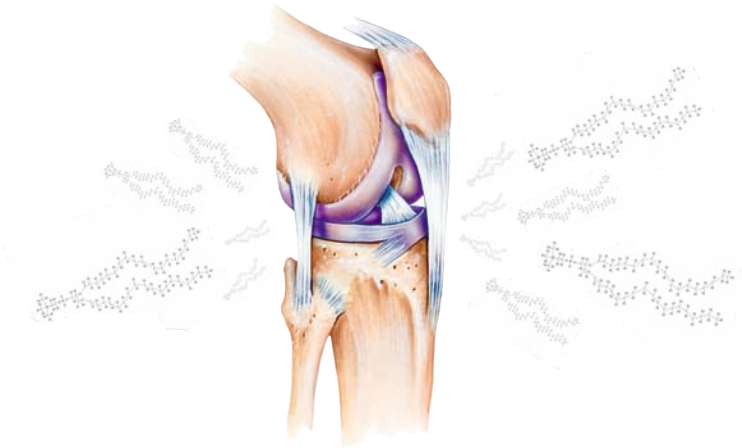
Boundary Lubricants in Osteoarthritic Synovial Fluid

Marta Krystyna Kosińska



INAUGURAL DISSERTATION submitted to the Faculty of Medicine
in partial fulfilment of the requirements for the PhD-Degree of the Faculties of
Veterinary Medicine and Medicine of the Justus-Liebig-University Giessen

édition scientifique
VVB LAUFERSWEILER VERLAG

1. Auflage 2012

1st Edition 2012

Printed in Germany

édition scientifique
VVB LAUFERSWEILER VERLAG

STAUFENBERGRING 15, D-35396 GIESSEN
Tel: 0641-5599888 Fax: 0641-5599890
email: redaktion@doktorverlag.de

www.doktorverlag.de

Boundary lubricants in osteoarthritic synovial fluid

Inaugural Dissertation

submitted to the

Faculty of Medicine

in partial fulfilment of the requirements

for the PhD-Degree

of the Faculties of Veterinary Medicine and Medicine

of the Justus-Liebig-University Giessen

by

Kosińska, Marta Krystyna

of

Toruń, Poland

Giessen 2012

From the Laboratory of Experimental Orthopaedics

Head: Prof. Dr. rer. nat. Jürgen Steinmeyer

Department of Orthopaedic Surgery

Director / Chairman: Prof. Dr. med. Markus Rickert

of the Faculty of Medicine of the Justus-Liebig-University Giessen

First Supervisor and Committee Member: Prof. Dr. rer. nat. Jürgen Steinmeyer

Second Supervisor and Committee Member: Prof. Dr. med. vet. Heinz-Jürgen Thiel

Committee Members: Prof. Dr. rer. nat. Magali Madry (née Cucchiarini)

Prof. Dr. rer. nat. Katrin Susanne Lips

Date of Doctoral Defence: 28th of August 2012

I Table of contents

II List of figures

III List of tables

IV List of abbreviations

APS	Ammonium persulfate
β2-MG	β2-Microglobulins
bp	Base pairs
BSA	Bovine serum albumin
CE	Cholesterol ester
Cer	Ceramide
Dex	Dexamethasone
DihSPM	Dihydrosphingomyelin
cDNA	Complementary deoxyribonucleic acid
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
dNTP	Deoxy ribonucleotide triphosphate
DPPC	Di-palmitoyl-phosphatidylcholine
ECL	Enhanced Chemiluminescence
EDTA	Ethylendinitrilo-*N*,*N*,*N´*,*N´*,-tetra-acetate
ELISA	Enzyme-linked immunosorbent assay
ESI-MS/MS	Electrospray ionization tandem mass spectrometry
FA	Fatty acid
FACS	Fluorescence-activated cell sorting
FC	Free cholesterol
FCS	Fetal calf serum
FDR	False discovery rate
FLS	Fibroblast-like synoviocytes
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GluCer	Glycosylceramide
HA	Hyaluronic acid
HEPES	2-(-4-2-hydroxyethyl)-piperazinyl-1-ethansulfonate
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase
IPG	Immobilized pH gradient
ITS™	Insulin-Transferrin-Selenium
kDa	Kilo Dalton

K/L	Kellgren/Lawrence
LB	Lamellar Body
LPC	Lysophosphatidylcholine
mAb	Monoclonal antibody
MALDI-TOF-MS	Matrix-assisted laser desorption ionization time-of-flight mass spectrometry
MMLV	Moloney murine leukemia virus
MMP	Matrix metalloproteinases
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
NA	Not analysed
NS	Not significant
OA	Osteoarthritis
PBS	Phosphate-buffered saline
PC	Phosphatidylcholine
PC O	Ether-phosphatidylcholine
PCR	Polymerase chain reaction
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
PI	Phosphatidylinositol
PL	Phospholipid
PLPE	Phosphatidylethanolamine-based plasmalogen
PPI	Protease Phospholipase Inhibitors
PRG 4	Proteoglycan 4
PS	Phosphatidylserine
PVDF	Polyvinylidene difluoride
RA	Rheumatoid arthritis
RNA	Ribonucleic acid
RNase	Ribonuclease
ROS	Reactive oxidative species
RT	Room temperature
RT-PCR	Reverse transcription PCR
SAPL	Surface active phospholipid

SD	Standard deviation
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SF	Synovial fluid
SP-A	Surfactant protein A
SP-B	Surfactant protein B
SP-C	Surfactant protein C
SP-D	Surfactant protein D
SPE	Solid phase extraction
SPM	Sphingomyelin
SZP	Superficial zone protein
TBS	Tris buffered saline
TBST	Tris buffered saline + 0.1% Tween 20
TC	Total cholesterol
TEMED	*N*,*N*,*N'*,*N'*-tetramethyl-ethane-1,2-diamine
Tris	Tris(hydroxymethyl)aminomethan

V Summary

Osteoarthritis (OA) is a degenerative joint disease characterised among others by the progressive loss of articular cartilage. Lubricin, hyaluronic acid (HA) and surface-active phospholipids contribute to the boundary lubrication provided by synovial fluid (SF). During OA, alterations in the concentrations of lubricin and HA impair the boundary lubricating ability of SF and may lead to increased friction and ultimately greater cartilage damage. In this line, the main hypothesis of the current study was that OA SF contains altered amounts of individual PL species, which might contribute to cartilage destruction during OA, and that these alterations are dependent on the stage of the disease.

Before starting the experiments, approval by the University ethics commission and the written informed consent of the patients were obtained. Qualitative and quantitative analyses of all phospholipids (PL) species were performed using electrospray ionization mass spectrometry (ESI-MS/MS) for human SF from healthy, rheumatoid arthritic (RA) and OA knee joints at different stages of the disease. Moreover, healthy and OA canine SF were also analysed in order to evaluate whether a canine model of OA reflects composition of PL species as found in human. In addition, the protein and mRNA expression pattern of surfactant proteins (SP-A, -D) was obtained in joint cells and SF.

The results of our lipidomic study provide for the first time a detailed overview of all PL species being present in human SF. Our analyses of SF indicate differences between healthy, OA and RA SF. Interestingly, SF from late stage of OA exhibited significantly higher concentrations of most of PL species when compared with healthy SF. More importantly, we provide first evidence that significant differences in PL composition exist between early and late stage of OA indicating, that PL composition of SF can serve as biomarker to distinguish between stages of OA. Moreover, the results obtained from human were confirmed by analysis of SF from canine model of OA. Our results also indicate a possible pathologic function of PL in the development of OA as based on their role in the boundary lubrication of joints as well as their biological activities. In addition, we provide several experimental proofs that human SF does not contain SP-A and –D.

