failure.

2.2. What kind of associations are present between plasma N/OFQ levels and peripheral artery disease?

However endogenous N/OFQ and other nociceptin receptor agonists produce nitric oxide-mediated systemic hypotension, the role of N/OFQ in peripheral artery disease is yet unexplored. We aimed to describe the associations between N/OFQ levels and peripheral artery disease in our patient cohort.

2.3. Are there any correlations between N/OFQ levels and the clinical characteristics in our atherosclerotic patient cohort?

We investigated the correlation between plasma N/OFQ levels, clinical characteristics and laboratory parameters of patients with severe ischemic heart failure and peripheral artery disease.

2.4. Is there a correlation between N/OFQ levels and the clinical findings in our ACS patient cohort?

N/OFQ is a possible marker of disease severity since ACS is characterized by pain, inflammation, and loss of function.

In a cross-sectional study of atherosclerotic peripheral artery disease and carotid stenosis patients we aimed to answer the questions listed below:

2.5. Does any association exist between the stress reaction (described with sHsp70 levels) and the severity of calcification in atherosclerosis?

Serum Hsp70 might contribute to the calcification process observed in severe atherosclerosis. We aimed to measure the extent of the calcification in our patient cohort and correlate those data with serum Hsp70 levels.

2.6. Does the sHsp70 level correlate with other known risk factors of atherosclerosis? What kind of biological correlation does sHsp70 level have in patients suffering from carotid and lower extremity stenosis?

We aimed to investigate the *in vivo* biological correlations of the studied biomarker in atherosclerotic calcification. The aim of our study was to identify the biological correlates between known risk factors and Hsp70 serum levels in our patient cohort.

2.7. Are sHsp70 levels associated with the inflammatory markers of atherosclerosis?

Serum Hsp70, as a marker of cellular stress may play a role in the systemic inflammation observed in atherosclerosis, although we do not have data about the association between markers of inflammation, such as CRP and serum bilirubin and sHsp70 levels. The aim of our study was to describe, whether sHsp70 is correlated with CRP and bilirubin levels in our severe atherosclerotic patient groups.

3. METHODS

Nociceptin/orphanin FQ measurements in ischemic cardiovascular patients (1. and 2. patient group)

22 patients with chronic stable angina pectoris (SAP) and 12 patients with peripheral artery disease (PAD) admitted to our hospital were enrolled in this study. Nociceptin levels were measured in 7 patients who had stable angina pectoris because of multiple coronary artery stenosis (SAP-multiple) and in 5 patients who had degenerative calcific aortic valve stenosis (AS). The severity of their symptoms required cardiac surgery. 10 stable angina patients had only one coronary vessel affected (SAP-single), therefore they underwent percutaneous coronary intervention (PCI). SAP- multiple and SAP- AS patients presented severe angina pectoris symptoms Canadian Cardiovascular Society, (CCS III-IV.). Patients with SAP-single presented CCS II-III. grade of angina pectoris. Patients were asked to avoid any exertion. No patient had shown either chest pain or dyspnea within 1 week before the blood samples were taken. No stable angina patient had angina at rest. Nine PAD patients of atherosclerotic origin had severe intermittent claudication (<100 meters) and 3 patients had rest pain and gangrene, but none of them had night pain. Patients with claudication were asked to avoid any exertion. PAD patients with rest pain had been on analgetics (tramadol hydrochloride) for two weeks before blood samples were taken. No adverse effects or drug interactions were detected because of the analgetics. No patient had either claudication or rest pain within 1 week before the study. 14 healthy subjects without any cardiovascular diseases served as a control group.

In the ACS patient group, a total of 59 subjects were examined. 28 of them were admitted to the Heart Center of Semmelweis University. In 17 cases, acute coronary syndrome was the cause of admission, in which cases taking the patient history and completing a thorough physical examination immediately followed admission.

