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KASPAR TOOTSI

Cardiovascular and metabolomic profiling of osteoarthritis

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Department of Traumatology and Orthopedics, Institute of Clinical Medicine, University of Tartu, Tartu, Estonia.

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To my beloved ones

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LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following original publications referred to in the text by their Roman numerals (I–IV):

I Tootsi K, Märtson A, Zilmer M, Paapstel K, Kals J. Increased arterial stiffness in end-stage osteoarthritis: a case-control study. BMC Musculoskel Disord 2016; 17:335

II Tootsi K, Kals J, Zilmer M, Paapstel K, Märtson A. Severity of Osteoarthritis Is Associated with Increased Arterial Stiffness. Int J Rheumatol 2016; 2016/6402963

III Tootsi K, Märtson A, Kals J, Paapstel K, Zilmer M. Metabolic factors and oxidative stress in osteoarthritis: A case-control study. Scand J Clin Lab Invest 2017; 77:520–526

IV Tootsi K, Kals J, Zilmer M, Paapstel K, Ottas A, Märtson A. Medium- and long-chain acylcarnitines are associated with osteoarthritis severity and arterial stiffness in end-stage osteoarthritis patients: A case-control study. (submitted for publication)

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ABBREVIATIONS

aa	diacyl
ae	acyl-alkyl
ACEI	angiotensin-converting enzyme inhibitor
AIx	augmentation index
AIx@75	augmentation index corrected for a heart rate of 75 beats/minute
ARB	angiotensin receptor blocker
BMI	body mass index
BP	blood pressure
cf-PWV	carotid-femoral pulse wave velocity
CPT	carnitine palmitoyltransferase
cr-PWV	carotid-radial pulse wave velocity
CVD	cardiovascular disease
Cx:y	x denotes the number of carbons in the fatty acid side chains and y denotes the number of double bonds
DC	decarboxyl
ECG	electrocardiogram
eGFR	estimated glomerular filtration rate
ELISA	enzyme-linked immunosorbent assay
HDL	high-density lipoprotein
HHS	Harris Hip score
HOMA2 IR	homeostasis model assessment estimated insulin resistance
HR	heart rate
HSS	Hospital for Special Surgery
hsCRP	high sensitivity C-reactive protein
LDL	low-density lipoprotein
lysoPC a Cx:y	lysophosphatidylcholine
MAP	mean arterial pressure
MetS	metabolic syndrome
MMP	matrix metalloproteinase
MPO	myeloperoxidase
NO	nitric oxide
OH	hydroxyl
OA	osteoarthritis
OSI	oxidative stress index
OxLDL	oxidized low-density lipoprotein
OxS	oxidative stress
PC	phosphatidylcholine
PC aa Cx:y	diacyl-phosphatidylcholine
PC ae Cx:y	acyl-alkyl-phosphatidylcholine
PCA	principal component analysis
PUFA	polyunsaturated fatty acid

PWA	pulse wave analysis
PWV	pulse wave velocity
PWV-ratio	PWV-ratio (cf-PWV divided by cr-PWV)
SM	sphingomyelin
SM (OH) Cx:y	hydroxysphingomyelin
TAC	total antioxidant capacity
TNF-α	tumor necrosis factor alpha
WBC	white blood cell
W/H-ratio	waist to hip ratio

1. INTRODUCTION

Modern understanding of osteoarthritis (OA) has largely evolved during 250 years, previously all forms of chronic arthritis were considered different manifestations of gout. In 1782 William Heberden described distinctive osteoarthritic changes in fingers and was the first to draw attention to the fact that these were not related to gout (Dequeker & Luyten, 2008). Since that time OA has been of great interest in clinical practice and in medical research.

OA is a chronic progressive disease that might affect every diarthrodial joint, but clinically the most significant findings can be attributed to knee, hip, spine and hand OA. OA has high prevalence, which has doubled since the mid-twentieth century (Wallace *et al.* 2017). Solely knee OA affects more than 250 million people worldwide (Vos *et al.* 2012). The lifetime risk for developing symptomatic knee OA is 45%, which rises to 60% with body mass index (BMI) over 30 kg/m^2 (Murphy *et al.* 2008). Although OA has long been considered a degenerative cartilage 'wear and tear' disease, the paradigm has shifted during the recent years. OA damages the whole joint: cartilage, subchondral bone, joint capsule, synovial tissue and periarticular muscles. Typical changes in progression of OA include cartilage deterioration from superficial fibrillation to total denuding of the subchondral bone; osteophytes in the joint margins; cysts and attrition of subchondral bone; synovitis; atrophy of the periarticular muscles. These changes lead to development of pain (during physical activities and night-time at the end-stage), stiffness, swelling, deformity, muscle weakness and decreased range of motion. It has been noted in recent years that OA is also associated with systemic changes: systemic low-grade inflammation, obesity, oxidative stress (OxS), metabolic factors (Berenbaum, 2013; King *et al.* 2013; Courties *et al.* 2015; Lepetsos & Papavassiliou, 2016). The pathogenesis of OA is complex and the knowledge of its specific mechanisms is inconclusive. Therefore, there is no disease modifying therapy that could reverse progression of OA.

OA is associated with several comorbidities including cardiovascular diseases (CVD). It has been found that patients with OA have up to 24% increased CVD mortality (Barbour *et al.* 2015). However, the precise mechanisms behind the association between OA and CVD are still largely unknown. Several studies have proposed that vascular pathology (i.e. atherosclerosis) might be related to pathogenesis of OA (Saleh *et al.* 2007; Davies-Tuck *et al.* 2012; Belen *et al.* 2016). Atherosclerosis causes systemic arterial damage and leads to premature arterial stiffening (Palombo & Kozakova, 2016). Arterial stiffening is the term used to describe the structural and functional changes that occur in the arteries during ageing and in CVD. The elastic properties of the central arteries (aorta, iliac arteries and carotid arteries) are important for cushioning left ventricular blood ejection during systole and for the smooth non-pulsatile flow of blood to the periphery during diastole (Safar, 2004). In the case of stiffened arteries, the high energy pulsatile blood flow reaches the tissue capillaries and causes organ

damage. Arterial stiffening is an independent predictor of cardiovascular mortality and morbidity (Laurent *et al.* 2006). Many studies suggest that arterial stiffening is associated with systemic inflammation and different biochemical alterations (Park & Lakatta, 2012; Kals *et al.* 2008). However, it remains unclear whether arterial pathology induces OA or vice versa because low-grade systemic inflammation, a characteristic of OA, might accelerate atherosclerosis (Klein-Wieringa *et al.* 2016*;* Geng *et al.* 2016; Hoeven *et al.* 2013).

Patients with OA are often multimorbid, which has lead to studies aimed at finding potential association between OA and metabolic syndrome (MetS) (Sellam & Berenbaum, 2013; Wang *et al.* 2016a). MetS is a cluster of metabolic disorders that include insulin resistance, dyslipidemia, vascular pathology and central obesity. Several studies have established that MetS is strongly associated with increased state of oxidative stress (OxS) (Keaney *et al.* 2003; Furukawa *et al.* 2004; Roberts & Sindhu, 2009).

Obesity has been found to increase the risk of OA (Lementowski & Zelicof, 2008). Moreover, obesity has been found to be associated with hand OA, which suggests a systemic impact of obesity and not only the mechanical overload (Visser *et al.* 2014). Adipokines are white-adipose-tissue derived bioactive factors that might play an important role in the pathogenesis of OA (Calvet *et al.* 2016). Since adipokines are also associated with CVD risk, they might contribute to the association between OA and CVD. Thus, further research is required to determine the role of adipokines in the pathogenesis of OA and in CVD.

Metabolomics is a comprehensive analysis of low molecular weight metabolites, which reflect the state of the human organism at a certain timepoint. Metabolites are the intermediates and end-products of different metabolic pathways and resemble the end result of the genotype, phenotype and environmental factors. Metabolic changes are the most proximal indicators of the disease process and drug therapy. Thus, metabolomics is a promising tool in search for novel biomarkers that could improve early diagnosis of OA, prognosis of the disease and response to therapy.

The present thesis aims to investigate the role of arterial stiffness in OA and to propose new biomarkers that could help to diagnose OA early and predict the course of the disease.

2. REVIEW OF THE LITERATURE

2.1 General aspects of osteoarthritis

Osteoarthritis (OA) is one of the most prevalent chronic diseases of the synovial joint, which is associated with a large socioeconomic burden (Hunter *et al.* 2014). The most frequently affected joints are the hip, knee, spine and hand. OA is the fastest increasing musculoskeletal disorder and the prevalence of knee OA has been estimated at 250 million people worldwide (Vos *et al.* 2012). The lifetime risk for developing knee OA is 45%, and it is 61% for obese population (Murphy *et al.* 2008).

OA damages all components of the diarthrodial joint causing loss of articular cartilage, remodelling of subchondral bone, osteophyte formation, ligamentous laxity, synovial inflammation and weakening of the periarticular muscles (Hutton, 1989). The primary symptoms of OA are pain and decreased range of motion and stiffness of the affected joint. Since OA is a heterogeneous disease group, there are many approaches to stratify OA into different subtypes. One of the most common classifications divides OA into two groups depending on the mechanism of onset: primary (idiopathic) and secondary (post-traumatic, congenital or developmental diseases, metabolic, inflammatory and other). However, several other classifications, based on anatomical components (McGonagle *et al.* 2010) and based on clinical phenotypes (Bijlsma *et al.* 2011), have been proposed.

Assessment of OA severity

OA can be defined as clinical, radiological or subjective (self-reported). Many different techniques have been developed to assess presence of OA and to grade the radiological severity of the disease, among them the most widely used method is that of Kellgren and Lawrence (Kellgren & Lawrence, 1957). The severity of OA falls under 5 categories from 0 to 4 based on joint space width, appearance of osteophytes, subchondral sclerosis and cysts:

Grade 0: normal joint
Grade 1: doubtful narrowing of the joint space and possible osteophytic lipping
Grade 2: definite osteophytes and possible narrowing of the joint space
Grade 3: moderate multiple osteophyte, definite narrowing of the joint space, some sclerosis and possible deformity of bone ends
Grade 4: large osteophytes, marked narrowing of the joint space, severe sclerosis and definite deformity of bone ends (Kellgren & Lawrence, 1957).

Several scoring systems have been developed for the clinical evaluation of the function and disability of hip OA patients, among them Harris Hip score (HHS), Hip disability and Osteoarthritis Outcome Score, Oxford Hip Score, American Academy of Orthopaedic Surgeons (AAOS) Hip and Knee Questionnaire

(Harris, 1969; Riddle *et al.* 2008; Nilsdotter & Bremander, 2011). All of these scoring systems have been used in clinical studies, but HHS is one of the most frequently used physician reported outcome measures for hip joint clinical assessment (Hoeksma *et al.* 2003; Riddle *et al.* 2008; Shi *et al.* 2010). HHS covers 4 domains: pain, function, absence of deformity and range of motion. It has been recently questioned whether range of motion provides any additional predictive value (Edwards *et al.* 2016). Several methods have been devised for assessing knee function in OA; the most widely used tests include Hospital for Special Surgery (HSS) Knee Score, Western Ontario and McMaster Universities Osteoarthritis Index, Oxford Knee Score and others (Marx, 2003). HSS Knee Score is a scoring method used for clinical knee joint evaluation (Insall *et al.* 1976), which quantifies pain, function, gait, deformity and range of motion (Kovalak *et al.* 2015; Cholewinski *et al.* 2015).

Etiopathogenesis of OA

For long time OA was considered a chronic degenerative disease that was the consequence of ageing and "wear and tear" type of damage. Ageing, besides obesity, female gender and genetic predisposition, is undoubtedly one of the strongest risk factors for OA. However, recent studies have shown that systemic inflammation has an important role in development and progression of OA *(*Klein-Wieringa *et al. 2016;* Sohn *et al. 2012;* Stannus *et al.* 2013). The origin of inflammation is not clear, but several possible mechanisms have been proposed. One proposed scenario is that the components of damaged cartilage are released into the synovial fluid and bind to the receptors on synovial cells, which activate the production of cartilage-degrading enzymes (e.g. matrix metalloproteinases (MMP)) and inflammatory cytokines (Bondeson *et al.* 2006; Sohn *et al.* 2012). Some authors have supposed that inflammation is driven by ageing and introduced the term "inflamm-aging" (Franceschi *et al.* 2000). Inflamm-aging incorporates immunosenescence and age-related metabolic, endocrine and nutritional changes that promote the pro-inflammatory state (Calçada *et al.* 2014). According to another view, advanced glycation end-products, which accumulate in different tissues with age, (including the articular cartilage) are involved in the pathogenesis of OA (Sell & Monnier, 1989). Also, the role of obesity cannot be underestimated in OA. In addition to mechanical wear in the weight-bearing joints, obesity is associated with proinflammatory state. Multiple bioactive molecules derived from the adipose tissue have been identified, which might have an important role in the pathogenesis of OA (Grotle *et al.* 2008; Poonpet & Honsawek, 2014; Zheng *et al.* 2016).

Since OA and CVD share many risk factors, there might be a link between the two diseases (Haugen *et al.* 2015). There are other possible explanations that relate vascular pathology with OA. According to Findlay, OA is driven by vascular insufficiency (Findlay, 2007). The subchondral bone is highly susceptible to arterial blood flow disruption due to the fact that most of the arterial flow comes through epiphyseal artery. Atherosclerosis or embolus in the

epiphyseal artery has devastating effects on the subchondral bone and overlying cartilage. Another reason for perfusion inadequacy might arise from the venous stasis through increased intra-articular pressure, increased loading or thrombus formation in the bone vessels (Lucht *et al.* 1981; Wang *et al.* 2003). Studies have reported hypoxic conditions in the subchondral bone and bone remodelling due to hypoxia in OA joints (Pedersen *et al.* 1989; Chang *et al.* 2014). In addition, other vascular problems have been reported in OA patients (Davies-Tuck *et al.* 2012). In conclusion, there is growing body of evidence that vascular changes might play a significant role in development and progression of OA.

2.2 Osteoarthritis and cardiovascular diseases

OA is associated with many comorbidities and one of the most important disease groups related to OA is CVD (van Dijk *et al.* 2008; Leite *et al.* 2011). Recent evidence suggests that patients with severe CVD are more likely to require joint arthroplasty (Kadam *et al.* 2011). Finding out more details about the co-existence of OA and CVD might help to develop better CVD risk management strategies and to provide insights into the pathophysiology of OA.

2.2.1 Increased cardiovascular risk in osteoarthritis

Many studies have shown that OA patients have increased CVD prevalence and mortality (Rahman *et al.* 2013; Hawker *et al.* 2014; Hall *et al.* 2016; Wang *et al.* 2016b). The Rotterdam study found independent association between atherosclerotic changes and presence and progression of hip, knee and hand OA (Hoeven *et al.* 2013). A nation-wide population study with over 90,000 participants reported that OA carries an increased risk for acute coronary syndrome (Chung *et al.* 2016). The Framingham heart study demonstrated increased incidence of coronary heart disease in symptomatic hand OA patients (Haugen *et al.* 2015). Furthermore, the AGES Reykjavik study in which 5300 subjects' coronary calcifications and carotid plaque severity were assessed, found linear association between severity of hand OA and atherosclerosis (Jonsson *et al.* 2009). Consequently, CVD and OA seem to be related. In order to improve the treatment of OA patients, we need to find out more about the mechanisms behind the interplay of these two major health concerns. The overlapping of risk factors, including obesity, ageing, decreased physical activity, increased use of non-steroidal anti-inflammatory drugs, metabolic factors and inflammation, cannot be overlooked (Stürmer *et al.* 1998, 2001; Yusuf *et al.* 2010; Trelle *et al.* 2011). However, there might be direct relationship between OA and vascular damage (Ghosh & Cheras, 2001; Conaghan *et al.* 2005). A study investigating knee bone marrow lesions (a surrogate marker for OA) found wider retinal venular calibre in subjects with signs of early OA (Davies-Tuck *et al.* 2012).