In conclusion, our results support the hypothesis that alterations in composition and concentrations of PL species may also contribute to cartilage destruction during OA. Moreover, the specific PL pattern reflects stages of the disease. Hence, analysis of the SF lipidome can serve as a novel useful tool for diagnosis and prognosis of OA. Our data also indicate that surfactant proteins do not play any role in the boundary lubrication of joints.

VI Zusammenfassung

Arthrose, auch Osteoarthritis genannt, ist eine degenerative Gelenkerkrankung, die unter anderem durch einen fortschreitenden Verlust des Gelenkknorpels gekennzeichnet ist. Lubricin, Hyaluronsäure (HS) und oberflächenaktive Phospholipide (PL) der Synovialflüssigkeit (SF) sind an deren Fähigkeit zur Grenzflächenschmierung beteiligt Während der Arthrose kommt es zu Veränderungen in den Konzentrationen an Lubricin und HS, wodurch die Grenzflächenschmierung der SF beeinträchtigt wird, was zu einer erhöhten Reibung und letztlich zu größeren Knorpelschäden führen kann. Im Rahmen der vorliegenden Studie sollte daher die Hypothese geprüft werden, dass arthrotische SF veränderte Mengen an einzelnen PL-Spezies enthält, die möglicherweise zur Knorpelzerstörung während der Arthrose beitragen können und dass diese Veränderungen abhängig vom Stadium der Erkrankung sind.

Vor Beginn der Experimente wurde die Genehmigung durch die Ethikkommission der Universität und eine schriftliche Einwilligung der Patienten eingeholt. Qualitative und quantitative Analysen der PL-Spezies wurden mit Hilfe der Elektrospray-Ionisations-Massenspektrometrie (ESI-MS/MS) in humanen SF von gesunden, rheumatoid arthritischen und arthrotischen Kniegelenken verschiedener Krankheitsstadien durchgeführt. Darüber hinaus wurden gesunde und arthrotische SF von einem Hundemodell der Arthrose analysiert um zu beurteilen, ob die SF eine ähnliche Zusammensetzung der PL-Spezies aufweist wie humane SF. Zusätzlich wurde das Protein- und mRNA-Expressionsmuster der Oberflächenproteine (SP-A und -D) in verschiedenen Zellen des Kniegelenks und der SF bestimmt.

Das Ergebnis unserer Lipidomik-Studie zeigt zum ersten Mal eine detaillierte Übersicht über alle PL-Spezies, die in humaner SF vorhanden sind. Unsere Analyse ergab, dass es Unterschiede zwischen gesunder, arthrotischer und rheumatoid arthritischer SF gibt. Interessanterweise zeigt SF aus dem späten Stadium der Arthrose eine signifikant höhere Konzentration der meisten PL-Spezies im Vergleich zur SF gesunder Spender. Noch wichtiger ist, dass der erste Nachweis erbracht werden konnte, dass signifikante Unterschiede in der PL-Zusammensetzung zwischen frühen und späten Stadien der Arthrose bestehen und dass die PL-Zusammensetzung der SF möglicherweise als Biomarker genutzt werden kann, um zwischen den einzelnen Stadien der Arthrose unterscheiden zu können. Darüber hinaus wurden die Ergebnisse aus der Untersuchung der humanen SF durch die Analyse der SF im Hundemodell der Arthrose bestätigt. Unsere

Daten deuten außerdem auf eine mögliche Beteiligung der PL in der Pathogenese der Arthrose hin, da PL eine Rolle bei der Grenzflächenschmierung der Gelenke spielen und sogar biologische Aktivitäten entfalten können. Darüber hinaus können wir mehrere experimentelle Beweise erbringen, dass humane SF kein SP-A und -D enthält.

Abschließend läβt sich sagen, dass unsere Ergebnisse die Hypothese stützen, dass Veränderungen in der Zusammensetzung und Konzentration der PL-Spezies zum Knorpelabbau während einer Arthrose beitragen können. Zudem reflektiert das spezifische PL-Muster der einzelnen Krankheitsstadien das Fortschreiten der Arthrose. Daher kann vermutlich eine Bestimmung des Lipidoms, zumindest aber der PL-Zusammensetzung für die Diagnose und Prognose der Arthrose genutzt werden. Unsere Daten zeigen auch, dass die Oberflächenproteine wie SP-A und -D an der Grenzflächenschmierung der Gelenke nicht beteiligt sind.

1. Introduction

1.1 Osteoarthritis (OA)

Osteoarthritis (OA) is one of the most common forms of joint disorder characterised by the progressive loss of articular cartilage. The prevalence of OA is worldwide and approximately 5-10 millions of patients in Germany have radiological discernible OA alterations of at least one articular joint [1]. In Europe, every 1.5 min. one total joint replacement surgery due to OA is reported. In the United States, 500,000 joint replacements are performed per year [2-3]. Due to ageing of the population and the epidemic of obesity, the prevalence of OA is expected to increase. Current estimates are showing that in the United States nearly 27 million people have symptomatic OA and this number is expected to increase. Surveys from the World Health Organization assessed that symptomatic OA affects worldwide 10% of men and 18% of women aged over 60 years. Estimated annual costs of OA in the USA are around 60-89 billion dollars [2, 4].

1.1.1 Symptoms and risk factors of OA

The primary clinical symptoms of OA are pain, stiffness and dysfunction of joints. This is the main reason why patients are seeking medical help. As the disease progresses the pain becomes stronger, begins immediately after using the joint and may also occur during rest and night. The pain is often described as deep aching, poorly localised discomfort that accompanies for years and can increase with changes in the weather [5]. The cause of joint pain in OA is still not fully understood. In most of the patients with diagnosed OA movement of the knee joint is limited. Many of them have also crepitus with motion and joint effusion. In advanced stages OA can lead to joint deformations and subluxations. Twenty five percent of OA patients have limited abilities to perform their normal activities of daily living. Patients who suffer from OA have problems with walking, climbing stairs and they also complain of early morning stiffness of joints. This can lead to impaired performance in the workplace, social isolation and depression [6]. Interestingly, some people with radiographic evidence of advanced OA have minimal symptoms, whereas people with minimal radiographic changes may have acute pain and severe other symptoms [5, 7].

The main risk factor for OA is ageing, nevertheless, other risk factors can be also specified like obesity, gender (more women than men suffer from OA), hormonal status of women (postmenopausal versus premenopausal), genetic predisposition, joint trauma, previous joint injury, abnormal joint shape, muscle weakness and joint laxity [8-14].

1.1.2 Diagnosis and therapy

OA can affect small and large joints, either singly or in combinations. However, the most common joints involved are the large weight-bearing joints like the knee and the hip. OA is also often diagnosed in the hand, where mainly distal and proximal interphalangeal joints and the carpometacarpal joint of the thumb are affected [15].

Frequently, the diagnosis of OA is based on the patient's history and physical findings. Therefore, OA is often diagnosed in more advanced stages of the disease. The most widely used radiological method for diagnosis of OA is based on the characteristic changes of joints visible on plain X-rays. The grading system used both for diagnosis as well as clinical studies was first described by Kellgren and Lawrence in 1957 [16]. The so called Kellgren/Lawrence (K/L) score (range 0-4) is based on estimating the narrowing of joint space and changes in subchondral bone like occurrence of osteophytes and bone sclerosis. However, it has been suggested that the described scoring method has some limitations especially due to limited sensitivity to discriminate between healthy individuals, patients with early phases and late phases of the disease [17].

