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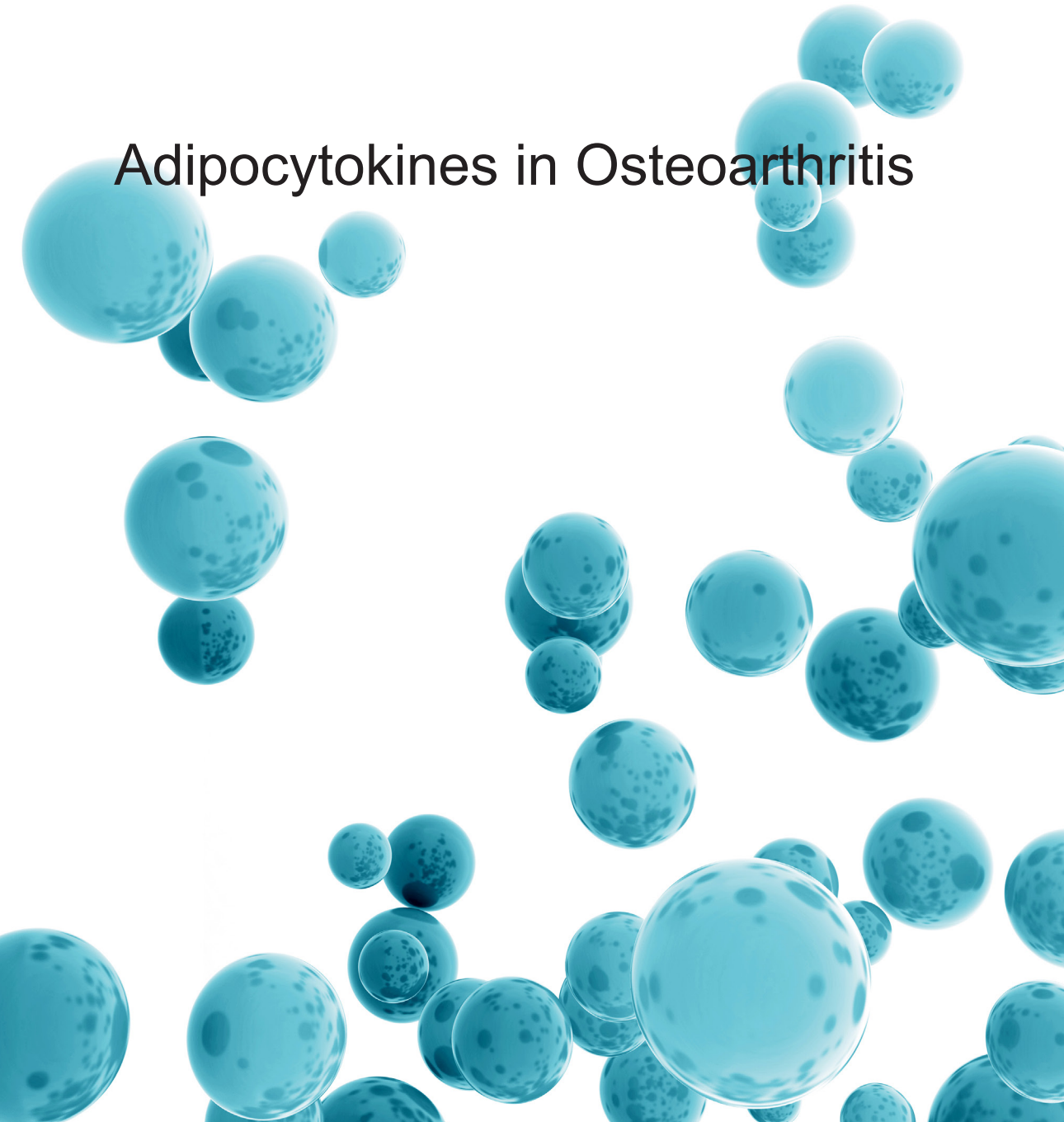
Adipocytokines in Osteoarthritis



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ACADEMIC DISSERTATION
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University of Tampere, Faculty of Medicine and Life Sciences
Tampere University Hospital
Finland

Supervised by
Professor Eeva Moilanen
University of Tampere
Finland
Docent Katriina Vuolteenaho
University of Tampere
Finland

Reviewed by
Professor Aspasia Tsezou
University of Thessaly
Greece
Professor Mikko Lammi
Umeå University
Sweden

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Contents

List of original communications

This thesis is based on the following original communications, referred to in the text by their roman numerals I-VI.

I A. Koskinen, S. Juslin, R. Nieminen, T. Moilanen, K. Vuolteenaho and E. Moilanen (2011): Adiponectin associates with markers of cartilage degradation in osteoarthritis and induces production of proinflammatory and catabolic factors through mitogen activated protein kinase pathways. Arthritis Res Ther. 13(6):R184.

II K. Vuolteenaho, A. Koskinen, M. Kukkonen, R. Nieminen, U. Päivärinta, T. Moilanen and E. Moilanen (2009): Leptin enhances synthesis of proinflammatory mediators in human osteoarthritic cartilage - a mediator role of NO in leptin-induced PGE_2, IL-6 and IL-8 production. Mediat Inflamm. Article ID 345838, 2009.

III A. Koskinen, K. Vuolteenaho, R. Nieminen, T. Moilanen and E. Moilanen (2011): Leptin enhances MMP-1, MMP-3 and MMP-13 production in human osteoarthritic cartilage and correlates with MMP-1 and MMP-3 in synovial fluid from OA patients. Clin Exp Rheumatol. 29(1):57-64.

IV K. Vuolteenaho, A. Koskinen, T. Moilanen and E. Moilanen (2012): Leptin levels are increased and its negative regulators, SOCS-3 and sOb-R are decreased in obese patients with osteoarthritis: a link between obesity and osteoarthritis. Ann Rheum Dis. 71(11):1912-3.

V A. Koskinen-Kolasa, K. Vuolteenaho, R. Korhonen, T. Moilanen, and E. Moilanen (2016): Catabolic and proinflammatory effects of leptin in

chondrocytes are regulated by suppressor of cytokine signaling-3. Arthritis Res Ther. 18(1):215.

VI A. Koskinen, K. Vuolteenaho, T. Moilanen and E. Moilanen (2014): Resistin as a factor in osteoarthritis: Synovial fluid resistin concentrations correlate positively with interleukin 6 and matrix metalloproteinases MMP-1 and MMP-3. Scand J Rheumatol 43(3):249-53.

In addition, some unpublished data are presented.

Abbreviations

1400W	N-[aminopethyl)benzyl]acetamidine, inducible nitric oxide synthase (iNOS) inhibitor
ADAMTS	a disintegrin and metalloproteinase with thrombospondin motifs
BMI	body mass index
CNS	central nervous system
COMP	cartilage oligomeric matrix protein
COX	cyclooxygenase
ECM	extracellular matrix
ELISA	enzyme-linked immunosorbent assay
Erk1/2	extracellular signal-regulated kinase 1 and 2
ESR	erythrocyte sedimentation rate
gp130	glycoprotein 130
HA	hyaluronan
HMW	high molecular weight
IFN	interferon
IGF-1	insulin-like growth factor
IL	interleukin
iNOS	inducible nitric oxide synthase
IQR	inter quartile range
JAK	janus kinase
JNK	c-Jun N-terminal kinase
KSS	Knee Society Score
ln	natural logarithm
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
MMP	matrix metalloproteinase
MRI	magnetic resonance imaging
NF-κB	nuclear factor κB

NO	nitric oxide
NOS	nitric oxide synthase
OA	osteoarthritis
Ob-Rb	leptin receptor, isoform b (functional leptin receptor)
p	plasma
PBMC	peripheral-blood mononuclear cell
PGE_2	prostaglandin E_2
PI3K	phosphoinositide 3-kinase
PKC	protein kinase C
RA	rheumatoid arthritis
s	serum
SEM	standard error of the mean
SF	synovial fluid
SD	stardard deviation
siRNA	small interfering ribonucleic acid
sOb-R	soluble leptin receptor
SOCS-3	suppressor of cytokine signaling-3
STAT	signal transducer and activator of transcription
TGF-β	transforming growth factor β
TIMP	tissue inhibitor of metalloproteinase
TLR	toll-like receptor
TNF-α	tumor necrosis factor-α
WAT	white adipose tissue

Abstract

Osteoarthritis (OA) is the most common joint disease in the world, but at present, there are no disease modifying drugs available. Although the risk factors of OA are well known, the detailed mechanisms underpinning the disease are poorly understood. There is a clear need to clarify the molecular pathways in the pathogenesis of OA in order to discover novel targets for drug development.

Obesity is a significant risk factor of OA; the increased load on weight-bearing joints partly explains this connection. However, obesity is also a risk factor for hand OA. This points to the existence of some obesity-related metabolic factor(s) that could systemically mediate the effect of obesity on joints.

Adipocytokines, also known as adipokines, are hormones produced by adipose tissue. Adipokines were originally discovered as regulators of food intake and energy expenditure. More recently, they have been observed also to participate in the regulation of other body functions, such as in the regulation of the immune system. Adipokines have also been detected in osteoarthritic joints and they have been hypothesized to have a role in the pathogenesis of joint diseases.

The aim of the present study was to investigate the role of three adipokines i.e., adiponectin, leptin and resistin, in OA and to consider the possibility that they act as mediators between obesity and OA. The ultimate goal of this study was to produce information on possible novel drug targets for the treatment or prevention of OA.

Blood, cartilage and synovial fluid samples as well as clinical data from 100 OA patients undergoing knee replacement surgery in Coxa hospital for Joint Replacement, Tampere, Finland were collected for the study. In addition, intracellular mechanisms were investigated in additional cartilage and cell cultures.

Adiponectin and leptin concentrations in synovial fluid and in the circulation, and the levels of all three adipokines released from cultured cartilage were higher in females than males. The leptin concentration in all compartments correlated strongly with body mass index (BMI).

Adiponectin levels correlated with nitric oxide (NO), interleukin-6 (IL-6) and matrix metalloproteinase (MMP) enzymes MMP-1, MMP-3 and MMP-13 measured in the synovial fluid or in the cartilage culture media. Adiponectin increased the production of these factors in cultured OA cartilage and chondrocytes through mitogen-activated protein kinase (MAPK) mediated signaling pathways. Adiponectin was associated with circulating biomarkers of OA, namely cartilage oligomeric matrix protein (COMP) and MMP-3, as well as with the radiographic severity of OA.

Leptin concentration correlated with those of MMP-1 and MMP-3 in synovial fluid from obese (BMI > 30 kg/m^2) OA patients. The leptin level was higher in patients with more severe findings/symptoms of OA as assessed by the Knee Society Score (KSS) rating of OA. Leptin increased the production of NO, IL-6, IL-8, prostaglandin E_2 (PGE_2), MMP-1, MMP-3 and MMP-13 in cartilage cultures through MAPK, protein kinase C (PKC), janus kinase 3 (JAK3) and nuclear factor κB (NF-κB) signaling pathways. Suppressor of cytokine signaling-3 (SOCS-3) was identified as a novel factor downregulating leptin responsiveness in chondrocytes. The expression of SOCS-3 and the level of soluble leptin receptor (sOb-R) that binds active free leptin into an inactive complex, were found to be lower in obese (BMI > 30 kg/m^2) than in non-obese (BMI < 30 kg/m^2) OA patients.

Resistin was present in OA synovial fluid and it was released from cultured OA cartilage. The levels of resistin correlated with those of NO, IL-6, MMP-1, MMP-3 and MMP-13 in synovial fluid and/or in cartilage culture media.

The correlations of all studied adipocytokines with proinflammatory and catabolic factors, as well as the proinflammatory and catabolic effects of adiponectin and leptin on cartilage, point to detrimental role for these adipokines in the pathogenesis of OA. Since the leptin concentration correlated with BMI and showed BMI-dependent clinical associations, it seems reasonable to postulate that it may act as a metabolic mediator between obesity and OA. Obese patients had also disturbed mechanisms to buffer leptin's actions, i.e. decreased sOb-R concentrations and SOCS-3 expression, suggesting that obese individuals might be particularly prone to the detrimental effects of leptin. The findings propose that the studied adipokines could be investigated as novel drug targets in the prevention/treatment of OA in the future, especially leptin in obese individuals.

Tiivistelmä

Nivelrikko on maailman yleisin nivelsairaus. Toistaiseksi siihen ei ole onnistuttu kehittämään parantavaa tai taudin etenemistä hidastavaa hoitoa. Nivelrikon riskitekijät tunnetaan hyvin, mutta niiden yhteys tautiprosessiin on monelta osin tuntematon. Nivelrikkoa hidastavien lääkeaineiden kehityksen kannalta on tärkeää saada uutta tietoa nivelrikkoon johtavista molekyylitason mekanismeista.

Lihavuus on merkittävä nivelrikon riskitekijä. Lihavuuden yhteyttä nivelrikkoon selittää osin kantaville nivelille kohdistuva ylimääräinen kuorma. Lihavuus on kuitenkin myös sorminivelrikon riskitekijä. Tämä viittaa siihen, että on olemassa jokin lihavuuteen liittyvä metabolinen tekijä (tai tekijöitä), joka välittää lihavuuden vaikutuksen niveliin systeemisesti, ts. verenkierron, ja edelleen nivelnesteen kautta.

Adiposytokiinit, eli adipokiinit, ovat rasvakudoksen erittämiä hormoneja. Alun perin adipokiinien havaittiin säätelevän ruokahalua ja toimivan kehon energiatasapainoa säätelevinä molekyyleinä. Sittemmin adipokiinien on huomattu säätelevän myös muita kehon toimintoja, kuten tulehdusreaktiota. Adipokiineja on löydetty myös nivelrikkonivelistä, ja niiden on hypotisoitu osallistuvan nivelsairauksien patogeneesiin.

Tämän tutkimuksen tarkoituksena oli tutkia kolmen adipokiinin, adiponektiinin, leptiinin ja resistiinin merkitystä nivelrikon patogeneesissa, ja niiden roolia lihavuutta ja nivelrikkoa yhdistävinä tekijöinä. Tavoitteena oli näin tuottaa uutta tietoa mahdollisista uusista nivelrikon lääkehoidon kohteista.

Tutkimusta varten kerättiin poikkileikkausaineisto sadalta polven tekonivelleikkauspotilaalta, sisältäen kliiniset tiedot, veri- ja nivelnestenäytteet, sekä rustonäytteet rustoviljelmää varten. Näytteistä analysoitiin adiponektiinin, leptiinin ja resistiinin pitoisuudet, ja verrattiin niitä nivelrikon patogeneesin kannalta tärkeiden tulehduksellisten välittäjäaineiden, mukaan lukien typpioksidin (engl. nitric oxide, NO), interleukiini-6:n (IL-6), ja katabolisten rustoa hajottavien entsyymien, matriksimetalloproteinaasien (MMP), pitoisuuksiin. Aineiston perusteella tutkittiin myös adipokiinien yhteyttä nivelrikon radiologiseen vakavuusasteeseen, nivelrikon

biomarkkereihin sekä nivelrikon kliiniseen vaikeusastemittariin (Knee Society Score, KSS). Lisäksi adiponektiinin ja leptiinin vaikutuksia, sekä niitä välittäviä solunsisäisiä signalointireittejä ja säätelymekanismeja tutkittiin nivelrikkorusto- ja rustosoluviljelmissä.

Adiponektiini- ja leptiinipitoisuudet verenkierrossa ja nivelnesteessä, ja kaikkien kolmen adipokiinin pitoisuudet rustoviljelmässä olivat suurempia naisilla kuin miehillä. Leptiini korreloi vahvasti painoindeksin (BMI, body mass index) kanssa niin verenkierrosta, kuin myös nivelnesteestä ja rustoviljelmästä mitattuna.

Adiponektiinipitoisuus korreloi NO:n, IL-6:n, MMP-1:n, MMP-3:n ja MMP-13:n kanssa nivelnesteessä ja/tai rustoviljelmän elatusnesteessä. Adiponektiini lisäsi näiden tekijöiden tuottoa viljellyssä nivelrikkorustossa ja rustosoluissa. Vaikutusta välitti solunsisäinen MAPK (engl. mitogen-activated protein kinase) signalointireitti. Adiponektiini assosioitui myös nivelrikon verestä mitattaviin biomarkkereihin, COMP:iin (eng. cartilage oligomeric matrix protein) ja MMP-3:een sekä nivelrikon radiologiseen vaikeusasteeseen.

Leptiini korreloi MMP-1:n ja MMP-3:n pitoisuuksien kanssa nivelnesteessä lihavilla (BMI > 30 kg/m^2) nivelrikkopotilailla. Leptiinipitoisuudet olivat korkeampia potilailla, joilla oli vakavampi nivelrikko KSS-luokituksen mukaisesti. Leptiini lisäsi NO:n, IL-6:n, IL-8:n, prostaglandiini E_2 (PGE_2):n, MMP-1:n, MMP-3:n ja MMP-13:n tuottoa rustoviljelmissä. Vaikutusta välittivät solunsisäiset MAPK, proteiinikinaasi C (PKC), janus kinaasi 3 (JAK3) ja NF-κB (engl. nuclear factor κB) -signalointireitit. Aiemmin sytokiinien vaikutuksia säätelevänä tunnetun solunsisäisen proteiinin, SOCS-3:n (engl. suppressor of cytokine signaling-3), todettiin estävän tutkimuksessa löydettyjä leptiinin haitallisia vaikutuksia rustosoluissa. SOCS-3:n ilmentyminen nivelrikkopotilaiden rustossa, kuten myös bioaktiivista leptiiniä sitovan liukoisen leptiinireseptorin (sOb-R:n) pitoisuus, olivat pienempiä lihavilla potilailla verrattuna muuhun potilasjoukkoon.

Nivelrikkoruston todettiin vapauttavan resistiiniä. Resistiinipitoisuus korreloi NO:n, IL-6:n, MMP-1:n, MMP-3:n ja MMP-13:n pitoisuuksiin nivelnesteessä ja/tai rustoviljelmän elatusnesteessä.

Tutkimuksessa löydetyt korrelaatiot adipokiinien ja tulehduksen välittäjäaineiden sekä rustoa hajottavien entsyymien välillä sekä havaitut adiponektiinin ja leptiinin tulehdusta lisäävät ja rustotuhoa voimistat vaikutukset viittaavat siihen, että näillä kolmella adipokiinilla on yhteys ja todennäköisesti haitallinen rooli nivelrikon

taudinkulussa. Leptiini korreloi vahvasti painoindeksiin ja sen löydetyt yhteydet katabolisiin entsyymeihin riippuivat painoindeksistä. Tämä viittaa siihen, että leptiini on haitallinen tekijä nivelrikon kannalta etenkin lihavilla yksilöillä. Lihavilla potilailla havaittiin myös häiriytyneitä mekanismeja puskuroida leptiinin vaikutuksia: pienempi sOb-R-pitoisuus ja matalampi SOCS-3:n ilmentyminen rustossa muuhun potilasjoukkoon (BMI < 30 kg/m^2) verrattuna. Tämä viittaa siihen, että lihavat henkilöt voivat ovat erityisen alttiita leptiinin haitallisille vaikutuksille.

Tutkimus loi uutta tietoa nivelrikon mekanismeista sekä lihavuuden, adipokiinien ja nivelrikon yhteyksistä. Tutkituilla adiponiineilla havaittiin olevan nivelrustovauriota edistäviä vaikutuksia ja kyseisiä adipokiineja sekä niiden vaikutusreittejä kannattaa jatkossa tutkia uusina lääkevaikutuskohteina nivelrikon ehkäisyssä / hoidossa, leptiiniä erityisesti lihavilla yksilöillä.

Introduction

Osteoarthritis (OA) is the most common joint disease worldwide and the leading cause of chronic disability (Moskowitz et al. 2007; Glyn-Jones et al. 2015). Osteoarthritis is a late-onset disease. The incidence of knee OA peaks at around 60 years, the prevalence of symptomatic knee OA being around 10% (Arokoski et al. 2007; Losina et al. 2013). As many as 45% of individuals aged over 85 years are estimated to have symptomatic knee OA (Arokoski et al. 2007; Murphy et al. 2008; Losina et al. 2013). OA exerts significant impacts on the quality of life and on the ability to function, and it is also responsible for significant losses of income for individuals, as well as being a financial burden for society (Heliövaara et al. 2008).

Osteoarthritis is a slowly progressing disease that leads to a loss of articular cartilage, impaired joint function and pain. The inflammatory process in joint tissues is central in the pathogenesis of OA; it is thought to shift the balance in chondrocyte metabolism away from low turnover state towards catabolia, leading to the degradation of the cartilage matrix. It is still unclear how the inflammatory process in cartilage and joint tissues is initiated. Articular cartilage has a low capability to repair itself, and once damaged, its structure does not return to its original state.

Obesity is a well-recognized risk factor of OA and according to different studies, it increases the risk of developing knee OA, with values ranging from doubling to as much as eightfold (Blagojevic et al. 2010; Toivanen et al. 2010; Lee and Kean 2012; Losina et al. 2013). As obesity is an increasing global health problem, it is likely that the prevalence rates of OA, and also disability and the need for replacement surgery, with the associated costs, will increase dramatically in the future.

Traditionally the increased risk of OA in obese individuals has been explained by increased load on weight bearing joints, particularly on the knee and hip joints. However, obesity does not only enhance the frequency of knee and hip OA, but it also increases the risk of hand OA (Haara et al. 2003; Yusuf et al. 2010; Kloppenburg and Kwok 2011), which cannot be attributed by mechanical effects. The link between

obesity and hand OA suggests that some obesity-related metabolic factor / factors might contribute to the pathogenesis of OA.

The underlying mechanisms in the onset of OA are not truly understood, and so far, the pharmaceutical industry has failed in developing effective therapies to slow down the progression of OA. The current pharmacological treatment options for OA are capable to reduce joint pain to some extent in terms of both severity and duration. In late stage disease, joint replacement surgery may be required.

Adipocytokines (e.g. leptin, resistin and adiponectin) are hormones secreted into the circulation by adipose tissue. They have physiological functions in controlling appetite, body weight and metabolism (Ouchi et al. 2011; Park and Ahima 2015). The circulating leptin level correlates strongly with BMI, and resistin levels have also been reported to be elevated in obesity. Adiponectin has been reported to display a negative correlation with body mass index (BMI) and to be potentially anti-inflammatory (Ouchi et al. 2011). Recently adipokines have been recognized to be involved also in the inflammation process and to regulate immune functions. The first studies that investigated adipokine concentrations in inflammatory joint diseases were published at the time when the present research project was started; they demonstrated that adiponectin, leptin and resistin could be detected in synovial fluid and that their levels are higher in rheumatoid arthritis (RA) than in OA patients and correlate with CRP/erythrocyte sedimentation rate (ESR)/white blood cell count (Schaffler et al. 2003; Presle et al. 2006; Senolt et al. 2007; Ibrahim et al. 2008). Otherwise the subject remained largely unstudied. Interest towards adipokines has increased substantially during the last few years, and the amount of research data on their properties in OA has grown tremendously. The latest results from other studies in the same field in relation to our own findings will be evaluated in the discussion section.

The aim of the present study was to investigate the role of three adipocytokines, namely leptin, adiponectin and resistin in the pathogenesis of OA, and as possible factors connecting obesity and OA. Furthermore, intracellular mechanisms of action of leptin and adiponectin as potential targets of future drug development were examined.

Review of literature

1 Osteoarthritis

Osteoarthritis is characterized by a gradual, progressive loss of articular cartilage in synovial joints that happens over years, even decades, of time. Changes are seen in all tissues of the joint, but the most prominent feature is a degradation of the articular cartilage. Traditionally, OA was believed to be a degenerative wear-and-tear disease. However, according to present knowledge, it is now generally accepted that inflammatory mechanisms drive the destructive events in cartilage and joint tissues. Chondrocytes, the only cell type found in the cartilage, are crucially involved in the production of catabolic factors, and subsequent cartilage degradation. Nonetheless, the actual mechanisms that initiate the inflammatory processes in joint tissues are poorly understood.

1.1 Etiology of OA

Risk factors of OA are well documented (Table 1), and they vary between different joints to some extent. The risk factors of OA can be divided into local and systemic factors. Ageing, female gender and genetic factors are considered as systemic factors, whereas obesity, previous joint injury, congenital abnormalities, malalignment and extreme physical loading of the joint are considered as local risk factors. The fact that obesity is a risk factor, not only for OA of weight bearing joints, but also for hand OA (Haara et al. 2003; Yusuf et al. 2010; Kloppenburg and Kwok 2011), suggests that obesity is not only a local but also a systemic risk factor for OA. Thus, it is likely that there exists some yet unrecognized metabolic factor(s) that would mediate the effect of obesity, or the fat stored in the adipose tissue, on cartilage.

Table 1 Risk factors of OA

knee OA	hip OA	hand OA
age	age	age
obesity	obesity	obesity
female gender		female gender
previous injury	previous injury	previous injury
occupational activity*	occupational activity*	occupational activity*
high intensity / competitive sports	high intensity / competitive sports	
genetic factors	genetic factors	genetic factors
malalignment	congenital abnormalities	
meniscectomy		

Modified from Osteoarthritis of knee and hip: Current Care Guidelines Abstract 2012 and from Waris et al. 2012. *high exposure to knee bending, lifting heavy items or frequent hand-straining tasks.

1.2 Clinical presentation and diagnosis of OA

The joints most frequently affected by OA are the knee, hip, hand interphalangeal and carpometacarpal I joints, as well as the spine (Moskowitz et al. 2007). Bilateral manifestation of the disease is common although the onset of the disease might be unilateral and the symptoms on one side progress ahead of the other side.

Pain is the leading symptom of OA. Other symptoms are stiffness and limitations in extension of movement of the joint. As the disease progresses, the changes in the cartilage and bone can eventually lead to joint deformations and subluxations. The diagnosis of OA is based on symptoms and radiographs of the symptomized joints. The Finnish Current Care Guidelines (Knee and hip osteoarthritis: Current Care Guidelines Abstract, 2014) recommends clinical and radiographic criteria of American College of Rheumatology (Figure 1) to be used in the diagnostics of knee and hip OA.

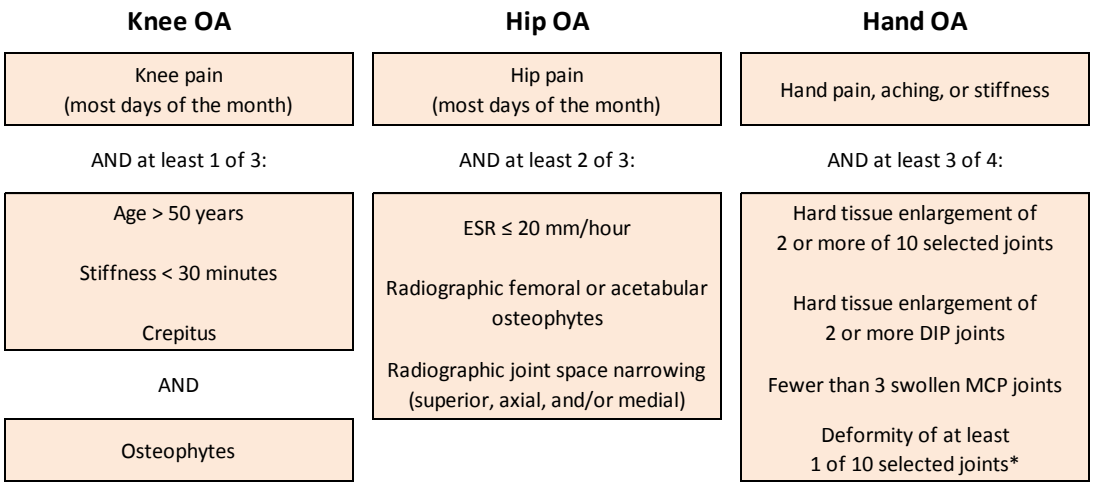

Figure 1 American College of Rheumatology diagnosis criteria for osteoarthritis of knee (Altman et al. 1986), hip (Altman et al. 1991) and hand (Altman et al. 1990). *The 10 selected joints are the second and the third distal interphalangeal, the second and third proximal interphalangea and the first carpometacarpal joints of both hands; MCP, metacarpophalangeal; ESR, erythrocyte sedimentation rate.

1.2.1 Radiographic findings in osteoarthritis

Joint space narrowing in the radiograph refers to loss of articular cartilage as cartilage is not visualized by X-rays. Other radiographic changes that can be seen in conventional radiographs of osteoarthritic joint include osteophyte formation, subchondral sclerosis, bone resorption, subchondral cysts and malalignment of joints (Figure 2). Radiographic findings are evident in progressed disease with remarkable loss of cartilage and related changes, but in early OA the structural changes are not necessarily visualized by conventional radiographs.

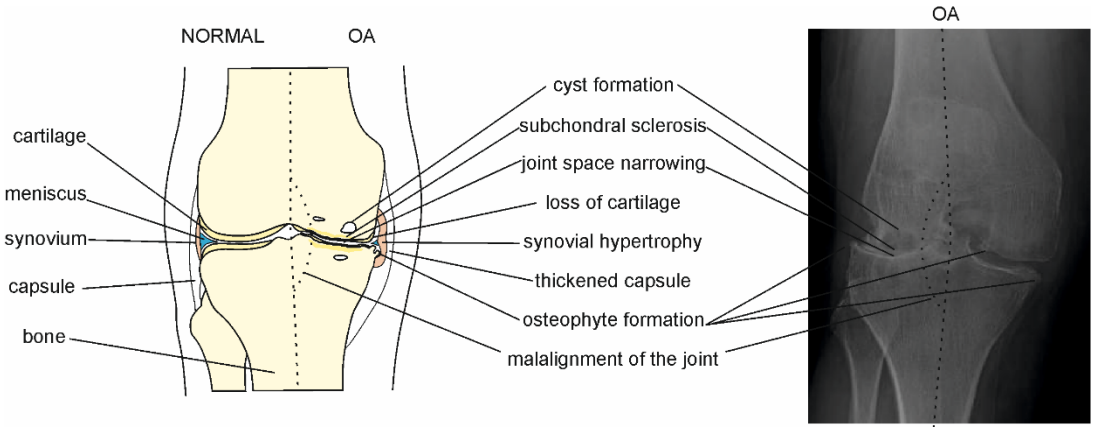

Figure 2 Schematic representation and an example of a conventional radiograph showing macroscopic changes caused by OA in a knee joint.

Different radiographic scales have been used to evaluate the severity of radiographic changes relative to OA. The most widely used scaling system is the Kellgren and Lawrence system (Kellgren and Lawrence 1957); this scale is also recommended in the Finnish Current Care Guidelines to be used in the diagnostics of knee and hip OA. Another scaling system, Ahlbäck grading, is based on five grades by joint space narrowing and subchondral changes (Ahlback 1968). The criteria of both scaling systems are presented in Table 2.

Table 2 Radiographic classification of knee OA by the Ahlbäck classification and the Kellgren and Lawrence grading scale

	grade	definition
Ahlbäck		
	I	joint space narrowing
	II	joint space obliteration
	III	minor bone attrition
	IV	moderate bone attrition
	V	severe bone attrition
Kellgren & Lawrence		
	1	doubtful narrowing of joint space and possible osteophytic lipping
	2	definite osteophytes, definite narrowing of joint space
	3	moderate multiple osteophytes, definite narrowing of joint space, some sclerosis and possible deformity of bone contour
	4	large osteophytes, marked narrowing of joint space, severe sclerosis and definite deformity of bone contour

Radiographic changes and symptoms do not correlate well with each other. This has been explained by the fact that cartilage is not innervated, and pain occurs only after bone and synovium are affected as well as in the case of joint effusion. Further, the individual sensation of pain is also affected by changes in the neuronal pain tract and by psychosocial factors (Moskowitz et al. 2007; Glyn-Jones et al. 2015). Magnetic resonance imaging (MRI) can reveal OA-related changes during an earlier phase and it represents a more accurate way to measure structural changes of the joint than conventional radiographs. However, at the present, there is rarely a need for MRI in clinical work as the decisions concerning treatment with the current treatment possibilities can be made based on symptoms and conventional radiographs.

1.3 Treatment of OA

Current treatment possibilities of OA, both non-pharmacological and pharmacological, according to Finnish Current Care Guidelines, are shown in Figure 3. These guidelines are in line with the recommendations issued by the American College of Rheumatology (ACR) (Hochberg et al. 2012) and the Osteoarthritis Research Society International (OARSI) (McAlindon et al. 2014). To date, no (undisputably proven) disease progression slowing medical therapeutics for OA are available. The disease-related pain is treated with analgesics in an on-demand based manner. Primarily, acetaminophen (paracetamol) and nonsteroidal anti-inflammatory drugs (NSAIDs) are used, and complemented with mild opioids when the first line medication is no longer sufficient. Topical NSAIDs may also be used as first-line medication for hand and knee OA (Hochberg et al. 2012; McAlindon et al. 2014). Intra-articular glucocorticoid injections have been quite generally used in the treatment of exacerbation of joint inflammation and joint pain in OA. They tend to alleviate pain for 2-3 weeks, but have not been shown to slow down the development of structural changes of joints (Raynauld et al. 2003; McAlindon et al. 2014).

Intra-articularly administered hyaluronan (HA) containing products have been on the market for about a decade. Their effect on pain has been demonstrated as either slightly better or no better than placebo in different studies (Rutjes et al. 2012; McAlindon et al. 2014). However, there is no evidence of their efficacy on structural changes. Other pharmaceuticals that might be useful in treating OA-related pain and that are recommended by the international guidelines include oral duloxetine (multiple-joint OA with chronical pain)(McAlindon et al. 2014) and topical capsaicin (Hochberg et al. 2012; McAlindon et al. 2014). Other products on the market include orally administered glucosamine and chondroitin sulphate. The existing research data and recommendations do not encourage their use in the treatment of OA (Hochberg et al. 2012; McAlindon et al. 2014).

Joint replacement surgery reduces OA-related pain and improves ability to function in end-stage OA (Knee and hip osteoarthritis: Current Care Guidelines Abstract, 2014). However, with respect to possible complications, surgery is considered only after the other treatment options have become insufficient, in practice meaning progressed disease that is accompanied with significant pain and disability. Joint replacement surgery also requires rather long recovery times and large financial

resources, even though, it has proven to be cost effective (Heliövaara et al. 2008). Other surgical methods include arthrodeses (small joints) and osteotomies. Arthroscopic debridement or lavage procedures have been demonstrated not to be advantageous in the treatment of knee OA, although previously widely used (Zhang et al. 2010a). Weight reduction has been recommended for overweight/ obese patients with knee/hip OA as it has been shown to decrease pain and disability in these individuals (Zhang et al. 2010a; Atukorala et al. 2016).

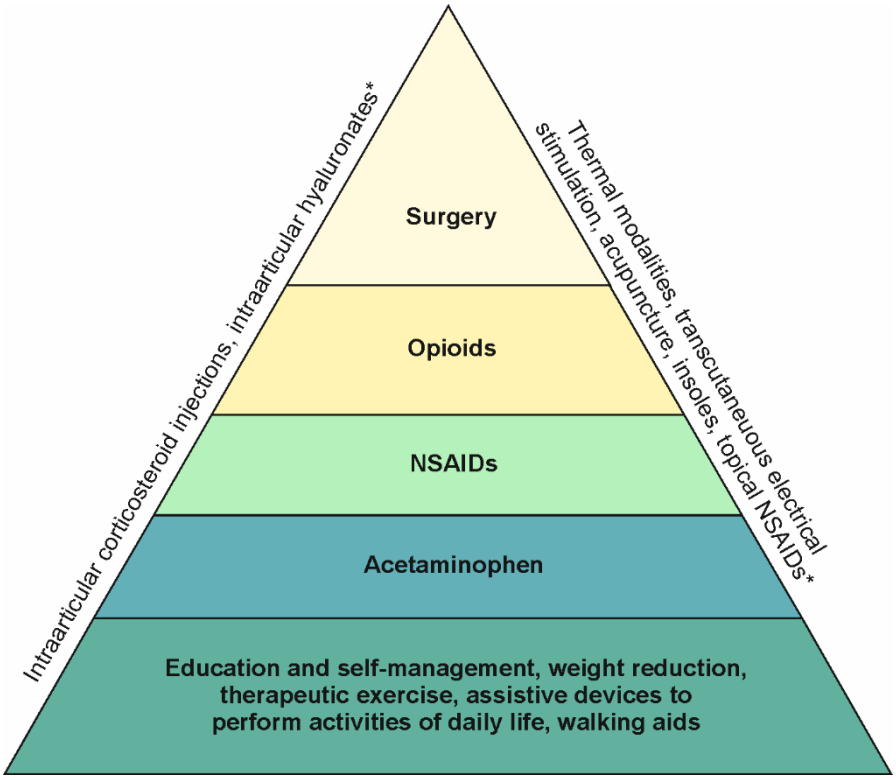

Figure 3 Treatment options of knee and hip OA according to the Finnish Current Care Guidelines. Modified from Knee and hip osteoarthritis: Current Care Guidelines Abstract 2014. *Recommended only for knee OA; NSDAIDs, nonsteroidal anti-inflammatory drugs.

1.4 Overview of cartilage composition and joint anatomy

Hyaline cartilage covers the bony ends in synovial joints. Cartilage provides a low friction surface for the joint; it participates in the lubrication of the joint and distributes the applied forces to the underlying bone (Ross et al. 2003). Hyaline cartilage consists of chondrocytes, the only cell type present in cartilage, and the extracellular matrix (ECM). There are macromolecules in ECM including collagens and components of HA proteoglycan aggregates. Collagens, as well as water that is

bound by proteoglycans, confer on cartilage its viscoelastic properties and tensile strength. (Ross et al. 2003; Pearle et al. 2005; Moskowitz et al. 2007)

Chondrocytes comprise only 1-3% of the total cartilage volume. Chondrocytes in adult cartilage are normally quiescent and maintain the matrix in a low turnover state by producing the structural components of ECM and matrix-degrading proteases. Cartilage has a limited capacity to repair, but under normal circumstances, it maintains its structure throughout the lifespan. (Ross et al. 2003; Moskowitz et al. 2007; Aigner et al. 2007; Goldring 2012)

Articular cartilage is organized as a layered structure so that the organization and composition of macromolecules and cells is different in the distinct zones. In the superficial zone, collagen fibrils are densely aligned parallel to the articular surface and the chondrocytes have an elongated appearance and express relatively high amounts of lubricating proteins but relatively little proteoglycan. In the middle zone, which encompasses most of the cartilage thickness, the collagen fibers are less organized in a rather oblique arrangement. These collagen fibers in the middle zone are thicker and more loosely packed. The deep zone contains the highest amount of proteoglycan, and in this zone the collagen fibers are oriented perpendicularly to the articular surface. (Pearle et al. 2005; Moskowitz et al. 2007)

The main type of collagen in cartilage is type II collagen. There are also other fibrillar collagen types, such as IX, X and XI collagen molecules; these are all considered as cartilage-specific collagen molecules, since they are found in significant amounts only in the cartilage matrix. In addition, non-fibrillar collagen VI is found in cartilage, and it is thought to help to attach cells to the matrix framework. Intra- and inter-molecular crosslinking of collagen fibrils serves to stabilize the matrix. (Ross et al. 2003; Pearle et al. 2005; Moskowitz et al. 2007)

Aggrecan, the major proteoglycan of cartilage is organized into large molecules, HA proteoglycan aggregates, consisting of HA and aggrecan molecules. Aggrecans are bottle brush-like molecules that consist of an aggrecan core protein binding many chondroitin sulphate and keratan sulphate glycosaminoglycan chains. Glycosaminoglycans are long polysaccharides composed of repeating disaccharide units. Proteoglycan binds water because of its negative charge. Cartilage consists between 60-80% of water. The water content allows diffusion of nutrients and other molecules to and from chondrocytes in the avascular cartilage, and movement of

water molecules allows the matrix to response to varying pressure loads. (Ross et al. 2003; Moskowitz et al. 2007)

In addition, cartilage contains a large number of other proteins and proteoglycans that have different functions in maintaining the assembly of the cartilage. These include link protein, thrombospondins, matrilins, cartilage intermediate layer protein, leucine-rich repeat proteins (such as decorin, biglycan and fibromodulin), tenascin-C, fibulin, fibrillin and fibronectin (Moskowitz et al. 2007). Cartilage oligomeric matrix protein (COMP) is a member of the thrombospondin family and one of the major proteins in cartilage. COMP binds to different ECM components and interacts with growth factors, and it is thought to be important in cell attachment, proliferation and differentiation (Acharya et al. 2014).

The joint capsule surrounds the hyaline cartilage-covered bone ends in the synovial joint. The joint capsule consists of an inner, thin synovial membrane and an outer strong fibrous membrane that is formed of dense connective tissue. The highly vascular and innervated synovial membrane, together with chondrocytes, release synovial fluid into the joint cavity inside the capsule. Normally, there is small volume of synovial fluid in the joint cavity. Synovial fluid is a filtrate of plasma that contains HA and many other molecules produced by synoviocytes and chondrocytes. The synovial fluid lubricates the articular surfaces and nourishes the avascular cartilage. (Moskowitz et al. 2007)

The synovial membrane is attached to the margins of the articular surfaces and also to the margins of some additional structures inside the joint cavity. These include fibrocartilaginous articular discs present, for example, in mandibular joint, menisci of the knee joints and labrum encircling glenoid in shoulder, and acetabulum in hip joint. The synovial membrane lines certain ligaments (e.g., cruciate ligaments in the knee joint, tendon of long head of biceps brachii muscle in the glenohumeral joint and the ligament of the femoral head) and fat pads (e.g., the infrapatellar fat pad located at the anterior of the knee joint and the fat pad located in acetabular fossa in the hip joint) that are located inside the fibrous capsule. (Moore and Dalley 1999; Drake et al. 2010)

1.5 Pathogenesis of OA

1.5.1 Structural changes in cartilage and joint tissues

The first changes in OA cartilage are seen as damage in the collagen network of the ECM, and as a reduction of its proteoglycan content and composition. Proteoglycan loses its level of aggregation (the "bottle brushes" become unbound) and the chain length of proteoglycans is reduced due to proteolytic degradation. These alterations compromise the formation of macromolecular complexes, resulting in reduced compressive stiffness of the tissue. The loss of proteoglycan aggrecation and damage of the collagen network can be observed first in the superficial zone as softening of the cartilage. In the second phase, the chondrocytes attempt to repair the damaged cartilage structure by synthesizing new matrix components. At the same time, the degradation of the matrix becomes accelerated. Finally, the synthetic activity of chondrocytes is reduced and the contents of the ECM decline, e.g., there is degradation and loss of type II collagen. Macroscopically, the degradation of ECM is seen as decreased thickness, fibrillation and ulceration of the cartilage (Figure 2) (Pearle et al. 2005; Moskowitz et al. 2007). It is believed that the loss of aggrecan occurs before collagen degradation, and that aggrecan loss can be reversed whereas collagen degradation is irreversible (Fosang and Beier 2011; Troeberg and Nagase 2012).

The changes in the subchondral bone are seen as thickening and stiffening, and are due to increased osteoblast activity. As a consequence of increased bone turnover, the vascularity of subchondral bone increases and cyst formation and resorption of bone are also commonly present in the progressed disease (Figure 2). Osteophytes are formed as a consequence of the proliferation of periosteal cells at the joint margin and at the insertion of ligaments and tendons. Osteophytes are formed from fibrocartilage; they can be seen in X-ray only after they have undergone endochondral ossification to form bony outgrowing structures. (Goldring and Goldring 2010)

The joint capsule is affected by synovitis which is characterized by hyperplasia of the synovial membrane, infiltration of macrophages and lymphocytes, and increased vascularity. Synovial inflammatory infiltrates might occur even in early stage OA. However, the prevalence of synovitis increases as the disease advances (Loeser et al. 2012).

Pathological changes in ligaments and other fibrous structures, including menisci of knee joints, are also common in OA. The same kind of disruption of connective tissue is seen in these structures as is seen in articular cartilage. In addition, the penetration of vasculature and nerves has been noted in the menisci of OA-affected knee joints.

In secondary OA, an injury in the cartilage, menisci or cruciate ligaments is thought to initiate the OA process (Loeser et al. 2012). Previously meniscal tears were treated by meniscectomy. However that procedure led to premature OA, and total meniscectomy is no longer recommended (Moskowitz et al. 2007).

1.5.2 Mediators of inflammation and cartilage degradation

Inflammatory processes in joint tissues are a central feature in the pathogenesis of OA. It can be observed as increased expression of proinflammatory cytokines and catabolic enzymes by chondrocytes, and as elevated concentrations of these factors in synovial fluid (Beekhuizen et al. 2013; Tsuchida et al. 2014). The clinical manifestation of inflammation related to OA is generally not very intense, meaning that the classical signs of inflammation, including rubor, tumor, calor, dolor and functio laesa – redness, swelling, heat, pain and impaired function, are not regularly or necessarily present. This is thought to be related to the avascular and aneural nature of cartilage, as OA is primarily a disease of the articular cartilage that secondarily affects subchondral bone and synovial membrane (Konttinen et al. 2012).

Proinflammatory cytokines increase the production of catabolic enzymes and downregulate the synthesis of ECM components in chondrocytes. Chondrocytes are thought to be the crucial cells that produce inflammatory and catabolic factors in OA. However, cytokines and catabolic enzymes can be released also by synovial cells, and by macrophages and lymphocytes infiltrated into the synovium and synovial fluid, as well as being released by bone (Berenbaum 2013).

In normal cartilage, chondrocytes maintain cartilage matrix so that the synthesis and degradation of ECM is in equilibrium at a low turnover state (Cawston 1996). In OA, chondrocytes are thought to undergo a phenotypic shift to hypertrophic OA chondrocytes (Berenbaum 2013), to produce increased amounts of proinflammatory mediators, and catabolic factors in response to proinflammatory stimuli. Anabolic

activity is also increased in chondrocytes in patients with OA, but the overall balance, particularly in advanced OA, lies on the catabolic side (Aigner et al. 2007).

The molecular events that initiate the inflammatory process in the cartilage or in other joint tissues and lead to the phenotypic shift of chondrocytes are incompletely understood. Activation of toll-like receptors (TLRs) by endogenous damage-associated molecular patterns (DAMPS) is thought to be one mechanism contributing to the increased production of cytokines and chemokines by chondrocytes and synovial fibroblasts (Konttinen et al. 2012; Scanzello and Goldring 2012). The endogenous ligands of TLRs include degraded cartilage fragments, such as fibronectin isoforms, low molecular weight species of HA, biglycan and tenascin C (Konttinen et al. 2012; Scanzello and Goldring 2012). In addition, complement activation has been implicated in the initiation of OA (Scanzello and Goldring 2012; Glyn-Jones et al. 2015).

The etiology of OA is multidimensional and it is possible that different stimuli, including tissue degradation due to trauma or mechanical stress, altered chondrocyte metabolism due to hormonal or metabolic mediators, or changes associated with ageing, might contribute to, or lead independently to the same kind of end result: inflammation and consequential loss of ECM and destruction of cartilage. The inflammatory process including upregulated production of cytokines and cartilage degrading enzymes is thought to be involved in the pathogenesis, irrespective of the initiating event. The next section describes some of the inflammatory mediators considered to be important in the pathogenesis of OA.

1.5.2.1 Proinflammatory mediators

Interleukin-1β (IL-1β) is a proinflammatory cytokine that is considered to be a central mediator in the pathophysiology of OA and in other inflammatory joint diseases. It was shown in the eighties that a protein called ‘catabolin’ that was extracted from conditioned media of cultured pig mononuclear cells (Saklatvala et al. 1983), inhibited the synthesis of proteoglycans (Tyler 1985a) and stimulated proteoglycan release (Tyler 1985b) in pig cartilage. Later it was confirmed that this protein was IL-1. IL-1β increases the production of MMPs, IL-6, IL-8, NO and cyclooxygenase-2 (COX-2), and suppresses synthesis of proteoglycans and type II

collagen in chondrocytes (Kapoor et al. 2011; Haseeb and Haqqi 2013). IL-1β has also been attributed to cause apoptosis of chondrocytes (Haseeb and Haqqi 2013).

IL-1β transduces its effects through cell membrane-located receptor IL-1R1. Another receptor, IL-1R2 also binds IL-1β, but is unable to transduce a signal. IL-1 receptor antagonist (IL-1Ra) is a natural inhibitor of IL-1β that binds to both IL-1R1 and IL-1R2 without transducing a signal. (Kapoor et al. 2011)

Even though the proinflammatory and catabolic effects of IL-1β on cartilage are undisputable, the levels of IL-1β are not consistently elevated or detectable in all OA patients (Scanzello and Goldring 2012; Chevalier et al. 2013; Beekhuizen et al. 2013; Tsuchida et al. 2014). Anti-IL-1β therapy via recombinant IL-IRa has been shown to inhibit the structural changes associated with OA in animal models, but unfortunately, in human studies only minimal symptom-reducing efficacy was achieved (Kapoor et al. 2011; Scanzello and Goldring 2012; Chevalier et al. 2013). Considering the lack of efficacy of anti-IL-1β therapy and the inconsistently elevated IL-1β levels is synovial fluid in OA patients, questions have been raised about the critical importance of this cytokine in the pathogenesis of OA, at least in all subtypes of the disease (Scanzello and Goldring 2012; Chevalier et al. 2013).

Tumor necrosis factor-α (TNF-α) is thought to be another cytokine of major importance in the pathogenesis of OA. Similar to IL-1β, TNF-α is elevated in the synovial fluid in some, but not in all, OA patients (Sauerschnig et al. 2014; Tsuchida et al. 2014). TNF-α exerts clear effects on increasing the production of catabolic enzymes and proinflammatory factors. It also downregulates the production of proteoglycans and type II collagen. (Kapoor et al. 2011; Haseeb and Haqqi 2013)

TNF-α mediates its effects through two cell membrane-located receptors, TNFR1 and TNFR2, of which TNFR1 expression is known to be increased in OA chondrocytes and synovial fibroblasts (Kapoor et al. 2011). The efficacy of TNF blockers has revolutionized the treatment of RA, however, the results in the treatment of OA in the clinical studies have been disappointing (Kapoor et al. 2011; Chevalier et al. 2013; Chevalier et al. 2014).

Interleukin-6 (IL-6) is a proinflammatory cytokine known to be involved in inflammation in rheumatic diseases. IL-6, like IL-1β, is an important factor in inducing hepatic acute-phase reaction in inflammatory conditions related to tissue

damage, including bacterial infection and chronic inflammatory diseases, such as rheumatoid arthritis. IL-6 binds to its receptor IL-6R on cell membrane, and intracellular signaling is initiated as the IL-6-IL6R complex associates with a transmembrane protein gp130 which then dimerizes and initiates intracellular signaling (Calabrese and Rose-John 2014). IL-6R occurs also in soluble form (sIL-6R) that binds IL-6 with the same affinity as cell membrane located IL-6R (Calabrese and Rose-John 2014). IL-6-sIL-6R complex is able to start intracellular signaling through association with ubiquitously expressed gp130 in cells which do not express the membrane located IL-6R (Calabrese and Rose-John 2014).

The levels of IL-6 and its soluble receptor sIL-6R have been reported to be elevated in synovial fluid and serum in OA patients (Beekhuizen et al. 2013; Kapoor et al. 2011; Tsuchida et al. 2014), and they are associated with joint effusion, arthroscopic synovitis and joint degeneration (Pearle et al. 2005). IL-6 is produced by chondrocytes, synoviocytes, macrophages, T cells and osteophytes in OA joints (Chevalier et al. 2013). In chondrocytes, IL-6 production is known to be increased by a number of cytokines and growth factors, including IL-1β, TNF-α, type II collagen and prostaglandin E_2 (PGE_2) (Kapoor et al. 2011). IL-6 has been shown to upregulate the production of MMP-1 and MMP-13 in bovine cartilage in combination with IL-1β or oncostatin M (Haseeb and Haqqi 2013; Kapoor et al. 2011). IL-6 also increases the number of inflammatory cells in synovial tissue (Pearle et al. 2005) and it has been reported to downregulate collagen type II synthesis in chondrocytes (Haseeb and Haqqi 2013; Kapoor et al. 2011). In RA, IL-6 levels correlate with disease activity (Alten and Maleitzke 2013). The biological anti-IL-6 drug, IL-6 receptor antibody tocilizumab, that is indicated for the treatment of RA since 2011 (in USA), has proven to be effective in the treatment for RA and also in some other rheumatic diseases (Alten and Maleitzke 2013). High circulating levels of IL-6 together with high BMI was reported to predict development of radiographic knee OA, and IL-6 also correlated positively with the radiographic scaling of OA in a prospective study (Livshits et al. 2009). To date, anti-IL-6 therapy has not been studied in the treatment of OA in clinical trials or in animal models.

Nitric oxide (NO) is a gaseous molecule that acts as a mediator in many physiological functions in mammals and is also involved in pathological processes. The physiological functions of NO include regulation of vasodilatation,

neurotransmission, thrombocyte aggregation and immune response. The vasodilating effect of NO is utilized in drugs indicated for pulmonary hypertension, angina pectoris and erectile dysfunction. In response to infections, NO exerts cytotoxic effects against microorganisms. Unreasonably high production of NO can also cause tissue destruction, for example in inflammatory diseases. In addition to the toxic effects, reactive nitrogen species and their end products are also able to modify protein activity, including activation of COX-2 and MMPs. Most of the physiological actions of NO, as well as the intense vasodilatation in septic shock, result from activation of soluble guanylate cyclase (sGC) by direct binding of NO to the ferrous iron (Fe2+) of sGC, and the subsequent elevation in intracellular cyclic guanylate mono phosphate levels. (Vuolteenaho et al. 2007; Abramson 2008)

NO is derived from L-arginine and oxygen in the reaction catalyzed by nitric oxide synthase (NOS) enzymes. Constitutive NOS is responsible for production of NO in physiological functions and includes two subtypes, epithelial NOS and neuronal NOS. Enhanced NO production in inflammation depends on the expression of inducible NOS (iNOS). iNOS is not present normally in resting cells, but it is readily synthesized due to inflammatory stimuli, like lipopolysaccharide (LPS) or proinflammatory cytokines. The transcription of iNOS is induced through intracellular signaling that includes activation of nuclear factor kappa B (NF-κB) or/and janus kinase/signal transducer and activation of transcription (JAK/STAT) pathway (Vuolteenaho et al. 2007). iNOS is expressed in various cell types, such as macrophages, epithelial cells, hepatocytes, smooth muscle cells and chondrocytes. Chondrocytes from osteoarthritic cartilage spontaneously express iNOS and produce NO (Vuolteenaho et al. 2001). NO production in chondrocytes is further increased by IL-1β, TNF-α, LPS (Vuolteenaho et al. 2001) and interferon-γ (IFN-γ) (Henrotin et al. 2000). It has been shown that certain NF-κB enhancer elements in iNOS gene are demethylated in OA chondrocytes (de Andres et al. 2013), possibly explaining the spontaneously expressed iNOS by epigenetically regulated mechanisms in OA. Furthermore, post-transcriptional mechanisms can influence iNOS expression. For example, reduced iNOS mRNA stability has been observed after treatment with transforming growth factor β (TGF-β), dexamethasone, intracellular calcium elevating agents and mitogen-activated protein kinase (MAPK) p38 inhibitor SB220025 (Vuolteenaho et al. 2007). Protein kinase C (PKC) and c-Jun N-terminal

kinase (JNK) pathways have been implicated in the regulation of iNOS mRNA (Vuolteenaho et al. 2007).

In OA, there are several pathogenic mechanisms to account for NO's properties to promote cartilage destruction. NO has been shown to activate MMPs (Murrell et al. 1995) and COX, to inhibit synthesis of proteoglycans and type II collagen, and to promote chondrocyte apoptosis (Scher et al. 2007; Vuolteenaho et al. 2007). NO (through the formation of peroxynitrite) has also been shown to sustain the activation of NF-κB, and subsequently promoting actions of proinflammatory cytokines (Clancy et al. 2004).

In animal models of OA, inhibitors of iNOS have shown promising results indicating that iNOS inhibitors could be capable of slowing down the structural changes related to OA (Vuolteenaho et al. 2007; Scher et al. 2007). However, a placebo-controlled trial that tested the effect of a selective iNOS inhibitor, cindunistat hydrochloride maleate (SD-6010), failed to show efficacy of this drug on joint space narrowing during a follow-up lasting two years (Hellio le Graverand et al. 2013). It remains an unanswered question whether iNOS inhibitors could prevent cartilage destruction, for example, in an earlier phase of OA, in a longer follow-up, in a selected group of patients, or perhaps in combination with other pharmacological treatments.