These results suggest systemic vascular involvement already in the early phase of OA. Another study determined aortic elasticity, using echocardiography in knee OA patients and found decreased aortic elasticity in OA patients (Belen *et al.* 2016). Furthermore, hand OA has been associated with increased arterial stiffness even though it was largely attributable to the confounding effect of age (Saleh *et al.* 2007). However, Goldsmith *et al.* (Goldsmith *et al.* 2014) found that large artery elasticity characteristics were not associated with the size or presence of bone marrow lesions. Altogether, there are only a few studies investigating the associations between OA and arterial stiffness and the results are inconsistent. Hence, drawing valid conclusions at the present is difficult and new studies are highly warranted.

The current thesis aims to provide a broader cardiovascular profile of OA patients with emphasis on arterial stiffness, but also taking into account the traditional risk factors (obesity, lipid metabolism, glucose metabolism, hypertension, tobacco use and different inflammatory markers).

2.2.2 Arterial function and cardiovascular diseases

The paramount role of vascular function in the pathogenesis of CVD has been undoubtedly established. Arterial stiffness has emerged as one of the earliest and independent determinants of cardiovascular morbidity and mortality (Laurent *et al.* 2001; Vlachopoulos *et al.* 2010). Increase in aortic stiffness is a hallmark of the ageing process and the consequence of many diseases like atherosclerosis, diabetes and chronic renal insufficiency (Lyle & Raaz, 2017). However, the precise mechanisms behind the development of arterial stiffening are largely unknown and remain to be discovered.

Arteries are the conduit vessels that provide the blood flow from the heart to the peripheral organs. The parameters of the arteries vary in wall structure and function depending on the location. The central arteries are more elastic and the peripheral arteries contain a thicker muscle layer and produce more resistance (Zieman *et al.* 2005). The arterial wall consists of 3 layers: intima, media and adventitia. These layers work together sensing and responding to acute changes in blood pressure (BP) by dilation and constriction (Zieman *et al.* 2005). The intima is the innermost layer and consists of a single layer of endothelial cells and functions as antithrombotic surface. The media consists of elastic lamellae, collagen and vascular smooth muscle cells and is responsible for the elastic properties of the arterial wall and the ability to dilate and contract. The outermost layer is the adventitia which is composed of fibroblasts and collagen (Lyle & Raaz, 2017). Elastin bears most of the load at lower pressures and collagen supports the arterial wall at higher pressures. In the case of increased arterial stiffness, the content of elastic elastin fibres is reduced and the content of stiffer collagen fibres is increased, which has also been observed in the inflammatory milieu (Johnson *et al.* 2001). In addition, cellular content is changed including infiltration of smooth muscle cells and macrophages and

increased expression of MMP, transforming growth factor beta and cytokines (Lakatta, 2003). Another functional determinant of arterial stiffness is mediated by paracrine mediators such as angiotensin (Dzau, 1986), endothelin (Yanagisawa *et al.* 1988) and nitric oxide (NO) (d'Alessio, 2004). Impaired production and action of NO lead to endothelial dysfunction that is a feature of arterial stiffness (Matz *et al.* 2000).

The pathogenesis of arterial stiffness is complex involving several established mechanisms. Ageing is one of the strongest risk factors for arterial stiffness (Laurent *et al.* 2006). The potential pathways through which ageing affects arterial stiffening are accumulation of advanced glycation end-products, inflammation, elastin fractures and calcification (Wang *et al.* 2007; Lyle & Raaz, 2017). Another condition that is closely related to arterial stiffness is hypertension. However, whether arterial stiffness is induced by hypertension (increased elastin degradation from pulsatile aortic wall stress (O'Rourke & Mancia, 1999)), or whether hypertension is the result of increased arterial stiffness is a matter of debate (Dernellis & Panaretou, 2005).

Metabolic disorders too have been linked to increased arterial stiffness. Increased arterial stiffening is one of the major complications of diabetes mellitus (Stehouwer *et al.* 2008). An important mechanism that leads to organ damage in MetS is generated by OxS (Roberts & Sindhu, 2009). OxS has been found to induce endothelial dysfunction by decreasing NO bioavailability due to reactive oxygen species (Lyle & Raaz, 2017). A possible source for the excessive amount of reactive oxygen species might be the mitochondria (Zhou *et al.* 2012). Another major reversible risk factor for arterial stiffness is smoking (Mahmud & Feely, 2003). However, the effects of cigarette smoking on the arterial stiffness of different segments of arteries are still unclear and need further research (McEvoy *et al.* 2015).

2.2.2.1 Measures of arterial stiffness

Arterial stiffness can be quantified using invasive and non-invasive techniques. Owing to the rapid development of the non-invasive methodology that enables to measure arterial stiffness relatively fast with high validity, in the majority of studies use non-invasive methods (Laurent *et al.* 2006). Pulse wave velocity (PWV) resembles the speed of a pressure wave travelling through the arterial tree and is directly associated with the elastic modulus of the vessel. In stiffer arteries PWV is increased. Carotid to femoral pulse wave velocity (cf-PWV) is considered the 'gold standard method' for measuring arterial stiffness (Townsend *et al.* 2015). There are various techniques for measuring PWV: 1) using a tonometer or a probe to acquire the pulse wave with a transducer; 2) using cuffs placed around the neck or limbs to determine the arrival of the pulse wave oscillometrically; 3) using ultrasonography; 4) using the magnetic resonance imaging-based approach (Townsend *et al.* 2015). One of the most widespread techniques for determining the cf-PWV is applanation tonometry.

The arterial pressure waves generated by the heart travel through the arterial system. The pressure wave is a composite of the forward travelling waves and wave reflections (Koh *et al.* 1998). Waves are reflected mainly from the branching points of arteries and other impedance mismatch sites (Laurent *et al.* 2006). In healthy vessels, reflected waves return to the aortic root in diastole, thereby increasing diastolic pressure. However, in stiffened arteries, waves arrive earlier in end-systole and augment systolic pressure (Nichols & Edwards, 2001). This can be quantified by means of augmentation index (Aix) (Townsend *et al.* 2015). However, some studies that account of the reservoir function of aorta, which markedly reduces the contribution of wave reflections to central BP augmentation, thus questioning the magnitude of impact from reflected waves (Tyberg *et al.* 2009; Davies *et al.* 2010; Davies-Tuck *et al.* 2012). Measurement of central hemodynamics can be made invasively or non-invasively, the latter being more appropriate in the clinical setting. Non-invasive pulse wave analysis (PWA) uses pressure waves from the carotid, brachial or radial artery and central pressure waveforms are calculated using transfer function or special algorithms (Townsend *et al.* 2015). Measurement of peripheral BP is of great importance for calibration and accurate results. Using pressure waves and simultaneously obtained or estimated flow waves, separation analysis can be applied to record the amplitudes of forward and backward travelling waves (Kips *et al.* 2009). Another method has been developed based on the electric circuit (employing a modified Windkessel model), which enables to measure proximal capacitive and peripheral oscillatory compliance (Cohn *et al.* 1995). In the case of this technique, a sensor on the radial artery identifies reflections in diastole as a decaying sinusoidal wave that is largely affected by the decreased buffering function of the aorta (Cohn *et al.* 1995; McVeigh, 2003).

2.2.2.2 Prognostic value of arterial stiffness

Arterial stiffness has been found to predict multiple end-organ complications. Arterial stiffness measured as cf-PWV is an independent predictor of cardiovascular and all-cause mortality (Laurent *et al.* 2001; Vlachopoulos *et al.* 2010; Townsend *et al.* 2015). However, data about carotid to radial PWV (cr-PWV) and its possible predictive properties are inconsistent (Pannier *et al.* 2005; Young-Soo *et al.* 2006; Townsend *et al.* 2015). Recently, increased aortic stiffness coupled with decreased brachial stiffness independently predicted mortality in dialysis population, thus challenging the 'gold standard' (Fortier *et al.* 2015; Covic & Siriopol, 2015). Increased arterial stiffness leads to an increase in systolic arterial pressure and a decrease in diastolic arterial pressure, resulting in isolated systolic hypertension, the most common form of hypertension in the elderly, causing excess morbidity and mortality (Franklin *et al.* 1997). Apart from being the cause of causing heart failure (Tsao *et al.* 2015), increased aortic stiffness leads to higher pulsatile pressures in the vulnerable

microcirculation of many organs. Therefore, increased arterial stiffness is related to cerebral small vessel disease and cognitive impairment (Fukuhara *et al.* 2006; Ding *et al.* 2015; Pase *et al.* 2016). Atherosclerosis leads to increased arterial stiffening and loss of small artery elasticity, which is an independent prognostic factor in patients with peripheral arterial disease (Kals *et al.* 2014). Also, arterial stiffening has been shown to accelerate renal insufficiency in chronic kidney disease (Selwaness *et al.* 2014). In conclusion, increased arterial stiffness is associated with not only increased CVD but also with other end-organ dysfunctions (Lyle & Raaz, 2017).

2.3 The role of adipokines in osteoarthritis and cardiovascular diseases

The former view of the adipose tissue as a passive reservoir for storing energy is no longer valid. The adipose tissue is now acknowledged as a metabolically active tissue that has great importance in regulating homeostasis in normal and disease conditions (Raucci *et al.* 2013). Adipokines are mainly white adipose tissue derived proteins that might be associated with OA and CVD (Hu *et al.* 2011; Mattu & Randeva, 2013). The most thoroughly studied and clinically promising adipokines are leptin, adiponectin and resistin.

2.3.1 Leptin

Leptin is a 16 kDa non-glycosylated protein that is encoded by the LEP gene and was the first identified adipokine (Zhang *et al.* 1994). After leptin, hundreds of adipose tissue synthesised bioactive molecules have been discovered (Raucci *et al.* 2013). Leptin acts through its receptor OB-Rb that is encoded by the diabetes gene. This adipokine is mainly produced by adipocytes and its circulating levels are highly dependent on white adipose tissue mass (Conde *et al.* 2011). One of the most important roles of leptin is regulation of appetite and energy expenditure by inducing anorexigenic and supressing orexigenic factors (Ahima *et al.* 1996). In obese subjects the levels of leptin are high, which correlates with increased adipose tissue mass; however, normal appetite controlling mechanisms do not function properly and resistance to leptin develops (Ahima *et al.* 2006). Leptin has been associated with immunomodulation (Lam & Lu, 2007) and inflammation (Iikuni *et al.* 2008).

During the last decade interest in the association between leptin and OA has grown significantly. OA patients have increased levels of leptin in the synovial fluid as well as in the serum (de Boer *et al.* 2012). It has been suggested that leptin serves as the link between OA and obesity since the risk for developing OA does not increase in obese leptin gene knockout mice (Aspden, 2011). Leptin has been found to have an anabolic effect on cartilage in obese OA patients, however, there is accumulating evidence suggesting its proinflam-

matory action (Dumond *et al.* 2003; Hu *et al.* 2011). Leptin increases the levels of proteolytic enzymes like MMP (Toussirot *et al.* 2007). In addition, this adipokine has increased expression in osteoblasts (Mutabaruka *et al.* 2010). Furthermore, a recent study found that leptin levels were associated with knee OA progression and total joint replacement (Martel-Pelletier *et al.* 2016).

Besides OA, leptin has been found to be associated with CVD. The possible interaction with CVD is attributable its proinflammatory properties and to obesity related changes (Martin *et al.* 2008). Leptin has been linked to coronary heart disease and heart failure in prospective studies (Sattar *et al.* 2009; Wannamethee *et al.* 2011), as well as with arterial stiffness (Tsai *et al.* 2016), insulin resistance (Mantzoros *et al.* 1998) and OxS (Bouloumie *et al.* 1999). However, several studies question the role of leptin in development of CVD (Bidulescu *et al.* 2013; Martin *et al.* 2015). Altogether, leptin might play a key role in the pathogenesis of OA and may also be involved in development of CVD.

2.3.2 Adiponectin

Adiponectin is a 30 kDa protein that is mostly produced in the adipose tissue and is present in the serum in three forms: trimer, hexamer and high molecular weight (Kadowaki *et al.* 2006). Adiponectin acts through two major functionally distinct receptors, AdipoR1 and AdipoR2 (Kadowaki & Yamauchi, 2005). Adiponectin circulates in serum at high concentrations 5-30 μg/mL. Unlike leptin, adiponectin is inversely correlated with BMI and the plasma levels of leptin are decreased in obese patients (De Rosa *et al.* 2013). Adiponectin is widely recognised by its anti-inflammatory, anti-atherogenic and anti-diabetic properties and is differently expressed in men and women (Kern *et al.* 2003; Ohashi *et al.* 2012). This adipokine serves many functions in the human organism, such as energy metabolism (Lee & Shao, 2014) and inflammatory response modulation (Takemura *et al.* 2007).

The results of studies assessing adiponectin in relation to OA provide contradictory results. Adiponectin has been negatively associated with radiographic OA severity, which suggests the protective role of this adipokine in OA (Honsawek & Chayanupatkul, 2010; Zheng *et al.* 2016). However, higher adiponectin levels have been found in radiographically most severe OA (Koskinen *et al.* 2011) and in patients with erosive hand OA compared with non-erosive OA (Filková *et al.* 2009). These conflicting results might be caused by different isoforms of adiponectin, which act differently. Adiponectin has been found to upregulate MMP and inflammatory markers in primary human chondrocytes (Koskinen *et al.* 2011). Thus, the role of adiponectin, which is often thought of as protective adipokine, needs further research.

Furthermore, adiponectin seems to play role in the pathogenesis of CVD as well. Lower adiponectin levels have been found in symptomatic coronary artery disease patients (Kumada *et al.* 2003). Yet, high adiponectin levels at baseline

in prospective studies have been associated with increased mortality and CVD morbidity (Wu *et al.* 2014; Witberg *et al.* 2016). Since adiponectin has many anti-inflammatory properties (Ouchi *et al.* 1999; Kobashi *et al.* 2005), the upregulation might be secondary reaction in order to protect cells from excessive inflammatory cascade activation. Plasma adiponectin levels have been associated with progression of arterial stiffness in hypertensive patients (Youn *et al.* 2013). Hypoadiponectinemia has been found to be an independent correlate of excess arterial stiffness in young black adults (Nguyen *et al.* 2008). The role of adiponectin requires further research involving different disease and population groups.

2.3.3 Resistin

Resistin is a 12.5 kDa cysteine-rich protein that was first described in 2001 as a mediator of insulin resistance in obese mice (Steppan *et al.* 2001a). Resistin belongs to a family of tissue-specific resistin-like molecules (Steppan *et al.* 2001b). It consists of 108 amino acid prepeptides that circulate as dimeric proteins. The main source of resistin in the human body is mononuclear cells, outside the adipose tissue (Filková *et al.* 2013). Resistin is involved in the secretion of pro-inflammatory mediators and recruitment of other immune cells which associates this adipokine with many inflammatory conditions (Bokarewa *et al.* 2005; Silswal *et al.* 2005).

Higher levels of resistin have been found in OA patients compared with non-OA controls (Li *et al.* 2014). Resistin is expressed in the synovial fluid of OA joints (Presle *et al.* 2006). This adipokine is associated with increased release of inflammatory mediators from articular cartilage and with activation of MMP leading to cartilage degradation (Lee *et al.* 2009; Koskinen *et al.* 2014). Resistin has even been proposed as a possible drug target in OA (Koskinen *et al.* 2014).