Magnetic resonance imaging (MRI) is supposed to be a better tool to diagnose early OA, but still not perfect, since clear quantification of especially small cartilage lesions is not possible [18]. Diagnostic strategies other than plain radiography and MRI for instance, bone scans, CT scans or even arthroscopic examination of joint surfaces are sometimes also applied depending on the diagnostic and/or therapeutic purposes [19].

Currently no therapies are available to stop or slow down the progression of OA. Therefore, the final therapeutic approach for OA is total joint replacement surgery. The main goal of OA treatment is pain relief and improvement of joint function. Pharmacological therapies use non-steroidal anti-inflammatory drugs, opioids, glucosamine, chondroitin sulfate and intraarticular injection of glucocorticoids or hyaluronic acid (HA) [20]. Non-pharmacologic therapies are also applied, such as physical therapy, manual therapy, acupuncture, different kinds of orthopaedic devices, education and self-management, weight loss, transcutaneous electrostimulation, and thermal

modalities [21-23]. Although, the progression of OA often leads to surgery followed by rehabilitation, many therapeutical strategies can be helpful to delay the need for joint replacement surgery by reducing the pain and inflammation and thus, improve the quality of life.

1.1.3 Pathophysiology of OA

OA is not a single disease entity but rather a complex disease with different underlying pathophysiological mechanisms. It is unlikely that one single factor causes this disorder. Historically, OA was considered to be caused only by mechanical factors with articular cartilage being the afflicted tissue. Indeed, OA is mostly characterised by a progressive and irreparable loss of articular cartilage. However, the present knowledge shows that OA affects the whole joint like synovium, subchondral bone and cartilage leading to joint degeneration (Figure 1.1).

In the past, OA was considered as a non-inflammatory disease. However, present knowledge indicates that OA is a unique inflammatory disease. OA cartilage does not have the characteristic signs of inflammation such as pain, redness, swelling and increased temperature since no nerves and blood vessels are present in cartilage. However, several studies revealed that many pro-inflammatory mediators are upregulated in OA affected chondrocytes similar as in a classic inflammatory cell like macrophages, as it was reviewed by Attur *et al.* in 2002 [24].

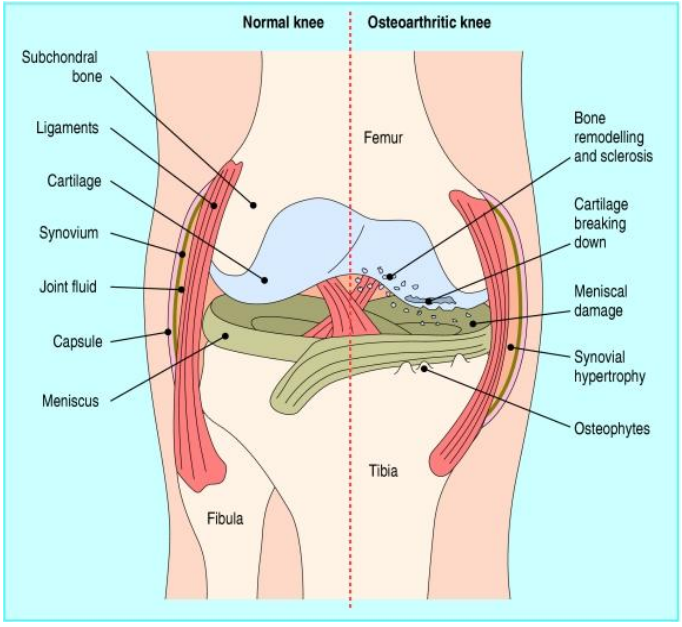


Figure 1.1. Pathologic alterations in OA. Picture obtained with permission from BMJ [25].

1.1.3.1 Articular cartilage

Normal healthy cartilage is an avascular, alymphatic and aneural tissue found at the end of articulating bones. It is responsible for providing a load-bearing, low friction interface for diarthrodial joints. Histologically, the cartilage tissue is classified into four zones: superficial or tangential, middle or transitional, deep or radial and calcified zone, where each of those zones is characterised by depth-varying cellular density and extracellular matrix properties [26]. The cell density of the superficial zone is high and chondrocytes are arranged parallel to the surface of cartilage. Chondrocytes are randomly dispersed in the middle zone and a lower cellular density can be observed. In the deep zone, chondrocytes have a columnar organisation [27]. It has been demonstrated that a tight interaction exists between those zones to regulate cell proliferation and secretion [28]. All zones are build by chondrocytes, which are sparsely distributed within the negatively charged cartilaginous extracellular matrix [29].

Proteoglycans (PGs) and collagens, mainly type II, VI, IX and XI are the main solid constituents of extracellular matrix, building a stable network within the cartilage. The most abundant cartilage PG is aggrecan which can bind to link protein and HA to form macromolecules embedded in a collagen net. Each monomer of macromolecule consists of core protein to which chains of chondroitin sulfate and keratan sulfate are attached. This polysaccharide side chains contribute to the hydrophilic nature of extracellular matrix of cartilage [30-32].

The most important process which occurs during initiation and progression of OA is an increased activity of proteolytic enzymes degrading the extracellular matrix. *In vitro* and *in vivo* studies have shown that aggrecanases are the primary enzymes responsible for aggrecan degradation of cartilage in the early stage of OA, even before aggrecan and collagen are cleaved by matrix metalloproteinases (MMPs). Collagen type II cleavage is primarily mediated by MMP-13 after aggrecan is enzymatically degraded by ADAMTS-4 and -5 (a disintegrin and metalloproteinase with trombospondin motif) and MMPs [33]. Activity of MMPs is revealed by generated VDIPEN fragments whereas ADAMTS-4 and -5 enzymatically produce NITEGE fragments. Both of those fragments can be found in RA and OA cartilage and synovial fluid (SF), thus, it is still not fully understood which of those enzymes play the major role in aggrecan degradation [34]. Several studies with use of ADAMTS-4 and -5 have implicated that they are the major factors responsible for cartilage degradation in OA [33, 35]. However, it has also been shown that expression of

ADAMTS-4 is induced and directly correlated with the level of cartilage destruction in OA, whereas expression of ADAMTS-5 is constitutive and comparable in both, normal and OA cartilage [36-37]. Moreover, a study using inhibitors for ADAMSTS-4 and -5 in human cartilage explants cultures show reduced production of prostaglandin E_2, which is known to be involved in PG loss from cartilage [38]. It has been postulated that higher levels of PG degradation products such as keratan sulfate and chondroitin sulfate epitopes in SF can be an early sign of OA [39-41]. Breakdown products of cartilage catabolism have therefore been studied as potential biomarkers for OA [42].

1.1.3.2 Synovium

The synovial membrane of healthy joints is only one to three cell layers thick and is composed of two cell types loosely embedded in an extracellular matrix: less numbered bone marrow-derived macrophage-like (type A) synoviocytes and locally derived, more abundant fibroblast-like (type B) synoviocytes (FLS) [43]. Below the synovial membrane no basement membrane is present, but the intercellular space contains type IV collagen, laminin and nidogen, major components of basement membranes. Such an organisation is unique only for the articular joints. The function of the synovial membrane is to provide nutrients and oxygen diffused from the blood vessels to avascular cartilage and to remove metabolic waste products from cartilage to the lymphatic vessels.

Several studies using electron microscopic methods provided evidence that FLS have lamellar bodies (LBs) [44-45]. This feature is unique and different from other classes of fibroblasts [43, 46]. It is known that synovial cells, mainly FLS actively produce HA [43, 47] and together with cells from the superficial zone of cartilage produce lubricin [48-49]. It has been also hypothesised that these cells are also responsible for the production of surface active phospholipids (SAPL) [44-45, 50-51] however, no experimental proof has been provided until today.