Prostaglandin E_2 (PGE_2) is considered to be a major contributor to inflammatory pain in acute inflammation and in arthritic conditions (Lee et al. 2013). PGE_2 is synthesized in a process where arachidonic acid is first converted to prostaglandin endoperoxide, prostaglandin H_2 (PGH_2) by COX enzymes, and PGH_2 is then converted to PGE_2 by prostaglandin E synthases (PGES). The COX enzymes exist in two subtypes of which COX-1 is responsible for physiological prostanoid production, and COX-2 is highly expressed in inflammation. PGES is known to be present in three subtypes, of which microsomal PGES-1 is induced by inflammatory factors and thought to be functionally related to COX-2. Synthesis of these two enzymes, followed by PGE_2 production, can be induced by pro-inflammatory cytokines, growth factors or endotoxin (Korotkova and Jakobsson 2014). Chondrocytes from OA cartilage express high levels of COX-2, microsomal PGES-1, and subsequently produce PGE_2 (Korotkova and Jakobsson 2014; Tuure et al. 2015). PGE_2 production by cartilage has been related to enhanced MMP-3, MMP-13 and a disintegrin and metalloproteinase with thrombospondin motifs-5 (ADAMTS-5), also known as

aggrecanase, expression and decreased synthesis of proteoglycans and type II collagen (Abramson and Attur 2009; Lee et al. 2013). PGE_2 has also been shown to have direct effects on chondrocytes by enhancing IL-6 and iNOS expression when administered together with IL-1β (Lee et al. 2013).

1.5.2.2 Proteinases

Proteolytic enzymes are the direct source of tissue degradation in OA. There are four distinct types of proteolytic enzymes in cartilage, namely cysteine, aspartate, serine and metalloproteinases; the first two of these act intracellularly and the latter two extracellularly. These proteinases have functions in cartilage development and ECM remodeling, however, many of them are produced in increased amounts and contribute to cartilage degradation in OA. (Cawston and Wilson 2006; Moskowitz et al. 2007)

Matrix metalloproteinases (MMPs) and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTSs) belong to the family of metalloproteinases. They are named metalloproteinases because they bind a metal ion, usually zinc, in their catalytic site. They are involved in the normal turnover of connective tissue including in reproduction, angiogenesis and bone turnover, as well as in pathological conditions, such as wound healing, tumor growth and metastasis, atherosclerosis and cartilage and bone destruction. These enzymes cleave the macromolecules of cartilage ECM at specific sites. (Cawston and Wilson 2006)

The most importantly considered proteinases in OA pathogenesis include collagenases MMP-1, MMP-8 and MMP-13 (collagenase-1, -2 and -3) that are primarily involved in type II collagen degradation, and stromelysin-1 (MMP-3) that degrades aggrecan, but is also involved in collagen and fibronectin degradation and in activating pro-collagenases (Figure 4). The expression of these MMPs is low in normal cells whereas OA chondrocytes express increased amounts of these enzymes (Tetlow et al. 2001). The production of these MMPs is known to be upregulated by proinflammatory cytokines found in OA and RA joints, such as IL-1, TNF-α and IL-17. In addition, fragments of the ECM macromolecules can induce MMP production in chondrocytes. (Ishiguro et al. 2002; Pearle et al. 2005; Cawston and Wilson 2006; Loeser et al. 2012) ADAMTS-4 and ADAMTS-5 have been more recently found to

be important in the cleavage of aggrecan in animal models. Their role in OA in humans is not thoroughly elucidated (Troeberg and Nagase 2012).

Overproduction of MMPs is the main mechanism related to cartilage degradation in OA (Baici et al. 2005). In addition to increased synthesis, the actions of metalloproteinases are also regulated through activation and inhibition of these enzymes. Metalloproteinases are produced as inactive proenzymes that can be activated by proteolytic removal of their pro-peptides, either intracellularly or extracellularly. The pro-peptide contains a conserved cysteine residue that interacts with the zinc in the active site of MMPs and prevents binding and cleavage of the substrate. However, the activators of MMPs are not fully known. Certain chondrocyte-derived serine proteinases including matriptase-1 and activated protein C have been shown to activate pro-MMP-1, -2, -3 and -9. Whereas MMP-2 and MMP-3 are known to activate other pro-MMPs (Fosang and Beier 2011; Troeberg and Nagase 2012). The aggrecanases, ADAMTS-4 and ADAMTS-5, are directly activated by serine protease protein convertase family members (Fosang and Beier 2011).

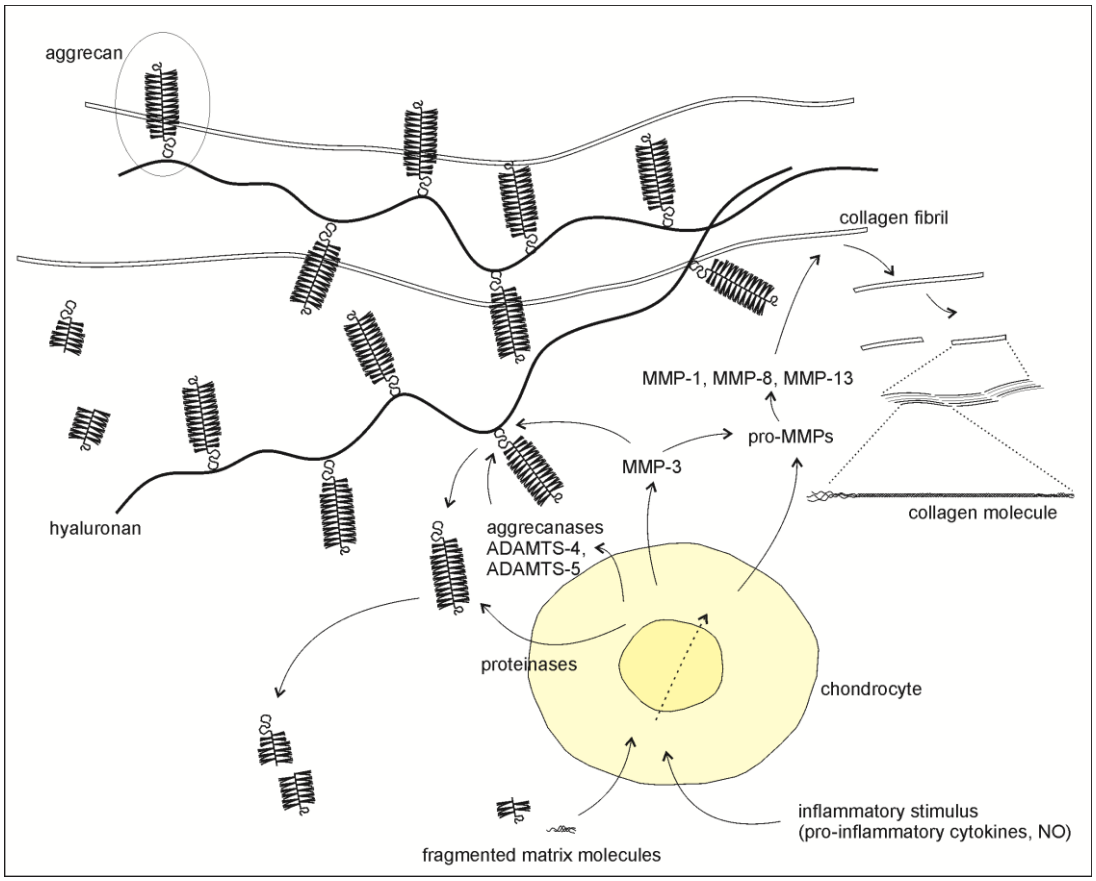

Figure 4 Schematic presentation of macromolecules of articular cartilage and proinflammatory stimulus-driven proteolytic effects of metalloproteinases on extracellular matrix. MMP, matrix metalloproteinase; ADAMTS, a disintegrin and metalloproteinase with thrombospondin motifs; NO, nitric oxide.

Active MMPs and ADAMTs are inhibited by endogenously produced tissue inhibitors of metalloproteinases (TIMPs) including TIMP-1, -2, -3 and -4. MMPs are inhibited by all TIMPs whereas ADAMTS-4 and -5 are mainly inhibited by TIMP-3 (Cawston and Wilson 2006; Troeberg and Nagase 2012).

MMP-1 and MMP-13 are produced by several cell types in the joint, including chondrocytes, synovial cells and white blood cells. The concentration of MMP-13 in synovial fluid is usually lower than that of the other MMPs. However, it is thought that MMP-13 hydrolyzes type II collagen more efficiently than other collagenases. MMP-3 is expressed by many cell types and its concentrations are among the highest of MMPs in synovial fluid. MMP-8 is thought to be mainly released from stimulated neutrophils, however, also chondrocytes have been shown to express MMP-8. (Cawston and Wilson 2006; Vincenti and Brinckerhoff 2002)

Since MMPs are responsible for the ECM break-down in OA, they are seemingly an attractive target for pharmacological intervention. Unfortunately, despite the

promising results emerging from work done in animal models, inhibitors of MMPs have failed to achieve the promised results in human studies and, furthermore the use of MMP inhibitors has been associated with adverse musculoskeletal events (Cawston and Wilson 2006; Kapoor et al. 2011).

1.6 Biomarkers of OA

Because there are limitations with respect to the use of plain radiographs in the detection of early changes in OA and in the short-term follow-up of the disease, attempts have been made to develop/find biomarkers. Useful biomarkers of OA could help to provide a way to objectively measure progression of the disease, to detect early structural changes in the joint or to predict prognosis or a response to some treatment. To date, biomarkers of OA are not in the clinical use. At the moment, there is a demand for biomarkers that could be used as surrogate endpoint measures instead of radiographic data or symptom questionnaires in clinical trials. (Kraus et al. 2011)

A number of molecules have been studied as potential biomarkers of OA; two of them are presented below as they were measured in the present study, namely cartilage oligomeric matrix protein (COMP) and MMP-3. Both of these compounds can be measured in the circulation and have been shown to be associated with radiographic severity of knee OA. These biomarkers possibly reflect the actual disease activity whereas radiographic data illustrate the cumulative joint destruction. However, it has to be noted that the ability of a systemic biomarker to measure disease activity of one particular joint is confounded by possible OA in other joints.

1.6.1 COMP

Cartilage oligomeric matrix protein (COMP), also known as thrombospondin 5 is a 524 kDa glycoprotein that is abundantly expressed in cartilage. Its function is thought to be a stabilizer of the fibre network and to facilitate fibrillogenesis (Jordan 2004; Garvican et al. 2010). COMP is also thought be important in chondrocyte proliferation and differentiation (Acharya et al. 2014), but it can also activate the alternative complement pathway (Happonen et al. 2010). Apart from cartilage, it is also present in synovium, and in menisci (Jordan 2004; Garvican et al. 2010)

In osteoarthritic cartilage, there is a lower concentration of COMP when compared to healthy cartilage (Acharya et al. 2014) whereas the circulating COMP level is higher in individuals with OA than in those without OA (Jordan 2004). Circulating COMP has been reported to predict incidence (Petersson et al. 1998) and progression (Vilim et al. 2002) of knee OA. It has also been reported to associate with radiographic severity of OA in cross-sectional (Clark et al. 1999) and in longitudinal (Sharif et al. 2004) studies and it also associates with synovitis (Vilim et al. 2001). Other factors that are reported to affect COMP levels include age and hormone replacement therapy (Jordan 2004).

1.6.2 MMP-3

MMP-3, when measured in the circulation, can be used as a biomarker of OA. The concentration of MMP-3 has been shown to be higher in the circulation of patients with OA compared to control subjects (Manicourt et al. 1994). It has been reported to predict joint space narrowing in patients with knee OA (Lohmander et al. 2005) and its level was also higher in patients with rapidly progressing hip OA compared with patients with averagely progressing disease, all undergoing total hip arthroplasty (Masuhara et al. 2002).

MMP-3 is also released from skeletal muscle. Apparently, regular physical training in athletes does not significantly increase MMP-3 levels (Roos et al. 1995), whereas running a marathon was found to double the circulating concentration of MMP-3 (Vuolteenaho et al. 2014). MMP-3 has also been found to correlate with age, and it has been reported to be higher in males than females (Manicourt et al. 1994).

1.7 Obesity as a risk factor of osteoarthritis

Obesity is an increasing health problem in the developed countries (NCD Risk Factor Collaboration (NCD-RisC) 2016). Approximately 70% of men and 57% of women of the Finnish population aged 25 to 74 years are overweight or obese (BMI > 25 kg/m^2) and 22% of men and 23% of women are obese (BMI > 30 kg/m^2) (Peltonen et al. 2008). In the high-income English-speaking countries, the prevalence of overweight and obesity is even higher (NCD Risk Factor Collaboration (NCD-RisC) 2016).

Obesity predisposes the individual to a variety of chronic diseases, including asthma, cardiovascular disease and stroke, many cancers, dementia, depression, diabetes, gout, infertility and pregnancy complications, kidney, liver, spleen and pancreas disease, sleep apnea, and osteoarthritis (Clinical guidelines on the identification, evaluation, and treatment of overweight and obesity in adults-the evidence report. National institutes of health. 1998).

Obesity has been thought to be associated with diabetes and cardiovascular disease as it is a chronic low-grade inflammation state, characterized by increased macrophage infiltration into adipose tissue and chronically increased circulating levels of inflammatory mediators (Ouchi et al. 2011).

Body mass index is the most widely used measure of obesity. It can be calculated by dividing weight (kg) by squared height (m). The World Health Organization has set cut-off values for normal weight (18.5 to 25 kg/m^2), overweight (BMI 25 to 30 kg/m^2) and obesity (BMI $\geq$ 30 kg/m^2) based on the increased incidence of variety of diseases outside the normal BMI range (World Health Organization: Global database on body mass index). BMI is independent of age and the same cut-off points are used for both genders. It correlates rather well with body adiposity measured by different methodologies (World Health Organization: Global database on body mass index). Body proportions might sometimes hamper the ability of BMI to measure body adiposity so that the general BMI cut-offs underestimate adiposity in certain Asian populations. On the contrary, in athletic young men, the amount of body fat stores by BMI is overestimated (World Health Organization: Global database on body mass index). According to a meta-analysis that collected studies comparing BMI and body fat percent measurement, the specificity of BMI to detect body adiposity was 90%, whereas the sensitivity was only 50% (Okorodudu et al. 2010), meaning that individuals who are determined as obese by BMI mostly have increased adiposity, whereas within the normal BMI range, there are a significant amount of individuals who also have increased body adiposity when measured by some other methodology.

Obesity is a clear risk factor for OA. An overweight person has approximately two-fold, and an obese individual has an over three-fold risk to develop knee OA as compared to a normal weighted person (Muthuri et al. 2011). The corresponding risk for an overweight or obese person to develop OA of hand or hip is somewhat lower, however, significantly increased (Yusuf et al. 2010; Jiang et al. 2011; Reyes et al. 2016). It has been estimated that in the countries with the highest prevalence of

obesity, the incidence of knee OA could be reduced by approximately up to 60% if obesity could be prevented (Muthuri et al. 2011).

Since being normal weighted is clearly a protective factor against development of OA, it is not clear how either duration or timing of obesity contribute to the incidence or progression rate of OA. In a Finnish case control study, it was shown that weight gain after the age of 20 carries a higher risk of severe knee OA than constant overweight (Manninen et al. 2004). In the light of the fact that structural changes in OA proceed relatively slowly over the years, it seems likely that weight loss or prevention of obesity should happen already at young age. A British prospective cohort study showed that the risk of knee OA accumulates from exposure to a high BMI value (Wills et al. 2012). In females, a high BMI already at 11 years of age, and in males at 20 years of age, were associated with the presence of symptomatic OA at the age of 53. If overweight did not remain constant, a high BMI at 43 years had a stronger association with knee OA than overweight at 26 years (Wills et al. 2012). Once OA is diagnosed, it is not clear whether or not weight loss can slow down the progression of OA, as no clinical trials have been conducted investigating that subject. However, weight loss has been proven to decrease knee OA-related pain and disability (Christensen et al. 2007).

Increased load on weight bearing joints does not fully explain the obesity-related risk of OA, based on the fact that obesity increases also the risk for hand OA. This explains why it is believed that there exists some metabolic factor(s) that could mediate the effect of obesity systemically. Adipokines have been suggested as candidates linking obesity and OA.

2 Adipocytokines

Adipocytokines or adipokines are proteins secreted by white adipose tissue (WAT). According to this definition, also cytokines like TNF-α, IL-6, IL-1 or chemokines like IL-8 or MCP-1 can be and sometimes are considered as adipokines. More commonly, and also in the present thesis, the term adipocytokine / adipokine is restricted to refer to the more recently found WAT produced proteins that are thought to have functions in the maintenance of metabolic homeostasis, such as leptin, adiponectin, resistin, adipsin or visfatin. Even though initially found in the fat tissue, adipokines are not

exclusively produced by adipose tissue, but many of them have been found to be expressed also by other tissues and cell types. Many of the adipokines have been discovered in the search for factors that could explain obesity-related disorders, such as diabetes and low-grade inflammation. Today it is thought that the imbalance between proinflammatory and anti-inflammatory adipokines contribute to the metabolic dysfunction observed in obesity (Ouchi et al. 2011).

In the present study, the three best known adipokines i.e. leptin, adiponectin and resistin were chosen to be studied. These adipokines are presented below.

2.1 Adiponectin

The discovery of adiponectin, or adipocyte complement-related protein of 30 kDa (Acrp30) as it was initially named, was first published in 1995 (Scherer et al. 1995). This adipokine was demonstrated to be produced by adipocytes and to be abundantly present in the circulation (Scherer et al. 1995). Soon afterwards, also three other groups reported that they had identified a new (actually the same) adipokine, naming it AdipoQ (Hu et al. 1996), apM1 (adipose most abundant gene transcript 1) (Maeda et al. 1996) and GBP28 (gelatin-binding protein of 28) (Nakano et al. 1996). At the time of the discovery of adiponectin, its biological function was unclear, but its transcription was shown to be upregulated during adipocyte differentiation (Scherer et al. 1995). Thereafter, adiponectin has been shown to improve insulin sensitivity (Fruebis et al. 2001; Tsao et al. 2002) and to have anti-atherogenic and anti-carcinogenic properties (Brochu-Gaudreau et al. 2010).

Adiponectin was first thought to be produced exclusively by white adipocytes (Scherer et al. 1995). More recently, also bone marrow, osteoblasts, fetal tissue, myocytes, cardiomyocytes, salivary epithelial cells and different tissues of osteoarthritic joints, including synovium, infrapatellar fat pad, meniscus, osteophytes, cartilage and bone, have been shown to produce adiponectin (Brochu-Gaudreau et al. 2010; Presle et al. 2006).

Adiponectin is present in the circulation in different oligomeric isoforms. The adiponectin molecule consists of an amino-terminal domain followed by a collagenous domain that allows oligomerization of the protein via disulfide bonds, and a globular carboxyl-terminal domain that is structurally similar to the complement

factor C1q and TNF-α (Brochu-Gaudreau et al. 2010). The different isoforms of adiponectin include globular adiponectin that is a proteolytic fragment corresponding globular domain of the protein, the trimer [also called low molecular weight (LMW) adiponectin] that can associate into hexamers [middle molecular weight (MMW) adiponectin] and finally into multimers of high molecular weight (HMW) species consisting of 12-18 monomers. The most abundant forms in circulation are the hexamers and HMW species that both have longer half-lives than the globular and the trimer forms (Brochu-Gaudreau et al. 2010). The different forms do not interconvert in the circulation (Liu and Liu 2014).

There are three known receptors that bind different isoforms of adiponectin with differing affinities, which in part is also thought to define the cell-specific effects of this adipokine. Two receptors that are able to transduce intracellular signaling are known for adiponectin, namely AdipoR1 and AdipoR2. Interestingly, these receptors seem to have contrasting roles in energy metabolism. AdipoR1-deficient mice become obese and glucose-intolerant, whereas AdipoR2-deficient mice are lean and resistant to diet-induced obesity (Bjursell et al. 2007). In human, AdipoR1 is highly expressed in skeletal muscle and AdipoR2 in skeletal muscle and liver (Brochu-Gaudreau et al. 2010). AdipoR1 has a high affinity to globular adiponectin whereas AdipoR2 binds globular and full-length adiponectin with intermediate affinity (Brochu-Gaudreau et al. 2010; Luo and Liu 2016).

In addition, a third receptor, T-cadherin, highly expressed in heart and large arteries, is known to bind hexameric and HMW adiponectin. It lacks intracellular domain and is thus unable to transduce intracellular signals. However it is believed to participate in regulating the effects of adiponectin by competing with AdipoR1 and AdipoR2 for adiponectin binding (Brochu-Gaudreau et al. 2010).

Circulating adiponectin levels have been reported to be decreased in obesity and in type 2 diabetes, and to correlate negatively with visceral fat accumulation (Brochu-Gaudreau et al. 2010; Ouchi et al. 2011), while under certain inflammatory conditions, there have been reports that its levels are elevated (Fantuzzi and Faggioni 2000; Ouchi et al. 2011). Adiponectin production in adipocytes has been shown to be down-regulated by insulin, TNF-α, prolactin, growth hormone and glucocorticoids, whereas peroxisome proliferator-activated receptor agonists can increase plasma levels of adiponectin (Brochu-Gaudreau et al. 2010). The antidiabetic effect of adiponectin is

thought to be mediated via decreased gluconeogenesis in the liver and increased fatty acid oxidation and energy consumption (Lago et al. 2007; Ouchi et al. 2011).

2.1.1 Adiponectin in inflammation and in rheumatic diseases

Whereas decreased levels of adiponectin have been observed in obesity, cardiovascular disease and in type 2 diabetes, increased levels have been reported in patients with autoimmune or chronic inflammatory diseases, including RA, systemic lupus erythematosus (SLE), type 1 diabetes and chronic obstructive pulmonary disease (COPD) (Fantuzzi 2013).

Under experimental conditions, adiponectin has been described to have both anti-inflammatory and proinflammatory effects. It is not clear whether the effects of this adipokine are target cell-specific or specific for the distinct adiponectin isoforms or adiponectin receptors. In macrophages and other monocyte-derived cells, adiponectin has been shown to increase expression of the anti-inflammatory cytokines IL-10 and IL-1Ra and to decrease production of IL-6, TNF-α and IFN-γ through inhibition of NF-κB pathway (Brochu-Gaudreau et al. 2010). In synovial fibroblasts from patients with RA and OA, adiponectin has been shown to increase the production of IL-6 (Ehling et al. 2006). In the study of Neumeier et al, the anti-inflammatory effect of adiponectin on monocytes was induced by LMW adiponectin, whereas HMW adiponectin increased the production of IL-6 in these cells (Neumeier et al. 2006).

Adiponectin is present in the synovial fluid of OA patients (Presle et al. 2006; Schaffler et al. 2003). Joint tissues including synovium, meniscus, osteophytes, cartilage, bone and fat, have been reported to produce adiponectin (Ehling et al. 2006; Presle et al. 2006; Tan et al. 2009). Adiponectin receptors AdipoR1 and AdipoR2 have been shown to be expressed in cartilage, bone and synovial tissue (Chen et al. 2006; Kang et al. 2010). At the time of the beginning of the present study, there were two contradictory research findings published concerning the effects of adiponectin on cartilage/chondrocytes. One group had been shown that adiponectin increases the expression of iNOS, IL-6, MMP-3 and MMP-9 in murine chondrocytes (Lago et al. 2008) whereas another group reported that intra-articularly injected adiponectin in mouse led to decreased expression of TNF-α, IL-1 and MMP-3 in cartilage (Lee et al. 2008).

2.2 Leptin

Leptin is a 16 kDa protein mainly produced by WAT. It was first identified in 1994 to be the product of the obesity (ob) gene (Zhang et al. 1994). Leptin controls food intake by functioning as a negative feedback loop in hypothalamus. It is produced in proportion to the amount of WAT, so that in the circulation its concentration correlates closely with BMI. Leptin is also expressed at low levels in intestine, gastric epithelium, placenta, mammary glands, skeletal muscle, brain, bone, cartilage and inflammatory cells (La Cava and Matarese 2004; Scotece et al. 2014).

Leptin and its receptor (Ob-R) are structurally similar with the class I cytokines and their receptors, which contain gp130 (Fantuzzi and Faggioni 2000; Munzberg and Morrison 2015). Six alternatively spliced isoforms of leptin receptor (Ob-Ra - Ob-Rf) are known. All the isoforms share identical extracellular and transmembrane domains except for Ob-Re, the soluble form of leptin receptor (better known as sOb-R) that does not have a transmembrane domain. Only the long form, Ob-Rb, is able to transduce an intracellular signal to the nucleus. Intracellular signaling is initiated due to leptin binding -induced conformational change of the receptor, subsequent JAK2 phosphorylation, and following tyrosine kinase activation of the receptor (Peelman et al. 2014; Munzberg and Morrison 2015).

Ob-Rb is expressed in a broad range of cells in both the central nervous system and peripheral tissues. Ob-Ra and Ob-Rc are expressed at high levels in choroid plexus and brain microvessels and are suspected to have a role in transport of leptin across the blood-brain barrier (Peelman et al. 2014). The other short forms of leptin receptor (Ob-Rd and Ob-Rf) have no known physiological functions. The soluble form sOb-R is at least partly responsible for the transport of leptin in the circulation (Sinha et al. 1996), and modulates the bioavailability of leptin by binding free active leptin (Peelman et al. 2014; Munzberg and Morrison 2015). In lean individuals, up to 95% of circulating leptin is in bound form (bound to sOb-R or to other plasma proteins), whereas in extremely obese subjects as much as 95% of leptin exists in an unbound, free form (Sinha et al. 1996).

Leptin is thought to be constitutively produced by adipocytes (Fantuzzi and Faggioni 2000), explaining its long-term fat mass-dependent levels. In addition, leptin synthesis is regulated by nutrition and hormones. After a meal, circulating leptin levels peak approximately 3 hours later. The amino acids and insulin present

after a meal activate the mammalian target of rapamycin pathway, which leads to an increase in leptin synthesis. Leptin mRNA levels are increased by glucocorticoids and decreased by catecholamines, whereas insulin stimulates and adrenergic agonists attenuate leptin secretion (Lee and Fried 2009). Estrogen increases and testosterone inhibits leptin synthesis; this is reflected in the slightly higher levels of leptin in females than males with similar fat mass (La Cava and Matarese 2004). Leptin also displays a circadian rhythm: leptin levels are highest at midnight and lowest in the afternoon (Park and Ahima 2014). The immune system affects leptin levels. Increased levels of leptin have been reported in sepsis and in fever (La Cava and Matarese 2004).

2.2.1 Discovery of leptin

The history of finding leptin leads to studies concerning obesity, diabetes and regulation of food intake. Already in 1942, it was observed that lesions in the ventromedial nuclei of hypothalamus in rat and other animals lead to obesity if the animal was allowed free access to food (Hervey 1959). There were a few theories on how long-term food intake could be regulated. One of the theories suggested that control centers in the hypothalamus are sensitive to the concentrations of some unknown metabolite that are in equilibrium with the stored fat. This theory was supported by the findings of Hervey who conducted experiments where ventromedial nuclei lesioned rats were parabiosed (surgical joining of two animals in order to create a cross circulation between the two individuals) with normal rats. In those experiments, the "normal" rat gradually stopped eating, lost weight and died because of starvation. However, the appetite could be restored by lesioning also the brain of the "normal" rat (Hervey 1959).

The theory of existence of satiety factor was later supported by the work of Douglas Coleman. He was asked to assist in characterizing a newly found obese mutant *db/db* mouse (Hummel et al. 1966) and to compare it to the earlier found obese mutant *ob/ob* mouse (Ingalls et al. 1950). Based on his parabiosis experiments in obese mutant *ob* and *db* mice, he concluded that the *db/db* mice overproduced, but could not respond to a satiety factor, which in turn the *ob/ob* mice recognized and responded to but could not produce (Coleman 2010). In his further studies with

ventromedial nucleus / arcuate nucleus lesioned mice (Coleman and Hummel 1970), he showed that this still unidentified product of the *ob* gene was likely to regulate appetite in these brain regions (Coleman and Hummel 1970). The product of the *ob* gene was identified and cloned 24 years later by Jeffrey Friedman and coworkers (Zhang et al. 1994) and named by Friedman's group as leptin, according to the Greek root leptós, meaning thin (Halaas et al. 1995). According to the findings of this group, leptin was suggested to be mainly produced by adipose tissue (Zhang et al. 1994). Soon afterwards, leptin receptor was cloned (Tartaglia et al. 1995) and shown to be coded by the *db* gene (Lee et al. 1996). The long form of the leptin receptor splice variants was highly expressed in hypothalamus, and shown to be abnormally spliced in *db/db* mice, confirming that *db/db* mice express a mutant leptin receptor that cannot mediate intracellular signal transduction (Lee et al. 1996).

2.2.2 Physiological functions of leptin

The main physiological function of leptin is to signal states of negative energy balance and decreased energy stores (Rosenbaum and Leibel 2014). The levels of circulating leptin are elevated in a fed state, after a meal or in obesity, and decrease due to fasting or loss of body weight. Signals originating from leptin-sensitive brain regions influence neuroendocrine functions, autonomic efferents, and food-related behaviors, such that the decline in leptin levels increase food intake and decrease energy expenditure (Rosenbaum and Leibel 2014; Park and Ahima 2015). The intensity of neuronal signaling is proportional to the ambient leptin concentration and is also influenced by the nutritional state (Rosenbaum and Leibel 2014).

In addition to the role of leptin as the body's "fat sensor" in the areas of the brain that regulate energy intake, this adipokine is also thought to mediate the information about changes in energy balance to other physiological systems (Friedman 2014). *Ob/ob* and *db/db* mice, as well as a rare subset of humans with a mutation of leptin or leptin receptor gene (Dubern and Clement 2012), have marked abnormalities in reproduction, haematopoiesis, angiogenesis, insulin secretion, bone metabolism and innate and adaptive immunity, suggesting that leptin has a significant role in the regulation of these systems (La Cava and Matarese 2004).

2.2.3 Leptin in inflammation

Leptin is known to exert effects on both the innate and adaptive immune systems. Leptin-deficient mice display a number of abnormalities in the activity of their immune system, manifested as thymic atrophy, increased susceptibility to bacterial infections and impaired wound healing (Fantuzzi and Faggioni 2000). Leptin promotes activation of monocytes/macrophages and their secretion / expression of leukotriene B_4, NO, COX-2 and pro-inflammatory cytokines. Leptin can also induce chemotaxis of neutrophils, probably through indirect mechanisms, and affect natural killer cell development and activation. Leptin also stimulates growth hormone production by peripheral-blood mononuclear cells (PBMCs), which is thought to influence the survival and proliferation of immune cells. (La Cava and Matarese 2004).

Leptin increases the production of T helper 1 (T_H1) cytokines IL-2 and IFN-γ, and suppresses the expression of the T_H2 cytokine IL-4 in T cells and in mononuclear cells, thus promoting T_H1 polarization (Ouchi et al. 2011). T_H cells play important role in the regulation of the adaptive immune system by releasing cytokines that regulate the function of other immune cells. T_H cells are classically divided into T_H1, T_H2 and T_H17 cells. T_H1 cells help to direct the immune response towards viral and bacterial infection and are also involved in the pathogenesis of autoimmune diseases. T_H2 cells regulate immune response towards parasites and are also implicated in allergic inflammation. In addition, there are regulatory T cells, a subpopulation of T_H cells that suppress the immune response and help to maintain tolerance to self-antigens and prevent autoimmune disease. *Ob/ob* mice are resistant to many experimentally induced autoimmune diseases; this is thought to be related to reduced secretion of IL-2, IFN-γ, TNF-α and IL-18. Leptin has been also shown to inhibit the proliferation of regulatory T cells, while stimulating T_H1 cells, thus possibly protecting from infection (Park and Ahima 2015).

2.2.4 Leptin and cartilage

The knowledge of leptin in rheumatic diseases has increased greatly during the past 10 years. At the time of beginning of the present study, articular cartilage had been found to produce leptin (Dumond et al. 2003; Presle et al. 2006) and to express its

functional receptor (Figenschau et al. 2001). Leptin was found to be overexpressed in OA cartilage in comparison to normal cartilage (Dumond et al. 2003), and its concentration in synovial fluid had been reported to be higher in patients with OA than in controls (Ku et al. 2009). Leptin had been shown to potentiate the effect of IL-1 and IFN-γ by inducing NO production in chondrocytes via intracellular pathways involving JAK2, phosphoinositide 3-kinase (PI3K), MEK-1 and p38 (Otero et al. 2003; Otero et al. 2005; Otero et al. 2007). Leptin had been also shown to increase the production of IL-1β and MMP-9 and -13 in human primary chondrocytes in long term culture, suggesting that the effect was perhaps not direct (Simopoulou et al. 2007). Furthermore, effects considered as anabolic were reported, as leptin injection into the rat knee was reported to increase the production of insulin-like growth factor (IGF-1) and TGF-β (Dumond et al. 2003). Subsequently, the research data on the subject has been extended and will be discussed in connection to the results of this thesis in the Discussion section.

2.2.5 Leptin resistance

Leptin deficiency in *ob/ob* mice and in humans with congenital leptin deficiency lead to severe obesity and associated metabolic dysfunction that are reversible with leptin substitution. Genetic leptin deficiencies, as well as leptin receptor mutation-related obesity in humans are, however, extremely rare (Dubern and Clement 2012).

Generally, obese individuals have elevated leptin levels in their circulation that fail to reduce the excess adiposity. This is thought to be a consequence of leptin resistance in the leptin responsive areas in hypothalamus. The elevated expression of suppressor of cytokine signaling (SOCS-3) in central nervous system (CNS) is thought to be the primary mechanism that attenuates leptin signaling, thus, causing leptin resistance and subsequent failure in controlling food intake in obesity (St-Pierre and Tremblay 2012). Diet-induced obese mice exhibit elevated SOCS-3 expression in hypothalamus in comparison to control mice (White and Nicola 2013). SOCS-3 deletion in neurons lead to prolonged STAT activation and resistance to diet-induced obesity in mice (Mori et al. 2004). It has been reported that STAT-3 knock-out in brain leads to severe obesity, indicating that the effects of leptin on the control of food intake are mediated through STAT-3 (Allison and Myers 2014). However, SOCS-3 overexpression

evokes only mild obesity or even slight leanness (Reed et al. 2010), depending on the neuron type where SOCS-3 is overexpressed. This indicates that the function of SOCS-3 is not as straightforward as initially thought, and that increased SOCS-3 expression is a consequence of obesity rather than its cause (Allison and Myers 2014).

Other factors thought to contribute to the development of leptin resistance include protein tyrosine phosphatases that also affect the intracellular signaling cascade of leptin, impaired transport of leptin across the blood brain barrier, endoplasmic reticulum stress and inflammation (St-Pierre and Tremblay 2012).

2.2.5.1 SOCS-3

SOCS proteins are the principal regulators of the JAK-STAT (janus kinase – the signal transducers and activators of transcription) pathway, intracellular signaling cascade activated by many cytokines. SOCS proteins are regulatory factors that limit the duration of cytokine signaling, and are also thought to be involved in the specificity of cytokine signaling (Babon and Nicola 2012). In human genome, there are eight members of SOCS family [SOCS-1 - SOCS-7 and CIS (cytokine-inducible SH2 protein)] of which SOCS-1 and SOCS-3 are known to directly inhibit JAKs (Babon and Nicola 2012). In addition, SOCS proteins possess ubiquitin ligase activity, although this is not thought to be the main mode-of-action of SOCS-3 (Babon and Nicola 2012; White and Nicola 2013).

SOCS-3 is induced by and inhibits the actions of IL-6, IL-11, leukemia inhibitory factor, oncostatin M, ciliary neutrophic factor, granulocyte colony stimulating factor and leptin (White and Nicola 2013). Synthesis of SOCS-3 is induced rapidly by these proteins through the JAK-STAT pathway. SOCS-3 then inhibits further JAK-STAT signaling by binding to JAK and the cytoplasmic domain of the cytokine receptor, thus forming a negative feedback loop for the above-mentioned mediators (Kershaw et al. 2013). SOCS-3 is also induced by factors and pathways that are not subsequently inhibited by SOCS-3. For example, the anti-inflammatory cytokine IL-10 induces SOCS-3 without being subsequently inhibited itself (Babon and Nicola 2012). Other factors / pathways known to induce SOCS-3 include TNF-α, LPS, growth hormone, cAMP, IFN-γ, IL-1, IL-9 and IL-21 (White and Nicola 2013).

SOCS-3 has been shown to exert a protective role in animal models of arthritis (Shouda et al. 2001; Veenbergen et al. 2011; Wong et al. 2006). Interestingly, in

addition to neurons in CNS and the cells of immune system, SOCS-3 is also expressed by chondrocytes (de Andres et al. 2011; Smeets et al. 2006; van de Loo et al. 2012). Van de loo et al. reported that the overexpression of SOCS-3 inhibits LPS-induced NO production in chondrocytes (van de Loo et al. 2012) suggesting that SOCS-3 has an immunosuppressive role not only in the cells of immune system, but also in chondrocytes. SOCS-3 might also inhibit anabolic responses in chondrocytes, as increased SOCS-3 expression has been reported to explain the non-responsiveness of OA chondrocytes toward IGF-1 stimulation, and overexpression of SOCS-3 was shown to inhibit aggrecan synthesis (Smeets et al. 2006; van de Loo et al. 2012).

2.3 Resistin

2.3.1 Discovery of resistin

Resistin is a member of resistin-like molecules (RELMs) family specific for a cysteine rich sequence. Resistin and the RELM family were characterized in 2001 by three different research groups (Holcomb et al. 2000; Kim et al. 2001; Steppan et al. 2001a). Mouse resistin (also called found in inflammatory zone 3, FIZZ3 or adipose tissue-specific factor, ADSF) was found to be expressed mainly in adipose tissue (in variety of organs), its serum levels being elevated in obese mice (diet-induced obesity and genetic *ob/ob* and *db/db* mice) (Steppan et al. 2001b).

The name resistin that has become established in use, was chosen by the Lazar group based on the effects of resistin on diabetes in mice and refers to resistance to insulin (Steppan et al. 2001a; Steppan et al. 2001b). This group identified the molecule in an attempt to identify factors that are produced by adipocytes, and that are down-regulated by the anti-diabetic drugs thiazolidinediones, aiming to find a factor connecting obesity and type 2 diabetes. They found an adipose tissue-derived molecule, to be named resistin, the expression of which was downregulated by thiazolidinediones. More interestingly, immuno-neutralization of resistin was shown to improve blood glucose levels and insulin sensitivity in a mouse diabetes 2 model, whereas administration of resistin impaired glucose tolerance and insulin sensitivity. (Steppan et al. 2001b)

Kim et al. identified resistin (or ADSF) in a microarray analysis of adipose tissue-specific genes. Their results showed that resistin expression is increased during adipocyte differentiation and suggested that resistin serves as a negative feedback molecule in adipogenesis. (Kim et al. 2001)

2.3.2 Resistin in human

Human resistin is a 12.5 kDa protein. It shares only a 59% similarity with mouse resistin at amino acid level (Filkova et al. 2009a). Resistin circulates in oligomeric and trimeric forms. The balance between the oligomeric and trimeric forms is related to its concentration-dependent conformational shift from the α-helical to the β-sheet form, which is postulated to regulate the bioactivity of resistin (Aruna et al. 2008). No specific resistin receptor has been recognized to date, but resistin has been shown to bind to toll-like receptor 4 (TLR-4), and to activate MAPK, NF-κB and PI3K signaling pathways (Tarkowski et al. 2010).

In spite of the encouraging data of the involvement of resistin in insulin resistance and diabetes in mouse models found by Steppan et al. (Steppan et al. 2001a; Steppan et al. 2001b), the evidence of the contribution of resistin in diabetes in human is controversial (Schwartz and Lazar 2011). Human resistin is produced by adipose tissue, however, the latest reports suggest that it is produced by mononuclear cells and other bone marrow rooted cells, and very unlikely synthesized by adipocytes (Filkova et al. 2009a). In addition to the non-fat fraction of adipose tissue, resistin is highly expressed in bone marrow, and can be also found in placenta, pancreas, synovial tissue, synovial fluid, monocytes/ macrophages inside atherosclerotic plaques and in circulation (Filkova et al. 2009a; Kunnari et al. 2009). In humans, no clear correlation has been found between resistin and BMI, but in morbidly obese patients, resistin expression in adipose tissue is reported to be significantly higher than in lean subjects, probably due to the higher amount of mononuclear cells in adipose tissue of the obese (Filkova et al. 2009a).

In humans, blood resistin levels seem to be determined by inflammatory stimuli and resistin also has cytokine-like effects. Elevated levels of circulating resistin have been found in inflammatory conditions like asthma, sepsis, rheumatoid arthritis and inflammatory bowel disease (Filkova et al. 2009a). An injection of LPS into healthy

volunteers was shown to cause an elevation in the circulating levels of resistin that returned close to baseline within one day after the injection (Lehrke et al. 2004). *In vitro*, resistin expression in monocytes and neutrophils has been shown to be increased by LPS, TNF-α (Kunnari et al. 2009), IL-1β and IL-6 (Filkova et al. 2009a). Resistin levels in blood have also been observed to increase after knee injury (Lee et al. 2009) and coronary artery bypass graft surgery (CABG) (Laurikka et al. 2014). Resistin has been shown to increase cytokine production (IL-6, IL-8, TNF-α) in PBMCs, adipocytes (Filkova et al. 2009a; Nagaev et al. 2006) and in joint tissues (see below).

2.3.3 Resistin in rheumatic diseases

Circulating, as well as synovial fluid levels of resistin are increased in patients with RA (Bokarewa et al. 2005; Migita et al. 2006; Schaffler et al. 2003; Senolt et al. 2007). Interestingly, in RA, the synovial fluid resistin concentrations are higher than in blood (Bokarewa et al. 2005; Bostrom et al. 2011; Schaffler et al. 2003; Senolt et al. 2007), suggesting that in RA resistin is produced in the inflamed joints. Moreover, resistin has been shown to induce catabolic effects in joints and in cartilage. Resistin was shown to induce cartilage destruction and synovial inflammation when injected into the knee joints of healthy mice (Bokarewa et al. 2005). *In vitro*, resistin has been reported to enhance the expression of cytokines, MMP-1 and MMP-13, and ADAMTS-4 in human primary chondrocytes (Zhang et al. 2010b), and to upregulate PGE_2, IL-6 and IL-8 production in mouse cartilage (Lee et al. 2009), as well as to reduce the production of proteoglycans in mouse and in human cartilage (Lee et al. 2009). Resistin has also been shown to up-regulate the expression of IL-6 and TNF-α in human macrophages and in cells extracted from synovial fluid of patients with acute synovitis (Bokarewa et al. 2005; Leivo-Korpela et al. 2011).

Aims of the study

Obesity is a major risk factor of OA. This association is not explained solely by the increased weight on joints, pointing to the existence of some obesity-related metabolic factor/ factors mediating the link between obesity and OA.

The aim of the present study was to investigate the role of adipocytokines adiponectin, leptin and resistin in the pathogenesis of OA, as possible mediators between obesity and OA, and as possible targets of disease-modifying drugs for OA.

The detailed aims were:

1. to study the relationships of the levels of three adiopocytokines, adiponectin, leptin and resistin, measured in the circulation, synovial fluid and in cartilage, with the levels of inflammatory and catabolic factors, biomarkers of OA and radiographic findings of OA in a cross-sectional study population consisting of 100 OA patients (I, III, VI)
2. to examine the effects of adipokines adiponectin and leptin on the production of proinflammatory and catabolic factors in human OA cartilage and the intracellular signaling pathways involved (I, II, III)
3. to determine whether leptin responsiveness in cartilage can be explained by some clinical or biological factor (IV, V)
4. to investigate whether SOCS-3 expression in chondrocytes regulates leptin-induced proinflammatory and catabolic effects (V)

Materials and methods

1 Patients

Cartilage samples, synovial fluid and blood samples, as well as clinical data, were collected from 100 OA patients undergoing knee replacement surgery in Coxa Hospital for Joint Replacement, Tampere, Finland. All patients were diagnosed to have clinical and radiographic knee OA according to the American College of Rheumatology criteria (Altman et al. 1986). Clinical data was collected about BMI, age, diabetic status, joint pain and mobility data as measured by the KSS (Insall et al. 1989), and preoperative radiographs.

Concentrations of adipokines, catabolic and proinflammatory factors, biomarkers of OA and regulatory factors were measured as described below. Blood samples were fasting samples collected in the morning of the surgery. Synovial fluid samples were collected by the operating surgeon by joint puncture before opening of the knee. They were centrifuged at 4000 g at 4˚C and the supernatants were kept at -70˚C until assayed. Preoperative CRP was analyzed in plasma by an immunochemical method in Fimlab, Tampere, Finland as a part of the routine procedure.

Blood samples were received from all 100 patients. An adequate amount of cartilage for the experiments and culture was received from 97 of the 100 patients, whereas synovial fluid sample amount was sufficient from 91 patients.

Radiographs were evaluated by two observers (Anna Koskinen and Teemu Moilanen) by Ahlbäck scale (Ahlback 1968).

The Knee Society Score (Insall et al. 1989) was evaluated preoperatively by an orthopedic surgeon in the operating hospital and the score was available from 90 of the 100 patients.

In the experimental part of the study, additional cartilage samples from OA patients undergoing knee replacement surgery outside the above-mentioned group were also obtained in order to study the effects of leptin and adiponectin on cartilage (I, II and III).

The study was approved by the ethics committee of Tampere University Hospital, and the patients gave their written approval.

2 Reagents

2.1 Chemicals

Alexis Corporation, Lausen, Switzerland:

Ro 31-8220 (2-{1-[3-(Amidinothio)propyl]-1H-indol-3-yl}-3-(1-methylindol-3-yl)maleimide methanesulfonate salt; PKC inhibitor)

BioVendor Research and Diagnostic Products, Modřice, Czech Republic:

recombinant human adiponectin

Calbiochem, Merck, Darmstadt, Germany:

SP600125 (Anthra[1,9-cd]pyrazol-6(2H)-one, 1,9-pyrazoloanthrone; JNK inhibitor)

SB220025 (5-(2-Aminopyrimidin-4-yl)-4-(4-fluorophenyl)-1-(4-piperidinyl)imidazole trihydrochloride; p38 inhibitor)

AG 490 ((E)-2-Cyano-3-(3,4-dihydrophenyl)-N-(phenylmethyl)-2-propenamide; JAK2 inhibitor)

WHI-P154 (2-Bromo-4-[(6,7-dimethoxy-4-quinazolinyl)amino]phenol; JAK3 inhibitor)

Cayman Chemical, Ann Arbor, MI, USA:

SNAP (S-Nitroso-N-Acetyl-D,L-Penicillamine; NO donor)

Promega, Madison, WI, USA:

PD 98059 (2-(2-Amino-3-methoxyphenyl)-4H-1-benzopyran-4-one; extracellular signal-regulated kinase 1 and 2 (Erk1/2) inhibitor)

R&D Systems Europe Ltd, Abingdon, UK:

recombinant human leptin

recombinant human IL-1 beta

recombinant mouse leptin

Sigma Chemical Co, St Louis, MO, USA:

PDTC (ammonium pyrrolidinedithiocarbamate; NF-κB inhibitor)

Tocris Bioscience, Ellisville, MO, USA:

MG 132 (N-[(Phenylmethoxy)carbonyl]-L-leucyl-N-[(1S)-1-formyl-3-methylbutyl]-L-leucinamide; NF-κB inhibitor)

1400W (N-[[3-(aminomethyl)phenyl]methyl]-ethanimidamide, dihydrochloride; iNOS inhibitor) was kindly given by Dr Richard G Knowles, Glaxo Smith Kline Research & Development, Stevenage, UK.

2.2 Immunoassays

2.2.1 ELISA kits

BioVendor Research and Diagnostic Products, Modřice, Czech Republic:

COMP

sOb-R

R&D Systems Europe Ltd, Abingdon, UK:

human adiponectin/arcp 30

human leptin

human resistin

human total MMP-1 (plasma, cartilage culture media/ articles IV-V)

human total MMP-3 (synovial fluid, plasma, cartilage culture media/ articles IV-V)

human total MMP-13 (cartilage culture media/article IV)

human IL-8

mouse total MMP-3

mouse IL-6

Sanquin, Amsterdam, The Netherlands:

human IL-6

2.2.2 Multiplex bead array, Fluorokine®

R&D Systems Europe Ltd, Abingdon, UK:

Human MMP Multi Analyte Profiling Base Kit

MMP-1 (synovial fluid and cartilage culture media/ article II)

MMP-3 (cartilage culture media/article II)

MMP-8 (synovial fluid (not detectable))

MMP-13 (synovial fluid (not detectable) and cartilage culture media/article II)

2.3 Antibodies used in Western blot analysis

Abcam, Cambridge, MA, USA:

rabbit polyclonal MMP-13 antibody (ab39012)

Cell Signaling Technology, Inc, Beverly, MA, USA:

rabbit polyclonal p38 antibody (#9212)

rabbit polyclonal phospho-p38 antibody (#9211)

rabbit polyclonal phospho-JNK (#9251)

rabbit polyclonal Erk1/2 antibody (#9102)

rabbit polyclonal phospho-Erk1/2 antibody (#9101)

Santa Cruz Biotechnology, Santa Cruz, CA, USA:

mouse monoclonal SOCS-3 antibody (sc-51699)

rabbit polyclonal iNOS antibodies (sc-651 and sc-650)

goat polyclonal COX-2 antibody (sc-1745)

rabbit polyclonal β-actin antibody (sc-1615R)

HRP-conjugated goat anti-mouse antibody (sc-2005)

HRP-conjugated goat anti-rabbit antibody (sc-2004)

HRP-conjugated donkey anti-goat antibody (sc-2020)

rabbit polyclonal JNK antibody (sc-571)

3 Tissue and cell cultures

3.1 Cartilage cultures

Cartilage tissue was obtained from the leftover pieces of total knee replacement surgery. The samples were washed with phosphate-buffered saline (PBS). Full thickness pieces of articular cartilage from femoral condyles, tibial plateaus, and patellar surfaces were removed aseptically from subchondral bone with a scalpel and cut into small pieces (about 2×2×2 mm). Three randomly selected cartilage cubes were placed in one well of a 6-well plate and incubated in 3 ml of tissue culture medium [Dulbecco's modified Eagle's medium (DMEM) with glutamax-I containing 10% heat inactivated fetal bovine serum, and penicillin (100 units/ml), streptomycin (100 μg/ml), and amphotericin B (250 ng/ml); all obtained from Invitrogen, Carlsbad, California, USA] with the reagents of interest at 37°C in 5% carbon dioxide atmosphere for 42 hours. The culture medium was removed and stored at -20˚C until assayed. Cartilage explants were weighed after the experiments before proceeding to protein extraction.

3.2 Primary chondrocyte experiments

The leftover pieces of OA cartilage were first processed in the same way as cartilage for cartilage cultures (see above). Cartilage pieces were then washed with PBS, and chondrocytes were isolated by enzymatic digestion for 16 hours at 37°C in a shaker by using a collagenase enzyme blend (1 mg/ml Liberase TM Research Grade medium; Roche, Mannheim, Germany). Isolated chondrocytes were washed, counted and plated on 24-well plates (1.5×10^5 cells/ml) in culture medium (DMEM with supplements; see above / cartilage cultures) containing 10% fetal bovine serum and reagents of interest.

3.3 Chondrocyte cell line

The immortalized murine H4 chondrocyte cell line (van Beuningen et al. 2002), developed in the Laboratory of Experimental Rheumatology, University Medical Center, Nijmegen, The Netherlands, was used to study the effect of downregulation of SOCS-3 by small interfering RNA (siRNA). The chondrocytes were cultured at 37°C in humidified 5% carbon dioxide atmosphere in Dulbecco's modified Eagle's medium (DMEM) with L-glutamine and Ham's F-12 medium (1:1) supplemented with 5% fetal bovine serum (all obtained from Lonza Group Ltd, Basel, Switzerland).

3.3.1 Down-regulation of SOCS-3 expression by siRNA

H4 murine chondrocytes were seeded at 1 x 10^5 cells/well in 24-well plates. Cells were incubated for 24 hours and transfected with SOCS-3 siRNA or with non-targeting control siRNA. On-Target SMART pool SOCS-3 specific siRNA (targeting sequences of GGCUAGGAGACUCGCCUUA, GGACCAAGAACCUACGCAU, CUAAUGAAACCUCGCAGAU, GAAGGGAGGCAGAUCAACA) and siGENOME Non-Targeting siRNA were used at 100 nM to transfect the cells using DharmaFECT 1. All transfection reagents were from Thermo Scientific Dharmacon (Lafayette, CO, USA) and transfection was carried out according to the manufacturer's protocol. The experiments were started 48 hours after the transfection by adding leptin (10 µg/ml) in fresh culture medium.

4 Immunoassays

The concentrations of adiponectin, leptin, resistin, MMP-1, MMP-3, MMP-8, MMP-13, IL-6, COMP and sOb-R were determined by enzyme linked immunosorbent assay (ELISA) or Multiplex bead array with commercial reagents according to the protocol provided by the manufacturer (see above 2.2 Immunoassays). The amount of PGE_2 was determined by radioimmunoassay, using reagents from the Institute of Isotopes (Budapest, Hungary).

5 Western blot analysis

After the experiments the cartilage specimen were snap-frozen in liquid nitrogen, milled and lysed in extraction buffer (10 mM Tris-HCl, 5 mM EDTA, 50 mM NaCl, 1% Triton-X-100, 0.5 mM phenylmethylsulfonylfluoride (PMSF), 1 mM sodium orthovanadate, 20 μg/ml leupeptin, 50 μg/ml aprotin, 5mM sodium fluoride, 2 mM sodium pyrophosphate, 10 μM n-octyl-beta-D-glucopyranoside). In the case of cell culture, after the removal of cell culture medium, the cells were rapidly washed with ice-cold PBS and lysed in the extraction buffer. Following incubation on ice for 15 minutes, samples were centrifuged and supernatants were mixed with sample buffer in ratio of 1 : 4 (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 0.025% bromophenol blue, and 5% β-mercaptoethanol) and stored at −20°C until analyzed. Coomassie blue method was used to measure the protein content of the samples (Bradford 1976). After boiling, protein samples were separated with SDS-polyacrylamide electrophoresis gels and transferred to Hybond enhanced chemiluminescence nitrocellulose membrane (Amersham Biosciences UK Limited, Buckinghamshire, UK). Proteins were identified by Western blotting using antibodies mentioned above (see reagents). Bound antibody was detected using SuperSignalWest Pico chemiluminescent substrate (Pierce, Rockford, IL, USA) and either by FluorChem 8800 imaging system (Alpha Innotech, San Leandro, CA, USA) or ImageQuantTM LAS mini (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Quantitation of the chemiluminescent signal was carried out with FluorChem software v.3.1 or ImageQuant TL 7.0 Image Analysis Software.

6 Nitrite assays

Nitrite, a stable metabolite of NO, was measured in the culture media by the Griess reaction (Green et al. 1982). The cartilage culture media samples were filtered through Amicon Ultra 10K filters (from Millipore, Cork, Ireland) at 14 000 g prior to the Griess analysis in order to remove large molecules that might interfere with the Griess analysis (article IV).