Increasing evidence associates resistin with atherogenesis (Reilly *et al.* 2005; Jung *et al.* 2006). Resistin has been found to independently predict myocardial infarction, cardiovascular death and restenosis in patients undergoing percutaneous coronary intervention (On *et al.* 2007; Momiyama *et al.* 2011; Kreçki *et al.* 2011; Fontana *et al.* 2015). In addition, resistin might be associated with worse outcome after ischaemic stroke independently of other predictors (Efstathiou *et al.* 2007). Plasma resistin levels have been found to correlate with determinants of MetS, a known CVD risk factor (Norata *et al.* 2007). In conclusion, resistin is related to many diseases including OA and CVD and the key mechanisms seem to be associated with inflammation.

2.4 Metabolic syndrome and metabolomic profiling of osteoarthritis

MetS is a composite of central obesity, hypertension, atherogenic dyslipidemia, hyperglycaemia and insulin resistance (Grundy *et al.* 2004). For diagnosis of MetS, at least 3 of the 5 characteristics must be present: increased waist circumference, high triglyceride level, low level of high-density lipoprotein (HDL) cholesterol, hypertension and increased fasting glucose (Grundy *et al.* 2005). MetS increases the risk for coronary heart disease and stroke threefold and cardiovascular mortality fivefold (Isomaa *et al.* 2001). Also high-grade OxS has been considered a hallmark of MetS (Ford *et al*. 2003; Fortuño *et al.* 2006). Even though MetS was first acknowledged as a major risk factor for CVD, recent studies have linked MetS with other diseases including OA (Le Clanche *et al.* 2016).

2.4.1 Metabolic factors and osteoarthritis

It has been reported that OA patients have increased prevalence of MetS (Puenpatom & Victor, 2009). According to the data of NHANES III, 59% of the patients with OA had MetS compared with 23% of the persons in the population without OA (Puenpatom & Victor, 2009). Thus the association between OA and MetS is worth exploring.

Hypertension is a prevalent condition in OA patients and it has been demonstrated that among 1000 patients with hip OA, 55% had hypertension or CVD (Marks & Allegrante, 2002). There are several possible explanations for how OA is linked to hypertension. OA patients have decreased physical activity that is a known risk factor for hypertension (Huai *et al.* 2013). OA patients use often non-steroidal anti-inflammatory drugs, which might be associated with hypertension or might interfere with hypertension treatment (Aljadhey *et al.* 2012). However, since vascular pathology presumably plays a role in the pathogenesis of OA, then OA might be the result of vascular damage (Findlay, 2007). Hypertension is also associated with other components of MetS, especially visceral obesity (Hayashi *et al.* 2004).

Central obesity is a growing health concern that has been associated with OA progression and increased disability (Felson *et al.* 1988; Cooper *et al.* 2000; Nishimura *et al.* 2011). Obesity results in increased loading of the weight-bearing joints that lead to activation of mechanoreceptors that induce the expression of cytokines and proteolytic enzymes (Pottie *et al.* 2006). However, obesity has also been associated with hand OA that is not affected by weight bearing (Visser *et al.* 2014). This suggests that the association between obesity and OA is more complex. Data from Osteoarthritis Initiative suggests that OA patients with normal BMI and central obesity have a higher risk of decline in physical activity and function, therefore, they might benefit from obesity related complication risk reclassification (Batsis *et al.* 2015). In addition, central

obesity is associated with the dysbalance of adipokines, which has impact on OA progression (Poonpet & Honsawek, 2014), CVD risk (Nakamura *et al.* 2014), insulin resistance, dyslipidemia and inflammation (Jung & Choi, 2014).

Dyslipidemia is an important component of MetS that is interrelated with the other components. MetS is associated with excess calories intake due to a high fat diet that leads to accumulation of lipids in different tissues (McGavock *et al.* 2006; Harasim *et al.* 2015). Articular chondrocytes express fatty acid and cholesterol transport proteins and receptors for low-density lipoprotein (LDL)-cholesterol. A characteristic feature of OA in chondrocytes is accumulation of cholesterol and fatty acids (Kosinska *et al.* 2013). One of the reasons for this might be impaired efflux of lipids in OA chondrocytes due to the decreased gene expression of efflux regulating genes (Tsezou *et al.* 2010). In addition, metabolism of free fatty acids can lead to high grade OxS that is associated with cartilage damage as well as with increased arterial stiffness (Yudoh *et al.* 2005; Kals *et al.* 2008). Serum cholesterol and triglyceride levels have been found to be associated with the incidence of bone marrow lesions in a prospective study (Davies-Tuck *et al.* 2012). HDL-cholesterol has anti-inflammatory properties and has been found to be protective of bone marrow lesions (Doré *et al.* 2012), however OA patients have lower levels of HDL-cholesterol (Karvonen-Gutierrez *et al.* 2012). In general, dyslipidemia might have an important role in the pathogenesis of OA, but the knowledge is insufficient and needs further research in order to determine, whether lipid metabolism biomarkers could be used as diagnostic and prognostic markers for OA.

Insulin resistance has been considered to be the core component of MetS. Glucose can modulate anabolic and catabolic genes including the genes of MMP 1 and 13 in articular chondrocytes (Rosa *et al.* 2011). In diabetic mice, chondrocytes became resistant to insulin growth factor I that mediates anabolic effects (Di Cola *et al.* 1997). Hyperglycemia results in development of a chronic pro-inflammatory environment and further interferes with the insulin receptor pathway, which leads to excessive OxS (Saudek & Kay, 2003; Rosa *et al.* 2009). A meta-analysis of the associations between OA and diabetes found that OA was more prevalent in diabetes patients (Louati *et al.* 2015). Hyperglycaemia has been found to be associated with increased symptoms of pain, disability and depression in OA patients (Li *et al.* 2016). Hyperglycemia might play central role in the pathogenesis of OA by causing low-grade inflammation (Stannus *et al.* 2010), cartilage damage, subchondral bone changes (Zhuo *et al.* 2012) and high-grade OxS (Rosa *et al.* 2009).

2.4.2 Oxidative stress and osteoarthritis

OxS is characterised by imbalance between the production and neutralisation of free radicals and active metabolites (Sies & Cadenas, 1985; Majima *et al.* 2016). Reactive oxygen species and reactive nitrogen species have an important role in normal physiological conditions (Nathan, 2003). However, in excessive

quantities they cause damage to proteins, carbohydrates, lipids and DNA, leading to possible cell death (Rahman *et al.* 2012). To cope with excessive OxS, the organism has several antioxidant mechanisms that are enzymatic and nonenzymatic and usually succeed in blocking the harmful effects of free radicals. The enzymatic antioxidant system consists of catalase, superoxide dismutase and glutathione peroxidase among others (Rahman *et al.* 2012). Since superoxide is the primary reactive oxygen species, its dismutation by superoxide dismutase is of primary importance for each cell (Birben *et al.* 2012). Nonenzymatic antioxidants include low-molecular-weight compounds such as vitamin C, vitamin E, glutathione, carotenoids and uric acid (Birben *et al.* 2012).

In pathological conditions the natural defence system fails to neutralise the reactive oxygen species and TAC is reduced. High grade OxS has been implicated into multiple pathological conditions including OA (Maneesh *et al.* 2005; Ziskoven *et al.* 2010). It has been noted that in OA the amount of total peroxides overwhelms antioxidant capacity, thus leading to overt OxS. Patients with OA have increased markers of OxS (Altindag *et al.* 2007). OxS has a major role in modulating chondrocyte function (Mazzetti *et al.* 2001), in inducing synovial fibroblast death (Jovanovic *et al.* 2002) and subchondral bone remodelling (Boileau *et al.* 2009), and in decreasing the resynthesis of collagen in OA (Altindag *et al.* 2007). Therefore, OxS might play a crucial role in development and progression of OA. Moreover, OxS has an important role in endothelial dysfunction and increased arterial stiffness (Kals *et al.* 2008; Kim *et al.* 2013).

Oxidized LDL (oxLDL) is the result of the impact of excessive OxS on LDL-cholesterol. OxLDL is a mixture of heterogeneously modified LDL particles that consist of different oxidized lipids and amino acid residues (Itabe, 2012). Precise mechanisms by which LDL is oxidized are largely unknown. oxLDL acts through several receptors that include the lectin-like oxLDL receptor-1 (Yoshida *et al.* 1998). The receptors of oxLDL are expressed on multiple cells including endothelial cells and macrophages (Kodama *et al.* 1988). Activation of these receptors by oxLDL leads to the phenotype shift into active inflammatory cytokine producing cells (van Tits *et al.* 2011). Therefore, oxLDL can also be viewed as a biomarker of inflammation (van Tits *et al.* 2011; Tekin *et al.* 2013; de Munter *et al.* 2013). Furthermore, oxLDL has a role in the pathogenesis of OA. After oxLDL has been taken up by chondrocytes, it induces OxS, which leads to cartilage damage (Akagi *et al.* 2009). Animal studies have demonstrated that LDL accumulates in the synovial lining and leads to activation of the synovium and osteophyte formation while the uptake of oxLDL by macrophages has been associated with activation of TGF-β (de Munter *et al.* 2013). In addition to being involved in the pathogenesis of OA, ox-LDL has also been associated with arterial stiffening (Brinkley *et al.* 2009), endothelial dysfunction (Cominacini *et al.* 2000) and CVD (Bliden *et al.* 2016).

2.4.3 Matrix metalloproteinase 3 and osteoarthritis

MMP3 is also known as stromelysin-1 and the MMP3 encoding gene is situated on chromosome 11. MMP3 is a 59/57 kDa zymogen that after secretion is proteolytically processed into 28 kDa and 45 kDa active forms (Chen *et al.* 2014). MMP3 belongs to the family of MMP that are responsible for degradation of different substrates and regulate cell-matrix composition (Chakraborti *et al.* 2003). The family of MMPs are zinc-dependent endopeptidases that have the ability to cleave extracellular matrix constituents and non-matrix proteins (Massova *et al.* 1998). MMPs regulate the remodelling and turnover of the extracellular matrix, which in addition to structural support, play an important role in signalling and participate in cell proliferation, differentiation and death (Chakraborti *et al.* 2003). The expression of MMPs is usually low in tissues and increases when remodeling is required. MMP3, like many other MMPs, is first secreted as a proenzyme and requires further activation. MMP3 can degrade collagens and matrix proteins such as laminin and proteoglycan (Chen *et al.* 2014). More importantly, MMP3 has the ability to activate other MMPs (e.g. MMP1, MMP9, MMP13) amplifying the proteolytic effect (Leong *et al.* 2010; Chen *et al.* 2014). The activity of MMPs is strictly controlled by tissue inhibitors of MMPs (Brew *et al.* 2000). MMP3 is associated with different diseases that include OA (Leong *et al.* 2010; Chen *et al.* 2014), myocardial infarction and morbidity after the infarction (Wang *et al.* 2011; Abd El-Aziz & Mohamed, 2016), as well as malignancies (Morrison *et al.* 2009).

The expression of MMP3 has been found to be increased in OA compared with healthy controls and the level of MMP3 correlates positively with cartilage damage (Lohmander *et al.* 1993; Fernandes *et al.* 2002) (Lohmander *et al.* 1993). Furthermore, MMP3 was highly expressed in the synovium, which suggests an important role of the synovium in MMP3 production and subsequent cartilage damage. Among other MMPs and inflammatory mediators, MMP3 is expressed in the damaged superficial cartilage layer in OA (Tetlow *et al.* 2001). Inflammation and adipokines can promote the production of MMP3 (Conde *et al.* 2011; Yang *et al.* 2013). It has been demonstrated that immobilisation increases MMP3 levels and mobilisation can decrease the level of MMP3, thus combating cartilage deterioration (Leong *et al.* 2010). In conclusion, MMP3 is associated with cartilage damage in OA and might prove to be an early biomarker of OA and reflect disease severity. However, further research is needed in order to clarify which the clinical relevance of MMP3 in OA.

2.4.4 Metabolomic profiling of osteoarthritis

Metabolomics is a novel methodology for assessing low-molecular weight molecules (<1.5 kDa) in a biological system at a specific point of time. The recent and most demanding challenge in systems biology is to incorporate genomics, transcriptomics, proteomics and metabolomics to provide a better

understanding of cellular biology. Metabolomics focuses on identification and quantification of small intermediary molecules and products of metabolism, the metabolome. The metabolome is the terminal downstream product of the genome and consists of the total amount of low-molecular weight molecules in an organism that are required for maintaining normal function in a specific physiological state. These metabolites include amino acids, peptides, carbohydrates, vitamins, thiols, lipids, nucleic acids and fatty acids (Zhang *et al.* 2012). Therefore, the metabolome represents a real time functional portrait of a living organism.

Several detection methods that are used to quantify changes in the concentrations of endogenous metabolites include, but are not limited to, ^{1}H-nuclear magnetic resonance, ^{13}C-nuclear magnetic resonance spectroscopy, gas-chromatography-mass spectrometry, direct infusion-mass spectrometry, gas chromatography-flame ionization detection and liquid chromatography-mass spectrometry (Roberts & Sindhu, 2009).

Two fundamentally distinct approaches can be taken to analyse the biofluids or tissues: targeted and non-targeted metabolomics (Nobeli & Thornton, 2006). For targeted metabolomics, quantitative values for only a preselected set of known metabolites are determined (Klein & Shearer, 2016). Also, internal standards must be used to calibrate sample concentrations by adding reference substances at known concentrations in order to obtain accurate and valid results. However, non-targeted analysis is the comprehensive analysis of all detectable metabolites in a sample. This includes a large number of unknown metabolites. Both approaches have pros and cons and the choice of the method depends on the study objectives (Christians *et al.* 2011).

Metabolomic profiling of OA is a novel approach for obtaining insights into the pathophysiological mechanisms that drive OA as well as for detecting new biomarkers. In recent years the field of metabolomic research is growing rapidly and scientists are sensing the potential of this methodology. There are several studies that have adopted this approach to analyse OA (Lamers *et al.* 2005; Zhang *et al.* 2014, 2015). Metabolomics is highly needed in the field of OA because of the heterogeneity of the disease and the recognition that no single biomarker can fully describe the pathological processes involved in the OA (Bay-Jensen *et al.* 2016). A study investigating the metabolite profile of urine in OA patients and controls found distinct changes in the profile of OA subjects and the profile was associated with radiographic severity (Lamers *et al.* 2005). Another study demonstrated that the serum ratios of valine and leucine to histidine were related to knee OA, which suggested the use of branched-chain amino acids as potential biomarkers (Zhai *et al.* 2010). In addition, perturbations of lipid metabolism have been described in OA (Adams *et al.* 2012; Zhang *et al.* 2014).

Acylcarnitines are the intermediates of the fatty acid β-oxidation process. L-carnitine is required to transport activated long-chain fatty acids from the cytosol into the mitochondrion, where fatty acid oxidation takes place. Inside the cell fatty acids are activated by esterification with co-enzyme-A.