The following changes in synovium might occur in up to 50% of OA patients and can be arthroscopically determined sometimes even in early OA: synovial hyperthrophy, synovial hyperplasia, increased number of lining cells, infiltration of the sublining tissue with scattered foci of lymphocytes and vascular hyperplasia [52-53].

1.1.3.3 Subchondral bone

The subchondral bone represents a nearly nonporous and poorly vascularised subchondral bone plate with an underlying porous trabecular bone containing hematopoietic bone marrow and fat [54]. Bone consists of organic components mainly type I and III collagens and of non-organic, mineral hydroxylapatite. Because of the presence of bone-forming cells namely osteoblasts and bone-resorbing cells named osteoclasts, the bone is able to remodel [55].

In advanced stages of OA the progressive loss of articular cartilage is accompanied by the formation of bony structure at the joint margins namely osteophytes. In addition, sclerosis of subchondral bone occurs. It has been suggested that osteophytes are the result of penetration of blood vessels into cartilage or result from abnormal healing after stress fractures [56].

Regarding the subchondral bone sclerosis, it has been suggested that probably excessive load cause microfractures of subchondral trabeculae that heal via callous formation and remodelling. Additionally, it is now known that some osteoblasts are phenotypically different in OA compared to normal cells. However, they can produce increased levels of cytokines and growth factors and play critical role in subchondral bone sclerosis [57-58].

1.1.3.4 Synovial fluid

Synovial fluid (SF) is an ultrafiltrate of blood with additives produced by surrounding tissues. The volume of SF lies in the range of 0.5-4 ml for healthy knees and 4.4-21 ml for OA joints [59]. The main functions of SF are lubrication of surfaces like articular cartilage, meniscus, tendon, ligaments as well as nutrition, load bearing and shock absorption in the joint.

Normal SF contains molecules postulated to play a key role alone or in combinations in boundary lubrication like

- proteoglycan 4 (PRG4, lubricin, SZP, megakaryocyte stimulating factor, 0.05-0.35 mg/ml in healthy SF) [60-62].
- hyaluronic acid (HA, 1-4 mg/ml in healthy SF, 0.1-1.3 mg/ml in OA SF) [63-65].

- surface-active phospholipids (SAPL, 0.1- 0.2 mg/ml in healthy SF, 0.2-0.3 mg/ml in OA SF) [51, 64].

Also, SF from rheumatoid arthritic joints contain elevated levels of cell-derived microparticles (MPs) mostly from monocytes, granulocytes and lymphocytes [66]. MPs represent the heterogeneous population of membrane-bound small vesicles released from the cell membranes during cell activation or early apoptosis. In contrast to other cell vesicles like exosomes and apoptotic bodies, MPs are bigger and their diameter ranges from 200-1000 nm. MPs can vary in size and biochemical composition depending on their parental cells. These MPs are thought to have a function in the development of many human diseases including rheumatoid arthritis, where they trigger fibroblast-like synoviocytes (FLS) to produce cytokines, chemokines and other pro-inflammatory mediators [67-69]. Similar, but to a lesser degree, MPs are expected to be present in OA and normal SF.

1.1.4 Animal models of OA

Because OA is often not diagnosed until alterations in the joints are already progressed leading to strong pain and changes in articular cartilage, synovial tissue and SF from early stages of OA are not easily available for researchers. Moreover, therapies able to stop the progression of OA are thought to be successful only when applied in early stages of the disease. For this purpose, over 30 animal models of OA have been developed to gain insight into the pathogenesis and to study treatment modalities. However, none of these models reproduce the full spectrum of all changes observed in human OA.

Two often used models of OA are the anterior cruciate ligament transection (ACLT) model, and recently, the canine "groove model of OA". The transection of the anterior ligament leads to a permanent instability within the knee joint of various species such as dogs, sheep, guinea pigs and rats. This instability can induce degenerative changes in articular cartilage and inflammatory alterations within synovial tissue, representing OA [70-71].

Another animal model of OA is the 'groove model' where the articular cartilage of femoral condyles is damaged with a Kirchner wire in that several grooves are cut into the cartilage. This initiates the development of OA without affecting the subchondral bone and is further enhanced by intensified loading of the affected joint [72-75]. Since in the present

study the SF from the ‘groove model’ was used, this OA model is described in further details in the section 3.1.4.2.

1.2 Rheumatoid arthritis (RA)

Rheumatoid arthritis (RA) is another joint disorder affecting a large number of patients worldwide. About 1% of the world's population is afflicted by RA, women three times more often than men. The onset often starts at the age of 35 to 45 years, but people of any age can also be affected [76].

RA attacks many tissues and organs including synovial joints. Without any treatment this disease can slowly destroy whole joints. RA is an aggressive inflammatory disease with the synovium being strongly inflamed, hyperplasia of synovial cells, swollen joint capsule, and invasion of FLS [77-78]. SF obtained from RA patients is characterised by increased number of neutrophiles, granulocytes and macrophages and has a reduced viscosity.

In our study RA has been classified according to the criteria of the American College of Rheumatology [79] and SF obtained was used as another control for OA being focused on.

1.3 The boundary lubrication system of articular joints

Several components of SF have been proposed to contribute to boundary lubrication. Schmidt *et al.* compared experimentally all SF constituents and their contribution to the boundary lubrication [80]. Lubricin, HA and SAPL were tested individually and in combinations, both at physiological and pathophysiological concentrations. The results of this study supported previous data that all components of SF significantly contribute alone or in combinations to the boundary lubrication by lowering the friction coefficient.

Until now, HA is considered to be the main SF component responsible for lubrication and thereby protecting the surface of articular cartilage. However, the major function of HA is not lubrication *per se* but providing high viscosity of SF. Indeed high concentration of HA makes SF very slippery, but doesn’t provide lubricating ability of SF. Several studies depleting HA with use of hyaluronidase showed that SF without HA has no altered lubricating properties and, furthermore, enzymatic digesting of HA resulted only in a lower viscosity of SF [81-82].

Similar experiments were performed to support the hypothesis that lubricin and SAPL are responsible for boundary lubrication. SAPL were digested with phospholipase A_2 (PLA_2) and lubricin was depleted from SF using trypsin. The results of these studies indicated that the major role in lubrication has been assigned with SAPL and lubricin. Digestion with both PLA_2 and trypsin significantly increased friction and wear of bovine cartilage [51, 81, 83].

Role, functions and characteristic of all SF components which are thought to contribute to boundary lubrication will be described in further details in the next sections.

1.3.1 Lubricin

Lubricin is a glycoprotein found only in synovial joints. Lubricin is detected on the surface of articular cartilage, synovial membrane, tendons, ligaments and within SF [84]. It was first isolated from culture media of superficial slices of bovine articular cartilage [49]. Cells responsible for production of this protein are FLS and chondrocytes from the superficial zone of cartilage, therefore, lubricin is also called the superficial zone protein (SZP) [84-85]. Six homologues due to alternative splicing and post-translational modifications of *Prg4* gene can be specified: SZP, PRG4, megakaryocyte stimulating factor precursor (MSF), camptodactyly-arthropathy-coxa vara-pericarditis syndrome (CACP) protein and lubricin [48, 84, 86]

Lubricin is a large, mucinous glycoprotein encoded by the *Prg4* gene [87] with observed molecular weight 227.5 kDa. Deglycosylation decrease the apparent molecular weight to 120 kDa [84]. Some studies also suggested that in the glycosylated form lubricin can have a molecular weight of even 345 kDa [49, 88]. Structurally, lubricin is composed of three main domains namely the cysteine-rich, somatomedin B-like N-terminal domain, the mucin-like O-linked oligosaccharide-rich repeat domain and a C-terminal domain [89].

