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

Doctoral dissertation

Dr. Miklós Krepuska
Semmelweis University
Doctoral School of Clinical Medicine

Tutor: Péter Sótonyi MD, PhD

Opponents: Prof. József Sándor MD, PhD
Ádám Remport MD, PhD

Final exam committee, chairman: Prof. Zsuzsanna Fürst, MD PhD
Final exam committee, members: Zsolt Csapó, MD PhD
Richárd Szmola, MD PhD

Budapest, 2013

APPENDIX

Abbreviations

CAD	carotid artery disease
CVD	cardiovascular diseases
HSP	heat shock protein
Hsp70	heat shock protein 70
IHF	ischemic heart failure
(ox)LDL	(oxidized) low-density lipoprotein
MHC	major histocompatibility complex
N/OFQ	nociceptin/ orphaninFQ
NOP	nociceptin/ orphaninFQ receptor (also named as ORL-1)
ORL-1	opioid receptor like receptor1 (also named as OP4/NOP)
PAD	peripheral artery disease
PMN	polymorphonuclear cells
PBMC	peripheral blood mononuclear cells
ScRs	scavenger receptors
TLRs	toll-like receptors
VLDL	very low-density lipoprotein

1. INTRODUCTION

Ischemic cardiovascular diseases (CVD) of atherosclerotic origin are still leading cause of death in the US (1) and in Europe (2). 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. (3).

The American Heart Association published the latest update on cardiovascular disease in the US: "Coronary heart disease caused ~1 of every 6 deaths in the United States in 2008." (Heart Disease and Stroke Statistics2012 Update: A Report From the American Heart Association). The European Heart Network states that "Each year cardiovascular disease (CVD) causes over 4.3 million deaths in Europe and over 2.0 million deaths in the European Union." (http://www.ehnheart.org/cvd-statistics.html)

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 (4).

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.

1.1. Atherosclerosis

1.1.1. Incidence and prevalence

The presence of extended calcification in peripheral artery disease (PAD) of the lower extremities and carotid artery disease (CAD) is associated with a 3-4-fold higher risk for mortality and cardiovascular events (5, 6). The adverse cardiovascular complications are higher in patients with more severe PAD, there is still a significant risk in persons with mild or even asymptomatic disease (7). Stress factors alone - such as smoking, hyperhomocysteinemia (6, 8) 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 (9). Novel biomarkers of arterial calcification are therefore urgently needed to avoid later major CVD complications.

1.1.2. Etiology and Pathophysiology

Atherosclerosis slowly progresses throughout a lifetime and may start early such as during adolescence - young adulthood. Fatty streaks, the hallmarks of early atherosclerosis can be prevalent in young adults. These asymptomatic lesions may develop later into established lesions, called atheromas or disappear, showing the vasculatures unique capability to heal itself.

Although the exact cause is unknown, atherosclerotic lesions, termed atheromas or atherosclerotic plaques, typically present as asymmetric focal thickening of the innermost layer of the artery, the intima. Atheromatic lesions consist of inflammatory and immune cells, smooth muscle cells (SMC) and vascular-endothelial cells. They are composed of lipids, connective tissue and debris as well. The atheroma represents a complex structural composition: macrophage foam cells, apoptotic and dead cells, lipid droplets build up a core surrounded and covered with SMCs and matrix.Activated T cells and macrophages migrate throughout the lesion and settle in the growing – shoulder - region of the atheroma and between cap and the core(10).

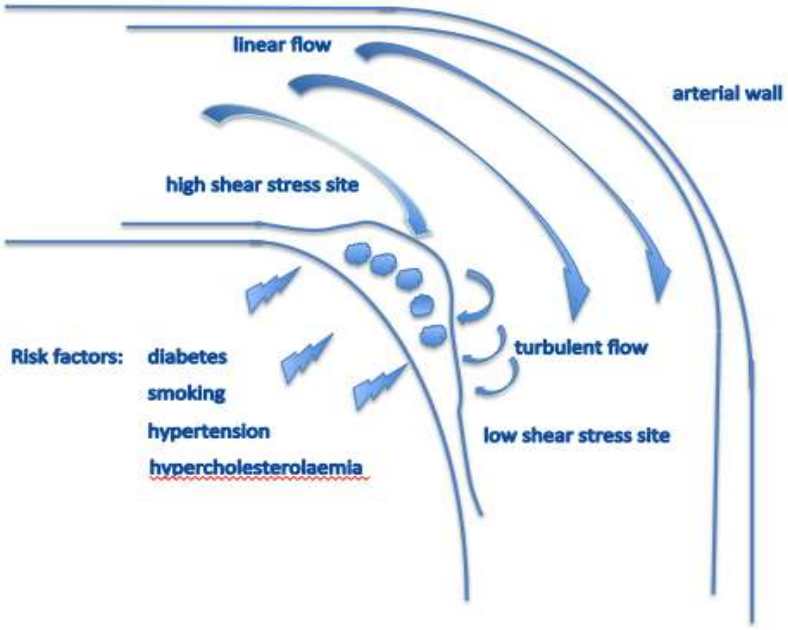
The inflammatory processes leading to the development from fatty streaks through atheromas to the calcified atherosclerotic plaque are traditionally described in *distinguished steps:*

1.1.2.1. Chronic inflammation in atherosclerosis

*Endothelial damage and activation:*The earliest step of atherosclerotic disease, leading to the established lesion – the atheroma, starts with the damage of the vascular endothelium. Two major causes of the disease, hypertonia and and hypercholesterolemia, cause endothelial activation in the arteries. In hypercholesterolemia, a disaese marked by higher levels of low-density lipoprotein (LDL), the lipoproteins infiltrate the endothelial layer and stuck in the extracellular matrix. The starting point of atherosclerosis is when the levels of lipoproteins exceeds the immune system`s capacity in eliminating them (10).The LDL becomes modified through the oxidation in the intima, where as a consequence,several activated phospholipids further activate the endothelial cells.

At a site activation, endothelial cells express vascular cell adhesion molecules (VCAM).Following VCAM expression, hematopoietic cells – such asmononuclear leukocytes –are able to roll on, adhere to and migrate through the endothelial layer. At sites of hemodynamic stress, cells become also activated. Atheroma build-up is preferred at low shear stress cytes with a turbulent blood flow. This strain causes further expression of inflammatory genes, VCAMs and promotes inflammation in the endothelium, Once adherent to the endothelial layer, monocytes are capable to migrate into the intimal layer upon chemoattractant stimuli(10, 11).

Picture 1: Development of an arterial atheromatic plaque. Common risk factors of atherosclerosis, such as smoking, diabetes, hypertension and hypercholesterolemia contribute to the disease. Low shear stress cytes favor monocyte-macrophage differentiation and plaque formation.



*Fatty streak - development of foam cells:*Monocytes are entering the endothelial wall and differentiate into macrophages. The inflamed intima produces macrophage-colony stimulating factor (M-CSF) to induce these changes. Monocyte-macrophage differentiation is a pivotal step in the forming plaque, because it is associated with pattern-recognition receptor upregulation. Toll-like receptors (TLRs) and scavenger receptors are capable to internalize multiple pathogenic molecules and particles. Apoptotic cells, viral, fungal and bacterial patterns and oxidative LDL particles are recognized, taken up and finally degraded through these patways. The modulation of these pathways causes furthere activation of pro-inflammatory cytokines, which further enhance the inflammatory processes in the intima. OxLDL will further derive into cholesterol particles in macrophages. If the cholesterol uptake exceeds the cells capacity to remove it, it will accumulate within the cell as cytosolic droplets. This process is supported by pro-inflammatory cytokines, since they inhibit the expression of ATP-

binding cassette transporters, which primary function is to remove cholesterol from the cell. These events lead to the macrophage turning into a foam cell, the characteristic cell of atherosclerosis (10, 11).

Established lesions -atheromas: Toll-like receptors initiate a signal cascade that mediate macrophage activation. Heat shock proteins and oxLDL may activate these receptors as well. Svensson et al concluded that Hsp70 is a paracrine inducer of cytokine production in oxLDL treated macrophages (12).

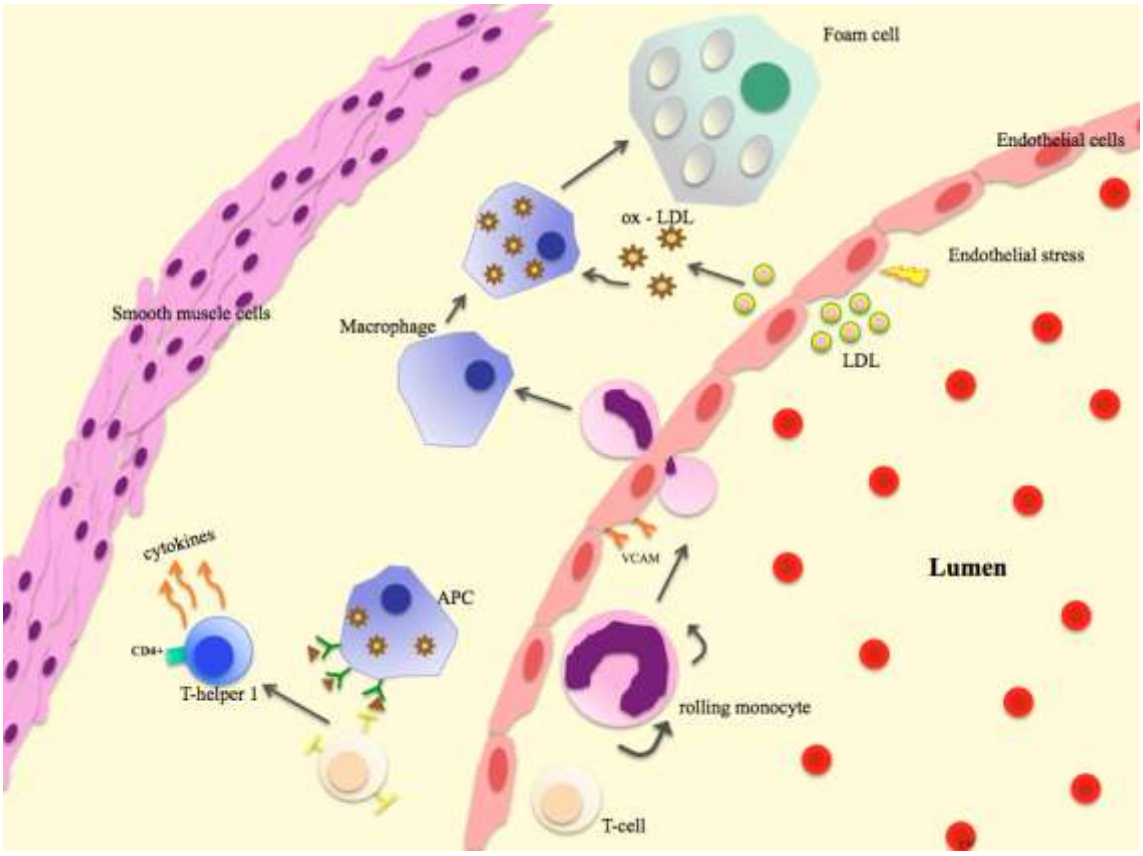
In the atheroma, cells contain a wide number of TLR types and regulate nuclear factor kappa B (NfκB). TLR2 and TLR4 act through the MyD88 and NfκB.Knockout gene deletion of TLR4 or MyD88, an intermediate molecule in the TLR signaling pathway, inhibits atherosclerosis in apoE KO mice(10, 11).
The activation of the macrophages has the following consequences: vasoactive molecules, such as nitric oxide, endothelins, and eicosanoids become released. They produce reactive oxygen species and proteolytic enzymes.Two initiating steps in cellular toxicity and matrix degradation. Cytotoxicity induced apoptosis-necrosis and the degradation of matrix components may lead to plaque instability and a risk for the rupture of the atherosclerotic plaque and subsequent thrombosis (10).

As other hematopietic cells, T cells are also activated in the atheroma after being recruited by adhesion molecules and chemokines. Macrophages become antigen-presenting cells (APCs) after they display local or foreign peptide agents, such as fragments of oxLDL, heat shock proteins or microbial antigens, with they major histocompatibility complexes (MHC). T cell activation leads to T helper-1 (Th1) response in the atheroma. The Th1 cells act mainly on macrophages with several cytokines (IFN-gamma, IL-2 and TGF-beta)and furthere promotes atheroma formation and plaque instability. Regulatory T cells inhibit this response.Natural killer T cells also react to lipid antigens presented by CD1 molecules and rapidly response by producing INF-gamma and IL-4.

There is a wide literature which antigen is the most abundant in atherosclerosis. Ox-LDL, HSPs, Chlamydia pneumoniae are among the antigens that can trigger atherosclerotic events. HSPs, such as human HSP60 and bacterial HSP 65/60 are recognized through the same TLR pathways. Other oral pathogens serve as bacterial foci and provide chronic inflammation which is associated with atherosclerosis. Cross-reactivity between the body`s own and external factors, called molecular mimicry, may lead to anti-self immune response and cause inflammatory reactivity in the atherosclerotic plaque (10, 13).

Atherosclerosis is a complex, chronic inflammatory disease, where several antigens are present and trigger immune responses, such as proinflammatory cytokines and may lead to disease morbidity(10, 13).

Picture 2: This graphic shows the steps towards fatty streaks and smooth muscle cell proliferation



1.1.2.2. Arterial calcification

Extraosseal calcification- ectopic mineral deposition-occurs in many diseases, including atherosclerosis. Calcification in the atheromatic plaque is a frequent sign in atherosclerosis. The calcium deposits are quantifiable using different advanced radiographic techniques such as coronaryCT, and and quantifiableserve as a surrogate marker for atherosclerosis, and predict a higher risk of later major negative outcomes such as acute coronary syndromes and mortality. There is an obvious need for understanding how plaque composition and stability influences morbidity and mortality. ionships between atheroma calcification and plaque stability has also been described. However, our knowledge is still limited how the radiographic measurements and the qualities of the plaque correlate (changes in calcium scans measure vs. progression and stability of the plaque)(14).

Risk factors, such as hypertension, diabetes and smoking, and other infectious stimuli, such as bacterial foci, initiate teh formation of the plaque. It begins to grow and might proceed toward major obstruction of the arterial lumen and eventually cause an unforeseen clinical event. It is highly variable among patients when the calcium deposition occurs and is rather episodic, higher rates of calcified atheroma build-up changing with long latent periods when growth of the plaque tends to be minimal.

*Vulnerable plaque:*The disease might be latent for longer period of time, however, at any point during a stable atheroma growth any individual lesion can become unstable. Atheroma intability leads to plaque rupture, after which thrombosis can occur. Plaque rupture itself can be asymptomatic or even harmless, but it can lead to fatal clinical events.Reorganization and remodeling of a ruptured plaque may be followed by a rapid plaque progression but may be also stabilized for an indeterminate time without causing a clinical event. Interestingly, this cycle seems to repeat independently from other atheroma sites. Calcification appears to develop linearly over time in the remodeling atheroma (14). It is suggested that arterial osteoblast and osteoclasts are involved in this process. Doherty et al. also summarize that atherosclerosis is a chronic vascular

inflammation, and arterial plaque calcification is best conceptualized as a convergence of bone biology with vascular inflammatory pathobiology(14).

1.1.2.3. Risk factors of atherosclerosis

Risk factors of atherosclerosis persist from the early childhood. In a large scale study(15), coronary artery and aorta specimens were investigated and correlated with known antemortem risk factors. The authors found that the extent of fatty streaks and fibrous plaques in the aorta and coronary arteries increased with age. The association between fatty streaks and fibrous plaques was much stronger in the coronary arteries than in the aorta(15).

Among the cardiovascular risk factors, body mass index, systolic and diastolic blood pressure, and serum concentrations of total cholesterol, triglycerides, low-density lipoprotein cholesterol, and high-density lipoprotein cholesterol, as a group, were strongly associated with the extent of lesions in the aorta and coronary arteries. In addition, cigarette smoking increased the percentage of the intimal surface involved with fibrous plaques in the aorta and fatty streaks in coronary vessels(15).

The authors concluded that as the number of cardiovascular risk factors increased, so did the severity of asymptomatic coronary and aortic atherosclerosis in young people (2-39 years of age)(15).

Understanding the significance of inflammation and its markers in the development of atherosclerosis, the American Heart Association investigated a number of assays of markers for potential clinical use(16). They concluded that among several cytokines, acute-phase reactants, and cellular responses to inflammatory stimuli potentially might be predictive of clinical disease, the best laboratory test to assess inflammation is limited to the measurement of high sensitive C-reactive protein.

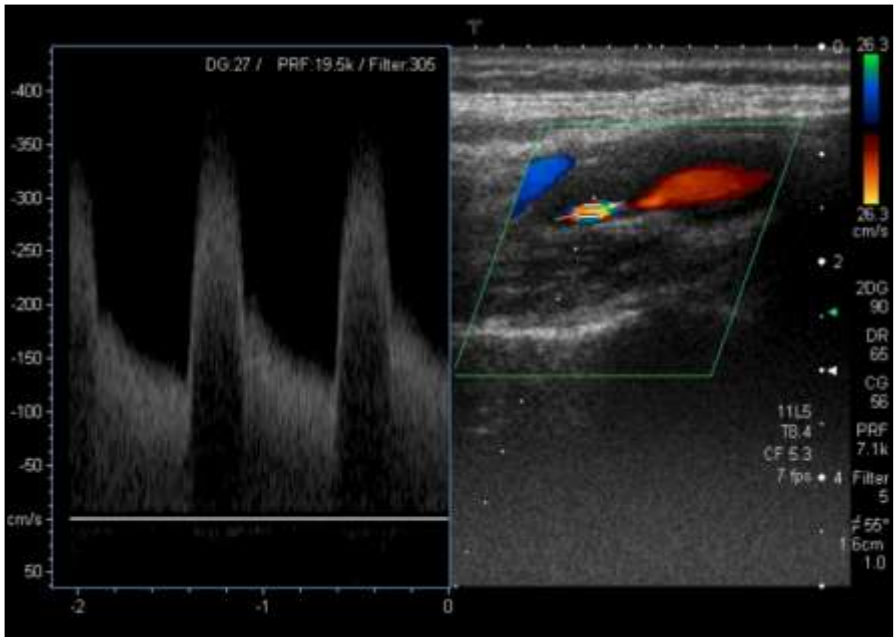
In the same year, others critically reviewed the usefulness of measurement of C-reactive protein with other factors such as lipoprotein-a, fibrinogen, and homocysteine(17). Reviewing the available epidemiological literature, they found independent associations for varying degrees, between these 4 candidate risk factors and atherosclerotic vascular

disease. However, the reviewers found relatively little data regarding the additive yield of screening for these factors over that of validated global risk assessment strategies in use. Furthermore, controlled intervention studies targeting individuals with these factors for proven risk-reduction therapies, or specifically treating these factors with available therapies, were few. They found that the explanatory power of the major, established cardiovascular risk factors has been systematically underestimated.