Acylcarnitines are produced via transfer of a fatty acyl-Co-enzyme-A to L-carnitine by carnitine transferases. After being transported through the mitochondrial membrane, the acyl-co-enzyme-A is converted to acylcarnitines by carnitine palmitoyltransferase 1 (CPT-1). CPT-1 is one of the most important regulators of long-chain fatty acid oxidation (McGarry *et al.* 1977). After being transported into the mitochondrion matrix, CPT-2 reconverts acylcarnitines back into free carnitine and long-chain acyl-co-enzyme-A that can then be oxidized (Ramsay *et al.* 2001). Acylcarnitines are transported through the cell membrane and circulate in plasma. The physiological role of acylcarnitine efflux to plasma is unclear. However, acylcarnitine formation prevents Co-enzyme-A trapping, thus enforcing continuation of Co-enzyme-A dependent metabolic processes (Lopaschuk *et al.* 1994). Acylcarnitines might also play a role in the detoxification process since they are found in urine and bile (Chalmers *et al.* 1984; Mueller *et al.* 2003). While the physiological role of plasma acylcarnitines remain to be elucidated, the level of acylcarnitines has been associated with several diseases including diabetes (Mihalik *et al.* 2010), encephalopathy (Murphy *et al.* 2007) and OA (Zhang *et al.* 2014) among others. Recent evidence suggests that acylcarnitines might be associated with activation of the pro-inflammatory signalling pathway (Rutkowsky *et al.* 2014). A serum assay of acylcarnitines has been performed for screening of genetic disorders of oxidation of fatty acid enzymes, which typically result in accumulation of acylcarnitines due to poor co-ordination between β-oxidation and the tricarboxylic acid cycle (Pourfarzam & Zadhoush, 2013). A nested case-control study has found that long-chain acylcarnitines predicted cardiovascular mortality in dialysis patients (Kalim *et al.* 2013). Another study found independent association between acylcarnitines and heart failure (Ahmad *et al.* 2016). In addition, the plasma level of acylcarnitines has been found to be associated with aortic stiffness in coronary artery disease patients (Paapstel *et al.* 2016). There are few small studies investigating the role of acylcarnitines in OA. A case-only study with 80 end-stage OA patients found that the patients can be divided into two distinct groups according to levels of acylcarnitines (Zhang *et al.* 2014). One of the groups had higher prevalence of hypertension, which might suggest that the CVD risk in these groups was different. Thus, acylcarnitines are associated with different disease groups and might provide insights into the pathologies involved in pathogenesis of the disease at a cellular level.

So far the disease-modifying treatment does not exist for OA and one of the pitfalls of developing new treatment is due to difficulties in accurate assessment of therapy response using radiographic techniques. Thus, there is urgent need for novel biomarkers that could describe disease status, prognosis and treatment response confidently in these patients. Metabolomics is close to ideal method for finding novel biomarkers. Moreover, studies published so far suggest that acylcarnitines might provide insights into the pathogenesis of OA and CVD.

2.5 Summary of the Literature

OA is a complex disease but still lacks a clear understanding of etiopathogenesis. There are several pieces of evidence suggesting increased CVD risk for OA patients, however the precise mechanisms are largely unknown. Only a few inconclusive studies with discordant results have investigated the association between OA and arterial stiffness, which is independent CVD risk factor. Another possible link between OA and CVD is obesity that promotes systemic low-grade inflammation and dysbalance in the level of adipokines. Therefore, further research is requiered to clarify the role of adipokines in OA and their potential as clinical biomarkers. In addition, there is no consensus on how MetS and OxS are linked to OA. The potential protective role of HDL-cholesterol and detrimental effects of triglycerides and LDL-cholesterol in OA needs to be elucidated. Furthermore, being able to detect changes in the low molecular weight metabolites might provide valuable information about the pathogenesis of OA, which helps to develop better diagnostic and prognostic biomarkers.

3. AIMS OF THE THESIS

The general aim of the thesis was to analyze arterial stiffness and metabolic biomarkers in end-stage osteoarthritis patients in comparison to controls. The current thesis was focused to explore the role of vascular function in osteoarthritis and to search new promising biomarkers for early diagnosis and disease course prediction.

Specific aims:

1. To measure arterial stiffness in osteoarthritis patients in comparison with asymptomatic controls and to assess the relationship between arterial stiffness and osteoarthritis severity.
2. To determine the levels of adipokines (leptin, adiponectin) and matrix metalloproteinase 3 in relation to osteoarthritis severity and arterial stiffness in end-stage osteoarthritis patients and in controls.
3. To assess the impact of metabolic syndrome and oxidative stress for osteoarthritis patients and for controls.
4. To determine the impact of acylcarnitines on osteoarthritis severity and on arterial stiffness in patients with end-stage osteoarthritis and in controls.

4. SUBJECTS AND METHODS

4.1 Subjects

4.1.1 Osteoarthritis patients

The study population included a total of 70 patients with end-stage knee and hip OA (n=48, n=70, n=55, n=70 in Papers I–IV, respectively). The study participants were prospectively recruited from the Department of Orthopaedic Surgery, Tartu University Hospital, Estonia, in 2014-2016. Only patients with primary OA were included according to the American College of Rheumatology criteria for knee and hip OA (Altman *et al.* 1986, 1991)**.** The exclusion criteria were posttraumatic OA, infectious and endocrine related arthropathy, any acute or chronic inflammatory disease, malignancy, renal insufficiency (estimated glomerular filtration rate (eGFR) < 60ml/min/1.73m^2), cardiac arrhythmia, clinically significant heart failure, valvular disease, diabetes. The presence of these conditions was determined based on interview with the study participant, clinical examination, blood tests and medical database.

4.1.2 Age- and gender-matched controls

A total of 82 age- and gender matched subjects (n=49, n=70, n=55, n=82 in Papers I–IV, respectively) were identified using assistance from local family physicians. The exclusion criteria for the control group were (based on interviews, clinical examinations and blood tests): any concomitant acute or chronic inflammatory disease, a visit to family practitioner due to hip or knee joint complaints, any persistent knee or hip joint pain, diabetes, symptomatic coronary artery disease, cardiac arrhythmia, cerebrovascular or peripheral artery disease, malignancies and renal insufficiency.

4.2 Study design and protocol

The medical history and lifestyle factors were recorded using a self-completed questionnaire and interview. Blood samples were collected between 07:00 and 11:00 after an overnight fast and abstinence from tobacco, coffee and alcohol. The height and weight of the patients were recorded and BMI was calculated. Waist circumference was measured with a tape at the end of a normal expirium at the narrowest part between the iliac crest and the lowest rib. HHS and HSS Knee score were assessed. The study participants were examined after at least 10 minutes of rest in a supine position in a quiet, temperature controlled room. Next, peripheral BP and PWV were measured and PWA was performed. Written informed consent was obtained from all study participants. The study was approved by the Ethics Committee on Human Research of the University of Tartu.

4.3 Methods

4.3.1 Measurement of osteoarthritis severity

Standard weight-bearing anteroposterior radiographs were taken from the hip and knee joint. The radiographic severity of OA was assessed using the Kellgren-Lawrence grading system (Kellgren & Lawrence, 1957). The radiographs were evaluated independently by two observers who were blinded to the clinical data and a consensus score was used. Intraclass correlation coefficient was used to assess consistency among the raters. The values of intraclass correlation coefficient under 0.75 are considered poor to moderate, over 0.75 good and over 0.90 reasonable for clinical measurements (Portney & Watkins, 2009). In addition, HHS (Harris, 1969) and HSS Knee score (Insall *et al.* 1976) were used as physician reported outcome measures for the hip and knee OA patients, respectively, and for the controls.

4.3.2 Analysis of biochemical markers

Peripheral venous blood samples were collected (into serum separator tubes BD SST II^{TM} Advance) and centrifuged at room temperature 30 minutes after collection. The serum was pipetted into Eppendorf tubes and stored at –70°C until analysis. The serum levels of triglycerides, total cholesterol, LDL-cholesterol, HDL-cholesterol, white blood cell count, high-sensitivity C-reactive protein (hs-CRP), serum creatinine, and eGFR were measured, using standard laboratory methods in the local clinical laboratory, with automated analyzers.

Serum adiponectin levels were measured using an enzyme-linked immunosorbent assay (ELISA) (Human Total Adiponectin/Acrp30 Immunoassay, R&D Systems Europe, Abingdon, UK). Human Total MMP3 Immunoassay kits available from R&D systems a Bio-Techne brand (catalogue number DMP 300) were used for quantitative determination of human active and promatrix metalloproteinase 3 (total pro-MMP3) concentrations. The level of serum leptin, resistin, C-peptide and insulin was measured using Evidence Investigator™ Metabolic Syndrome Array1, based on the sandwich chemoluminescent immunoassay (Randox Laboratories Ltd METS1 catalogue number EV 3755). The core technology is the Randox Biochip which contains an array of discrete test regions of immobilized antibodies specific for different biomarkers of MetS. The light signal generated from each of the test regions on the biochip is detected using the digital imaging technology. Insulin resistance was calculated from glucose and insulin levels, using updated nonlinear homeostasis model assessment estimated insulin resistance (HOMA2 IR) (Levy *et al.* 1998), which takes account of variations in hepatic and peripheral glucose resistance.

The total peroxide concentration (TPX) of the samples was determined using the OXYSTAT Assay Kit Cat. No BI-5007 (Biomedica Gruppe, Biomedica Medizinprodukte GmbH & Co Kg, Wien). Peroxide concentration was

determined by the reaction of biological peroxides with peroxidase and the subsequent colour reaction using TMB as the substrate. After addition of a stop solution, the coloured liquid was measured. A calibrator was used to calculate the concentration of circulating biological peroxides in the sample. TAC was measured applying an automated measurement method (Erel, 2004). In TAC measurement, a hydroxyl radical is produced. The ferrous ion solution in reagent 1 was mixed with H_2O_2 in reagent 2. Sequentially produced radicals include the brownish-coloured dianisidinyl radical cation. The antioxidative effect of the sample on potent free radical reactions was used to determine TAC. The percentage of the ratio of total plasma peroxide concentration to plasma TAC was accepted as oxidative stress index (OSI), which is an indicator of OxS. Commercially available ELISA kits were used to determine serum oxidized low-density lipoproteins (oxLDL) (Mercodia AB, Uppsala Sweden; catalogue number 10-1143-01) and myeloperoxidase (MPO) (Biocheck, Foster City, CA; catalogue number BC 1129).

4.3.3 Targeted analysis of metabolites

Serum for detection of metabolites was collected into plain tubes (Plain BD Vacutainer® Tubes). Samples were centrifuged for 60 minutes and the supernatant was pipetted into Eppendorf tubes and frozen at –70°C until analysis. The levels of metabolites in the serum were determined using the AbsoluteIDQ™ p180 kit (BIOCRATES Life Sciences AG, Innsbruck, Austria) according to the manufacturer's instructions. The assay procedures have been described in detail previously (Nkuipou-Kenfack *et al.* 2014). The validated assay allows comprehensive identification and quantification of 186 endogenous metabolites including 40 acylcarnitines, 14 lysophosphatidylcholines, 76 phosphatidylcholines, 19 biogenic amines, 21 amino acids, 15 sphingomyelins and the sum of hexoses from 10 µl of serum. The total number of carbons and double bonds present in the lipid fatty acid chains are denoted by C x:y, where x indicates the number of carbons and y indicates the number of double bonds. Glycerophospholipids are further differentiated according to the presence of ester (a) and ether (e) bonds in the glycerol moiety. Double letters (aa = diacyl, ae = acyl–alkyl) indicate that two glycerol positions are bound to the fatty acid residue, while a single letter (a = acyl or e = alkyl) indicates a bond with only one fatty acid residue. Identification and quantification of the metabolites was done using multiple reaction monitoring according to internal standards. Calculation of metabolite concentration was automatically performed using the MetIDQ™ software package integrated into the AbsoluteIDQ kit. The concentrations of metabolites were calculated in µM.

Table 1. Acylcarnitines that can be quantified with the AbsoluteIDQ p180 kit (http://www.biocrates.com/images/ListofMetabolites_p180.pdf).

Acylcarnitines			
C0	Carnitine	C10:1	Decenoylcarnitine
C2	Acetylcarnitine	C10:2	Decadienylcarnitine
C3	Propionylcarnitine	C12	Dodecanoylcarnitine
C3:1	Propenoylcarnitine	C12:1	Dodecenoylcarnitine
C3-OH	Hydroxypropionyl-carnitine	C12-DC	Dodecanedioylcarnitine
C4	Butyrylcarnitine	C14	Tetradecanoylcarnitine
C4:1	Butenylcarnitine	C14:1	Tetradecenoylcarnitine
C4-OH (C3-DC)	Hydroxybutyryl-carnitine	C14:1-OH	Hydroxytetradecenoylcarnitine
C5	Valerylcarnitine	C14:2	Tetradecadienylcarnitine
C5:1	Tiglylcarnitine	C14:2-OH	Hydroxytetradecadienylcarnitine
C5:1-DC	Glutaconylcarnitine	C16	Hexadecanoylcarnitine
C5-DC (C6-OH)	Glutarylcarnitine (Hydroxyhexanoyl-carnitine)	C16:1	Hexadecenoylcarnitine
C5-M-DC	Methylglutaryl-carnitine	C16:1-OH	Hydroxyhexadecenoylcarnitine
C5-OH (C3-DC-M)	Hydroxyvaleryl-carnitine (Methylmalonyl-carnitine)	C16:2	Hexadecadienylcarnitine
C6 (C4:1-DC)	Hexanoylcarnitine (Fumarylcarnitine)	C16:2-OH	Hydroxyhexadecadienylcarnitine
C6:1	Hexenoylcarnitine	C16-OH	Hydroxyhexadecanoylcarnitine
C7-DC	Pimelylcarnitine	C18	Octadecanoylcarnitine
C8	Octanoylcarnitine	C18:1	Octadecenoylcarnitine
C9	Nonaylcarnitine	C18:1-OH	Hydroxyoctadecenoylcarnitine
C10	Decanoylcarnitine	C18:2	Octadecadienylcarnitine

4.3.4 Measurement of arterial stiffness and central haemodynamics

Peripheral BP was measured using a digital oscillometric device (A&D UA-767; A&D Company Ltd., Tokyo, Japan) and the mean of three readings was recorded. Mean arterial pressure (MAP) was obtained by integration of the radial pressure waveform using the SphygmoCor software (SCOR Px, 7.0; AtCor Medical, Sydney, Australia).

Pulse wave velocity was determined from electrocardiogram (ECG)-gated sequential recordings of the carotid and radial or femoral pulse waveforms (SphygmoCor, AtCor Medical, Sydney, Australia) using an arterial tonometer (SPT-301B, Millar Instruments, USA). The R-wave in ECG was used as the reference in determining the travel time of the pulse wave between the recording sites. The PWV was automatically calculated by the Sphygmocor software as the ratio of the distance between the measuring sites to the transit

time (Townsend *et al.* 2015). For calculating the cf-PWV, the distance from the suprasternal notch over the umbilicus to the femoral artery minus minus the distance from suprasternal notch to the carotid artery was used. For calculating cr-PWV, the distance from the suprasternal notch to the radial artery measuring site carotid arterial length was obtained. Sequential recording was done over an approximate 20 sec of time with atleast 15 waveforms at one measuring site. All PWV measurements were made in duplicate and mean values were used in analysis. Recordings with a variation of more than 5 heart beats per minute were excluded.

Pulse wave analysis was employed for measuring central haemodynamics with the Sphygmocor device (version 7.1, AtCor Medical, Sydney, Australia). At least 15 sequential waveforms were measured at the subject's left wrist with a high fidelity micromanometer (SPT-301B, Millar Instruments, USA). Corresponding ascending aortic waveforms were then generated using a transfer function to calculate AIx and central haemodynamics (Pauca *et al.* 2001). AIx was defined as the difference between the second and the first systolic peaks of the central aortic wave, which was expressed as the percentage of central pulse pressure (Laurent *et al.* 2006). The AIx was adjusted automatically by means of the Sphygmocor software for 75 heart beats per minute (AIx@75).

In addition, arterial stiffness was measured using the diastolic part of the cardiac cycle. The pulse waveform was recorded from the right radial artery with a Cardiovascular Profiling Instrument (HDI/Pulse Wave CR-2000; Hypertension Diagnostics Inc, Eagan, USA) which measures large and small artery elasticity during 30 sec of recording. The subject's hand was supported with a wrist stabilizer for minimal movements and optimal positioning. The mean of two recordings was used for analysis.

4.3.5 Statistical analysis

Statistical analysis was performed using the Statistical Package of the Social Science software for Windows, version 22.0 (SPSS, Chicago, IL, USA). Categorical variables were expressed in percentages. Continuous variables were presented as mean ± SD, or as median and interquartile ranges. The Shapiro–Wilk test was employed to ascertain whether variables were normally distributed (Papers I–IV). Two tailed Student's t-test was used for comparing the means of normally distributed data and the Mann–Whitney U-test was used for non-parametric data.