1.3.1.1 Function of lubricin in OA

Jay *et al.* reported that lubricin adsorbs to the articular surfaces of synovial joints thereby providing a so called chondroprotection [90]. This hypothesis was intensively studied over the last years. It has been shown that *Prg4* knockout mice lack cartilage surface integrity. Their joints showed a higher friction than joints from wild-type mice and they developed histological abnormalities in articular cartilage with an irregular articular surface and disruption in the orientation of collagen fibrils [91]. Human CACP syndrome

is caused by mutation in *Prg4* gene so that no lubricin is expressed. However, SF obtained from CACP patients contain normal level of HA indicating that HA is unable to provide efficient boundary lubrication while lubricin seems to be a major lubricating component of SF [86]. Additionally, several studies using ELISA methods found decreased concentrations of lubricin in SF during development of OA in guinea pigs. Teeple *et al.* [92] reported that in the guinea pig model of OA, the lubricin concentration was 73% lower in OA knee that in normal knee joints. Observed decrease was from approximately 70 µg/ml in normal knee joint to 20 µg/ml in OA induced knee. The mechanism responsible for reduced level of lubricin still remains unknown, but it was postulated that it is due to decreased expression of this protein in FLS and chondrocytes from superficial zone cartilage of guinea pig knee [92-93].

Recombinant lubricin was proposed to be an efficient substance which might be used for intraarticular supplementation in OA treatment. Three animal studies using rats showed, that intraarticular injection of 200 or 500 µg/ml lubricin into rat knees may prevent cartilage from degradation. Results of the treatment were determined using K/L score. Five weeks after lubricin injection, K/L score (range 0-4), significantly decreased from 2.0 in PBS treated knees to 1.3 in lubricin treated knees [94-96]. Thus, lubricin seems to be a promising new agent to treat OA, however, this needs further confirmation in clinical studies.

1.3.2 Surfactant proteins

Surfactant protein A (SP-A), B (SP-B), C (SP-C) and D (SP-D) are members of the collectin family mostly found in the alveolar space, where they cover surfaces of alveoli and reduce surface tension [97]. All four collectins share similar chemical structure and are composed out of four regions, an amino terminal region, a collagenous region, an α-helical neck peptide and carboxy-terminal C-type carbohydrate recognition region [98].

All SPs present in the lung are expressed and secreted by alveolar type II cells and Clara cells [99] and each has individual function. SP-A and SP-D are water-soluble and hydrophilic, whereas SP-B and SP-C are extremely hydrophobic and strongly interact with pulmonary PL. SP-A is believed to be responsible for adsorption, binding and spreading of SAPL at alveoli surfaces, enhanced SAPL liposome uptake and inhibiting of SAPL secretion by type II cells. Takahashi *et al.* [100] reported other important functions of SP-A and SP-D namely as modulators of innate immunity in the lung [100].

Surfactant proteins have been considered to be present only in the lung. However, some investigators believe that these proteins can also be found within other organs where PL-rich surfaces are present. Cells which possess LBs for instance, tongue papillae, oral epithelium, the gastric and intestimal mucosae, the peritoneum, the pericardium, pleura and Eustachian tube are able to secrete those proteins [101-103]. During the past 15 years Kankavi provided some evidence that SP-A and SP-D are present in non-lung sites. He determined those proteins in human skin, human organ of Corti, human Eustachian tube, human and equine SF, human spermatozoa, human kidney and also in vagina, cervix, uterus, oviduct and ovaries of mares and prepuce, smegma, testis, prostate and vesicular glands of stallion [104-108].

There are only a few investigations supporting the hypothesis that SP-A and SP-D are present in the synovial joints [101, 109-111]. This hypothesis is based on previous observation that FLS similar like epithelial type II cells possesses LBs [44, 51]. Using immunoblotting methods, SP-A and SP-D were successfully detected in human and equine SF by Kankavi [109-110] and their function was hypothesised to be similar to those in the lung. In the lung, surfactant proteins were reported to build together with PL the monolayer at the liquid-air surface of alveoli lining. Therefore, the expression of SP-A and SP-D is abundant and necessary for normal lung function. Nevertheless, the question whether the surfactant system is similar in the joint as it is found in the lung still has to be determined.

1.3.3 Hyaluronic acid

High molecular weight HA is one major component of SF found on the surface of articular cartilage and in extracellular matrix. Meyer and Palmer [112] reported in 1934 that they have isolated HA for the first time from bovine vitreous humour. Furthermore, Weissman and Meyer [113] determined the HA structure as a long chain of polysaccharide with observed molecular weight of 2100 kDa. However, the molecular weight of HA has been shown to lie in wide range between 1.6×10^6 and 10.9×10^6 Da [114-115]. This polysaccharide is composed out of repeating disaccharide units of D-glucuronic acid and D-N acetylglucosamine, linked together via alternating β-1,4 and β-1,3 glycosidic bonds [113]. HA is synthesised by FLS at the inner layer of the cell membrane by membrane-spanning hyaluronan synthases (HAS 1-3) [116]. In synovial joints, HA has several functional properties with key roles, such as providing hydrodynamic viscous properties of

SF, mechano-protection, shock absorption, and being a buffer against fluid loss from cartilage during flexion, which prevent cartilage from drying [117].

It is well known that during OA influx of water can occur and the volume of SF may increase up to 20-fold resulting into a diminished viscoelastic property of SF. In OA both the concentration and the molecular weight of HA are decreased. The concentration of HA in normal SF varies between 1.45 and 3.12 mg/ml and is decreased in pathological SF down to 1.09-1.20 mg/ml [65]. Furthermore, the chain length of HA is enzymatically reduced from 7.0×10^6 to 5.0×10^6 Da during OA [114]. Treatment with intra-articular HA injection as viscosupplementation for OA shows conflicting clinical results. However, few recently published Cochrane reviews support in part the use of HA in the treatment of OA [118-119]. Wang *et al.* [120] reported that human derived FLS treated *in vitro* with 100 µg/ml high molecular HA down-regulated gene expression of OA-associated cytokines and enzymes, such as IL-6, IL-8, MMP-1, MMP-3 and MMP-13 which all have been shown to be involved in OA pathophysiology [120].

1.3.4 Lipids

Lipids are essential cellular and extracellular components which play important roles in mammalian cells. Lipids are part of biological membranes such as the cellular plasma membrane and the intracellular membranes of organelles like mitochondria, endoplasmic reticulum and vacuoles. As second messengers (e.g. diacylglycerols, ceramides, eicosanoids, lysolipids) lipids participate in cellular signalling and participate in interactions with membrane-associated proteins. Lipids are classified in classes such as glycerolipids, glycerophospholipids (phospholipids, PL), sphingolipids and sterol lipids. Within the last few years the interest in lipids, their classes, subclasses and individual molecular species has been increased constantly. Beside “genomics” and “proteomics”, “lipidomics” now also gained increased scientific attention.

1.3.4.1 Phospholipids

PL are characterised by the presence of glycerol backbone, whereas sphingolipids posses a sphingoid base. PL and sphingolipids are a group of molecules which contain a large number of molecular species with different length of carbon chains having different number of double bounds. It is assumed that 60% of all cellular lipids are PL [121].