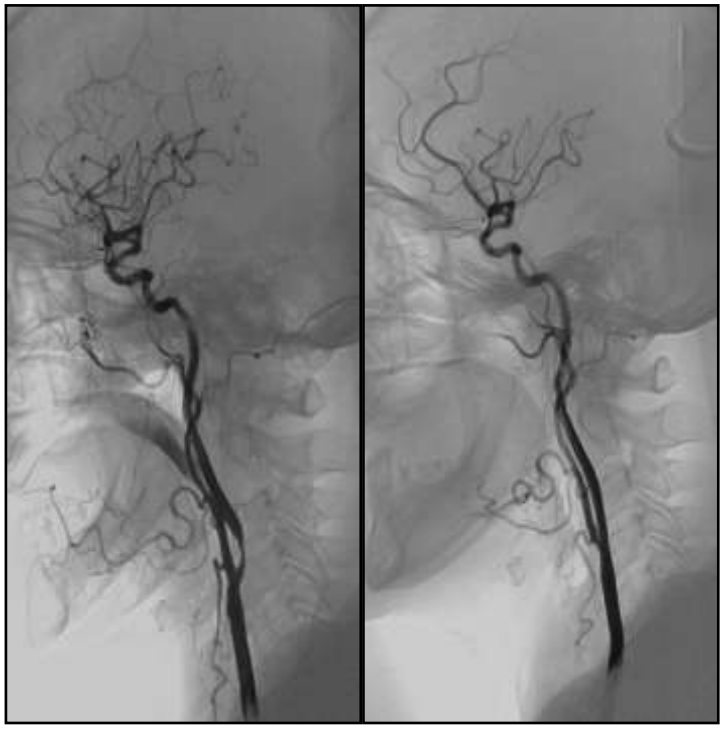
The authors concluded that although C-reactive protein, lipoprotein-a, fibrinogen, and homocysteine are associated with vascular disease risk, their optimal use in routine screening and risk stratification remains to be determined(17).

Picture 3.: Doppler-ultrasound (A) and angiography (B) confirmed carotid stenosis

(A)



(B)



Importance of clinical measurements:

Inflammatory stress factors alone - such as smoking, hyperhomocysteinemia (6, 8) 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 (9).

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.

1.1.3. Clinical features and diagnosis

1.1.3.1. Peripheral artery disease (lower extremity)

According to the ACC/ AHA practice guidelines: `Peripheral arterial disease is the preferred clinical term that is used to denote stenotic, occlusive, and aneurysmal diseases of the aorta and its branch arteries, exclusive of the coronary arteries`(18).
The guideline summarizes key components of the clinical preesentation of impaired vascular systems:: Any exertional pain, fatigue, aching, numbness which limitate the lower extremity muscles or any history of walking impairment. The primary site(s) of or non-healing woundsand discomfort of the lower extremity should be recorded, along with the relation of such discomfort to rest or exertion. Any pain at rest localized to the lower leg or foot and its association with the upright or recumbent positions. The traditional Fontaine classification is used to assess the clinical severity of the chronic lower extremity atherosclerotic disease (groups I, II/a, II/b, III, IV).

Classic Fontaine classification:

Stage 1 `No symptoms`
Stage 2 `Intermittent claudication subdivided into:
2a - without pain on resting, but with claudication at a distance of **greater** than 200 metres
2b - without pain on resting, but with a claudication distance of **less** than 200 metres`
Stage 3 `Nocturnal and / or resting pain`
Stage 4 `Necrosis (death of tissue) and/ or gangrene in the limb`

Known risk factors for atherosclerosis such as smoking, hypertension, diabetes, hyperecholestereolemia, and hyperhomocysteinemia increase the likelihood of developing peripheral artery disease. Lower extremity PAD is unfortunately a common syndrome that affects most adult populations worldwide. Peripheral arterial disease can be present in subclinical form as well, being atherosclerosis a silent disease, that can be detected by use of vascular imaging techniques, which may reveal subclinical

manifestations of arterial disease before it is detected by either ankle-brachial blood pressure measurements or manifested clinical symptoms.

By monitoring the intimal-medial thickness (IMT) in the carotid or femoral artery, early forms of asymptomatic PAD (20-50%) are easily detected in populations at risk. Other clinical manifestations are claudication (10-35%), atypical leg pain (40-50%) and critical limb ischemia (1-2%), whichdefines a significantly smaller subset of the total population with the disease(18).

The prognosis of patients with lower extremity PAD is poor, there is an increased risk for cardiovascular ischemic events due to concomitant coronary artery and cerebrovascular disease. These major negative cardiovascular morbidity is more frequent than ischemic limb events in any lower extremity PAD cohort, whether they present asymptomatic PAD or classic claudication/ critical limb ischemia(18).

1.1.3.2. Ischemic heart disease and aortic valve calcification

Our knowledge about ischemic heart diseasebeing mainly an alteration of haemodynamic status has largely changed. Nowadays, ischemic heart disease is more likely considered a complex syndrome where hypoxia, hypoperfusion and electrolytee alteration of the myocardial tissue affects the respiratory and renal system and leads to a multi organ failure. The describing parameters of the above mentioned pathways such as endothelin, brain natriuretic peptide are vasoactive biomarkers, helping to understand the pathomechanism of the disease and to determine the therapeutic pathways (Gombos et al. 2008). Other vasoactive peptides, such as nociceptin might be potential biomarkers for assessing cardiovascular risk in the future as well.

The common symptom of myocardial hypoxia in ischemic heart failure and in aortic valve calcification is stable angina pectoris (SAP). It is characterized by discomfort in the chest or arms, typically elicited by exertion or stress and relieved by rest or nitroglycerin. Less typically, discomfort may occur in the epigastric area(19).

Atherosclerosis is the cause of most coronary artery diseases, the convetional risk factors for the development of the disease: hypertension, hypercholesterolaemia, diabetes, and smoking have an adverse influence on prognosis and outcome in those

with subclinical or established disease, presumably through their effect on disease progression(19).
The diagnosis of angina pectoris is based upon a detailed patient history, electrocardiography and laboratory measurements (troponin, creatine kinase).

Classification of angina severity (20, 21)according to the Canadian Cardiovascular Society are:

Class I	'Ordinary activity does not cause angina'
Class II	'Slight limitation of ordinary activity'
Class III	'Marked limitation of ordinary physical activity'
Class IV	'Inability to carry out any physical activity without discomfort' or 'angina at rest'

Imaging techniques which assess the heart function are echocardiography and coronarography.

1.2. Nociceptin/orphanin FQ

1.2.1. Nociceptin/orphanin FQ and the cardiovascular system

Identification of the opioid receptor-like ORL1 receptor (recently named as OP4/NOP) and its endogenous peptide nociceptin/orphanin FQ (N/OFQ) (22, 23) 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 (24, 25).

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 (26-34) These findings were also confirmed in a study with spontaneously hypertensive rats (35), as well as in rats on chronic high-NaCl-diet, whereas prolonged effects have been seen after the administration of N/OFQ (36).

In an experimental model cerebrospinal fluid (CSF) nociceptin/orphanin FQ was found elevated after acute cerebral ischemia-reperfusion (I/R) and combined hypoxia and ischemia-reperfusion (H-I/R) injury, whereas values returned to control level within a few hours of reperfusion (37). In anaesthetized rats, acute myocardial ischemia up-regulated N/OFQ in the dorsal root ganglion and spinal cord (38).

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 (39). High plasma N/OFQ levels were found in acute unstable angina pectoris, but not during induced myocardial ischemia (40). 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.

1.2.2. Nociceptin/ orphanin FQ in inflammation

In a very up-to-date review, Serrano-Gomez et al. concluded that NOP and N/OFQ precursormRNA are found in monocytes, lymphocytes, and polymorphonuclear cells, suggesting a novel link between pain and inflammation(41-45). This is not the case with the other classical opioid receptors. In vitro studies show N/OFQ and its receptor are produced by immunocytes and the N/OFQ – NOP receptor crosstalk would cause immunomodulation, mainly pro-inflammatory stimulations, for example, induction of chemotaxis and proliferation of immune cells(44).However, some studies showed reduced immune cell proliferation and reduced chemokine production. These differences could be attributed to differences in techniques used to activate immune cells, cell population studied, and the response analysed(44).

Carvalho et al. found that in sepsis the administration of parenteral N/OFQ in rats exacerbated the inflammatory process and increased mortality(45). Animals treated with N/OFQ had hundred percentmortality, compared with those who wheretreated with NOP antagonistUFP-101 (50%) and with untreated group (70%). N/OFQ treatment also caused elevated plasma levels of TNFa and IL-1b(45). In addition, using anaesthetized (but non-septic) rats, Brookes and colleagues showed that N/OFQ produced an inflammatory response, and those effects were mediated by histamine(46). In mesenteric vessels, the authors found vasodilatation, macromolecular leak, and leucocyte adhesion. Conversely, intracerebroventricular administration of N/OFQ led to reduced cytokine production by peritoneal macrophages in rats undergoing exploratory laparotomy(46).

It is possible that there is a difference in the immune response to N/OFQ when adminsitered peripheralor central this is a possible new way for further investigation(44).

There are few human studies. Asmall study conducted of 21 critically ill patients admitted to the intensive care unit (ICU) with a diagnosis of sepsis. TheyPlasma N/OFQ concentrations were measured over four days. Plasma concentrations of N/OFQ at ICU admission were elevated in patients who subsequently died compared with the one`s who survived. The authors concluded that despite the strikingly new results, more data are required to confirm these findings (47).However, a novel study summoning a larger cancer patient group showed a lower expression of the nociceptin precursor pre-pronociceptin (pN/OFQ) in peripheral blood cells compared to healthy controls. The same finding was seen with intensive care unit patients(48).

In a study of nociceptin knockout mice with induced colitis compared wild-type mice it was found that the administration of oral dextran sulphate sodium (DSS) caused bloody diarrhoea in the NOP wt. group but had no effect in the NOP-KO group. Histology showed distortion in the colon crypts and elevated number of lymphocytes, macrophages, and neutrophilsin the KO group compared to wild type mice(49). This study showed that the absence of NOP significantly reduced the inflammatory response to a known pro-inflammatory stimulus in chemically-induced colitis(44).

These data suggest that elevated circulating nociceptin levels not necessarely correlate with the levels nociceptin transported by inflammatory cells. The other possible

explanation is that elevated plasma nociceptin levels down-regulate the nociceptin expressing genes.

Pain is mediated by prostaglandins, substance P, histamine, and other substances and is one of the prominent features of inflammation/ischemia. . Endogenous opioids provide analgesia by increasing the number of opioid receptors and the availability of endogenous opioid peptides at the site of inflammation(44). The immun-modulatory effects of nociceptin and its receptor may be similar to the effect of opioids and their receptors at the site of inflammated terminal nerve endings. Opioid receptors are upregulated at these locations, since there is an increased intra-axonal transport of receptor from the dorsal root ganglia, where they are synthetized.
This process, the transport of reeceptors to the terminal nerve endings, is mediated by interleukin 1beta (44).
Opioid-laden inflammatory cells - lymphocytes, monocytes, PMN, and macrophages - migrate to the inflamed tissue and release the endogenous opioids such as endorphin, met-enkephalin and dynorphin-A.They are stimulated by endotoxins, viruses, IL-1, and corticotropin releasing hormone (CRH). This novel theory of analgesia- endogenous opioids acting at peripheral sites - has been suggested as being part of a physiological neuro-immune-hormone axis(34, 50).

NOP has a similar distribution to classical opioid receptors in neuronal tissue and is also present in leucocytes. The antinociceptive effect of N/OFQ in an animal model of bowel inflammation has been examined by comparing peripherally N/OFQ.
Nociceptin administered intraperitonially decreased the painful response induced in rats with experimental colitis. Administration of N/OFQ antagonists increased the pain and diminished the positive anti-pain effect when given together with nociceptin(51). The authors observed an increased number of cells positive for NOP and myeloperoxidase activity - a marker of PMN granulation – confirming the infiltration of immunocytes into the inflamed tissue. Their findings supported two hypothesis: first that N/OFQ may act peripherally as an analgesic at sites of inflammation, second, that N/OFQ and its receptor NOP play a role in chemotaxis to the sites of inflammations. These actions of

nociceptin on PMN leukocytes and PBMCs should be further investigated in the chronic inflammation of the atherosclerotic plaque.

1.3. Heat shock proteins

Heat shock proteins (HSP) are phylogenetically highly conserved molecules in their molecular and immunological structure and biochemical properties. HSP maintains the homeostasis of cells and tissues. The members of the HSP family are categorised based on their molecular weight (kD), such as small Hsp, Hsp40, Hsp60, Hsp70, Hsp90 and Hsp110. The subtypes are expressed in various cell compartments and regulate structural proteins(52). One of thesee functions is `chaperoning`: they maintain the correct formation of proteins, even in circumstances of heat or oxiadative stress. Heat shock protein-1 regulates the synthesis of HSP, which can be activated by various stress signals such as hypoxia-ischemia, heat, or proinflammatory stress. HSP helps the cells to withstand the worsening environment (53).

1.3.1. 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 (54).

It has been recognised that Hsp70 are present in the peripheral circulation of normal individuals(55, 56)providing the first evidence that Hsp70 may be released into the extracellular environment not only in response to stress but also under physiologic conditions. Soluble Hsp70 showed no apparent endogenous circadian rhythm in a rested state (57). Elevated serum Hsp70 level was measured in pathologic pregnancies (58)such as preeclampsia and hemolytic anemia elevated liver enzymes low platelet count (HELLP) syndrome.Increased serum levels of Hsp 70 levels were reported in patients with peripheral and renal vascular disease (59) and after acute myocardial infarction (60, 61), whereas circulating Hsp70 levels were positively related to markers

of tissue damage including creatine kinase MB and cardiac troponin T. In chronic heart failure (CHF) patients increased serum Hsp70 levels were found compared to healthy controls (62). It was shown that Hsp70 levels positively correlate to NYHA (New York Heart Association) classes. Gombos et al. (63) completed these findings in CHF by showing significant associations between the Hsp70 and markers of disease severity such as lower ejection fraction and higher NT-proBNP levels. Elevated Hsp70 and heme oxygenase-1 (HO-1) levels have been reported in response to LPS-induced endothelial injury (64) and in brain stem death triggered by hypoxia (65).

The source of the serum Hsp70 has not been fully clarified; however, studies suggest that Hsp70 may be released from viable endothelial cells within exosomes (66). This mechanism can be induced by certain cytokines in breast adenocarcinoma (67). Hsp70 expression was investigated in vitro in monocytes of patients suffering from obstructive sleep apnea syndrome(68)and of peripheral artery disease (69, 70) where lower inducible monocyte Hsp70 levels were found compared to healthy controls. Hsp 70 is expressed in small arteries (71), explicated in dendritic cells of the arterial intima (72) and in smooth muscle cells (73). Inducible Hsp 70 can be released from the hepatosplanchnic tissue during exercise (74) or from the myocardium itself (75).

1.3.2. Heat shock protein 70 function in atherosclerosis – anti or proinflammatory?

There is an interesting debate in the literature whether Hsp70 is a pro-or antiinflammatory agent. In their sophisticated review, de Jong et al. (76)summarized the novel achievements of this debate, where they pointed out thatHsp70 has immunoregulatory properties with both pro- and anti-inflammatory effects in a cross-species manner(76). Interestingly it depends on the context and the actual source of Hsp70. Cytosolic Hsp70 is in charge to decrease inflammatory activity(76) In contrast, extracellular Hsp70 is immunostimulatory properties and has therefore been termed as a `chaperokine`(77). The latter is in agreement with the “danger model” proposed by Matzinger(78). This interesting model states that an exogenous or endogenous stress signal is needed for endogenous HSP upregulation, where they hamper inflammatory pathways and start tissue repair. On the other hand, the same receptors detect extracellular Hsp70 as well, such as CD40, CD91, CD94, CD195 (CCR5), oxidized

low-density lipoprotein receptor-1 and TLR-2 and TLR-4 (76). Monocytes, macrophages, dendritic cells, T-lymphocytes, NKcells and T-lymphocytes express those receptors where Hsp70 can can act as an immunmodulator(79). De Jong et al. summarizes that meanwhile intracellular Hsp70 functions as an anti-inflammatory agent, its extracellular action is largely pro-inflammatory(76). This might be a `dichotomous immunoregulatory effect`(76), however a possible explanation would be while intracellular Hsp70 tries to hamper proinflammatory stress in the living cells, there is one point when the already dying cells produce a waste number of Hsp70 and secrete it into the extracellular space and the circulation. The elevated levels of Hsp70 would act as `danger signals` for other cells where they react for the stress by enhancing their pro-inflammatory mechanisms. Therefore the measurement of inducible Hsp70 levels might be a good tool for evaluating inflammatory diseases such as atherosclerosis.