7 RNA isolation and quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Culture medium was removed at the indicated time points and total RNA extraction was carried out with GenElute^{TM} Mammalian Total RNA Miniprep kit (Sigma-Aldrich, St Louis, MO, USA). Total RNA was treated with DNAse (Fermentas UAB, Vilnius, Lithuania) and reverse-transcribed to cDNA using TaqMan Reverse Transcription reagents and random hexamers (Applied Biosystems, Foster City, CA, USA). cDNA obtained from the RT-reaction was diluted 1:20 with RNAse-free water and subjected to quantitative PCR using TaqMan Universal PCR Master Mix and ABI Prism 7000 Sequence detection system (Applied Biosystems). Primers and probes for SOCS-3, GAPDH, iNOS, IL-6 and MMP-13 were obtained from Metabion International AG (Martinsried, Germany). The primer and probe sequences and concentrations (Table 3) were optimized according to the manufacturer's instructions in TaqMan Universal PCR Master Mix Protocol part number 4304449 revision C. The expression of mouse MMP-3 mRNA was measured using TagMan Gene Expression Assay (Mm00440295_m1, Applied Biosystems). PCR reaction parameters were as follows: incubation at 50°C for 2 min, incubation at 95°C for 10 min, and thereafter 40 cycles of denaturation at 95°C for 15 s and annealing and extension at 60°C for 1 min. Each experimental reaction was performed in duplicate. The relative mRNA levels of SOCS-3, GAPDH, iNOS, IL-6 and MMP-13 were quantified using the standard curve method as described in Applied Biosystems User Bulletin number 2. mRNA levels were normalized against GAPDH. To calculate the relative expression of MMP-3 mRNA, the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001) was used. According to the method, the CT values for MMP-3 mRNA expression in each sample were normalized to the CT values of GAPDH mRNA in the same sample.

Table 3 Primer and probe sequences for quantitative RT-PCR

Gene	Oligunucleotide	Sequence	Conc. (nM)
Mouse GAPDH	Forward primer	GCATGGCCTTCCGTGTTC	300
	Reverse primer	GATGTCATCATACTTGGCAGGTTT	300
	Probe	TCGTGGATCTGACGTGCCGCC	150
Mouse SOCS-3	Forward primer	GCGGGCACCTTTCTTATCC	300
	Reverse primer	AAGCTGCCCCCCTCACA	300
	Probe	CTCGGACCAGCGCCACTTCTTCA	150
Mouse iNOS	Forward primer	CCTGGTACGGGCATTGCT	300
	Reverse primer	GCTCATGCGGCCTCCTT	300
	Probe	CAGCAGCGGCTCCATGACTCCC	150
Mouse IL-6	Forward primer	TCGGAGGCTTAATTACACATGTTC	900
	Reverse primer	CAAGTGCATCATCGTTGTTCATAC	300
	Probe	CAGAATTGCCATTGCACAACTCTTTTCTCA	200
Mouse MMP-13	Forward primer	TTGTGTTTGCAGAGCACTACTTGA	900
	Reverse primer	AACTGTGGAGGTCACTGTAGACTTCTT	900
	Probe	CATCCTGCGACTCTTGCGGGAATC	250

8 Statistical analysis

8.1 Demographics and clinical data

Data are expressed as mean (standard deviation, SD) or median (inter quartile range, IQR) depending on the distribution of the data. Chi-Square test, unpaired T-test or Mann-Whitney test (where appropriate) were used to analyze differences between subgroups of the patients. Wilcoxon test was used to determine differences between paired variables (adipokine levels between different compartments). Spearman correlation analysis was used for observing associations between continuous variables. Alternatively, Pearson correlation analysis was utilized for normally distributed variables or in cases where normal distribution was achieved by natural logarithm (ln) transformation.

Differences in adiponectin levels between radiographic subgroups were analyzed by analysis of variance (ANOVA) followed by Bonferroni post-test. Binary logistic regression was used to compute BMI or age-adjusted differences in adiponectin levels relative to radiographic grading (Ahlbäck grades 4 - 5 vs grades 1 - 3).

In order to observe differences of other adipokines relative to radiographic findings (unpublished data), the levels of these adipokines were compared between Ahlbäck grades 1 - 3 vs. 4 - 5 by calculating statistical significance by Mann-Whitney test.

The data were analyzed by IBM SPSS Statistics 19 (IBM Corporation, NY, USA).

8.2 Experimental data

The results are expressed as mean ± standard error of the mean (SEM). Statistical significance of the results concerning experiments on cartilage and primary chondrocytes was calculated by using paired t-test or by ANOVA with Bonferroni post-test where appropriate. The data were analyzed by Graph-Pad InStat version 3.00 software (GraphPad Software Inc., San Diego, CA, USA).

Statistical analysis concerning siRNA experiments was carried out by two-way ANOVA with Bonferroni multiple comparisons post-test using Graph-Pad Prism 5 for Windows version 5.04 (GraphPad Software Inc.).

8.3 Leptin responses in relation to SOCS-3 expression

To analyze the differences in leptin responsiveness in relation to SOCS-3 expression, protein samples extracted from cartilage (available from 97 patients) were run on 8 separate gels (samples from 12 to 13 patients on one gel). The samples on each western blot gel (run) were divided into two equal sized groups (low SOCS-3 or high SOCS-3) according to their SOCS-3 expression. Leptin-induced production of MMP-1, MMP-3, MMP-13, IL-6 and NO and expression of iNOS and COX-2 in cartilage cultures were compared between these two SOCS-3 expression groups. Inter-gel variation in SOCS-3 expression was controlled statistically by ANOVA modeling. Associations were further tested by adjusting for BMI and age.

The associations between MMPs or IL-6 and leptin in synovial fluid, and SOCS-3 expression in cartilage were also analyzed by ANOVA modeling. The analysis was done in BMI subgroups (obese, BMI > 30 kg/m^2; non-obese, BMI < 30 kg/m^2). Natural logarithms were calculated of the leptin response values, SOCS-3 expression levels and synovial fluid levels of the measured variables where appropriate in order to have normally distributed variables for the ANOVA modeling and for the correlation analyses. The data were analyzed by IBM SPSS Statistics 19 (IBM Corporation, NY, USA).

Summary of results

1 Demographic data and levels of adipocytokines in OA patients

Demographic data of the patients stratified by gender and in non-obese / obese subgroups are presented in table 4. Obese patients were younger and had slightly higher CRP values than their non-obese counterparts. In addition, a greater proportion of the obese patients had type 2 diabetes in comparison with the non-obese patients. (Table 4) None of the patients included in the study were diagnosed for type 1 diabetes. There were some gender-, age- and BMI-related differences in the levels of catabolic and proinflammatory factors measured: Females had higher synovial fluid levels of MMP-1 and MMP-3, higher cartilage produced MMP-1 and lower plasma MMP-3 than males (Table 4). Age correlated with cartilage culture media NO ($r=0.22$, $p=0.035$), SF-IL-6 ($r=0.29$, $p=0.005$) and p-MMP-3 ($r=0.38$, $p<0.001$). Age was also associated with more severe radiographic findings (Ahlbäck 1 to 3 vs. 4 to 5: $p=0.005$). There was also a weak positive correlation between BMI and p-IL-6 ($r=0.23$, $p=0.019$).

All of the studied adipokines were readily detectable in all collected samples and culture supernatants. The median concentrations of adipokines in the entire study population and in gender subgroups are shown in table 5. Levels of leptin and adiponectin in plasma, synovial fluid and cartilage culture media samples, and resistin in cartilage culture media were significantly higher in female patients. Levels of leptin in all compartments (plasma, synovial fluid and cartilage culture medium) showed strong positive correlation with BMI (Table 6). Resistin showed some correlation with BMI, but only in cartilage culture media (Table 6). Adiponectin levels did not correlate with BMI, except in females, in whom the plasma adiponectin concentration was weakly and inversely correlated with BMI (Table 6). There was also a positive correlation between age and adiponectin levels in all compartments in males (Table 6). Age and leptin concentrations in males correlated negatively (Table 6), perhaps

explained by negative correlation between age and BMI (r=-0.386, p=0.017) in this group.

The concentrations of all of the adipokines correlated with their levels measured in different compartments with the associations being strongest for leptin (Figure 5).

Table 4 Patient characteristics in the whole study population and compared across gender and body mass index subgroups

	all n=100	females n=62	males n=38	p	non-obese, BMI <30 kg/m^2 n=52	obese, BMI >30 kg/m^2 n=48	p
BMI (kg/m^2)[a]	**30.8** 5.9	**31.7** 6.4	**29.5** 4.8	0.97	**26.3** 2.4	**35.7** 4.6	**0.002**
age (years)[a]	**70.0** 10.0	**70.0** 10.4	**69.9** 9.3	0.055	**72.9** 9.6	**66.8** 9.4	**<0.001**
diabetes (number of patients with DM2)[b]	**17** 17.0	**12** 19.4	**5** 13.2	0.59	**4** 7.7	**13** 27.1	**0.015**
Ahlbäck radiographic score 1-2/3/4-5[b]	**41/40/19** 41/40/19	**30/24/8** 48/39/13	**11/16/11** 29/42/29	0.066	**20/21/11** 39/40/21	**21/19/8** 44/40/17	0.80
KSS (points from 0 to 100)[a]	**45.7** 15.7	**44.2** 15.4	**48.3** 16.1	0.23	**47.9** 13.8	**43.4** 17.3	0.18
s-COMP (U/l)[c]	**11.2** 5.4	**11.2** 5.0	**11.5** 6.5	0.65	**11.5** 6.2	**11.1** 4.3	0.40
CRP (mg/l)[c]	**1.4** 2.2	**1.8** 3.0	**1.1** 1.8	0.066	**1.3** 1.5	**2.2** 4.4	**0.002**
p-IL-6 (pg/ml)[c]	**3.1** 2.7	**3.3** 3.4	**3.0** 2.5	0.50	**3.0** 2.5	**3.5** 3.1	0.14
SF-IL-6 (pg/ml)[c, d]	**120** 194	**137** 230	**86** 199	0.24	**134** 205	**114** 280	0.86
cart-IL-6 (pg/mg cartilage)[c, e]	**9.2** 33.7	**13.0** 33.1	**8.1** 36.6	0.53	**8.1** 25.9	**15.6** 41.8	0.074
p-MMP-1 (pg/ml)[c]	**620** 719	**678** 705	**561** 741	0.43	**705** 675	**525** 780	0.20
SF-MMP-1 (ng/ml)[c, d]	**14.2** 25.4	**18.7** 24.7	**6.5** 11.9	**<0.001**	**10.5** 16.3	**18.1** 27.3	0.31
cart-MMP-1 (pg/mg cartilage)[c, e]	**78.9** 107	**86.1** 178	**74.2** 90.1	**0.030**	**74.2** 101	**89.9** 127	0.17
p-MMP-3 (ng/ml)[c]	**8.1** 4.5	**7.2** 4.7	**9.4** 4.9	**0.005**	**8.7** 5.7	**7.6** 4.4	0.18
SF-MMP-3 (ng/mg)[c, d]	**657** 923	**847** 938	**437** 505	**0.002**	**598** 568	**765** 1159	0.11
cart-MMP-3 (ng/mg cartilage)[c, e]	**8.0** 6.4	**8.3** 6.9	**7.8** 6.8	0.23	**7.6** 5.3	**8.6** 6.7	0.18
cart-MMP-13 (pg/mg cartilage)[c, e]	**6.7** 9.6	**6.6** 9.9	**6.7** 8.9	0.40	**6.4** 9.6	**6.9** 9.2	0.58
cart-NO_2 (pmol/mg cartilage)[c, e]	**114** 131	**109** 127	**125** 131	0.55	**106** 129	**122** 131	0.19

Values are [a]**mean** SD, [b]**number of patients** percentages or [c]**median** IQR. p values were calculated for comparison between female and male subjects or between non-obese and obese subjects using the unpaired t-test[a], the Chi-square test[b] or the Mann-Whitney test[c]. [d]Synovial fluid sample was obtained from 91 patients. [e]Cartilage sample was obtained from 97 patients. Statistically significant p values are written in bold. SD, standard deviation; IQR, inter quartile range; KSS, knee society score (score 80-100 indicate for excellent, 70-79 good, 60-69 fair and below 60 poor knee condition); p, plasma; SF, synovial fluid; cart, cartilage culture medium.

Table 5 Median concentrations of adipokines in plasma, synovial fluid and in cartilage culture supernatants in the whole study population and compared across gender subgroups

	all (n=100)		females (n=62)		males (n=38)		
	median	IQR	median	IQR	median	IQR	p
p-leptin (ng/ml)	**19.8**	22.4	**26.5**	36.7	**11.7**	10.5	**<0.001**
SF-leptin (ng/ml)	**13.3**	18.6	**18.3**	23.5	**5.85**	6.75	**<0.001**
cart-leptin (ng/ 10 mg cartilage)	**161**	206	**204**	263	**76.9**	118	**<0.001**
p-adiponectin (µg/ml)	**2.89**	2.51	**3.35**	2.52	**2.02**	2.11	**<0.001**
SF-adiponectin (µg/ml)	**0.482**	0.424	**0.588**	0.383	**0.336**	0.274	**0.001**
cart-adiponectin (pg/ mg cartilage)	**36.9**	30.6	**40.2**	36.1	**28.8**	26.2	**0.014**
p-resistin (ng/ml)	**4.09**	2.11	**4.02**	2.00	**4.12**	2.15	0.59
SF-resistin (ng/ml)	**0.752**	0.670	**0.742**	0.783	**0.790**	0.588	0.13
cart-resistin (ng/ 10 mg cartilage)	**117**	111	**159**	135	**105**	60.0	**0.012**

p value was calculated for comparison between female and male subjects using the Mann-Whitney test. Synovial fluid was analyzed according to availability, from 91 patients (60/31 females/males); cartilage samples were available and cultured from 97 patients (60/37 females/males). Statistically significant p values are printed in bold.

Table 6 Correlations between adipokines and BMI, and age, in the whole study population and in gender subgroups

		all (n=100)		females (n=62)		males (n=38)	
		BMI (kg/m2)	age (years)	BMI (kg/m^2)	age (years)	BMI (kg/m^2)	age (years)
p-leptin (ng/ml)	r	**0.67**	-0.20	**0.74**	-0.20	**0.59**	**-0.36**
	p	<0.001	0.052	<0.001	0.12	<0.001	0.025
SF-leptin (ng/ml)	r	**0.66**	-0.14	**0.73**	-0.16	**0.54**	-0.35
	p	<0.001	0.19	<0.001	0.22	0.002	0.050
cart-leptin (ng/10mg cartilage)	r	**0.67**	**-0.23**	**0.76**	-0.23	**0.48**	**-0.39**
	p	<0.001	0.026	<0.001	0.077	0.003	0.018
p-adiponectin (µg/ml)	r	-0.13	**0.25**	**-0.26**	0.13	-0.18	**0.47**
	p	0.22	0.012	0.041	0.31	0.31	0.005
SF-adiponectin (µg/ml)	r	-0.08	**0.28**	-0.22	0.18	-0.006	**0.46**
	p	0.48	0.009	0.086	0.18	0.97	0.012
cart-adiponectin (pg/mg cartilage)	r	0.04	**0.27**	-0.01	0.16	-0.04	**0.49**
	p	0.73	0.008	0.97	0.23	0.80	0.002
p-resistin (ng/ml)	r	0.16	0.01	0.22	0.01	0.13	-0.01
	p	0.11	0.92	0.086	0.94	0.42	0.95
SF-resistin (ng/ml)	r	0.08	**0.27**	0.05	**0.32**	0.05	0.06
	p	0.48	0.013	0.70	0.015	0.80	0.76
cart-resistin (ng/10mg cartilage)	r	**0.39**	0.07	**0.34**	0.06	0.27	0.12
	p	<0.001	0.51	0.012	0.66	0.11	0.48

r Spearman's rho; p statistical significance; SF synovial fluid; p plasma, cart cartilage released adipokine concentration measured in cartilage culture medium; Synovial fluid sample was available from 91 patients (60/31 females/males); cartilage sample was available from 97 patients (60/37 females/males); Statistically significant correlation coefficients are printed in bold.

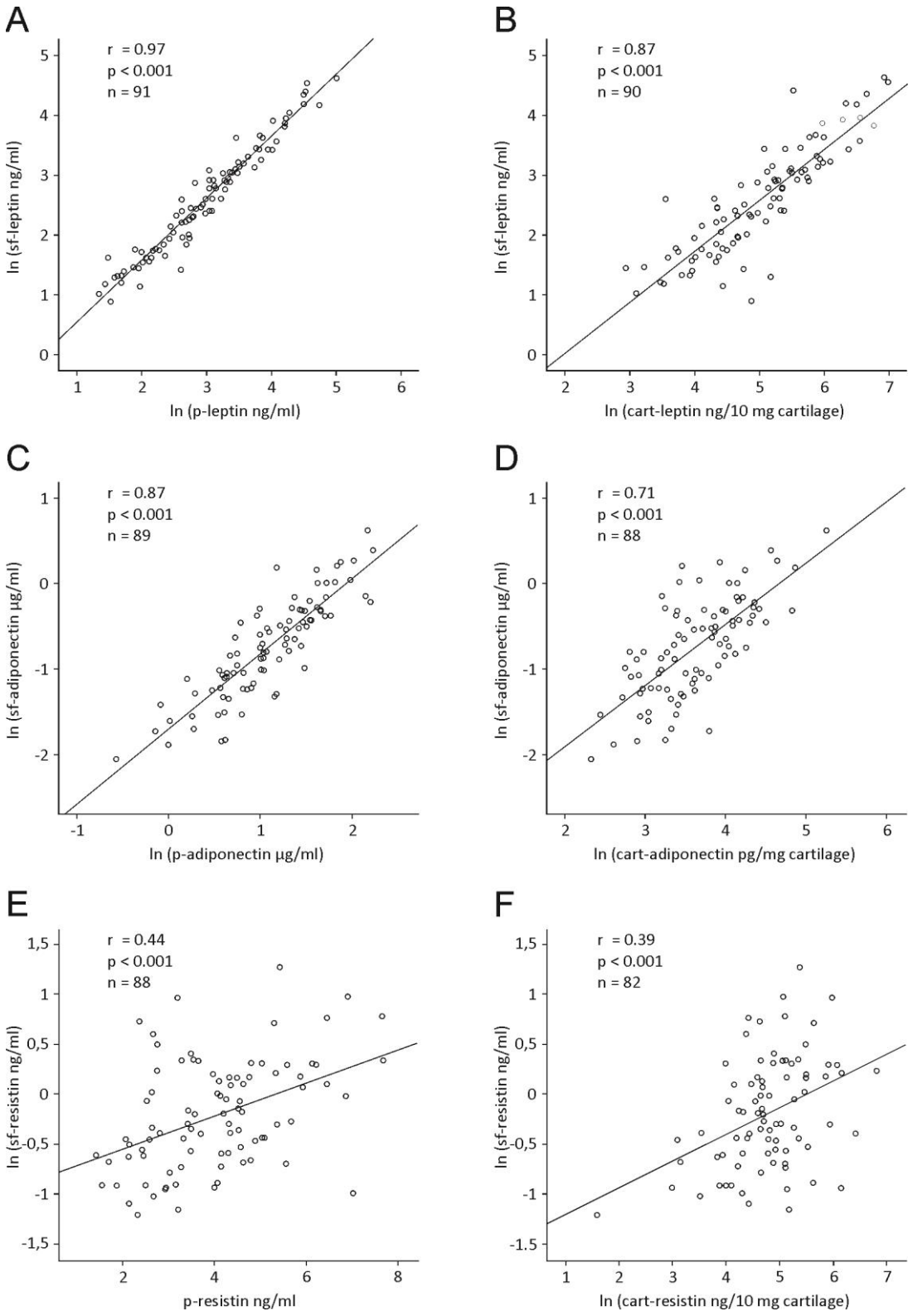

Figure 5 Scatterplots showing correlations of adipokines between different compartments. Pearson correlation coefficient (r), p value and n according to sample availability are indicated. Natural logarithm transformations were carried out where appropriate to obtain normally distributed variables. (E-F reprinted with permission from Koskinen et al. 2014, Scand J Rheumatol 43:249-53)

2 Associations of adipocytokines to clinical and biological markers of OA (I, III V-VI)

2.1 Associations of adipocytokines to catabolic and proinflammatory factors (I, III, V-VI)

2.1.1 Associations with MMPs

All of the levels of the measured adipokines in synovial fluid in the whole patient sample correlated with synovial fluid MMP-1 and MMP-3 amounts (Table 7). Because of the strong connection between leptin and BMI (Table 6), leptin was analyzed in BMI subgroups (obese > 30 kg/m^2; non-obese < 30 kg/m^2). In the BMI subgroups, leptin levels were found to correlate with the MMPs only in the obese group (Figure 6). It is noteworthy that no correlation was present between BMI and MMPs. Adiponectin was analyzed in gender subgroups since gender was the main demographic factor to explain adiponectin levels (Table 5). In synovial fluid, the adiponectin level correlated with MMP-1 and MMP-3 only in women ($r=0.30$, $p=0.023$; $r=0.30$, $p=0.023$, respectively).

In the cartilage culture media, adiponectin levels correlated statistically significantly with those of MMP-1 and MMP-3 in the entire patient sample (Table 7) and in females ($r=0.60$, $p<0.001$; $r=0.47$, $p<0.001$, respectively), and almost significantly with MMP-3 in males (Figure 7C). MMP-13 levels were detectable only in the cartilage culture media, and were found to correlate with cartilage produced adiponectin (in entire patient sample: Table 7, and in gender subgroups: males: $r=0.49$, $p=0.002$; females: $r=0.46$, $p<0.001$) and resistin (Table 7).

In the circulation, the concentrations of adipokines did not correlate with MMP-1 or MMP-3, except for the adiponectin level with MMP-3 in the male subgroup (see below / associations with biomarkers).

2.1.2 Associations with IL-6

IL-6 concentrations correlated with adiponectin levels (in the entire patient sample: Table 7 and in females: $r=0.32$, $p=0.002$) and with resistin levels in synovial fluid

(Table 7). The level of leptin correlated statistically significantly with that of IL-6 only in synovial fluid in the subgroup of patients in the highest quartile by BMI (BMI > 34.9 kg/m^2; r=0.46, p=0.030).

In the cartilage culture media, positive correlations were found between levels of adiponectin and IL-6 in the whole patient sample (Table 7) and in both gender subgroups (males: r=0.42, p=0.018, Figure 7B; females: r=0.41, p=0.001). In plasma, no correlations between the concentrations of adipokines and IL-6 were found except for a weak correlation with the plasma leptin level (Table 7).

2.1.3 Associations with NO

NO was measured in the cartilage culture media and its level was found to correlate with cartilage released adiponectin in the whole patient sample (Table 7) and in both gender subgroups (males: r=0.43, p<0.012, Figure 7A; females: r=0.47, p<0.001), as well as with resistin concentrations (Table 7).

Table 7 Correlations between adipokines and catabolic and proinflammatory factors measured in plasma, synovial fluid and cartilage culture medium int the whole study population

		Leptin			Adiponectin			Resistin		
		plasma (ng/ml)	SF (ng/ml)	cart (ng/10 mg cartilage)	plasma (µg/ml)	SF (µg/ml)	cart (pg/mg cartilage)	plasma (ng/ml)	SF (ng/ml)	cart (ng/10 mg cartilage)
p-MMP-1 (pg/ml)	r	-0.04			0.05			-0.13		
	p	0.671			0.609			0.200		
	n	98			95			98		
SF-MMP-1 (ng/ml)	r		**0.29**			**0.32**			**0.31**	
	p		0.006			0.003			0.004	
	n		87			85			85	
cart-MMP-1 (pg/mg cartilage)	r			0.16			**0.52**			**0.22**
	p			0.110			<0.001			0.033
	n			97			97			92
p-MMP-3 (ng/ml)	r	-0.20			0.03			0.17		
	p	0.044			0.748			0.086		
	n	100			97			100		
SF-MMP-3 (ng/ml)	r		**0.30**			**0.34**			**0.22**	
	p		0.004			0.001			0.037	
	n		90			88			87	
cart-MMP-3 (ng/mg cartilage)	r			0.12			**0.44**			0.10
	p			0.263			<0.001			0.330
	n			97			97			92
cart-MMP-13 (pg/mg cartilage)	r			0.10			**0.49**			**0.31**
	p			0.313			<0.001			0.002
	n			97			97			92
p-IL-6 (pg/ml)	r	**0.25**			-0.01			0.20		
	p	0.013			0.911			0.051		
	n	100			97			100		
SF-IL-6 (pg/ml)	r		0.18			**0.32**			**0.38**	
	p		0.097			0.002			<0.001	
	n		91			89			88	
cart-IL-6 (pg/mg cartilage)	r			0.13			**0.42**			0.20
	p			0.198			<0.001			0.062
	n			97			97			92
cart-NO2 (pmol/mg cartilage)	r			0.13			**0.45**			**0.33**
	p			0.222			<0.001			0.001
	n			97			97			92

r Spearman's correlation coefficient; p statistical significance; n number of cases according to the sample availability; p- plasma; SF synovial fluid; cart cartilage culture medium; statistically significant correlation coefficient values are written in bold.

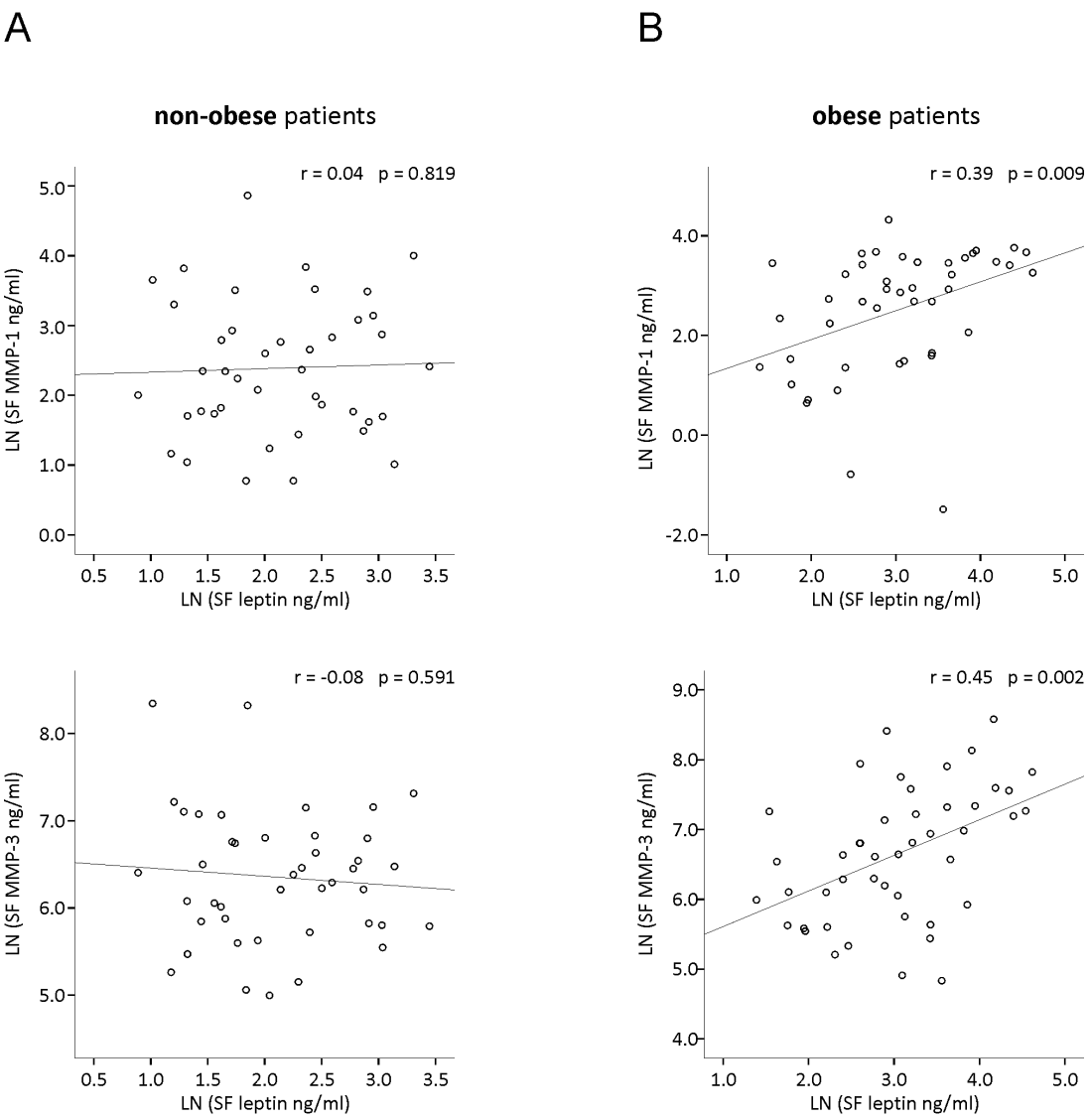

Figure 6 Scatterplots show the correlations between leptin and MMP-1 and MMP-3 in non-obese (A) and in obese (B) OA patients. Leptin and MMP levels in synovial fluid were measured by immunoassay. Natural logarithms (LN) were transformed from the synovial fluid levels of leptin and MMPs in order to have normally distributed variables for the Pearson correlation analysis. Correlation coefficients (r) and p-values are indicated. Samples were collected from 90 patients (non-obese, BMI < 30 kg/m^2, n = 44; obese, BMI > 30 kg/m^2, n = 46). (Reprinted with permission from Koskinen-Kolasa et al. Arthritis Res Ther. 2016, 18(1):215)

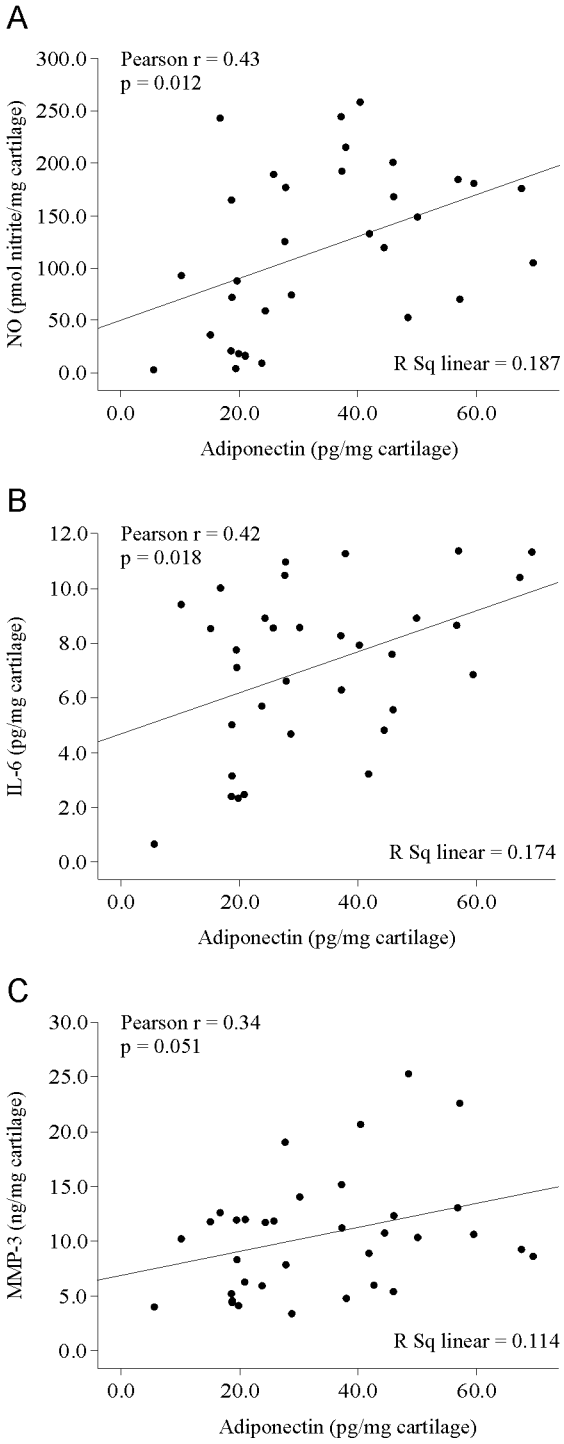

Figure 7 *Ex vivo* cartilage releases adiponectin and its concentration correlates with NO, IL-6 and MMP-3 production. Scatterplots show positive correlations between adiponectin and NO (A), IL-6 (B) and matrix metalloproteinase 3 (MMP-3) (C) released by osteoarthritis (OA) cartilage into the culture medium. Cartilage samples were collected from 35 OA patients undergoing knee replacement surgery. (Reprinted with permission from Koskinen et al. 2011, Arthritis Res Ther 13:R184)

2.2 Associations of adipocytokines to radiographic findings of OA and biomarkers of cartilage degradation (I)

Adiponectin concentrations relative to radiographic findings and biomarkers of OA were studied in the male subgroup. It was decided to analyse adiponectin in gender subgroups because gender was the main demographic factor as far as we were aware (Presle et al. 2006) and in our data to define adiponectin levels. The levels of adiponectin in plasma (Figure 8A) and in cartilage culture media (Figure 8B) were found to be the highest in the male patients with the most severe radiographic findings. After adjustment for age in the binary logistic regression analysis, a significant connection remained between the adiponectin level in cartilage culture media and radiographic severity of OA, whereas for adiponectin in plasma, the connection was not statistically significant (Table 8). Both of these associations remained significant after adjustment for BMI. In females, no differences in adiponectin levels in terms of radiographic findings were found.

There were no significant differences in leptin levels between radiographic subgroups whereas resistin concentrations in synovial fluid in females and in cartilage culture media in males were higher in the group with the radiographically most severe OA (p=0.04 and p=0.004, respectively).

Table 8 Association of adiponectin and radiographic severity of OA in males

Patient characteristics	Crude OR	95% CI	p-values	Adjusted OR	95% CI	p-values
p-adiponectin (µg/ml)	2.2	1.1 to 4.3	0.002	2.2[a]	1.1 to 4.4	0.022
				1.9[b]	0.9 to 3.8	0.090
cart-adiponectin (pg/mg cartilage)	1.1	1.0 to 1.2	0.007	1.1[a]	1.0 to 1.2	0.007
				1.1[b]	1.0 to 1.2	0.016
BMI (kg/m^2)	1	0.8 to 1.2	0.852			
age (years)	1.1	1.0 to 1.3	0.078			

[a]Adjusted for BMI, [b]Adjusted for age. Odds ratios (OR) are given for Ahlbäck grades 4 and 5 group vs grades 1 to 3 group. (Modified from Koskinen et al. 2011, Arthritis Res Ther 13:R184)

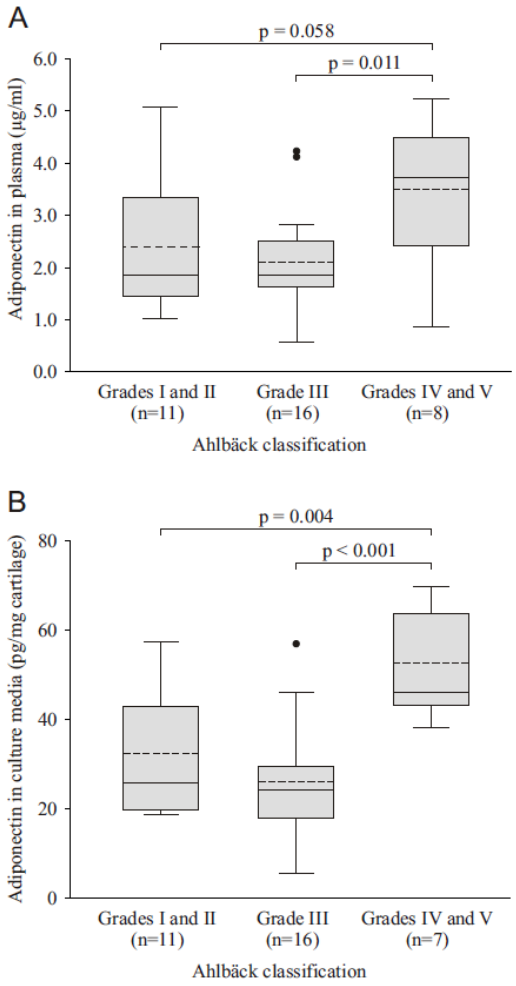

Figure 8 Adiponectin levels in plasma (A) and in cartilage culture medium (B) in OA patients classified according to the severity of knee OA evaluated using the Ahlbäck grading scale. Horizontal solid and dashed bars within the boxes represent the median and mean, respectively. Boxes represent the interquartile range. Lines outside boxes represent minimums and maximums. Outliers are indicated. (Reprinted with permission from Koskinen et al. 2011, Arthritis Res Ther 13:R184)

Plasma adiponectin also correlated with biomarkers of OA, serum COMP and plasma MMP-3 in males (Figure 9), but not in females. The levels of either COMP or p-MMP-3 did not correlate significantly with those of the other adipokines. The amounts of COMP and p-MMP-3 were both higher in the patients with the most advanced radiographic findings over the whole study population and in gender subgroups with the exception that there was no difference in the levels of p-MMP-3 in males (V).

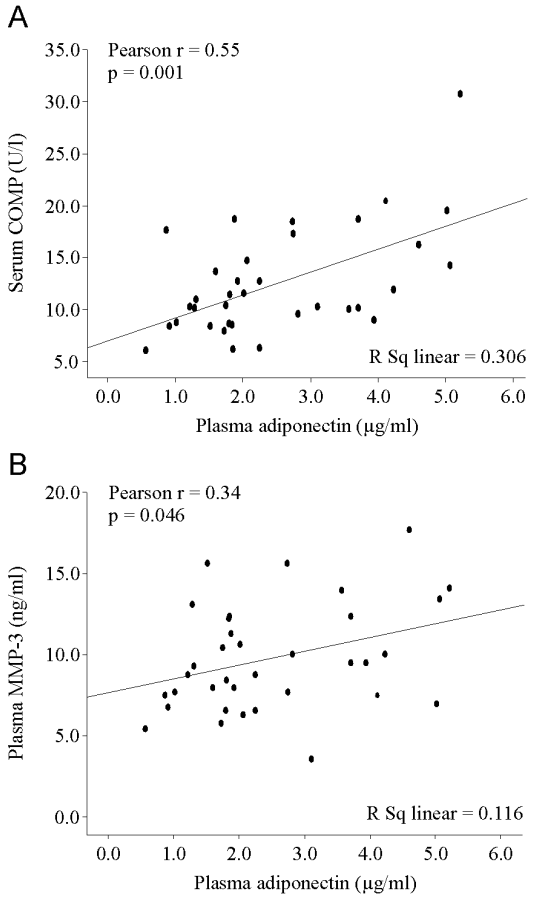

Figure 9 Scatterplot showing positive correlations between plasma adiponectin and biomarkers of osteoarthritis (OA), serum cartilage oligomeric matrix protein (COMP) (A) and plasma matrix metalloproteinase 3 (MMP-3) (B). n = 35 OA patients. (Reprinted with permission from Koskinen et al. 2011, Arthritis Res Ther 13:R184)

2.3 Association with clinical scoring of OA

We also had a clinical rating of OA, the Knee Society Score (Insall et al. 1989) available for 90 of our patients. KSS takes into consideration pain, extension of movement, alignment and stability of the osteoarthritic joint. The lower the score, the worse is the manifestation of the disease. KSS was found to have a negative correlation with the levels of leptin in the whole study population (p-leptin: r=-0.28, p=0.009; SF-leptin: r=-0.26, p=0.017; cart-leptin: r=-0.35, p=0.001), indicating that a high leptin level was associated with more severe disease. No correlation was found between KSS and the other adipokines, BMI, CRP, or biomarkers COMP and plasma MMP-3. There was also a weak negative correlation between KSS and p-IL-6 levels (r=-0.25, p=0.017). KSS was lower in the group of patients with the most serious radiographic findings (Ahlbäck grades IV-V vs grades I-III) in males (p=0.026) but not in females (p=0.83).

3 Effects of adiponectin on chondrocytes and cartilage (I)

The potential effects of adiponectin on cartilage were studied in primary chondrocytes and in cartilage explants, both obtained from OA patients undergoing knee replacement surgery. Adiponectin was shown to increase the production of NO, IL-6, MMP-1 and MMP-3 in a dose-dependent manner (Figure 10).

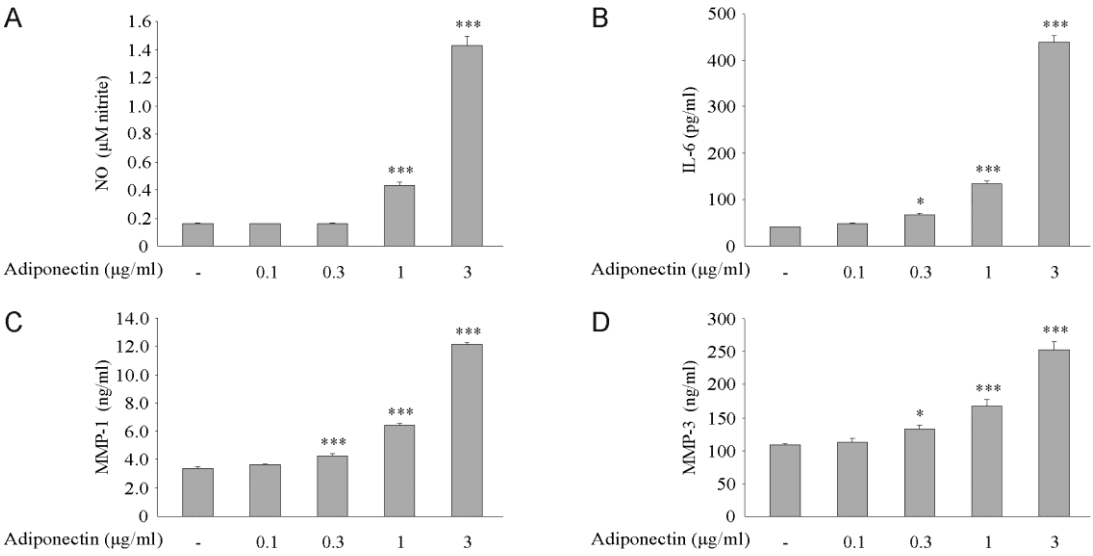

Figure 10 Adiponectin enhances NO, IL-6, MMP-1 and MMP-3 production in human primary chondrocytes in a dose-dependent manner. Chondrocytes obtained from patients with osteoarthritis (OA) were treated with increasing concentrations of adiponectin (0.1 to 3 μg/ml). Concentrations of NO (A) were measured by the Griess reaction), and those of IL-6 (B), matrix metalloproteinase 1 (MMP-1) (C) and MMP-3 (D) by ELISA in the culture medium after 24-hour incubation. The figure shows the results of a representative experiment which was repeated three times (i.e., with cells from three donors) with similar results. Results are expressed as means ± SEM (n = 4). *$p < 0.05$ and ***$p < 0.001$ compared to the control sample. (Reprinted with permission from Koskinen et al. 2011, Arthritis Res Ther 13:R184)

Since MAP kinases have been proposed as therapeutic targets in OA (Loeser et al. 2008; Saklatvala 2007), their role in mediating adiponectin-evoked responses in chondrocytes was studied. Adiponectin was shown to activate p38, Erk1/2 and JNK MAP kinases in human primary OA chondrocytes (Figure 11).

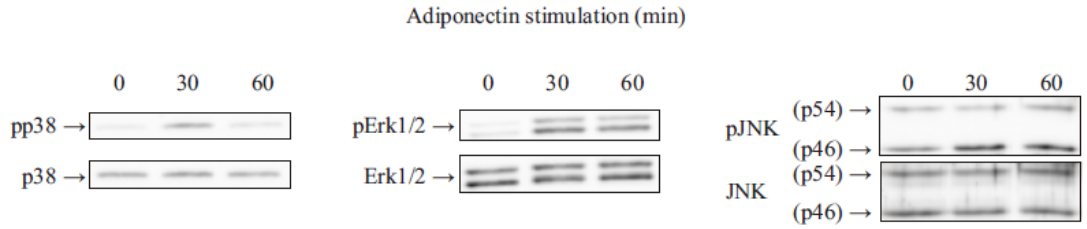

Figure 11 The effect of adiponectin (3 μg/ml) on mitogen-activated protein kinase (MAPK) phosphorylation in human primary chondrocytes obtained from patients with OA. The figure shows the results of a representative experiment which was repeated three times (i.e., with cells from three donors) with similar results. MAPKs were determined by Western blot analysis at baseline and at 30 and 60 minutes after addition of adiponectin. Erk1/2 = extracellular signal-regulated kinase 1/2; JNK: c-Jun N-terminal kinase. (Reprinted with permission from Koskinen et al. 2011, Arthritis Res Ther 13:R184)

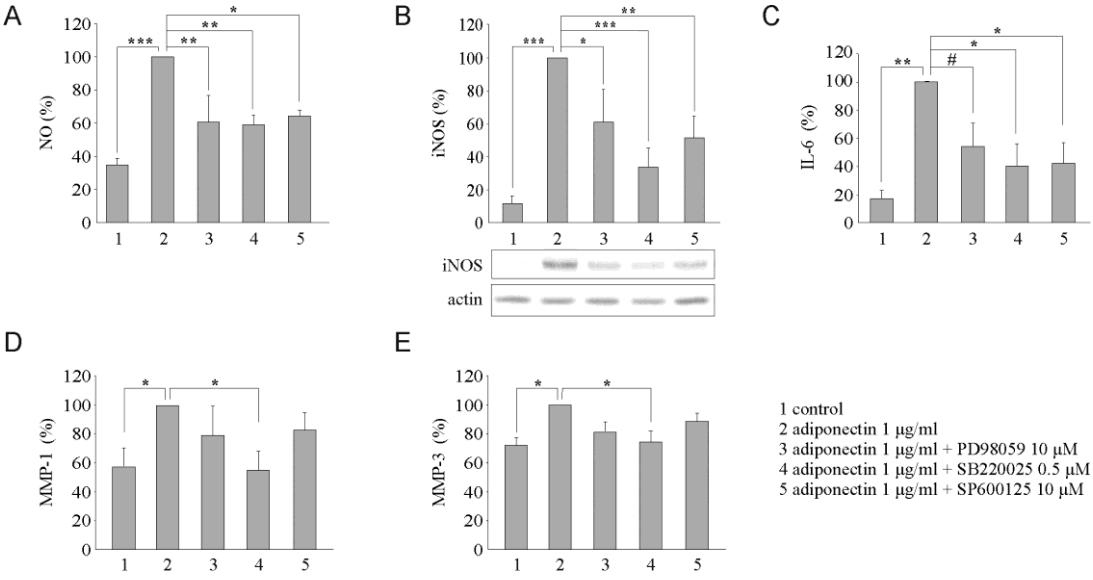

Figure 12 Mitogen-activated protein kinase pathways are involved in adiponectin induced NO, IL-6, MMP-1 and MMP-3 production in osteoarthritic cartilage. The effects of mitogen-activated protein kinase inhibitors on adiponectin-induced NO production (A), inducible nitric oxide synthase (iNOS) expression (B), IL-6 production (C), matrix metalloproteinase 1 (MMP-1) production (D) and MMP-3 production (E) in human osteoarthritic cartilage. Cartilage explants were incubated for 42 hours with adiponectin (1 μg/ml) with the indicated inhibitor. Samples were collected from six patients in (A) and (B) and from five patients in (C) through (E). Results are expressed as means ± SEM. #p < 0.1, *p <0.05, **p < 0.01 and ***p < 0.001 compared to explants treated with adiponectin alone. PD98059 = extracellular signal-regulated kinase ½ (Erk1/2) inhibitor; SB220025 = p38 inhibitor; SP600125 = c-Jun N-terminal kinase (JNK) inhibitor. (Reprinted with permission from Koskinen et al. 2011, Arthritis Res Ther 13:R184)

The inclusion of pharmacological inhibitors of MAPK pathways in the experiments revealed that activation of p38 was required for adiponectin-induced production of NO, IL-6, MMP-1 and MMP-3. In addition, Erk1/2 was involved in adiponectin-induced production of NO and IL-6, and JNK was involved in adiponectin-induced NO production (Figure 12). The expression of iNOS was inhibited by the MAPK inhibitors in the same manner as the production of NO (Figure 12), suggesting that adiponectin increases iNOS expression and subsequent NO production through MAPK activation.

4 Effects of leptin on chondrocytes and cartilage and regulatory mechanisms (II-V)

4.1 Effects of leptin on cartilage *ex vivo* (II-III)

The effects of leptin on the production of proinflammatory and catabolic factors were studied in cultured cartilage obtained from OA patients undergoing knee replacement surgery. Leptin increased the production of NO, PGE_2, IL-6, IL-8, MMP-1, MMP-3 and MMP-13, and expression of iNOS and COX-2 in cartilage (Figures 13-14).

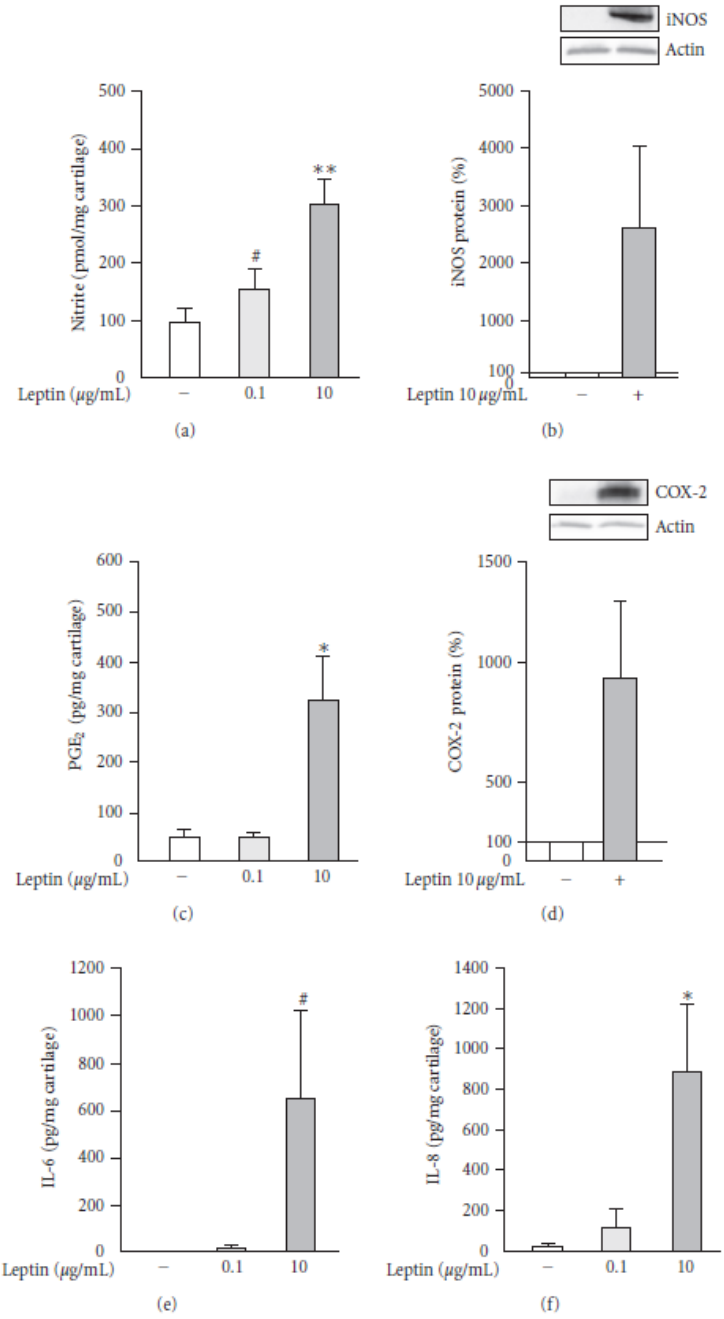

Figure 13 The effect of leptin on NO production (a), iNOS protein expression (b), PGE_2 production (c), COX-2 protein expression (d), IL-6 production (e), and IL-8 production (f) in human OA cartilage. Cartilage explants were incubated with leptin (0.1 μg/mL or 10 μg/mL) for 48 hours. NO production (a) was measured as nitrite accumulation in the culture medium by Griess reaction. Expression of iNOS protein (b) and COX-2 protein (d) were measured by Western blot. PGE_2 production (c) in the culture medium was measured by RIA. Concentrations of IL-6 (e), and IL-8 (f) in the culture medium were measured by ELISA. Results are expressed as pmol/mg cartilage (a), as percentages in comparison with control sample ((b) and (d)) or pg/mg cartilage ((c), (e), and (f)). Values are mean ± SEM. Samples were collected from 6 patients (n = 6) in (a) and (c), from 3 patients (n = 3) in (b) and (d), and from 7 patients (n = 7) in (e) and (f). #: $p < 0.2$, ∗: $p < 0.05$, and ∗∗: $p < 0.01$ as compared to control explants incubated in absence of exogenous leptin. (Reprinted with permission from Vuolteenaho et al. 2009, Mediators Inflamm. 2009;2009:345838)

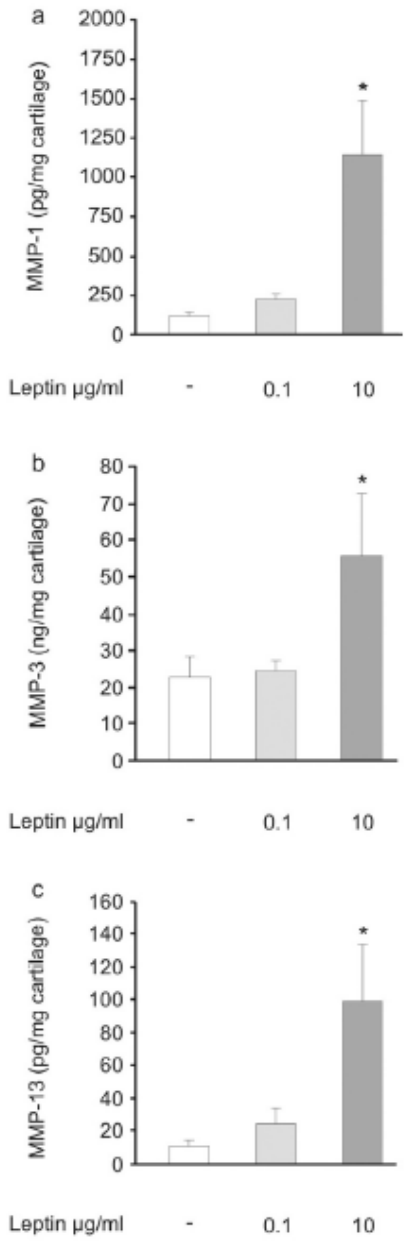

Figure 14 The effect of leptin on MMP-1 (a), MMP-3 (b), and MMP-13 (c) production in human OA cartilage. Cartilage explants were incubated with leptin (0.1 µg/ml or 10 µg/ml) for 48h. MMP concentrations in the culture medium were measured by immunoassay. Results are expressed as pg of MMP/mg of cartilage (a and c) or ng of MMP/mg of cartilage (b). Values are mean ± SEM. Cartilage samples were collected from 7 patients. *$p<0.05$ as compared to control explants incubated in the absence of exogenous leptin. (Reprinted with permission from Koskinen et al. 2011, Clin Exp Rheumalol 29:57-64)

According to the literature, leptin is known to signal through JAK-STAT, MAPK, PKC and NF-κB pathways in some cell types (Fruhbeck 2006; Sweeney 2002). We studied whether activation of these signaling pathways by leptin triggered the production of catabolic and proinflammatory factors by using pharmacological

inhibitors of these pathways. All studied leptin responses were dependent on NF-κB activation and on MAPK JNK activation. In addition, PKC was involved in leptin-induced NO, IL-6, IL-8, and MMP-1, MMP-3 and MMP-13 production; p38 in PGE_2, MMP-1 and MMP-13 production; Erk1/2 in PGE_2 production; and JAK3 in NO, MMP-3 and MMP-13 production (Figures 15-16).