Laboratory results showed enzyme positivity in 10 cases (study group No. 1: enzyme positive acute coronary syndrome [EPACS], n=10), the other patients were grouped as having enzyme negative acute coronary syndrome (study group No. 2: ENACS, n=7). Urgent coronarography was completed in each of the 17 cases by an experienced interventional cardiologist of our institute, who did not participate in this study.

Coronarography showed significant stenosis in 15 patients, in which cases percutaneous coronary intervention (PCI) was performed immediately.

The other 11 patients enrolled in this study were admitted to the Heart Center with a known ischemic heart disease (angina pectoris or myocardial infarction in patient history) in a quiescent phase for a control examination.Exclusion criterias concluded liver or kidney failure, severe inflammation or malignant disease in patient history, thrombolytic or immunosuppressive therapy, and admission after more than 6 hours after the onset of chest pains. A group of 31 healthy people served as the control group, who were not aware of any disease and did not take any medication [15 males, 16 females, age 36.2 (13.8), BMI 24.4 (4.0)]. We obtained a written informed consent from all participants. All patients were managed in accordance with the guidelines of the American College of Cardiology and the American Heart Association (3).

Written informed consent was obtained from all participants, and the protocol was approved by the Regional and Institutional Committee of Science and Research Ethics of Semmelweis University (205-1/2007). Research protocol was done in accordance with the principles of the Declaration of Helsinki.

Determination of soluble Hsp70 in peripheral artery disease patients with vascular calcification (3. patient group)

In this cross sectional study 180 consecutive patients were recruited at the outpatient clinic of the Department of Vascular Surgery of Semmelweis University Budapest between January and June 2009. Consecutive patients with history or present symptoms of atherosclerotic chronic lower limb ischemia or chronic carotid artery stenosis were considered for inclusion.. Patients with coexisting malignant tumour, hepatic disease, end stage renal disease (dialysis) or immune suppression were also excluded. The full clinical record of the patients was registered at inclusion with the detailed physical status and routine clinical laboratory tests. Systemic atherosclerosis and calcification was assessed by ultrasound (carotid intima-media thickness /IMT/, presence of calcification at the abdominal aorta, carotid and femoral bifurcations, aortic and mitral cardiac valves). Standard serum markers of inflammation, diabetes, renal function, ankle-brachial indexes and traditional risk factors for atherosclerosis were noted. Blood samples for the measurement of serum Hsp70 were also collected at inclusion before the

patients underwent surgery or percutaneous transluminal angioplasty (PTA). The study was carried out in accordance of the Helsinki Declaration at the Department of Vascular Surgery, Semmelweis University based on a study protocol approved by the Semmelweis University Regional and Institutional Committee of Science and Research Ethics. All patients provided written informed consent.

Clinical data

All patients had a medical history interview and physical examination. A study questionnaire was used for recording the relevant demographic and clinical data (age, weight, height, smoking habit, medications and concomitant disease). A careful exploration for the presence of symptoms (exertional shortness of breath, chest pain, claudication or rest pain) were then followed by echocardiography and coronary/ peripheral angiography. The traditional Fontaine classification was used to assess the clinical severity of the chronic lower extremity atherosclerotic disease (groups I, II/a, II/b, III, IV). Group II was separated to "a" and "b" subgroups at walking distance of 200 meters. Ankle-brachial index (ABI) measurement with Doppler ultrasound probe was performed by a medical doctor experienced in taking ABI. In the first study group, blood pressure and pulse was also measured on the same day before the blood samples were taken.One experienced cardiologist blinded for other study information performed transthoracic echocardiograms. According to the guidelines of the American Society of Echocardiography complete 2-dimensional examinations were performed including Doppler images in all standard views using phased array transducers (2.5-4.5MHz) of a Toshiba Xario and Philips IE 33 ultrasound system.