The calculation of intraclass correlation coefficient is based on absolute agreement and mixed model and single measures were used. Pearson's correlation coefficient and Spearman's rho were used to identify associations between continuous variables within the study groups. The Chi-square test or Fischer's exact test was used to compare group proportions.

Multiple linear regression analysis, using a forward and backward stepwise variable selection procedure, was performed to investigate independent

associations between variables. Cf-PWV (Papers I, II, IV) and ox-LDL (Paper III) were used as the dependent variables in multiple regression analysis. The variables inserted into the model were selected from univariate correlation analyses and previous observations. Analysis of covariance was used to adjust for BMI (Papers I, II, III), MAP (Paper I, II) and age (Paper I, II). Statistical significance was defined as $p<0.05$. The Benjamini-Hochberg procedure was used to account for false discovery rate at the of 0.05 level (Benjamini & Hochberg, 1995) (Papers III, IV).

Principal component analysis (PCA) was performed to reduce the large number of correlated metabolites to fewer uncorrelated components for the OA patients and the controls. Varimax rotation was used to identify interpretable factors. Only the factors that explained at least 5% of total variance were included in further analysis. Only the metabolites with absolute factor loadings of at least 0.50 were reported as the components of a given factor in order to avoid false discoveries. Next, for each subject, factor scores (weighted sum of the standardized metabolites within a given factor, weighted on the factor loading for each individual metabolite) were calculated and included in analysis.

5. RESULTS

5.1 Arterial stiffness in osteoarthritis patients and in controls (Papers I, II)

Baseline characteristics of the study groups

The baseline characteristics of the OA and control groups are presented in Table 2. There was no significant difference in age or male to female ratio between the study groups. The level of BMI and the W/H-ratio were significantly higher and the HHS and HSS Knee scores were lower in the OA group. Smoking status did not differ significantly between the study groups.

Table 2. General parameters of the osteoarthritis patients and the controls.

Variable	Osteoarthritis (*n=70*)	Controls (*n=70*)	p-value
Age (years)	62 ± 7	60 ± 7	0.066
Male/Female (n)	35 / 35	36 / 34	0.866
BMI (kg/m^2)	28 ± 3	26 ± 3	0.001
Waist circumference (cm)	96 ± 9	88 ± 11	<0.001
Hip circumference (cm)	104 ± 7	02 ± 6	0.033
W/H- ratio	0.92 ± 0.08	0.87 ± 0.09	0.001
Smoking (n, %)			0.127
Current smoker	15 (22)	8 (11)	
Former smoker	4 (6)	9 (13)	
Non-smoker	50 (72)	53 (76)	
Involved joint (n)			
Hip	41	0	
Knee	29	0	
HHS	37 (32–48)	100 (100–100)	<0.001
HSS Knee score	62 (49–65)	100 (100–100)	<0.001

Parameters of arterial stiffness in osteoarthritis patients and in controls

The parameters of arterial stiffness and central hemodynamics are presented in Table 3. After adjusting for BMI and MAP, the level of cf-PWV was significantly higher in the OA group compared with the controls. The level of small artery elasticity was significantly lower in the OA patients than in the control group. Also, MAP, peripheral BP and central BP were significantly higher in the OA group after adjusting for BMI. While in Paper I the level of AIx@75 was significantly higher in the OA group, the difference in the level of AIx@75 was not statistically significant in Paper II (p=0.078) involving a higher number of study participants. However, after adjusting for the gender proportions, the

AIx@75 was significantly higher in the OA group (p=0.033). There were no differences in large artery elasticity index between the study groups.

Table 3. Hemodynamic parameters of the osteoarthritis patients and the controls.

Variable	Osteoarthritis patients (*n=70*)	Controls (*n=70*)	p-value
Peripheral systolic BP (mmHg)	133 ± 18	126 ± 15	0.011
Peripheral diastolic BP (mmHg)	81 ± 8	78 ± 7	0.014
Central systolic BP (mmHg)	125 ± 17	118 ± 15	0.014
Central diastolic BP (mmHg)	82 ± 8	78 ± 8	0.019
MAP (mmHg)	100 ± 11	95 ± 10	0.010
Heart rate (bpm)	64 ± 11	61 ± 7	0.042
AIx@75 (%)	25 ± 9	22 ± 11	0.078
cf-PWV (m/s)	9.1 ± 2.2	8.2 ± 1.5	0.007[a]
C1 (ml/mmHg×100)	12.8 ± 4.4	14.1 ± 5.0	0.119
C2 (ml/mmHg×100)	4.2 ± 3.6	6.0 ± 2.7	0.002

All variables are adjusted for BMI
a- adjusted for mean arterial pressure

In univariate correlation analysis the level of serum urea was positively correlated with cf-PWV and with central systolic BP (Figure 1 and Figure 2) as reported in Paper I. There were no association between parameters of arterial stiffness and urea in the control group.

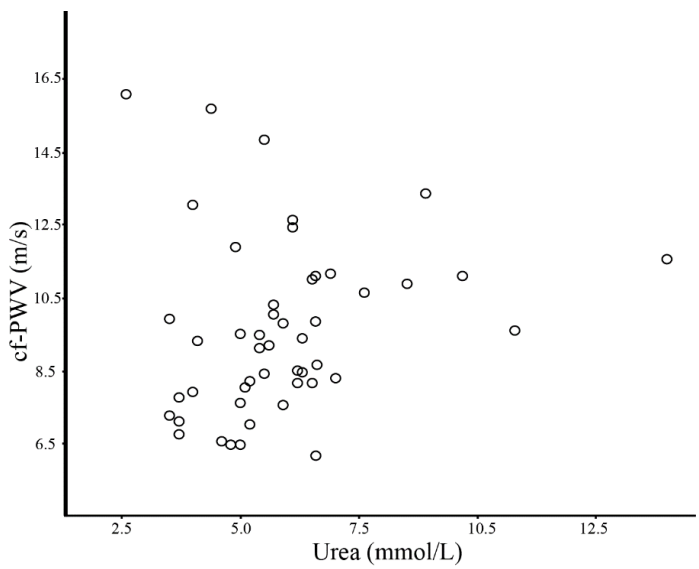

Figure 1. The association between cf-PWV and serum urea level (rho = 0.290, $p = 0.046$)

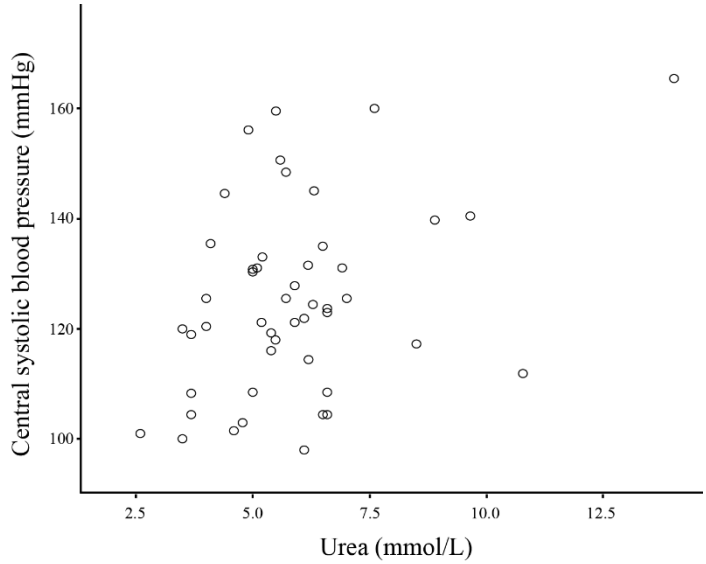

Figure 2. The association between central systolic pressure and serum urea level (rho = 0.318, $p = 0.029$)

In multiple regression analysis (Table 4), where cf-PWV was set as the dependent variable, age ($p < 0.001$), MAP ($p = <0.001$) and OA status ($p = 0.029$) were found to be independent predictors (Paper I).

Table 4. Multiple regression analysis with carotid-femoral pulse wave velocity as dependent variable (adjusted R^2=0.48).

Variable	Regression coefficient	Beta	SE	p- value
Constant	–6.68			<0.001
Age	0.15	0.53	0.02	<0.001
MAP	0.07	0.36	0.01	<0.001
OA status	0.74	0.17	0.33	0.029
Urea	–1.20	–0.09	0.10	0.233

In addition, a significant positive association was found between OA radiographic Kellgren-Lawrence grade and cf-PWV (Figure 3B) (Paper II). The association was further analyzed separately in the hip and knee subgroups. Cf-PWV was set as the dependent variable in the hip OA patient group, and OA grade, age, MAP and hs-CRP remained significant independent predictors (Table 5a). In the knee OA group, OA grade was not an independent predictor of cf-PWV after adjusting for the W/H-ratio and age (Table 5b).

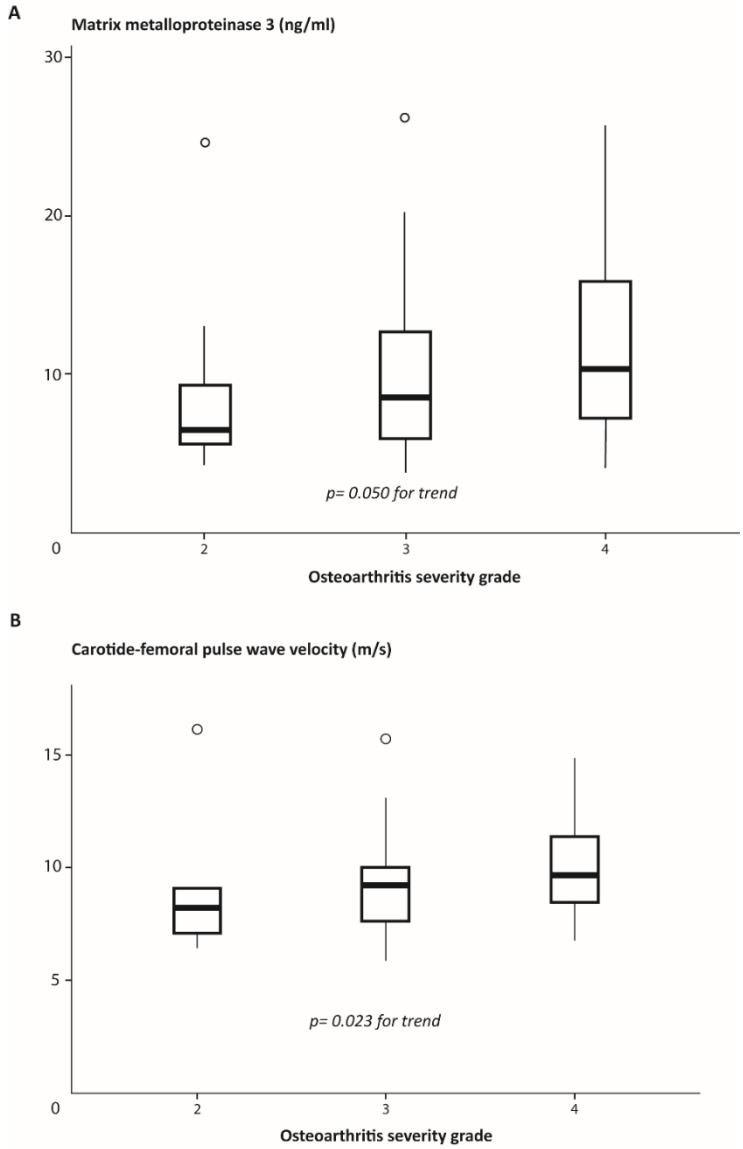

Figure 3. (A) Matrix metalloproteinase 3 levels for different osteoarthritis severity scores. (B) Boxplot describing cf-PWV in different groups of osteoarthritis severity.

Table 5a. Multiple regression analysis with cf-PWV as the dependent variable for the hip OA patients (adjusted R^2=0.655, p<0.001).

Variable	B	Std Error	Beta	t	p- value
Constant	–8.487	2.445		–3.471	0.001
OA grade	0.739	0.332	0.219	2.228	0.033
Age	0.125	0.029	0.422	4.302	<0.001
MAP	0.079	0.016	0.477	4.880	<0.001
hsCRP	–0.433	0.170	–0.246	–2.543	0.016

Table 5b. Multiple regression analysis with cf-PWV as the dependent variable for the knee OA patients (adjusted R^2=0.351, p=0.003).

Variable	B	Std Error	Beta	t	p- value
Constant	–10.450	5.357		–1.951	0.062
Age	0.164	0.047	0.556	3.492	0.002
W/H-ratio	11.326	5.345	0.344	2.119	0.044
OA grade	–0.227	0.695	–0.055	–0.327	0.746

5.2 Adipokines, matrix metalloproteinase 3 and arterial stiffness in osteoarthritis patients and in controls (Paper II)

The levels of leptin, WBC, hsCRP and urea were higher, and the levels of adiponectin and HDL-cholesterol were lower for the patient group (Table 6). However, after correcting for BMI, the difference in hsCRP and platelet count was no longer significant and the difference in adiponectin had borderline significance.

Table 6. Metabolic markers for the osteoarthritis and control groups.

Variable	Osteoarthritis patients (*n=70*)	Controls (*n=70*)	p-value
Triglycerides (mmol/l)	1.6 ± 1.2	1.4 ± 0.8	0.249
LDL- cholesterol (mmol/l)	4.0 ± 0.9	3.9 ± 1.0	0.630
HDL- cholesterol (mmol/l)	1.5 ± 0.4	1.7 ± 0.5	0.026
Total cholesterol (mmol/l)	5.8 ± 1.1	5.8 ± 1.2	0.892
hsCRP (mg/l)	1.89 ± 1.14	1.42 ± 0.96	0.093
eGFR (ml/mg/1.73m^2)	83 ± 15	83 ± 12	0.991
Urea (mmol/L)	6.0 ± 2.0	5.2 ± 1.2	0.017
White blood cells (10^9 / l)	6.5 ± 1.4	5.7 ± 1.9	0.010
Platelets (10^9 / l)	243 ± 57	227 ± 55	0.123
MMP-3 (ng/ml)	14.1 ± 6.54	10.9 ± 5.94	0.004
Adiponectin (ng/ml)	8131 ± 5681	9664 ± 3897	0.066
Leptin (ng/ml)	3.7 ± 5.14	2.20 ± 1.85	0.022

All variables are adjusted for BMI

In addition, Kellgren-Lawrence grade correlated positively with MMP3 (Figure 3A) and inversely with leptin. However, these associations did not remain significant after adjustment for potential confounders. Furthermore, we found that adiponectin was correlated with C1 and AIx@75 (Figure 4). In the model where adiponectin was set as the dependent variable, neither C1 nor AIx was an independent predictor when the potential confounders were included.