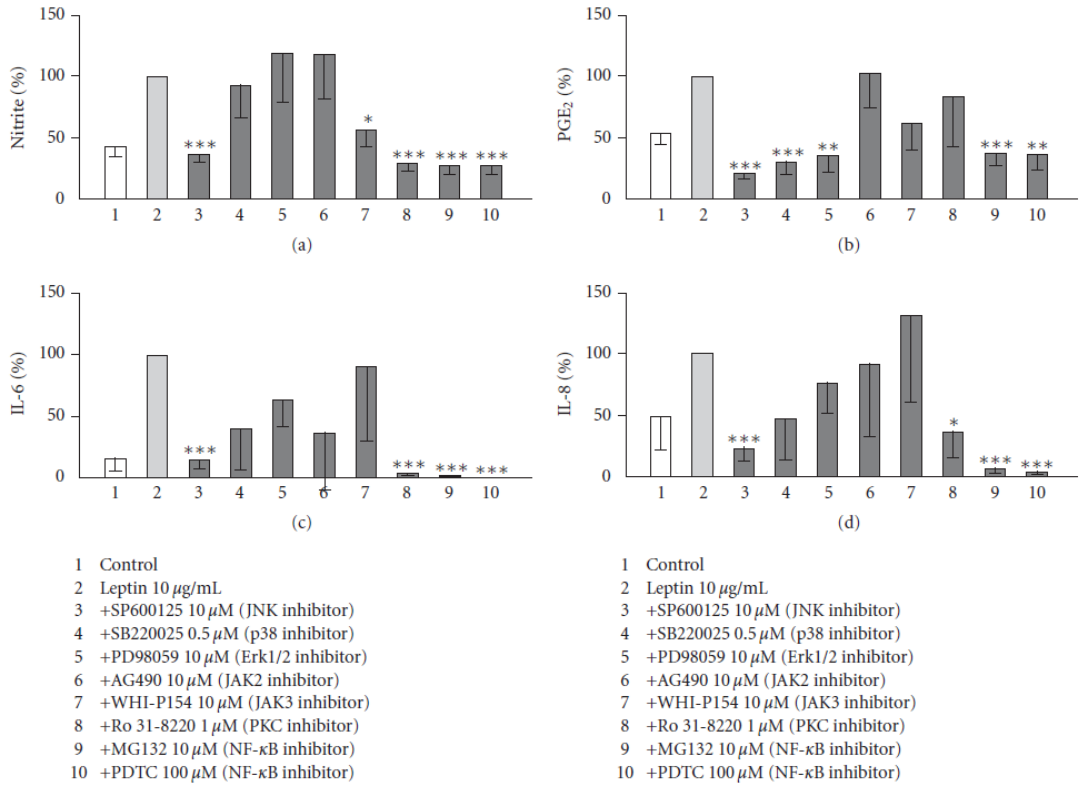

Figure 15 The effects of signaling pathway inhibitors on leptin-induced NO (a), PGE_2 (b), IL-6 (c), and IL-8 (d) production in human OA cartilage. Cartilage explants were incubated for 48 hours with leptin (10 μg/mL) and the inhibitor indicated. NO production (a) was measured as nitrite accumulation in the culture medium by Griess reaction. PGE_2 production (b) in the culture medium was measured by RIA. Levels of IL-6 (c), and IL-8 (d) in the culture medium were measured by ELISA. Leptin-induced NO/ PGE_2/IL-6/IL-8 production was set as 100%, and the other values were related to that. The results are expressed as mean ± SEM. Samples were collected from 7 patients in (a) and (b) (n = 7) and from 6 patients in (c) and (d) (n = 6). ∗: $p < .05$, ∗∗: $p < .01$, and ∗∗∗: $p < .001$ as compared to explants treated with leptin alone. (Reprinted with permission from Vuolteenaho et al. 2009, Mediators Inflamm. 2009;2009:345838)

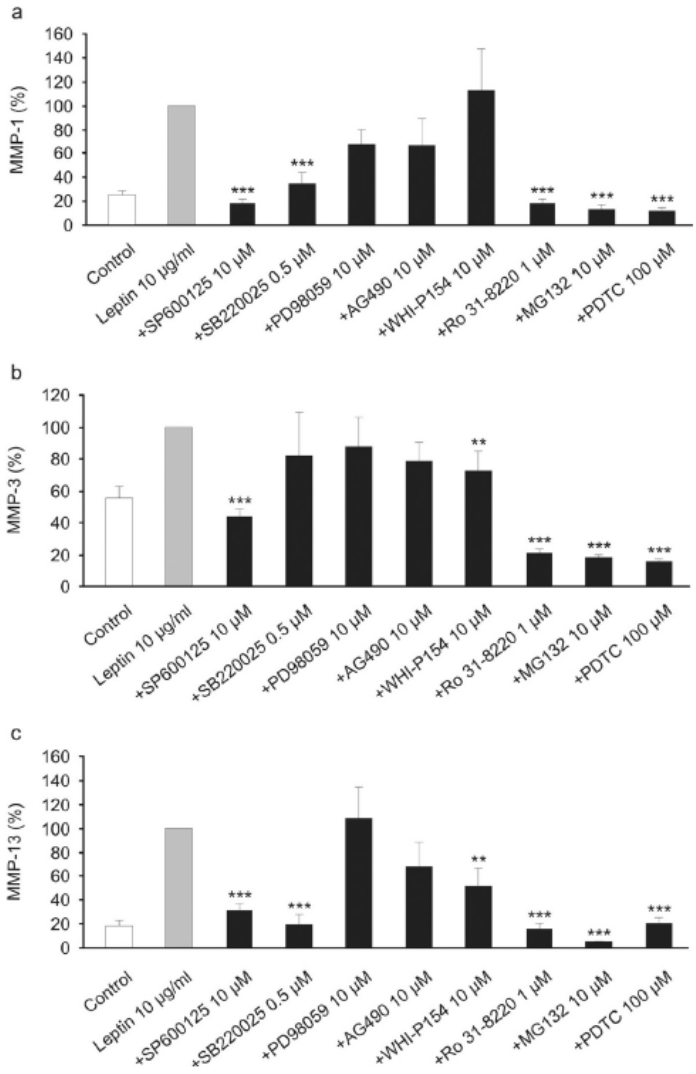

Figure 16 The effects of selected signal transduction inhibitors on leptin-induced MMP-1 (a), MMP-3 (b) and MMP-13 (c) production in human OA cartilage. Cartilage explants were incubated for 48 hours with leptin (10 µg/ml) and the inhibitor indicated. MMP concentrations in the culture medium were measured by immunoassay. Results are expressed as percentages in comparison with samples treated with leptin only. Values are mean ± SEM. Cartilage samples were collected from 5 patients. **p<0.01 and ***p<0.001 as compared to explants treated with leptin alone. SP600125 - JNK inhibitor; SB220025 - p38 inhibitor; PD98059 - Erk1/2 inhibitor; AG490 - JAK2 inhibitor; WHI-P154 - JAK3 inhibitor; Ro 31-8220 - PKC inhibitor; MG132 and PDTC - NF-κB inhibitors. (Reprinted with permission from Koskinen et al. 2011, Clin Exp Rheumalol 29:57-64)

The inhibitor of iNOS enzyme, 1400W, efficiently inhibited leptin induced NO production as expected. We observed that 1400W also inhibited leptin-induced PGE_2, IL-6 and IL-8 production suggesting that NO modulates the effect of leptin on the production PGE_2, IL-6 and IL-8. This was proven by inclusion of an NO donor into the incubation; this increased the production of PGE_2, IL-6 and IL-8 when iNOS activity was inhibited by 1400W (Figure 17).

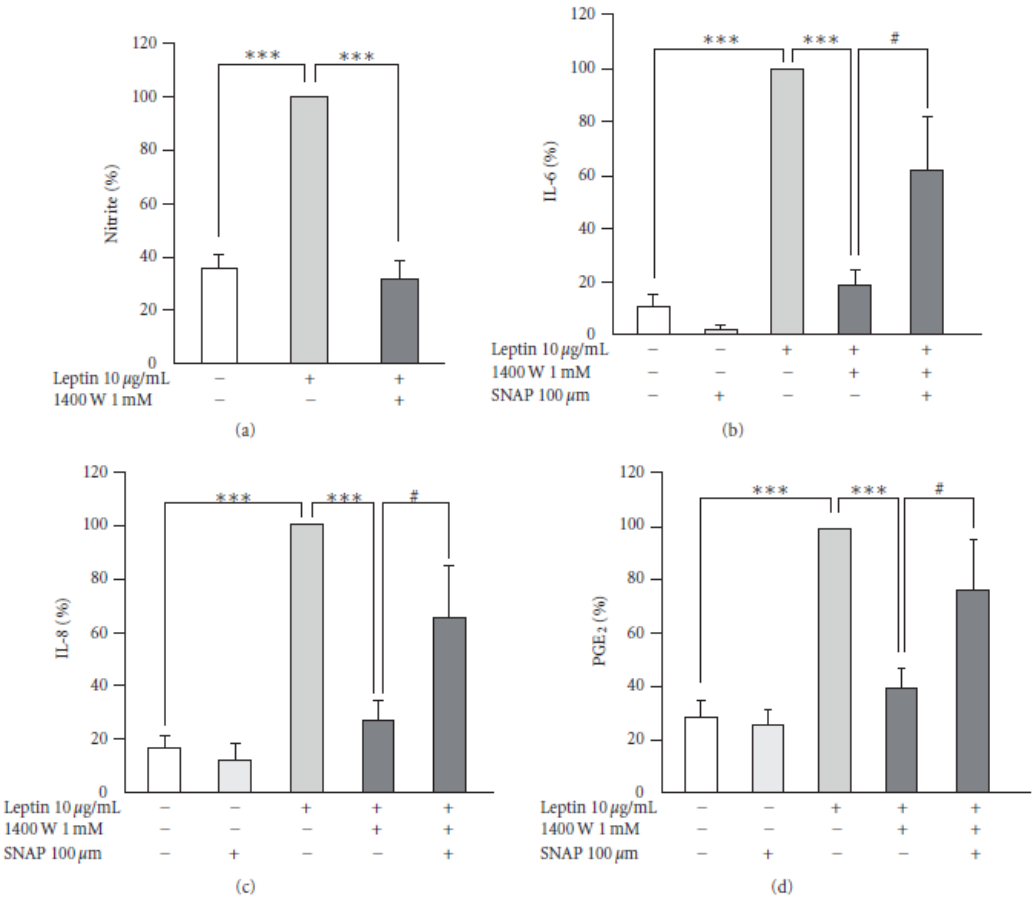

Figure 17 The effects of the selective iNOS inhibitor 1400W (1mM) ((a)–(d)) with and without the NO-donor SNAP ((b)–(d)) on leptin (10 μg/mL) induced NO (a), IL-6 (b), IL-8 (c), and PGE_2 (d) production in OA cartilage during 48 hours incubation. In the culture medium, NO production (a) was measured as nitrite accumulation by Griess reaction, levels of IL-6 (b) and IL-8 (c) were measured by ELISA, and PGE_2 production (d) was measured by RIA. Leptin-induced NO / PGE_2 / IL-6 / IL-8 production was set as 100%, and the other values were related to that. The results are expressed as mean ± SEM. Samples were collected from 8 patients in (a) (n = 8), from 6 patients in (b) (n = 6), from 8 patients in (c) (n = 8), and from 6 patients in (d) (n = 6). #: $p < 0.2$, ∗∗∗: $p < 0.001$. (Reprinted with permission from Vuolteenaho et al. 2009, Mediators Inflamm. 2009;2009:345838)

4.2 Regulation of the effects of leptin in chondrocytes (IV, V)

Already in the first study (II), we observed rather extensive variability in the magnitude of leptin-induced responses in the production of catabolic and proinflammatory factors between the cartilage samples from different donors. Therefore, we wanted to explore the possible mechanisms or clinical features which could explain this phenomenon.

None of the clinical factors measured clearly explained the variable leptin responses, as the responses did not correlate with BMI or age, and they did not differ

between genders, between diabetic vs non-diabetic patients or between groups formed by radiographic severity.

To examine possible biological factors contributing to the effects of leptin in chondrocytes, we studied soluble leptin receptor sOb-R and SOCS-3.

4.2.1 Soluble leptin receptor (sOb-R) (IV)

SOb-R modulates the bioavailability of leptin by binding free active leptin into inactive complexes (Peelman et al. 2014), and in that way, it determines the biological effects of leptin. In our study population, the level of sOb-R was higher in non-obese patients than in obese patients, both in circulation and in synovial fluid (Figure 18). There were no differences in the levels of sOb-R in synovial fluid or in circulation between genders (p=0.63; p=0.76, respectively). However, the ratio of leptin/sOb-R, both in synovial fluid and in circulation, was higher in females than males ($p < 0.001$ for both synovial fluid and circulation), pointing to higher active leptin levels in females. The leptin/sOb-R ratio in synovial fluid was more than twice the value of that in circulation ($p<0.001$) pointing to higher active leptin levels in synovial fluid than in circulation. Cartilage did not produce measurable amounts of sOb-R in the culture.

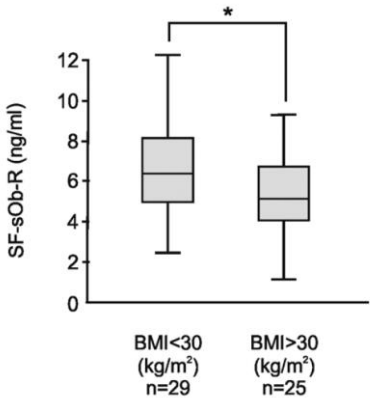

Figure 18 Levels of soluble leptin receptor (sOb-R) in synovial fluid samples were lower in obese (BMI > 30 kg/m^2) than in non-obese (BMI < 30 kg/m^2) osteoarthritis patients. sOb-R concentrations were measured by ELISA. The horizontal solid bar within the boxes represents the median; the boxes refer to the interquartile range and the lines outside boxes display minimum and maximum. Differences between groups were tested by unpaired t test. *p<0.01. (Reprinted with permission from Vuolteenaho et al. 2012, 71:1912-3.)

4.2.2 Suppressor of cytokine signaling-3 (SOCS-3) (IV, V)

Because SOCS-3 is an important regulator of cytokine effects and also known to regulate leptin responses in central nervous system, we hypothesized that SOCS-3 could regulate the effects of leptin also in chondrocytes. SOCS-3 was found to explain much of the variance in the leptin responses between cartilage specimens from different OA patients, as leptin responses were significantly higher in cartilage with low SOCS-3 expression (Figure 19). This suggests that SOCS-3 regulates leptin-induced proinflammatory and catabolic responses in chondrocytes. Statistical analysis revealed that not only SF-leptin, but also SOCS-3 explains the synovial fluid levels of MMP-1 and MMP-3 in the obese patients (Table 9).

Table 9 Associations between MMP-1, MMP-3 and leptin levels in synovial fluid and SOCS-3 expression in cartilage from non-obese and obese patients with OA

		non-obese, BMI < 30 kg/m^2		obese, BMI > 30 kg/m^2	
Dependent variable	Covariates	R^2adj.	p	R^2adj.	p
LN (SF MMP-1)		0.15		0.30	
	LN SOCS-3		0.818		**0.007**
	LN (SF leptin)		0.884		**0.023**
LN (SF MMP-3)		0.03		0.27	
	LN SOCS-3		0.608		**0.004**
	LN (SF leptin)		0.733		**0.015**

p values are calculated for covariates in ANOVA (analysis of variance) modeling. The model is controlled for inter-gel variation in SOCS-3 expression levels. Analysis was performed in BMI subgroups. Natural logarithms (LN) were formed where appropriate. (Modified from Koskinen-Kolasa et al. Arthritis Res Ther. 2016, 18(1):215)

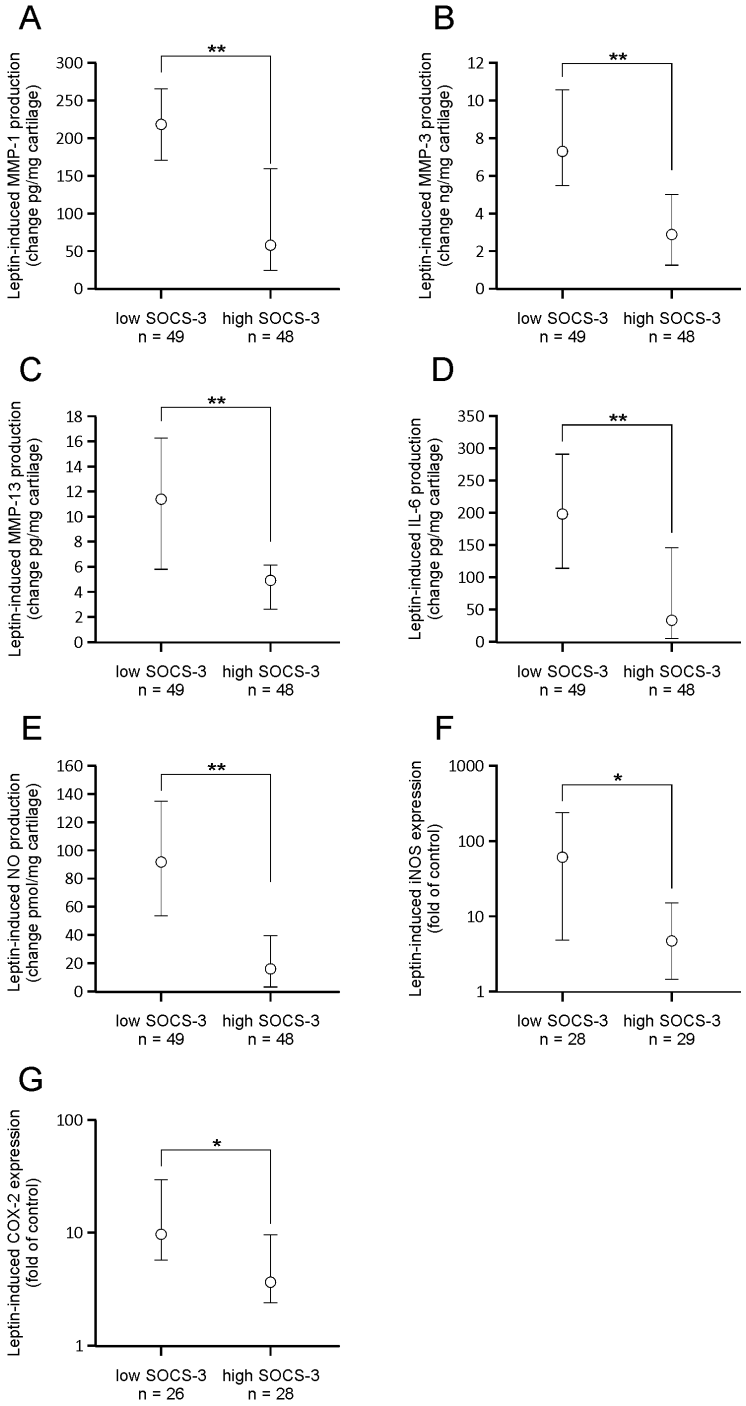

Figure 19 Leptin-induced production/expression of matrix metalloproteinase-1 (MMP-1) (a), MMP-3 (b), MMP-13 (c), interleukin-6 (IL-6) (d), nitric oxide (NO) (e), inducible nitric oxide synthase (iNOS) (f) and cyclooxygenase-2 (COX-2) (g) in cartilage from patients with OA in subgroups stratified by suppressor of cytokine signaling-3 (SOCS-3) expression in the non-treated cartilage. Human osteoarthritic cartilage was cultured with leptin (10 µg/ml) for 42 hours. Concentrations of MMP-1, MMP-3, MMP-13 and IL-6 were measured by ELISA, NO was determined as its metabolite nitrite by the Griess reaction and iNOS and COX-2 proteins were analyzed by western blotting. The circles represent the median change in the leptin-induced effects. The whiskers represent the 95% confidence interval of the median. Numbers of patients from whom the cartilage samples were collected are indicated. Statistical significance was calculated using the Mann–Whitney test; *$p < 0.05$, **$p < 0.01$. (Reprinted with permission from Koskinen-Kolasa et al. Arthritis Res Ther. 2016, 18(1):215)

In order to further test whether SOCS-3 regulates leptin-induced responses in chondrocytes, SOCS-3 was downregulated with siRNA in the H4 murine chondrocyte cell line. In these cells, proinflammatory and catabolic leptin responses, including expression of MMP-3, MMP-13, IL-6 and iNOS, both at mRNA and protein levels, were significantly higher than in wild type cells (Figure 20).

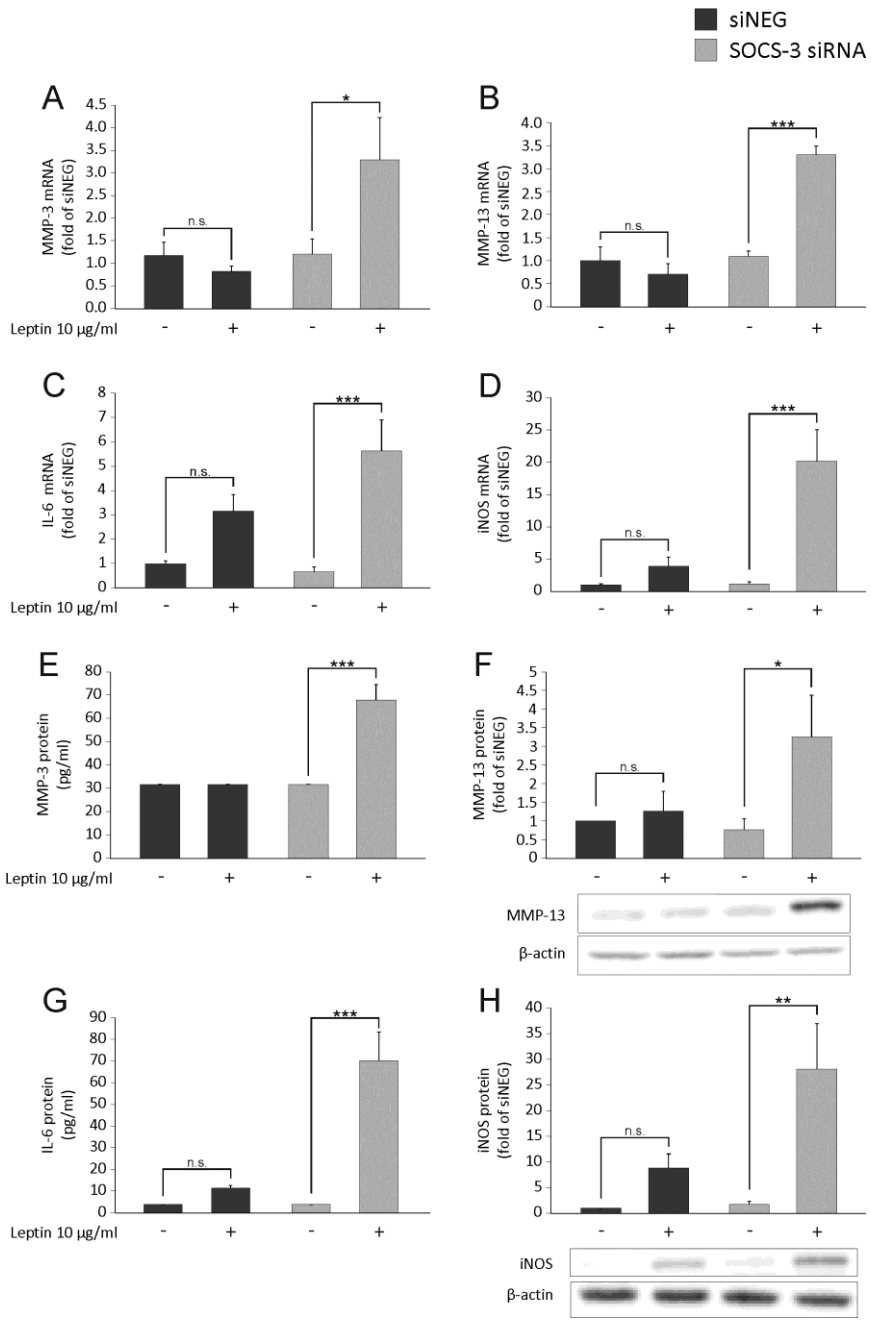

Figure 20 The effect of silencing of SOCS-3 by siRNA on leptin-induced mRNA expression levels of a) MMP-3, b) MMP-13, c) IL-6 and d) iNOS, and protein expression levels of e) MMP-3, f) MMP-13, g) IL-6 and h) iNOS in H4 murine chondrocytes. The cells were transfected with SOCS-3 siRNA or non-targeting siRNA (siNEG) and treated with leptin (10 µg/ml) for 4 (c and d), 8 (a, b and h) or 24 (e, f and g) hours. The time points were chosen based on the assumed time of peak / rise in the expression of the measured mRNAs / proteins. mRNA expression (a-d) was determined by quantitative RT-PCR, the levels of MMP-3 (e) and IL-6 (g) in the culture media supernatants by ELISAs, and MMP-13 and iNOS expression in the chondrocyte lysates by western blotting. Results are expressed as means ±SEM. n=6 in a-e and g, and n=3 in f and h. MMP-3 protein level in siNEG and in non-treated SOCS-3 siRNA samples was below the detection limit and is set as half of the lowest standard. Representative bands of the western blots are shown. Statistical analysis was carried out by two-way ANOVA with Bonferroni multiple comparisons post hoc test. *p < 0.05, **p < 0.01, ***p < 0.001, n.s. = not significant. (Reprinted with permission from Koskinen-Kolasa et al. Arthritis Res Ther. 2016, 18(1):215)

The relative SOCS-3 expression in cartilage in BMI subgroups was analyzed in part of the study population (the first 28 patients). The expression of SOCS-3 was found to be significantly lower in obese than non-obese patients (Figure 21), pointing to a dysregulation of this mechanism in leptin metabolism in obese individuals.

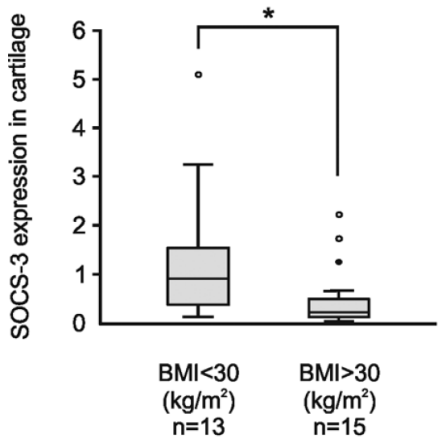

Figure 21 SOCS-3 expression levels in cartilage in obese (BMI > 30 kg/m2) and in non-obese (BMI < 30 kg/m2) patients with OA. SOCS-3 expression in cartilage samples was determined by Western blotting. Each Western blot gel was loaded with protein samples (20 µg) extracted from cartilage samples and a pooled control protein sample. Densitised SOCS-3 bands in individual patient samples were related against the SOCS-3 band, in the pooled control sample in the same Western blot gel resulting in comparable values of SOCS-3 expression levels between patient samples. The horizontal solid bar within the boxes represents the median; the boxes refer to the interquartile range and the lines outside boxes display minimum and maximum. Outliers are indicated as circles. Differences between groups were tested by unpaired t test. *p<0.01. (Reprinted with permission from Vuolteenaho et al. 2012, 71:1912-3.)

Discussion

1 Methodology

At the time when the present research project was started, very little was known about adipokines in rheumatic diseases. The aim of the present study was to investigate not only the effects of adipokines on cartilage, but also to develop a general view of adipokines in OA patients, i.e., to determine their levels in blood and in the joint cavity and their relationships with BMI and other characteristics of the patients. For these reasons, a cross-sectional study design was chosen. This study design introduces some limitations on the conclusions that can be drawn about causality of the found associations between adipokines and markers of inflammation and degradation. However, results emerging from experimental work support the results from the clinical data and provide insights into the causality. One of the advantages of the study is that synovial fluid, blood and cartilage samples were collected simultaneously from a comparatively large group of OA patients. In addition, leptin response experiments were carried out with the use of cartilage specimens collected from the same group of patients.

Cytokines, MMPs and NO were measured to evaluate the degree of inflammation and matrix degradation in cartilage / joint. Joint X-rays, biomarkers of OA and KSS were used to evaluate the stage, activity and symptoms of OA. The cytokines and MMPs produced by cartilage or found in the synovial fluid reflect the severity/ activity of ongoing inflammation / degradation process that might be of variable intensity in the course of time. Therefore, it is reasonable to compare levels of these factors with adipokine levels measured at the same moment in a cross- sectional design.

As we wanted to determine the relationship of adipokines with the disease stage, preoperative X-rays, available from all patients, were chosen to be analyzed. Nonetheless, it is not possible to draw conclusions about causality between adipokines and the process that led to OA in the present study design including patients with OA

with variable disease duration. However, it can be concluded that adiponectin, which was associated with radiographic severity in the present study, is either produced by more severely diseased joint or is associated with some other OA severity-related factor.

Another aspect to consider with respect to radiographic data in the present study is that all patients were going through arthroplasty, meaning that none of these individuals had early disease, instead all had severe knee OA. A narrow range in disease stage might lessen the differences that, if present, would be more easily seen in a group of patients with a larger range of the disease stage. In addition, differentiating patients with end-stage OA by their grading on a radiographic scale might have certain limitations. Though widely used, the Kellgren and Lawrence scaling system especially tends to rather roughly divide the patients with advanced OA. For this reason, the Ahlbäck grading scale was chosen for the present study, in order to divide the end-stage patients more carefully into smaller groups (Petersson et al. 1997).

Biomarkers of OA were used as a measure of ongoing disease activity in the present study. The aim of finding biomarkers has been that they could be used as a surrogate for imaging methods so that they could 1) provide more sensitive and specific information of early changes in OA, 2) predict the rate of progression of the disease or 3) serve as more sensitive outcome monitoring measures for therapeutic trials than radiologic modalities. They are also minimally invasive and easy to measure. In the present study, COMP and MMP-3 were chosen as the biomarkers of OA since both have been shown to predict radiographic progression of OA with their levels becoming more elevated as the disease becomes more severe (Jordan 2004; Lohmander et al. 2005). The weakness of systemic biomarkers is that they are not truly comparable in a disease with multiple joints involved. Because they are released by OA-affected joints, their levels tend to be higher in individuals with numerically more diseased joints. It also should be considered while interpreting the results of the present study that the level of MMP-3 is higher in males, and both, COMP and MMP-3 are associated with age, independently of OA (Manicourt et al. 1994; Jordan 2004; Lohmander et al. 2005). The number of OA joints could not be controlled in the present study which might have an impact on the results. Both biomarkers have been investigated in individuals with earlier phase OA (Jordan 2004; Lohmander et al. 2005) than in the present study; for this reason, it has to be considered that their

plasma levels do not necessarily behave in the same manner in end-stage patients. The level of COMP apparently parallels the degradation process that occurs in phases rather than steadily (Sharif et al. 2004) but there is not this kind of information available for MMP-3.

Adipokine levels were also compared against the KSS (Insall et al. 1989) that was available from 90 of the patients. This score is an indicator of clinical manifestation of OA. This scale has been developed to allow orthopedic surgeons to evaluate the need for knee replacement surgery. Since radiographic findings and symptoms of OA do not always correlate with each other, this clinical rating that considers pain (50% of the points) and clinical status of the knee joint (observed as stability, range of motion and alignment), is an interesting addition to the results. In the present study, the KSS was lower (indicating more severe symptoms) in patients with the most serious radiographic findings in males, but not in females.

There are some possible confounding factors in the present study including BMI, age and gender, which were all taken into account to the greatest possible extent. Other confounding factors include medication, metabolic diseases, particularly diabetes, other diseases and OA of other joints. BMI was not only a confounding factor but also a target of our investigation. Because of the strong correlation between BMI and leptin, adjustment of the leptin-related analysis by BMI would most likely lead to loss of statistical significance. In two recent longitudinal clinical studies, this issue was taken into account by means of statistical methods (Fowler-Brown et al. 2015; Karvonen-Gutierrez et al. 2013a). In the present study, a simple approach was used by observing the associations of leptin with measures of interest in BMI subgroups.

Adiponectin was chosen to be analyzed in gender-differentiated subgroups because gender is the main demographic factor which determines adiponectin levels. Since a positive correlation was present between adiponectin and age, adjustment for age was carried out when analyzing adiponectin levels in relation to radiographic findings.

As diabetes and the adipokines share connections, diabetes was examined as a possible confounding factor. In the present study settings, it did not explain adipokine levels or leptin responses *in vitro*. Corticosteroids might also influence adipokine levels. Charlier et al. reported that prednisolone increases the production of leptin in cultured human chondrocytes (Charlier et al. 2016). In the present study, three patients had been given intra-articular corticosteroids within three months of the study

period (but none of them within one month). The levels of adipokines, cytokines or metalloproteinases in joint cavity did not differ between these three individuals and the rest of the patients. Information about other medications, OA of other joints or other diseases of the patients was not available for this study.

In the experimental part of the study, cartilage tissue and primary chondrocytes from OA patients, as well as immortalized murine chondrocyte cell line, were used. The use of OA tissue/cells in comparison to healthy tissue/cells is advantageous when studying OA as the protein expression profile and metabolism in OA chondrocytes differs from that in the corresponding cells from healthy tissue. The benefit of using cartilage tissue in the experiments include that the chondrocytes live in their natural micro-environment, resembling the *in vivo* situation, and are thus less vulnerable to undergo a phenotypic change, that happens when chondrocytes are grown in monolayer culture. However, it is more difficult to control the number of cells than in monolayer cell culture. This undeniably introduces a challenge into the statistical evaluation and interpretation of the results, as there is more variability.

Use of primary cells is also closer to the *in vivo* situation than the use of cell lines, and cell number is easier to control compared to cartilage tissue culture. However, primary chondrocytes start to change their phenotype towards a fibrocytic state as soon as they are placed in the culture such that the production of chondrocyte-specific proteins declines with every passage (Darling and Athanasiou 2005). Because the amount of tissue and the number of cells that can be gathered from one individual are very limited, and expansion of the cells is not desirable for the above-mentioned reason, it is not possible to use OA cartilage or primary chondrocytes in large series of experiments without combining cells from several donors, which in turn, may induce changes in cellular responses.

For these reasons, the H4 murine chondrocyte cell line was chosen for the siRNA studies. These cells have a stable phenotype and similar responses to IL-1 as articular chondrocytes *ex vivo* (van Beuningen et al. 2002). Preliminary experiments conducted for the present study showed that these cells also produce nitric oxide in response to leptin exposure (not shown). We have also a human T/C28a2 chondrocytic cell line (Goldring et al. 1994) in use in our laboratory. However, according to our experience, H4 chondrocytes mimic the responses of human primary chondrocytes (e.g., in NO, MMP and cytokine production) more closely than the human T/C28a2 cell line. For these reasons, the H4 cell line was chosen over the human cell line.

siRNA is an effective and relatively simple method to silence a gene of interest. As there are no known antagonists for SOCS-3, siRNA was chosen to reduce the SOCS-3 expression and subsequently inhibit the effects of this protein.

Standard methods in cell biology, ELISA, western blotting and RT-PCR were used to detect protein and mRNA expression in the patient samples, in the culture media and in the cultured cartilage tissue/cells. As a result of the limited availability of human tissue/cells, only protein expression in the human tissue samples was determined, whereas for the cell line, also mRNA expression was determined and found to parallel the protein expression induced by leptin.

2 Levels of adipokines in patients with osteoarthritis

All three adipokines were found in synovial fluid of the osteoarthritic joints. They were also released by OA cartilage into the culture fluid. The concentrations of adiponectin and leptin correlated closely with their own levels between the different compartments, as also reported by Presle et al. (Presle et al. 2006). Adiponectin and leptin concentrations were higher in the blood than in the synovial fluid, consistently with previous findings (Presle et al. 2006; Senolt et al. 2006; Tan et al. 2009; Hao et al. 2010; Honsawek et al. 2011; Staikos et al. 2013; Bas et al. 2014) with one exception in leptin levels (Presle et al. 2006).

In females, the levels of adiponectin and leptin were higher than in males, both in the circulation and in synovial fluid, as also reported in the literature (Presle et al. 2006; Ibrahim et al. 2008; de Boer et al. 2012; Gross et al. 2014). In addition, the levels of these adipokines released by cultured cartilage were higher in females, which could contribute to their higher risk of OA. In male patients, the levels of adiponectin in all compartments correlated positively with age. Interestingly, deBoer et al. reported that serum adiponectin concentration correlates with age only in OA patients but not in control patients (de Boer et al. 2012), suggesting that the higher levels of adiponectin in older individuals could be related to more advanced disease but not to age itself.

Only leptin levels showed a clear correlation with BMI, and this correlation was present in all compartments, evidence that leptin could be a mediator between obesity and OA. The positive correlation between circulating / synovial fluid leptin and BMI

in OA patients has also been described in the literature (Dumond et al. 2003; Wislowska et al. 2007; Simopoulou et al. 2007; Gandhi et al. 2010; de Boer et al. 2012).

In our data, adiponectin did not correlate with BMI, with the exception of a weak negative correlation of plasma adiponectin in women. A weak negative correlation of circulating adiponectin with BMI in OA patients has also been described by others (de Boer et al. 2012; Yusuf et al. 2011), whereas some other groups did not detect any association between adiponectin levels and BMI (Laurberg et al. 2009; Hao et al. 2010). As in obesity/diabetes-related studies, more clear negative associations of adiponectin levels with BMI, and especially with visceral fat accumulation, have been found (Brochu-Gaudreau et al. 2010), it is possible that in patient samples consisting of OA patients, it is the disease itself that determines the adiponectin levels more than BMI.

In our data, resistin released from cartilage, but not in plasma or synovial fluid, correlated with BMI, whereas larger cohort studies have previously found elevated circulating levels of resistin in obese patients (Schwartz and Lazar 2011). It remains unclear why the association of resistin with BMI was only seen in cartilage culture media. Perhaps the increased load subjected to the joints in obese individuals could lead to increased resistin release, in view of the fact that elevated intra-articular resistin levels have also been observed following joint trauma (Lee et al. 2009).

It is not clear where the intra-articular adipokines are produced. Cartilage tissue was observed to release adipokines in the present study, and cartilage as well as other joint tissue explants including synovium, infrapatellar fat pad, meniscus, osteophytes and bone have been reported also by others to release adipokines *ex vivo* (Distel et al. 2009; Presle et al. 2006; Tsuchida et al. 2014). Based on the facts that the levels of adiponectin and leptin were higher in circulation than in synovial fluid, and the strong correlations between the levels in plasma and synovial fluid seen in the present study, it is probable that they were mainly produced outside the joint, e.g., in adipose tissue, and then diffused into synovial fluid from circulation. As there was a strong correlation also between the synovial fluid and the cartilage culture media concentrations of these adipokines, it is possible the adipokines released by cartilage partly originate from synovial fluid and diffuse into the cartilage. Joint tissues, including cartilage, evidently do synthesize some amounts of adiponectin and leptin since mRNA and protein expression of leptin, as well as adiponectin have been

detected in OA chondrocytes (Dumond et al. 2003; Morroni et al. 2004; Simopoulou et al. 2007; Iliopoulos et al. 2007; Francin et al. 2011; Tsuchida et al. 2014) and in synovial fibroblasts (Ehling et al. 2006; Tan et al. 2009). The expression of leptin has been shown to be higher in OA than in normal chondrocytes (Iliopoulos D, 2007; Simopoulou et al. 2007; Tsuchida et al. 2014), and in fact Iliapoulos et al. showed that epigenetic up-regulation of leptin in OA chondrocytes upregulates MMP-13 production (Iliopoulos et al. 2007). Accordingly, higher levels of leptin has been reported in synovial fluid of OA patients compared to control subjects (Beekhuizen et al. 2013; Ku et al. 2009). Leptin and adiponectin were higher also in circulation from OA than control patients (Laurberg et al. 2009; de Boer et al. 2012; Cuzdan Coskun et al. 2015). Thus, based on the present findings and literature, it can be concluded that adiponectin and leptin are apparently partly produced in the joint cavity, possibly in greater amounts by cells in OA affected than healthy joint, yet their intra-articular levels depend highly on circulating levels and BMI.

Correlations of resistin between different compartments were clear but not as strong as for the two other adipokines. A study by Senolt et al. also found a moderate correlation between resistin levels in plasma and synovial fluid (r=0.55) in OA patients whereas in RA patients, in whom synovial fluid levels of resistin were higher than in plasma, no correlation between synovial fluid and plasma existed (Senolt et al. 2007). In our data and also in other studies that included OA patients (Presle et al. 2006; Senolt et al. 2007) resistin was slightly higher in plasma than in synovial fluid, similarly to adiponectin and leptin. In addition to the study by Senolt et al., also two other studies reported higher levels of resistin in synovial fluid than in plasma in patients with RA (Bokarewa et al. 2005; Bostrom et al. 2011; Senolt et al. 2007), suggesting that in RA, resistin is produced intra-articularly. Considering that resistin in humans is thought to be produced exclusively by inflammatory cells (Filkova et al. 2009b; Schwartz and Lazar 2011) including those in adipose tissue, it is likely that the intra-articular levels of resistin in OA depend on both the levels in circulation and the amount produced by intra-articular/synovial leukocytes.

It should be noted that as resistin concentrations in the joint cavity are more independent of plasma concentrations than those of leptin and adiponectin, it may be problematic to study the association of resistin with joint diseases based on its plasma levels. In the case of adiponectin and leptin, plasma levels relate to synovial fluid

levels more closely, and thus are likely to be more reliable for use in studies investigating joint diseases.

Though it remains uncertain where the studied adipokines are produced, it is clear that these three adipokines do end up in the joint cavity where they seem to exert harmful effects on chondrocytes. The higher intra-articular levels of leptin and adiponectin in females found in the present study could be related to the increased risk of females to develop OA, whereas elevated leptin concentrations might explain the obesity-related risk of OA.

3 Adipokines as proinflammatory and catabolic factors in osteoarthritis

3.1 Adiponectin

The levels of adiponectin were found here to correlate with those of MMPs and IL-6 in synovial fluid and in cartilage culture medium, which was a novel finding. Positive associations between adiponectin and IL-6 in synovial fluid from OA patients have been published more recently also by others (Gross et al. 2014; Tsuchida et al. 2014) to support our findings.

Similar concentrations of adiponectin to those that were detected in synovial fluid have been reported to enhance the production of proinflammatory and catabolic factors, including IL-6, NO and MMPs, in primary chondrocytes and in OA cartilage *in vitro* in the present study. These effects were mediated, at least partly, by MAPKs, as evidenced by studies with pharmacological inhibitors. Similar catabolic/proinflammatory effects of adiponectin on murine and human chondrocytes have been reported also by other groups (Lago et al. 2008; Kang et al. 2010; Tong et al. 2011; Gomez et al. 2011). These experimental findings, together with the associations of adiponectin to proinflammatory and catabolic factors found in synovial fluid and in cartilage culture media in the present study, point to detrimental role for adiponectin in the pathogenesis of OA.

However, contrary experimental and clinical data have also been published. Lee et al. reported that intra-articularly injected adiponectin in mice mitigated the severity of collagen-induced arthritis and decreased expression of TNF, IL-1 and MMP-3 (Lee et al. 2008). In another study, adenovirus-mediated gene transfer of adiponectin reduced the progression of collagen-induced arthritis in mice (Ebina et al. 2009a). Chen et al. reported that adiponectin up-regulated TIMP-2 and down-regulated IL-1β and MMP-13 in human primary chondrocytes (Chen et al. 2006). An inverse association of plasma adiponectin concentrations with progression of hand OA during a six years' follow-up has been reported by a Dutch group (Klein-Wieringa et al. 2014; Yusuf et al. 2011). Another group reported an inverse association between the serum adiponectin level and cartilage volume loss (analyzed by MRI) with 2 years' follow-up in knee OA (Martel-Pelletier et al. 2016), whereas Berry et al. did not find any association between adiponectin concentrations and the change in cartilage volume loss in MRI in knee OA patients over two years' follow-up (Berry et al. 2011).

Whereas those follow-up studies investigating adiponectin in the progression of OA do not support the view that adiponectin exerts harmful effects in joint pathology, studies on RA patients point to an association between adiponectin and progression of RA (Ebina et al. 2009b; Klein-Wieringa et al. 2011; Giles et al. 2011; Klein-Wieringa et al. 2014). It might be that adiponectin has a greater role in RA than OA. Lee et al. reported that the adiponectin levels correlate with those of IL-6 and IL-8 in synovial fluid from RA but not from OA patients (Lee et al. 2012). The levels of adiponectin in synovial fluid have also been shown to be higher in RA than in OA patients (Schaffler et al. 2003; Senolt et al. 2006). On the other hand, adiponectin levels were found to be increased in RA patients treated with methotrexate (Laurberg et al. 2009) and disease-modifying antirheumatic drugs (Cansu et al. 2011). Thus, it is possible that the positive association between the adiponectin concentration and the progression of RA in the follow-up studies (Giles et al. 2011; Klein-Wieringa et al. 2011; Klein-Wieringa et al. 2014) could have been influenced by medication of those patients. However, the experimental data on the deleterious effects of adiponectin on chondrocytes (Lago et al. 2008; Kang et al. 2010; Tong et al. 2011; Gomez et al. 2011) and also on synovial fibroblasts (Ehling et al. 2006; Tang et al. 2007; Frommer et al. 2010; Frommer et al. 2012; Lee et al. 2012) support the concept that adiponectin is an active effector in the pathology of arthritis.

Adiponectin is present in the circulation in different oligomeric isoforms that bind to adiponectin receptors with different affinities. The contradictory findings in the studies investigating adiponectin in OA might also be related to the isoforms of adiponectin. In most studies, including ours, the clinical samples were analyzed for total adiponectin and not for the isoforms. In the study of Klein-Wieringa et al. total adiponectin, but not HMW adiponectin, associated positively with RA and negatively with OA (Klein-Wieringa et al. 2014). They concluded that some isoform other than HMW mediates the harmful effects of adiponectin in OA. In the experimental study of Frommer et al. all isoforms of adiponectin, including HMW, MMW, globular and the trimer form of adiponectin increased the production of proinflammatory mediators and MMPs in synovial fibroblasts, with the trimer being the least potent isoform (Frommer et al. 2012). In another study where primary chondrocytes were used, full length adiponectin, but not the globular form, increased MMP-13 activity and PGE_2 production (Francin et al. 2014). Chen et al. reported that the proportions of the different isoforms differ in synovial fluid vs. plasma (Chen et al. 2006), which could obviously impact on the results of clinical studies in which it is usually the circulating level that is measured.

It is also possible that the production of adiponectin in the joint cavity is compensatory to the catabolic state in the joint and that the balance in the adiponectin-induced effects lies on the side of anabolic and anti-inflammatory factors that were not measured in the present study (Korkmaz 2012).

Adiponectin was associated with the severity of radiographic findings, as well as with the circulating biomarkers of OA, MMP-3 and COMP in the present study. Within the framework of the study design, conclusions about causality are not justified, but the results do suggest that adiponectin may be a promising biomarker of OA. Similar associations between adiponectin levels and radiographic findings in knee OA (Cuzdan Coskun et al. 2015) and in hand OA (Filkova et al. 2009b), and biomarkers including s-COMP (Van Spil et al. 2012) and degradation markers of aggrecan (AGG1 and AGG2) (Hao et al. 2010) were found by others in recent studies. Serum adiponectin levels have also been reported to be higher in patients with OA than in control patients (Laurberg et al. 2009; de Boer et al. 2012; Cuzdan Coskun et al. 2015). Nonetheless, contradictory findings exist, for example, Zheng et al. reported a negative association between the serum adiponectin level and radiographic scoring of OA in plain radiographs in patients with early OA (Zheng et al. 2016). However,

in that same cross-sectional study, the adiponectin concentration was not associated with cartilage volume in magnetic resonance imaging (MRI). No association was found between the adiponectin level and native radiographs by Van Spil et al. (Van Spil et al. 2012) in early knee OA patients and nor was any relationship detected by Choe et al. in patients with hand OA (Choe et al. 2012), whereas Honsawek et al. found a negative association in knee OA patients (Honsawek et al. 2011). The results of the above-mentioned and the present study appear to be partly conflicting and might be related to study design including the radiological scale used, the phase and type of OA, the gender of the patients, as well as the assay used to detect adiponectin in terms of adiponectin isoforms.

To sum up, the present results, i.e., the pro-degradative effects of adiponectin on cartilage, and the clinical associations of this adipokine found with catabolic and proinflammatory factors and biomarkers of OA, together with data from experimental studies by other groups point to a deleterious role for adiponectin in OA and joint pathology. However, the findings from longitudinal studies in OA patients as well as from animal models are conflicting. The differences could be explained by variable proportions and effects of the different isoforms of adiponectin and this issue should be investigated in future studies. As the adiponectin level in plasma was associated with radiographic severity and biomarkers of OA, it could be studied as a biomarker of OA in future.

3.2 Leptin

The synovial fluid leptin level correlated with the amounts of the cartilage degrading enzymes MMP-1 and MMP-3 in the present study, suggesting an association between leptin and cartilage damage. This finding became evident in the obese (BMI > 30 kg/m^2) subgroup of the patients. The association of the leptin level with those of the MMPs in synovial fluid is a novel finding. In the experimental studies, leptin was shown to increase MMP production by OA cartilage, suggesting that the associations found in synovial fluid could be explained by a catabolic effect of leptin on the cartilage. Leptin was also shown to increase the production of proinflammatory factors NO, IL-6, IL-8 and PGE_2, and to enhance the expression of iNOS and COX-2 in human OA cartilage. These results are supported by other studies, as leptin has

been shown to enhance the expression of MMP-1, MMP-3, MMP-9 and MMP-13, as well as IL-1β, IL-8, COX-2, and to cause glycosaminoglycan release in cultured chondrocytes / cartilage (Simopoulou et al. 2007; Iliopoulos et al. 2007; Pallu et al. 2010; Gomez et al. 2011; Hui et al. 2012). Otero et al showed that leptin together with IFN-γ (Otero et al. 2003) or IL-1β (Otero et al. 2005), but not on its own, increased iNOS mRNA expression and NO production in a murine chondrocyte cell line and in human primary chondrocytes. Two groups that investigated the effect of leptin on cartilage of animal origin reported that leptin had no effect on the MMP activity, NO production or proteoglycan release in porcine or bovine cartilage (McNulty et al. 2011; Nishimuta and Levenston 2015). Leptin was found to increase NO production and proteoglycan release in bovine meniscus in one of these studies (Nishimuta and Levenston 2015), while in a more recent publication of the same authors, lower concentration of leptin (1 μg/ml) was reported to have no effect on proteoglycan release in bovine meniscus (Nishimuta and Levenston 2017).

In *in vivo* animal studies, leptin has been shown to have both catabolic and anabolic effects as Dumond et al showed that leptin injection into rat knee induced the synthesis of IGF-1 and TGF-β1, pointing to an anabolic role for leptin on cartilage (Dumond et al. 2003). Bao et al. reported increased expression of MMP-2, MMP-9, cathepsin D, type II collagen, ADAMTS-4 and ADAMTS-5 caused by injection of leptin into rat the knee pointing to catabolic effects of leptin on cartilage (Bao et al. 2009). Thus, it is plausible that leptin has both anabolic and catabolic functions in the cartilage. Griffin et al claimed that leptin was critically involved in the pathogenesis of OA in obese mice as leptin-deficient obese mice did not develop OA unlike their wild type littermates with diet-induced obesity (Griffin et al. 2009).

In longitudinal clinical studies, leptin has been shown to be associated with cartilage volume loss (Martel-Pelletier et al. 2016; Stannus et al. 2015) and the incidence (Karvonen-Gutierrez et al. 2013b) and the progression of OA in terms of progression of radiographic changes (Van Spil et al. 2012) and the incidence of total knee replacement surgery (Martel-Pelletier et al. 2016). Exceptions also exist, e.g., Berry et al (Berry et al. 2011) did not detect any association between leptin and OA-related changes in MRI of knee OA patients over 2 years. In the study by Yusuf et al., there was a tendency, although not statistically significant, towards higher leptin levels in hand OA progressors vs non-progressors over 6 years (Yusuf et al. 2011).

In the first published sub-study (II), major differences in the leptin-induced effects between cultured cartilage explants from different patients were observed. Therefore, it was decided to collect cartilage samples and clinical information from a larger group of OA patients to investigate whether some feature of the patients, such as obesity, could explain the variable leptin responses and to explore possible mechanisms regulating leptin responsiveness. Obesity was not found to explain the leptin responsiveness *in vitro* in our study design, unlike in the study by Pallu et al. who reported that the response of primary chondrocytes to express MMP-13, type II collagen, TIMP-2, COX-2 and IGF-1 depended on both the leptin dose and obesity status (non-obese vs. obese) of the patients from whom the cells had been collected (Pallu et al. 2010). Furthermore, we did not find that any other clinical factor measured in our study could explain the dispersion in leptin responses.

Next, in order to explore possible mechanisms regulating leptin responsiveness, SOCS-3 was determined in the cartilage samples. Interestingly, an inverse association was found between SOCS-3 expression and leptin responsiveness. In further experiments with the help of siRNA to downregulate SOCS-3 in chondrocytes, SOCS-3 was shown to inhibit leptin-induced catabolic and proinflammatory effects. SOCS-3 has been previously investigated in chondrocytes in only a few studies (Smeets et al. 2006; van de Loo et al. 2012; Liu et al. 2014; Liu et al. 2015). In these studies, SOCS-3 was reported to inhibit LPS-induced NO production (van de Loo et al. 2012) and IGF-1 mediated cell signaling (Smeets et al. 2006). Liu et al recently examined chondrocytes from mice with a conditional knock-out of SOCS-3, and found that SOCS-3 regulated the effects of gp130 cytokines including oncostatin M, IL-6, IL-11 and leukemia inhibitory factor (Liu et al. 2014). The present study is the first to show that the effects of leptin in chondrocytes are regulated by SOCS-3. SOCS-3 has been reported to exert a protective role in animal models of arthritis (Shouda et al. 2001; Wong et al. 2006; Veenbergen et al. 2011). In these studies, the target cells of SOCS-3 over-expression / deletion were thought to be of hematopoietic and endothelial origin. The results of the present study suggest a protective role for SOCS-3 also in chondrocytes and in OA.

Other possible mechanisms behind the variable leptin responses are the amount of soluble and functional leptin receptors expressed by chondrocytes. In the present study, the former was found to be decreased in synovial fluid in obesity, increasing further the susceptibility of obese individuals to the effects of bioactive leptin. The

latter has been shown to be increased in OA vs normal cartilage (Simopoulou et al. 2007). There might be also intracellular regulators other than SOCS-3, which determine the fate of intracellular signaling in response to a leptin stimulus.

It is not clear why relatively high concentrations of leptin were needed in the present study in order to obtain significant effects. In studies conducted by other groups, relatively high levels of leptin were most often needed (Gomez et al. 2011; Hui et al. 2012; Otero M, 2005; Otero et al. 2003). If we examine the values from individual patient samples in study I, we can see that in most samples, a leptin concentration 100 ng/ml was adequate to at least double the production of proinflammatory and catabolic factors. However, the changes were not statistically significant because of the large inter-patient variation in the responses. The higher concentration, i.e., 10 µg/ml, was high enough to induce these effects in almost all samples, so that in spite of the extensive variability in the responses, the result became statistically significant. Pallu et al. reported that only 20 ng/ml of leptin, which approximates to the level of leptin that can be found in the synovial fluid of an overweight individual, was enough to induce MMP-13 expression in chondrocytes from obese patients, but not in the chondrocytes from normal weight patients, in whom as much 500 ng/ml did not increase MMP-13 expression significantly (Pallu et al. 2010). Unlike those investigators, we did not find significant differences in the responses between samples from non-obese and obese patients, which might be due to the higher leptin concentration (10 µg/ml) used (sub-study V). There are also other issues that might explain why high levels of leptin were needed in the culture: Phenotypic modulation depending on culture conditions might have influenced the chondrocyte responsiveness to leptin (Francin et al. 2011). Simopoulou et al. showed also that chondrocytes from normal cartilage (fracture patients) vs from OA patients needed a higher level of leptin in order to increase IL-1β production (Simopoulou et al. 2007), suggesting that OA chondrocytes are more susceptible to harmful effects of leptin. It is also possible that culture conditions induce SOCS-3, and, thus create some level of leptin resistance in cultured cartilage / chondrocytes.

JAK-STAT signaling pathway is thought to be essential for leptin signaling in CNS (Myers et al. 2012). Otero et al. reported that iNOS expression and subsequent NO production in murine ATDC5 chondrogenic cells were mediated through the activation of JAK2 when leptin was utilized in combination with either interferon-γ

(Otero et al. 2007) or IL-1 (Otero et al. 2005). In another study, it was shown that leptin increased the production of IL-8 in synovial fibroblasts from OA patients through JAK2/STAT3 and IRS-1 (insulin receptor substrate 1)/PI3K/Akt/NF-κB-dependent pathways (Tong et al. 2008). Based on the present studies with pharmacological inhibitors, the activation of JAK2 was not involved in the leptin-induced catabolic and proinflammatory effects in chondrocytes, instead MAPKs and NF-κB, as well as PKC, were found to mediate these responses. In addition, JAK3 was involved in leptin-induced NO, MMP-3 and MMP-13 production. MAPKs p38 and Erk have been shown by others to be involved in leptin-induced expression of MMP-1 and MMP-13 in chondrocytes (Hui et al. 2012). SOCS-3 is known to be induced through the JAK-STAT pathway and to inhibit cytokine signaling by binding simultaneously to the cytokine receptor and either JAK1, JAK2 or TYK2 (tyrosine kinase 2), but not JAK3 (Babon et al. 2012; Yoshimura et al. 2007). In this respect, it is not clear at which site in the leptin signaling SOCS-3 acts to inhibit the leptin-induced effects in chondrocytes; this remains an issue to be studied in the future.