Structurally all PL share similar chemical pattern with presence of a polar head attached to the glycerol backbone via a phosphodiester bound. Various fatty acids are linked to the glycerol backbone at *sn-1* and *sn-2* position via either two acyl linkages or one acyl and one alkyl linkage [121]. Exceptions are lysophospholipids (monoacylglycerophospholipids) which have only one fatty acid linked to either *sn-1* or *sn-2*. According to differences in the head group PL are divided into classes, such as phosphatidylcholine (PC), lysophosphatidylcholine (LPC), phosphatidylethanolamine (PE), phosphatidylethanolamine-based plasmalogen (PLPE), phosphatidylinositol (PI), phosphatidylserine (PS) and phosphatidylglycerol (PG). Each PL class is further divided into subclasses according to chain length which are typically 14-22 carbons long and the number of double bonds within the fatty acids which are introduced at various positions (Figure 1.2).

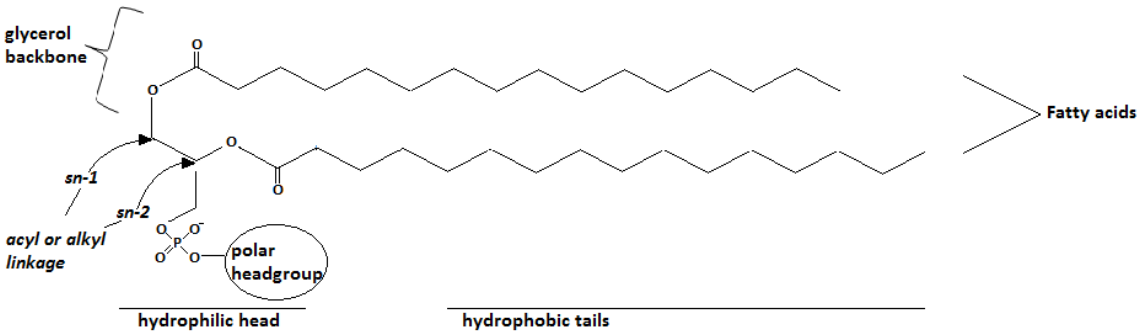


Figure 1.2. General chemical structure of glycerophospholipids.

1.3.4.1.1 Boundary lubrication provided by PL

As it has been reviewed by Postle [122], PL can be involved in the development of many lung diseases like asthma, cystic fibrosis, viral infections and acute respiratory distress syndrome, brain diseases like Alzheimer's disease and Down's syndrome, heart failure, and kidney disorders. Several studies performed by Hills and co-workers indicated that PL play also a role in the development of OA [50, 123].

SAPLs in the lung often named as "surfactants" are produced by alveolar type II cells, which are characterised by presence of LBs. Because several studies already showed that LBs can be microscopically identified in FLS, it was suggested that those cells in the joint are responsible for SAPL synthesis, release and secretion [44-45, 51]. SAPL has also been found in many other organs beside the lung such as joints, stomach, Eustachian tube, peritoneal cavity and pleural cavity [102, 124-126]. The main component of surfactant

found in the lung is the saturated form of PC named di-palmitoyl-phosphatidylcholine (DPPC) (PC 16:0/16:0) providing most of the surfactant activities in the lung. Two unsaturated PC species are also present in high concentrations in the extrapulmonary sites namely palmitoyl-linoleoyl-phosphatidylcholine and palmitoyl-oleoyl-phosphatidylcholine. Originally, DPPC was believed to be the main PL responsible for boundary lubrication, similar as found for the lung. Indeed, it has been demonstrated that DPPC is able to reduce friction on the surfaces of articular cartilage. However, it was also reported that unsaturated PC species posses better lubrication properties than those of DPPC [127]. Some studies using gas chromatography investigated the PL composition of articular cartilage surfaces. These studies also support the finding that the most abundant species on the surfaces of for instance bovine and human articular cartilage is unsaturated PC [128-129]. Chen *et al.* [130] reported also that PC species play a role in the water transport within the articular cartilage. PC molecule can bind to the negatively charged surface of cartilage and acts as effective semipermeable membrane which allows for transport of water into the cartilage. When the amount of PC lining on the surfaces is decreased, increased accumulation of fluid in joint might appear contributing to joint effusion [129-130].

SAPLs can be reversibly adsorbed onto the surface of articular cartilage, therefore, rendering the surface of articular cartilage hydrophobic. SAPLs cover the surface of cartilage thereby leading to a microscopically thick hydrophobic bio-film, which is believed to contribute to boundary lubrication (Figure 1.3) [131-132].

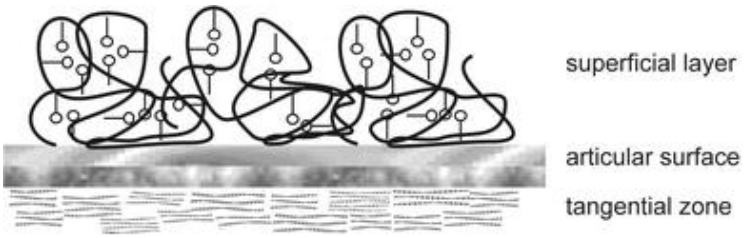


Figure 1.3. Scheme of superficial layer of cartilage contains HA and SAPL. Picture obtained with permission from Springer [132].

SAPL has been demonstrated to possess the excellent anti-wear properties and to act as a cartilage protective agent. In this line, SF was aspirated from sheep knee joints and was tested together with PL extracted from SF and synthetic SF in a wear test using a "four ball extreme-pressure lubricant machine" [133]. This experiment demonstrated that SF,

synthetic SF containing SAPL and HA, and extracted PL reduce kinetic friction as well as wear in the wear tests [133].

PLs, which can be found within SF and on the surface of articular cartilage, seem to be similar to those in the lung (SAPL). However, the question, if the functions of SAPL which have been found in the lung can be extrapolated to the joint, still remains to be determined. SAPLs, for instance plasmalogens, in the lung were reported to function as a scavenger of free radicals [134]. It is not known whether SAPL can play a similar function in the joint. However, this would be important to know since several studies already implicated that activated oxygen species play a critical role in cartilage destruction and chondrocytes apoptosis during OA [135].

Little is known about the biosynthesis of PL in the joint and their regulation. Several studies indicated that intraarticular injections of glucocorticoids may have a prolonged anti-inflammatory effect on OA in humans [136] and horses [123]. Because it is already known that glucocorticoids promote the secretion of SAPLs in the lung [137], it has been also suggested that in the joint glucocorticoids can also induce SAPLs production [123]. This hypothesis would help to explain the long term therapeutic effect of corticosteroids which are drugs having a short half-life lying in the range of hours. Hills *et al.* also hypothesised that during the development of OA SAPL are becoming deficient on articular surfaces [138]. Using methylprednisdolone acetate (MPA) Hills *et al.* [123] demonstrated, that after intra-articular injection of this corticosteroid, the quantity of SAPLs in SF of equine knee joints was elevated. The authors injected 100 mg of MPA in 2.5 ml of saline into the right knee joints of 5 healthy horses. Two and half ml of saline was injected to the left knee joints which served as a control. PL content was determined using the phosphate assay, 16 h and 32 h post injection. The phosphate assay oxidise phosphorus to phosphate using perchloric acid and then determines the quantity in a spectrophotometer after addition of ammonium molybdate as an indicator. This study revealed that the amount of PL increased in the treated knee joint from 1.48 to 3.25 mg/ml SF, whereas in the control knee it increased only from 2.25 to 2.56 mg/ml SF suggesting that intraarticular injection of corticosteroids might promote PL secretion [123].

In the next sections all PL classes which were investigated in this study will be introduced in more detail.