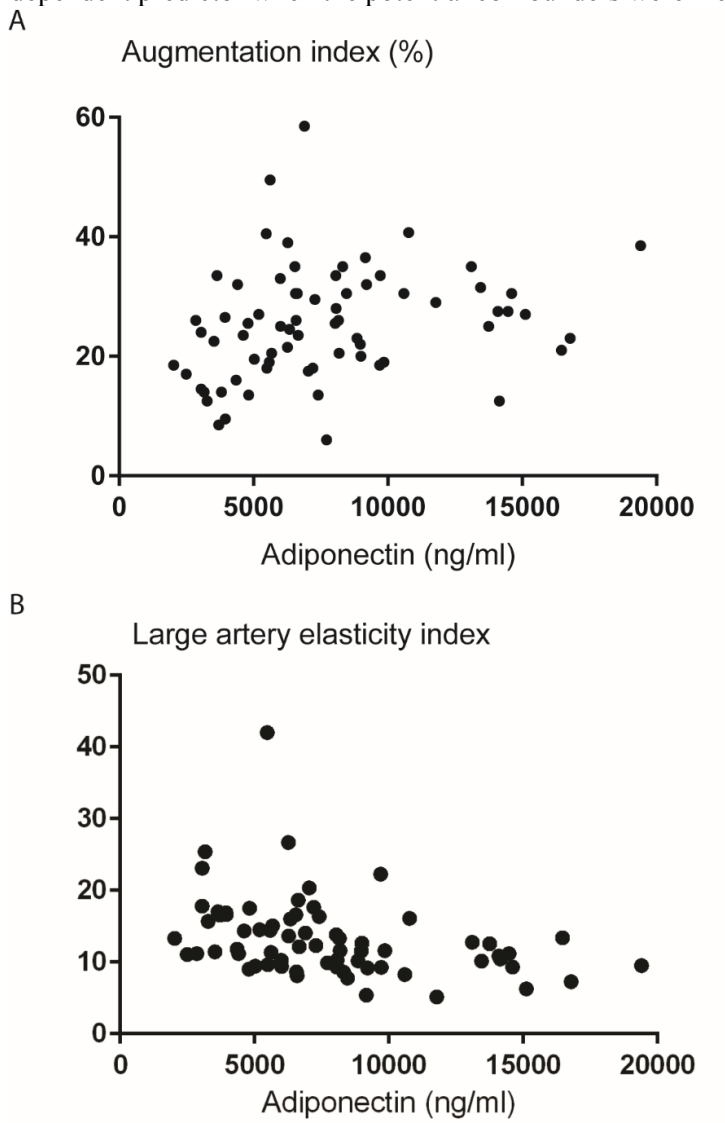

Figure 4. Associations of adiponectin with (A) augmentation index (r=0. 293, p=0.006) and (B) large artery elasticity index (r=–0.249, p=0.003).

5.3 Oxidative stress and metabolic syndrome in osteoarthritis patients and in healthy controls (Paper III)

General parameters of osteoarthritis patients and controls

A total of 110 participants, who were divided into two groups according to presence of OA, were evaluated and the general parameters are presented in Table 7. The study groups did not differ significantly with regard to the age, gender proportions, BMI or the W/H-ratio. The mean age of the OA patients was 63 ± 7 years and the proportion of male subjects was 53%. The mean age of the controls was 61 ± 8 years and the proportion of males was 51%. Thirty-five patients had predominantly hip OA and 20 patients had knee OA. The proportion of the smoking status was not significantly different across the study groups (Table 7). The study groups did not differ significantly with regard to waist circumference, fasting glucose level, triglycerides level, HDL level or peripheral systolic BP.

The subjects in the OA group had significantly higher insulin and C-peptide levels and their HOMA2 IR levels were higher with borderline significance (p=0.050). The level of resistin was lower in the OA group with borderline significance (p=0.056). The differences in triglycerides, HOMA2 IR and insulin were no longer significant after adjusting for BMI and accounting for multiple testing. The level of TPX and OSI was significantly higher in the OA group while total antioxidant capacity (TAC) was significantly lower in this group. MPO and oxLDL did not differ significantly between the study groups.

The prevalence of MetS was 9% in the control group and 20% in the OA group (p=0.105). The proportions of the MetS components in the OA and control groups are presented in Figure 5.

Table 7. General and metabolic parameters of the osteoarthritis patients and the controls.

Variable	Osteoarthritis (*n=55*)	Controls (*n=55*)	*p-value*
Age (years)	63 ± 7	61 ± 8	*0.301*
Male/Female (n)	29 / 26	28 / 27	*0.849*
BMI (kg/m^2)	27 ± 3	26 ± 3	*0.112*
Waist circumference (cm)	96 ± 10	91 ± 12	*0.163*[a]
W/H- ratio	0.92 ± 0.08	0.89 ± 0.09	*0.096*
HSS Knee score	54 (49–65)	100 (99–100)	*0.001*
HHS	37 (32–48)	100 (100–100)	*0.001*
OA severity	3 (3–4)	N/A	
Smoking (n, %)			*0.280*[a]
Current smoker	11 (20)	7 (13)	
Former smoker	3 (6)	9 (16)	
Non-smoker	41 (75)	39 (71)	

Table 7. Continuation

Variable	Osteoarthritis (*n=55*)	Controls (*n=55*)	*p-value*
Hypertension (%)	55	38	*0.085*
Peripheral systolic BP (mmHg)	134 ± 18	129 ± 18	*0.353* [a]
LDL-cholesterol (mmol/L)	3.9 ± 0.9	3.9 ± 1.0	*0.899* [a]
Triglycerides (mmol/L)	1.4 (1.03–1.91	1.2 (0.83-1.57)	*0.280* [a]
HDL-cholesterol (mmol/L)	1.5 ± 0.5	1.7 ± 0.5	*0.290* [a]
Fasting glucose (mmol/)	5.8 ± 0.7	5.7 ± 0.4	*0.704* [a]
Insulin (pmol/L)	52 (35–72)	41 (32–53)	*0.353* [a]
HOMA2 IR	1.0 (0.7–1.4)	0.8(0.6–1.0	*0.420* [a]
C-peptide (ng/ml)	1.6 (0.94–2.47)	0.9 (0.46–1.42)	*0.003* [a]
Resistin (ng/ml)	2.6 ± 0.9	3.3 ± 1.8	*0.054* [a]
oxLDL (U/L)	78 ± 22	73 ± 24	*0.578* [a]
MPO (ng/ml)	37 ± 20	42 (22–55)	*0.463* [a]
TPX (µmol/L)	357 (250–612)	286 (168–442)	*0.011* [a]
TAC (mmol trolox equivalent/L)	1.49 ± 0.27	1.66 ± 0.27	*0.008* [a]
OSI (%)	25 (17–51)	18 (11–28)	*0.002* [a]
ACEI (n, %)	5 (9)	12 (22)	*0.112*
ARB(n, %)	7 (4)	2 (13)	*0.161*
Aspirin (n, %)	2 (4)	3 (6)	*1.0*
Ca channel blocker (n,%)	6 (11)	1 (2)	*0.113*
Beta-blocker (n, %)	5 (9)	6 (11)	*1.0*
Diuretics (n, %)	7 (4)	1 (2)	*0.363*
Statins (n, %)	1 (2)	0 (0)	*1.0*

a- the p-value has been adjusted for BMI and multiple testing

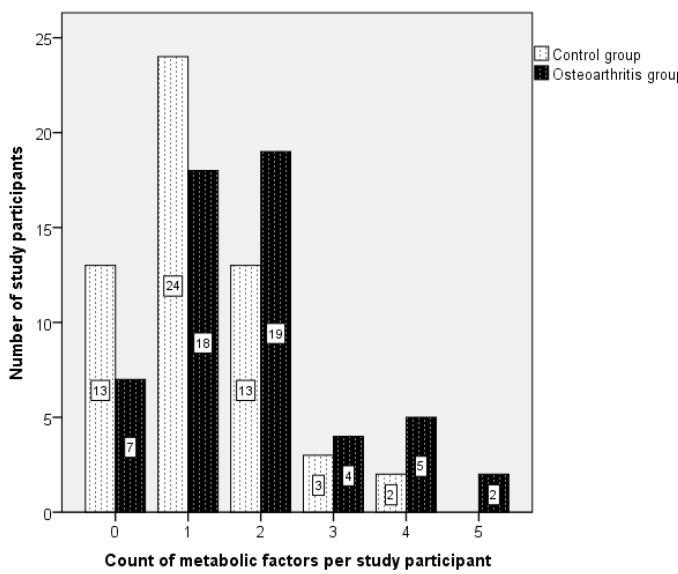

Figure 5. The count of metabolic factors (hypertension, central obesity, high triglyceride level, low HDL-cholesterol level, high fasting glucose level) per study participant among the osteoarthritis group and the control group.

Associations between metabolic markers and osteoarthritis

The associations between OA severity, metabolic factors and OxS parameters are presented in Table 8. In univariate analysis, waist circumference, LDL-cholesterol, insulin, HOMA2 IR and oxLDL were positively associated with OA severity. After adjusting for the potential confounders (BMI, age, gender) and accounting for multiple testing, OA severity was associated with LDL-cholesterol and oxLDL. There was no significant association between OA severity and W/H-ratio (Rho=0.24, p=0.087) in the present study.

Table 8. Associations between osteoarthritis severity, components of metabolic syndrome, glucose metabolism and oxidative stress in osteoarthritis patients.

			OA severity	
	Rho	***p-value***	***BMI and age adjusted p-value***	***BMI, age and gender adjusted p-value*** [a]
Metabolic syndrome components				
Waist circumference	**0.31**	*0.022*	*0.076**	*0.119**
Peripheral systolic BP	**0.20**	*0.135*	*0.107*	*0.078*
Triglycerides	**0.22**	*0.107*	*0.111*	*0.155*
HDL-cholesterol	**–0.20**	*0.153*	*0.517*	*0.522*
Fasting glucose	**0.06**	*0.681*	*0.821*	*0.412*
Dyslipidemia				
LDL-cholesterol	**0.40**	*0.003*	*0.017*	*0.007*
Glucose metabolism markers				
HOMA2 IR	**0.29**	*0.033*	*0.056*	*0.081*
Insulin	**0.28**	*0.039*	*0.141*	*0.165*
C-peptide	**0.23**	*0.089*	*0.283*	*0.265*
Oxidative stress markers				
OxLDL	**0.37**	*0.006*	*0.019*	*0.022*
MPO	**–0.16**	*0.260*	*0.863*	*0.492*
TPX	**–0.19**	*0.160*	*0.133*	*0.226*
TAC	**0.24**	*0.084*	*0.195*	*0.250*
OSI	**–0.25**	*0.062*	*0.077*	*0.154*

** waist circumference was not adjusted for BMI*
a- the p-values have been accounted for multiple testing

In multiple regression analysis, OxLDL was independently associated with OA severity (p=0.040) and with MetS score (p=0.008) when BMI and smoking status were taken into account (Table 9). None of the components of MetS was significantly associated with oxLDL when they were entered into the model together with OA severity and BMI.

Table 9. Regression model with oxLDL as the dependent variable in osteoarthritis patients.

Variable	B	Std Error	Beta	*p- value*
Constant	17.91	25.00		*0.477*
MetS score	6.51	2.36	0.36	*0.008*
OA severity	8.51	4.04	0.25	*0.040*
Smoking status	7.25	4.58	0.19	*0.119*
BMI	0.68	0.91	0.10	*0.458*

5.4 Medium- and long-chain acylcarnitines in osteoarthritis patients and in controls (Paper IV)

Characteristics of the study population

In Paper IV a total of 152 participants were evaluated (70 with OA vs 82 controls). General, hemodynamic and biochemical variables are presented in Table 10. The study groups did not differ significantly in regard to age, male to female ratio, cr-PWV, LDL-cholesterol or fasting glucose levels. The levels of BMI, W/H-ratio, peripheral and central BP, cf-PWV and PWV-ratio were significantly higher in the patient group. In addition, inflammatory markers (hs-CRP, WBC, TNF-α) were significantly higher in the OA group. After adjusting for gender ratio and BMI, the level of AIx@75 was significantly higher in the OA group (p=0.008).

Table 10. General and hemodynamic parameters.

Variable	Osteoarthritis (*n=70)*	Controls (*n=82*)	*p-value*
Age (years)	62 ± 7	61 ± 8	*0.217*
Male/Female (n)	36 / 34	37 / 45	*0.515*
BMI (kg/m^2)	27.8 ± 3.2	26.0 ± 3.5	*0.001*
Hypertension (n (%))	33 (45)	11 (13)	*0.001*
Peripheral systolic BP (mmHg)	134 ± 18	127 ± 16	*0.013*
Peripheral diastolic BP (mmHg)	81 ± 8	78 ± 8	*0.010*
Central systolic BP (mmHg)	126 ± 17	119 ± 16	*0.011*
Central diastolic BP (mmHg)	82 ± 8	79 ± 9	*0.011*
MAP (mmHg)	100 ± 11	95 ± 11	*0.005*
HR (bpm)	64 ± 8	62 ± 8	*0.041*
AIx@75 (%)	25 ± 9	22 ± 11	*0.110*
cf-PWV (m/s)	9.3 ± 2.2	8.2 ± 1.7	*0.001*
cr-PWV (m/s)	8.7 ± 1.1	8.6 ± 1.1	*0.549*
PWV-ratio	1.07 ± 0.22	0.96 ± 0.19	*0.001*

Table 10. Continuation

Variable	Osteoarthritis (*n=70)*	Controls (*n=82*)	*p-value*
hs-CRP (mg/l)	1.9 ± 1.1	1.5 ± 1.2	*0.014*
WBC (10^9/l)	6.5 ± 1.4	5.7 ± 1.9	*0.001*
TNF-α (pg/ml)	7.5 ± 2.1	6.3 ± 1.9	*0.001*
LDL-cholesterol (mmol/l)	4.0 ± 0.9	3.9 ± 1.1	*0.804*
Triglycerides (mmol/l)	1.7 ± 0.8	1.4 ± 1.1	*0.001*
HDL-cholesterol (mmol/l)	1.5 ± 0.5	1.7 ± 0.5	*0.001*
Total cholesterol (mmol/l)	5.8 ± 1.1	5.7 ± 1.3	*0.539*
Fasting glucose (mmol/l)	5.7 ± 0.7	5.7 ± 0.5	*0.509*

The levels of acylcarnitines in the osteoarthritis patients and in the controls

Table 11 presents the acylcarnitines derived from PCA. The levels of C10:1, C10:2, C12, C12:1, C14, C14:2, C14:1-OH, CPT1-ratio and total AC/C0 were found to be significantly lower in the OA group after adjusting for BMI and accounting for multiple testing. PCA determined 4 distinct components that are presented in Table 12.

Table 11. Acylcarnitines in the osteoarthritis group and in the control group.

Variable	Osteoarthritis (n=70)	Controls (*n=82*)	*p-value* [a]
C10:1	**0.131 ± 0.069**	**0.151 ± 0.060**	***0.014***
C10:2	**0.069 ± 0.011**	**0.072 ± 0.016**	***0.050***
C12	**0.110 ± 0.045**	**0.135 ± 0.050**	***0.004***
C12:1	**0.125 ± 0.049**	**0.140 ± 0.045**	***0.043***
C14	**0.024 ± 0.006**	**0.027 ± 0.009**	***0.014***
C14:1	0.046 ± 0.023	0.054 ± 0.025	*0.052*
C14:2	**0.017 ± 0.007**	**0.021 ± 0.009**	***0.011***
C14:1-OH	**0.013 ± 0.003**	**0.015 ± 0.004**	***0.004***
C14:2-OH	0.008 ± 0.002	0.008 ± 0.002	*0.052*
C16	0.114 ± 0.024	0.107 ± 0.029	*0.220*
C16:1	0.032 ± 0.009	0.032 ± 0.010	*0.630*
C16:1-OH	0.013 ± 0.003	0.013 ± 0.003	*0.236*
C18	0.053 ± 0.013	0.053 ± 0.015	*0.807*
C18:1	0.143 ± 0.033	0.132 ± 0.036	*0.088*
Total AC / C0	**0.261 ± 0.065**	**0.320 ± 0.078**	***0.004***
CPT1 ratio	**0.004 ± 0.001**	**0.005 ± 0.001**	***0.050***

All variables are adjusted for BMI and accounted for multiple testing. Concentrations of all acylcarnitines are presented as μM.
a- Benjamini-Hochberg adjusted p-value

Table 12. Principal component analysis for the osteoarthritis group.