The leptin level was not associated with radiographic findings or with circulating biomarkers COMP or MMP-3 in the present study population. In our data, patients with less severe radiographic changes tended to have higher BMI, which might explain the leptin levels in the radiographic subgroups rather than the possibility that the increased leptin production would be related to cartilage pathology seen in the study by Simopoulou et al. (Simopoulou et al. 2007). Some of the previous cross-sectional studies have reported positive associations between circulating (Staikos et al. 2013; Stannus et al. 2010) or synovial fluid leptin levels (Ku et al. 2010) and radiographic severity of OA although negative results (i.e. no association) have also been published (Iwamoto et al. 2011; Massengale et al. 2012; Zheng et al. 2016). In population based studies and in studies comparing OA and control patients, the leptin level has been shown to be associated with the prevalence of radiographic OA (Ku et al. 2010; Van Spil et al. 2012; de Boer et al. 2012; Fowler-Brown et al. 2015) and with reduced cartilage volume/thickness as measured by MRI (Ding et al. 2008; Stannus et al. 2015). Van Spil et al. reported also an association between circulating leptin and s-COMP, as well as some other biomarkers of OA, including uCTX-II (urinary C-terminal telopeptide of type II collagen), sPIIANP (serum N-terminal propeptide of type IIA procollagen), sHA and sPIIINP (serum N-terminal propeptide

of type III procollagen), in a large sample of OA patients with early, symptomatic disease.

Since, according to follow-up and experimental studies, leptin seems to be the cause but perhaps not the consequence of OA, a cross-sectional analysis of OA patients with variable disease duration is not necessarily capable of revealing associations of this adipokine to OA when these are compared with parameters of the severity of the disease. Furthermore, in order to obtain reliable results, careful attention has to be paid to the close relationship between leptin and BMI. These issues probably explain much of the discrepancies in the above mentioned studies, including ours. One can speculate that the effects of leptin in the body are dependent not only on the leptin concentration, but also on the leptin response; this latter phenomenon is regulated by other factors (including sOb-R and SOCS-3). This means that a leptin concentration that would be harmful for one individual might not be harmful to some other person.

We found an association between the leptin level and worse symptoms / clinical manifestation of OA, as measured by KSS. Leptin has been associated with OA-related pain also in other studies recently (Massengale et al. 2012; Bas et al. 2014; Lubbeke et al. 2013; Gandhi et al. 2013; Perruccio et al. 2014). The mechanism behind this association remains unknown. In our study, in addition to leptin, also plasma IL-6, but not, for example, synovial fluid IL-6, NO or MMPs, correlated with the KSS, indicating that if leptin does increase pain, the effect is perhaps not mediated through overall increased inflammatory activity but rather via more specific mechanisms. Indeed in the experimental part of the present study, leptin was found to increase the production of PGE_2 in cartilage, i.e., it is possible that leptin is involved in OA pain through increased PGE_2 production. One can also speculate that leptin increases pain sensation by some other mechanisms, for instance in the central nervous system. However, the association between the KSS and leptin cannot be explained by BMI since the BMI value did not correlate with the score.

Taken together, in the present study, leptin was shown to increase the production of proinflammatory and catabolic factors, and associate with MMP-1 and MMP-3 levels in synovial fluid. SOCS-3 was identified as a novel factor regulating the effects of leptin in chondrocytes. Leptin levels in synovial fluid and those released from cartilage were highly dependent on BMI. In addition, the amount of the soluble leptin receptor was decreased in the obese patients, which is thought to increase the

availability of bioactive leptin in these individuals. Based on the present results and the existing longitudinal research data published by others (see above), it seems likely that leptin is a significant factor in the pathogenesis of OA and a mediator between obesity an OA.

3.3 Resistin

We found that intra-articular levels of resistin correlated with those of MMP-1 and MMP-3, and with IL-6. Resistin was also found to correlate with MMP-13 in cartilage culture media. These correlations are novel findings. Our results are supported by the findings of Gross et al. (Gross et al. 2014) who reported that resistin levels correlated with those of IL-6 in synovial fluid of OA patients. Schäffler et al. reported a positive correlation also between resistin levels and CRP, and ESR in synovial fluid collected from OA patients in an outpatient clinic (Schaffler et al. 2003). Neither in our data nor in the study by Senolt et al. (Senolt et al. 2007), was such a correlation between resistin and CRP found in OA patients. This could be explained by the timing of sampling as it is likely that the synovial fluid samples in the study of Schäffler et al. (Schaffler et al. 2003) were taken at the time of exacerbation of the symptoms and intra-articular inflammation causing a possible elevation in CRP, ESR and possibly also in resistin levels, whereas synovial fluid samples for the present study were collected during the knee replacement surgery. In addition, in RA patients, positive correlations of serum resistin with TNF-α and IL-6 (Klein-Wieringa et al. 2011), as well as with CRP and ESR (Senolt et al. 2007), have been found. Interestingly, an LPS injection has been shown to cause a sharp increase in resistin levels (Lehrke et al. 2004) and synovial fluid resistin has been reported to increase due to joint trauma (Lee et al. 2009). These findings together suggest that resistin concentrations could behave in a similar manner to those of acute phase proteins. Thus, resistin could be a factor mediating the effect of inflammation, infection, trauma and obesity on cartilage.

In the present study, resistin levels were not found to associate with radiographic severity or biomarkers of OA. A small number of studies have investigated the association of circulating resistin with radiographic severity or progression of OA, and in most of them this kind of association was not found (Filkova et al. 2009a;

Yusuf et al. 2011; Berry et al. 2011; Massengale et al. 2012; Martel-Pelletier et al. 2016; Zheng et al. 2016) with a few exceptions (Choe et al. 2012; Van Spil et al. 2012). One problem with these studies is that the serum resistin level might differ significantly from that in synovial fluid, so that the possible association could be lost by measuring resistin in the wrong fluid.

The effects of resistin on chondrocytes were not studied in the present study but in studies by other groups catabolic and proinflammatory effects have been shown: Resistin has been shown to enhance the expression of cytokines, such as IL-1β, IL-6, IL-8 and TNF-α, and MMP-1, MMP-13 and ADAMTS-4, in human chondrocytes (Zhang et al. 2010b). In mouse chondrocytes, resistin has been reported to increase production of PGE_2, IL-6 and IL-8 and to decrease procuction of proteoglycans (Lee et al. 2009). Resistin was also found to increase proteoglycan release and increase NO production in bovine cartilage and meniscus *ex vivo* (Nishimuta and Levenston 2015). The nature of a specific resistin receptor is not known, but resistin has been shown to signal through TLR-4 in PBMCs to increase production of IL-6, IL-1β, and TNF-α (Tarkowski et al. 2010).

In chondrocytes it is unclear to which receptor resistin binds. TLR-4 receptor expression has been shown to be up-regulated in OA cartilage and its activation by LPS has been reported to enhance MMP-1, MMP-3, MMP-13, and NO production in human OA chondrocytes/cartilage (Vuolteenaho et al. 2003; Kim et al. 2006). Thus, it is possible that the catabolic and proinflammatory effects of resistin in chondrocytes are mediated through TLR-4 and resistin could be an endogenous activator of TLR-4.

The existing, relatively small amount of research data point to a potential harmful role for resistin in the pathogenesis of OA. New study designs will be needed in the future to clarify the role of resistin not only in OA but also in other rheumatic diseases.

4 Clinical aspects

At present, there are no effective treatments which can either prevent or slow down the structural changes that take place in the course of OA. Therefore, there is an urgent need to identify novel drug targets.

The difficulty in finding novel drugs might be related to the fact that OA is a multifactorial disease. In the future, it may be necessary to identify different etiologies of OA and to devise personalized treatment options for each phenotype of the disease (Siebuhr et al. 2016). Different phenotypes of OA have been suggested to be classified for example as metabolic, ageing-driven, cartilage-driven, traumatic injury-driven, subchondral bone and synovitis-driven inflammatory phenotypes (Mobasheri et al. 2017).

Next, since cartilage has a limited ability to regenerate, it will be probably necessary to start the treatment at the onset of the disease, or before cartilage destruction has taken place.

Another difficulty is to diagnose the onset of the disease or to identify the subjects who are at risk of developing symptomatic OA in order to find the subjects who would benefit from the treatment. To achieve this goal, appropriate biomarkers or more sensitive imaging methods will need to be developed.

In the present study, adiponectin and leptin were found to activate human OA chondrocytes to increase their production of catabolic and proinflammatory factors that are central in the pathogenesis of OA. Similar effects on cartilage by resistin have been described by others (Lee et al. 2009; Nishimuta and Levenston 2015; Zhang et al. 2010b). These experimental data together with the associations of these three adipokines with the proinflammatory and catabolic factors in OA patients found in this study point to a significant role of these adipokines in the pathogenesis of OA. Thus, these adipokines could be novel targets to prevent/treat OA. Their role as biomarkers of OA should also be further studied.

Adiponectin and leptin were both shown to signal through MAPK pathways and also resistin has been shown to activate MAPKs (Tarkowski et al. 2010). Thus, inhibiting MAPKs could be one option to reduce the effects of adipokines on cartilage.

Obesity is common; approximately every second person in the Western countries is overweight (BMI > 25 kg/m^2) and 20 to 25 % are obese (BMI > 30 kg/m^2) (NCD Risk Factor Collaboration (NCD-RisC) 2016). There is clear evidence that obesity increases the risk of OA. It has been estimated that the incidence of symptomatic knee OA in Finland could be reduced by 30% if obesity could be prevented (Muthuri et al. 2011). Leptin levels, and especially active free leptin, are increased in synovial fluid of obese individuals. Thus, it would be interesting to investigate whether leptin could

be a pharmacological target in this group of OA patients. As leptin regulates many physiological functions, inhibiting leptin systemically, otherwise than by losing weight, could lead to unwanted side effects. Intra-articularly administered leptin antibody could be worthwhile studying in future. Interestingly, leptin antibody has been investigated and shown to decrease MMP-1 and MMP-3 expression in chondrocytes that were grown in conditioned media from cultured white adipose tissue (Hui et al. 2012). Another way to target leptin-driven proinflammatory and catabolic effects could be to increase SOCS-3 expression in chondrocytes (or in other joint tissue cells). For instance, the anti-inflammatory cytokine, IL-10, as well as statins and drugs that elevate cAMP, have been shown to induce SOCS-3 expression (White and Nicola 2013), and could be also examined as a possible modulators of leptin's effects in chondrocytes.

Adiponectin is thought to be a protective factor in terms of diabetes and atherosclerosis. Thus, if one considers adiponectin as a target of pharmacological therapy, it would not be beneficial to inhibit the effects of this adipokine throughout the body. In addition, more information should be obtained about the different isoforms of adiponectin and the possible group of patients in whom focused targeting of adiponectin would be appropriate. As adiponectin has been found to correlate with biomarkers of OA and with radiographic severity of OA, this adipokine would be worthwhile studying as a novel biomarker of OA in the future. Nonetheless, the fact that adiponectin is not only produced by joint tissues but by different cell types in multiple locations might complicate the use of adiponectin as a biomarker of OA.

The amounts of resistin have been shown to increase in synovial fluid due to trauma (Lee et al. 2009). Resistin could be perhaps viewed as a target of preventing OA related to joint trauma, e.g., by local administration of a drug that would inhibit the cartilage-damaging effects of resistin. Clearly, the resistin receptor and its signaling in chondrocytes will need to be identified in order to find sites for pharmacological inhibition. The resistin receptor has still not been characterized, but resistin has been shown to bind to TLR-4 to increase expression of proinflammatory cytokines in PBMCs (Tarkowski et al. 2010).

The present study extends our knowledge of adipocytokines adiponectin, leptin and resistin as harmful factors in the pathogenesis of OA. These adipokines or their signaling mechanisms could represent promising novel drug targets of OA in future.

This study also found evidence that leptin might be a mediator between obesity and OA.

Clearly, much work and research will need to be conducted before there are novel, effective pharmacological treatments to prevent or treat OA. However, there is an existing intervention known to decrease elevated leptin levels and also to prevent OA. That is, to lose excess weight.

Summary and conclusions

The aim of the present study was to investigate the role of adipocytokines as pathogenic factors in OA, as possible factors connecting obesity and OA, and as possible targets of disease-modifying drugs for OA. The study was based on the analysis of levels of three adipokines, adiponectin, leptin and resistin, as well as different markers of joint inflammation and cartilage degradation in samples collected from a group of 100 OA patients undergoing knee arthroplasty. In addition, an experimental approach was used by studying the effects and mechanisms of actions of adiponectin and leptin on chondrocytes in cell and cartilage cultures. The findings and conclusions are as follows:

1. The three adipocytokines, adiponectin, leptin and resistin, were detectable in the circulation, in the synovial fluid and in the growth media of cultured cartilage collected from OA-affected joints. Their concentrations correlated between the different compartments (blood, synovial fluid and cartilage culture media). Leptin levels in all three compartments, and resistin levels in cartilage culture media correlated with BMI. Leptin and adiponectin levels in all compartments, and resistin levels in cartilage culture media were higher in females than males. The adiponectin concentrations in males correlated with age.

   The levels of three adipocytokines, adiponectin, leptin and resistin, associated with markers of inflammation / cartilage degradation, and measures of OA severity in OA patients as follows:

   - Adiponectin levels correlated with NO, IL-6 and MMPs in the cartilage culture media and in the synovial fluid. Adiponectin levels also correlated with circulating biomarkers of OA, COMP and MMP-3. The plasma level of adiponectin and that released by cultured cartilage were

higher in samples from patients with more severe radiographic findings. There were gender-specific differences in these associations. (Figure 22)

- Leptin levels in synovial fluid correlated positively with those of MMP-1 and MMP-3 in obese patients (BMI > 30 kg/m^2) and with IL-6 levels in the subgroup of patients in the highest quartile by BMI (BMI > 34.9 kg/m^2). Leptin levels correlated with the KSS such that the patients with the highest leptin concentrations had the worst manifestation of OA assessed by the KSS. (Figure 22)

- Resistin levels correlated with NO, IL-6 and MMPs in cartilage culture media and in synovial fluid. (Figure 22)

2. Adiponectin and leptin enhanced the expression of proinflammatory and degradative factors, including iNOS, NO, COX-2, PGE_2, IL-6, IL-8, MMP-1, MMP-3 and MMP-13, in cultured OA cartilage; these effects were mediated through MAPK-, NF-κB-, PKC- and JAK3-dependent intracellular pathways (Figure 23).

3. Of the studied clinical and biological factors, only the level of SOCS-3 expression explained the variability in leptin-induced production of catabolic and proinflammaroty factors in cartilage *in vitro*. Obese patients as compared with non-obese patients had disturbed mechanisms to buffer the actions of leptin, i.e., lower SOCS-3 expression in cartilage and a lower sOb-R concentration in synovial fluid.

4. SOCS-3 was shown to negatively regulate the leptin-induced responses in chondrocytes (Figure 23).

All of the studied adipokines were detectable in synovial fluid and in cartilage culture fluid, and associated with proinflammatory / catabolic activity of cartilage. These data together with the direct proinflammatory effects of leptin and adiponectin found in this study and of resistin reported by others, the findings suggest catabolic roles for

these three adipokines i.e. leptin, adiponectin and resistin, in the pathophysiology of OA.

Leptin associated with obesity, and with proinflammatory / catabolic factors especially in the obese patients. The elevated leptin concentrations together with altered SOCS-3 expression and sOb-R concentrations in obese individuals are likely to increase the detrimental effects of leptin in cartilage. These findings point to a role for leptin as a factor connecting obesity with OA. The mechanisms found to regulate and to be involved in leptin signaling could be promising drug targets to prevent or treat OA in obese individuals.

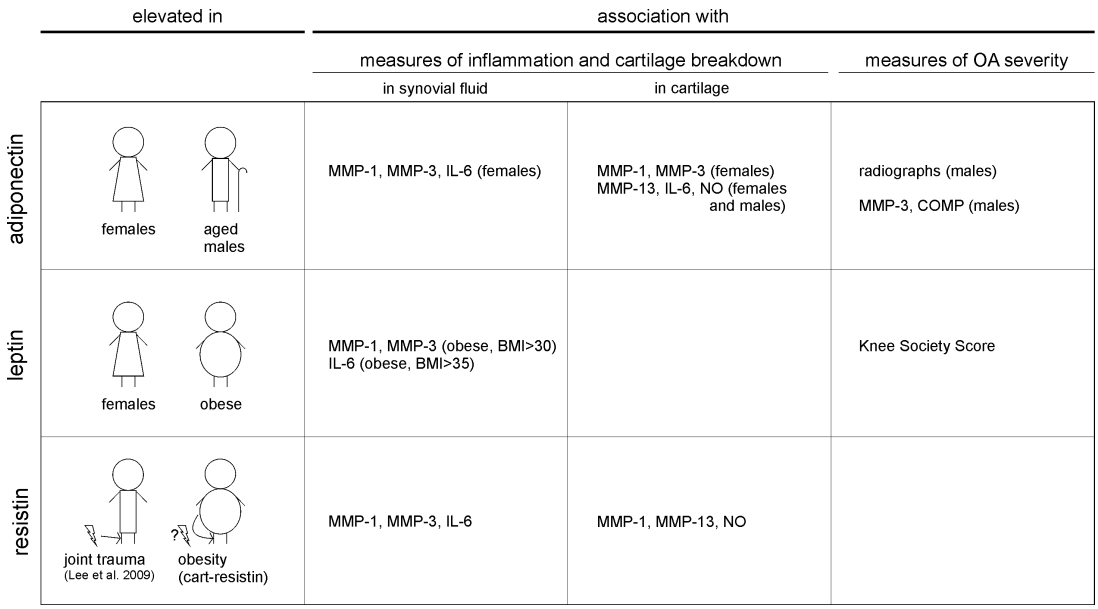

Figure 22 Summary of associations of the studied adipokines with catabolic and proinflammatory factors measured in synovial fluid and in cartilage culture media, and with measures of OA severity in 100 OA patients.

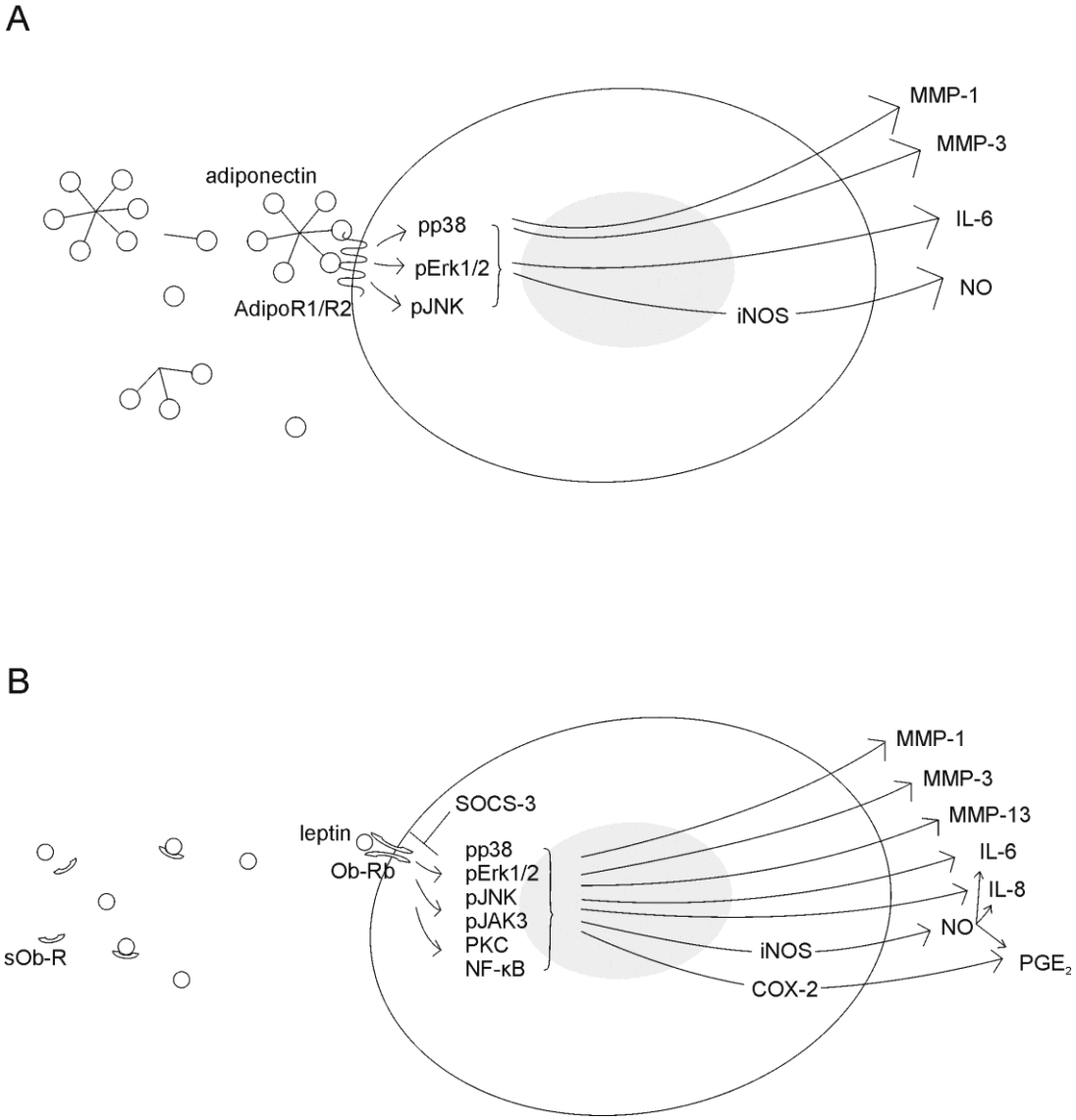

Figure 23 Summary of the effects of adiponectin (A) and leptin (B) on chondrocytes. AdipoR1/R2, adiponectin receptor R1 / R2; COX-2, cyclooxygenase-2; Erk1/2, extracellular signal-regulated kinase 1 and 2; IL, interleukin; iNOS, inducible nitric oxide synthase; JNK, c-Jun N-terminal kinase; MMP, matrix metalloproteinase; NF-κB, nuclear factor κB; NO, nitric oxide; Ob-Rb, functional leptin receptor; PGE_2, prostaglandin E_2; PKC, protein kinase C; sOb-R, soluble leptin receptor; SOCS-3, suppressor of cytokine signaling-3.

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Adiponectin associates with markers of cartilage degradation in osteoarthritis and induces production of proinflammatory and catabolic factors through mitogen-activated protein kinase pathways

Anna Koskinen[1], Sami Juslin[1], Riina Nieminen[1], Teemu Moilanen[1,2], Katriina Vuolteenaho[1*] and Eeva Moilanen[1]

Abstract

Introduction: Adiponectin is an adipokine that regulates energy metabolism and insulin sensitivity, but recent studies have pointed also to a role in inflammation and arthritis. The purpose of the present study was to investigate the association and effects of adiponectin on inflammation and cartilage destruction in osteoarthritis (OA).

Methods: Cartilage and blood samples were collected from 35 male OA patients undergoing total knee replacement surgery. Preoperative radiographs were evaluated using Ahlbäck classification criteria for knee OA. Circulating concentrations of adiponectin and biomarkers of OA, that is, cartilage oligomeric matrix protein (COMP) and matrix metalloproteinase 3 (MMP-3), were measured. Cartilage samples obtained at the time of surgery were cultured *ex vivo*, and the levels of adiponectin, nitric oxide (NO), IL-6, MMP-1 and MMP-3 were determined in the culture media. In addition, the effects of adiponectin on the production of NO, IL-6, MMP-1 and MMP-3 were studied in cartilage and in primary chondrocyte cultures.

Results: Plasma adiponectin levels and adiponectin released from OA cartilage were higher in patients with the radiologically most severe OA (Ahlbäck grades 4 and 5) than in patients with less severe disease (Ahlbäck grades 1 to 3). Plasma adiponectin concentrations correlated positively with biomarkers of OA, that is, COMP ($r = 0.55$, $P = 0.001$) and MMP-3 ($r = 0.34$, $P = 0.046$). Adiponectin was released by OA cartilage *ex vivo*, and it correlated positively with production of NO ($r = 0.43$, $P = 0.012$), IL-6 ($r = 0.42$, $P = 0.018$) and MMP-3 ($r = 0.34$, $P = 0.051$). Furthermore, adiponectin enhanced production of NO, IL-6, MMP-1 and MMP-3 in OA cartilage and in primary chondrocytes *in vitro* in a mitogen-activated protein kinase (MAPK)-dependent manner.

Conclusions: The findings of this study show that adiponectin is associated with, and possibly mediates, cartilage destruction in OA.

Introduction

Adiponectin belongs to the adipokine hormones, which were initially found to be synthesized by white adipose tissue and to control appetite and metabolism. Adiponectin was discovered in 1995 by Scherer *et al.* [1], and it was first named Acrp30 (adipocyte complement-related protein of 30 kDa). Adiponectin has been found to improve insulin sensitivity [2,3] and to have antiarthrogenic properties [4]. Interestingly, adiponectin has also been identified as a regulatory factor in inflammation and arthritis [5-8].

Adiponectin can be found in synovial fluid from osteoarthritis (OA) patients [9,10]. Tissues in the joint, including synovium, meniscus, osteophytes, cartilage,

* Correspondence: katriina.vuolteenaho@uta.fi
[1]The Immunopharmacology Research Group, University of Tampere School of Medicine and Tampere University Hospital, Medisiinarinkatu 3, Tampere, FI-33014, Finland
Full list of author information is available at the end of the article

bone and fat, have been reported to produce adiponectin [10-12]. The biological effects of adiponectin are mediated through two adiponectin receptor subtypes, adiponectin receptor type 1 (AdipoR1) and adiponectin receptor type 2 (AdipoR2), which have been shown to be expressed in articular cartilage, bone and synovial tissue [13,14].

In arthritis models and in joint tissues, adiponectin has been postulated to have both pro- and anti-inflammatory effects. Adiponectin has been reported to increase the production of cartilage-degrading matrix metalloproteinase (MMP) enzymes, cytokines and prostaglandin E_2 in chondrocytes and in synovial fibroblasts [11,14-19]. By contrast, intraarticularly injected adiponectin has been reported to mitigate the severity of collagen-induced arthritis in the mouse and to decrease immunohistochemically detected expression of TNF, IL-1 and MMP-3 [20]. Recently, high circulating adiponectin was found to correlate with cartilage degradation in patients with rheumatoid arthritis (RA) [21-23], although partly contradictory results have also been published [24,25].

Adiponectin has emerged as a regulator of immune responses and inflammatory arthritis [5-7], but its role in OA and cartilage degradation is controversial and, in many aspects, poorly known. The purpose of the present study was to investigate whether adiponectin is associated with radiographic severity or biomarkers of OA or with inflammatory and/or destructive factors released by cartilage samples obtained from OA patients. Since mitogen-activated protein kinase (MAPK) pathways have been proposed as therapeutic targets in OA [26,27], we decided also to study the possible involvement of these pathways in adiponectin-induced responses in OA cartilage.

Materials and methods

Patients and clinical studies

The patients in this study fulfilled the American College of Rheumatology classification criteria for OA [28]. Preoperative radiographs, blood samples and cartilage tissue were collected from 35 male patients with OA (means ± SEM: age = 69.5 ± 1.6 years, body mass index (BMI) = 29.3 ± 0.8 kg/m^2) undergoing total knee replacement surgery at Coxa Hospital for Joint Replacement, Tampere, Finland. Radiographs were evaluated according to the Ahlbäck criteria, grades I to V, with grade V representing the most severe findings [29]. Plasma and serum samples were stored at -80°C until analyzed for cartilage oligomeric matrix protein (COMP), MMP-3 and adiponectin. Cartilage samples were processed as described below, and the amounts of adiponectin, NO, IL-6, MMP-1 and MMP-3 released by the cartilage *ex vivo* during a 42-hour incubation were measured as described below. The study was approved by the Ethics Committee of Tampere University Hospital and carried out in accordance with the Declaration of Helsinki. Written informed consent was obtained from the patients.

Cartilage cultures

Leftover pieces of OA cartilage from knee joint replacement surgery were used. Full-thickness pieces of articular cartilage from femoral condyles, tibial plateaus and patellar surfaces showing macroscopic features of early OA were removed aseptically from subchondral bone with a scalpel, cut into small pieces and cultured in DMEM with GIBCO GlutaMAX-I supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml) and amphotericin B (250 ng/ml) (all from Invitrogen/Life Technologies, Carlsbad, CA, USA) at 37°C in a humidified 5% carbon dioxide atmosphere.

Cartilage samples were incubated for 42 hours with or without adiponectin (recombinant human adiponectin produced in HEK cells; BioVendor Research and Diagnostic Products, Modřice, Czech Republic) and the MAPK inhibitors PD98059 (Erk1/2 inhibitor, 10 μM; Promega, Madison, WI, USA), SB220025 (p38 inhibitor, 0.5 μM; Calbiochem/Merck KGaA, Darmstadt, Germany) and SP600125 (JNK inhibitor, 10 μM; Calbiochem/Merck KGaA). The concentrations of adiponectin and MAPK inhibitors used in the experiments were based on preliminary experiments and studies previously carried out in our laboratory [30-32]. After the experiments, the cartilage explants were weighed and the results were expressed per milligram of cartilage. The culture media were kept at -20°C until analyzed.

Primary chondrocyte experiments

The leftover pieces of OA cartilage were processed the same way as cartilage for cartilage cultures (see above). Cartilage pieces were washed with PBS, and chondrocytes were isolated by enzymatic digestion for 16 hours at 37°C in a shaker by using a collagenase enzyme blend (1 mg/ml Liberase TM Research Grade medium; Roche, Mannheim, Germany). Isolated chondrocytes were washed and plated on 24-well plates (1.5×10^5 cells/ml) in culture medium (DMEM with supplements; see above) containing 10% fetal bovine serum. Cells were treated with increasing concentrations of adiponectin (0.1 to 3 μg/ml) for 24 hours. The culture media were kept at -20°C until analyzed. Concentrations of NO, IL-6, MMP-1 and MMP-3 were determined in culture media as described below. To investigate MAPK activation (phosphorylation), cells were treated with adiponectin for 30 or 60 minutes and processed for Western blot analysis.

NO production

Concentrations of nitrite, a stable metabolite of NO in aqueous solutions, were measured by Griess reaction [33].

Measurement of adiponectin, COMP, MMP-1, MMP-3 and IL-6

Concentrations of adiponectin, COMP, MMP-1, MMP-3 and IL-6 in plasma, serum and/or medium samples were determined by performing ELISA with commercial reagents (adiponectin, MMP-1 and MMP-3: R&D Systems Europe Ltd, Abindgon, UK; COMP: BioVendor; IL-6: Sanquin, Amsterdam, The Netherlands).

Western blot analysis

Western blot analysis was performed as previously described [34] using the following antibodies: rabbit anti-human pAb inducible nitric oxide synthase (iNOS), actin and c-Jun N-terminal kinase (JNK) antibodies, and horseradish-conjugated goat anti-rabbit immunoglobulin G antibody from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and rabbit anti-human pAb p38, phospho-p38, phospho-JNK, extracellular signal-regulated kinase 1/2 (Erk1/2) and phospho-Erk1/2 antibodies from Cell Signaling Technology, Inc (Beverly, MA, USA).

Statistical analysis

Data were analyzed using SPSS version 17.0 for Windows software (SPSS Inc, Chicago, IL, USA) and GraphPad InStat version 3.00 software (GraphPad Software, San Diego, CA, USA). The results are presented as means ± SEM unless otherwise indicated. Pearson's correlation analysis was carried out, and r values over +0.3 and under -0.3 were considered to indicate a correlation. Differences between groups were tested by one-way analysis of variance (ANOVA) or repeated-measures ANOVA, followed by Fisher's least significant difference test or the Bonferroni correction for multiple comparisons when appropriate. P-values less than 0.05 were considered significant. Standard multiple regression analysis was used to predict circulating biomarker levels (COMP and MMP-3) when adiponectin, age and BMI were set as independent variables. Nonstandardized regression coefficients (β) and coefficients of determination squared (R^2) with the related P-values were calculated. Binary logistic regression was used to compute (BMI or age-adjusted) ORs for plasma adiponectin and adiponectin released by cartilage to predict the most severe radiographic findings (Ahlbäck grade 4 or 5 vs grades 1 to 3). A statistician was consulted regarding the statistical analysis.

Results

Correlation between plasma adiponectin and biomarkers of osteoarthritis

Thirty-five male OA patients were included in the study. Mean adiponectin concentration in plasma was 2.5 ± 0.2 μg/ml, and no correlation between plasma adiponectin and BMI was found (r = -0.15, P = 0.379). Interestingly, adiponectin correlated positively with the biomarkers of OA, that is, COMP (r = 0.55, P = 0.001) (Figure 1A) and MMP-3 (r = 0.34, P = 0.046) (Figure 1B), pointing to a possible connection between adiponectin and cartilage matrix degradation.

In multiple regression analysis, where serum COMP was set as a dependent variable and plasma adiponectin, age and BMI were set as predictive variables,

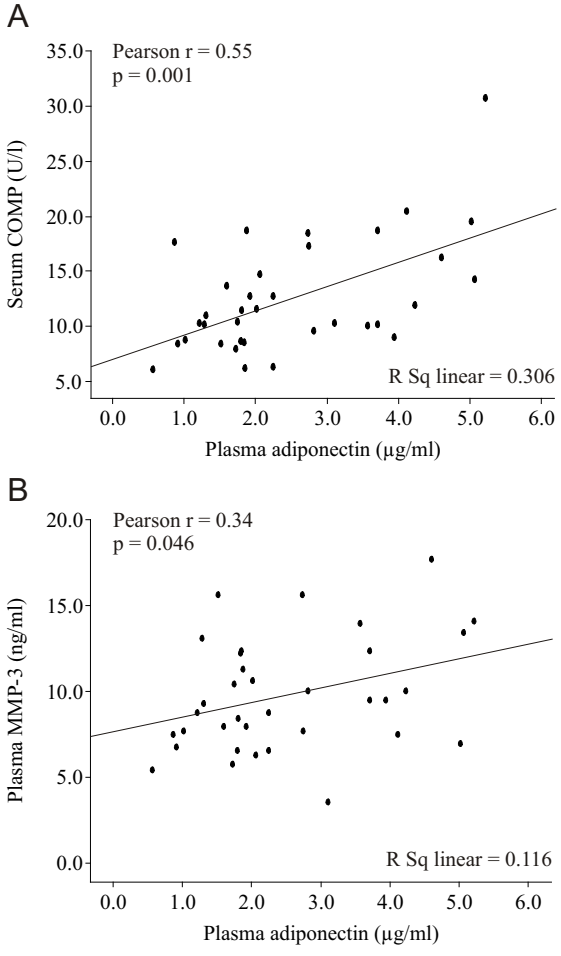

Figure 1 Adiponectin correlates with biomarkers of OA. Scatterplot showing positive correlations between plasma adiponectin and biomarkers of osteoarthritis (OA), serum cartilage oligomeric matrix protein (COMP) **(A)** and plasma matrix metalloproteinase 3 (MMP-3) **(B)**. N = 35 OA patients.

adiponectin (β (that is, expected change in COMP with 1-U change in adiponectin) = 1.7; P = 0.010), but not BMI (β = -0.10, P = 0.566) or age (β = 0.14, P = 0.160), was a significant determinant of COMP (R^2 = 0.39 and P = 0.001 for model). Also, adiponectin was a significant determinant of MMP-3 when it was set alone as an independent variable (β = 0.85, P = 0.046; R^2 = 0.12 and P = 0.046 for model). Addition of BMI or age as independent variables did not improve the model (R^2 = 0.15, P = 0.070 or R^2 = 0.14, P = 0.089, respectively).

Plasma adiponectin levels and radiographic severity of osteoarthritis

Preoperative radiographs of the knees were evaluated by Ahlbäck classification from grades 1 to 5, with grade 5 representing the most severe findings [29]. Grades 1 and 2, and 4 and 5 were combined to create more equally distributed subgroups. Mean plasma adiponectin concentrations were higher in the grades 4 and 5 group than in the grade 3 and grades 1 and 2 groups (Figure 2A), but there was no difference between the grades 1 and 2 group and the grade 3 group. There were no significant differences in age or BMI between the radiographic subgroups, but serum COMP was higher in the grades 4 and 5 group than in the grade 3 group and the grades 1 and 2 group (P = 0.012 and P = 0.006, respectively) (Table 1). Binary logistic regression analysis was used to further evaluate whether adiponectin is associated with the radiographic severity of OA (Ahlbäck grades 4 and 5 group vs Ahlbäck grades 1 to 3 group). When set alone in the model, plasma adiponectin and cartilage culture medium adiponectin, but not BMI or age, were significant explanatory factors of radiographic severity (Table 2). After adjusting for BMI, plasma adiponectin was a significant predictor of disease severity and almost statistically significant after adjusting for age (Table 2). Adiponectin measured in the cartilage culture media was a significant predictor of OA severity after controlling for age and BMI (Table 2).

Production of adiponectin and inflammatory and/or degrading factors by osteoarthritis cartilage *ex vivo*

Cartilage samples were obtained during joint replacement surgery from the same patients from whom the preoperative radiographs and the blood samples had been collected (see above), and tissue culture experiments were carried out. The amounts of adiponectin, NO, IL-6, MMP-1 and MMP-3 released from the cartilage into the culture medium during 42-hour incubation were measured. Adiponectin release was increased in patients with the radiographically most severe OA (Ahlbäck grades 4 and 5) as compared to patients in grades 1 and 2 and those in grade 3 (P = 0.004 and P < 0.001, respectively) (Figure 2B). Interestingly, adiponectin levels in the cartilage culture media correlated positively with those of NO (r = 0.43, P = 0.012) (Figure 3A), IL-6 (r = 0.42, P = 0.018) (Figure 3B) and MMP-3 (r = 0.34, P = 0.051) (Figure 3C), whereas no correlation between adiponectin and MMP-1 production was found (r = 0.17, P = 0.31).

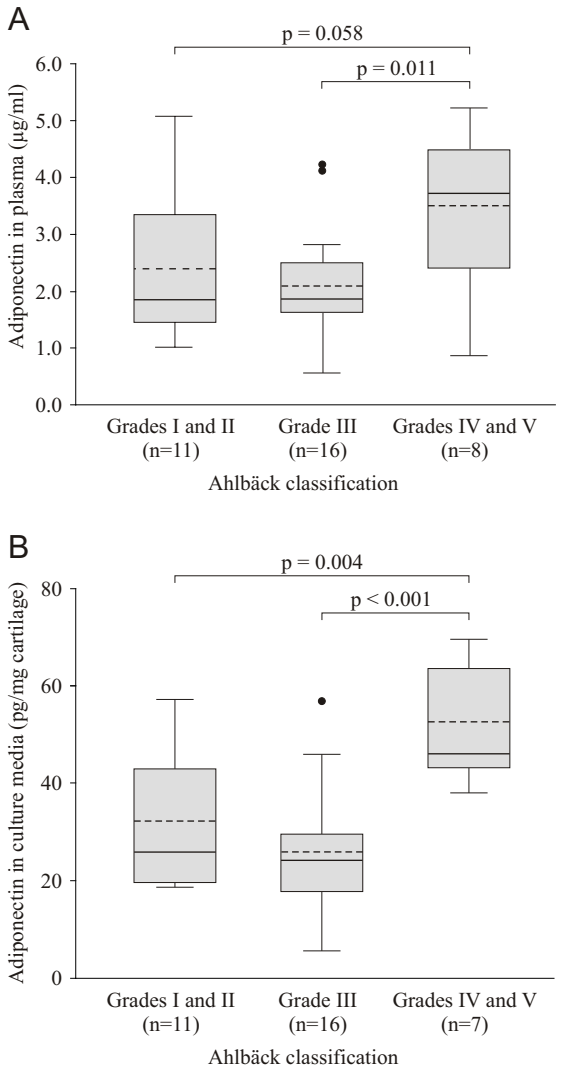

Figure 2 Adiponectin levels in plasma and those released by cultured cartilage were higher in patients with radiologically more severe osteoarthritis. Adiponectin levels in plasma **(A)** and the amount of adiponectin released by osteoarthritis (OA) cartilage samples into culture media **(B)** in OA patients classified according to the severity of knee OA evaluated using the Ahlbäck grading scale. Horizontal solid and dashed bars within the boxes represent the median and mean, respectively. Boxes represent the interquartile range. Lines outside boxes represent minimums and maximums. Outliers are indicated.

Effect of adiponectin on osteoarthritis cartilage and primary chondrocytes *in vitro*

To further evaluate the role of adiponectin in OA, we studied the effect of this adipokine on MAPK

Table 1 Plasma adiponectin levels and clinical characteristics of patients in radiographic subgroups

Patient characteristics	Radiographic severity of osteoarthritis by Ahlbäck classification			Total (*N* = 35)	*P*-values
	Grades 1 and 2 (*n* = 11)	Grade 3 (*n* = 16)	Grades 4 and 5 (*n* = 8)		
Adiponectin (μg/ml)	2.4 (0.4)	2.1 (0.2)	3.5 (0.5)	2.5 (0.2)	0.03
Age (years)	68.7 (3.0)	67.3 (2.6)	75.0 (2.0)	69.5 (1.6)	0.17
BMI (kg/m^2)	28.7 (1.1)	29.8 (1.4)	29.0 (1.6)	29.3 (0.8)	0.84
COMP (U/L)	10.6 (1.1)	11.7 (1.0)	17.0 (2.4)	12.6 (0.9)	0.01
MMP-3 (ng/ml)	9.6 (1.1)	9.2 (0.6)	10.9 (1.5)	9.7 (0.6)	0.52

BMI = body mass index; COMP = cartilage oligomeric matrix protein; MMP-3 = matrix metalloproteinase 3. Values are means (SEM). *P*-values refer to the significance of the comparison of the three groups calculated by analysis of variance.

phosphorylation (that is, activation) and on NO, IL-6, MMP-1 and MMP-3 production in primary chondrocytes from OA patients. Adiponectin treatment resulted in time-dependent phosphorylation of p38, Erk1/2 and JNK in primary OA chondrocytes that was obvious within 30 minutes and decreased toward baseline by 60 minutes (Figure 4). Adiponectin also enhanced NO, IL-6, MMP-1 and MMP-3 production in primary OA chondrocytes in a dose-dependent manner (Figure 5). Because cartilage matrix is an important regulator of chondrocyte metabolism, we wanted to investigate the effects of adiponectin on OA cartilage in tissue culture. Owing to the limited amount of tissue available for the experiments, one concentration of adiponectin (1 μg/ml) was selected based on the cell culture studies (Figure 5) and previously published data on adiponectin levels in OA synovial fluid [9,10,12,35]. Adiponectin enhanced NO, IL-6, MMP-1 and MMP-3 production and iNOS expression in OA cartilage culture, and their production was suppressed by the p38 MAPK inhibitor SB220025 (0.5 μM) (Figure 6). In addition, the Erk1/2 inhibitor PD98059 (10 μM) and the JNK inhibitor SP600125 (10 μM) inhibited adiponectin-induced production of IL-6 and NO, as well as expression of iNOS, in a statistically significant manner, whereas their effect on MMP-1 and MMP-3 was smaller and did not reach statistical significance (Figure 6).

Discussion

Adiponectin is found in OA joints, and proinflammatory and catabolic effects have been reported [9,10,13-19]. On the basis of our findings of the present study, we show for the first time that the circulating adiponectin concentrations correlate positively with the levels of the widely used biomarkers of OA, that is, COMP and MMP-3, and that plasma adiponectin levels, as well as adiponectin levels released by cultured cartilage, are associated with the radiographic severity of OA. Interestingly, the amount of adiponectin released by OA cartilage *ex vivo* also correlated positively with the production of inflammatory mediators NO and IL-6 and with the matrix-degrading enzyme MMP-3. Furthermore, adiponectin, when added at physiological concentrations to cultures of intact human OA cartilage or primary OA chondrocytes, enhanced the production of inflammatory and/or destructive factors NO, IL-6, MMP-1 and MMP-3. These findings suggest that adiponectin is associated with cartilage matrix degradation and has a role in the pathogenesis or as a biomarker in OA.

In the present study, we measured circulating levels of COMP and MMP-3 to evaluate the degree of ongoing cartilage destruction in OA [36]. The level of serum COMP has been shown to correlate with the grade of OA assessed by the radiological score [37], which we also observed in this study. Also, the concentrations of MMP-3 have been reported to associate with joint space narrowing [38]. The present results demonstrate for the first time that plasma adiponectin levels correlate with COMP and MMP-3, suggesting an association between adiponectin and the degree of ongoing cartilage matrix degradation.

Table 2 Association of adiponectin and radiographic severity of osteoarthritis

Patient characteristics	Ahlbäck grades 4 and 5			Ahlbäck grades 1 to 3		
	Crude OR	95% CI	*P*-values	Adjusted OR	95% CI	*P*-values
Plasma adiponectin (μg/ml)	2.2	1.1 to 4.3	0.022	2.2[a]	1.1 to 4.4	0.022
				1.9[b]	0.9 to 3.8	0.090
Culture media adiponectin (pg/mg cartilage)	1.1	1.0 to 1.2	0.007	1.1[a]	1.0 to 1.2	0.007
				1.1[b]	1.0 to 1.2	0.016
BMI (kg/m^2)	1.0	0.8 to 1.2	0.852			
Age (years)	1.1	1.0 to 1.3	0.078			

[a]Adjusted for BMI. [b]Adjusted for age. Odds ratios are given for Ahlbäck grades 4 and 5 group vs grades 1 to 3 group.

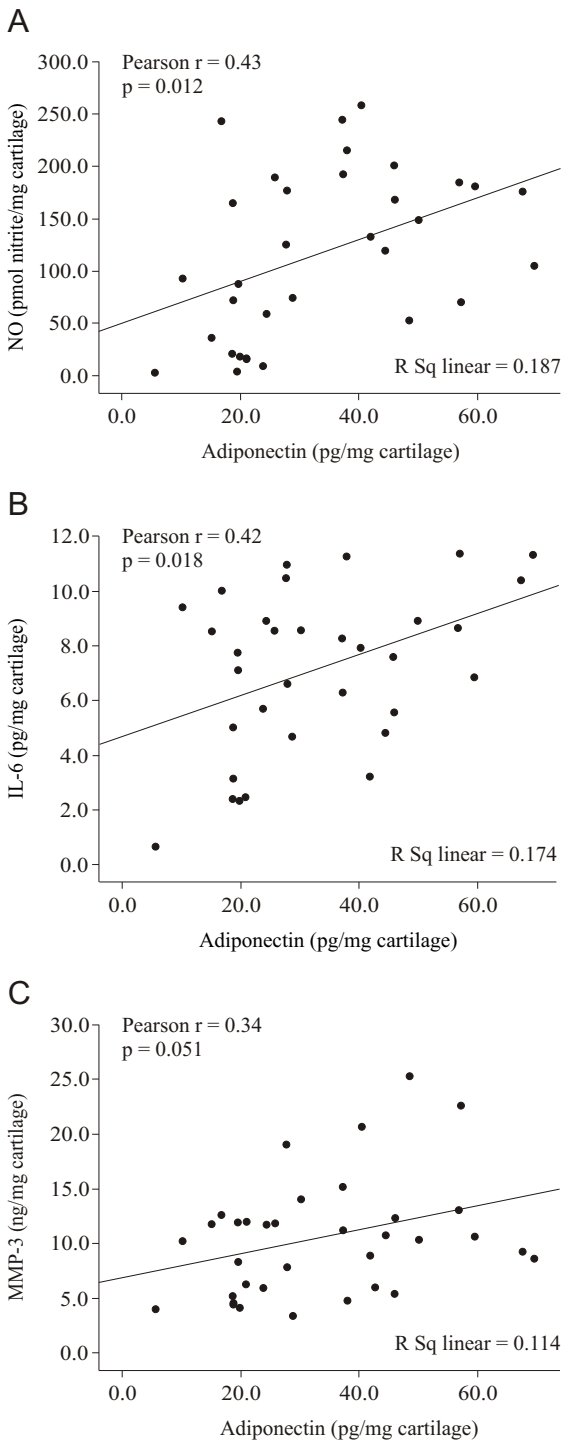

Figure 3 *Ex vivo* cartilage releases adiponectin and its concentration correlates with NO, IL-6 and MMP-3 production. Scatterplots show positive correlations between adiponectin and NO **(A)**, IL-6 **(B)** and matrix metalloproteinase 3 (MMP-3) **(C)** released by osteoarthritis (OA) cartilage into the culture medium. Cartilage samples were collected from 35 OA patients undergoing knee replacement surgery.

We also found that plasma adiponectin levels and adiponectin amounts released by cultured OA cartilage *ex vivo* were higher in patients with radiographically advanced OA (grades 4 and 5 according to the Ahlbäck classification system) than in patients with less severe disease (Ahlbäck grades 1 to 3). This suggests that adiponectin is associated with cartilage degradation in patients with OA. Our results are supported by the recent studies by Ebina *et al.* [21], Giles *et al.* [22] and Klein-Wieringa *et al.* [23], who showed that circulating adiponectin correlates with joint erosions in RA patients. Additional support for our findings is provided by the study of Laurberg *et al.* [39], who reported elevated plasma adiponectin concentrations in OA patients as compared to healthy controls. In our study, adiponectin released by cultured cartilage also correlated positively with NO, IL-6 and MMP-3 production in the cartilage.

Two recent studies have reported somewhat different findings on the association between plasma adiponectin levels and radiographic findings. In the study by Honsawek *et al.* [24], adiponectin concentrations in plasma and synovial fluid were lower in patients with more severe knee OA measured according to the Kellgren-Lawrence Grading Scale. After they adjusted for gender, age and BMI, their plasma findings became nonsignificant, but the differences between adiponectin levels in synovial fluid within the radiographic groups remained significant. Most of their patients were female, which may at least partly explain the differences between their results and ours. Also, the two different radiographic scaling systems emphasize different findings. We chose to use Ahlbäck grading, since it tends to divide the end-stage OA patients less roughly than the Kellgren-Lawrence Grading Scale, as reported by, for example, Petersson *et al.* [40]. Accordingly, most (80%) of our patients were scaled into the most severe Kellgren-Lawrence grade (grade 4). We included only male patients in the present study because gender is likely to be a confounding factor.

A study by Yusuf *et al.* [25] revealed that higher levels of plasma adiponectin decreased the risk for hand OA progression during a 6-year follow-up period as measured by radiographic changes. The findings of that study appear to be somewhat contradictory to our results and to those reported by the other research groups mentioned above. The differences may be explained by many factors, including different methodologies used to measure adiponectin, differences in patient characteristics and study protocols, gender differences (most of the patients in the study by Yusuf *et al.* were women) and possibly even by differences in the pathophysiologies of hand and knee OA. It is also possible

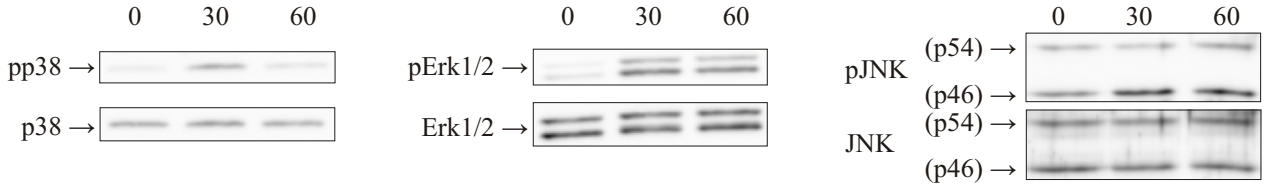

Figure 4 Adiponectin induces activation of mitogen-activated protein kinases in human primary chondrocytes. The effect of adiponectin (3 μg/ml) on mitogen-activated protein kinase (MAPK) phosphorylation in human primary chondrocytes obtained from patients with OA. The figure shows the results of a representative experiment which was repeated three times (that is, with cells from three donors) with similar results. MAPKs were determined by Western blot analysis at baseline and at 30 and 60 minutes after addition of adiponectin. Erk1/2 = extracellular signal-regulated kinase 1/2; JNK: c-Jun N-terminal kinase.

that the significance of adiponectin varies according to the phase and severity of the OA process. It is noteworthy that all our patients had advanced OA and were undergoing joint replacement surgery. This made it possible to obtain simultaneous cartilage and blood samples. Lack of patients with less severe OA, however, limits our ability to generalize the results to milder cases.

An inverse relationship between adiponectin levels and BMI, especially visceral fat, has been reported in studies in which an endocrinological approach was used [41]. However, no correlation between adiponectin and BMI was found in several recent clinical studies in which patients with OA or RA were investigated [35,39,42]. This was also the case within our group of OA patients. This may be explained by the fact that circulating concentrations of adiponectin can be regulated by various hormonal, nutritional or pharmacological factors and that adiponectin is produced not only by white adipose tissue but by other tissues as well [41]. The question remains open whether there is such a systemic factor that affects the adiponectin levels in patients with

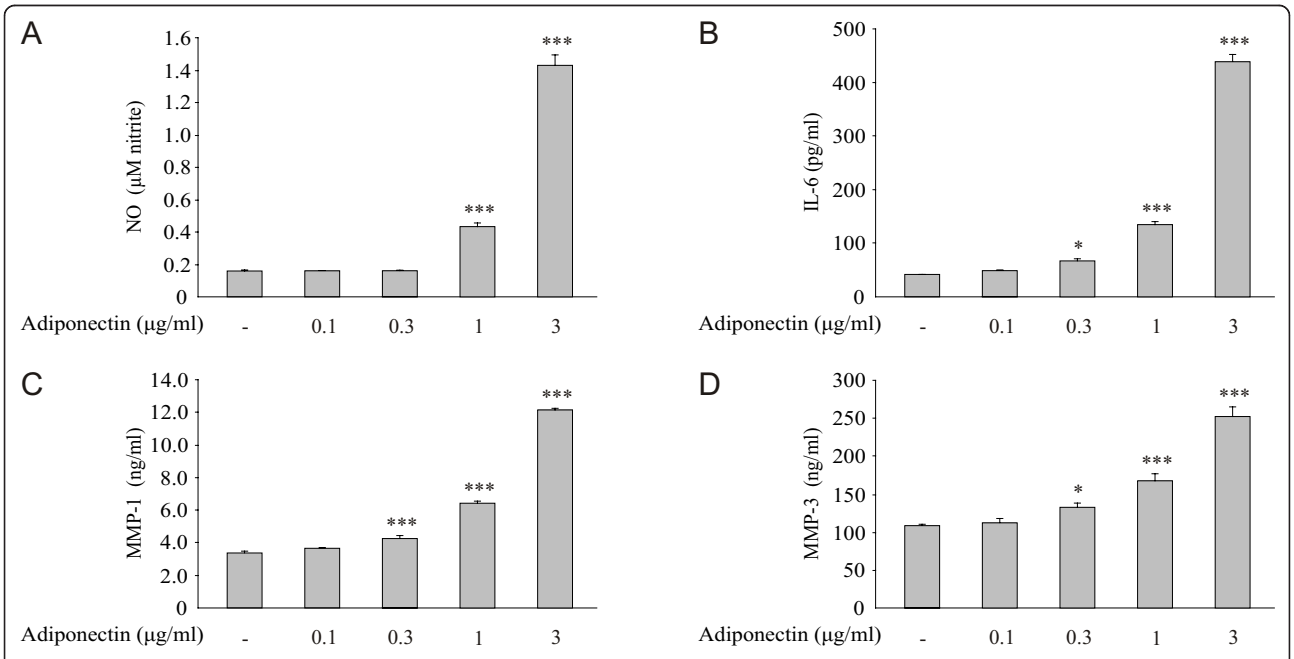

Figure 5 Adiponectin enhances NO, IL-6, MMP-1 and MMP-3 production in human primary chondrocytes in a dose-dependent manner. Chondrocytes obtained from patients with osteoarthritis (OA) were treated with increasing concentrations of adiponectin (0.1 to 3 μg/ml). Concentrations of NO **(A)**, IL-6 **(B)**, matrix metalloproteinase 1 (MMP-1) **(C)** and MMP-3 **(D)** were measured by the Griess reaction and ELISA in the culture medium after 24-hour incubation. The figure shows the results of a representative experiment which was repeated three times (that is, with cells from three donors) with similar results. Results are expressed as means ± SEM ($n = 4$). *$P < 0.05$ and ***$P < 0.001$ compared to the control sample.