To further support this hypothesis, it has been shown that a number of cell types overexpress Hsp70 in advanced atheroma, including inflammatory cells and SMC-s as well. In the early lesions, only dendritic cells seem to overexpress Hsp70, which might activate T cells in the plaque. Hsp70 could facilitate the APC-s lipid presentation by acting locally in the atheroma. It was suggested that the Hsp70 overexpressing dendritic cells might be responsible for the earliest steps towards atherogenesis in the intima (72).

Two very sophisticated study by Asea et al. also investigated whether macrophage-derived Hsp70 can induce cytokine production in naive macrophages and what are the receptors for this effect(80, 81). They showed a previously unknown function of Hsp70, namely the exogenous Hsp70 might be major paracrine inducer of cytokine expression and secretion through a CD14, TLR2/4 and Myd88 dependant pathway in human oxLDL-induced macrophages and provide a link between the establishment of lipid-laden foam cells and the initiation of inflammatory processes in atherosclerotis(82). However, Zhu et al. showed quite the opposite findings in a clinical study, where higher serum levels of Hsp70 were associated with a lower risk of coronary artery disease(83).

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 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.

In a study with acute coronary syndrome patients we aimed to answer the following question:

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?

Despite multiple recent studies focused on Hsp70 as a marker of the stress changes in the body, less is known about its biological correlates in the clinical setting. 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

3.1. Study populations and research design

3.1.1. Nociceptin/orphanin FQ measurements in ischemic cardiovascular patients

3.1.1.a Chronic heart failure and peripheral artery disease patients (1. 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.) (20, 21). 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. Exclusion criteria included unstable angina, elevated cardiac troponin enzymes, pulmonary disease, kidney or liver failure, systemic inflammatory processes or chronic musculoskeletal disease.

3.1.1.b Acute coronary syndrome patients (2. patient group)

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

Electrocardiography was made to determine if there was an ST elevation (>1 mm). We used a troponin-T quick test as a specific marker of myocardial necrosis. Blood samples were collected for usual laboratory measurements and enzyme diagnostics (Table II). 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. Post-interventional coronarography showed good hemodynamic results.

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. None of these patients suffered any pain related to their cardiac disease during the previous week of admission. Seven of them underwent elective coronarography; intervention was indicated and performed in four cases. All cardiologic patients underwent ultrasonography before being released from the hospital, all completed by the same cardiologist experienced in echocardiography. 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 (21).
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.

3.1.2. 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 who had acute onset of lower limb ischemia, clinical or laboratory signs of acute infection,myocardial infarction, stroke, trauma and surgical procedure in the last 6 months were excluded in this study. 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.

3.2. Clinical data

Diagnosis of multiple or single coronary stenosis, aortic valve stenosis and peripheral artery disease as well as the indication for cardiac and/or vascular surgery (coronary artery bypass grafting, aortic valve repair, endarterectomy or arterial bypass) was evaluated by independent cardiologists, cardiac- and vascular surgeons, who were not participated in this study.

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. The patients lied in supine position after resting at normal room temperature; measurements were taken at each ankle over the posterior tibial and dorsal pedal arteries. ABI was calculated as the lowest pressure of the ankles divided by the higher of the left and right arm pressures (84, 85).

In the first study group, blood pressure and pulse was also measured on the same day before the blood samples were taken.

Transthoracic echocardiograms were performed by one experienced cardiologist blinded for other study information. 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 cm²

was considered severe (86). 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) and convex (3.5-5MHz) transducer of a Toshiba Aplio SSA-770 ultrasound system. The mean value and the maximum IMT was used for calculations (87). At the same examination carotid stenosis was also determined (88), 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 (89, 90).

3.2.1. 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 (ABLE 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).

3.2.2. 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.(91) 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.

3.3. Statistics

3.3.1. 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. Data are presented in the text as median and interquartile range (IQR). $P<0.05$ was considered as statistically significant. Statistical analyses were carried out by Statistica 8.0 (StatSoft Inc. Tulsa, OK, USA), SPSS 15.0.1 (SPSS Inc. Chicago, IL, USA) and self-devised scripts running in the MATLAB software environment (The MathWorks Inc., Natick, MA, USA).

3.3.2. Statistical analysis - Hsp70 study

As most of the variables had non-Gaussian distributions, data are presented in the text and in the tables as median (25th–75th percentile) or as number (percent). 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. 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

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

The baseline clinical and laboratory characteristics of the study population are reported in Table 1 and in Table 2.

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 as visualized on Fig. 1 and Fig. 2.

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 ($p=0.014$) (Fig. 1).

Nociceptin/ orphanin FQ plasma levels showed a marked difference between PAD and control groups ($p=0.001$) (Fig. 2).

Table 1. Demographic findings – nociceptin study

	Stable angina pectoris n=22	Peripheral artery disease n=12
Gender (M/F)	14/8	8/4
Age (years)	72.5 (67.8-76)	65 (59-68)
BMI (kg/m²)	26.2 (24.6-28.7)	25.8 (24.5-30.5)
Risk factors		
Diabetes mellitus	7 (32%)	4 (33%)
Hypertension	20 (91%)	11 (92%)
Hyperlipidemia	15 (68%)	5 (42%)
Current smoking	7 (32%)	6 (50%)
Drug therapy		
Aspirin	7 (32%)	11 (92%)
Clopidogrel	4 (18%)	2 (17%)
LMWH	4 (18%)	1 (8%)
Oral anticoagulants	6 (27%)	2 (17%)
ACE-inhibitors	14 (64%)	5 (42%)
Angiotensin receptor blockers	8 (36%)	-
β-Blockers	14 (64%)	5 (42%)
Calcium antagonists	6 (27%)	6 (50%)
Nitrates	5 (23%)	2 (17%)
Glycosides	2 (9%)	-
Statins	13 (59%)	5 (42%)
Oral hypoglycemics	4 (18%)	2 (17%)
Diuretics	12 (54%)	2 (17%)

This table shows the demographic characteristics of stable angina pectoris and peripheral artery disease patients enrolled in our first patient cohort.

Table 2. Laboratory parameters – nociceptin study

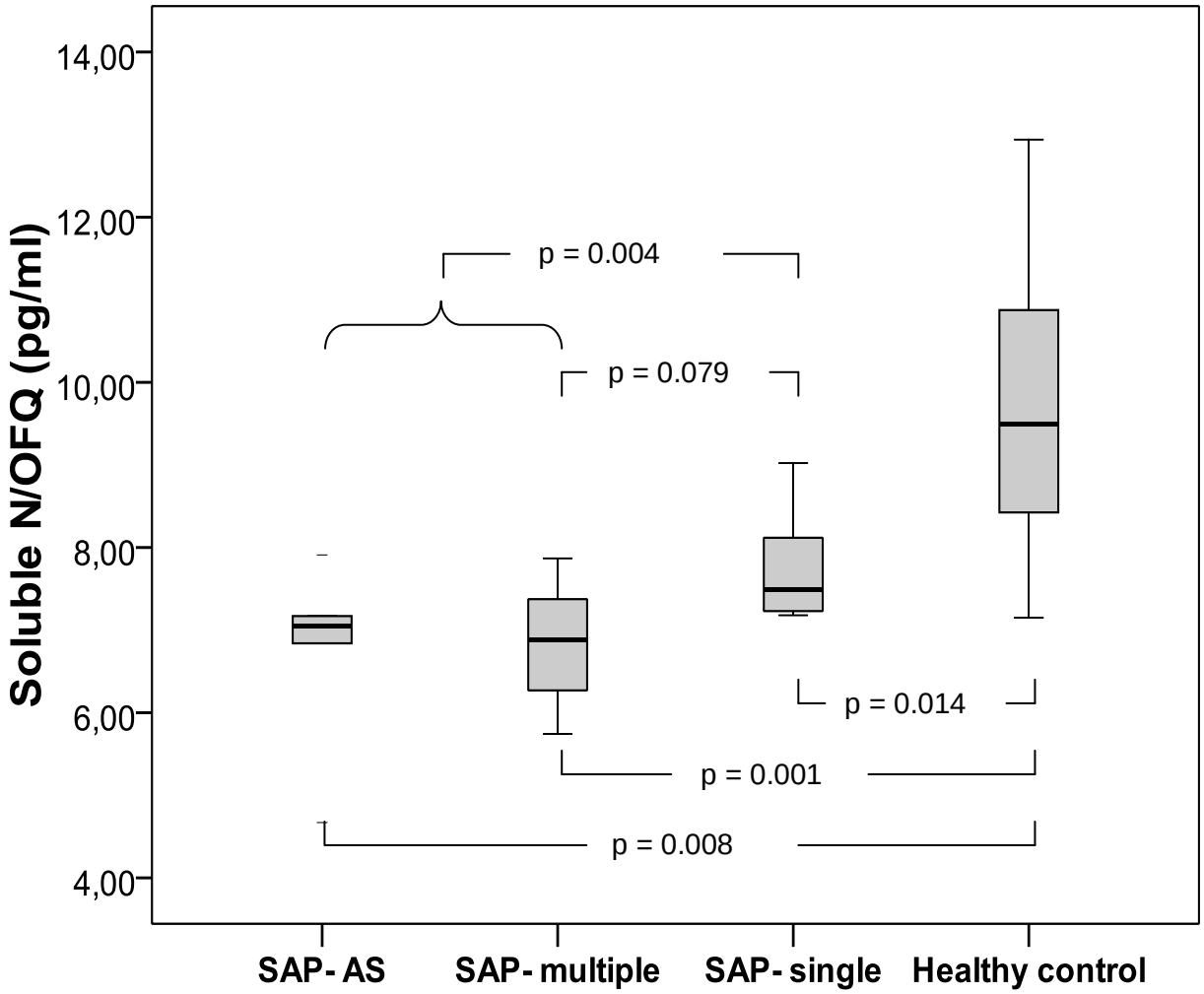
	Stable angina pectoris n=22	Peripheral artery disease n=12
White blood count (G/l)	10 (6-13)	8 (6-12)
Thrombocytes (G/l)	140 (102-204)	229 (188-251)
Hemoglobin (mmol/l)	12.3 (9.7-13.5)	14.5 (13.9-15.1)
Serum sodium (mmol/l)	137 (133-140)	141 (138-143)
Serum potassium (mmol/l)	4.4 (4.1-4.9)	4.5 (4.3-4.8)
Serum creatinine (µmol/l)	98.0 (81.0-118.0)	71 (65-93)
Serum carbamide (mmol/l)	7.7 (5.5-10.8)	6.6 (5.20-7.85)
eGFR (ml/min)	60 (50-69)	88 (74-110)
Triglyceride (mmol/l)	1.6 (1.3-2.1)	1.3 (1-2.5)
Total cholesterol (mmol/l)	4.5 (3.7-5.1)	4.9 (4-6.5)

This table shows the clinical laboratory results of stable angina pectoris and peripheral artery disease patients enrolled in our first patient cohort. The samples were simultaneously taken with the plasma samples for the nociceptin/ orphanin FQ measurements.

eGFR estimated glomerular filtration rate

Values are median (interquartile range) or number (%)

Figure. 1. Box and whiskers plots show plasma N/OFQ levels in stable angina pectoris expressed with median, maximum and minimum values and interquartile ranges

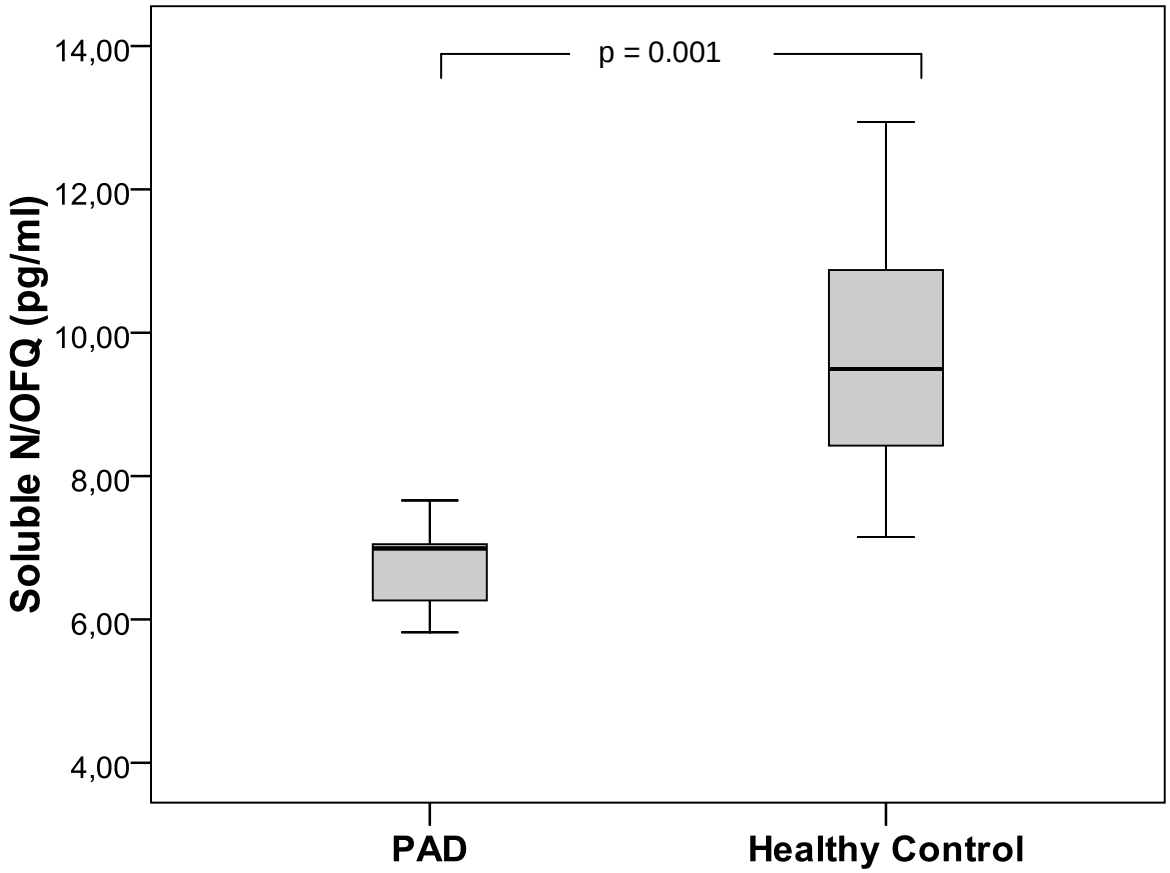


SAP-AS: Stable angina pectoris – aortic stenosis

SAP-multiple: Stable angina pectoris – multiple coronary stenosis

SAP-single: Stable angina pectoris – single coronary stenosis

Fig. 2. Box and whiskers plots shows plasma N/OFQ levels in peripheral artery disease expressed with median, maximum and minimum values and interquartile ranges



PAD: peripheral artery disease

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.

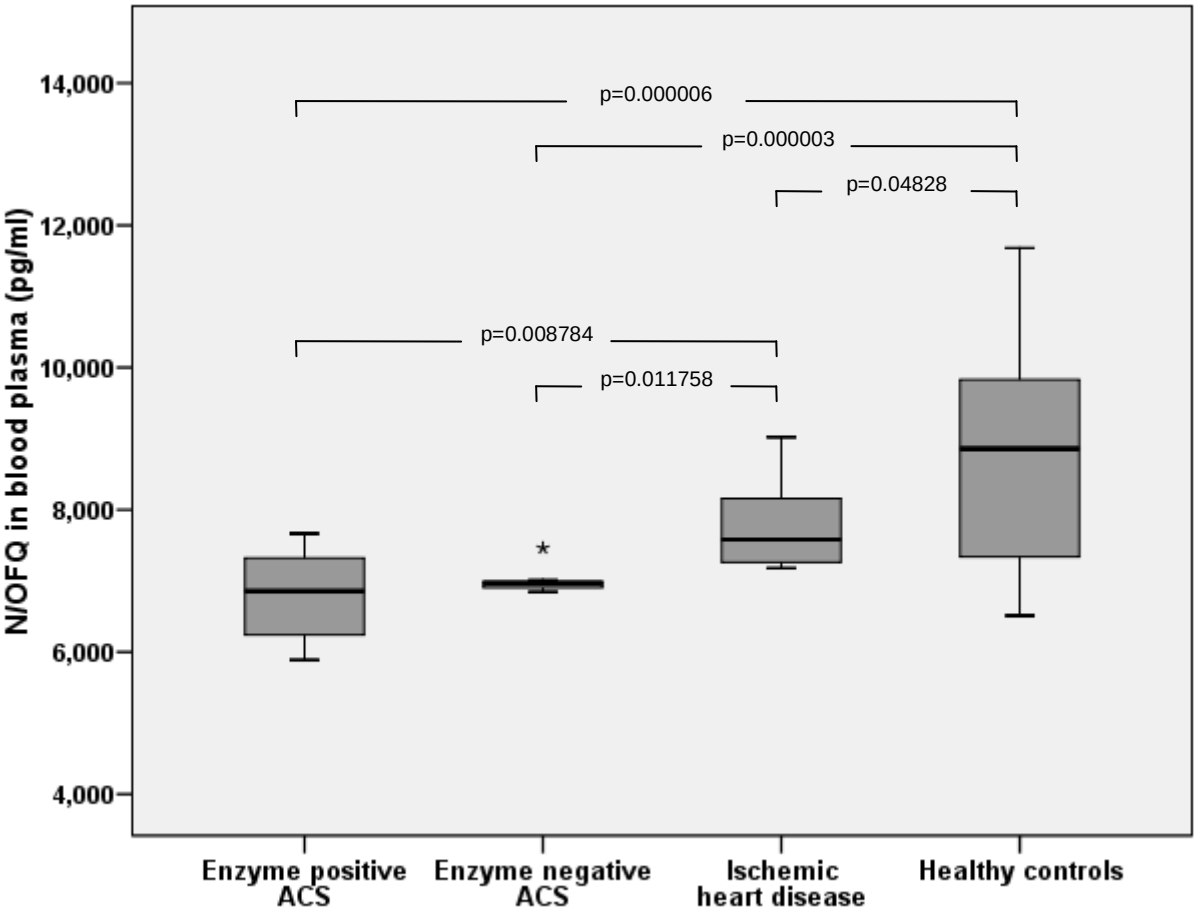
Exploratory factor analysis with varimax rotation was used to identify the underlying hidden structure of the relationships between the following simultaneously measured variables: N/OFQ, age, WBC, THR, Hb, cholesterol, triglyceride, glucose A, eGFR, creatinine, systolic blood pressure, and diastolic 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.