In the first study group, echocardiography was also performed to measure left ventricular ejection fraction in patients suffering from chronic ischemic heart disease and to quantify degree of aortic valve stenosis. AS with a valve area (less than)<1.0 cm^2 was considered severe. Electrocardiography was recorded in all cases to exclude cardiac arrhythmias and acute myocardial infarction.

In the second study group, patients underwent ultrasonography during their ICU stay and before being released from the hospital, all completed by the same cardiologist experienced in echocardiography.

In the third study group, mitral valve calcification was defined as an echodense (reaching epicardial density) structure of the anterior and posterior mitral leaflet and the mitral annulus on the parasternal short and long axis and apical four chamber view. Aortic valve calcification was determined if echodense structure (reaching epicardial density) was noticed at the aortic root on the parasternal short and long axis and apical five chamber view. Carotid IMT and the noncardiac part of the general calcification score was determined by a single experienced radiologist who was blinded to patients' clinical information. IMT was measured at three points on a plaque free area of the dorsal wall of both common carotid arteries using linear (7.5-11MHz) transducer of a Toshiba Aplio SSA-770 ultrasound system. The mean value and the maximum IMT was used for calculations. At the same examination carotid stenosis was also determined, stenosis with 70 % or more were determined as significant. After having measured both carotid arteries, the more stenotic artery was determined as the maximal carotid stenosis. Body mass index (BMI) was calculated as weight (kg) / height^2 (m). To assess the overall extent of systemic atherosclerosis a calcification score (CS) was calculated after examining the vascular system at seven sites: both carotid bifurcations, the infrarenal aorta, both common femoral arteries, aortic and mitral valves by B-mode ultrasound (see technical details above at carotid IMT measurements). If calcification was noted, the spot was rated as 1. Sites with no calcification received 0, so the calcification range was 0-7.

Blood samples for nociceptin/ orphanin FQ measurements

Blood samples (3.0 ml) were taken for nociceptin/ orphanin FQ measurements from fasting chronic heart disease or PAD subjects along with the clinical laboratory samples on the second day after admission before surgery or PCI. For N/OFQ measurements in acute coronary syndrome patients we collected blood samples two days after coronarography. Blood was collected in vacutainer tubes containing K-EDTA as anticoagulant. 100 µl aprotinin was added immediately as protease inhibitor per tube (0.6 TIU/ml, Calbiochem). Plasma was separated by centrifugation (Janetzky K70, 1600 g x 15 min at 40C) and samples were kept frozen at –80 0C until direct analysis. 1000 µl aliquots of plasma samples were mixed with equal volume of 1% v/v trifluoroacetic acid (TFA I.), centrifuged at 1600 g for 20 min at 4 0C. The acidified samples were

loaded onto C18 Sep-Pack cartridges (ABĻE JASCO Hungary Ltd), washed twice with TFA I., then eluted with 60% acetonitrile in 0.1% TFA. Samples were freeze dried by centrifugation (SAVANT, USA). The reconstituted eluate was subjected to radioimmunoassay. Nociceptin was measured by a validated radioimmunoassay (125I-Nociceptin kit, Phoenix Pharmaceuticals, Phoenix, CA, USA) with minimum sensitivity of 1 pg/ml, as described before [28]. The assay was performed blind to the subject groups. Data were evaluated by RIA-Mat 280 (Byk-Sangtec, Dietzenbach, Germany). Significance was counted by using Origin-program (based on the Student t-test). Fasting blood samples were also used to examine standard clinical laboratory parameters and troponin-T levels in all cases to exclude acute myocardial ischemia, renal and hepatic failure, or any inflammatory processes. Clinical chemistry was performed in the local and as well as in the core laboratories of Semmelweis University (diagnostic instruments: D-Cell 5D - Diagon Ltd., Cobas Integra 400 – Roche, STA-Compact - Diagnostica Stago).