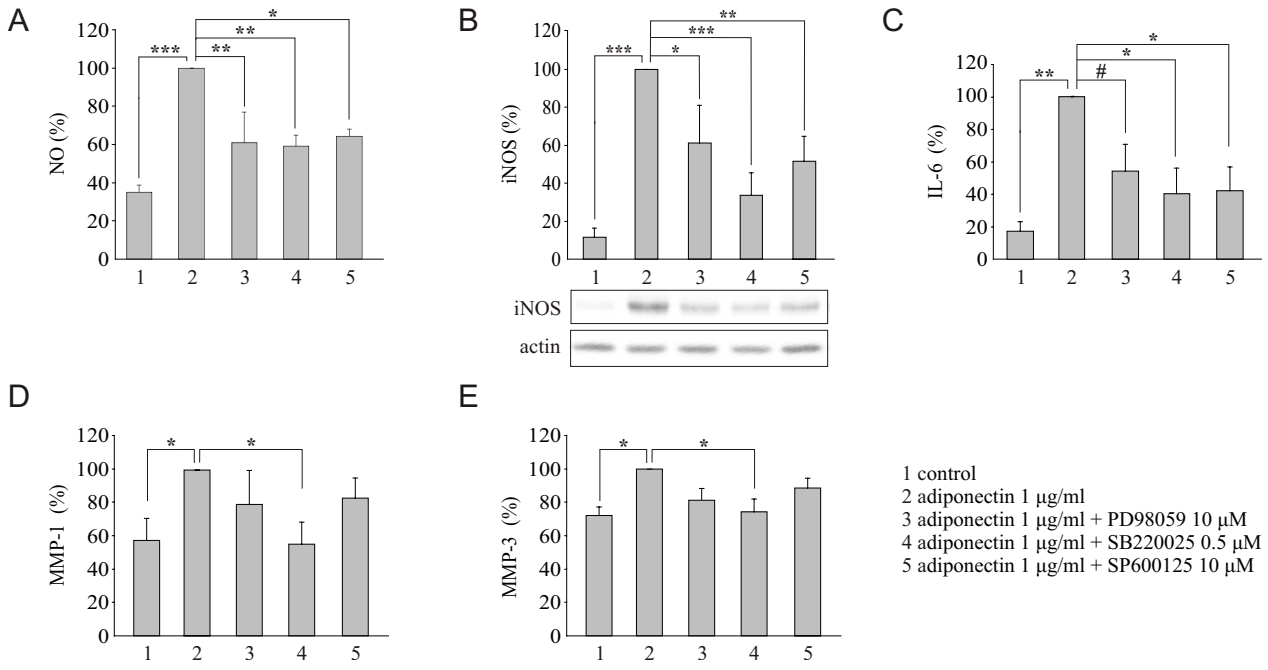

Figure 6 Mitogen-activated protein kinase pathways are involved in adiponectin induced NO, IL-6, MMP-1 and MMP-3 production in osteoarthritis cartilage. The effects of mitogen-activated protein kinase inhibitors on adiponectin-induced NO production **(A)**, inducible nitric oxide synthase (iNOS) expression **(B)**, IL-6 production **(C)**, matrix metalloproteinase 1 (MMP-1) production **(D)** and MMP-3 production **(E)** in human osteoarthritis cartilage. Cartilage explants were incubated for 42 hours with adiponectin (1 μg/ml) and the inhibitor indicated. Samples were collected from six patients in **(A)** and **(B)** and from five patients in **(C)** through **(E)**. Results are expressed as means ± SEM. $^{\#}P < 0.1$, $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared to explants treated with adiponectin alone. PD98059 = extracellular signal-regulated kinase 1/2 (Erk1/2) inhibitor; SB220025 = p38 inhibitor; SP600125 = c-Jun N-terminal kinase (JNK) inhibitor. The inhibitor concentrations used were based on previous studies in our laboratory [30-32].

arthritis or whether the joint disease itself rather than BMI might be a greater explanatory factor in defining the adiponectin levels in these patients.

As the clinical data suggest that the amount of adiponectin released by cartilage is related to the severity of cartilage degradation, we decided to study its possible mechanisms in OA cartilage. In agreement with recent findings [11,14-16,18], we found that adiponectin stimulated human OA cartilage and primary OA chondrocytes to produce NO, IL-6, MMP-1 and MMP-3, which are proinflammatory and catabolic mediators in OA [43-50]. In agreement with these findings, adiponectin was very recently reported to increase the production of chemokine IL-8 in human chondrocytes [19]. MAPK inhibitors are under development for treatment of OA [26,27], and MAPK pathways have been reported to be activated by adiponectin [51,52]. Therefore, we studied whether MAPK signaling pathways are also activated by adiponectin in articular chondrocytes and whether they might mediate adiponectin's effects on NO, IL-6, MMP-1 and MMP-3 production. Adiponectin was found to activate the kinases p38, JNK and Erk1/2 at physiologically relevant concentrations. The p38 inhibitor decreased the production of all factors studied in a statistically significant manner, whereas Erk1/2 was involved in adiponectin-induced iNOS expression and NO production and JNK was involved in NO, iNOS and IL-6 production. These results, together with recently published findings [14,18], show that MAPKs, especially p38, are significant pathways in adiponectin signaling in chondrocytes. Also, MAPK inhibitors are likely to attenuate adiponectin-induced gene expression in OA cartilage.

Adipokines, that is, hormones secreted by adipose tissue, have emerged as important modulating agents, not only in energy metabolism and appetite but also in the immune system and inflammation [53], and they are likely to have a role in mediating the connection between obesity and chronic inflammatory diseases. The actions of adiponectin, leptin, resistin and other, less studied adipokines in OA and other rheumatic diseases have recently been reviewed by Gómez *et al*. [7] and by Neumann *et al*. [8]. The most studied adipokine in the pathophysiology of arthritis is leptin, which has been proven to have proinflammatory and catabolic roles in OA [8,19,54-58]. Knowledge about adiponectin in joint diseases has accumulated only lately. The present results, together with those described in the other recent

reports, strongly suggest a proinflammatory and catabolic role for adiponectin in OA and RA cartilage.

Conclusions

We found that adiponectin was associated with markers and signs of cartilage degradation, that is, with circulating concentrations of COMP and MMP-3 and with radiographic severity of OA. Adiponectin was released by OA cartilage *ex vivo*, and it correlated with production of NO, IL-6 and MMP-3, which are important mediators in the pathogenesis of OA. Subsequent *in vitro* studies demonstrated that adiponectin, when added to the culture media, enhanced the production of NO, IL-6, MMP-1 and MMP-3 in OA cartilage and primary OA chondrocytes. Adiponectin also activated p38, Erk1/2 and JNK in chondrocytes, and the adiponectin-induced production of NO, IL-6, MMP-1 and MMP-3 were mediated by MAPKs, especially by p38. These findings strongly suggest that adiponectin is involved in the pathogenesis of joint inflammation and cartilage destruction in OA and may be a target for disease-modifying drug development.

Abbreviations

BMI: body mass index; COMP: cartilage oligomeric matrix protein; DMEM: Dulbecco's modified Eagle's medium; ELISA: enzyme-linked immunosorbent assay; Erk1/2: extracellular signal-regulated kinase 1/2; HEK cells: human embryonic kidney cells; IL: interleukin; iNOS: inducible nitric oxide synthase; JNK: c-Jun N-terminal kinase; MAPK: mitogen-activated protein kinase; MMP: matrix metalloproteinase; NO: nitric oxide; OA: osteoarthritis; pAb: polyclonal antibody; PBS: phosphate-buffered saline; RA: rheumatoid arthritis; TNF: tumor necrosis factor.

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Author details

[1]The Immunopharmacology Research Group, University of Tampere School of Medicine and Tampere University Hospital, Medisiinarinkatu 3, Tampere, FI-33014, Finland. [2]Coxa Hospital for Joint Replacement, Biokatu 6b, Tampere, FI-33520, Finland.

Authors' contributions

AK, SJ and KV were involved in the conception and design of the study, the laboratory analyses, calculation of the results and interpretation of the data, and they drafted the manuscript. RN was involved in the conception and design of the study, the laboratory analyses, the interpretation of the data and revising the manuscript. TM was involved in the conception and design of the study, selecting the patients, acquiring the patient samples, the interpretation of the data and revising the manuscript. EM was involved in the conception and design of the study, the interpretation of the data and writing the manuscript. All authors approved the final version of the manuscript.

Competing interests

The authors declare that they have no competing interests.

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Research Article

Leptin Enhances Synthesis of Proinflammatory Mediators in Human Osteoarthritic Cartilage—Mediator Role of NO in Leptin-Induced PGE_2, IL-6, and IL-8 Production

Katriina Vuolteenaho,[1] Anna Koskinen,[1] Meiju Kukkonen,[1] Riina Nieminen,[1] Unto Päivärinta,[2] Teemu Moilanen,[1,2] and Eeva Moilanen[1]

[1] *The Immunopharmacology Research Group, Medical School, University of Tampere and Research Unit, Tampere University Hospital, 33014 University of Tampere, Tampere, Finland*
[2] *Coxa Hospital for Joint Replacement, P.O. Box 652, 33101 Tampere, Finland*

Correspondence should be addressed to Eeva Moilanen, eeva.moilanen@uta.fi

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Obesity is an important risk factor for osteoarthritis (OA) in weight-bearing joints, but also in hand joints, pointing to an obesity-related metabolic factor that influences on the pathogenesis of OA. Leptin is an adipokine regulating energy balance, and it has recently been related also to arthritis and inflammation as a proinflammatory factor. In the present paper, the effects of leptin on human OA cartilage were studied. Leptin alone or in combination with IL-1 enhanced the expression of iNOS and COX-2, and production of NO, PGE_2, IL-6, and IL-8. The results suggest that the effects of leptin are mediated through activation of transcription factor nuclear factor κB (NF-κB) and mitogen-activated protein kinase (MAPK) pathway c-Jun NH_2-terminal kinase (JNK). Interestingly, inhibition of leptin-induced NO production with a selective iNOS inhibitor 1400 W inhibited also the production of IL-6, IL-8, and PGE_2, and this was reversed by exogenously added NO-donor SNAP, suggesting that the effects of leptin on IL-6, IL-8, and PGE_2 production are dependent on NO. These findings support the idea of leptin as a factor enhancing the production of proinflammatory factors in OA cartilage and as an agent contributing to the obesity-associated increased risk for osteoarthritis.

1. Introduction

Leptin is a hormone that was initially found to be synthesized by white adipocytes and has a strong correlation with the amount of adipose tissue and with body mass index (BMI). Leptin was first discovered in 1994 and found to act as a signal for the central nervous system to inhibit food intake and to stimulate energy expenditure [1]. More recent findings on the ubiquitous expression of leptin receptors in almost all tissues and on its cellular effects have revealed that leptin is also involved in the regulation of a variety of biological functions related to immune responses and inflammatory diseases, and to cardiovascular and respiratory pathophysiology [2–5].

Obesity is an important risk factor for osteoarthritis (OA) [6]. Increased joint loading and altered mechanic loading axis has been proposed to explain the increased risk of OA in weight-bearing joints, including hip and knee joints. Surprisingly, there is also a positive association between obesity and OA in the hand, pointing to an obesity related metabolic factor that acts as a risk factor for OA. Recently, it has been shown that synovial fluid (SF) from OA patients contains leptin concentrations that are similar or higher than those measured in serum [7–9]. Furthermore, low soluble leptin receptor (sOb-R) level in SF potentiates the biological activity of leptin in the joint as compared to that in serum [8]. Articular cartilage has been reported to produce leptin [8, 10] and express functional leptin receptor Ob-R [11], and the expression of these two is further increased in advanced OA correlating with BMI of the patients [9].

In vitro, leptin has been shown to potentiate interleukin-1 (IL-1) and interferon γ (IFNγ)-induced production of

nitric oxide (NO) in chondrocytes, which is a proinflammatory and destructive mediator in cartilage [12, 13]. Leptin has been shown to decrease chondrocyte proliferation and to increase production of proinflammatory cytokine IL-1β and destructive matrix metalloproteinases 9 and 13 (MMP-9 and 13) in human chondrocytes [9, 14]. On the other hand, leptin has also been reported to increase proliferation and to enhance proteoglycan and collagen synthesis in human chondrocytes [11]. In vivo, leptin injection into rat knee was reported to increase synthesis of insulin-like growth factor 1 (IGF-I) and transforming growth factor β (TGFβ) both contributing to increased proteoglycan synthesis [7]. These effects are linked to increased cartilage matrix production, and also to osteophyte formation.

NO is related to the pathogenesis of OA as a destructive mediator [15]. Inducible nitric oxide synthase (iNOS) is expressed in OA cartilage, and there are markers of enhanced NO production in OA joints [15, 16]. Prostaglandins (PGs), especially PGE_2, mediate inflammation, tissue destruction, and pain in OA and in OA joints they are formed by cyclooxygenase (COX) enzymes (particularly COX-2) and prostaglandin synthases [17]. Interleukin-6 (IL-6) and interleukin-8 (IL-8) are produced by OA cartilage and have a proinflammatory and modulatory role in the pathogenesis of OA [10, 18].

The presence of bioactive leptin in OA joint and the effects of leptin on cartilage metabolism point to a pathophysiological role for leptin in OA. The aim of the present study was to investigate the effects of leptin on mediators of cartilage metabolism by measuring its effects on the production of NO, PGE_2, IL-6, and IL-8 in OA cartilage and by evaluating the signaling mechanisms involved in these effects by pharmacological means.

2. Materials and Methods

2.1. Patients and Cartilage Cultures. Cartilage tissue was obtained from the leftover pieces of total knee replacement surgery. The study was approved by the Ethics Committee of Tampere University Hospital, and the patients gave their written approval. The donor patients, age ranging from 53 to 87 years and body mass index ranging from 20 to 32, were all diagnosed to have osteoarthritis.

Cartilage samples were washed with phosphate buffered saline (PBS) and processed for the experiments within two hours after the operation. Full thickness pieces of articular cartilage from femoral condyles, tibial plateaus, and patellar surfaces showing macroscopical features of early OA were removed aseptically from subchondral bone with a scalpel and cut into small pieces (about $2 \times 2 \times 2$ mm). Cartilage cubes randomly selected from 3 different areas of the joint were incubated in one well of a 6-well plate in 3 mL of tissue culture medium (Dulbecco's modified Eagle's medium (DMEM) with glutamax-I containing 10% heat-inactivated fetal bovine serum, penicillin (100 units/mL), streptomysin (100 μg/mL), and amphotericin B (250 ng/mL); all obtained from Invitrogen, Carlsbad, Calif, USA)) at 37°C in humidified 5% carbon dioxide atmosphere.

In the first two series of experiments, OA cartilage explants from 8 patients were incubated with leptin (0.1 μg/mL or 10 μg/mL) alone or in combination with IL-1β (10 pg/mL) for 48 hours. Concentrations of NO, PGE_2, IL-6, and IL-8 were determined in the culture medium. In the third series of experiments OA explants from 3 patients were incubated with leptin (0.1 μg/mL or 10 μg/mL) alone or in combination with IL-1β (10 pg/mL) for 48 hours. Cartilage samples were used to determine expression of iNOS and COX-2 protein. In the fourth series of experiments, signaling mechanisms involved in the leptin-induced NO, PGE_2, IL-6, and IL-8 production were studied using pharmacological inhibitors. OA explants from 7 patients were incubated for 48 hours with leptin (10 μg/mL) and following signaling pathway inhibitors: SP600125 10 μM (JNK inhibitor), SB220025 0.5 μM (p38 inhibitor), PD98059 10 μM (Erk1/2 inhibitor), AG490 10 μM (JAK2 inhibitor), WHI-P154 10 μM (JAK3 inhibitor), Ro 31-8220 1 μM (PKC inhibitor), MG132 10 μM (NF-κB inhibitor), and PDTC 10 μM (NF-κB inhibitor). Concentrations of NO, PGE_2, IL-6, and IL-8 were determined in the culture medium. In the fifth series of experiments, the effect of NO on leptin-induced (10 μg/mL) IL-6, IL-8, and PGE_2 production was studied by inhibiting leptin-induced endogenous NO production with a selective iNOS inhibitor 1400 W (1 mM) during a 48 hour incubation of OA explants from 8 patients. The effect of NO was further investigated by studying if exogenous NO could reverse the effects of iNOS inhibitor 1400 W in leptin-treated cartilage. This was made by adding NO-donor SNAP (100 μM) together with 1400 W and leptin in the cartilage cultures. Concentrations of NO, PGE_2, IL-6, and IL-8 were determined in the culture medium.

After the experiment the cartilage explants were weighed, and the results were expressed per mg of cartilage. Aliquots of the culture media were kept at −20°C until assayed, and cartilage samples for Western blotting were first snap frozen in liquid nitrogen and analysed as described below.

2.2. NO Production. Concentrations of nitrite, a stable product of NO in aqueous solutions, were measured using the Griess reaction [19]. The results were expressed as pmol of nitrite/mg of cartilage.

2.3. Prostaglandin E_2 Assays. The amount of PGE_2 released into the incubation medium was determined by radioimmunoassay, using reagents from the Institute of Isotopes (Budapest, Hungary). The results were expressed as pg of PGE_2/mg of cartilage.

2.4. IL-6 and IL-8 Assays. The concentrations of IL-6 (Sanquin, PeliPair, Amsterdam, The Netherlands) and IL-8 (R&D Systems, Minneapolis, MN, USA) in the culture medium were determined by ELISA. The results were expressed as pg of IL-6/mg of cartilage, or pg of IL-8/mg of cartilage.

2.5. Western Blot Analysis. After incubations, the cartilage specimen were snap frozen in liquid nitrogen, milled and lysed in extraction buffer (10 mM Tris-HCl, 5 mM

EDTA, 50 mM NaCl, 1% Triton-X-100, 0.5 mM phenylmethylsulfonylfluoride (PMSF), 1 mM sodiumorthovanadate, 20 μg/mL leupeptin, 50 μg/mL aprotin, 5 mM sodium fluoride, 2 mM sodium pyrophosphate, 10 μM n-octyl-beta-D-glucopyranoside). Following incubation on ice for 15 minutes, samples were centrifuged and supernatants were mixed with sample buffer 1 : 4 (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 0.025% bromophenol blue, and 5% β-mercaptoethanol) and stored at -20°C until analyzed. Coomassie blue method was used to measure the protein content of the samples [20]. After boiling, protein samples (20 μg) were separated with 8% SDS-polyacrylamide electrophoresis gels and transferred to Hybond enhanced chemiluminescence nitrocellulose membrane (Amersham Biosciences UK Limited, Buckinghamshire, UK). Proteins were identified by Western blotting using rabbit polyclonal antibody for human iNOS and goat polyclonal antibody for human COX-2 (both obtained from Santa Cruz Biotechnology, Santa Cruz, Calif, USA). Actin was analysed as a loading control by using rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, Calif, USA). Bound antibody was detected using SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL, USA) and FluorChem 8800 imaging system (Alpha Innotech, San Leandro, Calif, USA). Quantitation of the chemiluminescent signal was carried out with FluorChem software v.3.1.

2.6. Statistical Analysis. Results are expressed as mean ± standard error of the mean (SEM). Statistical significance of the results was calculated by using paired t-test.

2.7. Materials. Recombinant Human Leptin and Recombinant Human IL-1 beta were purchased from R&D Systems; manufacturer ensures low endotoxin level of the products (<1.0 EU per 1 μg of the recombinant protein), and that amount in relation to the leptin concentrations used in the present study was tested to have no effect in our tissue culture conditions. Other reagents were obtained as follows: SP600125, SB220025, AG 490 and WHI-P154 were from Calbiochem (Merck, Darmstadt, Germany); PD 98059 was from Promega (Madison, WI, USA); Ro 31-8220 was from Alexis Corporation (Lausen, Switzerland); MG 132 was from Tocris Bioscience (Ellisville, MO, USA); PDTC was from Sigma Chemical Co (St Louis, MO, USA); SNAP was from Cayman Chemical (Ann Arbor, MI, USA). 1400 W was kindly given by Dr Richard G Knowles, Glaxo SmithKline Research & Development, Stevenage, UK.

3. Results

3.1. The Effects of Leptin on NO, PGE_2, IL-6, and IL-8 Production in Human OA Cartilage. Leptin (0.1 μg/mL and 10 μg/mL) enhanced NO production in OA cartilage in a dose-dependent manner (Figure 1(a)). Western blot analysis with human iNOS antibody showed that leptin (10 μg/mL) induced also iNOS expression in cultured cartilage tissue (Figure 1(b)). In addition, leptin (10 μg/mL) increased PGE_2 production and COX-2 expression, and IL-6 and IL-8 production in human OA cartilage during 48 hours incubation (Figures 1(c), 1(d), 1(e), and 1(f)).

A low concentration of proinflammatory cytokine IL-1β (10 pg/mL) had a slight stimulatory effect on NO, PGE_2, IL-6, and IL-8 production and iNOS and COX-2 expression (Figure 2). Leptin (10 μg/mL) enhanced NO, PGE_2, IL-6, and IL-8 production, and iNOS and COX-2 expression in OA cartilage also in the presence of IL-1β (Figure 2).

3.2. Signaling Mechanisms Involved in the Leptin-Induced NO, PGE_2, IL-6, and IL-8 Production. The involvement of signaling pathways (JNK, p38, and Erk1/2 MAP-kinases, JAK2 and JAK3, PKC, and transcription factor NF-κB) in leptin-stimulated NO, PGE_2, IL-6, and IL-8 production in OA cartilage was studied by pharmacological means. Inhibitors of transcription factor NF-κB, MG 132 (10 μM) and PDTC (100 μM), and JNK inhibitor SP600125 (10 μM) significantly inhibited leptin-induced NO, PGE_2, IL-6, and IL-8 production (Figure 3). In addition to the effect of JNK inhibitor, inhibitors of other MAP-kinases, that is, SB220025 (p38 inhibitor; 0.5 μM) and PD 98059 (Erk1/2 inhibitor; 10 μM) inhibited leptin-induced PGE_2 production, but had no effect on NO, IL-6, or IL-8 production. JAK2 inhibitor AG 490 (10 μM) had no effect on leptin-induced NO, PGE_2, IL-6, or IL-8 production, whereas JAK3 inhibitor WHI-P154 (10 μM) inhibited leptin-induced NO synthesis, but not PGE_2, IL-6, or IL-8 production. Leptin-induced NO, IL-6, and IL-8 production was inhibitable with protein kinase C inhibitor Ro 31-8220 (1 μM), while it had no effect on PGE_2 production.

3.3. The Effect of NO on Leptin-Induced IL-6, IL-8, and PGE_2 Production. A selective iNOS inhibitor 1400 W (1 mM) inhibited leptin-induced NO production almost completely indicating that it was synthesized through iNOS pathway (Figure 4(a)). Interestingly, inhibition of NO production with 1400 W reduced also the production of IL-6, IL-8, and PGE_2 (Figures 4(b)–4(d)). The effect of 1400 W was reversed when NO-donor SNAP (100 μM) was added into the culture. Those results suggest that the increasing effect of leptin on IL-6, IL-8, and PGE_2 production in human OA cartilage is dependent on NO.

4. Discussion

Osteoarthritis is a chronic disease characterised by gradual loss of the articular cartilage. The course of the destructive process is determined by the balance between anabolic and catabolic mediators and their regulators in the joint, and the local distribution of these mediators in the cartilage [18]. Leptin is an obesity related mediator, which has been suggested to take part in the regulation of anabolic and catabolic processes within the osteoarthritic joint and to play a role in the pathogenesis of OA [21]. In the present study, we found that leptin induced the production of NO, PGE_2, IL-6, and IL-8 in human osteoarthritic cartilage and that leptin-induced PGE_2, IL-6, and IL-8 production was dependent on NO. These findings support the role of leptin in the pathogenesis of OA.

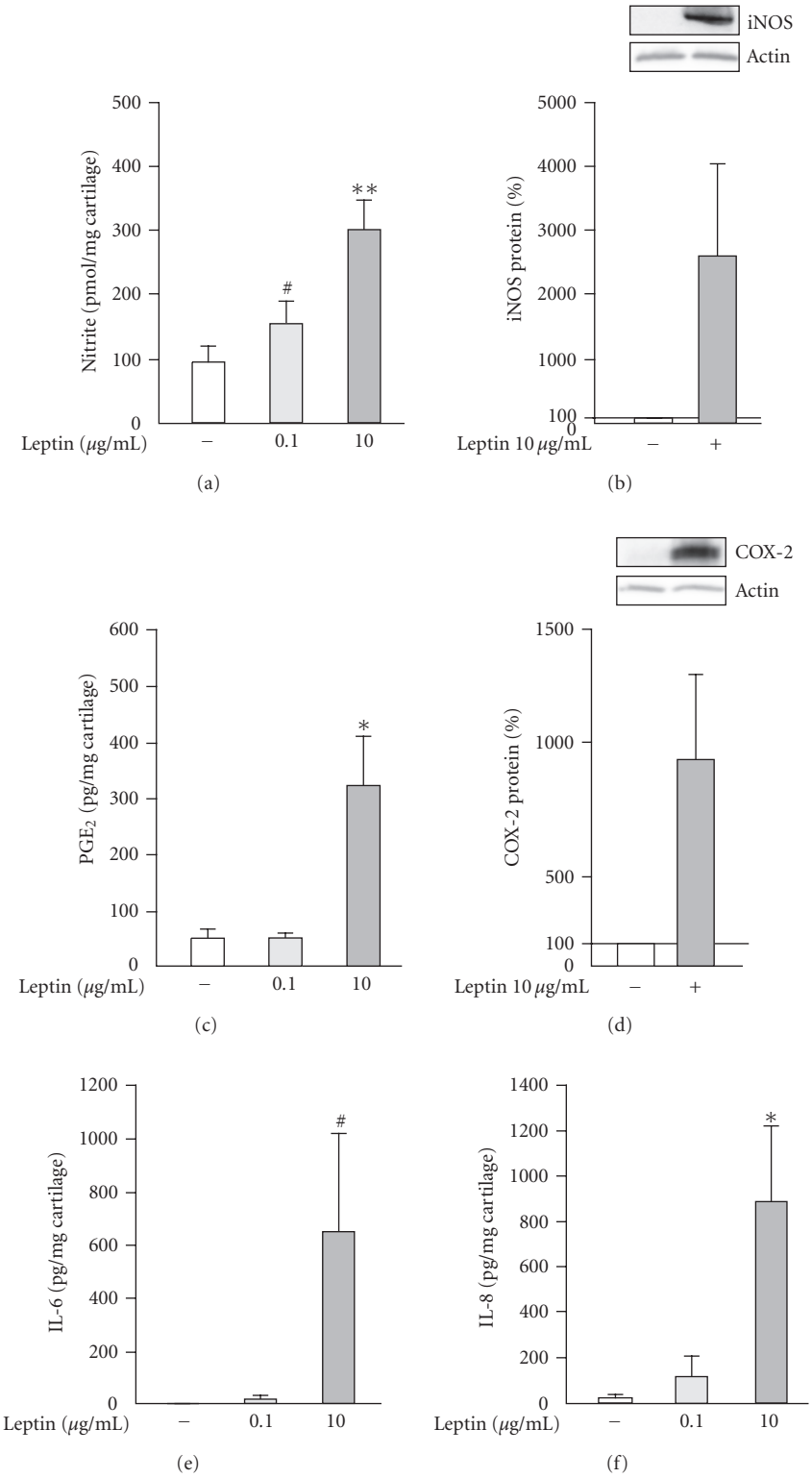

Figure 1: The effect of leptin on NO production (a), iNOS protein expression (b), PGE_2 production (c), COX-2 protein expression (d), IL-6 production (e), and IL-8 production (f) in human OA cartilage. Cartilage explants were incubated with leptin (0.1 μg/mL or 10 μg/mL) for 48 hours. NO production (a) was measured as nitrite accumulation in the culture medium by Griess reaction. Expression of iNOS protein (b) and COX-2 protein (d) were measured by Western blot. PGE_2 production (c) in the culture medium was measured by RIA. Concentrations of IL-6 (e), and IL-8 (f) in the culture medium were measured by ELISA. Results are expressed as pmol/mg cartilage (a), as percentages in comparison with control sample ((b) and (d)) or pg/mg cartilage ((c), (e), and (f)). Values are mean ± SEM. Samples were collected from 6 patients ($n = 6$) in (a) and (c), from 3 patients ($n = 3$) in (b) and (d), and from 7 patients ($n = 7$) in (e) and (f). #: $P < .2$, ∗: $P < .05$, and ∗∗: $P < .01$ as compared to control explants incubated in absence of exogenous leptin.

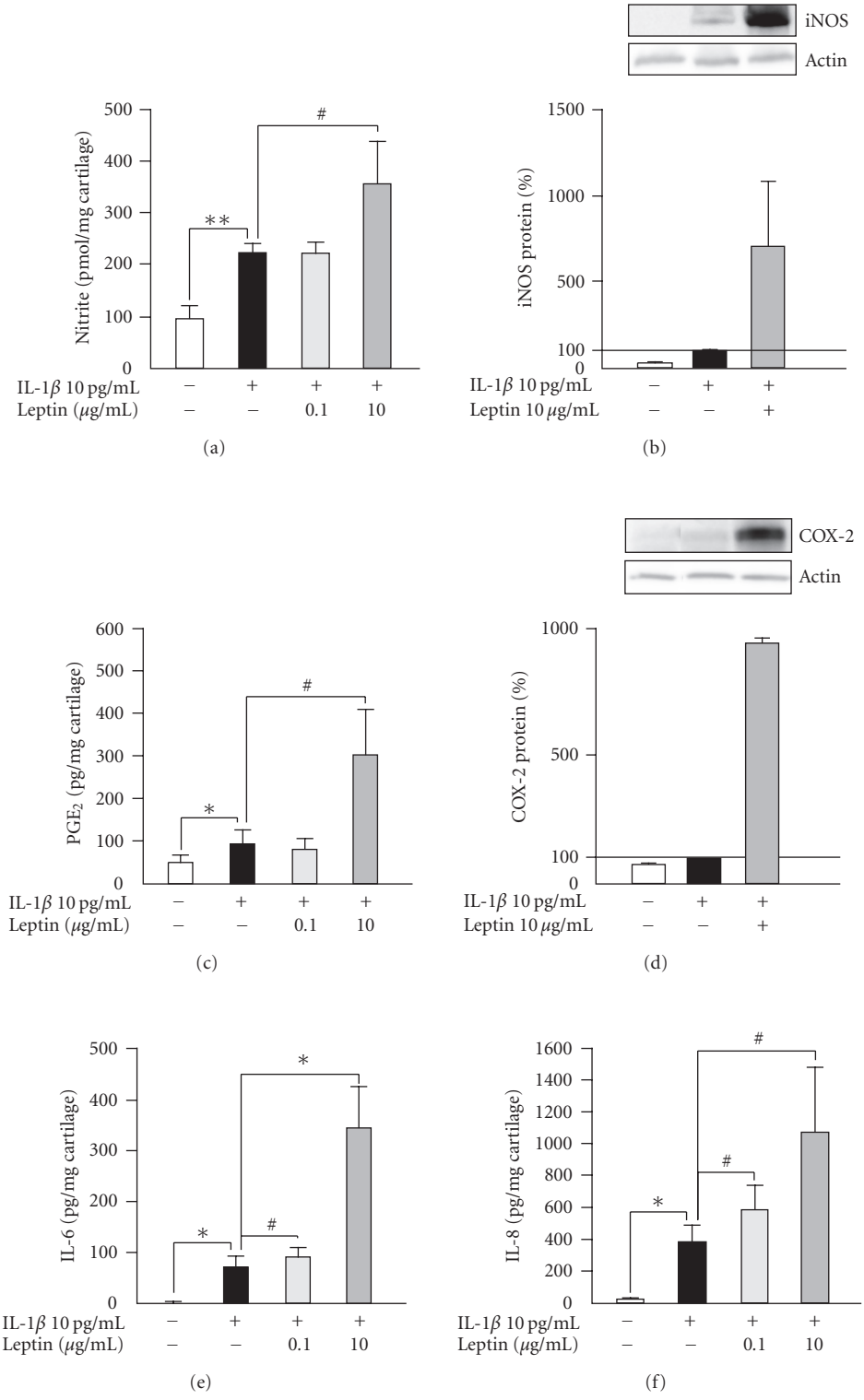

Figure 2: The effect of leptin on NO production (a), iNOS protein expression (b), PGE_2 production (c), COX-2 protein expression (d), IL-6 production (e), and IL-8 production (f) in human OA cartilage in the presence of IL-1β. Cartilage explants were incubated with IL-1β (10 pg/mL) alone or in combination with leptin (0.1 μg/mL or 10 μg/mL) for 48 hours. NO production (a) was measured as nitrite accumulation in the culture medium by Griess reaction. Expression of iNOS protein (b) and COX-2 protein (d) were measured by Western blot. PGE_2 production (c) in the culture medium was measured by RIA. Concentrations of IL-6 (e) and IL-8 (f) in the culture medium were measured by ELISA. Results are expressed as pmol/mg cartilage (a), as percentages in comparison with control sample ((b) and (d)) or pg/mg cartilage ((c), (e), and (f)). Values are mean ± SEM. Samples were collected from 6 patients ($n = 6$) in (a) and (c), from 3 patients ($n = 3$) in (b) and (d), and from 7 patients ($n = 7$) in (e) and (f). #: $P < .2$, ∗: $P < .05$, and ∗∗: $P < .01$.

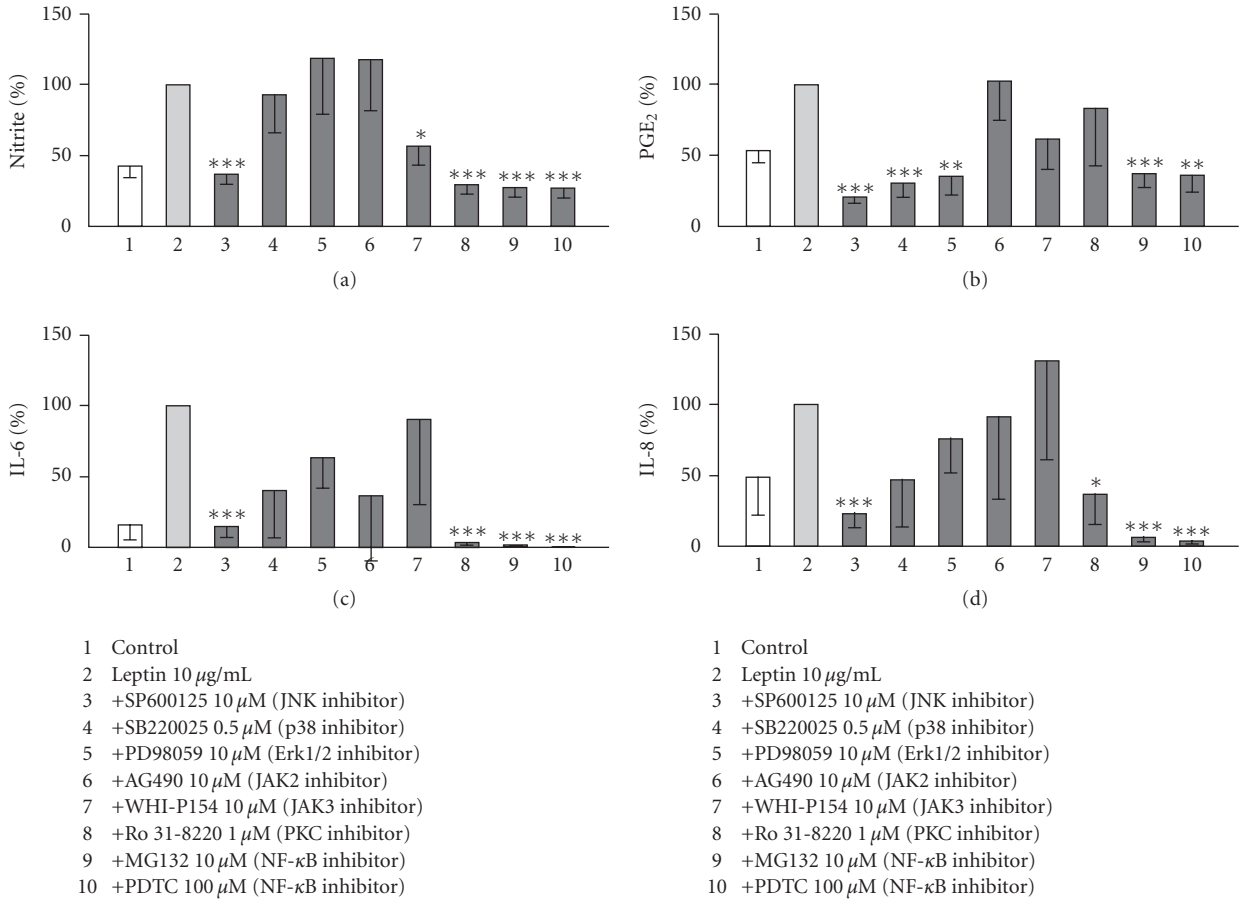

Figure 3: The effects of signaling pathway inhibitors on leptin induced NO (a), PGE_2 (b), IL-6 (c), and IL-8 (d) production in human OA cartilage. Cartilage explants were incubated for 48 hours with leptin (10 μg/mL) and the inhibitor indicated. NO production (a) was measured as nitrite accumulation in the culture medium by Griess reaction. PGE_2 production (b) in the culture medium was measured by RIA. Levels of IL-6 (c), and IL-8 (d) in the culture medium were measured by ELISA. Leptin-induced NO/PGE_2/IL-6/IL-8 production was set as 100%, and the other values were related to that. The results are expressed as mean ± SEM. Samples were collected from 7 patients in (a) and (b) ($n = 7$) and from 6 patients in (c) and (d) ($n = 6$). $*$: $P < .05$, $**$: $P < .01$, and $***$: $P < .001$ as compared to explants treated with leptin alone.

NO mediates many of the destructive effects of IL-1 in inflamed joints [15, 16]. NO has been reported to increase the production of matrix metalloproteinases (MMPs) and to activate them [10, 22, 23], to inhibit proteoglycan [24–26] and collagen [27] synthesis and to induce chondrocyte cell death [28, 29]. NO is also involved in the progress of inflammation by reducing the production of anti-inflammatory/anabolic factors TGF-β [30], endogenous IL-1 receptor antagonist (IL-1ra), and IL-10 in chondrocytes [10, 31, 32], and by contributing to the resistance against anabolic effects of IGF-1 [33]. NO has also been shown to sustain activation of NF-κB providing a prolonged transcription of NF-κB dependent genes [34]. In support, Pelletier et al. reported reduced destruction of the articular cartilage by using iNOS-inhibitor L-NIL in instability-induced OA in dogs [35]. In further studies with this model, L-NIL was shown to reduce the levels of matrix metalloproteinase-1 and -3 (MMP-1 and -3) [23], to inhibit chondrocyte apoptosis [36] and to reduce the interleukin-1 converting enzyme (ICE) levels [37]. Van den Berg et al. studied the development of experimental osteoarthritis induced with intra-articular collagenase injection in iNOS knock-out mice. In this model, iNOS deficiency prevented the degree of cartilage destruction and osteophyte formation [38]. NO has several catabolic and antianabolic actions in cartilage and thus it is identified as a possible target of treatment in osteoarthritis.

Leptin has been shown to induce or to potentiate together with interferon γ (IFNγ), with tumor necrosis factor α (TNFα) or with IFNγ and IL-1β, NO production in murine J774A.1 macrophages, rat adipocytes, C6 glioma cell-line, and rat vascular smooth muscle cells (VSMCs) [39–42]. In human chondrocytes, leptin was reported to enhance interleukin-1 (IL-1) and interferon γ (IFNγ)-induced production of NO [12, 13]. In the present study, we showed that leptin alone is sufficient to induce the iNOS expression and NO production in human OA cartilage, and an enhancing effect was seen also in the presence of low concentrations of IL-1β (Figures 1(a), 1(b), and 2(a), 2(b)).

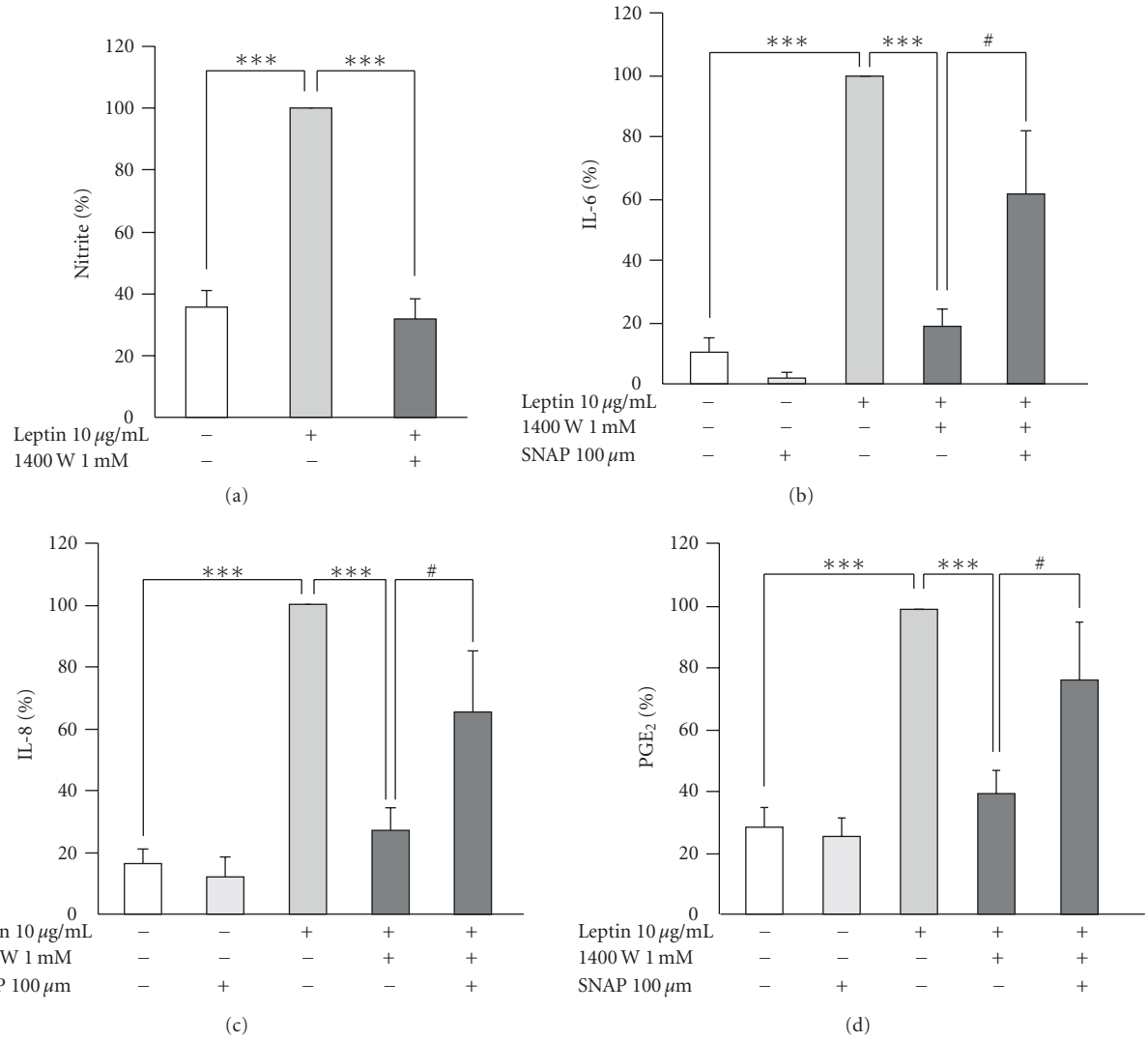

Figure 4: The effects of a selective iNOS inhibitor 1400 W (1 mM) ((a)–(d)) with and without NO-donor SNAP ((b)–(d)) on leptin (10 μg/mL) induced NO (a), IL-6 (b), IL-8 (c), and PGE_2 (d) production in OA cartilage during 48 hours incubation. In the culture medium, NO production (a) was measured as nitrite accumulation by Griess reaction, levels of IL-6 (b) and IL-8 (c) were measured by ELISA, and PGE_2 production (d) was measured by RIA. Leptin-induced NO/PGE_2/IL-6/IL-8 production was set as 100%, and the other values were related to that. The results are expressed as mean ± SEM. Samples were collected from 8 patients in (a) ($n = 8$), from 6 patients in (b) ($n = 6$), from 8 patients in (c) ($n = 8$), and from 6 patients in (d) ($n = 6$). #: $P < .2$, ∗ ∗ ∗: $P < .001$.

Prostaglandins (especially PGE_2) are produced in high amounts in OA cartilage and are modulators of inflammation, tissue destruction, and inflammatory pain. Prostaglandins are formed from arachidonic acid by the prostaglandin synthesizing cyclooxygenase (COX) enzymes and prostaglandin synthases [17]. COX-2 is highly expressed in OA cartilage and is induced by various cytokines that are involved in destructive processes in OA cartilage, for example, IL-1 and TNFα [43–45]. Current drug therapy of OA is based on nonsteroidal anti-inflammatory drugs (NSAIDs). NSAIDs inhibit COX enzymes and prostanoid production, and they are used to relieve OA pain [46, 47]. To our knowledge, we show here for the first time that leptin increases COX-2 expression and PGE_2 production in human OA cartilage in the absence and in the presence of IL-1 (Figures 1(c), 1(d) and 2(c), 2(d)). Previously, leptin has been shown to induce PGE_2 production in OE33 Barret's esophageal adenocarcinoma (EAC) cell-line and in murine J774A.1 macrophages [39, 48].

Proinflammatory and regulatory cytokines IL-6 and IL-8 are found in SF from OA patients [49]. OA cartilage produces IL-6 and IL-8, and quantitative RT-PCR studies have shown elevated IL-6 and IL-8 mRNA levels in OA cartilage as compared to normal cartilage [10, 50]. Cytokines IL-1β and TNFα which induce destructive effects in cartilage both induce IL-6 and IL-8 production in human articular chondrocytes [51]. IL-6 and IL-8 may promote inflammation and cartilage destruction induced by IL-1 or TNFα and have a modulatory role in the pathogenesis of OA [18, 52–54]. In the present study, we show for the first time that leptin

enhances IL-6 and IL-8 production in human OA cartilage (Figures 1(e), 1(f) and 2(e), 2(f)). Leptin has previously been shown to induce IL-6 production in human dendritic cells, human monocytes, human endometrial stromal cells (ESC), and in epithelial cells [55–57], and to stimulate IL-8 production in human endometrial stromal cells (ESCs) and in epithelial cells [56].

Leptin signaling through leptin receptors (Ob-R) is thought to be mediated through janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway and in addition to this, also mitogen-activated protein kinases (MAPKs), protein kinase C (PKC), phosphatidylinositol 3-kinase (PI3K), and nuclear factor κB (NF-κB) pathways have been reported to mediate some effects of leptin, depending on the cell type [3, 58]. In chondrocytes, Otero et al. showed by using pharmacological inhibitors that induction of NO production with a combination of leptin and IL-1 or IFNγ was dependent on JAK2, PI3K, Erk1/2, and p38 [12, 13, 59], but these studies did not show signaling pathways involved in the responses induced by leptin alone. We studied the effects of inhibitors of JAK (JAK2 and JAK3), MAPK (Erk1/2, p38, JNK), PKC and NF-κB pathways on leptin-induced NO, PGE_2, IL-6, and IL-8 production in OA cartilage. There seems to be some differences in the signaling pathways important for production of the four different leptin-induced inflammatory molecules in OA cartilage. Leptin-induced NO production was suppressed by inhibitors of JNK, JAK3, PKC, and NF-κB, while leptin-induced PGE_2 production was reduced by inhibitors of JNK, p38, and Erk1/2 MAPkinases and transcription factor NF-κB. Leptin-induced IL-6 and IL-8 production was reduced by inhibitors of JNK, PKC, and NF-κB (Figure 3).

In the present study, inhibition of leptin-induced endogenous NO production in OA cartilage with a selective iNOS inhibitor 1400 W also suppressed the effects of leptin on PGE_2, IL-6, and IL-8 production. The effect was reversed with exogenously added NO (NO-donor SNAP) (Figure 4). Those results suggest that leptin induces PGE_2, IL-6, and IL-8 production in OA cartilage by an NO-dependent manner. The mediator role of NO in leptin-induced metabolic changes in human OA cartilage has not been reported previously. It is, however, supported by the previous findings on the involvement of NO in the effects of leptin in some other organ systems, that is, in the secretion of luteinizing hormone-releasing hormone from the pituitary gland, in the control of blood pressure, and in the gastroprotection upon vagal activity [60–62].

In conclusion, OA cartilage was shown to respond to leptin by producing increased amounts of NO, PGE_2, IL-6, IL-8, and all those effects can be considered harmful in cartilage metabolism. Those effects of leptin seem to be dependent on activation of transcription factor NF-κB and the MAPK pathway c-Jun NH_2-terminal kinase (JNK) in human OA cartilage. In addition, JAK3 signaling seems to be involved in leptin-induced NO production, p38, and Erk1/2 MAPK pathways in leptin-induced PGE_2 production, and PKC pathway in leptin-induced NO, IL-6, and IL-8 production. Inhibition of NO production reduced the effects of leptin on PGE_2, IL-6, and IL-8 production pointing to a mediator role of NO in these leptin-induced changes in cartilage metabolism and to a possible beneficial effect of iNOS inhibitors on OA cartilage. These findings support the idea of leptin as a factor in the pathogenesis of osteoarthritis, and as a possible link between obesity and osteoarthritis.

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Leptin enhances MMP-1, MMP-3 and MMP-13 production in human osteoarthritic cartilage and correlates with MMP-1 and MMP-3 in synovial fluid from OA patients

A. Koskinen[1], K. Vuolteenaho[1], R. Nieminen[1], T. Moilanen[1,2], E. Moilanen[1]

[1]The Immunopharmacology Research Group, University of Tampere School of Medicine and Tampere University Hospital, Tampere, Finland; [2]Coxa Hospital for Joint Replacement, Tampere, Finland.

Abstract

Objective

In the present study, we investigated the role of adipocytokine leptin in the pathogenesis of osteoarthritis (OA) by measuring its effects on matrix metalloproteinase (MMP) production in human OA cartilage. In addition, the correlations between leptin and MMP concentrations in synovial fluid from OA patients were studied.

Design

Cartilage tissue obtained from leftover pieces of total knee replacement surgery from patients with OA was used in the experiments. Production of collagenases MMP-1, MMP-8 and MMP-13, and stromelysin-1 (MMP-3) in the cartilage was measured by immunoassay and the signalling pathways were explored by pharmacological means. In addition, synovial fluid samples were collected from 84 OA patients undergoing knee replacement surgery. The concentrations of leptin and MMPs in synovial fluid were measured by immunoassay.

Results

Leptin alone and in combination with IL-1β enhanced production of MMP-1, MMP-3, and MMP-13 in human OA cartilage, while MMP-8 concentrations remained undetectable. The effects of leptin on MMP-1, MMP-3 and MMP-13 production were mediated through transcription factor NF-κβ, and through protein kinase C and MAP kinase pathways. Interestingly, leptin concentrations in synovial fluid from OA patients correlated positively with MMP-3 ($r=0.51$, $p<0.001$) and MMP-1 ($r=0.41$, $p<0.001$) levels.

Conclusions

To our knowledge, this is the first study to show that leptin up-regulates MMP-1 and MMP-3 production in human OA cartilage and correlates positively to MMP-1 and MMP-3 in synovial fluid from OA patients. The findings suggest that leptin has catabolic effects in OA joints by increasing MMP production in cartilage.

Key words

leptin, adipokines, osteoarthritis, matrix metalloproteinases, cartilage

Anna Koskinen, MD
Katriina Vuolteenaho, MD, PhD
Riina Nieminen, PhD, Assoc. Prof.
Teemu Moilanen, MD, PhD, Assoc. Prof.
Eeva Moilanen, MD, PhD, Professor

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Please address correspondence and reprint requests to:
Prof. Eeva Moilanen,
The Immunopharmacology Research Group, University of Tampere School of Medicine, 33014 Tampere, Finland.
E-mail: eeva.moilanen@uta.fi

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Competing interests : none declared.

Introduction

Leptin is a hormone that was initially found to be synthesised by white adipocytes and to regulate food intake and energy expenditure (1). Obese people have increased circulating levels of leptin as compared to non-obese individuals (2), and in murine models leptin deficiency causes morbid obesity (1). However, due to leptin resistance in hypothalamus, the increased levels of leptin in the circulation of obese subjects fail to induce expected responses, *i.e.* reduced food intake and body weight (3). In addition to the regulatory function in energy homeostasis, leptin plays a role in other physiological functions such as neuroendocrine function, reproduction, angiogenesis and bone formation. More recently, leptin has been recognised as a cytokine-like hormone with pleiotropic actions also in immune response and inflammation (4-6).

Osteoarthritis (OA) is the most common joint disease worldwide. It is characterised with progressive degeneration of articular cartilage, limited intra-articular inflammation, pain, stiffness and loss of mobility. The etiology of OA is not established but the risk factors are well known including obesity as one of the main risk factors. Traditionally obesity has been thought to contribute to the development of OA by increased load on the weight-bearing joints. However, it does not offer full explanation for the connection between obesity and OA because obesity is also connected to OA of hand joints (7-9). This suggests that obesity is associated with the development of OA also by systemic/metabolic influence, in addition to biomechanical effect.

Recent studies provide evidence for leptin as a possible link between obesity and OA. Leptin has been shown to be produced by chondrocytes (10-14) as well as by other tissues in the joint including synovium, infrapatellar fat pad, osteophytes, meniscus and bone (10-12, 15). Leptin is found in synovial fluid (SF) (10, 12, 13, 16, 17), and its levels in synovial fluid have been reported to be higher in OA patients than in controls (16). Leptin concentration in SF, and leptin mRNA expression in OA cartilage have been found to correlate positively with BMI (10, 13) and the levels of leptin and leptin receptor (Ob-Rb) expression in human OA cartilage have been found to be related to the severity of cartilage destruction (10, 13). Griffin *et al.* recently showed that extreme obesity in leptin deficient mice does not increase the incidence of OA as compared to lean wild type mice (18). A recent study by Ding *et al.* demonstrated an association of serum leptin level to cartilage volume loss in 190 randomly selected subjects (19). These findings further support the role of leptin in the pathogenesis of OA. There is also some evidence of increased leptin levels in patients with radiographically severe OA as compared to control patients and patients with radiographically less severe OA (16, 20).

Cartilage destruction in OA is due to disequilibrium between the catabolic and anabolic factors produced by chondrocytes and other cells in the joint (21). Chondrocytes play an important role in the degenerative processes by releasing inflammatory and destructive mediators including proinflammatory cytokines, nitric oxide (NO), prostaglandins and catabolic enzymes (22, 23). Matrix metalloproteinases (MMPs) are extracellular matrix degrading enzymes known to have essential role in the pathogenesis of OA. IL-1β and TNF-α are considered as the main mediators inducing synthesis and secretion of MMPs, NO and proinflammatory cytokines (21). Recent studies have shown that also leptin might be a factor that regulates chondrocyte functions. We have previously demonstrated that leptin induces IL-6, IL-8, NO and prostaglandin E_2 (PGE_2) production, and expression of inflammatory enzymes iNOS and COX-2 in human OA cartilage, and, that leptin-induced IL-6, IL-8 and COX-2 expression is mediated by NO (24). Exogenous leptin has also been reported to enhance IL-1β, MMP-9 and MMP-13 production, and downregulation of leptin with small interference RNA has been found to inhibit MMP-13 expression in human OA cartilage (13, 25).