4.1.2. **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 (Fig. 1. showing the interquartile ranges) analysis of variances were performed by Brown-Forsythe ANOVA. 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 ($p=0.012$).

Fig. 1. Box and whisker plot of plasma N/OFQ levels shows the median, minimum and maximum values and the interquartile ranges in the groups of enzyme positive ACS (n=10), enzyme negative ACS (n=7), ischemic heart diseases (n=11) and in healthy control subjects (n=31).



Statistical analyses of patient characteristics found the following variables also showing difference between the three patient groups (Table 1.). 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 (Table 2.) 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 (Table 2.).

Strong linear association was detected in the enzyme positive group between plasma N/OFQ levels and white blood cell (WBC) and platelet (PLT) count (Fig. 2., r=0.93, p=0.0001 for WBC, and 0.69 with p=0.03 for PLT) (Fig. 1.).

Table I. Patient characteristics

	Enzyme positive ACS (n=10)	Enzyme negative ACS (n=7)	Ischemic heart disease (n=11)
Sex (male/female)	4/6	4/3	6/5
Age (years)	71 (62-79)	57 (52-65) *	75 (62-76)
BMI (kg/m^2)	23.8 (23.5-31.2)	25.3 (23.2-34.9)	25.3 (23.8-28)
Systolic pressure (mmHg)	128 (100-140)	132 (114-143)	120 (112-140)
Diastolic pressure (mmHg)	70 (50-72)	75 (65-79)	70 (70-77)
Heart rate (beats/min)	80 (73-85)	72 (65-75)	72 (67-81)
Ejection fraction (%)	50 (42.5-52.5)	56 (55-57.75) *	45 (32-55)
Risk factors			
Previous myocardial infarction	3 (30%)	1 (14%)	5 (45%)
Previous PCI	3 (30%)	2 (29%)	7 (64%)
Previous stroke	1 (10%)	0 (0%)	2 (18%)
Current smoking	3 (30%)	2 (29%)	2 (18%)
Diabetes mellitus	6 (60%)	1 (14%)	2 (18%)
Hypertension	9 (90%)	7 (100%)	11 (100%)
Hyperlipidaemia	7 (70%)	4 (57%)	9 (82%)

Drug therapy			
Aspirin	4 (40%)	4 (57%)	6 (55%)
Ticlopidine	1 (10%)	0 (0%)	1 (9%)
Clopidogrel	2 (20%)	3 (43%)	2 (18%)
Oral anticoagulants	0 (0%)	2 (29%)	3 (27%)
Beta-blockers	5 (50%)	5 (71%)	6 (55%)
Calcium antagonists	3 (30%)	2 (29%)	3 (27%)
ACE-inhibitors	7 (70%)	5 (71%)	7 (64%)
Angiotensin-II antagonists	2 (20%)	3 (43%)	5 (45%)
Nitrates	2 (20%)	2 (29%)	4 (36%)
Statins	5 (50%)	4 (57%)	8 (73%)
Oral antidiabetics	4 (40%)	1 (14%)	2 (18%)
Insulin	1 (10%)	0 (0%)	0 (0%)
Diuretics	4 (40%)	4 (57%)	7 (64%)
Antiarrhythmics	3 (30%)	3 (43%)	6 (55%)

Values are expressed as median (interquartile ranges) or as the number of patients (percentages)

P values show differences compared to the enzyme positive ACS group ($p<0.05$ *).

Table II. Clinical chemistry

	Enzyme positive ACS (n=10)	Enzyme negative ACS (n=7)	Ischemic heart disease (n=11)
Creatinine kinase (U/l)	818.50 (211.25-2861.50)	132.00 (81.50-148.00) *	95.00 (89.50-110.25) *
LDH (U/l)	802.50 (488.50-2475.00)	399.00 (333.00-483.50) *	552.00 (319.25-673.75)
CRP (mg/l)	26.630 (2.63-101.73)	2.50 (2.40-67.40)	2.70 (0.40-5.00)
HBDH (U/ml)	347.50 (230.25-1071.25)	167.50 (151.00-206.50)	191.50 (131.50-268.75)
INR	1.18 (1.03-1.20)	1.50 (1.10-1.89)	1.53 (1.05-2.00)
White blood cell count (G/l)	10.50 (6.23-19.08)	9.60 (7.40-13.20)	6.80 (5.30-10.00)
Platelet count (G/l)	214.50 (160.00-254.25)	223.00 (165.50-290.00)	214.00 (201.00 - 230.00)
Hb (g/l)	12.15 (10.33-13.00)	14.50 (11.80-15.55)	13.20 (12.50-13.90)
Serum sodium (mmol/l)	141.00 (137.75-142.75)	143.00 (140.50-146.50)	138.00 (137.00-141.00)
Serum potassium (mmol/l)	4.35 (3.93-4.53)	4.30 (3.95-4.40)	4.30 (4.10-4.90)
Cholesterol (mmol/l)	5.00 (2.50-5.55)	4.45 (4.40-5.25)	3.90 (3.35-4.55)
Tryglicerid (mmol/l)	0.60 (0.50-1.05)	1.45 (0.80-2.48)	1.27 (0.90-1.50)
LDL (mmol/l)	4.10 (1.40-4.90)	3.30 (3.30-4.00)	2.80 (2.80-3.20)
HDL (mmol/l)	1.20 (0.90-1.70)	1.30 (0.83-1.48)	1.10 (0.93-1.58)
Admission glucose (mmol/l)	11.45 (6.25-17.45)	5.20 (5.03-6.43) *	5.70 (4.78-7.50) *
Fasting glucose (mmol/l)	6.40 (4.85-9.25)	6.25 (4.80-7.70)	5.70 (5.70-5.70)
ALP (U/l)	177.50 (92.25-193.00)	254.50 (171.50-	187.00 (134.50-

		302.25)	253.00)
GOT (U/l)	35.00 (24.75-208.50)	20.00 (17.50-23.50)	23.00 (18.75-27.25)
GPT (U/l)	24.00 (17.50-44.50)	18.00 (14.50-33.00)	19.50 (13.50-33.00)
Serum creatinine (µmol/l)	95.50 (79.50-244.25)	90.00 (65.50-118.50)	109.00 (81.00-178.00)
Serum carbamide (mmol/l)	9.00 (5.38-19.13)	6.30 (4.90-10.35)	6.60 (5.25-10.33)
Nociceptin/ orphanin FQ (pg/ml)	6.86 (6.24-7.32)	6.97 (6.87-7.01)	7.58 (7.23-8.20) **

Values are expressed as median (interquartile ranges) or as the number of patients (percentages)

LDH lactate dehydrogenase CRP C-reactive protein, HBDH hydroxybutyrate dehydrogenase, INR International Normalized Ratio, Hb hemoglobin, LDL low-density lipoprotein- cholesterol, HDL high-density lipoprotein-cholesterol, ALP (alkaline phosphatase), GOT (glutamate oxalacetate transaminase), GPT (glutamic pyruvic transaminase)

P values show differences compared to the enzyme positive ACS group ($p<0.05$ *, $p<0.01$ **).

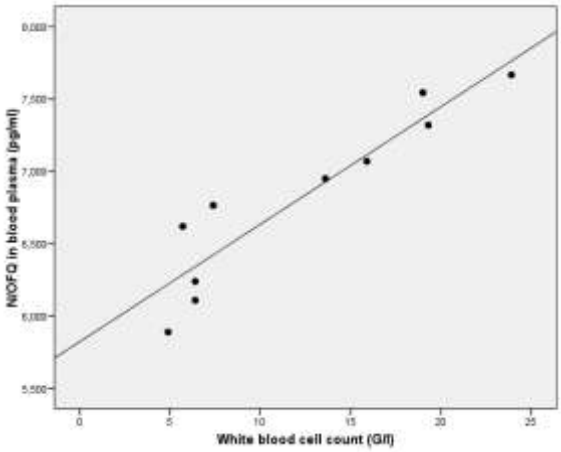
We noticed further significant correlations between N/OFQ levels of the subjects in the same group of enzyme positive ACS and creatine-kinase (CK), and plasma cholesterol level (correlation coefficients and p values were 0.73 with $p=0.02$ for CK, and -0.66 with $p=0.05$ for cholesterol) (Fig. 3.). Significant 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).

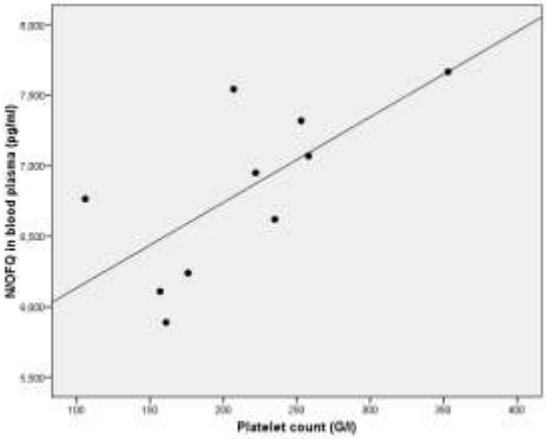
Exploratory factor analysis classified the 25 patient characteristic variable together with the plasma N/OFQ into three factors wich explain nearly equal proportions of the total variance. The factors represent common underlying hidden phenomena and are responsible for the covariation between the observed 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.

Fig. 2. Scatterplots showing the linear regression between the N/OFQ level and other laboratory parameters in the group of enzyme positive acute coronary syndrome (EPACS).**A**: N/OFQ and WBC count (r=0.93, p=0.0001). **B**: N/OFQ and platelet count (r=0.69, p=0.03) **C**: N/OFQ and serum CK (r=0.73, p=0.02).

A



B



C

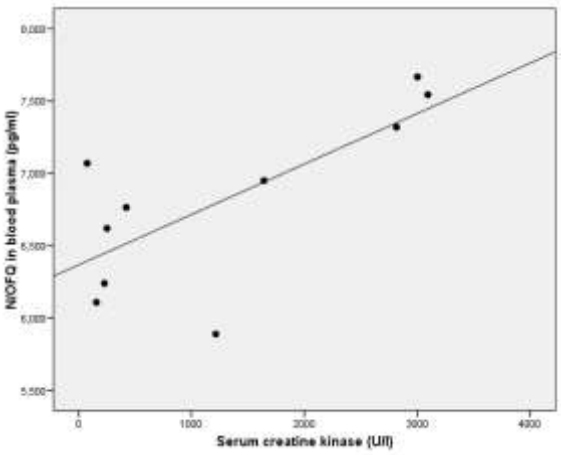
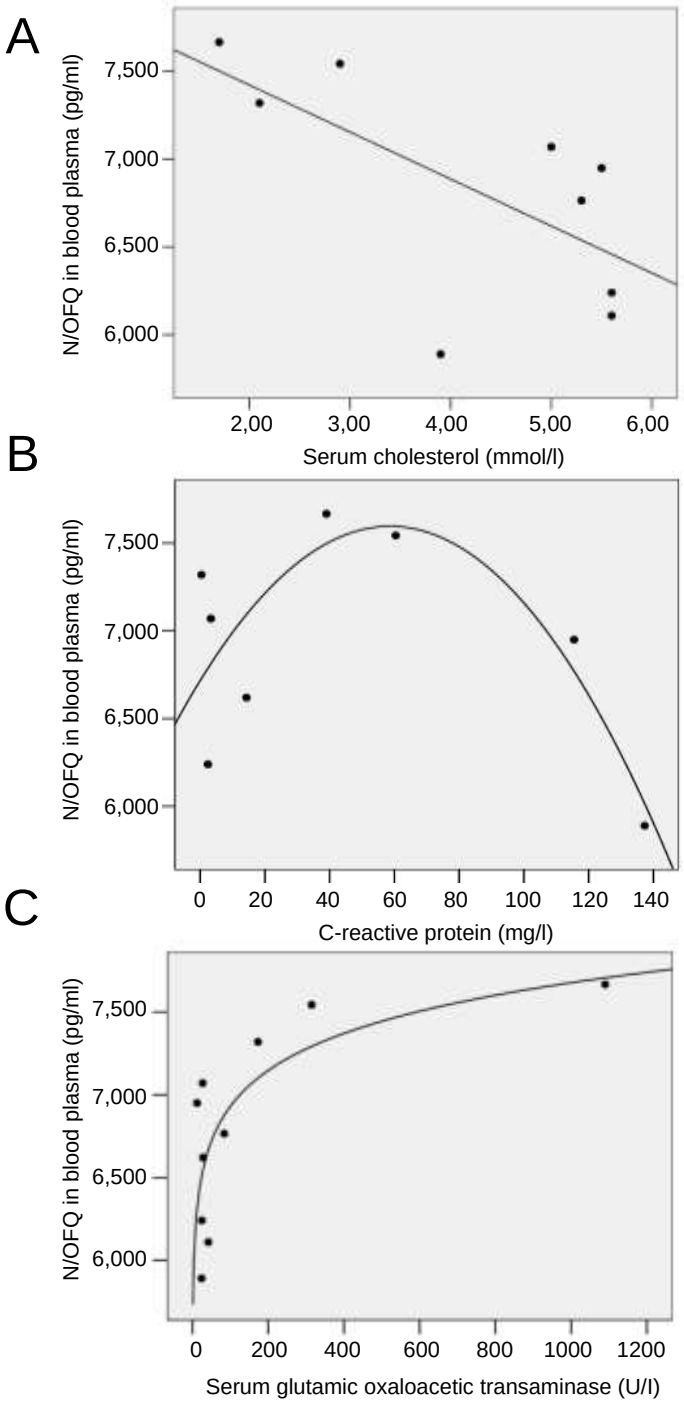


Fig. 3. Scatterplots showing the linear regression between A: N/OFQ and cholesterol (r=-0.66, p=0.05) and nonlinear regression between **B**: N/OFQ and c-reactive protein (R^2=0.629, p=0.07). **C**: N/OFQ and serum GOT (R^2=0.49, p=0.03)Outliers are not shown.



4.2. Association of soluble heat shock protein 70 with vascular calcification

4.2.1 Patient characteristics

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. The baseline clinical and laboratory characteristics of the study population are reported in Table 1. 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 values 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.

Table 1. Clinical patient characteristics (n=180) – sHsp70 study

Variables	
Demographics and risk factors	
Age (years)	64.1 (57.04-70.66)
Gender (male)	124 (68.89%)
BMI	26.1 (23.88-28.99)
Smoking (years)	35 (25-41)
Consumed cigarettes/day	20 (20-30)
Hypertension	140 (77.78%)
Diabetes	62 (34.44%)
Ischemic heart disease	65 (36.11%)
Carotid stenosis significant (%)	95 (52.78%)
Carotid stenosis maximum	0.6 (0-0.9)
Fontaine stages I, II/a, II/b, III, IV (n=143)	14, 21, 83, 12, 13
ABI	0.50 (0.26-0.76)
IMT mean (mm)	0.83 (0.70-0.97)
IMT maximum (mm)	1.00 (0.80-1.30)
Calcification score	5 (4-6)

Table 1. Clinical patient characteristics (n=180) – sHsp70 study

Drug therapy	
Aspirin	118 (65.56%)
Clopidogrel	49 (27.22%)
Oral anticoagulants	12 (6.67%)
ACE-inhibitors	102 (56.67%)
Angiotensin receptor blockers	102 (56.67%)
β-blockers	83 (46.11%)
Oral hypoglycemics	43 (23.89%)
Insulin	18 (10.00%)
Statins	105 (58.33%
Laboratory findings	
Serum sodium (mmol/l)	141 (138-143)
Serum potassium (mmol/l)	4.5 (4.3-4.8)
Serum calcium (mmol/l)	2.47 (2.38-2.53)
Serum phosphate (mmol/l)	1.11 (0.97-1.21)
Serum creatinine (μmol/l)	89 (80-103)
Serum carbamide (mmol/l)	6.6 (5.20-7.85)
eGFR (ml/min)	75.8 (57.17-94.36)
Serum bilirubin (μmol/l)	9.65 (7.25-13.05)

Table 1. Clinical patient characteristics (n=180) – sHsp70 study

Laboratory findings (continued) AST (U/l)	 21 (17-26)
ALT (U/l)	21 (14-28)
Gamma-GT (U/l)	33 (22-55)
Serum albumin (g/l)	46.5 (43.20-49.00)
CRP (mg/l)	2.7 (0.9-6.5)
Homocysteine (µmol/l)	16.1 (13.20-19.30)
Triglyceride (mmol/l)	1.7 (1.20-2.40)
Total cholesterol (mmol/l)	5.2 (4.30-6.40)
LDL (mmol/l)	3.1 (2.61-4.07)
HDL (mmol/l)	1.4 (1.20-1.58)
Fetuin-a (µg/l)	732 (648-804)
Soluble Hsp70 (ng/ml)	0.63 (0.52-0.81)

BMI Body mass index *ABI* Ankle-brachial index, *IMT* intima-media thickness, *AST*aspartate aminotransferase, *ALT*alanine aminotransferase, *Gamma-GT* gamma-glutamyl transpeptidase, *CRP*C-reactive protein, *eGFR* estimated glomerular filtration rate, *LDL* low-density lipoprotein- cholesterol, *HDL* high-density lipoprotein-cholesterol. Values are median (interquartile range) or number (%)

4.2.2 Correlation of soluble Hsp70 levels with calcification severity

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 (Table 2)

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 (Table 2).

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, $p<0.02$). The significant relationships between soluble Hsp70 levels and homocysteine, serum bilirubin levels and calcification scores are visualized on Figure 1 (Fig.1. a,b,c).