Factor	Description	Components	Eigenvalue	Variance (%)
1	Phosphatidyl-cholines, sphingomyelins	Total PC ae, PC ae C40:3, Total SM, PC ae C36:2, PC ae C34:1, PC ae C36:1, Total SM-OH, Total SM-non OH, SM C16:0, SM (OH) C22:2, PC ae C34:0, SM C16:1, SM (OH) C16:1, SM (OH) C14:1, PC ae C40:5, PC ae C40:6, SM (OH) C22:1, PC aa C32:3, PC aa C28:1, PC ae C40:2, PC ae C38:3, PC ae C42:2, SFA (PC), PC ae C34:2, PC ae C32:2, PC ae C40:4, PC ae C38:2, PC ae C42:4, SM C18:1, PC ae C38:0, SM C18:0, PC ae C34:3, PC ae C32:1, PC aa C40:3, PC ae C36:0, PC ae C38:6, PC aa C38:0, lysoPC a C17:0, SM (OH) C24:1, PC ae C36:3, PC aa C42:1, PC ae C42:5, PC aa C42:0, PC aa C42:4, PC aa C40:1, PC ae C44:6, PC aa C36:0, PC ae C38:4, SM C24:1, PC ae C36:5, PC ae C44:4, PC ae C38:5, SM C26:0, PC aa C32:0, PC aa C34:2, PC ae C30:0, SM C20:2, PC aa C38:1, lysoPC a C18:1, Total lysoPC, PC aa C40:2, PC aa C42:2, lysoPC a C18:0, PC aa C36:5, Total PC aa, Tyr, Total PC, PUFA (PC), Total (PC+SM), MUFA (PC), PC aa C38:5, PC aa C36:1, SM C24:0, PC ae C42:3, PC ae C40:1	50	22
2	Aminoacids, phophatidyl-cholines	PC aa C40:5, AAA, PC aa C40:4, PC aa C38:3, Essential AA, PC aa C36:3, Total PC aa, Tyr, Total PC, PUFA (PC), PC aa C38:4, Total AA, Trp, PC aa C32:1, Total (PC+SM), BCAA, Val, His, PC aa C34:4, Ala, Total SM / Total PC, Total SM / Total (SM+PC), PC aa C36:4, Phe, Leu, Met, lysoPC a C14:0, Ile, MUFA (PC), Lys, lysoPC a C20:3, PC aa C38:5, PC aa C34:1, alpha-AAA, PC aa C36:1, SM C24:0, PC aa C30:0, PC aa C32:2, Pro, H1, PC aa C36:2, Glu, PC aa C34:3, lysoPC a C16:1	23	10
3	Short-, medium- and long-chain acylcarnitines	C14:1, C12:1, C2, Total AC-DC / Total AC, C14, C16, C16:1, C12, C14:2, C7-DC, C18:1, Total AC-OH / Total AC, C14:1-OH, C16:1-OH, Total AC / C0, C10:1, C10, C2 / C0, (C2+C3) / C0, C8, C6 (C4:1-DC), C9, C14:2-OH, C10:2, C18, C5-DC (C6-OH)	16	7
4	Phosphatidyl-cholines, lysophosphatidyl-cholines	lysoPC a C26:1, lysoPC a C24:0, lysoPC a C28:1, PC aa C24:0, PC ae C30:1, Spermine, PC ae C44:3, PC ae C42:1, PC aa C26:0, lysoPC a C26:0, lysoPC a C28:0, SDMA, PC ae C30:2, PC ae C42:0,Met-SO / Met, PC ae C42:3, PC ae C40:1, Asp, Met-SO, Total DMA / Arg	14	6

Associations between acylcarnitines and arterial stiffness in osteoarthritis patients

Cr-PWV and cf-PWV were positively correlated with several acylcarnitines in the OA group (Table 13a). Furthermore, C14-OH, C16, C16:1-OH and C18 were significantly associated with radiographic severity of OA after accounting for multiple testing. Component scores of component 3 were positively correlated with cf-PWV (r=0.25, p=0.037) and cr-PWV (r=0.35, p=0.003) in the OA group. In the control group neither the acylcarnitines nor the component scores derived from PCA were significantly associated with markers of arterial stiffness (Table 13b).

Table 13a. Spearman correlation coefficients for the acylcarnitines associated with arterial stiffness in the osteoarthritis group.

Variable	cf-PWV (m/s)	cr-PWV (m/s)	PWV-ratio (m/s)	OA-severity
C12:1	0.247	**0.309***	–0.059	0.157
C14	0.156	**0.302***	0.001	0.272
C14:1-OH	**0.321***	0.216	–0.168	**0.337***
C14:2	**0.316***	**0.315***	–0.112	0.240
C16	0.176	**0.304***	–0.027	**0.306***
C16:1	0.238	0.263	–0.113	0.099
C16:1-OH	0.117	**0.367***	0.052	**0.351***
C18	0.036	**0.290***	0.123	**0.332***

Table 13b. Spearman correlation coefficients for the acylcarnitines associated with arterial stiffness in the control group.

Variable	cf-PWV (m/s) m	cr-PWV (m/s)	PWV-ratio
C12:1	0.096	0.074	–0.018
C14	0.175	0.004	0.092
C14:1-OH	0.223	–0.064	0.204
C14:2	0.104	0.153	–0.017
C16	0.316	0.123	0.185
C16:1	0.229	0.052	0.146
C16:1-OH	0.278	0.112	0.164
C18	0.122	–0.076	0.142

*Statistically significant ($p<0.05$) correlation coefficients are shown in bold and marked with *. P-values are accounted for multiple testing using the Benjamini-Hochberg method.*

Even though none of the long- and medium-chained acylcarnitines presented in Table 13 was associated with PWV-ratio in the univariate analysis, acylcarnitines were significantly associated with PWV-ratio in the stepwise multiple regression analysis. After adjusting for potential confounders (Table 14), component 3 (acylcarnitines), TNF-α, age, gender and W/H-ratio remained independently associated with PWV-ratio in the OA group (R^2=0.46, p<0.001).

Table 14. Table describing independent predictors of PWV-ratio in osteoarthritis patients.

Variable	Standardized coefficient	Standard error	p value
Age	0.58	0.01	0.001
TNF-a	0.28	0.01	0.007
W/H-ratio	0.25	0.29	0.014
Component 3 (acylcarnitines)	–0.30	0.02	0.026
Gender	–0.22	0.02	0.026

R^2=0.46, p<0.001

6. DISCUSSION

6.1 Arterial stiffness and central haemodynamics in osteoarthritis (Papers I, II)

Evidence has emerged that patients with OA have increased cardiovascular morbidity and mortality (Nüesch *et al.* 2011; Rahman *et al.* 2013), however, very little is known about the exact mechanism underlying this association. Arterial stiffness has been shown to be an independent determinant of cardiovascular morbidity and mortality and has been proposed as a surrogate endpoint for CVD (Townsend *et al.* 2015). We demonstrated in Paper I, II and IV that OA patients have increased central arterial stiffness compared to non-OA controls.

In Paper I we found significantly higher cf-PWV in the OA patients compared to the controls of approximately the same age and BMI. Also, the level of AIx@75 was increased in the OA group after adjusting for potential confounders. In Paper II we demonstrated independent association between the severity of OA and cf-PWV. Parameters of arterial stiffness have rarely been studied in OA patients. A study (Saleh *et al.* 2007) found association between hand OA and cf-PWV but it was largely attributable to the confounding effect of age. Another study (Goldsmith *et al.* 2014) found no difference in arterial stiffness between subjects with and without knee bone marrow lesions. The discrepancy between their results and ours might at least partly be explained by the fact that our study patients had more advanced OA. Because of pain and functional disability, the level of physical activity is limited in the advanced stages of OA. Since physical activity is associated with arterial stiffness (Ashor *et al.* 2014), increased pain and higher functional disability status might also partly explain the increased arterial stiffness in OA patients. In support of our hypothesis, a recent study has also found elevated stiffness of the aorta in end-stage OA patients (Belen *et al.* 2016). Many factors, such as reduced physical activity, inflammation and OxS, which are characteristic of end-stage OA patients, might be responsible for the stiffer arteries. Accumulation of advanced glycation end-products is a feature of ageing that is present in both diseases (Goldin *et al.* 2006). Obesity is a known risk factor for both OA and CVD.

Another plausible explanation for the association of CVD with OA is that vascular pathology might be involved in development of OA. Vascular damage to the subchondral bone has been proposed as a possible initiator of OA (Findlay, 2007). Blood supply to the subchondral bone region may be disturbed by microemboli and venous stasis. Highly vascularized epiphysis is mainly supplied with blood via the epiphyseal artery, which makes this region of high nutrient demand particularly susceptible to perfusion insufficiencies. In support of this hypothesis, a study (Chang *et al.* 2014) found that osteoblasts and chondrocytes from osteoarthritic joints suffer from hypoxia. Furthermore, they also found that hypoxia induced production of MMP 9 and proangiogenic

factors and caused reduction in osteoblast mineralized bone nodule formation, which are all characteristic of OA.

In Paper I, cf-PWV was positively correlated with urea in OA patients. Since urea is the end-product of amino acid metabolism, this association highlights the possible role of altered amino acid metabolism in OA patients. Changes in amino acid profiles and impaired kidney function have been shown to contribute to increased arterial stiffness (Chue *et al.* 2010; Jung *et al.* 2014). On the basis of eGFR levels, which were approximately the same across both groups in this study, there was no difference in the kidney function. After adjusting for the effects of age and MAP, the association between urea and cf-PWV was no longer significant. Higher applied forces (higher BP) in the aorta and ageing are strong independent predictors of cf-PWV (McEniery *et al.* 2007). It is therefore important to adjust for mean pressure and age when evaluating predictors of cf-PWV. In the present study, the association between urea level and cf-PWV was largely due to the confounding effects of age and MAP. Additional analysis exploring the effects of body composition, smoking status, functional status and use of anti inflammatory drugs on arterial stiffness in OA patients should be within the scope of future studies. Altogether, our study suggests that increased central arterial stiffness is associated with OA and might at least partly be responsible for the higher CVD risk in this population.

6.2 The impact of adipokines and matrix metalloproteinase 3 in osteoarthritis and in cardiovascular diseases (Paper II)

Paper II demonstrates associations between adipokines, MMP3, arterial stiffness and OA severity in older adults with hip and knee OA in comparison with asymptomatic controls.

The adipokines are a growing family of white-adipose-tissue derived factors that have multiple functions through different pathways and are involved in inflammation and modulation of immunological response but also in glucose and lipid metabolism (Gualillo *et al.* 2007). Many adipokines have been associated with OA. Leptin has proinflammatory properties and complex actions on chondrocytes. Leptin upregulates MMPs and induces cartilage loss (Koskinen *et al.* 2014); however, its anabolic effects have also been described (Berry *et al.* 2011). Although leptin has been associated with OA severity (Ku *et al.* 2009), the pathophysiological pathways are not fully understood. In addition to bone and cartilage, leptin also influences the cardiovascular system. It is an independent predictor of myocardial infarction and stroke and has been associated with increased arterial stiffness (Gonzalez *et al.* 2013) through promoting inflammation and MMP upregulation (Scotece *et al.* 2012). In accord with previous studies, we found elevated expression of leptin in OA patients and, in addition, inverse association with OA radiographic grade, which might indicate

the anabolic effect of leptin. These findings point to the possible key role of leptin in the pathogenesis of OA and allow to suggest leptin as a link between OA and vascular damage.

Adiponectin is prevalently synthesized in the adipose tissue and circulates in blood in large quantities (Kadowaki & Yamauchi, 2005). Adiponectin has anti-inflammatory properties and hypoadiponectinemia has a detrimental effect on aortic stiffness (Tsioufis *et al.* 2007). In Paper II, adiponectin correlated inversely with large artery elasticity index and positively with AIx@75. Findings similar to ours have been reported for essential hypertension (Mahmud & Feely, 2005), but inverse association was recently found in juvenile idiopathic arthritis patients (Ilisson *et al.* 2015). Our results suggest that adiponectin as well as leptin might link OA with increased arterial stiffness. These findings describe the pleiotropic role of adipokines in OA and require further research to determine their potential as therapeutic targets.

MMP3 is a catabolic enzyme that has not only the ability to degrade the extracellular matrix but plays a central role in activating other members of the MMP family (Chen *et al.* 2014). In Paper II we demonstrated higher level of MMP3 in OA patients compared to controls as well as positive correlation between radiographic severity of OA and serum level of MMP3 these patients. Activation and secretion of MMP3 are driven by inflammatory cytokines, which in turn enhances the production of inflammatory mediators like interleukin-1 (Chen *et al.* 2014). This is in line with our results, according to which the OA group also showed higher values of WBC, which indicates a systemic inflammatory state. Furthermore, MMP3 is likely to influence arterial stiffness and has been found to be elevated in the atherosclerotic aorta (Knox *et al.* 1997; Agrotis, 2005). In conclusion, MMP3 might be a marker that links vascular pathology to OA.

6.3 Metabolic syndrome and oxidative stress in osteoarthritis (Paper III)

MetS is more prevalent among OA patients than in the general population (Singh *et al.* 2002). Several studies have found association between OA and components of MetS (Yoshimura *et al.* 2011, 2012; Shin, 2014). Dyslipidemia is an important feature of MetS and many epidemiological studies have reported altered serum lipid profiles among the OA population (Hart *et al.* 1995; Al-Arfaj, 2003). However, the exact mechanisms involved are poorly yet understood. In Paper III we found independent association between LDL-cholesterol and OA severity. These findings suggest the potential role of LDL-cholesterol in OA progression. LDL-cholesterol has been extensively studied in CVD while studies focusing on LDL-cholesterol and OA are far more infrequent. Animal studies have indicated that cholesterol is involved in the pathogenesis of OA (Gierman *et al.* 2014). In healthy cells, LDL-receptors are down-regulated after activation, but in OA the regulatory mechanism is aberrant and leads to cholesterol accumulation (de Munter *et al.* 2013). LDL-cholesterol has also

been associated with synovial activation and osteophyte formation (de Munter *et al.* 2013). According to a recent study, LDL-receptor-related protein 1 is a modulator of MMP13 extracellular levels, which might also contribute to the association between OA severity and LDL-cholesterol (Yamamoto *et al.* 2016). Altogether, LDL-cholesterol seems to play an important role in the pathogenesis of OA.

Furthermore, the particles of LDL are susceptible to ROS, which and results in increased formation of oxLDL particles that might be even more important in the pathogenesis of OA. The oxLDL is taken up via scavenger receptors, such as lectin type oxLDL receptor 1 (LOX-1), which leads to activation and secretion of different proteases and inflammatory mediators (de Munter *et al.* 2016). The receptors of oxLDL are expressed on macrophages, endothelial cells, chondrocytes and fibroblasts. In Paper III we found that oxLDL was associated with OA severity as well as with MetS score. These results suggest that OA severity is associated with OxS independently of the effect of MetS. Moreover, the association between OxS and OA severity remained significant after correcting for BMI, age, gender and accounting for multiple testing, while the association between components of MetS and OA severity did not. The present study suggests that OxS is the result of both the increased amount of ROS and decreased antioxidative capacity. The sources of ROS contributing to oxidative damage include free radicals generated from aerobic metabolism and in response to specific stimuli like cytokines and growth factors. Several components of MetS have been found to be associated with OxS (Roberts & Sindhu, 2009). Since TAC is a relatively new marker and has rarely been studied in OA patients, the results are inconclusive (Altindag *et al.* 2007; Altay *et al.* 2015). Decreased antioxidative capacity might result from the high amount of ROS as well as from the dysfunction of the regulatory mechanisms of antioxidants (Lepetsos & Papavassiliou, 2016). In conclusion, we found that OxS was associated with OA, but further research is required for a better understanding of the role of OxS in OA and for possible clinical implications.

Altered glucose metabolism is the core pathology of MetS and has been the focus of several epidemiological studies investigating metabolic OA (Hart *et al.* 1995; Yoshimura *et al.* 2011, 2012). However, the results concerning insulin resistance and OA are inconsistent; nor have many studies adjusted for the effect of body composition and age, which are important confounders. The present thesis (Paper III) describes a trend towards positive correlation between radiographic severity of OA and insulin resistance.