Serum samples for Heat shock protein 70 measurements

Soluble Hsp70 level was measured by using R&D System (USA, Cat No. DYC1663E) enzyme-linked immunosorbent assay (ELISA) kit. For Hsp70 family nomenclature, the suggestions of Tavaria et al. were used. Ninety-six-well microtitre plates were coated with mouse anti-human Hsp70 capture antibodies (100 µl, 2 µg/ml) in carbonate buffer (pH 9.5) overnight at 4°C. Plates were washed with phosphate-buffered saline (PBS) containing 0.1% Tween 20 three times and non-specific binding sites blocked by incubation with 200 µl of PBS containing 0.5% gelatine and Tween 20 for 1 h at room temperature (RT). After washing, 100 µl of the reference preparation (recombinant human Hsp70, 0–10 ng/ml) or undiluted serum samples were added, and the plates were incubated for 2 h at RT. Plates were subsequently washed, and Hsp70 binding was determined using biotinylated rabbit antihuman antibodies (100 µl, 0.5 µg/ml) in PBS gelatine. After 1.5 h at room temperature, plates were washed and incubated with streptavidin–horseradish peroxidase (1:200) in PBS gelatine for 20 min at RT. Plates were washed, and 100 µl of o-phenylene-diamine (Sigma, St. Louis, MO, USA) in citrate buffer was added. The optical density was measured at λ=490 nm (reference at λ=620 nm). The detection range of the assay was 0.05–10 ng/ml, the intra/ inter-assay

variability <10/<16%, respectively.Fasting serum samples were also used to examine standard clinical laboratory measurements, CRP and homocysteine levels in the core laboratory of Semmelweis University (diagnostic instruments: D-Cell 5D - Diagon Ltd., Cobas Integra 400 – Roche, STA-Compact - Diagnostica Stago).We used the Cockcroft-Gault formula for the calculation ofglomerular filtration rate. Estimated glomerular filtration rate = ([140-age] X weight in kg) X constant/ (serum creatinine (μmol/L), whereconstant was 1.23 for men and 1.04 for women.

STATISTICS

Statistical analysis – N/OFQ study

Brown-Forsythe ANOVA accompanied by Games-Howell post hoc tests was used to compare plasma N/OFQ level means between the groups. The relationships of binary patient characteristic variables (coded by 0 and 1) with plasma N/OFQ were examined by logit model. Factor analysis was used to classify patient characteristic variables together with plasma N/OFQ and in order to reveal underlying hidden factors. Associations between continous patient characteristic variables and N/OFQ were explored by linear and nonlinear regression technique.

Statistical analysis - Hsp70 study

Non-parametric tests were used for group comparisons; continuous variables between two groups were compared with the Mann–Whitney U test. Spearman rank correlation coefficients were calculated for estimation of interrelations between sHsp70 and other variables. A Power calculation was used to estimate the sample size in the correlation analysis between sHsp70 levels and CS (P=0.62). Multiple logistic regression analysis was applied to estimate interrelationship between variables as categorical predictors and severity of peripheral artery disease. Data are presented in the text as median and interquartile range (IQR). Analyses were carried out using STATISTICA 8.0 (StatSoft Inc., Tulsa, OK, USA), Prism for Windows 5.01 (GraphPad Software, San Diego, CA, USA) and SPSS for Windows 15.0.1 (SPSS Inc., Chicago, IL) statistical software

products. All statistical analyses were performed two-tailed and $p<0.05$ was considered as significant.

4. RESULTS

Association of plasma nociceptin/orphanin FQ levels with chronic ischemic cardiovascular diseases

The severity of symptoms of our patients required surgical or interventional treatment in all cases. The median value of LVEF in the stable angina group was 50 (43-56). Mean arterial pressure/ pulse in the SAP and PAD groups were 90 (82-100) mmHg/ 69 (64-72) beats/min and 96 (88-105) mmHg/ 85 (78-91) beats/min, respectively. Lipid profile results showed moderately higher than normal levels of triglyceride and cholesterol. Creatinine sample medians and interquartile ranges were as follows 109 (81-178) μmol/l for the SAP-single, 98 (90-117) μmol/l for the SAP-multiple, 89 (79-99) μmol/l for SAP-AS and 71 (65-93) μmol/l for the PAD patient groups.