Table 2. Correlation coefficients with p values between serum Hsp70 levels and clinical and laboratory variables (total n=180)

Variables	**r**	**p-value**
Age	***0.150549***	***0.045485***
Gender (male)	-0.026178	0.729442
BMI	0.083219	0.270798
Smoking (years)	0.090170	0.269263
Consumed cigarettes/day	0.031008	0.704520
Hypertension	0.097433	0.196998
Diabetes	-0.007125	0.925012
Ischemic heart disease	0.099890	0.185885
Carotid stenosis significant (%)	0.055731	0.461263
Carotid stenosis maximum	0.061359	0.417191
Fontaine stages I, II/a, II/b, III, IV (n=143)	-0.157684	0.062791
ABI	0.062841	0.406005
IMT mean (mm)	0.076109	0.325362
IMT maximum (mm)	-0.031641	0.682991
Calcification score	***0.168998***	***0.024535***
Serum creatinine (µmol/l)	0.111446	0.139731
Serum carbamide (mmol/l) Urea	0.030041	0.691422
eGFR (ml/min)	-0.094085	0.212905

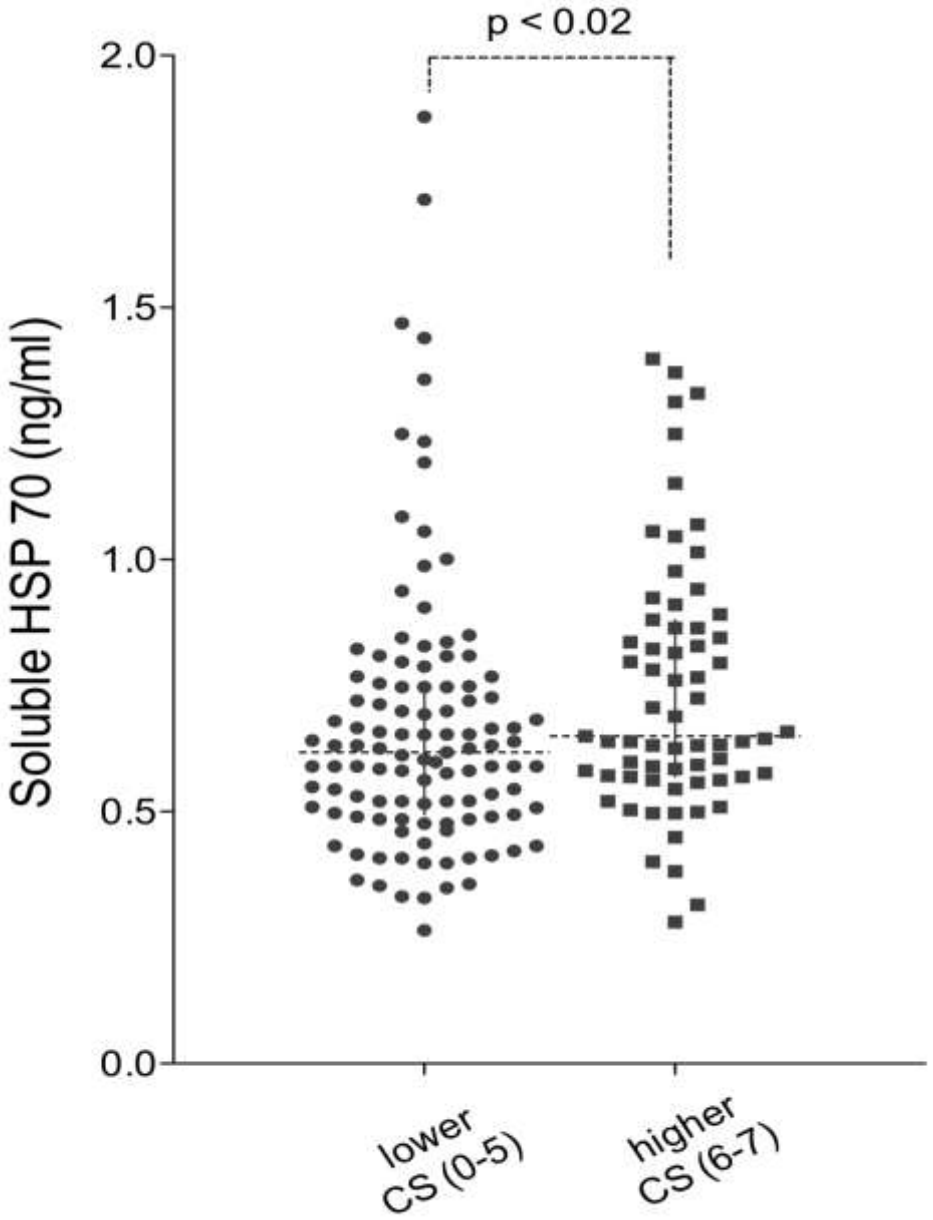
Table 2. Correlation coefficients with p values between serum Hsp70 levels and clinical and laboratory variables (total n=180)

Variables	**r**	**p-value**
Serum bilirubin (µmol/l)	***0.233000***	***0.002036***
AST (U/l) GOT	0.126953	0.094095
ALT (U/l) GPT	0.101128	0.184258
Gamma-GT (U/l)	0.150425	0.046923
Serum albumin (g/l)	-0.045852	0.551502
CRP (mg/l)	-0.085874	0.262677
Homocysteine (µmol/l)	***0.179437***	***0.017829***
Triglyceride (mmol/l)	-0.026359	0.729904
Total cholesterol (mmol/l)	-0.028605	0.707898
LDL (mmol/l)	-0.027531	0.728840
HDL (mmol/l)	0.020420	0.795849
Fetuin-a (µg/l)	0.003335	0.964965

BMI Body mass index, *ABI* Ankle-brachial index, *IMT* intima-media thickness, *AST*aspartate aminotransferase, *ALT*alanine aminotransferase, *Gamma-GT* gamma-glutamyl transpeptidase, *CRP*C-reactive protein, *eGFR* estimated glomerular filtration rate, *LDL* low-density lipoprotein- cholesterol, *HDL* high-density lipoprotein-cholesterol.

Figure 1 (Fig.1. a,b,c) Association of serum Hsp70 levels with calcification score (a) and homocysteine levels (b) and serum bilirubin levels (c) in patients with peripheral artery disease and carotid stenosis.

Figure 1. a. Association of serum Hsp70 levels with calcification score (CS)



This figure shows that sHsp70 levels are elevated in patients with higher calcifications scores compared to those with lower calcification scores.

Figure 1. b. Association of serum Hsp70 levels with homocysteine levels

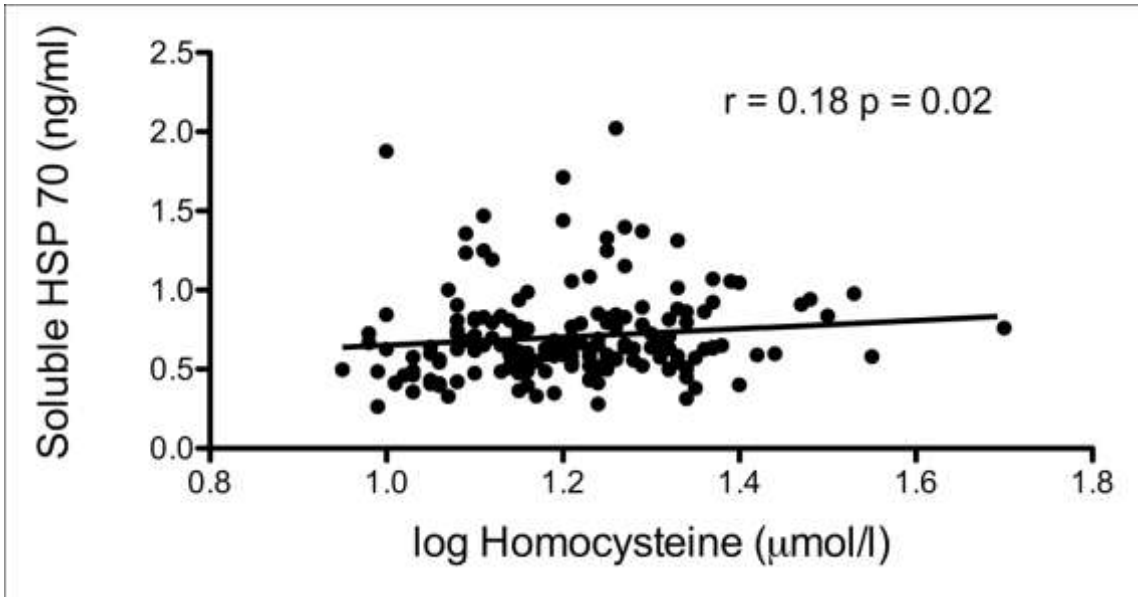
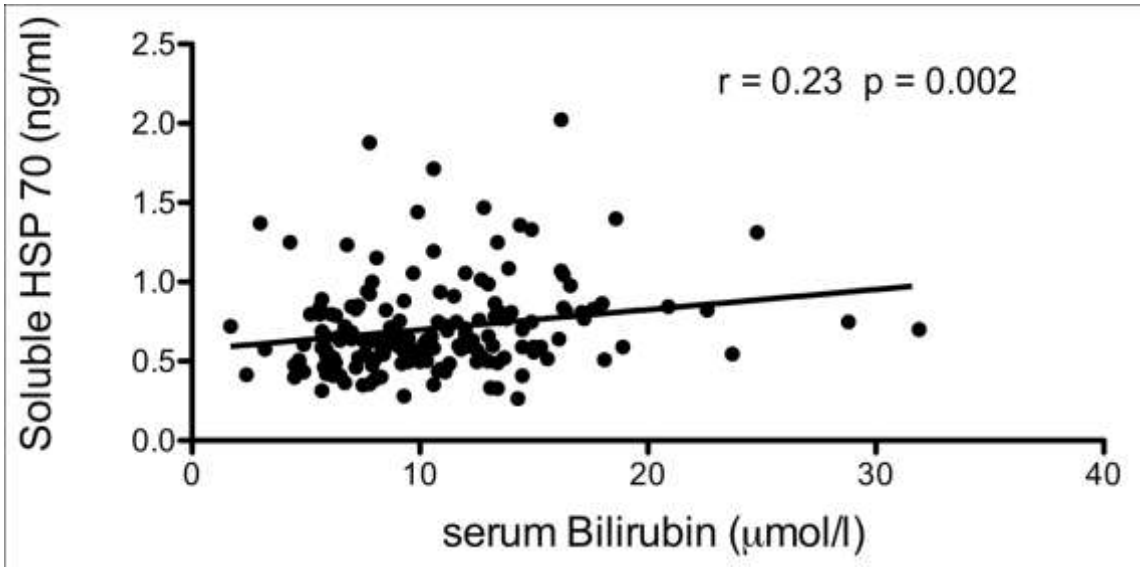


Figure 1. c. Association of serum Hsp70 levels with serum bilirubin levels



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.

Table 3. Association between sHsp70 and arterial calcification score

	Odds of more severe arterial calcification	*p-value*
unadjusted	2.189 (1.156-4.144)	0.016
Model 1	2.110 (1.066-4.175)	0.032
Model 2	2.233 (1.054-4.730)	0.036
Model 3	2.403 (1.115-5.181)	0.025
Model 4	2.264 (1.021-5.020)	0.044

Odds ratio (OR) and 95 % confidence intervals (CI) were obtained by logistic regression model

Model 1. Demographics: age, gender, eGFR

Model 2. Model 1+Smoking (years)

Model 3. Model 2+CRP

Model 4. Model 3+Homocysteine

5. DISCUSSION

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

Plasma nociceptin/orphanin FQ levels were significantly lower in patients with chronic angina pectoris and peripheral artery disease than in healthy controls. Nociceptin levels did not significantly differ in patients with aortic stenosis, multiple coronary stenosis compared to patients with intermittent claudication or rest pain medicated with analgetics. None showed any discomfort or chest/ limb pain during the last one week before the fasting blood samples for N/OFQ levels were taken. The novel finding of this study is the striking decrease of nociceptin/orphanin FQ plasma levels in our patient groups with severe chronic ischemic cardiovascular diseases due to atherosclerosis.

N/OFQ plays a major role in pain management in experimental settings, however changes in human nociceptin levels are controversial in pain-related diseases. Clinical reports on changes in endogenous N/OFQ levels concentrated so far mainly on acute, subacute and chronic pain-related states/disorders. Brooks et al. were the first identifying nociceptin in human CSF and plasma by ^{125}I-RIA technique. They reported that acute pain of labor has no association with the nociceptin levels (92). However Ko et al. found in patients with acute (within 4 weeks), subacute (4 weeks to 6 months), and chronic (> 6 months) pain states of different etiology that serum concentration of nociceptin was significantly higher in the patients with pain than in normal healthy subjects (93). N/OFQ level was significantly higher in the chronic pain group than acute pain group. Plasma nociceptin level of female cyclic patients with fibromyalgia syndrome in the luteal phase was found lower than in corresponding controls (94). It was concluded that perturbed plasma N/OFQ levels may be linked to both the sex hormones and to the stress system. This finding is in line with the results of Xie et al. (95) who found that transcription of the N/OFQ gene was enhanced by estrogen.Plasma N/OFQ was found to be elevated in chronic liver disorders such as Wilson disease (96), primary biliary cirrhosis (97), and hepatocellular carcinoma (98). In primary neurovascular headaches (migraine and cluster headache) plasma nociceptin levels were significantly lower than in age-, and sex-matched controls. Functional role of circulating

N/OFQ levels in the trigeminal sensory neurotransmission was suggested. After the termination of the cluster period N/OFQ levels were not statistically different from controls. During the migraine attack decrease in plasma N/OFQ level was even more pronounced (99). In an animal model, long-term, streptozotocin-induced diabetes in rats (used as a metabolic model of neuropathic pain) was shown not to influence N/OFQ levels neither in the plasma nor in the CSF (100).

In our study setting chronic CVD patients with severe atherosclerosis were investigated. It could be excluded that factors other than chronic ischemia affected plasma N/OFQ levels in our patient groups. For two weeks, chronic angina and peripheral disease patients were asked to avoid any exertion. They reported no chest or limb pain during these periods before the fasting blood samples for nociceptin/orphanin FQ were taken. We did not find any correlation between subject age/ gender and N/OFQ level, which is in agreement with the previous findings in humans made by Ertsey et al (101).

In this study N/OFQ proved to be a separate significant factor accounting for nearly one third of the total variance of patient's laboratory parameters. This finding can be interpreted as evidence for a substantive phenomenon underlying the N/OFQ regulation.

On the other hand, marked correlations were found between plasma N/OFQ levels and creatinine in all patient groups, and between N/OFQ and urea levels in the SAP-single group. These findings, the renal effects of N/OFQ, are in line with previous experimental studies. Although the association found between creatinine and nociceptin/orphanin FQ is not very high, but statistically significant while its clinical significance remains to be proven.A possible limitation of our study was that our patients with peripheral artery disease suffering from rest pain were given tramadol hydrochloride as analgetic, which possesses agonist actions at the μ-opioid receptor. In vivo reports implicated μ-opioid receptor involvement in N/OFQ-mediated analgesia and hyperalgesia in the central nervous system(102-104). However there are no data from the literature according to our knowledge that central agonist actions on μ-opioid receptor might affect peripheral N/OFQ levels. On the other hand, more data and more studies with larger patient numbers are needed to clarify the possible role of dysregulated N/OFQ system in CVD. This is an emerging issue because some nociceptin/ orphanin FQ agonists were already filed for clinical trials.

Literature data on the relation between N/OFQ and pain in human studies are inconclusive. Our data suggest that not only pain sensation, but ischemia/ reperfusion (I/R) participates also in the cardiovascular regulatory effects of nociceptin/ orphanin FQ. An elegant experimental study in anaesthetized rats showed that acute myocardial ischemia up-regulates nociceptin/orphanin FQ in dorsal root ganglion and spinal cord. Pain sensation was excluded in this setting because of anaesthesia (38). In a human study, elevated plasma NC levels in 26 acute stroke (influencing the carotis or the lacunar region) and 6 transient ischemic attack (TIA) patients were reported. The conclusion of the study was that the increase in N/OFQ levels is a consequence of the neural ischemic injury as similar values were measured both in healthy control subjects and in patients recovered from previous ischemic stroke (39). These studies showed another evidence of the regulatory effects of acute I/R in the nociceptin/orphanin FQ system, and are not in contradiction with our findings, as our study is the first examing chronic ischemic CVD. Other possible explanation is that lower plasma levels of nociceptin/orphanin FQ are a consequence of chronic and severe atherosclerosis, or a symptom of dysregulated physiological mechanism due to myocardial / skeletal muscle ischemia. The changes in inflammatory system after I/R or shock might also contribute to the better understanding of N/OFQ system. Inflammatory response in sepsis also involves among others cardiovascular derangements. Plasma N/OFQ concentrations were higher in critically ill patients in sepsis who died within 30 days compared with survivors (47). Therefore are studies of huge importance which suggest participation of peripheral nociceptinerg system in a neuro-vascular–immune axis in humans (105). Both *in vitro* (106) and *in vivo* studies (107)NOP and N/OFQ were shown to be expressed on peripheral mononuclear cells(42-44), and neutrophils(41). The possible role of the nociceptin system in the chronic inflammation of atherosclerosis needs to be further elucidated.

Study limitations: The major limitation of our study is the relatively small number of the whole patient cohort. However, this is the first, small number investigation related to chronic vascular calcification and plasma nociceptin/ orphanin FQ levels. Further investigations are needed and a larger number of cardiovascular atherosclerotic cohorts with differing extents of the disease should be investigated in the future to confirm the novel findings of our study.

5.1.2. Association of plasma nociceptin/orphanin FQ levels with acute coronary syndromes

We noticed significantly lower plasma nociceptin/orphanin FQ levels 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 finding suggests that N/OFQ levels of the plasma depends not only on the actual level of pain, but is very much dependent on whether there is an ischemic injury or not, followed by necrosis.