In the present study, we investigated the effect of leptin on the production of four matrix metalloproteinases centrally involved in cartilage damage in OA,

i.e. collagenases MMP-1, MMP-8 and MMP-13, and stromelysin MMP-3, in human OA cartilage. These metalloproteinases are produced by chondrocytes and are present in diseased cartilage. The collagenases are able to cleave type II collagen, which is the predominant collagen type present in cartilage, while MMP-3 is the major enzyme that cleaves aggrecan and it is also able to activate other MMPs. The association of increased production of these MMPs with cartilage damage has been established (21, 26-28). In addition, signalling pathways mediating leptin induced MMP expression were evaluated by pharmagological means. We found out that leptin enhances the production of MMP-1, MMP-3 and MMP-13 in OA cartilage. In order to evaluate the clinical relevance of this finding, leptin and MMP levels were measured in the SF from OA patients, and leptin was found to correlate with MMP-1 and MMP-3.

Materials and methods

Materials

Reagents were obtained as follows: recombinant human leptin and IL-1β were purchased from R&D systems (Minneapolis, MN, USA); SP600125, SB220025, AG 490 and WHI-P154 were from Calbiochem (Merck, Darmstadt, Germany); PD 98059 was from Promega (Madison, WI, USA); Ro 31-8220 was from Alexis Corporation (Lausen, Switzerland); MG 132 was from Tocris Bioscience (Ellisville, MO, USA); and PDTC was from Sigma Chemical Co (St Louis, MO, USA).

Patients

Samples were obtained from 96 OA patients (63 female and 33 male, BMI 30.2±0.6 kg/m^2, age 70.6±0.9 years; mean±SEM) undergoing total knee replacement surgery in Coxa Hospital for Joint Replacement, Tampere, Finland. All patients were diagnosed to have clinical and radiographic knee OA according to the American College of Rheumatology criteria (29). The radiological severity of OA in these patients was scored to stages III or IV according to the Kellgren and Lawrence scaling (30) at the time of surgery. Cartilage samples in the tissue culture experiments were collected from 12 patients, and synovial fluid samples from 84 patients. The study was approved by the ethics committee of Tampere University Hospital and the patients gave their written approval.

Tissue cultures

Cartilage tissue was obtained from the leftover pieces of total knee replacement surgery. The samples were washed with phosphate buffered saline (PBS). Full thickness pieces of articular cartilage from femoral condyles, tibial plateaus, and patellar surfaces were removed aseptically from subchondral bone with a scalpel and cut into small pieces (about 2×2×2 mm). Three randomly selected cartilage cubes were placed in one well of a 6-well plate and incubated in 3mL of tissue culture medium [Dulbecco's modified Eagle's medium (DMEM) with glutamax-I containing 10% heat-inactivated fetal bovine serum, and penicillin (100 units/mL), streptomysin (100 μg/mL), and amphotericin B (250 ng/mL); all obtained from Invitrogen, Carlsbad, California, USA] at 37°C in 5% carbon dioxide atmosphere for the time indicated.

In the first two series of experiments, OA cartilage samples from 7 patients were used. Explants were incubated with leptin (0.1 μg/ml or 10 μg/ml) alone or in combination with IL-1β (10 pg/ml) for 48h. In the third series of experiments, explants from subsequent 5 patients were incubated for 48 hours with leptin (10 μg/ml) alone or with leptin in combination with each of the following signalling pathway inhibitors added into the culture media together with leptin: SP600125 10 μM (JNK inhibitor), SB220025 0.5 μM (p38 inhibitor), PD98059 10 μM (Erk1/2 inhibitor), AG490 10 μM (JAK2 inhibitor), WHI-P154 10 μM (JAK3 inhibitor), Ro 31-8220 1 μM (PKC inhibitor), MG132 10 μM (NF-κB inhibitor) and PDTC 100 μM (NF-κB inhibitor). Due to limited amount of cartilage that can be obtained from one donor, we used only one concentration of each inhibitor in the experiments. The concentrations used in the present experiments, were selected on the basis of the studies previously carried out in our laboratory, and on data collected from literature (31-33). The stock concentrations of the inhibitors were made in DMSO (when appropriate), and all treatments as well as controls contained same concentration of the diluent (final concentration of DMSO 1:1000 in all wells). After the experiments the cartilage explants were weighed and the results were counted per mg of cartilage. Aliquots of the culture media were kept at -20°C until assayed. Concentrations of MMP-1, MMP-3, MMP-8 and MMP-13 were determined in the culture medium by Multiplex bead array (Fluorokine® Human MMP Multi Analyte Profiling Base Kit, R&D systems, Minneapolis, MN, USA).

Synovial fluid samples

Synovial fluid samples were punctured aseptically in the operation room before knee replacement surgery from 84 OA patients. The samples were stored at -70ºC until assayed. MMP-1 and MMP-13 concentrations in the synovial fluid were determined by Multiplex bead array (Fluorokine® Human MMP Multi Analyte Profiling Base Kit, R&D systems, Minneapolis, MN, USA), and leptin and MMP-3 concentrations were assessed by Enzyme-linked immunosorbent assay (R&D Systems, Inc, Minneapolis, USA).

Statistical analysis

Results are expressed as mean ± standard error of the mean (SEM). Statistical significance of the results was calculated by paired *t*-test or by repeated measures analysis of variance with Bonferroni post-test when appropriate. Pearson's *r* was used to analyse the correlations. $R \geq 0.3$ or ≤ -0.3 was considered to indicate a meaningful correlation (r=0.3 to 0.5 or r=0.5 to 1.0 indicating medium or strong correlations, respectively, according to Cohen (34)). p-values <0.05 were considered as significant. Standard multiple regression modeling was used to evaluate the relationships between MMPs and leptin in synovial fluid from OA patients. The independent variables entered into the models were gender, BMI, age and leptin, and MMP-1 or MMP-3 as dependant variable. Multi collinearity was

assessed with tolerance and variance inflation factor methods. Instat (GraphPad Software, La Jolla, California, USA) and SPSS 16.0 software (SPSS Inc., Chicago, Illinois, USA) were used in the statistical analysis.

Results

The effects of leptin on MMP-1, MMP-3, MMP-8 and MMP-13 production in human OA cartilage

OA cartilage produced low levels of MMP-1, MMP-3 and MMP-13 in the tissue culture medium, and their production was significantly increased when leptin (10 μg/ml) was added into the culture (Fig. 1). Lower concentration of leptin (0.1 μg/ml) had a slight enhancing effect on MMP-1 and MMP-13, but it did not reach statistical significance. The effect of leptin was also investigated in the presence of low concentration of IL-1β (10 pg/ml) mimicking the inflammatory condition in OA joints. Leptin enhanced MMP-1, MMP-3 and MMP-13 production also in the presence of IL-1β (Fig. 2). MMP-8 concentrations remained undetectable in control samples and under leptin or/and IL-1β treatment with the detection limit of 90 pg/ml.

Signalling pathways involved in the leptin-induced MMP-1, MMP-3 and MMP-13 production

The signalling pathways involved in leptin-stimulated MMP-1, MMP-3 and MMP-13 production in OA cartilage were studied by pharmacological means. Inhibitors of transcription factor NF-κB, MG 132 (10 μM) and PDTC (100 μM), protein kinase C inhibitor Ro 31-8220 (1 μM), and JNK inhibitor SP600125 (10 μM) inhibited leptin-induced MMP-1, MMP-3 and MMP-13 production in a statistically significant manner (Fig. 3). In addition, leptin-induced MMP-1 and MMP-13 production was inhibited by p38 inhibitor SB220025 (0.5 μM). MMP-13 and to a lesser extent MMP-3 production was also reduced by JAK3 inhibitor WHI-P154 (10 μM). Erk1/2 inhibitor PD 98059 (10 μM) and JAK2 inhibitor AG 490 (10 μM) did not have statistically significant effect on leptin-induced MMP-1, MMP-3 or MMP-13 production.

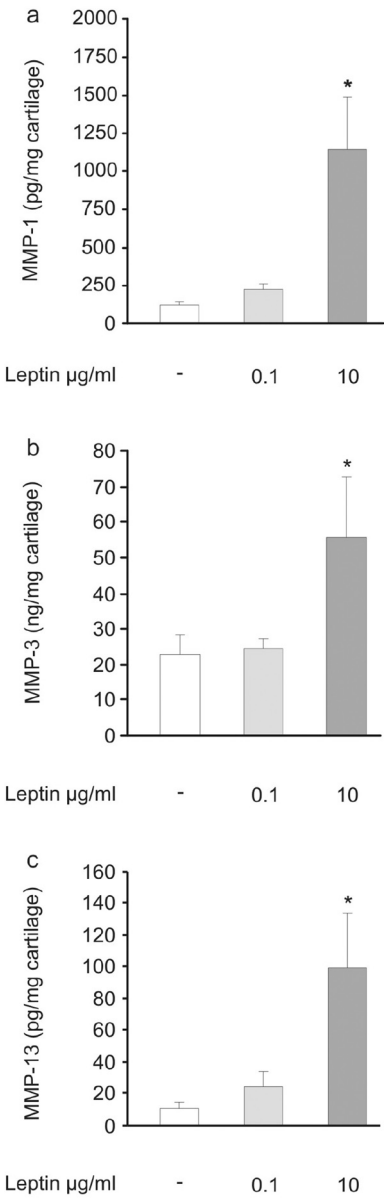

Fig. 1. The effect of leptin on MMP-1 (**a**), MMP-3 (**b**), and MMP-13 (**c**) production in human OA cartilage. Cartilage explants were incubated with leptin (0.1 μg/ml or 10 μg/ml) for 48h. MMP concentrations in the culture medium were measured by immunoassay. Results are expressed as pg of MMP/mg of cartilage (**a** and **c**) or ng of MMP/mg of cartilage (**b**). Values are mean ± SEM. Cartilage samples were collected from 7 patients. *$p<0.05$ as compared to control explants incubated in the absence of exogenous leptin.

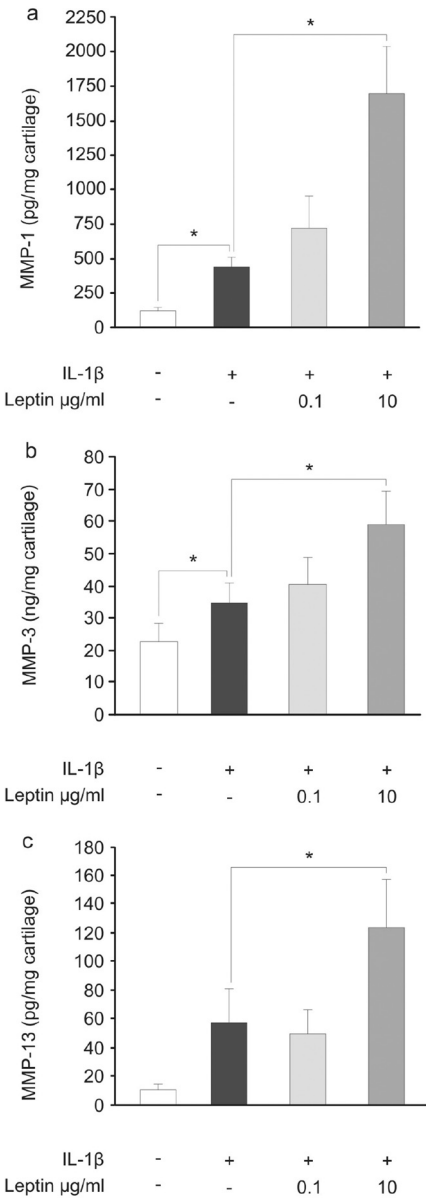

Fig. 2. The effect of leptin in combination with IL-1β on MMP-1 (**a**), MMP-3 (**b**), and MMP-13 (**c**) production in human OA cartilage. Cartilage explants were incubated with IL-1β (10 pg/ml) alone or in combination with leptin (0.1 μg/ml or 10 μg/ml) for 48h. MMP concentrations in the culture medium were measured by immunoassay. Results are expressed as pg of MMP/mg of cartilage (**a** and **c**) or ng of MMP/mg of cartilage (**b**). Values are mean ± SEM. Cartilage samples were collected from 7 patients. *$p<0.05$.

The relationships between leptin and MMP-1, MMP-3 and MMP-13 in synovial fluid from OA patients

Because leptin enhanced MMP-1, MMP-3 and MMP-13 production in OA cartilage *in vitro*, the concentrations of leptin and these MMPs were determined in synovial fluid from OA patients to investigate if there is a relation between leptin and MMPs *in vivo*. Leptin concentrations (20.4±2.2 ng/ml) correlated positively with MMP-3 ($r=0.51$, $p<0.001$, 823.2±73.3 ng/ml) and MMP-1 ($r=0.41$, $p<0.001$, 16.7±1.5 ng/ml) levels (Fig. 4). MMP-13 remained undetectable in synovial fluid with the detection limit of 90 pg/ml. In the standard multiple regression model,

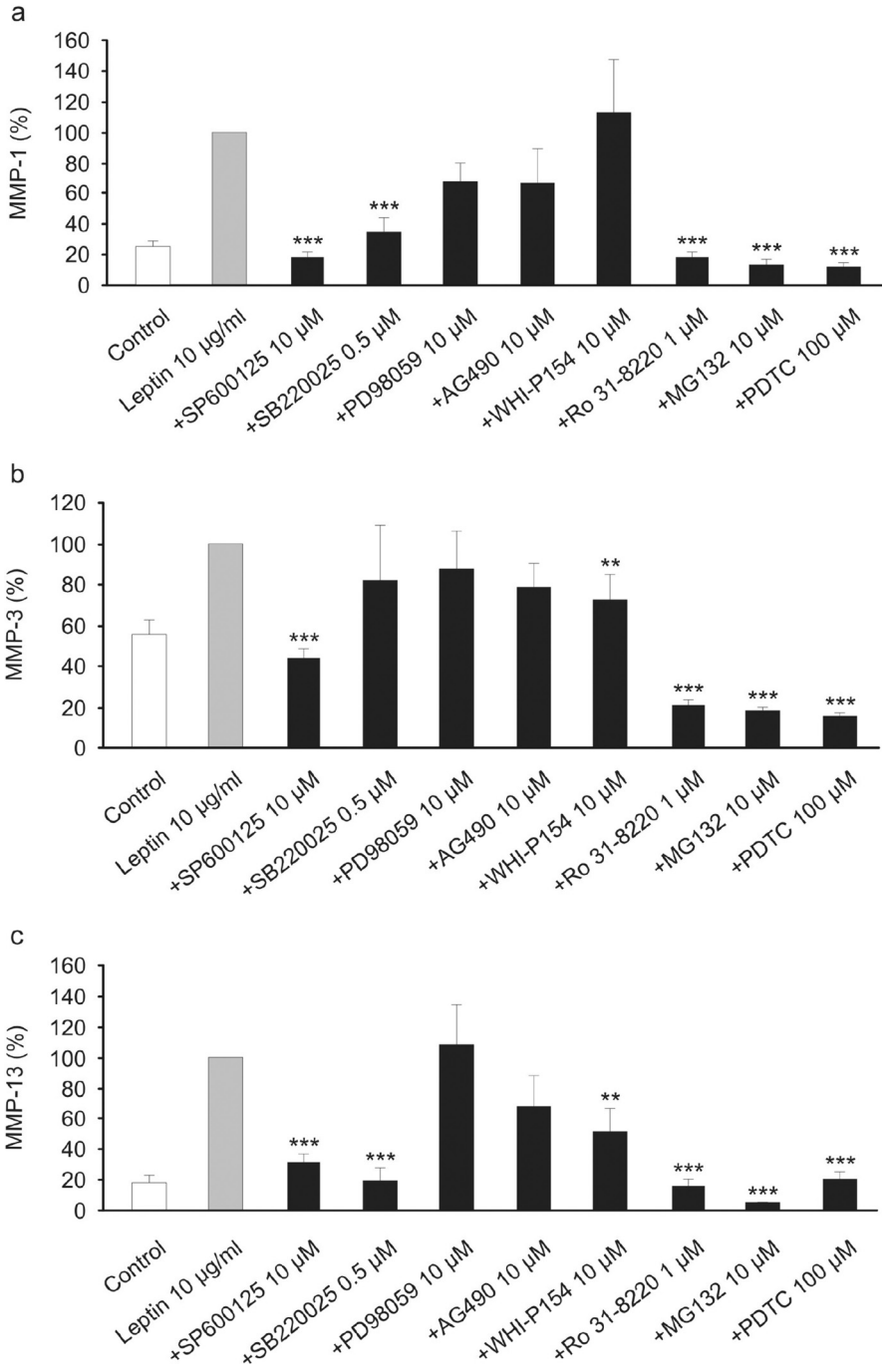

Fig. 3. The effects of selected signal transduction inhibitors on leptin induced MMP-1 (**a**), MMP-3 (**b**) and MMP-13 (**c**) production in human OA cartilage. Cartilage explants were incubated for 48h with leptin (10 µg/ml) and the inhibitor indicated. MMP concentrations in the culture medium were measured by immunoassay. Results are expressed as percentages in comparison with samples treated with leptin only. Values are mean ± SEM. Cartilage samples were collected from 5 patients. $^{**}p<0.01$ and $^{***}p<0.001$ as compared to explants treated with leptin alone. SP600125 - JNK inhibitor; SB220025 - p38 inhibitor; PD98059 - Erk1/2 inhibitor; AG490 - JAK2 inhibitor; WHI-P154 - JAK3 inhibitor; Ro 31-8220 - PKC inhibitor; MG132 and PDTC - NF-κB inhibitors.

leptin and female gender, but not age or BMI, were significant determinants of MMP-1; and leptin, but not gender, age or BMI, was a significant predictor for MMP-3 in synovial fluid. Standardised beta was 0.320 (p=0.023) for leptin predicting MMP-1, and 0.380 (p=0.006) for leptin predicting MMP-3 (Table I). In these models, leptin explained 5.2% and 7.3% (square of semipartial correlation coefficient) of variation in MMP-1 and MMP-3 concentration, respectively.

Discussion

Obesity is a major risk factor for OA, which has been explained by increased load on the weight-bearing joints, and by systemic factor(s) associated with obesity. Adipocytokines, especially leptin, are present in OA synovial fluid (10, 12, 13, 16, 17), and chondrocytes express functional receptors for leptin (13, 35). In the present study, we investigated the action of leptin on OA cartilage by measuring its effects on the production of the matrix metalloproteinases that play a central role in the pathogenesis of osteoarthritis. To our knowledge, this is the first study to show that leptin upregulates the production of MMP-1 and MMP-3 in human OA-cartilage. We also found that leptin enhances MMP-13 production in OA-cartilage which is supported by the previous findings (13, 36). The levels of MMP-8 remained under detection limit in our study. Further, we investigated if the relation between leptin and MMPs could be translated to an *in vivo* situation. Interestingly, we found a positive correlation between leptin and MMP-1 and MMP-3 levels in synovial fluid from OA patients. These findings support the idea of leptin having a role in regulating MMP production in OA cartilage.

The present results together with recently published findings provide evidence for leptin, either produced in the joint or transported there from the circulation, as a factor in the pathogenesis of OA. Leptin was recently found to enhance the production of proinflammatory/destructive mediators NO, IL-6, IL-8, IL-1β and PGE_2 in articular chondrocytes (13, 24, 37-39), and to decrease proliferation of cultured human chondrocytes (13). On the other hand, Figenschau *et al*. demonstrated increased proliferation of chondrocytes and enhanced synthesis of proteoglycans and collagen under leptin exposure (35) suggesting that leptin has also anabolic properties in the joint or that the catabolic effects of leptin trigger compensatory anabolic responses which is typical for the early OA process. Dumond *et al*. reported that leptin injection to rat knee led to increased synthesis of insulin-like growth factor 1 (IGF-I), transforming growth factor β

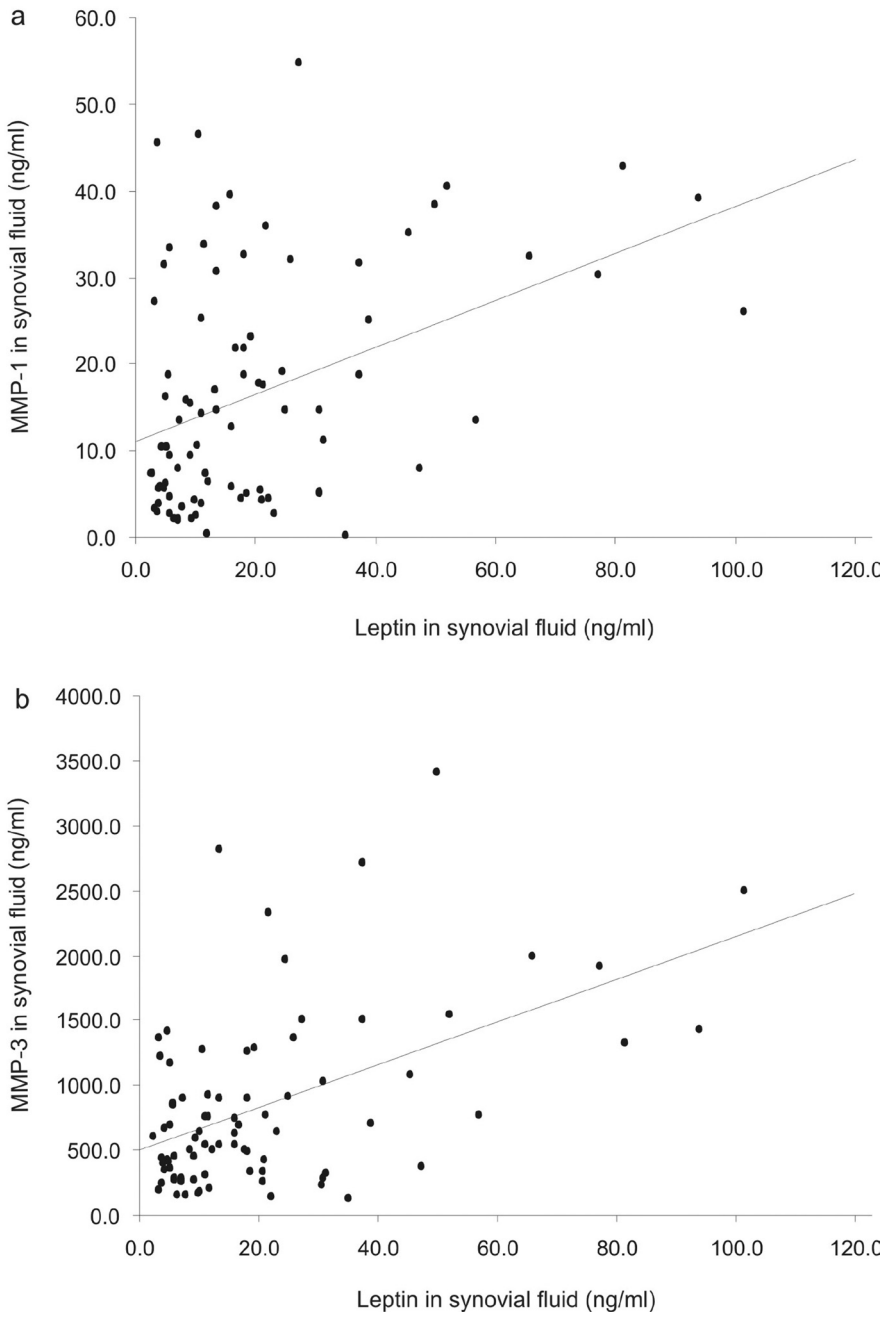

Fig. 4. Scatter plot for the association between synovial fluid levels of leptin and MMP-1 (**a**), and leptin and MMP-3 (**b**). Leptin was significantly correlated with MMP-1 (r=0.41, p<0.001) and with MMP-3 (r=0.51, p<0.001) in Pearson correlation analysis. n=84.

(TGFβ) and leptin itself (10). While in a study by Bao *et al*. (40) intra-acticular leptin was found to increase the expression of MMP-2, MMP-9, cathepsin D, collagen II, ADAMTS -4 and -5 genes and to reduce bFGF and proteogycan expression. The somewhat contradictory effects of leptin may be explained by differences in experimental conditions, by possible differences in responses between healthy and OA cartilage and by differences between animal species used in the experiments and vs human cartilage. The current findings support the catabolic role of leptin in human OA joints through increased MMP expression in OA cartilage and positive correlations between leptin and MMP-1 and MMP-3 concentrations in synovial fluid from OA affected joints.

Leptin transduces its signal by binding to leptin receptor (OB-Rb). Intracellular signalling mechanisms which leptin has been described to activate include JAK-STAT pathway, phosphoinositide 3-kinase (PI3K), nuclear factor κB (NF-κB), protein kinase C (PKC) and mitogen-activated protein kinases (MAPK), the latter including extracellular signal-regulated kinase (Erk1/2), p38 kinase and c-jun N-terminal kinase (JNK) (6). In OA, MAPK pathways and NF-κB are known to be activated in response to inflammatory cytokines to enhance MMP expression (41). We have reported previously that leptin enhances the production of the proinflammatory mediators NO, IL-6, IL-8 and PGE_2 in OA cartilage through activation of NF-κB and JNK pathways. In addition, p38, Erk1/2, PKC and JAK3 were involved in some responses (24). Tong *et al*. reported that leptin increases IL-8 production in human synovial fibroblasts via JAK2/STAT3- and IRS1/PI3K/Akt/NF-κB-dependent manner (42). Otero *et al*. have shown in mouse chondrogenic ATDC5 cell line and in human primary chondrocytes that leptin when given together with IL-1β or IFNγ, but not alone, induced NO production and nitric oxide synthase II (NOSII) expression through pathways involving JAK2, PI3K, MEK-1 and p38 (37-39). Figenschau *et al*. reported that leptin binding to Ob-Rb resulted in activation of STAT1 and STAT5 in cultured human articular chondrocytes (35). To our knowledge, the signalling pathways activated by leptin to enhance MMP production in cartilage have not been studied before. According to our present results, leptin-induced MMP-1, MMP-3 and MMP-13 production is mediated through NF-κB, PKC and JNK pathways. Also, activation of p38 kinase is involved in MMP-1 and MMP-13 production, and JAK3 pathway mediates MMP-3 and MMP-13 production. JAK2 and Erk1/2 inhibitors had no effect on the production of the MMPs studied.

Leptin has previously been shown to enhance production of NO, IL-6, IL-8 and PGE_2 in human OA cartilage (4, 23, 37, 39). In addition to their other effects in OA joints, IL-6 (21, 43) and IL-8 (44) have been also reported to enhance MMP production, and NO has been shown to activate MMPs (45). Therefore it is possible that these me-

Table I. Standard multiple regression predicting synovial fluid MMP-1 and MMP-3 by gender, age, BMI and synovial fluid leptin.

	Standardised beta for predicting synovial fluid MMP-1 (ng/ml)	*p*-value	Standardised beta for predicting synovial fluid MMP-3 (ng/ml)	*p*-value
Female gender	0.262	0.021	0.118	0.277
Age (years)	0.038	0.718	0.033	0.751
BMI (kg/m^2)	-0.027	0.838	0.127	0.329
SF leptin (ng/ml)	0.320	0.023	0.380	0.006

diators positively regulate the synthesis of each other in OA cartilage exposed to leptin, further activating the OA process. According to our preliminary results, iNOS inhibitor 1400W was found to downregulate leptin-induced MMP-3 production suggesting that it may be mediated by NO. However, further studies are needed to understand the complex network of these mediators and leptin in degradative processes in OA cartilage.

According to the literature, mean / median leptin concentrations measured in synovial fluid samples from OA-affected joints vary between 4.4-28.5 ng/ml (10, 12, 16, 17). SF leptin concentrations, like plasma leptin levels, correlate with BMI (10, 13, 17) and also leptin's mRNA expression in advanced OA cartilage has been shown to correlate with BMI (13). It is likely that SF leptin may originate partly from the circulation and is partly synthesized in the joint. In this study we measured leptin concentration of 20.4±2.2 ng/ml (mean±SEM) ranging from 2.4 to 101 ng/ml in synovial fluid from OA-patients, and that is comparable to previously published data (10, 12, 13, 16, 17).

In the *in vitro* studies, leptin concentrations starting from the highest levels found in OA synovial fluid (100 ng/ml) going to 10 ug/ml were needed to enhance MMP production. That raises a question if the *in vitro* findings could be translated to an *in vivo* situation in OA joints. That could be supported by the observations that cultured cartilage explants in general require elevated levels of exogenously added protein mediators compared to physiological levels to be responsive. High molecular size proteins (MW for leptin is 16 kDa) are not easily diffusible into the cartilage matrix, and therefore the concentration of exogenously added proteins likely remains clearly lower in the vicinity of the chondrocytes in tissue culture than that added into the culture medium (46). In the physiological situation, chondrocytes are thought to produce a significant proportion of the leptin present in the synovial fluid, and respond to it in autocrine/paracrine manner. If that is the case, leptin concentrations in the vicinity of the chondrocytes are most likely much higher than those measured in synovial fluid. To clarify if there might be an association between leptin and MMPs in OA joints *in vivo*, we also measured leptin and MMP levels in synovial fluid samples. Interestingly, leptin correlated positively with MMP-1 and MMP-3 suggesting that leptin may enhance the production of these MMP enzymes also *in vivo*.

MMP-1, MMP-3 and MMP-13 are involved in matrix degradation in OA (27, 47). MMP-3 is also used as a biomarker of cartilage degradation in OA patients (48). In the present study, leptin correlated with MMP-1 and MMP-3 in synovial fluid, whereas MMP-13 remained below the detection limit. Although leptin has been reported to be associated with BMI, leptin but not BMI was found to be a significant determinant of MMP-1 and MMP-3 in SF according to standard multiple regression modeling. To our knowledge, the positive correlations between synovial fluid leptin and MMP-1 and MMP-3 have not been reported previously, and they support the view of leptin as an intra-articular factor involved in the pathogenesis of OA.

In conclusion, OA cartilage was shown to respond to leptin by producing increased amounts of MMP-1, MMP-3 and MMP-13 enzymes which have a central role in the pathogenesis of cartilage degradation in OA. The enhancing effect of leptin in inducing MMP-1, MMP-3 and MMP-13 expression was dependent on activation of transcription factor NF-κB, and on PKC and JNK pathways in human OA cartilage, and activation of p38 kinase was involved in MMP-1 and MMP-13 production. The clinical significance of the novel finding of leptin as an MMP enhancing factor in cartilage was supported by the positive correlation between leptin and MMP-1, and MMP-3 in synovial fluid from OA patients. These findings support the role of leptin as a factor in the pathogenesis of osteoarthritis through increased MMP production in OA affected joints.

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Leptin levels are increased and its negative regulators, SOCS-3 and sOb-R are decreased in obese patients with osteoarthritis: a link between obesity and osteoarthritis

Leptin is a hormone originally discovered in white adipocytes which regulates energy metabolism and appetite. Obese individuals have increased levels of circulating leptin, as compared with their non-obese counterparts, and in mouse models, leptin deficiency causes morbid obesity.[1] However, due to the appearance of leptin resistance in the hypothalamus, increased blood levels of leptin in obese subjects fail to induce the expected responses to high leptin, that is, increased energy expenditure, reduced food intake and decreased body weight.[2] Leptin resistance has been shown to be mediated by an increased expression of the suppressor of cytokine signalling-3 (SOCS-3).[1 2]

Obesity has been thought to contribute to the development of osteoarthritis (OA) by increasing the load on weight-bearing joints. However, this appears to be an over-simplification, since obesity is also linked to OA in the hand and finger joints.[3] Recent studies show that leptin is a possible link between obesity and OA, since leptin is increased in obesity and induces the production of matrix metalloproteinases, proinflammatory cytokines and nitric oxide in chondrocytes.[1 4–7] It is not known whether the obesity related mechanisms, mediating leptin responses/resistance in the hypothalamus, also exist and regulate the proinflammatory and catabolic effects of leptin inside osteoarthritic joints. In fact, the findings of Pallu *et al* pointed in the opposite direction: the peripheral responsiveness to leptin was increased in chondrocytes, this data was obtained from obese OA patients (body mass index (BMI) >30 kg/m^2).[8] Since SOCS-3 acts as an intracellular negative feedback regulator in leptin signalling and the soluble leptin receptor (sOb-R) binds to leptin, modulating its biological activity, we measured leptin and sOb-R concentrations in the synovial fluid, and SOCS-3 expression in the cartilage from OA patients, to investigate whether there are endogenous leptin suppressors inside the OA joints.

Synovial fluid samples were obtained from 54 OA patients (35 women, BMI 30.3±0.8 kg/m^2, age 70±1 years; mean±SEM) undergoing total knee replacement surgery and cartilage samples were obtained from 28 of these patients. The study was approved by the Ethics Committee of Tampere University Hospital, and patients gave their written approval, according to the Declaration of Helsinki. Leptin and sOb-R concentrations in synovial fluid were measured by ELISA, and SOCS-3 expression in the cartilage samples was determined by Western blotting. Leptin concentrations, in synovial fluid, were 22.3±3.2 ng/ml (mean±SEM). Interestingly, leptin concentrations in synovial fluid exhibited a clear positive correlation with patients' BMI (Spearman's r=0.57, p<0.001, figure 1A). Synovial fluid also contained sOb-R, which can bind and inactivate leptin. Therefore, we estimated the levels of free bioactive leptin, by dividing molar leptin concentrations

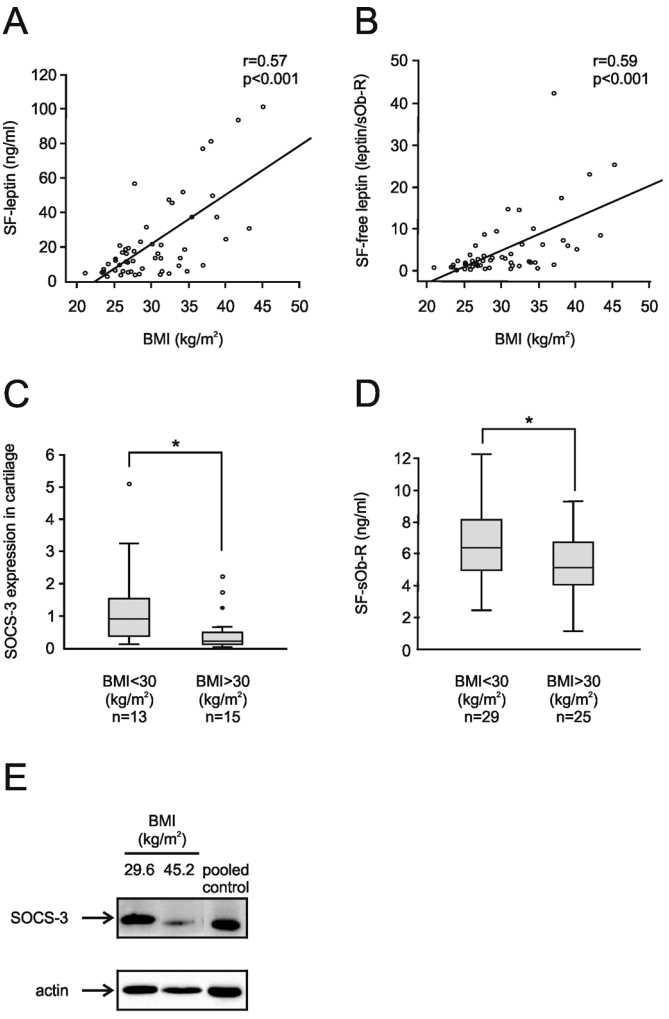

Figure 1 Association between leptin, soluble leptin receptor (sOb-R) and suppressor of cytokine signalling (SOCS-3) within the osteoarthritic joint and body mass index (BMI) values of the subjects. Scatter plots show the positive correlation between BMI and synovial fluid (SF) leptin (A) and between BMI and free leptin (molar ratio of leptin to soluble leptin receptor (sOb-R)) (B). SOCS-3 expression levels in cartilage (C, E) and sOb-R levels in synovial fluid samples (D) were lower in obese (BMI>30 kg/m^2) than in non-obese (BMI<30 kg/m^2) osteoarthritis patients. Leptin and sOb-R concentrations, in synovial fluid were measured by ELISA (A, B and D), and SOCS-3 expression in cartilage samples was determined by Western blotting (C, E). Each Western blot gel was loaded with protein samples (20 μg) extracted from cartilage samples and a pooled control protein sample. Densitised SOCS-3 bands in individual patient samples were related against the SOCS-3 band, in the pooled control sample in the same Western blot gel resulting in comparable values of SOCS-3 expression levels between patient samples (C). An example of such gels is shown (E). The horizontal solid bar within the boxes represents the median; the boxes refer to the interquartile range and the lines outside boxes display minimum and maximum. Outliers are indicated as circles (C and D). Data were analysed using SPSS V.19.0 software for Windows (SPSS, Chicago, Illinois, USA). Spearman's correlation analysis was carried out (A and B); differences between groups were tested by Mann–Whitney test (C) and unpaired t test (D) as appropriate according to the distribution of the data. *p<0.01.

by sOb-R levels. As seen in figure 1B, synovial fluid free leptin levels positively correlated with BMI (r=0.59, p<0.001). We then divided patients into two groups based on their BMI (non-obese, BMI<30 kg/m^2 and obese, BMI>30 kg/m^2). Interestingly, SOCS-3 expression in the cartilage and sOb-R levels in the synovial fluid were significantly lower in obese patients (BMI>30 kg/m^2) (figure 1C,D).

In OA patients, leptin levels have been reported to be higher in synovial fluid than in the serum,[9 10] and our study showed that synovial fluid leptin concentrations correlate with BMI, which confirm the previous findings.[10] More interestingly, we report here for the first time, a negative association between synovial fluid sOb-R and BMI. Furthermore, SOCS-3 expression (which suppresses leptin-induced signalling in the cell) was lower in cartilage from obese patients. These results indicate that in obese OA patients, levels of bioactive leptin in synovial fluid are elevated, while SOCS-3 expression in cartilage is decreased, supporting the concept that leptin is a possible factor linking obesity to OA.

Katriina Vuolteenaho,[1] Anna Koskinen,[1] Teemu Moilanen,[1,2] Eeva Moilanen[1]

[1]The Immunopharmacology Research Group, University of Tampere School of Medicine and Tampere University Hospital, Tampere, Finland
[2]Coxa Hospital for Joint Replacement, Tampere, Finland

Correspondence to Dr Katriina Vuolteenaho, The Immunopharmacology Research Group, University of Tampere, School of Medicine, FI-33014, Tampere, Finland; katriina.vuolteenaho@uta.fi

Contributors KV, AK, TM and EM were involved in the conception and design of the study. TM was involved in selecting the patients and in acquiring patient samples. KV and AK were involved in the laboratory analyses and calculating the results and along with TM and EM contributed to data interpretation. KV and EM contributed to writing the manuscript. All authors revised the manuscript and approved the final version.

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RESEARCH ARTICLE Open Access

Catabolic and proinflammatory effects of leptin in chondrocytes are regulated by suppressor of cytokine signaling-3

Anna Koskinen-Kolasa[1], Katriina Vuolteenaho[1], Riku Korhonen[1], Teemu Moilanen[1,2] and Eeva Moilanen[1*]

Abstract

Background: Previous studies provide evidence that adipokine leptin increases production of catabolic and proinflammatory factors in chondrocytes and serves as a link between obesity and osteoarthritis (OA). However, the magnitude of the response to leptin treatment varies greatly between chondrocytes from different donor patients. In the present study, we investigated the regulatory role of suppressor of cytokine signaling-3 (SOCS-3) in the leptin-induced responses in OA cartilage.

Methods: Cartilage and synovial fluid samples from 97 patients with OA undergoing knee replacement surgery were collected. Cartilage samples were cultured with leptin (10 μg/ml), and the levels of proinflammatory and catabolic factors in synovial fluid and in the cartilage culture media, and SOCS-3 expression in the cartilage were measured. The role of SOCS-3 in leptin signaling was further studied in H4 murine chondrocytes by downregulating SOCS-3 with siRNA.

Results: Leptin-induced expression of matrix metalloproteinases MMP-1, MMP-3, MMP-13, interleukin-6 (IL-6), inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) were higher in the cartilage samples with low SOCS-3 expression. Accordingly, downregulation of SOCS-3 by siRNA in H4 chondrocytes led to enhanced leptin-induced expression of MMP-3, MMP-13, IL-6 and iNOS. Synovial fluid leptin was associated positively, and cartilage SOCS-3 negatively with synovial fluid levels of MMPs in a multivariate model in obese (body mass index (BMI) >30 kg/m^2) but not in non-obese (BMI <30 kg/m^2) patients.

Conclusions: Our results show, for the first time, that SOCS-3 regulates leptin-induced responses in cartilage, and could thus be a future drug target in the treatment or prevention of OA, especially in obese patients.

Keywords: Leptin, Adipokine, SOCS-3, Osteoarthritis, Chondrocytes, Obesity

Background

Adipokines are cytokine-like hormones produced by adipose tissue and originally discovered to regulate energy metabolism [1, 2]. Their role in inflammation and obesity-related disease, such as type 2 diabetes mellitus and cardiovascular disease, and also in rheumatic disease has attracted increasing interest during the past decade. Leptin was first characterized in 1994 [3] and to date it is probably the most studied adipokine. The circulating levels of leptin are closely associated with the amount of stored body fat and with body mass index (BMI) [4]. Leptin is, however, not only produced by adipose tissue, but also by several other tissues, including cartilage and other joint tissues [5–7]. Interestingly, synovial fluid leptin levels are also correlated with BMI and leptin expression in chondrocytes is increased in obese individuals with OA [5, 6, 8]. The expression of leptin and its functional receptor Ob-Rb is also reported to be increased in chondrocytes in OA, in comparison to healthy chondrocytes [6].

Obesity is a major risk factor for OA [9]. Traditionally obesity has been thought to explain the risk of developing OA due to increased wear-and-tear on weight-bearing joints. However, obesity is also a risk factor for hand OA

* Correspondence: eeva.moilanen@uta.fi
[1]The Immunopharmacology Research Group, University of Tampere School of Medicine and Tampere University Hospital, Tampere, Finland
Full list of author information is available at the end of the article

[10], which points to a systemic factor or factors that mediate the obesity-related impact on cartilage. Leptin, with its strong positive association with body fat stores, fits well in this picture; in fact, increasing evidence supports the role of leptin as a significant factor in the pathogenesis of OA. Leptin has been shown to have direct proinflammatory and catabolic effects on cartilage in experimental settings. We and others have previously shown that leptin enhances production of catabolic enzymes, including matrix metalloproteinase 1 (MMP-1), MMP-2, MMP-3, MMP-9, MMP-13, a disintegrin and metalloproteinase with thrombospondin motifs 4 (ADAMTS-4) and ADAMTS-5 and proinflammatory mediators, such as nitric oxide (NO), interleukin 6 (IL-6), IL-1β, IL-8 and prostaglandin E_2 (PGE_2) in chondrocytes, synoviocytes and in cartilage [6, 11–19]. These findings suggest that leptin is not only a bystander of cartilage breakdown, but an active detrimental factor in the pathogenesis of OA.

According to our experience, cartilage from different donor patients respond to leptin treatment in a quite versatile manner: some of the samples produce large amounts of catabolic/proinflammatory mediators like MMPs, IL-6 and NO following leptin treatment, while in some samples leptin-induced changes in the production of these factors are very small. Similar wide variation in the response to leptin is also supported by other studies [17]. A study by Pallu et al. showed that primary chondrocytes received from obese patients with OA respond to smaller amounts of leptin to enhance MMP-13 production than chondrocytes obtained from non-obese patients [17], suggesting that obese individuals might be more susceptible to the harmful effects of leptin on cartilage. However, the mechanisms regulating leptin responsiveness in chondrocytes remain unknown.

Suppressor of cytokine signaling 3 (SOCS-3) belongs to SOCS proteins, which are intracellular molecules that have an important function of limiting excessive inflammatory activation of the innate and adaptive immune system [20]. In inflammatory cells SOCS-3 expression is induced by type I and type II cytokine receptors via the JAK-STAT pathway. SOCS-3 binds to the gp130 subunit of those receptors and inhibits the JAK-STAT pathway, thus forming a negative feedback loop to limit cytokine actions [21]. Interestingly, SOCS-3 is also involved in regulating leptin responsiveness in the central nervous system (CNS) [22].

The metabolic function of leptin is to serve as a sensor of body fat stores for the CNS. Elevation of blood leptin due to calorie intake, whether short-term or long-term, in a lean person normally suppresses food intake, whereas decreased leptin levels due to fasting or loss of adipose tissue lead to increased food intake [23]. In obesity however, elevated leptin does not lead to the expected responses in weight control. This is thought to be due to disturbed leptin signaling, also called leptin resistance. Elevated SOCS-3 expression in the CNS is proposed to be the primary mechanism that causes leptin resistance and subsequent failure in controlling food intake in obesity [22]. Consistently, leptin-deficient mice develop severe obesity [24], whereas SOCS-3 conditional knockout mice are resistant to diet-induced obesity [25]. In humans, genetic leptin deficiency also causes severe obesity, though leptin and leptin-receptor-related mutations are extremely rare [26].

SOCS-3 is also expressed in cartilage [27–29], and we reported previously that its expression is lower in cartilage from obese patients with OA than from non-obese patients [8]. That led us to hypothesize that SOCS-3 could be a significant mechanism behind the variable leptin responsiveness in cartilage samples from different donor patients. We addressed the hypothesis by investigating SOCS-3 expression and leptin responsiveness in cartilage samples obtained from 97 patients with OA. In addition, the role of SOCS-3 expression in leptin signaling was studied by downregulating SOCS-3 by siRNA in chondrocyte cultures.

Methods

Cartilage and cell cultures

Cartilage and synovial fluid (SF) samples were collected from 97 patients with OA who were undergoing knee replacement surgery. All patients fulfilled the American College of Rheumatology classification criteria for OA [30]. Cartilage samples were processed for tissue culture as previously described [15]. Cartilage pieces were incubated for 42 hours with or without leptin (10 μg/ml). The concentration of leptin used was chosen based on our previous studies and on existing literature [15, 17–19]. Recombinant human leptin was purchased from R&D Systems Europe Ltd, Abindgon, UK. Synovial fluid (SF) samples from the corresponding patients were also collected at the beginning of the arthroplasty. The SF samples were centrifuged at 4000 g at 4 °C and supernatants were collected and kept at −70 °C until assayed.

The immortalized murine H4 chondrocyte cell line [31], developed in the Laboratory of Experimental Rheumatology, University Medical Center, Nijmegen, The Netherlands, was used in the siRNA experiments. The chondrocytes were cultured at 37 °C in humidified 5 % carbon dioxide atmosphere in Dulbecco's modified Eagle's medium (DMEM) with L-glutamine and Ham's F-12 medium (1:1) supplemented with 5 % fetal bovine serum (all obtained from Lonza Group Ltd, Basel, Switzerland).

Immunoassays and nitrite measurements

Concentrations of MMP-1, MMP-3, MMP-13 and IL-6 were determined by immunoassays with commercial reagents according to the protocol provided by the manufacturer (human total MMP-1, human total MMP-3,

human total MMP-13, mouse total MMP-3 and mouse IL-6 ELISA kits were from R&D Systems; human IL-6 ELISA kit was from Sanquin, Amsterdam, The Netherlands; MMP-1 in SF was determined by Multiplex bead array, Fluorokine® Human MMP Multi Analyte Profiling Base Kit, purchased from R&D systems). Nitrite, stable metabolite of nitric oxide (NO), was measured in the culture media by the Griess reaction [32]. The cartilage culture media samples were filtered through Amicon Ultra 10-K filters (from Millipore, Cork, Ireland) at 14,000 g prior to the Griess analysis in order to remove large proteins that might interfere with the Griess analysis.

RNA isolation and quantitative reverse transcription/polymerase chain reaction

Culture medium was removed at the indicated time points and total RNA of H4 chondrocytes was extracted with GenElute™ Mammalian Total RNA Miniprep kit (Sigma-Aldrich, St Louis, MO, USA). Total RNA was treated with DNAse (Fermentas UAB, Vilnius, Lithuania) and reverse-transcribed to cDNA using TaqMan Reverse Transcription reagents and random hexamers (Applied Biosystems, Foster City, CA, USA). cDNA obtained from the RT reaction was diluted 1:20 with RNAse-free water and subjected to quantitative PCR using TaqMan Universal PCR Master Mix and the ABI Prism 7000 Sequence detection system (Applied Biosystems). Primers and probes for SOCS-3, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), iNOS, IL-6 and MMP-13 were obtained from Metabion International AG (Martinsried, Germany). The primer and probe sequences and concentrations (Table 1) were optimized according to the manufacturer's instructions in TaqMan Universal PCR Master Mix Protocol part number 4304449 revision C. The expression of mouse MMP-3 mRNA was measured using TagMan Gene Expression Assay (Mm00440295_m1, Applied Biosystems).

PCR reaction parameters were as follows: incubation at 50 °C for 2 minutes, incubation at 95 °C for 10 minutes, and thereafter 40 cycles of denaturation at 95 °C for 15 s and annealing and extension at 60 °C for 1 minute. Each experimental reaction was performed in duplicate. The relative mRNA levels of SOCS-3, GAPDH, iNOS, IL-6 and MMP-13 were quantified using the standard curve method as described in Applied Biosystems User Bulletin number 2. To calculate the relative expression of MMP-3 mRNA, the $2^{(-\Delta\Delta CT)}$ method [33] was used. According to the method, the cycle threshold (C_T) values for MMP-3 mRNA expression in each sample were normalized to the C_T values of GAPDH mRNA in the same sample.

Western blot

Preparation of cell lysates, SDS-polyacrylamide gel electrophoresis and western blot analysis were carried out as previously described [15]. Mouse monoclonal SOCS-3 antibody (sc-51699), rabbit polyclonal iNOS antibodies (sc-651 and sc-650), goat polyclonal cyclooxygenase-2 (COX-2) antibody (sc-1745) and rabbit polyclonal β-actin antibody (sc-1615R), and secondary horseradish peroxidase (HRP)-conjugated goat anti-mouse (sc-2005), goat anti-rabbit (sc-2004) and donkey anti-goat (sc-2020) antibodies were all from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit polyclonal MMP-13 antibody (ab39012) was from Abcam (Cambridge, MA, USA). Leptin-induced iNOS and COX-2 expression was determined by running the control and leptin-induced samples side by side and the result is given as fold of change in the β-actin-normalized densitometry value of the leptin-induced versus the control sample.

Downregulation of SOCS-3 expression by siRNA

H4 murine chondrocytes were seeded at 1×10^5 cells/well in 24-well plates. Cells were incubated for 24 hours and transfected with SOCS-3 siRNA or with non-targeting control siRNA. On-Target SMART pool SOCS-3-specific siRNA (targeting sequences of GGCUAGGAGACUCGC CUUA, GGACCAAGAACCUACGCAU, CUAAUGAAA CCUCGCAGAU and GAAGGGAGGCAGAUCAACA) and siGENOME Non-Targeting siRNA were used at 100 nM to transfect the cells using DharmaFECT 1. All transfection reagents were from Thermo Scientific Dharmacon (Lafayette, CO, USA) and transfection was carried out according to the manufacturer's protocol. The experiments were started 48 hours after the transfection by adding leptin (10 μg/ml) (mouse recombinant leptin from R&D systems) in fresh culture medium.

Statistical analysis

The chi-square test, unpaired *t* test and Mann–Whitney test (where appropriate) were used to analyze differences between subgroups of the patients. The Wilcoxon test was used to calculate the significance of leptin-induced effects in the cartilage culture.

To analyze the differences in leptin responsiveness in relation to SOCS-3 expression, the samples on each western blot gel were divided to two equal sized groups (low SOCS-3 or high SOCS-3) according to SOCS-3 expression. Median leptin responses, measured as change in the production of MMP-1, MMP-3, MMP-13, IL-6 and NO in the leptin-treated versus control sample, and as fold of change in the expression of iNOS and COX-2, were compared between the low SOCS-3 and the high SOCS-3 groups. Possible intergel differences in SOCS-3 expression were controlled by analysis of variance (ANOVA) in which the leptin response variable (e.g., leptin-induced change in production of MMP-1) was set as a dependent variable, western blot gel (1 to 8) as a grouping variable and SOCS-3

Table 1 Primer and probe sequences for quantitative RT-PCR

Gene	Oligunucleotide	Sequence	Conc. (nM)
Mouse GAPDH	Forward primer	GCATGGCCTTCCGTGTTC	300
	Reverse primer	GATGTCATCATACTTGGCAGGTTT	300
	Probe	TCGTGGATCTGACGTGCCGCC	150
Mouse SOCS-3	Forward primer	GCGGGCACCTTTCTTATCC	300
	Reverse primer	AAGCTGCCCCCCTCACA	300
	Probe	CTCGGACCAGCGCCACTTCTTCA	150
Mouse iNOS	Forward primer	CCTGGTACGGGCATTGCT	300
	Reverse primer	GCTCATGCGGCCTCCTT	300
	Probe	CAGCAGCGGCTCCATGACTCCC	150
Mouse IL-6	Forward primer	TCGGAGGCTTAATTACACATGTTC	900
	Reverse primer	CAAGTGCATCATCGTTGTTCATAC	300
	Probe	CAGAATTGCCATTGCACAACTCTTTTCTCA	200
Mouse MMP-13	Forward primer	TTGTGTTTGCAGAGCACTACTTGA	900
	Reverse primer	AACTGTGGAGGTCACTGTAGACTTCTT	900
	Probe	CATCCTGCGACTCTTGCGGGAATC	250

SOCS-3 suppressor of cytokine signaling-3, *iNOS* inducible nitric oxide synthase, *IL-6* interleukin-6, *MMP-13* matrix metalloproteinase-13, *Conc.* concentration

expression as a continuous variable as a covariate. Associations were further tested by adjusting for BMI and age.

Correlation between the factors of interest in SF were determined by Pearson's correlation analysis. The associations between MMPs or IL-6 and leptin in SF, and SOCS-3 expression in cartilage were further analyzed by ANOVA modeling, by including the variable of interest (SF MMP-1, MMP-3 or IL-6) as a dependent variable, leptin in SF and SOCS-3 expression in the cartilage as covariates and gel number as a grouping factor. The analysis was done in BMI subgroups (obese, BMI >30 kg/m^2; non-obese, BMI <30 kg/m^2). Natural logarithms were formed of the leptin response values, SOCS-3 expression levels and SF levels of the measured variables where appropriate in order to have normally distributed variables for the ANOVA modeling and for the correlation analyses.

The data were analyzed by IBM SPSS Statistics 19 (IBM Corporation, NY, USA) and Graph-Pad InStat version 3.00 software (GraphPad Software Inc., San Diego, CA, USA). The results of the siRNA experiments are presented as means (SEM). The statistical significance of these data was calculated by two-way ANOVA with Bonferroni multiple comparisons post-test using Graph-Pad Prism 5 for Windows version 5.04 (GraphPad Software Inc.). Differences were considered statistically significant at $p < 0.05$.

Results

Leptin-induced production of proinflammatory and catabolic factors in osteoarthritic cartilage in relation to clinical factors and SOCS-3 expression

Patient characteristics and leptin responses in the cultured cartilage across the whole study population and in the obese (BMI >30 kg/m^2) and non-obese subgroups are presented in Table 2. Leptin significantly enhanced the expression of MMP-1, MMP-3, MMP-13, IL-6, iNOS and COX-2 and NO production in OA cartilage *ex vivo* (Fig. 1). However, there was considerable variation in these responses between the samples from different donor patients (Table 2). There were no statistically significant differences in the leptin responses between obese and non-obese patients (Table 2), and neither did the leptin responses correlate with age, sex or radiographic scaling of OA.

When the patients were divided into subgroups according to SOCS-3 expression in the cartilage, leptin-induced changes in the expression/production of MMP-1, MMP-3, MMP-13, IL-6, NO, iNOS and COX-2 in the cartilage were significantly greater in the samples with low SOCS-3 expression than in the samples with high SOCS-3 expression (Fig. 2). This suggests that the level of SOCS-3 expression determinates the magnitude of leptin-induced inflammatory responses. The results remained statistically significant ($p < 0.05$) for the responses in the expression of MMP-3, MMP-13, IL-6, NO, iNOS and COX-2, and almost significant for response in the expression of MMP-1 ($p = 0.10$) in the ANOVA modeling after controlling for intergel variation, BMI and age.