N/OFQ level showed non-normal (skewed) distribution with different skewnesses in the control and patient groups. The following median (IQR) plasma N/OFQ levels were found for each groups - SAP-single: 7.49 (7.23-8.12) pg/ml; SAP-multiple 6.88 (6.27-7.46) pg/ml; SAP-AS: 7.05 (5.75-7.54) pg/ml; PAD: 6.99 (6.16-7.05) pg/ml and healthy control: 9.50 (8.43-10.88) pg/ml. Grouped by more severe CCS score, SAP-multiple/ SAP-AS together had a 6.96 (6.27-7.38) pg/ml plasma N/OFQ level. Median-centered Fligner-Killeen median test demostrated ($F=5.18$, $df_1=3$, $df_2=42$, $p=0.004$) that the data violated the assumption of homoscedasticity. Therefore the Brown-Forsythe ANOVA was used which showed a significant main effect, $F(3,23.9)= 20.41$, $p<0.001$. Post hoc multiple comparisons detected significant difference between SAP-single and SAP-multiple/ SAP-AS patient groups ($p=0.04$). Nociceptin/Orphanin FQ levels did not differ from each other between SAP-multiple and SAP-AS groups (p= n.s.). SAP-single and SAP-multiple groups tended to, but did not differ significantly from each other either ($p=0.079$). SAP-multiple and SAP- AS patient's N/OFQ levels proved to be significantly lower than that of the healthy control's ($p=0.001$ and 0.008 respectively). This difference was also true by SAP-single vs. healthy controls ($p=0.014$)Nociceptin/

orphanin FQ plasma levels showed a marked difference between PAD and control groups (p=0.001)

We investigated the correlation between plasma N/OFQ levels, clinical characteristics and laboratory parameters of patients with SAP and peripheral artery disease. N/OFQ levelscovariate with creatinine (r=0.38, p=0.04) and the linear regression explains 14% of N/OFQ level variance in the patient groups.

Linear association was detected also in SAP-single group, interestingly enough, between patients' plasma N/OFQ levels and arterial pulse (r= - 0.72, p=0.04), the higher the N/OFQ levels the lower the pulse. Significant nonlinear (second order polynomial) regression describes the association between urea and N/OFQ level (R^2=0.91, p=0.03) and between N/OFQ and SBP (R^2=0.77, p=0.02) both in the SAP-single group.

Exploratory factor analysis with varimax rotation was used to identify the underlying hidden structure of the relationships between the following simultaneously measured variables: N/OFQ, age, WBC, THR, Hb, cholesterol, triglyceride, glucose A, eGFR, creatinine, systolic blood pressure, and diastolic blood pressure. Four factors were found which together account for 72% of the variance. N/OFQ level proved to be a separate factor explaining 31.9% of the total variance, with factor loading 0.92. The second factor explaines 17.4 % of the total variance and consist of the cholesterol, triglyceride and glucose, with factor loadings 0.85, 0.87, and 0.71, respectively. The third factor comprised eGFR and creatinine with loadings 0.85 and -0.,83, respectively and accounts for 13.4% of the total variance. Finally the fourth factor was associated to WBC, with loading -0.76 and explains further 9.2% of the variance.