1.3.4.1.2 Phospholipids containing choline

Phosphatidylcholine

Phosphatidylcholine (PC) is a class of PL that incorporates choline as a head group. PC is the major PL class within SAPL (Figure 1.4). Two fatty acids are needed to form an intact saturated PC or unsaturated PC molecules. DPPC is an example of saturated PC which requires two saturated fatty acids, i.e. palmitic acid. Palmitoyl-linoleoyl-phosphatidylcholine and palmitoyl-oleoyl-phosphatidylcholine are examples of unsaturated PC which require one saturated fatty acid namely either palmitic or stearic acid and one unsaturated fatty acid either namely linoleic or oleic acid.

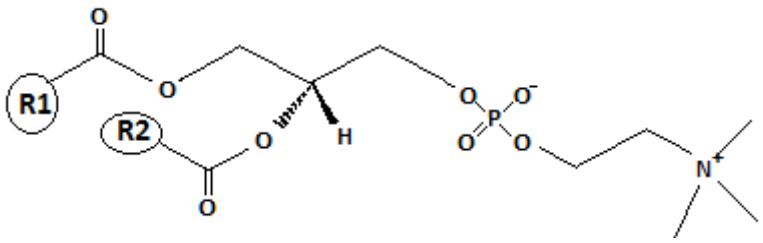


Figure 1.4. General chemical structure of phosphatidylcholine. R1 and R2–fatty acids.

PC plays various functions in the human organism for instance, PC is a major component of biological membranes and functions as a SAPL in the lung and probably also in the joint [129, 139]. It is known that DPPC is more abundant in the lung than the unsaturated form of PC and serves as a surfactant in this organ. Chen *et al.* [129] demonstrated that in the joint the unsaturated form of PC is more abundant as compared to the saturated form of PC [129]. The study of Eros *et al.* [140] shows that oral treatment of mice with PC exhibit anti-inflammatory properties. In this study, arthritis was induced by collagen injection (CIA) in group of 10 mice. The animals received PC-enriched diet either prophylactically before induction of CIA or after induction of CIA. The results indicated that PC decreased the inflammatory activation of leukocytes and reduced the elevated expression of inducible nitric oxide synthase. The study revealed also a decreased degree of synovial angiogenesis and less pain, redness and swelling. Taken together, these results suggest that PC possess anti-inflammatory properties and may reduce the damages within the joint accompanied with the RA disease [140].

Lysophosphatidylcholine

Lysophosphatidylcholine (LPC) is a class of PL which is mainly generated by partial hydrolysis of the membrane PC by phospholipase A_2 (PLA_2). LPC is an exceptional PL since it possess only one fatty acid in either *sn-1* or *sn-2* position (Figure 1.5). LPC is the major component of plasma membranes and is recognised as an important cell signalling molecule. [141].

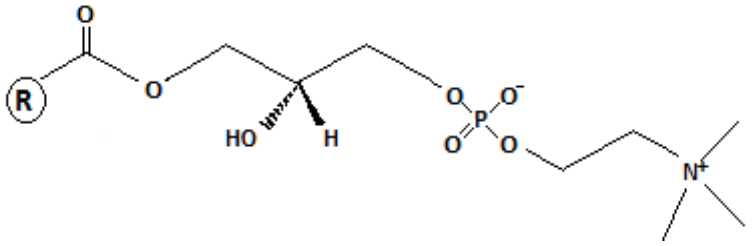


Figure 1.5. General chemical structure of lysophosphatidylcholine. R – fatty acid.

LPC can act as a pro-inflammatory mediator and the PC/LPC ratio in the serum or in the SF might be a good diagnostic marker for RA. SF and plasma samples were obtained from 9 patients with RA and plasma samples was obtained from 14 patients with RA undergoing anti-TNF-α therapy. PC and LPC were determined with MALDI-TOF/MS and high resolution ^{31}P-NMR with subsequent calculation of the PC/LPC ratio. The PC/LPC ratio was expected to be about 20 for healthy individuals and decreased in RA patients to 9.7±5 prior to therapy. Seven months after therapy the ratio increased 2-fold (19±5) [142].

1.3.4.1.3 Phosphatidylethanolamine and PE-based plasmalogen

Phosphatidylethanolamine

Phosphatidylethanolamine (PE) is the second most abundant component of mammalian cell membranes with ethanolamine as a head group (Figure 1.6).

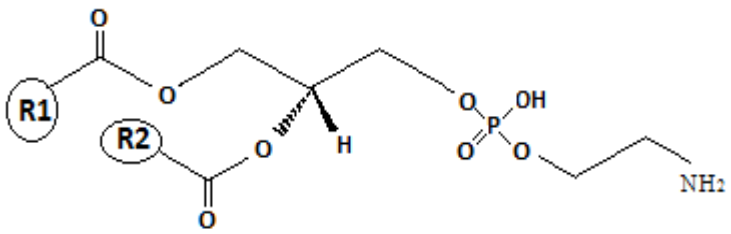


Figure 1.6. General chemical structure of phosphatidylethanolamine. R1 and R2-fatty acids.

PE together with PS belongs to aminophospholipid group and can be synthesised by two independent pathways. In the first pathway the mitochondrial enzyme PS decarboxylase converts PS to PE. The second pathway is called the CDP-ethanolamine pathway which consists of three steps and which needs ethanolamine from dietary sources. This pathway is catalysed by ethanolamine kinase, phosphoethanolamine cytidyltransferase and diacylglycerol ethanolaminephosphotransferase [143].

PE-based plasmalogen

Plasmalogens represent a large group of PL species and make up approximately 18% of all PL in human body. However, in our study only PE-based plasmalogen (PLPE) was measured as one major group of plasmalogens (Figure 1.7). This PL is characterised by a vinyl ether linkage at the *sn-1* position and an ester linkage at the *sn-2* position. Plasmalogen can also appear as PC-based, PS-based or PI-based molecules.

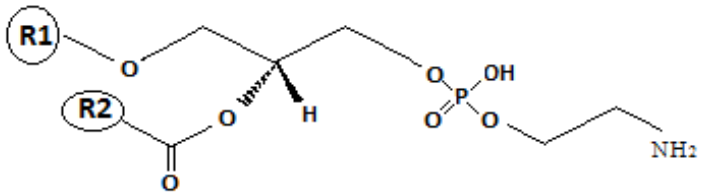


Figure 1.7. General chemical structure of PE-based plasmalogen. R1 and R2-fatty acids.

Nagan and Zoeller in their review [144] highlighted the most important functions of plasmalogens, such as intracellular signalling, a protective role during oxidant-induced stress against reactive oxygen species for instance in the Chinese hamster ovary cells, rat neurons and murine macrophage-like cells. A study using plasmalogen-deficient human fibroblasts suggested that plasmalogens influence membrane trafficking by promotion the fusions of membranes thus, playing a role in endocytosis and secretion [145]. Human genetic disorders accompanied by lack of plasmalogen in the tissues, may trigger diseases such as, severe mental retardation, hypotonicity, adrenal dysfunction, cataracts, deafness, facial dysmorphism, and chondrodysplasia [144].

1.3.4.1.4 Phosphatidylserine

Phosphatidylserine (PS) is quantitatively a minor PL which makes up to 2-10% of the total PL. It is primarily localised on the inner leaflet of plasma membranes [146]. PS is synthesised by replacement of the head group of existing PL by serine (Figure 1.8). For

instance, the choline head group from PC or ethanolamine group from PE can be replaced by a serine group. These reactions are catalysed by PS synthase 1 and 2, respectively [147].