In our previous studies, we have shown that chronic ischemic heart disease patients, suffering from chronic angina pectoris, have decreased nociceptin/ orphanin FQ levels compared to healthy volunteers (108). We also concluded that the severity of the atherosclerosis in the heart is in inverse relationship with circulating N/OFQ levels. In an elegant study, Fontana et al. (40) found that N/OFQ levels were elevated in patients with unstable angina. The authors implicated that higher nociceptin levels were due to repeated spontaneous episodes of angina. They also accomplished a plausible stress test with adenosine infusion, which showed that induced myocardial ischemia does not have any effect on the plasma N/OFQ levels. In our study, we noticed that enzyme positive acute coronary syndrome is attached to a lower level of N/OFQ compared to enzyme negative ACS, although the difference did not reach the level of significance. We also found that after ACS, nociceptin levels are significantly correlated with higher GOT levels during the cardiac ischemia-necrosis. However, all these findings are not in contradiction with the important study of Fontana et al.(40), since our patients were examined for nociceptin two days after the onset of acute coronary syndrome and their symptoms abolished after undergoing PCI. However, our results indicate that not only pain, but acute ischemic injury/necrosis also plays a role in the regulation of N/OFQ, as the only difference between enzyme positive ACS and the other disease groups was the presence of the acute ischemic injury in the heart.

We found a negative correlation between plasma nociceptin and cholesterol levels in the EPACS group. This result is in possible contradiction with recent studies, where intracerebroventricular nociceptin treatment was shown to increase cholesterol levels in mice (109). Stressful stimuli have also been shown to elevate total plasma cholesterol

levels and activate endogenous opioid systems (110). Further studies are needed for explaining the relationship between plasma nociceptin and cholesterol levels during acute stress in humans. Guo et al. noticed that myocardial ischemia up-regulates N/OFQ in dorsal root ganglia and spinal cord of rats (38). These interesting findings are in line with our results, although the possible correlation of neural and blood plasma N/OFQ levels have not been examined yet.

Stamer et al. found lower pN/OFQ expression in the peripheral blood cells (PBC) of end-stage cancer patients and septic patients compared with healthy controls (48)**.** They also showed that increased plasma procalcitonin, a marker of inflammation, was associated with decreased pN/OFQ in all patient groups. However, NOP expression was upregulated in the same patient groups. The same study included postoperative patients with acute surgical stress. Interestingly, changes in postoperative patients were found to be minor compared to septic and cancer patients.

Plasma N/OFQ levels proved to be also elevated in ischemic stroke and transient ischemic attack patients (39). The conclusion of the study was that the increase in N/OFQ levels is the consequence of the neural ischemic injury. This finding also confirmed that ischemic injury is in connection with the N/OFQ levels.

Human studies carried out on females in the luteal phase suffering in fibromyalgia syndrome have shown that N/OFQ level is linked to sex hormones (94). This is in agreement with the result of a previous in vitrostudy which has shown that estrogen up-regulates the transcription of the N/OFQ precursor (95). Human studies made so far did not find any significant difference between male and female patients suffering from cluster headache or from bipolar disorders (101, 111). We can affirm this statement as we did not find any significant difference between male and female subjects either in the patients' groups or the controls.

We found significant correlations of N/OFQ and white blood cell 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. Previous findings of studies made on rodents have shown both in vivo and in vitro that the NOP receptor and N/OFQ are expressed on peripheral mononuclear cells and neutrophils(41, 43). The connection between N/OFQ and inflammation has

been also confirmed in a human study conducted on critically ill patients with sepsis (47). Plasma N/OFQ concentrations were found higher in patients who died within 30 days compared to survivors, however no correlation was detected between plasma N/OFQ concentrations and markers of illness severity and organ dysfunction. How decreased plasma nociceptin/orphanin FQ levels after acute coronary syndromes affect immune/ inflammatory functions remains to be further elucidated. It is not obvious whether lower N/OFQ levels would mean impaired immune functions after ACS, since WBC nociceptin receptor expression levels have not yet been investigated during acute cardiac ischemia/reperfusion. However, temporary impairment of immune functions could be beneficial by modulating the healing process of the myocardium after an acute ischemic event.

Study limitations: The major limitation of our study is the relatively small number of the whole patient cohort. However, this is the first, small number investigation related to ischemia/necrosis and plasma nociceptin/ orphanin FQ levels after acute myocardial infarction. Further investigations are needed and a larger number of acute coronary syndrome cohorts with differing extents of the disease should be investigated in the future to confirm the novel findings of our study.

Our findings indicate that the presence of acute coronary syndromes is closely associated with lower plasma N/OFQ levels after coronarography. Both enzyme positive and negative ACS are attached to a very low level of plasma N/OFQ, even compared to quiescent ischemic heart diseases. As we found correlation between N/OFQ and CK and WBC count in the enzyme positive ACS group, it seems that not only pain sensation, but also ischemic injury and the concomitant inflammatory response may have a role in regulating the plasma level of N/OFQ. Further studies are needed to examine the relevance and the effect of acute ischemic stress on the N/OFQ system and to decide whether the lower N/OFQ level is a cause or a consequence of the acute ischemic heart disease.

5.2. Association of soluble heat shock protein 70 with vascular calcification

The novel finding of the present study is that we reported a significant increase in serum heat shock protein 70 levels in patients with more severe systemic arterial calcification scores in a cohort with severe chronic lower extremity atherosclerosis and severe carotid stenosis. 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 and serum bilirubin. There was, however, no relationship between soluble Hsp70 and the acute phase reactants C-reactive protein and fetuin-a.

A soluble heat-shock mediated component to cardiovascular disease has been suggested by a number of studies. Previous works have shown an inverse correlation between circulating Hsp70 levels and atherosclerotic disease progression in cardiac (83, 112)and in extracardiac vascular calcifications, such as peripheral artery disease of the lower extremities and carotid artery disease (59, 113). In their elegant study, Martin-Ventura et al. (113) showed significantly decreased Hsp70 levels in plasma of patients with CAD in relation to matched healthy subjects. In contrast, Wright et al. had shown that Hsp70 serum levels were increased in patients with PAD (59). This discrepancy could have been due to the degree and localization of atherosclerosis (peripheral versus carotid) other samples (serum versus plasma) and different type of ELISA used. However, potential mechanisms which could explain this inverse relation have not yet been explored.

Our observations corroborate the results of Wright et al. (59) and Zhang et al. (112) inasmuch as the higher concentration of Hsp70 was related to disease severity in peripheral artery and in acute coronary syndrome patients. In our study group, Hsp70 levels significantly correlated to the extent of arterial calcification in patients with PAD, carotid artery disease or in those suffering from both localizations of atherosclerosis.

The increasing serum concentration of Hsp70 with the severity of arterial calcification seemed to be independent of the condition of the kidneys and the inflammation, proven by a logistic regression model. Patient groups divided according to lower and higher calcification score classes (1-5 vs. 6-7) showed marked significant difference in sHsp70

levels and were compared using a logistic regression adjusted for the following variables: age, gender, estimated glomerular filtration rate, smoking, diabetes type II, C-reactive protein and homocysteine levels. The risk to belong to the more severe calcified group was more than two times higher for those having high (>75% percentile, 0.7296 ng/ml) serum Hsp70 levels as compared to those with low levels. This association was independent from the above mentioned variables. Furthermore, lack of significant correlation was observed between sHsp70 and serum creatinine and serum carbamide, indicating that the elevation of sHsp70 parallel with calcification severity is not due to the impaired renal clearance of Hsp70.

In a prospective study, Pockley et al. demonstrated that increased concentrations of circulating Hsp70 correlated with decreased changes in intima/media thickness in hypertensive patients(114). It was also implicated by the same authors that serum Hsp70 levels reflect tissue expression, and elevated levels might therefore reflect the presence of an antiatherogenic state in the vasculature. We could not strengthen these findings with IMT because of our cross-sectional study design and more severe atherosclerotic cohort. In their well-designed study Dulin et al.(115)have not detected any correlations between the concentrations of circulating Hsp70 and classical vascular risk factors such as homocysteine and C-reactive protein. In our present study, a positive correlation between serum Hsp70 and homocysteine levels was found. Hyperhomocysteinemia is a well-known and independent risk factor for vascular disease (8) and triggers atherosclerotic lesion development in apolipoprotein E-deficient mice involving Hsp70 stress pathway (116). An other interesting animal study showed that heat shock protein 70 enhanced vascular bone morphogenetic protein-4 (BMP) signaling by binding matrix Gla protein,enhanced BMP-induced calcium deposition. In addition, Hsp70 mediated the procalcific effect of interleukin-6 on calcifying vascular cells (117).

The source of the serum Hsp70 has not been fully elucidated. Febbraio et al. (74)found inducible Hsp 70 release from splanchnic tissues into the blood stream during exercise. This elevation in plasma Hsp70 levels after acute exercise stress was disappeared after longer intensive exercise and heat acclimation suggesting a thermoregulatory role on the cellular level(118). This finding is not in contradiction with our study since we aimed to investigate the possible chronic effects of hsp70 in vascular calcification. Our patients

were investigated and their blood samples collected in a rested state. No patients had surgical or traumatic stress or suffered from any acute disease in the last 6 months before their blood samples were taken.

We also observed positive correlation between soluble Hsp70 and serum bilirubin levels. This result may reflect the possible relation between hepatosplanchnic tissue damage and circulating Hsp 70, suggesting that one source of heat shock protein 70 is maybe in the liver. This hypothesis is in concordance with our previous results (63, 119), however other markers of hepatic damage were not correlated with sHsp70 in our present study. The positive correlation between elevated soluble Hsp70 and serum bilirubin levels indicates that both factors increase due to chronic atherosclerotic inflammatory and oxidative stress. HO-1, also known as Hsp32, an enzyme in heme degradation, indirectly elevates blood bilirubin levels in response to oxidative stress (120, 121). Bilirubin is a potent antioxidant by scavenging RONS directly, and suppressing the activity of NADPH oxidase indirectly(122, 123). Sources of circulating Hsp70 can be endothelial cells induced by oxidative low density lipoprotein and homocysteine (66). Hyperhomocysteinemia triggers apoptosis in vascular endothelial cells acting as an endoplasmic reticulum stress-inducer (124). Moreover, this extracellular serum Hsp70 may represent a danger signal of cellular death or lysis-activating innate immunity through a strong classical complement pathway activation (125). These elevated levels of circulating Hsp70 may also origin from necrotic cells, and stimulate macrophages to secrete cytokines, and induce expression of antigen-presenting and co-stimulatory molecules on the dendritic cells, in response to cell death.

The role of sHsp70 in atherosclerosis is controversial. It is not clear, whether higher serum Hsp70 levels are a causative or an atheroprotective factor due to severe systemic calcification. Evidence suggest that soluble Hsp70 is likely to be involved in cytoprotection (82) due to the chronic stress of atherosclerosis (114). The significance of this inverse relation between Hsp70 and atherosclerosis remains to be clarified.

Study limitations: There are two major limitations of our study. The first one is the different patient numbers in the three cohorts. The different pathology of carotid and lower extremity atherosclerosis is well known and widely investigated. The relatively low number of our carotid artery patient group compared to the other two groups and

the severity of the atherosclerotic disease in our whole cohort might have influenced our analysis regarding this issue. The other limitation of our study is the relatively small number of the whole patient cohort. However, this is the first, small number investigation related to vascular calcification and serum Hsp70 levels. Further investigations are needed and a larger number of atherosclerotic cohorts with differing extents of the disease should be investigated in the future to confirm the novel findings of our study.

6. CONCLUSIONS

The major findings of our studies answer the questions detailed in the Aims part.

6.1. 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(108).

6.2. 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(108).

6.3. 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.

On the other hand, marked correlations were found between plasma N/OFQ levels and creatinine in all patient groups, and between N/OFQ and urea levels in the SAP-single group. These findings, the renal effects of N/OFQ, are in line with previous experimental studies.

Clinical studies on patients with different types of CVDs are still very limited in number. Improvement in our understanding of the dysregulated NOP-N/OFQ system in

cardiovascular disorders is needed in order to clarify whether low circulating levels of N/OFQ are a consequence of chronic ischemia or a predisposing factor for acute cardiovascular syndromes.

6.4. 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(126). This novel finding 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, followed by necrosis.

We found significant correlations of N/OFQ and white blood cell 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(126).

6.5. Elevated sHsp70 levels are associated with more severe vascular calcification

This is the first human study investigating serum heat shock protein 70 in association with the extent of arterial calcification in atherosclerosis. 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(127).

6.6. 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(127).

6.7. 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(127). However, these findings are need to be further investigated by larger studies in the future.

7. SUMMARY

Genetic predisposition and harmful environmental factors do not always explain the origin and the progression atherosclerosis and arterial calcification.There is a growing literature that chronic inflammatory processes are also involved in the development of the disease. In this work we investigated nociceptin/ orphaninFQ and heat shock protein 70 in chronic atherosclerotic patient cohorts and N/OFQ in acute cardiac patiens. N/OFQ and Hsp70 are both thought to play an important role in stress and inflammation. We demonstrated that lower N/OFQ levels are correlated to more severe ischemic heart disease and peripheral artery disease. We suggest that lower levels of nociceptin/ orphanin FQ indeed reflect the disturbed immune-inflammatory system response to the various stress factors during atherosclerosis (Krepuska M, 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). In acute coronary syndrome patients, our findings indicate that the presence of ACS is closely associated with lower plasma N/OFQ levels after coronarography. Both enzyme positive and negative ACS groups are attached to a very low level of plasma N/OFQ, even compared to quiescent ischemic heart diseases. As we found correlation between N/OFQ and CK and WBC count in the enzyme positive ACS group, it seems that not only pain sensation, but also ischemic injury and the concomitant inflammatory response may have a role in regulating the plasma level of N/OFQ(Csobay-Novák C, 2012, Decreased plasma nociceptin/orphanin FQ levels after acute coronary syndromes, Acta Physiologica Hungarica, 99(2): 99-110). We showed that there is a positive correlation between serum Hsp70 levels and the severity of arterial calcification in patients with chronic atherosclerotic lower extremity and carotid disease. The novel finding of our study was that heat shock protein-70 serum levels significantly correlate with the degree of arterial calcification independently of other known atherosclerotic risk factors (Krepuska M, 2011, Serum level of soluble Hsp70 is associated with vascular calcification, Cell Stress Chaperones. 16(3):257-65).

The above mentioned hypothesis need to be further elucidated, and larger scale studies are needed to confirm our preliminary results presented in this work.

7. ÖSSZEFOGLALÁS

A genetikai hajlam és a káros környezeti tényezők nem mindig magyarázzák az atheroszklerózis és az arteriális kalcifikáció eredetét.A növekvő irodalomi adatok szerint szerint krónikus gyulladásos folyamatok is részt vesznek a betegség kialakulásában. Munkánk során a nociceptin/ orphanin FQ és a hősokk fehérje 70 szerepét vizsgáltuk krónikusatheroszklerotikus betegcsoportokban valamint a N/OFQ szerepét akut szívbetegekben (ACS). A N/OFQ valamint a Hsp70 fontos szerepet játszanak a stressz és a gyulladás kialakulásában.Kimutattuk, hogy az alacsonyabb N/OFQ szintek korreláltak az ischaemiás szívbetegség és a perifériás artériás betegség (PAD) súlyosságával. Azt javasoljuk, hogy alacsonyabb nociceptin/ orphaninFQ valóban tükrözik a megzavart immun-inflammatorikus rendszer választ különböző stressz-faktorok hatására atheroszklerózisban(Krepuska M, 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).Az akut koronária szindrómás betegeknél, eredményeink azt mutatják, hogy az ACS szorosan összefügg az alacsonyabb plazma N/OFQ szintekkel koronarográfia után. Mind a pozitív, mind a negatív enzim ACS csoportban nagyon alacsonyak a plazma N/OFQ szintek nyugalmi ischaemiás szívbetegekhez képest. Az enzim pozitív ACS csoportban a N/OFQ valamint a CK és a fehérvérsejtszám között talált korreláció alapján úgy tűnik, hogy nemcsak a fájdalomérzet, hanem az ischaemiás sérülés és az azzal együtt járó gyulladásos válasznak is szerepe lehet a N/OFQ plazmaszintek szabályozásában(Csobay-Novák C, 2012, Decreased plasma nociceptin/orphanin FQ levels after acute coronary syndromes, Acta Physiologica Hungarica, 99(2): 99-110).Pozitív korrelációt találtunk a szérum Hsp70 szintek és az artériás meszesedés a súlyossága között PAD és carotis betegekben. Tanulmányunk új megállapítása az volt, hogy a Hsp70 szérumszintje szignifikánsan korrelál az artériás meszesedés súlyosságával, függetlenül az egyéb ismert atherosclerosis kockázati tényezőktől(Krepuska M, 2011, Serum level of soluble Hsp70 is associated with vascular calcification, Cell Stress Chaperones. 16(3):257-65).

A fenti hipotézisek megerősítésére további, nagyobb léptékű tanulmányokra van szükség.