Association of plasma nociceptin/orphanin FQ levels with disease characteristics after acute coronary syndromes

Median (IQR) plasma N/OFQ levels were found as follows: 6.86 (6.24-7.32) pg/ml for the group of enzyme positive acute coronary syndrome, 6.97 (6.87-7.01) pg/ml for enzyme negative acute coronary syndrome and 7.58 (7.23-8.20) pg/ml for ischemic heart diseases. We measured a level of 8.86 (7.27-9.83) pg/ml in the control group. We

did not detect any significant difference between N/OFQ levels measured in male and female subjects. Analysis of plasma N/OFQ levels in the control group showed a much wider interquartile range compared to the other three groups. Further investigations are needed to clarify whether or not healthy people can be divided into subgroups regarding the plasma N/OFQ levels.Owing to the apparent heteroscedasticity Brown-Forsythe ANOVA was performed. The main effect for N/OFQ was significant, $F(3,50.8)=21.6$, $p<0.000$. Post hoc tests revealed that N/OFQ was significantly lower in all cardiovascular patients compared to the control group (EPACS $p<0.000$, ENACS $p<0.000$, IHD $p=0.05$). Plasma N/OFQ levels in ischemic heart diseases at the same time were significantly higher than what we measured in enzyme positive acute coronary syndrome ($p=0.009$) and in enzyme negative acute coronary syndrome ($p=0.012$).

Statistical analyses of patient characteristics found that age was significantly higher in the enzyme positive ACS group compared to enzyme negative ACS ($p=0.02$). In spite of this age difference together with the fact that all the patient group's age were higher than that of the healthy control group age is not a covariate, thus, there was no need to control for this variable in N/OFQ ANOVA. Laboratory results showed that CK was significantly higher in the enzyme positive ACS group compared to enzyme negative ACS and to the ischemic heart disease group (both with $p=0.04$). Admission glucose level was significantly higher in the enzyme positive acute coronary syndrome compared to the other two groups ($p=0.03$ for ENACS and $p=0.04$ for OHD). LDH difference between EPACS and ENACS groups were also significant ($p=0.05$). Echocardiography completed two days after admission showed that ejection fraction was significantly lower in the EPACS group compared to the ENACS ($p=0.016$). Other patient characteristic variables did not show significant differences.
Strong linear association was detected in the enzyme positive group between plasma N/OFQ levels and white blood cell (WBC) and platelet (PLT) count ($r=0.93$, $p=0.0001$ for WBC, and 0.69 with $p=0.03$ for PLT).

We noticed further significant correlations between N/OFQ levels of the subjects in the same group of enzyme positive ACS and creatine-kinase (CK), and plasma cholesterol level (correlation coefficients and p values were 0.73 with $p=0.02$ for CK, and -0.66

with p=0.05 for cholesterol). Significant nonlinear (power) regression was found between N/OFQ and glutamate oxalacetate transaminase (GOT) in EPACS group (R^2=0.49, p=0.03). N/OFQ has a nearly significant nonlinear (quadratic) association with C-reactive protein (CRP) (R^2=0.629, p=0.07).Based on the N/OFQ level measured in patient groups two binary variables could be predicted, pain and necroenzyme efflux, using logistic regression. Low N/OFQ level predicted pain (Chi^2=27.5, df=1, p<0.000) and necroenzyme efflux (Chi^2=7.8, df=1, p=0.005).

Exploratory factor analysis classified the 25 patient characteristic variable together with the plasma N/OFQ into three factors wich explain nearly equal proportions of the total variance. The factors represent common underlying hidden phenomena and are responsible for the covariation between the observed variables. The first latent factor has an r=0.92 correlation with N/OFQ and the other variables in this factor are INR, platelet count, TG, SGOT and SGPT. The members of the second factor are age, heart rate, CRP, Hb, cholesterol, HDL, GGT, creatinine and urea. The third factor consists of the following patient characteristic variables: systolic blood pressure, serum CK, LDH, HBDH, EF, LDL, GLC admission, and ALP.

Association of soluble heat shock protein 70 with vascular calcification

The mean age was 64 years in our 180 patient study population, 56 (31.1 %) were female, 98 (54.4 %) were current smokers and 55 (30.6%) were past smokers, only 27 (15%) patients smoked never before. Thirty-seven (20.6 %) patients suffered from significant carotid stenosis only, 91 (50.6%) participants had only lower extremity arterial disease, whereas 52 (28.9%) patients suffered from both diseases.