Hypertension has been associated with OA in many studies, but only a few of them have found significant association after adjusting for age and BMI (Yoshimura *et al.* 2012; Monira Hussain *et al.* 2014). In the current study we found a trend towards positive correlation between systolic BP and OA after correcting for the confounders and multiple testing. However, the arterial pathology accompanying OA and MetS is probably more complex than hypertension. Assessment of arterial stiffness, besides hypertension, might provide a more profound insight into the arterial pathology involved in metabolic OA.

6.4 The role of acylcarnitines as biomarkers in osteoarthritis (Paper IV)

In Paper IV we found independent association between arterial stiffness and serum acylcarnitines in end-stage OA patients. Several serum medium- and long-chain acylcarnitines were associated with radiographic severity of OA. Furthermore, the levels of acylcarnitines were significantly lower in the OA patients than in the age- and gender-matched controls.

Acylcarnitines are essential for transport of fatty acids into mitochondria during the process of β-oxidation, which is a major source of energy. We found that several medium- and long-chain acylcarnitines such as C14, C14:2, C14:1-OH, C16, C16:1-OH and C18 were significantly lower in the OA group. Similar findings have been found in early rheumatoid arthritis patients (Surowiec *et al.* 2016). The lower level of acylcarnitines might be caused by increased energy consumption in OA. One of the causes of increased energy expenditure is inflammation. Low-grade systemic inflammation has an important role in the pathogenesis of OA (Berenbaum, 2013) and has also been associated with arterial stiffening and endothelial dysfunction (Steyers & Miller, 2014). In support, we found that TNF-α was independently associated with arterial stiffness in the linear regression model and the serum levels of TNF-α, hs-CRP and WBC were higher in the OA patients compared with the controls. It has been noted that in end-stage OA, chondrocytes become hypertrophic as metabolic activity increases (van der Kraan & van den Berg, 2012). These changes might also be associated with inflammation involving increased production of inflammatory mediators and proteolytic enzymes (e.g. MMPs).

Recently, a study (Zhang *et al.* 2014) described two different subtypes of knee OA based on different synovial fluid acylcarnitine concentrations and activity of acetyltransferase. In support, our study found that acylcarnitines were positively correlated with OA radiographic severity. These changes are in accordance with a study reporting accumulation of lipid particles in the chondrocytes in the later stages of OA (Villalvilla *et al.* 2013). In addition to lower acylcarnitine levels, we also found decreased levels of the CPT-ratio and total AC/C0 ratio in the OA patients. These ratios describe the activity of enzymes responsible for acylcarnitine metabolism. Moreover, CPT is responsible for condensation of activated long-chain fatty acids into carnitine to form acylcarnitines, thereby regulating the entry of acyl-coenzyme-A into the mitochondrial matrix (Turner *et al.* 2007). The level of the CPT-1-ratio describes the activity of CPT-1, which is a rate-limiting enzyme for β-oxidation of long-chain fatty acids. Thus, the decreased levels of serum acylcarnitines in the OA patients might be related to dysfunctional enzymes of fatty acid β-oxidation.

We found that acylcarnitines were positively correlated with arterial stiffness. In regression analysis, component 3 (acylcarnitines) was an independent predictor of the PWV-ratio. Cr-PWV describes stiffness of the peripheral muscular arteries; as it had stronger association with acylcarnitines than cf-PWV, it might have greater influence on the association between the PWV-ratio

and acylcarnitines in OA patients. The PWV-ratio describes the arterial stiffness mismatch phenomenon. The peripheral arteries are physiologically stiffer than the aorta. The physiological gradient of stiffness creates backward travelling pulse waves from the reflection sites. Thereafter, the forward travelling pressure wave is dampened and peripheral microcirculation is protected from high pulsatile pressure. Ageing and different diseases increase aortic stiffness to a point where it is higher than peripheral muscular artery stiffness. This phenomenon is described as stiffness mismatch and it results in higher pressure and energy transmission to the microcirculation (McEniery *et al.* 2005; Mitchell *et al.* 2010). Our study showed for the first time that OA patients' serum acylcarnitine levels were associated with arterial stiffness and stiffness mismatch, while there was no such association in the control group. Acylcarnitines have been found to be predictive of future CVD events (Shah *et al.* 2012). Altogether, medium- and long-chain acylcarnitines entail great potential for CVD risk assessment and might also explain the association between OA and CVD.

6.5 Limitations

The present thesis (Papers I–IV) has several limitations that must be acknowledged when interpreting the results. Firstly, the cross-sectional study design is not appropriate for proving causal relationships. Thus, the results of the study should be confirmed or overruled in future prospective studies. Secondly, as the control group was not radiographically tested for absence of OA, it might have included asymptomatic OA patients, which might introduce a type II error. Another limitation of the study is the potential confounding role of different uses of nonsteroidal anti-inflammatory drugs across the study groups. End-stage OA patients use several medications to alleviate pain, which include NSAIDs. Since many NSAIDs increase the CVD risk, this might also have influenced the results of the study.

7. CONCLUSIONS

1. Carotid-femoral pulse wave velocity (cf-PWV) were significantly increased and small artery elasticity index was significantly decreased in the end-stage osteoarthritis patients compared with age- and gender matched controls. The level of cf-PWV was independently associated with radiographic severity of osteoarthritis. Thus, arterial stiffening is linked to osteoarthritis and might play a role in the increased cardiovascular risk in these patients.
2. The serum levels of leptin and matrix metalloproteinase 3 (MMP3) were significantly higher and the serum levels of adiponectin were significantly lower in the end-stage osteoarthritis patients compared with the controls. The serum level of adiponectin was significantly correlated with augmentation index and large artery elasticity and the level of MMP3 correlated with severity of osteoarthritis in the osteoarthritis patients. Thus, measurement of adipokines and MMP3 provides relevant clinical information about the vascular function and severity of osteoarthritis.
3. The serum levels of LDL-cholesterol and oxidized LDL-cholesterol were increased and independently correlated with osteoarthritis severity in the end-stage osteoarthritis patients. Elevated serum C-peptide, total peroxide concentrations and lower total antioxidant capacity were found in the osteoarthritis patients. High grade oxidative stress and dyslipidemia might influence the clinical course of osteoarthritis and could therefore serve as important therapeutic targets and diagnostic biomarkers.
4. The levels of long- and medium-chain acylcarnitines were significantly lower in the end-stage osteoarthritis group. These acylcarnitines were independently associated with the pulse wave velocity ratio and osteoarthritis severity in the osteoarthritis patients. Therefore, shifts in lipid metabolism provide valuable information for a better understanding of how metabolic pathways are involved in the pathogenesis of osteoarthritis and arterial stiffening.

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SUMMARY IN ESTONIAN

Osteoartroosi kardiovaskulaarne ja metaboloomiline profileerimine

Osteoartroos (OA) on krooniline progresseeruv liigeshaigus, mis haarab sagedamini põlve, puusa ja käe liigeseid ning lülisammast. Muskuloskeletaalhaigused on üks suuremaid haigena elatud aastate põhjustajaid. Juba ainuüksi põlve OA mõjutab 250 miljonit inimest. Lisaks on leitud, et praktiliselt igal teisel inimesel tekib elu jooksul sümptomaatiline põlve OA. Sellest tulenevalt on haiguse sotsio-ökonoomiline mõju suur. Vaatamata suurele huvile pole tänaseni võimalik OA patsienti terveks ravida. Parim ravi keskendub valu vähendamisele ja funktsiooni säilitamisele ning lõppstaadiumis endoproteesimisele, mis on küll väga efektiivne patsientide elukvaliteedi parandamisel, aga toob endaga kaasa mitmed riskid. Seetõttu on hädavajalik haiguse protsesside täiendav uurimine, et leida paremaid ravimeetodeid.

OA on heterogeenne haiguse grupp, mis omab mitmeid seoseid teiste haigustega. Üheks oluliseks OA riskifaktoriks on rasvumine. Tegemist on arenenud maailmas üha sageneva probleemiga. Ühest küljest avaldab ülekaal otseselt mehhaanilist survet raskust kandvatele liigestele, mis tõstab liigese traumaatilise kahjustuse riski ja aktiveerib ka põletiku kaskaadi. Teisest küljest on leitud, et rasvumine on seotud ka raskust mitte kandvate liigeste OA-ga. Selles seoses võivad olulist rolli mängida adipokiinid, mis on peamiselt valge rasvkoe poolt sünteesitud bioaktiivsed ained. Adipokiinid, mille tuntumad esindajad on leptiin, adiponektiin ja resistiin, omavad organismi homöostaasi säilitamisel olulist rolli ning rasvumise korral on nende normaalne tasakaal häirunud. OA korral on mitmete adipokiinide tase muutunud. Adipokiine on seostatud OA raskusastme ja haiguse progresseerumisega. Seetõttu omavad nad suurt potentsiaali aitamaks diagnoosida OA varasemas staadiumis ning ennustada haiguse edasist kulgu. Lisaks muutustele adipokiinide tasemes tõstab rasvumine oksüdatiivse stressi taset, mis väljub organismi antioksüdatiivse süsteemi kontrolli alt ja põhjustab kudede kahjustusi.

Varasemad uuringud on näidanud, et OA patsientidel esineb rohkem südame- ja veresoonkonna haigusi. Selle taga võivad olla ühised riskifaktorid nagu vanus, ülekaalulisus, ravimite kõrvaltoimed ja vähene füüsiline aktiivsus. Teisest küljest on OA ja ateroskleroosi patogeneesis mitmeid ühiseid radu. Seetõttu võivad OA ja veresoonte haigused olla omavahel seotud. Tegemist on uudse lähenemisega ja vähe uuritud valdkonnaga. Üheks südame- ja veresoonkonna haiguste riskimarkeriks on arterite jäikus. Arterite jäikuse mõõtmine mitteinvasiivselt on lihtne ja usaldusväärne metoodika, mis võimaldab hinnata organismi veresoonte seisundit. Arterite jäikuse mõõtmise kuldseks standardiks loetakse aordi pulsilaine levikukiiruse (cf-PWV) kindlaks tegemist. cf-PWV on sõltumatu südame ja veresoonkonna haiguste ja suremuse ennustaja.

Nii veresoonte jäigenemise kui ka OA korral toimuvad organismi ainevahetuses mitmed muutused, mille selgitamine võimaldaks täiustada teadmisi OA

olemusest ning pakkuda välja uusi ründepunkte haiguse ravimiseks. Metaboloomika on uus ja kiiresti arenev teadusvaldkond, mis hindab organismi madalmolekulaarsete metaboliitide profiili. See on nn "oomika" teaduste viimane lüli, mis annab ülevaate organismi hetkeolukorrast, kui mõju on avaldanud nii geneetilised tegurid, epigeneetilised tegurid kui ka keskkond. Metaboloomika annab võimaluse uurida OA ja südame- ning veresoonkonna haiguste omavahelisi seoseid uuel tasandil.

Uurimuse eesmärgid

Antud doktoritöö üldiseks eesmärgiks oli uurida osteoartroosiga patsientide arterite jäikust, metaboolseid kõrvalekaldeid, oksüdatiivset stressi ning madalmolekulaarsete metaboliitide profiili ning võrrelda neid vanuse ning soo poolest sobitatud kontrollgrupiga. See on vajalik, et selgitada kardiovaskulaarse riski seost osteoartroosiga ning otsida uusi biomarkereid osteoartroosi varaseks diagnoosimiseks ja haiguskulu ennustamiseks.

Uuringu täpsemad eesmärgid:

1. Mõõta arterite jäikust osteoartroosiga patsientidel võrreldes kontrollgrupiga ning uurida arterite jäikuse seoseid osteoartroosi raskusega.
2. Määrata adipokiinide (leptiin, adiponektiin) ja maatriksi metalloproteinaas 3 taset osteoartroosiga patsientidel ja kontrollgrupil ning uurida nende biomolekulide seoseid arterite jäikuse ja osteoartroosi raskusega.
3. Hinnata metaboolse sündroomi ja oksüdatiivse stressi mõju osteoartroosiga patsientidele ja kontrollgrupile.
4. Määrata atsüülkarnitiinide mõju arterite jäikusele ning osteoartroosi raskusega osteoartroosiga patsientidel ning kontrollgrupil.

Uuringu meetodid

Uuringusse kaasati 70 lõpp-staadiumis põlve ja puusa primaarse OA patsienti (Tartu Ülikooli Kliinikumi ortopeedia osakonnast) ja 82 vanuse ning soo poolest sobitatud kontrollgrupi liiget (perearstide nimistutest). Arterite jäikust hinnati pulsilaine leviku kiiruse ja pulsilaine analüüsi metoodikaga mitteinvasiivselt Endoteeli Keskuses. OA raskusastet hinnati radioloogiliselt Kellgren-Lawrence klassifikatsiooni järgi. Biomarkerite ja madalmolekulaarsete metaboliitide mõõtmine tehti Tartu Ülikooli bio- ja siirdemeditsiini instituudi biokeemia osakonnas ning Tartu Ülikooli Kliinikumi Ühendlaboris.

Tulemused ja järeldused

1. Osteoartroosiga patsientide arterite jäikus oli märgatavalt kõrgem kui vanuse ja soo poolest sobitatud kontrollgrupil. Lisaks esines sõltumatu seos

osteoartroosi raskusastme ja arterite jäikuse vahel, mis viitab veresoonte kahjustuse rollile osteoartroosi arengus.

2. Osteoartroosiga patsientide leptiini tase oli kõrgem ja adiponektiini tase madalam kui kontrollgrupil. Selgus, et osteoartroosi raskusaste on seotud maatriksi metalloproteinaas 3 ja leptiini tasemega ning adiponektiin on seotud arterite jäikusega osteoartroosiga patsientidel. Seega võivad need valgud tulevikus osutuda osteoartroosi biomarkeriteks.
3. Osteoartroosiga patsientide oksüdatiivse stressi tase oli oluliselt kõrgem kui kontrollgrupil. Metaboolse sündroomi ükski komponent ei olnud sõltumatult seotud osteoartroosi raskusastmega, aga C-peptiidi tase oli oluliselt kõrgem osteoartroosiga patsientidel ning LDL-kolesterooli tase oli seotud osteoartroosi raskusastmega. Seega näitasid uuringu tulemused liigset oksüdatiivset stressi ja lipiidide ning glükoosi ainevahetuse häireid osteoartroosiga patsientidel.
4. Osteoartroosiga patsientide keskmise ja pika ahelaga atsüülkarnitiinide tasemed oli madalamad võrreldes kontrollgrupiga. Lisaks esines sõltumatu seos atsüülkarnitiinide ja arterite jäikuse vahel. Seega võivad atsüülkarnitiinid osutuda tulevikus olulisteks osteoartroosi biomarkeriteks, olles abiks haiguse diagnoosimisel ja edasise kulu ennustamises.

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PUBLICATIONS

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2) Tootsi K, Kals J, Zilmer M, Paapstel K, Märtson A. Severity of Osteo-arthritis Is Associated with Increased Arterial Stiffness. Int J Rheumatol 2016; 2016:6402963.
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Teadustöö:
Minu teadustöö põhilisteks suundadeks on osteoartoosi ja arterite jäikuse, metaboolsete tegurite, oksüdatiivse stressi ja metaboloomika vaheliste seoste uurimine. Olen osalenud 8 rahvusvahelise teadusartikli publitseerimisel ning teinud 8 ettekannet rahvusvahelistel teaduskonverentsidel.

Publikatsioonid:

1) Tootsi K, Märtson, A, Kals J, Zilmer M, Paapstel K. Metabolic factors and oxidative stress in osteoarthritis: A case-control study. Scand J Clin Lab Invest
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