8. REFERENCES

1. www.americanheart.org
2. www.heartstats.org
3. Hansson (2005) Inflammation, atherosclerosis, and coronary artery disease. *N Engl J Med. 2005 Apr 21;352(16):1685-95.*
4. Lahoute C, Herbin O, Mallat Z, & Tedgui A (2011) Adaptive immunity in atherosclerosis: mechanisms and future therapeutic targets. *Nat Rev Cardiol* 8(6):348-358
5. Rennenberg RJ, Kessels AG, Schurgers LJ, van Engelshoven JM, de Leeuw PW, & Kroon AA (2009) Vascular calcifications as a marker of increased cardiovascular risk: a meta-analysis. *Vasc Health Risk Manag* 5(1):185-197
6. Norgren L, Hiatt WR, Dormandy JA, Nehler MR, Harris KA, Fowkes FG, & Group TIW (2007) Inter-Society Consensus for the Management of Peripheral Arterial Disease (TASC II). *J Vasc Surg* 45 Suppl S:S5-67
7. Eberhardt RT & Coffman JD (2004) Cardiovascular morbidity and mortality in peripheral arterial disease. *Curr Drug Targets Cardiovasc Haematol Disord* 4(3):209-217
8. Clarke R, Daly L, Robinson K, Naughten E, Cahalane S, Fowler B, & Graham I (1991) Hyperhomocysteinemia: an independent risk factor for vascular disease. *N Engl J Med* 324(17):1149-1155
9. Cao JJ, Arnold AM, Manolio TA, Polak JF, Psaty BM, Hirsch CH, Kuller LH, & Cushman M (2007) Association of carotid artery intima-media thickness, plaques, and C-reactive protein with future cardiovascular disease and all-cause mortality: the Cardiovascular Health Study. *Circulation* 116(1):32-38
10. Hansson GK, Robertson AK, & Soderberg-Naucler C (2006) Inflammation and atherosclerosis. *Annu Rev Pathol* 1:297-329
11. Hansson GK (2009) Inflammatory mechanisms in atherosclerosis. *J Thromb Haemost* 7 Suppl 1:328-331
12. Svensson PA, Asea A, Englund MC, Bausero MA, Jernas M, Wiklund O, Ohlsson BG, Carlsson LM, & Carlsson B (2006) Major role of HSP70 as a

paracrine inducer of cytokine production in human oxidized LDL treated macrophages. *Atherosclerosis* 185(1):32-38

13. Moore KJ & Tabas I (2011) Macrophages in the pathogenesis of atherosclerosis. *Cell* 145(3):341-355

14. Doherty TM, Asotra K, Fitzpatrick LA, Qiao JH, Wilkin DJ, Detrano RC, Dunstan CR, Shah PK, & Rajavashisth TB (2003) Calcification in atherosclerosis: bone biology and chronic inflammation at the arterial crossroads. *Proc Natl Acad Sci U S A* 100(20):11201-11206

15. Berenson GS, Srinivasan SR, Bao W, Newman WP, 3rd, Tracy RE, & Wattigney WA (1998) Association between multiple cardiovascular risk factors and atherosclerosis in children and young adults. The Bogalusa Heart Study. *N Engl J Med* 338(23):1650-1656

16. Pearson TA, Mensah GA, Alexander RW, Anderson JL, Cannon RO, 3rd, Criqui M, Fadl YY, Fortmann SP, Hong Y, Myers GL, Rifai N, Smith SC, Jr., Taubert K, Tracy RP, Vinicor F, Centers for Disease C, Prevention, & American Heart A (2003) Markers of inflammation and cardiovascular disease: application to clinical and public health practice: A statement for healthcare professionals from the Centers for Disease Control and Prevention and the American Heart Association. *Circulation* 107(3):499-511

17. Hackam DG & Anand SS (2003) Emerging risk factors for atherosclerotic vascular disease: a critical review of the evidence. *JAMA* 290(7):932-940

18. Hirsch AT, Haskal ZJ, Hertzer NR, Bakal CW, Creager MA, Halperin JL, Hiratzka LF, Murphy WR, Olin JW, Puschett JB, Rosenfield KA, Sacks D, Stanley JC, Taylor LM, Jr., White CJ, White J, White RA, Antman EM, Smith SC, Jr., Adams CD, Anderson JL, Faxon DP, Fuster V, Gibbons RJ, Hunt SA, Jacobs AK, Nishimura R, Ornato JP, Page RL, Riegel B, American Association for Vascular S, Society for Vascular S, Society for Cardiovascular A, Interventions, Society for Vascular M, Biology, Society of Interventional R, Disease AATFoPGWCtDGftMoPWPA, American Association of C, Pulmonary R, National Heart L, Blood I, Society for Vascular N, TransAtlantic Inter-Society C, & Vascular Disease F (2006) ACC/AHA 2005 Practice Guidelines for the management of patients with peripheral arterial disease (lower extremity,

renal, mesenteric, and abdominal aortic): a collaborative report from the American Association for Vascular Surgery/Society for Vascular Surgery, Society for Cardiovascular Angiography and Interventions, Society for Vascular Medicine and Biology, Society of Interventional Radiology, and the ACC/AHA Task Force on Practice Guidelines (Writing Committee to Develop Guidelines for the Management of Patients With Peripheral Arterial Disease): endorsed by the American Association of Cardiovascular and Pulmonary Rehabilitation; National Heart, Lung, and Blood Institute; Society for Vascular Nursing; TransAtlantic Inter-Society Consensus; and Vascular Disease Foundation. *Circulation* 113(11):e463-654

19. Fox K, Garcia MA, Ardissino D, Buszman P, Camici PG, Crea F, Daly C, De Backer G, Hjemdahl P, Lopez-Sendon J, Marco J, Morais J, Pepper J, Sechtem U, Simoons M, Thygesen K, Priori SG, Blanc JJ, Budaj A, Camm J, Dean V, Deckers J, Dickstein K, Lekakis J, McGregor K, Metra M, Osterspey A, Tamargo J, Zamorano JL, Task Force on the Management of Stable Angina Pectoris of the European Society of C, & Guidelines ESCCfP (2006) Guidelines on the management of stable angina pectoris: executive summary: The Task Force on the Management of Stable Angina Pectoris of the European Society of Cardiology. *Eur Heart J* 27(11):1341-1381

20. Campeau L (1976) Letter: Grading of angina pectoris. *Circulation* 54(3):522-523

21. Fraker TD, Jr., Fihn SD, Chronic Stable Angina Writing C, American College of C, American Heart A, Gibbons RJ, Abrams J, Chatterjee K, Daley J, Deedwania PC, Douglas JS, Ferguson TB, Jr., Gardin JM, O'Rourke RA, Williams SV, Smith SC, Jr., Jacobs AK, Adams CD, Anderson JL, Buller CE, Creager MA, Ettinger SM, Halperin JL, Hunt SA, Krumholz HM, Kushner FG, Lytle BW, Nishimura R, Page RL, Riegel B, Tarkington LG, & Yancy CW (2007) 2007 chronic angina focused update of the ACC/AHA 2002 guidelines for the management of patients with chronic stable angina: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines Writing Group to develop the focused update of the 2002 guidelines

for the management of patients with chronic stable angina. *J Am Coll Cardiol* 50(23):2264-2274

22. Reinscheid RK, Nothacker HP, Bourson A, Ardati A, Henningsen RA, Bunzow JR, Grandy DK, Langen H, Monsma FJ, Jr., & Civelli O (1995) Orphanin FQ: a neuropeptide that activates an opioidlike G protein-coupled receptor. *Science* 270(5237):792-794
23. Meunier JC, Mollereau C, Toll L, Suaudeau C, Moisand C, Alvinerie P, Butour JL, Guillemot JC, Ferrara P, &Monsarrat B (1995) Isolation and structure of the endogenous agonist of opioid receptor-like ORL1 receptor. *Nature* 377(6549):532-535
24. Nelson RM, Calo G, Guerrini R, Hainsworth AH, Green AR, & Lambert DG (2000) Nociceptin/orphanin FQ inhibits ischaemia-induced glutamate efflux from rat cerebrocortical slices. *Neuroreport* 11(17):3689-3692
25. Mogil JS & Pasternak GW (2001) The molecular and behavioral pharmacology of the orphanin FQ/nociceptin peptide and receptor family. *Pharmacol Rev* 53(3):381-415
26. Gumusel B, Hao Q, Hyman A, Chang JK, Kapusta DR, & Lippton H (1997) Nociceptin: an endogenous agonist for central opioid like1 (ORL1) receptors possesses systemic vasorelaxant properties. *Life Sci* 60(8):PL141-145
27. Giuliani S & Maggi CA (1997) Prejunctional modulation by nociceptin of nerve-mediated inotropic responses in guinea-pig left atrium. *Eur J Pharmacol* 332(3):231-236
28. Czapla MA, Champion HC, & Kadowitz PJ (1997) Nociceptin, an endogenous ligand for the ORL1 receptor, has vasodilator activity in the hindquarters vascular bed of the rat. *Peptides* 18(6):793-795
29. Czapla MA, Champion HC, & Kadowitz PJ (1997) Decreases in systemic arterial and hindquarters perfusion pressure in response to nociceptin are not inhibited by naloxone in the rat. *Peptides* 18(8):1197-1200
30. Champion HC, Czapla MA, & Kadowitz PJ (1997) Nociceptin, an endogenous ligand for the ORL1 receptor, decreases cardiac output and total peripheral resistance in the rat. *Peptides* 18(5):729-732

31. Malinowska B, Godlewski G, & Schlicker E (2002) Function of nociceptin and opioid OP4 receptors in the regulation of the cardiovascular system. *J Physiol Pharmacol* 53(3):301-324

32. Kapusta DR, Burmeister MA, Calo G, Guerrini R, Gottlieb HB, & Kenigs VA (2005) Functional selectivity of nociceptin/orphanin FQ peptide receptor partial agonists on cardiovascular and renal function. *J Pharmacol Exp Ther* 314(2):643-651

33. Doggrell SA (2007) Cardiovascular and renal effects of nociceptin/orphanin FQ: a new mediator to target? *Curr Opin Investig Drugs* 8(9):742-749

34. Lambert DG (2008) The nociceptin/orphanin FQ receptor: a target with broad therapeutic potential. *Nat Rev Drug Discov* 7(8):694-710

35. Kapusta DR, Dayan LA, & Kenigs VA (2002) Nociceptin/orphanin FQ modulates the cardiovascular, but not renal, responses to stress in spontaneously hypertensive rats. *Clin Exp Pharmacol Physiol* 29(3):254-259

36. Wainford RD & Kapusta DR (2009) Chronic high-NaCl intake prolongs the cardiorenal responses to central N/OFQ and produces regional changes in the endogenous brain NOP receptor system. *Am J Physiol Regul Integr Comp Physiol* 296(2):R280-288

37. Armstead WM (2000) NOC/oFQ PKC-dependent superoxide generation contributes to hypoxic-ischemic impairment of NMDA cerebrovasodilation. *Am J Physiol Heart Circ Physiol* 279(6):H2678-2684

38. Guo Z, Yao TP, Wang JP, & Ding JY (2008) Acute myocardial ischemia up-regulates nociceptin/orphanin FQ in dorsal root ganglion and spinal cord of rats. *Neurosci Lett* 433(3):274-278

39. Tekes K, Hantos M, Bator G, Gyenge M, Laufer R, & Folyovich A (2006) [Endogenous nociceptin level in ischemic stroke: connection to serotonin system]. *Neuropsychopharmacol Hung* 8(2):53-59

40. Fontana F, Bernardi P, Pizzi C, Spampinato S, Bedini A, & Pich EM (2009) Plasma nociceptin/orphanin FQ levels rise after spontaneous episodes of angina, but not during induced myocardial ischemia. *Peptides* 30(9):1705-1709

41. Fiset ME, Gilbert C, Poubelle PE, & Pouliot M (2003) Human neutrophils as a source of nociceptin: a novel link between pain and inflammation. *Biochemistry* 42(35):10498-10505

42. Williams JP, Thompson JP, McDonald J, Barnes TA, Cote T, Rowbotham DJ, & Lambert DG (2007) Human peripheral blood mononuclear cells express nociceptin/orphanin FQ, but not mu, delta, or kappa opioid receptors. *Anesth Analg* 105(4):998-1005

43. Williams JP, Thompson JP, Rowbotham DJ, & Lambert DG (2008) Human peripheral blood mononuclear cells produce pre-pro-nociceptin/orphanin FQ mRNA. *Anesth Analg* 106(3):865-866

44. Serrano-Gomez A, Thompson JP, & Lambert DG (2011) Nociceptin/orphanin FQ in inflammation and sepsis. *Br J Anaesth* 106(1):6-12

45. Carvalho D, Petronilho F, Vuolo F, Machado RA, Constantino L, Guerrini R, Calo G, Gavioli EC, Streck EL, & Dal-Pizzol F (2008) The nociceptin/orphanin FQ-NOP receptor antagonist effects on an animal model of sepsis. *Intensive Care Med* 34(12):2284-2290

46. Brookes ZL, Stedman EN, Guerrini R, Lawton BK, Calo G, & Lambert DG (2007) Proinflammatory and vasodilator effects of nociceptin/orphanin FQ in the rat mesenteric microcirculation are mediated by histamine. *Am J Physiol Heart Circ Physiol* 293(5):H2977-2985

47. Williams JP, Thompson JP, Young SP, Gold SJ, McDonald J, Rowbotham DJ, & Lambert DG (2008) Nociceptin and urotensin-II concentrations in critically ill patients with sepsis. *Br J Anaesth* 100(6):810-814

48. Stamer UM, Book M, Comos C, Zhang L, Nauck F, & Stuber F (2011) Expression of the nociceptin precursor and nociceptin receptor is modulated in cancer and septic patients. *Br J Anaesth* 106(4):566-572

49. Kato S, Tsuzuki Y, Hokari R, Okada Y, Miyazaki J, Matsuzaki K, Iwai A, Kawaguchi A, Nagao S, Itoh K, Suzuki H, Nabeshima T, & Miura S (2005) Role of nociceptin/orphanin FQ (Noc/oFQ) in murine experimental colitis. *J Neuroimmunol* 161(1-2):21-28

50. Stein C, Schafer M, & Machelska H (2003) Attacking pain at its source: new perspectives on opioids. *Nat Med* 9(8):1003-1008

51. Agostini S, Eutamene H, Broccardo M, Improta G, Petrella C, Theodorou V, & Bueno L (2009) Peripheral anti-nociceptive effect of nociceptin/orphanin FQ in inflammation and stress-induced colonic hyperalgesia in rats. *Pain* 141(3):292-299
52. Benjamin IJ & McMillan DR (1998) Stress (heat shock) proteins: molecular chaperones in cardiovascular biology and disease. *Circ Res* 83(2):117-132
53. Latchman DS (2001) Heat shock proteins and cardiac protection. *Cardiovasc Res* 51(4):637-646
54. Prohaszka Z & Fust G (2004) Immunological aspects of heat-shock proteins-the optimum stress of life. *Mol Immunol* 41(1):29-44
55. Pockley AG, Shepherd J, & Corton JM (1998) Detection of heat shock protein 70 (Hsp70) and anti-Hsp70 antibodies in the serum of normal individuals. *Immunol Invest* 27(6):367-377
56. Dhingra R, Larson MG, Benjamin EJ, Lipinska I, Gona P, Corey D, Keaney JF, Jr., & Vasan RS (2006) Cross-sectional correlates of serum heat shock protein 70 in the community. *Am J Hypertens* 19(2):227-231;
57. Fortes MB & Whitham M (2009) No endogenous circadian rhythm in resting plasma Hsp72 concentration in humans. *Cell Stress Chaperones* 14(3):273-280
58. Molvarec A, Derzsy Z, Kocsis J, Boze T, Nagy B, Balogh K, Mako V, Cervenak L, Mezes M, Karadi I, Prohaszka Z, & Rigo J, Jr. (2009) Circulating anti-heat-shock-protein antibodies in normal pregnancy and preeclampsia. *Cell Stress Chaperones* 14(5):491-498
59. Wright BH, Corton JM, El-Nahas AM, Wood RF, & Pockley AG (2000) Elevated levels of circulating heat shock protein 70 (Hsp70) in peripheral and renal vascular disease. *Heart Vessels* 15(1):18-22
60. Satoh M, Shimoda Y, Akatsu T, Ishikawa Y, Minami Y, & Nakamura M (2006) Elevated circulating levels of heat shock protein 70 are related to systemic inflammatory reaction through monocyte Toll signal in patients with heart failure after acute myocardial infarction. *Eur J Heart Fail* 8(8):810-815
61. Dybdahl B, Slordahl SA, Waage A, Kierulf P, Espevik T, & Sundan A (2005) Myocardial ischaemia and the inflammatory response: release of heat shock protein 70 after myocardial infarction. *Heart* 91(3):299-304

62. Genth-Zotz S, Bolger AP, Kalra PR, von Haehling S, Doehner W, Coats AJ, Volk HD, & Anker SD (2004) Heat shock protein 70 in patients with chronic heart failure: relation to disease severity and survival. *Int J Cardiol* 96(3):397-401

63. Gombos T, Forhecz Z, Pozsonyi Z, Janoskuti L, & Prohaszka Z (2008) Interaction of serum 70-kDa heat shock protein levels and HspA1B (+1267) gene polymorphism with disease severity in patients with chronic heart failure. *Cell Stress Chaperones* 13(2):199-206

64. Bernardini C, Zannoni A, Bacci ML, & Forni M (2010) Protective effect of carbon monoxide pre-conditioning on LPS-induced endothelial cell stress. *Cell Stress Chaperones* 15(2):219-224

65. Chang AY, Chan JY, Cheng HL, Tsai CY, & Chan SH (2009) Hypoxia-inducible factor 1/heme oxygenase 1 cascade as upstream signals in the prolife role of heat shock protein 70 at rostral ventrolateral medulla during experimental brain stem death. *Shock* 32(6):651-658

66. Zhan R, Leng X, Liu X, Wang X, Gong J, Yan L, Wang L, Wang Y, & Qian LJ (2009) Heat shock protein 70 is secreted from endothelial cells by a non-classical pathway involving exosomes. *Biochem Biophys Res Commun* 387(2):229-233

67. Bausero MA, Gastpar R, Multhoff G, & Asea A (2005) Alternative mechanism by which IFN-gamma enhances tumor recognition: active release of heat shock protein 72. *J Immunol* 175(5):2900-2912

68. Lavie L, Dyugovskaya L, Golan-Shany O, & Lavie P (2009) Heat-shock protein 70: expression in monocytes of patients with sleep apnoea and association with oxidative stress and tumour necrosis factor-alpha. *J Sleep Res* 19(1 Pt 2):139-147