The severity of symptoms of our patients required surgery or PTA in 30 (81%) carotid patients, in 80 (88%) PAD patients and in 50 (96%) patients suffering from the both diseases. According to the Fontaine classification (I-IV) of chronic atherosclerotic lower extremity arterial disease, 129 patients (71.7%) belonged to Fontaine II/b-IV groups. The median value of ankle-brachial index was0.50 (0.26-0.76), mean IMT was 0.83 mm (0.70-0.97) and worst IMT was 1.00 mm (0.80-1.30) respectively. Lipid profile results showed moderately higher than normal levels of triglyceride, cholesterol and low-density lipoprotein (LDL). The median calcification score (CS) was 5 (4-6), this corresponds with the severe systemic arterial calcification of our cohort.

We investigated the association between serum Hsp70 levels and calcification score, clinical characteristics and laboratory parameters of patients with peripheral artery disease. Analysis of clinical characteristics and other laboratory parameters revealed a significant correlation between serum heat shock protein 70 levels and age, serum bilirubin.There were no significant correlations between sHsp70 concentrations and other markers of liver injury [aspartate aminotransferase (AST), alanine aminotransferase (ALT), γGT].No significant association was found between serum Hsp70 levels and clinical characteristics such as gender, BMI, hypertension, diabetes, presence of ischemic heart disease, mean IMT, ankle-brachial index.Most importantly, no correlation with CRP, triglyceride, cholesterol, serum creatinine, serum carbamide levels and eGFR was observed.No correlation was found between sHsp70 levels and any of the medications used at the time the blood samples were taken (data not shown). No significant differences have been found between the three patient groups in age, gender, BMI, diabetes, smoking, or calcification scores (p= n.s., respectively).

The examination of Hsp70 levels (p=0.993) or calcification scores (p=0.909) in diabetic and non-diabetic patients resulted no differences.Patients with higher calcification scores had higher soluble heat shock protein levels (Mann-Whitney probe, $p<0.02$). In an univariate analysis, patients with sHsp70 level above the 75th percentile (0.7296 ng/ml) had an almost 2.2-fold risk to belong to the seriously calcified group (CS 6-7). To evaluate the association of sHsp70 level and the extent of arterial calcification, logistic regression analysis were performed. We examined the possible effect of three models (Model 1: age, gender, eGFR; model 2: model 1 + smoking (years); model 3: model 2 + CRP model 4: model 3 + homocysteine) on the odds ratio. After correction for those major confounding factors the significant correlation between circulating Hsp70 and arterial calcification still remained significant (Table 3.). Adjustment for type II diabetes mellitus with age, gender and eGFR has also not influenced the significant risk (OR= 2.136 (1.077-4.237) p=0.03) for extended calcification in patients with higher soluble Hsp70 levels.

5. CONCLUSIONS

Nociceptin/orphaninFQ levels correlate with the severity of ischemic heart failure: This is the first human pilot study measuring plasma nociceptin/ orphanin FQ levels in chronic cardiovascular disease. Our findings indicate that the presence chronic angina pectoris is attached to lower circulating levels of nociceptin/orphanin FQ in patients with severe atherosclerosis. More severe ischemic heart failure states are associated with lower plasma N/OFQ levels.

Peripheral artery disease is attached to lower levels of N/OFQ: We have found lower nociceptin/orphanin FQ levels in patients suffering from peripheral artery disease compared to healthy controls.

N/OFQ levels and clinical parameters in chronic cardiovascular patients: We did not find any correlation between subject age/ gender and N/OFQ level, which is in agreement with previous findings from the literature.

In this study N/OFQ proved to be a separate significant factor accounting for nearly one third of the total variance of the laboratory parameters in the whole patient cohort. This result might underline an independent role for nociceptin/orphanin FQ in the regulation of ischemic cardiovascular diseases. However, these findings need to be further elucidated.