Synovial fluid levels of MMPs and IL-6 in relation to SF leptin and SOCS-3 expression in cartilage from patients with OA

SF samples were obtained from 90 of the 97 patients. Obese patients had significantly higher SF leptin than non-obese patients, while SF MMP-1 and MMP-3 did not significantly differ between obese and non-obese

Table 2 Patient characteristics and leptin responses in cartilage cultures in the whole study population and compared across body mass index subgroups

	All		Non-obese, BMI <30 kg/m^2		Obese, BMI >30 kg/m^2		
	n = 97		*n* = 49		*n* = 48		*P*
Gender (female/male)[a]	60/37		26/23		34/14		0.072
Body mass index (kg/m^2)[b]	30.9	(6.1)	26.2	(2.4)	35.7	(4.6)	<0.001
Age (years)[b]	69.8	(10.0)	72.8	(9.7)	66.8	(9.4)	0.003
Synovial fluid leptin (ng/ml)[c, d]	12.8	(17.8)	7.6	(11.0)	21.5	(26.7)	<0.001
Synovial fluid IL-6 (pg/ml)[c, d]	118.9	(196.0)	126.8	(204.3)	114.0	(280.2)	0.784
Synovial fluid MMP-1 (ng/ml)[c, d]	14.4	(25.7)	10.4	(16.6)	18.1	(27.3)	0.325
Synovial fluid MMP-3 (ng/ml)[c, d]	649.5	(929.6)	591.6	(571.0)	764.9	(1159.0)	0.106
Leptin response in cartilage							
MMP-1 (change pg/mg cartilage)[c]	145.8	(247.1)	123.2	(253.6)	150.0	(258.3)	0.773
MMP-3 (change ng/mg cartilage)[c]	5.2	(8.8)	6.0	(8.6)	4.9	(10.2)	0.920
MMP-13 (change pg/mg cartilage)[c]	5.8	(13.6)	6.3	(11.8)	5.4	(15.9)	0.983
IL-6 (change pg/mg cartilage)[c]	123.2	(310.2)	114.6	(295.1)	130.9	(312.0)	0.740
NO (change pmol/mg cartilage)[c]	44.5	(133.4)	31.0	(125.8)	52.2	(140.0)	0.359
iNOS (fold of increase)[c, e]	11.7	(160.6)	5.4	(143.1)	15.2	(209.6)	0.501
COX-2 (fold of increase)[c, e]	6.9	(18.4)	7.1	(15.0)	6.4	(22.6)	0.748

[a]Values are numbers of female/male subjects; *p* value was calculated for comparison between non-obese and obese subjects using the chi-square test. [b]Values are mean (SD); *p* values were calculated for comparison between non-obese and obese subjects using the unpaired *t* test. [c]Values are median (IQR); *p* values were calculated for comparison between non-obese and obese subjects using the Mann–Whitney test. [d]Synovial fluid sample was obtained from 90 patients. [e]Numbers of patients (non-obese/obese) in the analysis were 26/31 for inducible nitric oxide synthase (iNOS) and 25/29 for cyclooxygenase-2 (COX-2). *MMP* matrix metalloproteinase, *IL* interleukin, *NO* nitric oxide

patients (Table 2). Leptin correlated positively with MMP-1 and with MMP-3 in SF from obese but not from non-obese patients (Fig. 3). In ANOVA modeling, leptin concentrations in SF and SOCS-3 expression in cartilage significantly explained levels of SF MMP-1 and MMP-3 in the obese but not in the non-obese group (Table 3) pointing to obesity-related association of leptin and SOCS-3 in OA pathophysiology. In addition, SF IL-6 levels were explained by SOCS-3 in the obese but not in the non-obese group, while leptin did not significantly explain SF IL-6 levels in either of the BMI subgroups (Table 3).

SOCS-3 modulates leptin responses in chondrocytes

In order to investigate further the role of SOCS-3 in the regulation of leptin-induced responses in chondrocytes, we used siRNA to downregulate SOCS-3 in the H4 chondrocyte cell line. H4 chondrocytes expressed SOCS-3 mRNA at relatively high levels and it was reduced by approximately 80 % in the SOCS-3-siRNA-treated cells when compared to the cells transfected with control siRNA. Leptin had a clear effect on inducing MMP-3, MMP-13, IL-6 and iNOS expression in the SOCS-3-deficient cells, whereas in the control siRNA-treated cells leptin did not have any statistically significant effect on the production of these factors (Fig. 4), confirming that SOCS-3 negatively regulates leptin-induced proinflammatory responses in chondrocytes.

Discussion

Leptin has been shown to have detrimental effects on cartilage metabolism in several studies [6, 11–19]. However, considerable variation in leptin responsiveness between cartilage/chondrocytes from different patients has been observed. Our present results indicate that a significant mechanism behind the differential leptin responsiveness could be SOCS-3.

SOCS-3 is a known negative regulator of inflammatory signals [20]. Its role in controlling the effects of leptin in chondrocytes has not been previously investigated, but it has been reported to regulate the responses of leptin in the CNS [22]. In the present study we show, for the first time, that SOCS-3 regulates the proinflammatory and catabolic effects of leptin in chondrocytes. This was demonstrated as greater leptin responsiveness in cartilage explants with low SOCS-3 expression in comparison to lower leptin responsiveness in the explants with high SOCS-3 expression. The causality of this association was illustrated by downregulation of SOCS-3 by siRNA in the chondrocyte cell line, which led to increased leptin-induced expression of proinflammatory and catabolic genes. In addition, SF leptin levels were shown to be positively associated, and cartilage SOCS-3 expression negatively associated with SF MMP levels in obese, but not in non-obese patients with OA. This points to dysregulation of the leptin-SOCS-3 axis, especially in obese

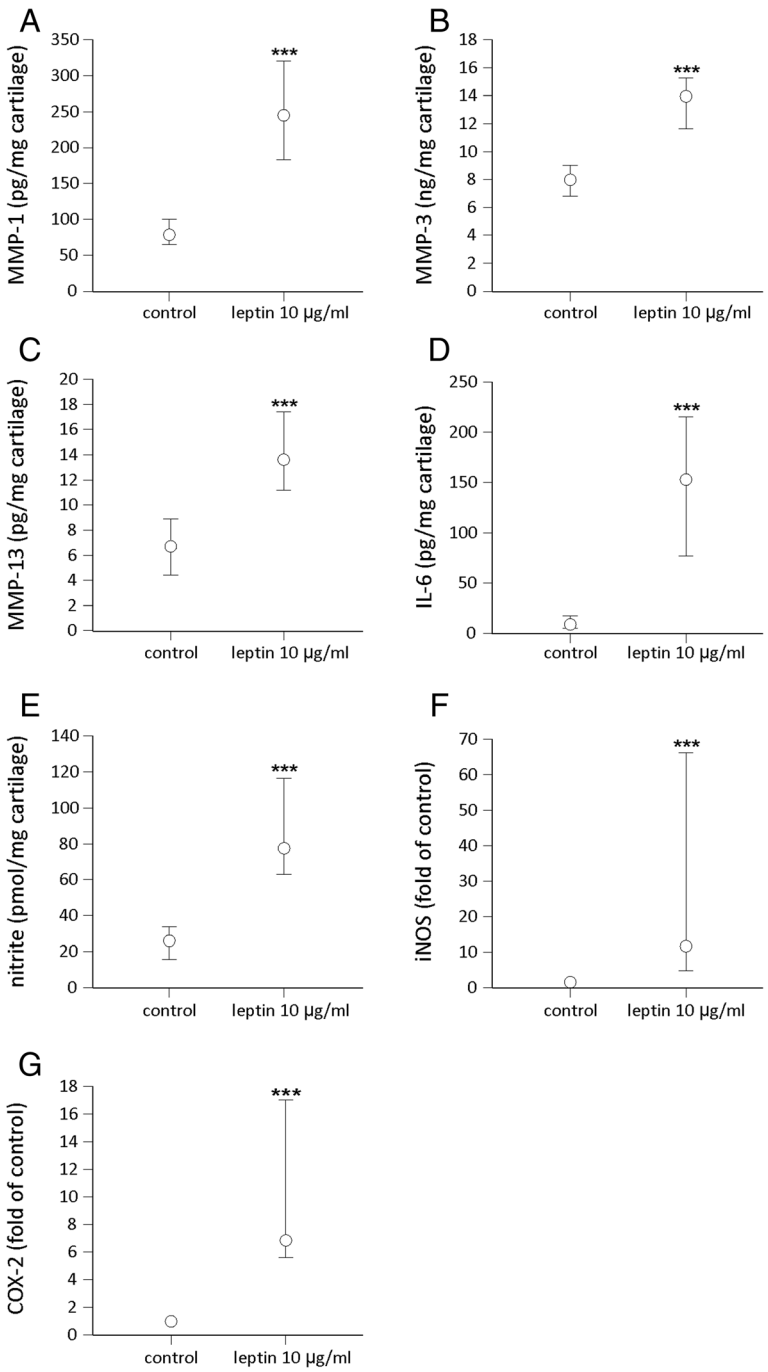

Fig. 1 Effect of leptin on the production of matrix metalloproteinase-1 (*MMP-1*) (**a**), MMP-3 (**b**), MMP-13 (**c**), interleukin-6 (*IL-6*) (**d**), nitric oxide (NO) (**e**) and on the expression of inducible nitric oxide synthase (*iNOS*) (**f**) and cyclooxygenase-2 (*COX-2*) (**g**) in cartilage from patients with osteoarthritis (OA). Cartilage samples from 97 patients with OA were cultured with and without leptin (10 μg/ml) for 42 hours. Concentrations of MMP-1, MMP-3, MMP-13 and IL-6 were measured by ELISA; NO production was determined as its metabolite nitrite by the Griess reaction and iNOS and COX-2 proteins by western blotting. The *circles* represent the medians. The *whiskers* represent 95 % confidence interval of the median. Statistical significance was calculated using the Wilcoxon test; ***$p < 0.001$

individuals, and to a possible obesity-related pathogenic mechanism in OA.

In the present study we observed a positive association between leptin levels and matrix metalloproteinases in SF that was only present in the obese patients with OA. However, obesity did not explain the differential leptin responsiveness in the cartilage culture experiments, unlike in the study by Pallu et al. where greater leptin

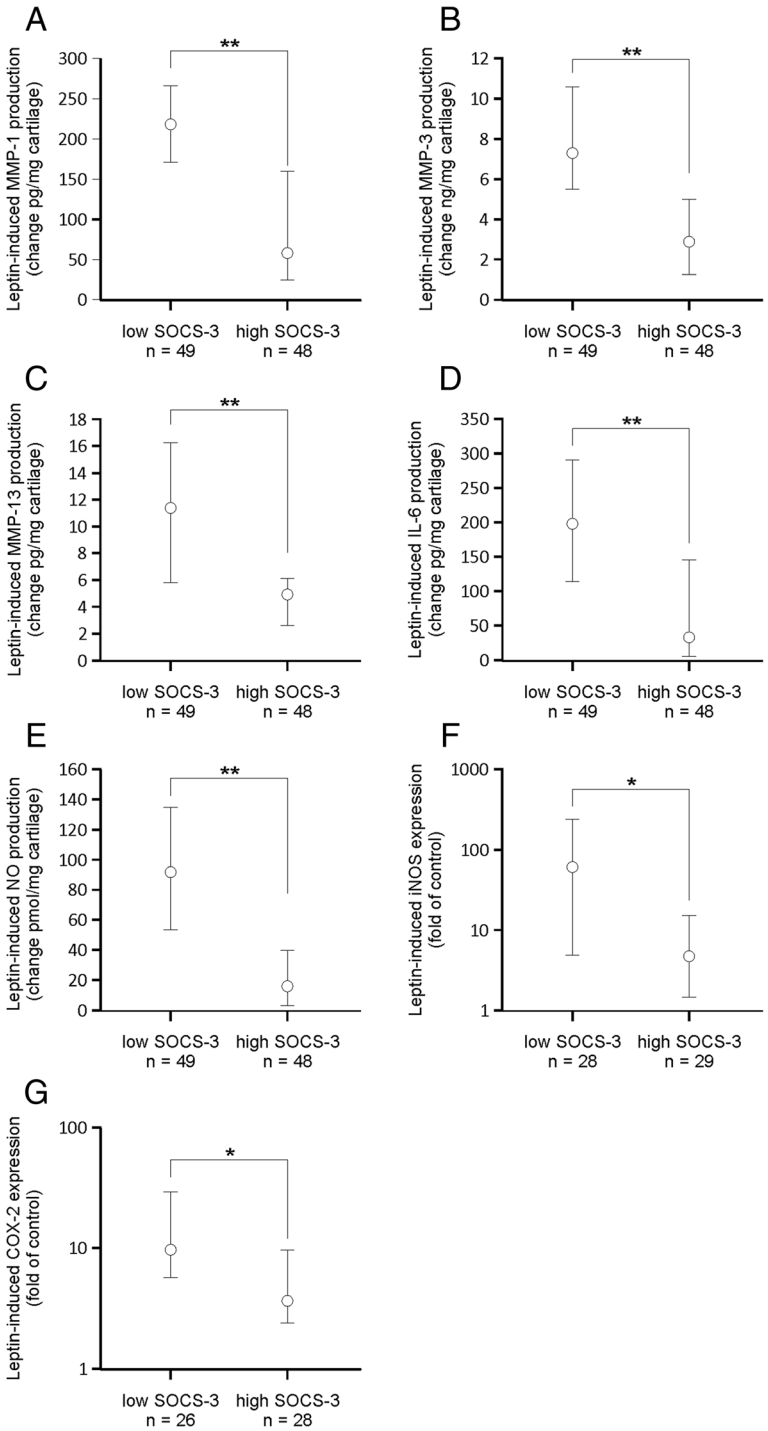

Fig. 2 Leptin-induced production/expression of matrix metalloproteinase-1 (*MMP-1*) (**a**), MMP-3 (**b**), MMP-13 (**c**), interleukin-6 (*IL-6*) (**d**), nitric oxide (*NO*) (**e**), inducible nitric oxide synthase (*iNOS*) (**f**) and cyclooxygenase-2 (*COX-2*) (**g**) in cartilage from patients with osteoarthritis (OA) in subgroups stratified by suppressor of cytokine signaling-3 (*SOCS-3*) expression in the non-treated cartilage. Human osteoarthritic cartilage was cultured with leptin (10 μg/ml) for 42 hours. Concentrations of MMP-1, MMP-3, MMP-13 and IL-6 were measured by ELISA, NO was determined as its metabolite nitrite by the Griess reaction and iNOS and COX-2 proteins were analyzed by western blotting. The *circles* represent the median change in the leptin-induced effects. The *whiskers* represent the 95 % confidence interval of the median. Numbers of patients from whom the cartilage samples were collected are indicated. Statistical significance was calculated using the Mann–Whitney test; *$p < 0.05$, **$p < 0.01$

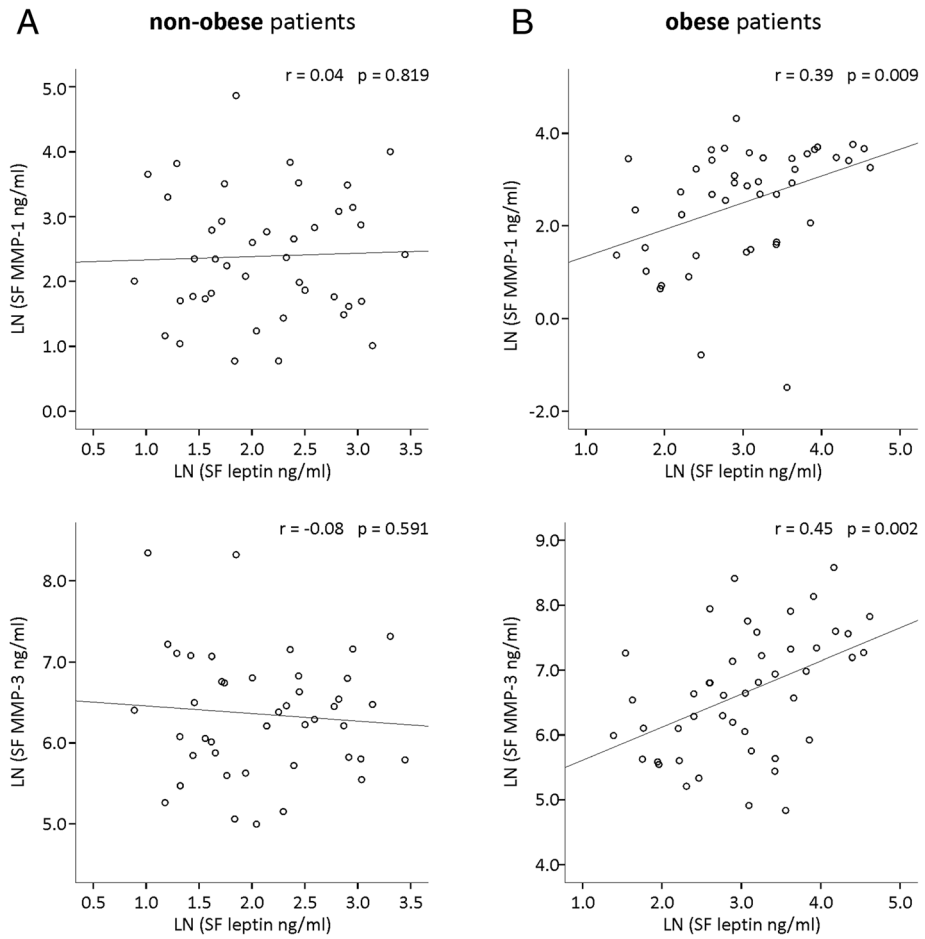

Fig. 3 Correlation between leptin and matrix metalloproteinase-1 (*MMP-1*) and MMP-3 in non-obese (**a**) and obese (**b**) patients with osteoarthritis. Leptin and MMPs were measured in synovial fluid (*SF*) by immunoassay. Natural logarithms (*LN*) were formed of the SF levels of leptin and MMPs in order to have normally distributed variables for the Pearson correlation analysis. Correlation coefficients (*r*) and *p* values are indicated. Samples were collected from 90 patients (non-obese, BMI <30 kg/m^2, $n = 44$; obese, BMI >30 kg/m^2, $n = 46$)

Table 3 Associations between interleukin-6 (IL-6), matrix metalloproteinase-1 (MMP-1), MMP-3 and leptin in synovial fluid and suppressor of cytokine signaling-3 (SOCS-3) expression in cartilage from non-obese and obese patients with osteoarthritis

		Non-obese, BMI <30 kg/m^2		Obese, BMI >30 kg/m^2	
Dependent variable	Covariates	R^2 adjusted	*P*	R^2 adjusted	*P*
LN (SF MMP-1)		0.15		0.30	
	LN SOCS-3		0.818		0.007
	LN (SF leptin)		0.884		0.023
LN (SF MMP-3)		0.03		0.27	
	LN SOCS-3		0.608		0.004
	LN (SF leptin)		0.733		0.015
LN (SF IL-6)		−0.05		0.20	
	LN SOCS-3		0.945		0.003
	LN (SF leptin)		0.808		0.466

P values are calculated for covariates in analysis of variance modeling. The model is controlled for intergel variation in SOCS-3 expression levels. Analysis was performed in body mass index (BMI) subgroups. Natural logarithms (LN) were formed where appropriate. *SF* synovial fluid

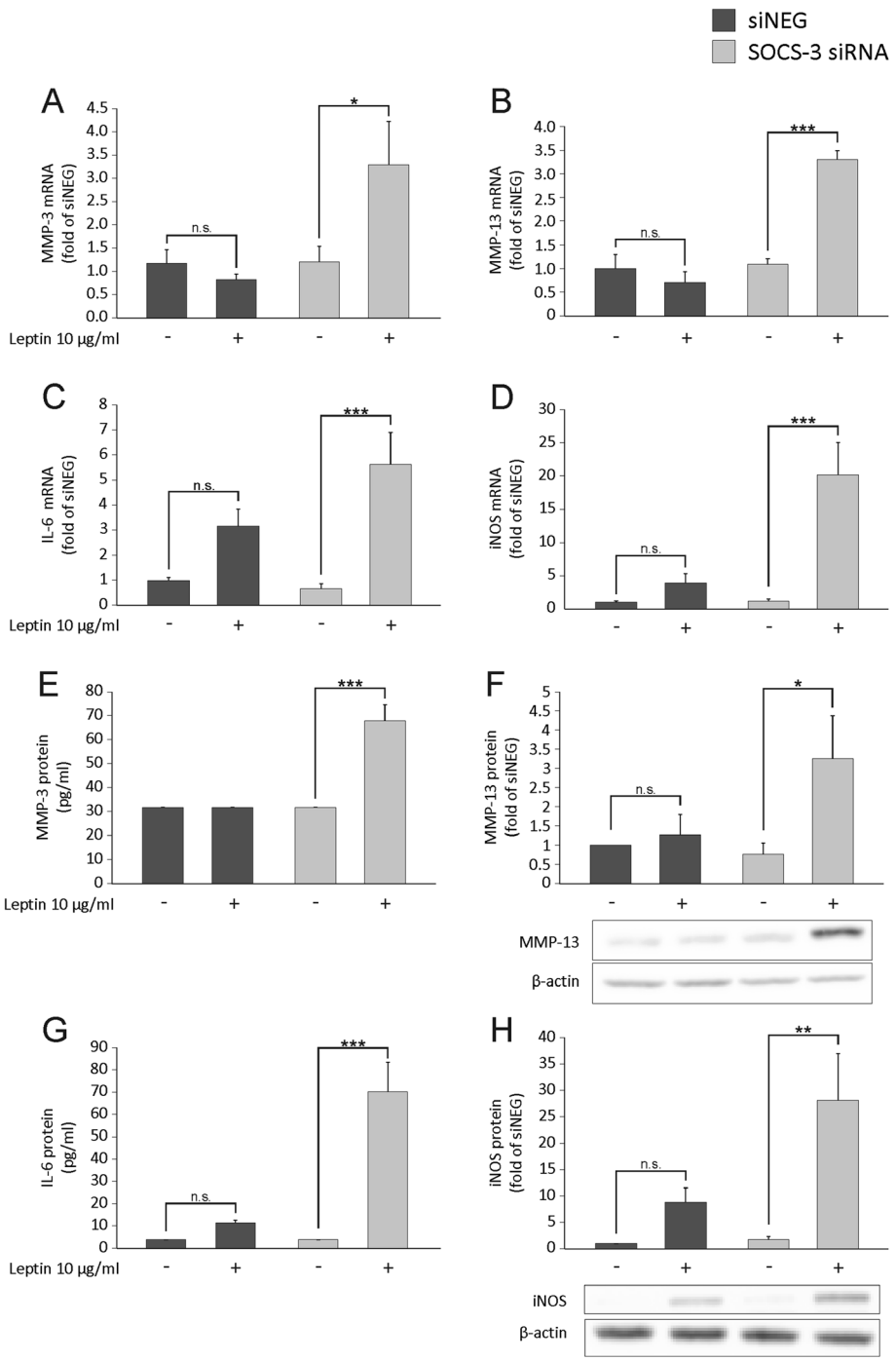

Fig. 4 The effect of silencing of suppressor of cytokine signaling-3 (*SOCS-3*) by siRNA on leptin-induced expression of matrix metalloproteinase-3 (*MMP-3*) (**a**, **e**), MMP-13 (**b**, **f**), interleukin-6 (*IL-6*) (**c**, **g**) and inducible nitric oxide synthase (*iNOS*) (**d**, **h**) in H4 murine chondrocytes. The cells were transfected with SOCS-3 siRNA or non-targeting siRNA (*siNEG*) and treated with leptin (10 µg/ml) for 4 (**c**, **d**), 8 (**a**, **b**, **h**) or 24 (**e**-**g**) hours. mRNA expression (**a**-**d**) was determined by quantitative RT-PCR, the levels of MMP-3 (**e**) and IL-6 (**g**) in the culture media supernatants by ELISA, and MMP-13 (**f**) and iNOS (**h**) expression in the chondrocyte lysates by western blotting. Results are expressed as means ± SEM; $n = 6$ (**a**-**e** and **g**) and $n = 3$ (**f**, **h**). MMP-3 protein level in siNEG and in non-treated SOCS-3 siRNA samples was below the detection limit and is set as half of the lowest standard. Representative bands of the western blots are shown. Statistical analysis was carried out by two-way analysis of variance with Bonferroni multiple comparisons post hoc test; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. *n.s.* not significant

response in MMP-13 mRNA expression in primary chondrocytes obtained from obese in comparison with non-obese patients with OA was reported [17]. The differences between these two studies could be explained by differential experimental conditions. Instead of primary chondrocytes we used pieces of cartilage, which provides a more natural environment for the chondrocytes in the culture. In addition, we used higher concentrations of leptin to ensure adequate penetration of leptin into the cartilage explant tissue. Of note, in the experimental studies, all cartilage explants were treated with the same leptin concentration in the culture in contrast to the in vivo situation where leptin concentration in SF is highly relative to BMI [5, 6, 8].

Other factors that might affect leptin responsiveness in vivo and that could contribute to the differential leptin responsiveness in cartilage from individual donors as seen in the present study, include functional leptin receptor (Ob-Rb) expression in chondrocytes and the amount of soluble leptin reseptor (sOb-R) in SF or in environment surrounding the chondrocytes. We previously reported that the level of soluble leptin receptor, that is thought to bind active leptin, is decreased in SF in obese subjects [8]. The expression of Ob-Rb has been shown to be unaffected in obesity [6, 17], whereas it has been reported to be increased in severely damaged cartilage [5, 6]. In vivo, all of these mechanisms are likely to contribute to the quantity of leptin-evoked effects, obesity seemingly favoring enhanced responses in many ways.

Leptin binding to its receptor leads to activation of multiple intracellular pathways including the Janus kinase-2 (JAK2)-STAT3, mitogen-activated protein kinase (MAPK), nuclear factor (NF)-kB and PI3K/Akt pathways, all of which have been shown to be involved in the leptin-induced production of proinflammatory factors by chondrocytes [11–15, 18, 19]. SOCS-3 has been shown to inhibit not only the STAT3 pathway, but also the extracellular signal-related kinase (Erk)1/2 and NF-kB pathways [34, 35], providing a possible mechanistic explanation for how leptin-induced responses could be modulated by SOCS-3 in chondrocytes. Interestingly Pallu et al. report higher leptin-induced activation of STAT3 in chondrocytes from obese than from non-obese patients [17], which could be a consequence of decreased SOCS-3 expression, and may explain the differential leptin responsiveness in obese individuals observed in their study. However, the involvement of SOCS-3 expression in explaining increased STAT3 activation in chondrocytes needs to be further studied.

According to the data in the present study, SOCS-3 appears to be a cartilage-protective factor in OA, as low SOCS-3 expression was associated with enhanced proinflammatory and catabolic effects of leptin. Here we investigated the role of SOCS-3 only in leptin signaling, but its role in OA may be much wider, as it can be involved in the regulation of the inflammatory responses induced by a variety of cytokines in multiple cell types in the joint [27, 36]. To our knowledge, only a few groups have previously investigated SOCS-3 expression in chondrocytes. Van de loo et al. reported that SOCS-3 overexpression inhibits lipopolysachharide (LPS)-induced NO production in chondrocytes [29] supporting the idea that SOCS-3 has a similar function in chondrocytes as in white blood cells, that is, to limit excessive inflammatory response. The role of SOCS-3 in arthritis has been studied in animal models by a few groups and the existing data support the idea that SOCS-3 has a protective role in arthritis.

In a study by Shouda et al. *SOCS-3* overexpression by intra-articular adenoviral gene transfer prevented the development of collagen-induced arthritis in mice [37]. Veenbergen et al. reported similar results [38]. In their study *SOCS-3* was delivered into the animals also by adenovirus gene transfer, but intravenously. Conversely, in a study by Wong et al. conditional deletion of *SOCS-3* in the hematopoietic and endothelial cell compartment led to particularly severe arthritis in a mouse model [39], supporting the importance of SOCS-3 as a negative regulator of inflammation. In the first two studies mentioned [37, 38] the target cells of SOCS-3 overexpression were supposed to be synoviocytes, antigen-presenting cells and possibly also B and T lymphocytes. In OA, chondrocytes are thought to be the central cell population that produces pathogenic factors; however, synoviocytes and inflammatory cells are also assumed to contribute to the inflammatory process. The present results indicate that SOCS-3 also modulates the inflammatory response in chondrocytes and thus, could be a promising drug target in the prevention/treatment of OA.

It is unclear, what explains the differential SOCS-3 expression in the cartilage from patients with OA in the present study. Cytokines including IL-1, IL-6, IFN-γ and TNF-α are all known inducers of SOCS-3 [40] and can be also found in the SF of affected joints in OA [41]. Interestingly, anti-inflammatory cytokine IL-10, statins and drugs that elevate cAMP are also known to induce SOCS-3 expression [40]. As it is induced by multiple factors, it is likely that the level of SOCS-3 expression in chondrocytes is defined by a complex net effect of proinflammatory and anti-inflammatory factors. SOCS-3 expression has been reported to be elevated in chondrocytes from patients with OA and RA in comparison to patients with femoral neck fracture without arthritis [29]; this is consistent with previous findings in other tissues, suggesting that SOCS-3 expression is elevated at the sites of inflammation, possibly as a regulatory mechanism to limit excessive inflammation response [42]. It is also possible that SOCS-3 expression would be affected by genetic polymorphisms in *SOCS-3*. There are two single nucleotide

polymorphisms (SNPs) previously described in human *SOCS-3*, one in the promoter region and another in the exon 1 of *SOCS-3*. However, in a large case–control study by Hölter et al. [43] these SNPs were found to have no effect on the expression/function of SOCS-3 and there were no differences in the frequency of these SNPs between overweight and underweight individuals.

In the present study leptin was positively associated and SOCS-3 was negatively associated with MMP levels in SF in obese but not in non-obese patients with OA, further confirming the importance of the leptin-SOCS-3 axis in cartilage metabolism and its possible significance in obesity-induced OA. Obesity is a significant risk factor for OA. In a Finnish population-based long-term follow-up study, a sevenfold risk of developing knee OA was reported in obese individuals with BMI >30 kg/m^2, as compared with subjects with BMI <25 kg/m^2 [44]. Several epidemiological and cross-sectional studies have investigated the association between leptin and the prevalence/incidence of OA, pain in OA and structural changes in the cartilage in OA [45–55].

Leptin is linked to OA in many of those studies but the results are partly conflicting. The differences might arise from the research frame and also from difficulty in differentiating the impact of body fat stores and leptin by statistical means, as these factors correlate strongly with one another. Karvonen-Gutirrez et al. used sophisticated statistical analysis, by adjusting their data for residuals from the regression of leptin on BMI, aiming to control their data for factors other than the metabolic component of BMI [54]. Interestingly, in their population-based study leptin predicted cartilage defects, as detected by magnetic resonance imaging (MRI) at the 10-year follow-up [54].

Another way to separate the effect of obesity and leptin was attempted by the use of leptin-deficient animals. In fact, Griffin et al. showed that leptin-deficient C57BL mice with diet-induced obesity did not develop OA like the corresponding wild-type mice, suggesting that obesity without increased leptin does not lead to OA [56]. In the present study we analyzed patients with OA in BMI subgroups (obese and non-obese) and interestingly, leptin correlated with MMP enzymes in SF only in the obese patients. This finding further supports the role of leptin in connecting obesity and OA. OA is a heterogenic disease with many known risk factors and it is likely that cartilage destruction in non-obese individuals is driven by pathogenic factors other than leptin. We also showed here that SOCS-3 expression in cartilage is negatively associated with SF levels of IL-6 and MMPs, and this was also seen only in the obese group, suggesting that SOCS-3 expression might be inadequate in the cartilage of obese individuals. Our previous finding that SOCS-3 expression in OA cartilage is decreased in obese compared to non-obese patients [8] supports this idea.

Conclusions

As a summary of this work, we demonstrated that SOCS-3 negatively modulates the pathogenic effects of leptin in chondrocytes. In addition, SOCS-3 expression in cartilage was negatively associated and synovial fluid leptin levels positively associated with synovial fluid MMP concentrations in obese, but not in non-obese patients with OA. This supports the harmful role of leptin and meaningful regulator role of SOCS-3 in obesity-related OA. Considering these factors in future studies could help to recognize new targets in the treatment and prevention of obesity-related OA.

Abbreviations

ADAMTS: a disintegrin and metalloproteinase with thrombospondin motifs; ANOVA: analysis of variance; BMI: body mass index; cDNA: complementary DNA; CNS: central nervous system; COX-2: cyclooxygenase-2; C_T: cycle threshold; DMEM: Dulbecco's modified Eagle's medium; ELISA: enzyme-linked immunosorbent assay; Erk1/2: extracellular signal-regulated kinase ½; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; gp130: glycoprotein 130; IFN: interferon; IL: interleukin; iNOS: inducible nitric oxide synthase; JAK: Janus kinase; LN: natural logarithm; LPS: lipopolysaccharide; MAPK: mitogen-activated protein kinase; MMP: matrix metalloproteinase; MRI: magnetic resonance imaging; mRNA: messenger RNA; NF-kB: nuclear factor kappa B; NO: nitric oxide; OA: osteoarthritis; Ob-Rb: functional leptin receptor; PCR: polymerase chain reaction; PGE_2: prostaglandin E_2; PI3K: phosphoinositide 3-kinase; SEM: standard error of the mean; SF: synovial fluid; siRNA: small interfering RNA; SNP: single nucleotide polymorphism; sOb-R: soluble leptin receptor; SOCS-3: suppressor of cytokine signaling-3; STAT: a signal transducer and activator of transcription; TNF: tumor necrosis factor

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Authors' contributions

AKK was involved in the conception and design of the study, the laboratory analyses, calculation of the results and interpretation of the data and she drafted the manuscript. KV was involved in the conception and design of the study, the laboratory analyses, calculation of the results, interpretation of the data and drafting the manuscript. RK was involved in the conception and design of the siRNA experiments, calculation of the results, interpretation of the results and revising the manuscript. TM was involved in the conception and design of the study, selecting the patients, acquiring the patient samples, interpretation of the data and revising the manuscript. EM was involved in the conception and design of the study, interpretation of the data and drafting the manuscript. All authors approved the final version of the manuscript.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Ethics approval and consent to participate

All patients gave their informed consent to the study. The study was approved by the Ethics Committee of Tampere University Hospital and it was carried out in accordance with the Declaration of Helsinki.

Author details
[1]The Immunopharmacology Research Group, University of Tampere School of Medicine and Tampere University Hospital, Tampere, Finland. [2]Coxa Hospital for Joint Replacement, Tampere, Finland.

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Brief Communication

Resistin as a factor in osteoarthritis: Synovial fluid resistin concentrations correlate positively with interleukin 6 and matrix metalloproteinases MMP-1 and MMP-3

Anna Koskinen, MD[1], Katriina Vuolteenaho, MD, PhD[1], Teemu Moilanen, MD, PhD[1,2] and Eeva Moilanen, MD, PhD, Professor[1]

[1] The Immunopharmacology Research Group, University of Tampere School of Medicine and Tampere University Hospital, Tampere, Finland

[2]Coxa Hospital for Joint Replacement, Tampere, Finland

The corresponding author:

Professor Eeva Moilanen

The Immunopharmacology Research Group

University of Tampere School of Medicine

33014 University of Tampere

FINLAND

Fax: +358 3 364 0558

e-mail: eeva.moilanen@uta.fi

Running head: Resistin in osteoarthritis

Abstract

Objectives: Resistin is an adipocytokine which has been related to inflammation and insulin resistance. Following knee injury, elevated levels of resistin have been found in synovial fluid (SF) while very little is known about the role of resistin in osteoarthritis (OA). The aim of the present study was to investigate resistin levels in OA joints and to determine if it is associated with inflammatory and catabolic factors in the joints.

Methods: Synovial fluid, plasma and cartilage samples were collected from 88 OA patients undergoing knee replacement surgery. Resistin levels were measured by enzyme-linked immunosorbent assay (ELISA) in SF, plasma and cartilage culture media.

Results: Significant levels of resistin [0.75 (0.67) ng/ml; median (IQR)] were found in SF from OA patients. Interestingly, resistin correlated positively with IL-6 ($r=0.39$, $p<0.001$) and with matrix metalloproteinases MMP-1 ($r=0.31$, $p=0.004$) and MMP-3 ($r=0.24$, $p=0.024$) in SF. Resistin was also released from cultured OA cartilage in correlation with its levels in SF ($r=0.39$, $p<0.001$). Resistin concentrations in SF correlated with those in plasma ($r=0.44$, $p<0.001$) as well, but were somewhat lower in SF. There were no differences in SF or plasma resistin concentrations between females and males or between non-diabetic and diabetic patients, and resistin did not correlate with body mass index (BMI).

Conclusions: Resistin is present in OA joints and is released from OA cartilage. Its levels in synovial fluid associate with inflammatory and catabolic factors suggesting that resistin is a factor with a role in the pathogenesis and as a drug target in OA.

Introduction

Resistin belongs to adipokine hormones and it was first described in 2001 (1). Originally, it was found to be produced by adipose tissue in proportion to fat mass and to promote insulin resistance in mice (1). Later studies have shown that in humans resistin is produced mainly by immune cells and only in minor amounts by adipose tissue (2,3). Further, resistin has been associated with multiple inflammatory diseases (2) while the evidence of its contribution to the development of insulin resistance in humans remains unclear (3).

The role of resistin as an inflammatory factor is supported by the finding that lipopolysaccharide (LPS) injection in healthy volunteers caused a rise in the circulating resistin levels which returned close to the baseline in one day after the injection (4). Accordingly, elevated circulating resistin concentrations have been reported in inflammatory conditions including sepsis, inflammatory bowel disease, rheumatoid arthritis (RA) and asthma (2). Specific receptors for resistin have not been identified, but a recent study revealed that resistin acts as an endogenous ligand of Toll-like receptor 4 (TLR-4) (5). As TLR-4 triggers major inflammatory pathways and is also activated by bacterial products like LPS, the finding further supports a significant role for resistin in mediating inflammatory responses.

There is also evidence to suggest that resistin may be involved in cellular events typical for the pathogenesis of osteoarthritis. Resistin levels in synovial fluid and in serum have been shown to increase in patients following knee injury (6) which is a known risk factor for osteoarthritis. Also, Bokarewa et al. found that resistin induced cartilage destruction and synovial inflammation when injected into knee joints of healthy mice (7). Cell and tissue culture studies have shown that resistin induces changes in chondrocyte metabolism which disturb the balance between catabolic and anabolic responses to favor the former: Resistin was reported to enhance cytokine, MMP-1, MMP-13 and ADAMTS-4 (a disintegrin and metalloproteinase with thrombospondin motifs 4) expression

in human primary chondrocytes (8), to upregulate prostaglandin E_2 ($PGE_{2)}$, IL-6 and IL-8 production in mouse cartilage (6) and to decrease production of proteoglycan in mouse and in human cartilage (6). Those findings led us to hypothesize that resistin may be involved in the pathogenesis of human osteoarthritis and to address that hypothesis by using clinical samples from patients with OA. The aim of the present study was to investigate whether resistin is present in OA joints and if it is associated with inflammatory and catabolic factors IL-6, MMP-1 and MMP-3 in OA patients.

Materials and methods

Resistin levels were measured in SF and plasma samples from 88 OA patients undergoing knee replacement surgery. Leftover pieces of cartilage were also obtained from 82 of those patients during surgery, and cultured as previously described (9). The tissue culture media were collected after 42 hours of culture and stored in -20˚C for further analysis. The levels of resistin, IL-6 and MMP-3 were measured by enzyme-linked immunosorbent assay (ELISA) using reagents from R&D Systems, Minneapolis, MN, USA (resistin and MMP-3) and Sanquin, PeliPair, Amsterdam, The Netherlands (IL-6). MMP-1 in SF was determined by Multiplex bead array (Fluorokine® Human MMP Multi Analyte Profiling Base Kit, R&D systems, Minneapolis, MN, USA). C-reactive protein (CRP) was measured in plasma by immunochemical method in Fimlab, Tampere, Finland.

The data were analyzed by IBM SPSS Statistics 19 (IBM Corporation, NY, USA). Results are expressed as mean ± standard deviation (SD) for normally distributed variables, and median (inter quartile range, IQR) in case of skewly distributed variables. Mann-Whitney Test was used to test the statistical significance of the differences in resistin levels between subgroups and Wilcoxon test to test statistical significance between different compartments. For correlation analysis, natural

logarithm (ln) transformations were formed where appropriate in order to have normally distributed variables. Correlations were assessed with the Pearson correlation coefficient.

The study was approved by the Ethics Committee of Tampere University Hospital, Tampere, Finland and carried out in accordance with the Declaration of Helsinki. Written informed consent was obtained from the patients.

Results

The study population consisted of 88 OA patients (58 females, 30 males; BMI 30.9 ± 5.8 kg/m^2; age 70.1 ± 9.8 years, mean ± SD; 15 patients with type 2 diabetes, 73 non-diabetics). Significant levels of resistin were found in SF [0.75 (0.67) ng/ml, median (IQR)] and in plasma [4.1 (2.0) ng/ml], the concentrations being lower in SF (Figure 1). Resistin in SF correlated with its plasma levels ($r=0.44$, $p<0.001$) (Figure 2a). In addition, cultured OA cartilage released resistin into the culture media [12.1 (11.6) ng/mg cartilage] and these resistin levels correlated with the SF resistin concentrations ($r=0.39$, $p<0.001$) (Figure 2b). There were no differences in SF or plasma levels of resistin between genders [SF: females 0.74 (0.78) ng/ml vs. males 0.79 (0.59) ng/ml, $p=0.13$; plasma: females 4.0 (2.0) ng/ml vs. males 4.1 (2.2) ng/ml, $p=0.71$] or between non-diabetic vs. diabetic patients [SF: non-diabetics 0.74 (0.69) ng/ml vs. diabetics 0.86 (0.62) ng/ml, $p=0.74$; plasma: non-diabetics 4.1 (2.0) ng/ml vs. diabetics 4.1 (2.3) ng/ml, $p=0.76$]. Resistin levels in SF or plasma did not correlate with BMI (SF: $r=0.09$, $p=0.42$; plasma: $r=0.15$, $p=0.16$).

Interestingly, SF resistin concentrations correlated positively with SF IL-6 [118.8 (200.2) pg/ml; $r=0.39$, $p<0.001$], MMP-1 [14.2 (25.1) ng/ml; $r=0.31$, $p=0.004$] and MMP-3 [649.5 (935.9) ng/ml; $r=0.24$, $p=0.024$] (Figure 3). No correlations were found between circulating levels of resistin and plasma CRP [1.6 (2.3) mg/l; $r=0.02$, $p=0.83$] or IL-6 [3.1 (2.5) pg/ml; $r=0.12$, $p=0.27$].

Discussion

The present results show that resistin is present in significant (ng/ml) amounts in OA joints. More interestingly, positive correlations were found between resistin and inflammatory cytokine IL-6, and between resistin and catabolic enzymes MMP-1 and MMP-3 in SF suggesting that resistin is linked to the local inflammatory process and cartilage destruction in OA joints.

The present clinical findings are supported by the previous data on the cellular effects of resistin in experimental models. Resistin has been reported to increase expression of MMP-1, MMP-13, ADAMTS-4, PGE_2, IL-1β, tumor necrosis factor alfa (TNF-α), IL-6 and IL-8 in murine cartilage and/or human primary chondrocytes (6,8). Resistin has also been shown to up-regulate the expression IL-6 and TNF-α in human macrophages and in the cells extracted from SF of patients with acute synovitis (7,10). Specific receptors for resistin have not been identified, but a recent study revealed that resistin signals through TLR-4 in peripheral blood mononuclear cells (PBMC) to increase production of IL-6, IL-1β and TNF-α (5). Interestingly, TLR-4 receptor expression is up-regulated in OA cartilage and its activation by LPS has been shown to enhance MMP-1, MMP-3, MMP-13 and nitric oxide production in human OA chondrocytes/cartilage (11,12). The correlations between resistin and IL-6 and MMPs in synovial fluid found in the present study could thus be explained by the impact of resistin on cartilage or on the leukocytes in SF or synovial membrane, possibly effecting through TLR-4 receptors.

To our knowledge, only few studies have investigated the role of resistin in OA patients (13-19), and most of them were based on small groups of patients (13-15) or investigated resistin levels in serum / plasma samples only (16-19). Our study is unique in its ability to investigate simultaneously resistin levels in plasma and synovial fluid and resistin release from OA cartilage. In a cross-sectional analysis by De Boer et al. circulating resistin was reported to be elevated in OA patients and, in line with our study, resistin was positively associated with histologically assessed

inflammation in synovial tissue (18). Schäffler et al. reported positive correlations between synovial fluid resistin and erythrocyte sedimentation rate (ESR) and CRP in a small cross-sectional study in OA patients (13), while Senolt et al. did not find correlation between SF resistin and CRP in OA patients (15). In the present study, resistin correlated positively with local parameters of inflammation and cartilage degradation at the site of inflammation, i.e. in SF from OA affected joint, while no correlations between resistin and systemic acute phase markers CRP or IL-6 in plasma samples were found. The discrepancy between the results of our study and the study by Schäffler et al. could arise from the timing of sample collection (SF sample taken at the outpatient clinic likely in the connection of exacerbation of the symptoms and synovitis vs. SF sample taken at the time of arthroplasty) or otherwise different patient characteristics. The pathogenic focus of OA is in the joint, and in spite of the ongoing local intra-articular inflammation, systemic acute phase markers such as ESR, CRP or IL-6 are mostly not elevated in OA patients (20). The local, but not systemic correlations found in the present study suggest that the possible involvement of resistin in OA is local and takes place in the pathological process in the joint. Whether SF resistin is produced in the joint or transported there from circulation cannot be concluded based on the present results. In RA patients the levels of resistin in synovial fluid have been reported to be higher than those in circulation (7,13,15,21), suggesting that resistin is produced in the inflamed joint. In OA patients, according to the present data and consistently with the previous findings by Presle et al. and Senolt et al.(14,15), resistin concentrations in SF were somewhat lower than those in the paired plasma samples. In our study SF resistin showed positive correlation with both resistin levels in plasma and resistin released from cartilage.

A few recent studies have investigated the association of serum resistin with radiographic findings of hand OA with conflicting results. In a cross-sectional study by Choe et al. serum resistin was associated with radiologically assessed subchondral erosion in patients with hand OA (17) while in another small cross-sectional study resistin did not associate with radiographic changes in hand OA

(19). In a longitudinal study by Yusuf et al. baseline serum resistin failed to predict radiographic progression of hand OA in a six year follow-up (16). As the levels of resistin markedly fluctuate in response to inflammatory stimuli (3) or trauma (6), it should be noted that one time point measurement of resistin in circulation might not represent the long-term levels, hampering the possible correlations with e.g. radiographic changes in OA joint. In addition, it should be noted that these studies measured serum resistin levels, not local intra-articular resistin concentrations.

Interestingly, also other adipokines in addition to resistin have been reported to have proinflammatory / degenerative effects on chondrocytes and joint tissues (22). There are a few possibilities to explain the similar effects. It's possible that one adipokine could influence on the expression of another adipokine. In fact, according to very recent findings by Tsiotra et al. leptin enhances release of resistin in human mononuclear cells (23). However, we have not found significant correlation between leptin and resistin in SF of OA patients (unpublished data), leaving the question unanswered if leptin, or other adipokines could influence on resistin levels in OA joint. Though this kind of interactions are possible, different adipokines, based on the responses seen in cell culture experiments (6,8,9,24,25), seem to have direct effects on chondrocytes which are not dependent on the other adipokines. Also, it should be noted that despite the original definition of adipokines as adipose tissue derived factors, it is now established that the sources of these molecules are multiple and diverge between different adipokines (22), and therefore their significance in inflammation / cartilage homeostasis may be distinct in different patient groups and conditions.

Positive association with BMI has been described for a couple of adipokines (22), leptin being the clearest example. In our relatively small patient population (n=88) neither BMI nor diabetic status was associated with resistin levels in synovial fluid or in circulation. In larger cohorts elevated circulating levels of resistin have been described in both diabetics as well as in obese individuals (3). However, data in accordance with the present findings also exists and it has been speculated

that inflammation driven influence on resistin levels might overrun the slight associations with diabetes and obesity (3). No conclusions can be drawn based on the present study whether possible obesity-related increase in resistin expression could be linked to obesity-induced OA. This interesting prospect should be studied in the future in larger patient groups.

In the present study we show positive correlations between resistin and inflammatory/catabolic factors in synovial fluid from OA patients suggesting that resistin could be a detrimental local factor in the pathogenesis of OA and a possible drug target.

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Figure legends

Figure 1. Resistin levels in synovial fluid and in circulation in OA patients. Resistin concentrations were measured by ELISA. Wilcoxon test was used to calculate the statistical significance. Horizontal bars within the boxes represent the medians. Boxes represent the interquartile ranges. Lines outside boxes represent minimums and maximums. Outliers are indicated. n=88.

Figure 2. Correlations between (A) resistin levels in plasma (p-resistin) and in the synovial fluid (SF-resistin), and between (B) resistin released by cultured cartilage and in synovial fluid in samples from OA patients. Ln transformations were formed of synovial fluid resistin and culture media resistin in order to have normally distributed variables. Pearson correlation analysis was assessed. n=88 (A); n=82 (B).

Figure 3. Correlations between synovial fluid levels of resistin and (A) IL-6, (B) MMP-1 and (C) MMP-3 in OA patients. Ln transformations were formed where appropriate in order to have normally distributed variables. Pearson correlation analysis was assessed. n=88.

Figure 1

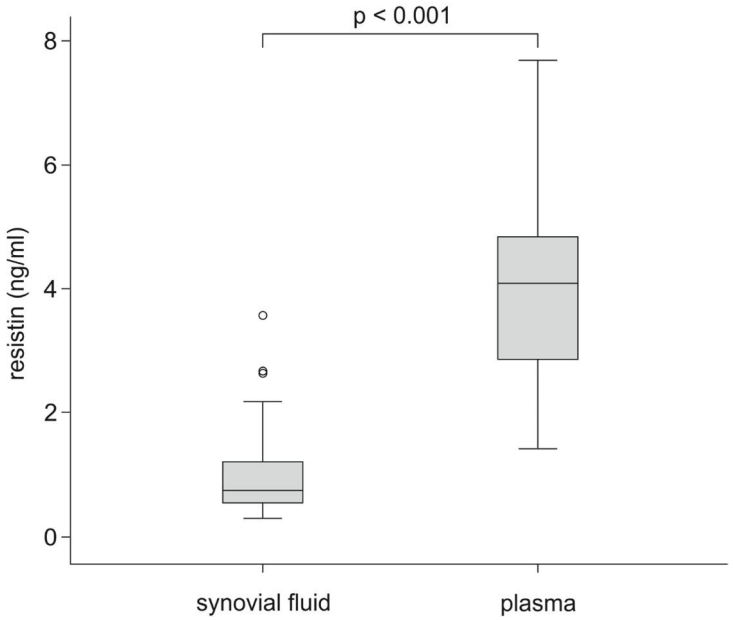
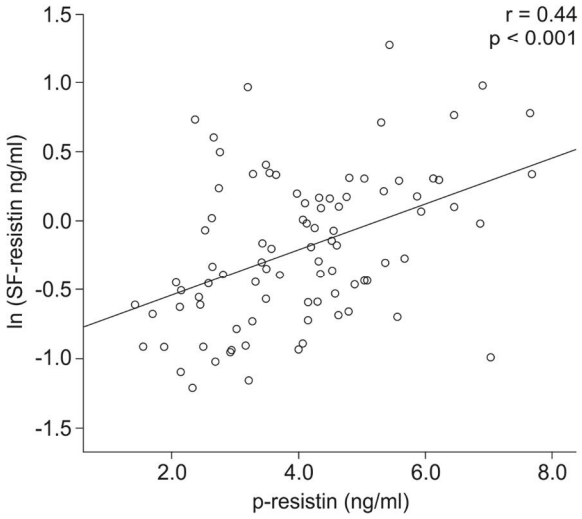


Figure 2

A



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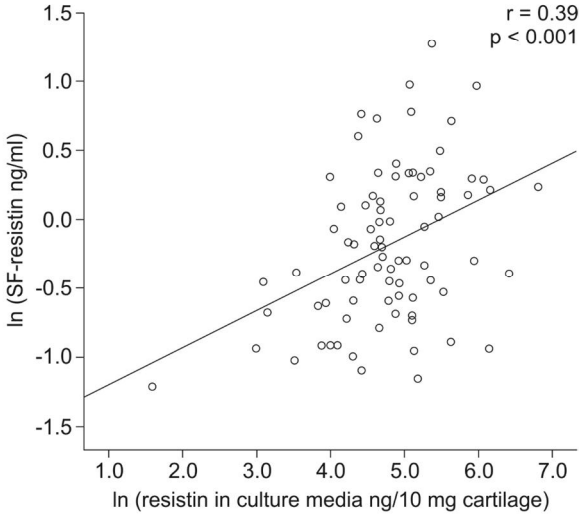
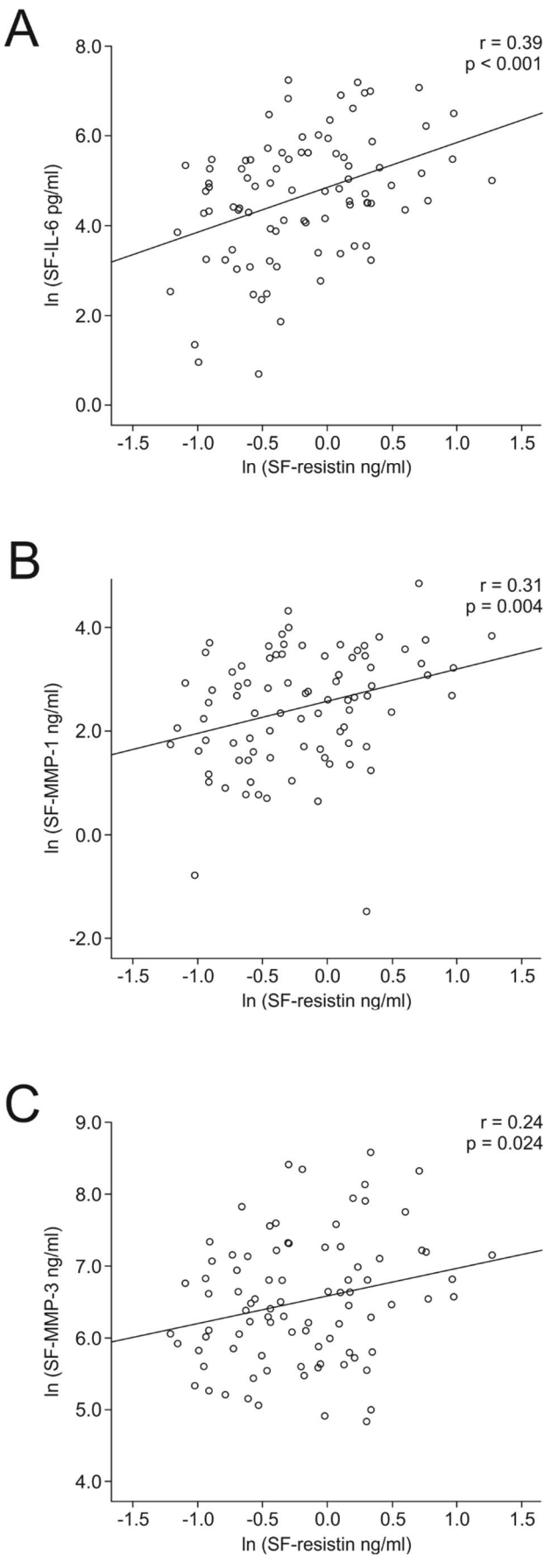


Figure 3


A
r = 0.39
p < 0.001
ln (SF-IL-6 pg/ml)
8.0
6.0
4.0
2.0
0.0
-1.5
-1.0
-0.5
0.0
0.5
1.0
1.5
ln (SF-resistin ng/ml)
B
r = 0.31
p = 0.004
ln (SF-MMP-1 ng/ml)
4.0
2.0
0.0
-2.0
ln (SF-resistin ng/ml)
C
r = 0.24
p = 0.024
ln (SF-MMP-3 ng/ml)
9.0
8.0
7.0
6.0
5.0
4.0
ln (SF-resistin ng/ml)