69. Luu NT, Madden J, Calder PC, Grimble RF, Shearman CP, Chan T, Tull SP, Dastur N, Rainger GE, & Nash GB (2007) Comparison of the pro-inflammatory potential of monocytes from healthy adults and those with peripheral arterial disease using an in vitro culture model. *Atherosclerosis* 193(2):259-268

70. Madden J, Coward JC, Shearman CP, Grimble RF, & Calder PC (2009) Hsp70 expression in monocytes from patients with peripheral arterial disease and healthy controls : Monocyte Hsp70 in PAD. *Cell Biol Toxicol*

71. Paier A, Agewall S, & Kublickiene K (2009) Expression of heat shock proteins and nitrotyrosine in small arteries from patients with coronary heart disease. *Heart Vessels* 24(4):260-266

72. Bobryshev YV & Lord RS (2002) Expression of heat shock protein-70 by dendritic cells in the arterial intima and its potential significance in atherogenesis. *J Vasc Surg* 35(2):368-375

73. Zhu WM, Roma P, Pirillo A, Pellegatta F, & Catapano AL (1995) Oxidized LDL induce hsp70 expression in human smooth muscle cells. *FEBS Lett* 372(1):1-5

74. Febbraio MA, Ott P, Nielsen HB, Steensberg A, Keller C, Krustrup P, Secher NH, & Pedersen BK (2002) Exercise induces hepatosplanchnic release of heat shock protein 72 in humans. *J Physiol* 544(Pt 3):957-962

75. St Rammos K, Koullias GJ, Hassan MO, Argyrakis NP, Voucharas CG, Scarupa SJ, & Cowte TG (2002) Low preoperative HSP70 atrial myocardial levels correlate significantly with high incidence of postoperative atrial fibrillation after cardiac surgery. *Cardiovasc Surg* 10(3):228-232

76. de Jong PR, Schadenberg AW, Jansen NJ, & Prakken BJ (2009) Hsp70 and cardiac surgery: molecular chaperone and inflammatory regulator with compartmentalized effects. *Cell Stress Chaperones* 14(2):117-131

77. Asea A (2005) Stress proteins and initiation of immune response: chaperokine activity of hsp72. *Exerc Immunol Rev* 11:34-45

78. Matzinger P (2002) The danger model: a renewed sense of self. *Science* 296(5566):301-305

79. Pockley AG & Multhoff G (2008) Cell stress proteins in extracellular fluids: friend or foe? *Novartis Found Symp* 291:86-95;

80. Asea A, Kraeft SK, Kurt-Jones EA, Stevenson MA, Chen LB, Finberg RW, Koo GC, & Calderwood SK (2000) HSP70 stimulates cytokine production through a CD14-dependant pathway, demonstrating its dual role as a chaperone and cytokine. *Nat Med* 6(4):435-442

81. Asea A, Rehli M, Kabingu E, Boch JA, Bare O, Auron PE, Stevenson MA, & Calderwood SK (2002) Novel signal transduction pathway utilized by extracellular HSP70: role of toll-like receptor (TLR) 2 and TLR4. *J Biol Chem* 277(17):15028-15034

82. Bielecka-Dabrowa A, Barylski M, Mikhailidis DP, Rysz J, & Banach M (2009) HSP 70 and atherosclerosis--protector or activator? *Expert Opin Ther Targets* 13(3):307-317

83. Zhu J, Quyyumi AA, Wu H, Csako G, Rott D, Zalles-Ganley A, Ogunmakinwa J, Halcox J, & Epstein SE (2003) Increased serum levels of heat shock protein 70 are associated with low risk of coronary artery disease. *Arterioscler Thromb Vasc Biol* 23(6):1055-1059

84. Fowkes FG, Murray GD, Butcher I, Heald CL, Lee RJ, Chambless LE, Folsom AR, Hirsch AT, Dramaix M, deBacker G, Wautrecht JC, Kornitzer M, Newman AB, Cushman M, Sutton-Tyrrell K, Fowkes FG, Lee AJ, Price JF, d'Agostino RB, Murabito JM, Norman PE, Jamrozik K, Curb JD, Masaki KH, Rodriguez BL, Dekker JM, Bouter LM, Heine RJ, Nijpels G, Stehouwer CD, Ferrucci L, McDermott MM, Stoffers HE, Hooi JD, Knottnerus JA, Ogren M, Hedblad B, Witteman JC, Breteler MM, Hunink MG, Hofman A, Criqui MH, Langer RD, Fronek A, Hiatt WR, Hamman R, Resnick HE, Guralnik J, & McDermott MM (2008) Ankle brachial index combined with Framingham Risk Score to predict cardiovascular events and mortality: a meta-analysis. *JAMA* 300(2):197-208

85. Espinola-Klein C, Rupprecht HJ, Bickel C, Lackner K, Savvidis S, Messow CM, Munzel T, & Blankenberg S (2008) Different calculations of ankle-brachial index and their impact on cardiovascular risk prediction. *Circulation* 118(9):961-967

86. Vahanian A, Baumgartner H, Bax J, Butchart E, Dion R, Filippatos G, Flachskampf F, Hall R, Iung B, Kasprzak J, Nataf P, Tornos P, Torracca L, Wenink A, Task Force on the Management of Valvular Hearth Disease of the European Society of C, & Guidelines ESCCfP (2007) Guidelines on the management of valvular heart disease: The Task Force on the Management of Valvular Heart Disease of the European Society of Cardiology. *Eur Heart J* 28(2):230-268

87. Higgins CL, Marvel SA, & Morrisett JD (2005) Quantification of calcification in atherosclerotic lesions. *Arterioscler Thromb Vasc Biol* 25(8):1567-1576

88. Grant EG, Benson CB, Moneta GL, Alexandrov AV, Baker JD, Bluth EI, Carroll BA, Eliasziw M, Gocke J, Hertzberg BS, Katanick S, Needleman L, Pellerito J, Polak JF, Rholl KS, Wooster DL, & Zierler RE (2003) Carotid artery stenosis: gray-scale and Doppler US diagnosis--Society of Radiologists in Ultrasound Consensus Conference. *Radiology* 229(2):340-346

89. Cozzolino M, Gallieni M, & Brancaccio D (2006) Vascular calcification in uremic conditions: new insights into pathogenesis. *Semin Nephrol* 26(1):33-37

90. Guerin AP, London GM, Marchais SJ, & Metivier F (2000) Arterial stiffening and vascular calcifications in end-stage renal disease. *Nephrol Dial Transplant* 15(7):1014-1021

91. Tavaria M, Gabriele T, Kola I, & Anderson RL (1996) A hitchhiker's guide to the human Hsp70 family. *Cell Stress Chaperones* 1(1):23-28

92. Brooks H, Elton CD, Smart D, Rowbotham DJ, McKnight AT, & Lambert DG (1998) Identification of nociceptin in human cerebrospinal fluid: comparison of levels in pain and non-pain states. *Pain* 78(1):71-73

93. Ko MH, Kim YH, Woo RS, & Kim KW (2002) Quantitative analysis of nociceptin in blood of patients with acute and chronic pain. *Neuroreport* 13(13):1631-1633

94. Anderberg UM, Liu Z, Berglund L, & Nyberg F (1998) Plasma levels on nociceptin in female fibromyalgia syndrome patients. *Z Rheumatol* 57 Suppl 2:77-80

95. Xie GX, Ito E, Maruyama K, Suzuki Y, Sugano S, Sharma M, Pietruck C, & Palmer PP (1999) The promoter region of human prepro-nociceptin gene and its regulation by cyclic AMP and steroid hormones. *Gene* 238(2):427-436

96. Hantos MB, Tekes K, & Szalay F (2002) [Peripheral nociceptin levels in Wilson disease]. *Acta Pharm Hung* 72(4):260-264

97. Horvath A, Folhoffer A, Lakatos PL, Halosz J, Illyes G, Schaff Z, Hantos MB, Tekes K, & Szalay F (2004) Rising plasma nociceptin level during development of HCC: a case report. *World J Gastroenterol* 10(1):152-154

98. Szalay F, Hantos MB, Horvath A, Lakatos PL, Folhoffer A, Dunkel K, Hegedus D, & Tekes K (2004) Increased nociceptin/orphanin FQ plasma levels in hepatocellular carcinoma. *World J Gastroenterol* 10(1):42-45

99. Ertsey C, Hantos M, Bozsik G, & Tekes K (2005) Plasma nociceptin levels are reduced in migraine without aura. *Cephalalgia* 25(4):261-266

100. Tekes KH, M.;Gyenge,M.; Bizderi,B.;Kecskeméti,V. (2005) Diabetes and endogenous orphanin FQ/nociceptin levels in rat CSF and plasma. *Int J Diab Metabol* (13):147-153

101. Ertsey C, Hantos M, Bozsik G, & Tekes K (2004) Circulating nociceptin levels during the cluster headache period. *Cephalalgia* 24(4):280-283

102. Mandyam CD, Thakker DR, & Standifer KM (2003) Mu-opioid-induced desensitization of opioid receptor-like 1 and mu-opioid receptors: differential intracellular signaling determines receptor sensitivity. *J Pharmacol Exp Ther* 306(3):965-972

103. Lutfy K & Maidment NT (2000) Blockade of mu-opioid receptors reveals the hyperalgesic effect of orphanin FQ/nociceptin in the rat hot plate test. *Br J Pharmacol* 131(8):1684-1688

104. Jhamandas KH, Sutak M, & Henderson G (1998) Antinociceptive and morphine modulatory actions of spinal orphanin FQ. *Can J Physiol Pharmacol* 76(3):314-324

105. Leggett JD, Dawe KL, Jessop DS, & Fulford AJ (2009) Endogenous nociceptin / orphanin FQ system involvement in hypothalamic-pituitary-adrenal axis responses: relevance to models of inflammation. *J Neuroendocrinol* 21(11):888-897

106. Waits PS, Purcell WM, Fulford AJ, & McLeod JD (2004) Nociceptin/orphanin FQ modulates human T cell function in vitro. *J Neuroimmunol* 149(1-2):110-120

107. Acosta C & Davies A (2008) Bacterial lipopolysaccharide regulates nociceptin expression in sensory neurons. *J Neurosci Res* 86(5):1077-1086

108. Krepuska M, Sotonyi P, Csobay-Novak C, Szeberin Z, Hartyanszky I, Zima E, Szilagyi N, Horkay F, Merkely B, Acsady G, & Tekes K (Plasma

nociceptin/orphanin FQ levels are lower in patients with chronic ischemic cardiovascular diseases--A pilot study. *Regul Pept* 169(1-3):1-5

109. Matsushita H, Ishihara A, Mashiko S, Tanaka T, Kanno T, Iwaasa H, Ohta H, & Kanatani A (2009) Chronic intracerebroventricular infusion of nociceptin/orphanin FQ produces body weight gain by affecting both feeding and energy metabolism in mice. *Endocrinology* 150(6):2668-2673

110. Bryant HU, Story JA, & Yim GK (1988) Assessment of endogenous opioid mediation in stress-induced hypercholesterolemia in the rat. *Psychosom Med* 50(6):576-585

111. Wang SJ, Hung CW, Fuh JL, Lirng JF, & Hwu CM (2009) Cranial autonomic symptoms in patients with pituitary adenoma presenting with headaches. *Acta Neurol Taiwan* 18(2):104-112

112. Zhang X, Xu Z, Zhou L, Chen Y, He M, Cheng L, Hu FB, Tanguay RM, & Wu T (2010) Plasma levels of Hsp70 and anti-Hsp70 antibody predict risk of acute coronary syndrome. *Cell Stress Chaperones*

113. Martin-Ventura JL, Leclercq A, Blanco-Colio LM, Egido J, Rossignol P, Meilhac O, & Michel JB (2007) Low plasma levels of HSP70 in patients with carotid atherosclerosis are associated with increased levels of proteolytic markers of neutrophil activation. *Atherosclerosis* 194(2):334-341

114. Pockley AG, Calderwood SK, & Multhoff G (2009) The atheroprotective properties of Hsp70: a role for Hsp70-endothelial interactions? *Cell Stress Chaperones* 14(6):545-553

115. Dulin E, Garcia-Barreno P, & Guisasola MC (2010) Extracellular heat shock protein 70 (HSPA1A) and classical vascular risk factors in a general population. *Cell Stress Chaperones* 15(6)::929-937

116. Zhou J, Werstuck GH, Lhotak S, de Koning AB, Sood SK, Hossain GS, Moller J, Ritskes-Hoitinga M, Falk E, Dayal S, Lentz SR, & Austin RC (2004) Association of multiple cellular stress pathways with accelerated atherosclerosis in hyperhomocysteinemic apolipoprotein E-deficient mice. *Circulation* 110(2):207-213

117. Yao Y, Watson AD, Ji S, & Bostrom KI (2009) Heat shock protein 70 enhances vascular bone morphogenetic protein-4 signaling by binding matrix Gla protein. *Circ Res* 105(6):575-584

118. Magalhaes FD, Amorim FT, Passos RL, Fonseca MA, Oliveira KP, Lima MR, Guimaraes JB, Ferreira-Junior JB, Martini AR, Lima NR, Soares DD, Oliveira EM, & Rodrigues LO (2010) Heat and exercise acclimation increases intracellular levels of Hsp72 and inhibits exercise-induced increase in intracellular and plasma Hsp72 in humans. *Cell Stress Chaperones* 15(6)::885-895.

119. Madach K, Molvarec A, Rigo J, Jr., Nagy B, Penzes I, Karadi I, & Prohaszka Z (2008) Elevated serum 70 kDa heat shock protein level reflects tissue damage and disease severity in the syndrome of hemolysis, elevated liver enzymes, and low platelet count. *Eur J Obstet Gynecol Reprod Biol* 139(2):133-138

120. Vitek L & Ostrow JD (2009) Bilirubin chemistry and metabolism; harmful and protective aspects. *Curr Pharm Des* 15(25):2869-2883

121. Grochot-Przeczek A, Dulak J, & Jozkowicz A (2010) Heme oxygenase-1 in neovascularisation: A diabetic perspective. *Thromb Haemost* 104(3):424-431

122. McCarty MF (2007) "Iatrogenic Gilbert syndrome"--a strategy for reducing vascular and cancer risk by increasing plasma unconjugated bilirubin. *Med Hypotheses* 69(5):974-994

123. Jansen T, Hortmann M, Oelze M, Opitz B, Steven S, Schell R, Knorr M, Karbach S, Schuhmacher S, Wenzel P, Munzel T, & Daiber A (2010) Conversion of biliverdin to bilirubin by biliverdin reductase contributes to endothelial cell protection by heme oxygenase-1-evidence for direct and indirect antioxidant actions of bilirubin. *J Mol Cell Cardiol* 49(2):186-195

124. Kyriakakis E, Philippova M, Joshi MB, Pfaff D, Bochkov V, Afonyushkin T, Erne P, & Resink TJ (2010) T-cadherin attenuates the PERK branch of the unfolded protein response and protects vascular endothelial cells from endoplasmic reticulum stress-induced apoptosis. *Cell Signal*

125. Prohaszka Z, Singh M, Nagy K, Kiss E, Lakos G, Duba J, & Fust G (2002) Heat shock protein 70 is a potent activator of the human complement system. *Cell Stress Chaperones* 7(1):17-22

126. Csobay-Novák C SP, 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 Physiol Hung.* 99(2):99-110.

127. Krepuska M, Szeberin Z, Sotonyi P, Sarkadi H, Fehervari M, Apor A, Rimely E, Prohaszka Z, & Acsady G (Serum level of soluble Hsp70 is associated with vascular calcification. *Cell Stress Chaperones* 16(3):257-265

9. PUBLICATION LIST

9.1. 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

9.2. 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.

Szeberin Z, Fehérvári M, Krepuska M, Apor A, Rimely E, Sarkadi H, Széplaki G, Prohászka Z, Kalabay L, Acsády G.(2011) Serum Fetuin-A Levels Inversely Correlate with the Severity of Arterial Calcification in Patients with Chronic Lower Extremity Atherosclerosis without Renal DiseaseInt Angiol.;30(5):474-50. **IF: 0.993**

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.

10. ACKNOWLEDGEMENTS

I am grateful to Professor György Acsády, for giving me the opportunity to conduct this research at the Department of Vascular Surgery, Semmelweis University, Budapest.
I am also grateful to Professor Béla Merkely for hiscontinuous supportand allowing me to conduct my research at his department ever since I started as a research scholar under his supervision.
I would like to give special thanks to Dr Péter Sótonyi for his endless patience and support during my scientific studies and for his friendship.
I would like to thank to Professor Kornélia Tekes for enabling me to work with her team on the nociceptin study.
I am grateful to Dr. Zoltán Prohászka and his collegues for enabling me to work on the Hsp70 study.
I would like to thank to Dr. Zoltán Szeberin for his help and his continuous encouragement during my PhD studies.
I would like to express my gratitude to all of the collegues and tutors, who helped and participated in my work: Dr. Csaba Csobay-Novák, Dr. Mátyás Fehérvári, Dr. Hunor Sarkadi, Dr. Astrid Apor, Dr. Endre Rimely, Dr. Endre Zima, Dr. István Hartyánszky, Professor Ferenc Horkay.
The elegant statistical analysis in the nociceptin study was conducted by Dr. Nóra Szilágyi.Dr. Péter Vargha and Dr. Gábor Széplaki gave advices on the Hsp70 statisticts.
I would also like to thank all of the work for the nurses, assistants, patients, and volunteers who participated in our studies. I am thankful to Dr. Edit Dosa and Dr. Gabor Szabo, who were the in-house opponents. Their interesting and valuable comments improved the dissertation.
Dr. Eva Mezey helped with the figures and the references and gave a lot of support here at NIH.

I would like to express my gratitude to all of my friends, parents and family for their endless encouragement. And last but not least, I am thankful to my wife, Dr. Nikoletta Lendvai, who prepared some of the pictures and who gives me tremendous love and support in our life.