N/OFQ levels are associated with markers of clinical severity after acute coronary syndromes:Lower plasma nociceptin/orphanin FQ levels were found in acute coronary syndrome patients after coronarography compared to healthy volunteers. Our results show that ACS is attached to the lowest levels of N/OFQ among the three disease groups examined. We found correlation between N/OFQ and GOT level in ACS as well. This suggests that N/OFQ levels of the plasma depends not only on the actual level of pain, but is dependent on whether there is an ischemic injury or not.We found significant correlations of N/OFQ and WBC counts, which provides further evidence of N/OFQ being regulated by immune cells beside the nervous system. The strong correlation between WBC and platelet counts and nociceptin levels suggest that plasma nociceptin levels might be WBC and platelet derived in ACS.

Elevated sHsp70 levels are associated with more severe vascular calcification:Our findings indicate that higher numbers of calcified plaques are closely correlated with higher Hsp70 levels. This correlation was undependent from major confounding risk factors such as age, gender, smoking habits, eGFR, CRP and homocysteine values.

Serum Hsp70 and homocysteine levels are significantly correlated: We also observed significant correlation between serum Hsp70 and homocysteine levels. The detailed characterisation of the patient population allowed us to identify significant correlations between sHsp70 levels and age as well.

Hsp70 levels are positively correlated with serum bilirubin levels but not related to CRP levels in our patient cohort: Inflammatory markers such as bilirubin are closely associated with circulating Hsp70. There was, however, no relationship between soluble Hsp70 and the acute phase reactant C-reactive protein. These results may explain an independent, anti-inflammatory role for soluble Hsp70 in severe vascular atherosclerosis, which need to be confirmed by later studies.

6. PUBLICATION LIST

6.1. Publications connected to the thesis

Krepuska M, Sótonyi P, Csobay-Novák C, Szeberin Z, Hartyánszky I, Zima E, Szilágyi N, Horkay F, Merkely B, Acsády G, Tekes K. (2011) Plasma nociceptin/orphanin FQ levels are lower in patients with chronic ischemic cardiovascular diseases-A pilot study., Regulatory Peptides;169(1-3):1-5) **IF: 2.473**

Krepuska M, Szeberin Z, Sótonyi P, Sarkadi H, Fehérvári M, Apor A, Rimely E, Prohászka Z, Acsády G. (2011) Serum level of soluble Hsp70 is associated with vascular calcification.Cell Stress Chaperones;16(3):257-65.

IF: 3.162

Csobay-Novák C, Sótonyi P, Krepuska M, Zima E, Szilágyi N, Tóth S, Szeberin Z, Acsády G, Merkely B, Tekes K. (2012) Decreased plasma nociceptin/orphanin FQ levels after acute coronary syndromes.Acta Physiologica Hungarica;99(2):99-110.

IF: 1.226

6.2. Other publications

Szeberin Z, Fehérvári M, Krepuska M, Apor A, Rimely E, Sarkadi H, Bíró G, Sótonyi P, Széplaki G, Szabolcs Z, Prohászka Z, Kalabay L, Acsády G. (2011) Fetuin-A serum levels in patients with aortic aneurysms of Marfan syndrome and atherosclerosis.Eur J Clin Invest;41(2):176-82. **IF: 2.736**

Szeberin Z, Firneisz G, Bíró G, Szabó GV, Sótonyi P, Windisch M, Krepuska M, Sípos F, Mihály E, Acsády G. (2009) [Surgical treatment of acute type-B aortic dissection associated with cocaine use]. Orv Hetil.;150(3):129-31. Hungarian.

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Sótonyi P, Csobay-Novák Cs, Balázs Gy, Krepuska M, Acsády Gy. (2010) A bal arteria carotis communis és subclavia okklúziója Vascularis Neurológia 2: pp. 52-55.