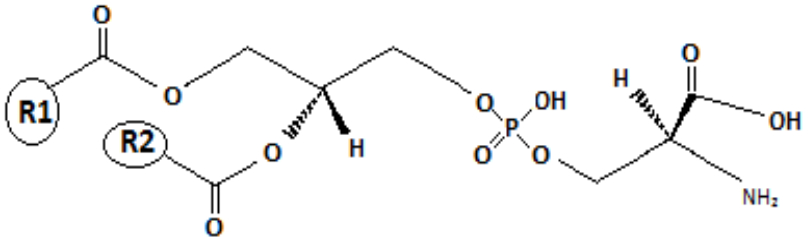


Figure 1.8. General chemical structure of phosphatidylserine. R1 and R2-fatty acids.

PS was first isolated from bovine brain and it was reported that oral administration of bovine cortex-derived PS improves cognitive disorders of patients with senile dementia [148]. In another study aged rats were given water with emulsified PS prepared from soybean lecithin and L-serine. Average daily intake was 60 mg/kg. Further, memory abilities of rats were tested. The results from the Morris water maze test show, that aged-related memory impairments of rats were improved [149]. Human neural cells from gray matter at the surface of cerebral cortex are also enriched with PS as compared to cells from other organs. Therefore, PS is considered to play an essential role in the development and function of the nervous system [150].

1.3.4.1.5 Phosphatidylinositol

Phosphatidylinositol (PI) is a minor component of cell membranes and is characterised by the presence of inositol as a head-group. PI can be rapidly phosphorylated to form phosphatidylinositol phosphate (PIP), phosphatidylinositol biphosphate (PIP_2) and phosphatidylinositol triphosphate (PIP_3). PIP, PIP_2 and PIP_3 are collectively called phosphoinositides (Figure 1.9).

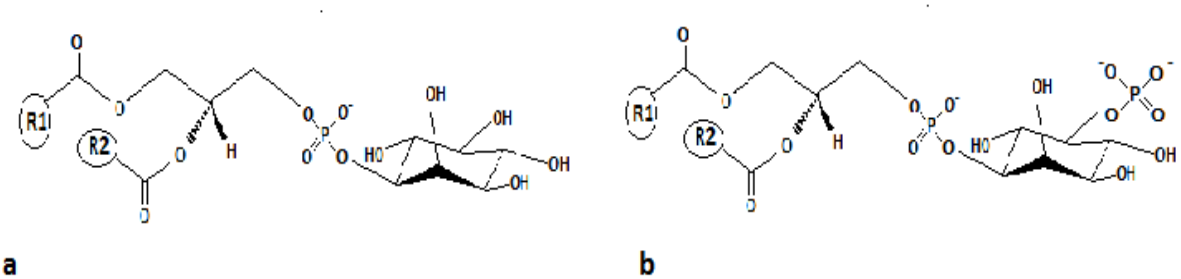


Figure 1.9. General chemical structure of (a) phosphatidylinositol and (b) phosphoinositide (phosphatidylinositol biphosphate). R1 and R2-fatty acids.

It has been shown that monophosphorylated PI is associated with intracellular membranes where it can play a role in the constitutive membrane trafficking. Because of the negative charge being present on the inositol ring, PI is able to bind to the membrane associated proteins and induce protein cycle between organelles from the donor membrane to the acceptor membrane. PI is also specifically incorporated in membranes from various cell organelles such as the Golgi complex, endosomes, liposomes and vacuoles and may play a key role in distinguishing the organelles [151-152].

The possible role of PI in the joints is not known, however, *in vitro* study using FLS derived from rabbit knees revealed, that FLS stimulated with 180 µg/ml of hydroxyapapite crystals, synthesised respectively released about 2-fold more of PI as compared to untreated control [153]. This result suggests that PI can play an important function within synovial joints.

1.3.4.1.6 Phosphatidylglycerol

Phosphatidylglycerols (PG) possess a glycerol as a head-group (Figure 1.10). Only a few molecular species can be determined in human samples. The highest concentration of PG in the body can be found in the lung where PG make up to 10% of all SAPLs being present in the lung. PG are thought to play an important role in the surfactant system of the lung, [154-155] where they interact with SP-B and maintain the alveolar surfactant layer [156].

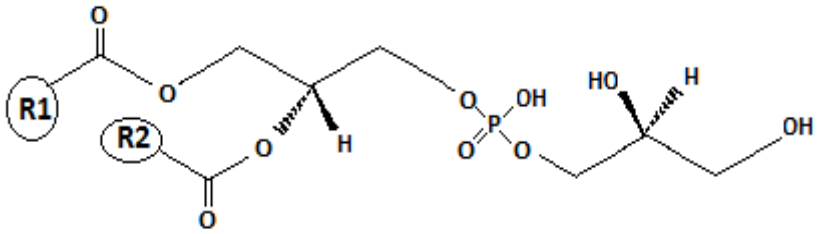


Figure 1.10. General chemical structure of phosphatidylglycerol. R1 and R2-fatty acids.

1.3.4.1.7 Sphingolipids

Sphingolipids are found in many living organisms and have been intensively investigated over the past years. Sphingolipids are a large group of lipids namely ceramide, glycosylceramide, lactosylceramide, sphingomyelin, dihydrosphingomyelin, sphinganine, sphinganine-1-phosphate, sphingosylphosphorylcholine, sphingosine and sphingosine-1-phosphate. The common structural feature of all sphingolipids is the sphingoid base composed of a hydrophobic moiety and a hydrophilic head-group [157].

Sphingomyelin (SPM)

Sphingomyelins (SPM) are amphiphiles consisting of a phosphocholine zwitterionic hydrophilic head-group and two saturated hydrocarbon chains which form the hydrophobic domain of this molecule (Figure 1.11). In the past, SPMs were considered to serve only as a component of the plasma membranes. Currently, SPM have been shown to play a more prominent role in cells, for instance, in cellular signal transduction, growth, proliferation, differentiation and survival of many cell types e.g. mouse fibroblasts, rat brain neurons, mouse epidermal keratinocytes, human neuronal and endothelial cells [158].

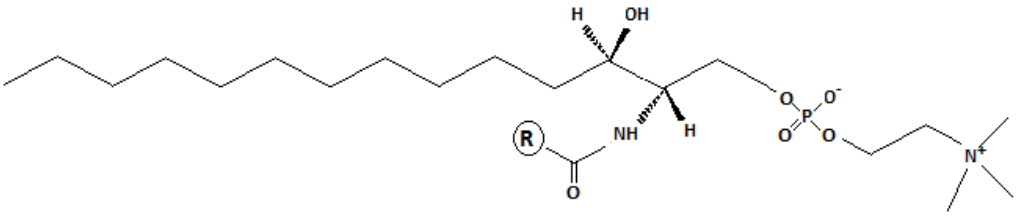


Figure 1.11. General chemical structure of sphingomyelin. R-fatty acid.

Gerritsen *et al.* [159] reported that SPM and sphingomyelinase pathway where ceramides (Cer) are produced, may play role in inhibition of cell cycle progression, thus inhibit proliferation and also induce apoptosis of cultured human FLS. They also presented that treatment with SPM or Cer significantly increased TNF-α production. These results indicate that SPM might play a role in development of RA, where apoptosis level is greater than in OA and pro-inflammatory cytokines are present in higher concentrations [159].

Ceramide

Ceramides (Cer) are composed of sphingosine and fatty acid (Figure 1.12). Cer are synthesized by cleavage of phosphorylcholine linkage in SPM, which is catalysed by neutral or acid sphingomyelinase.

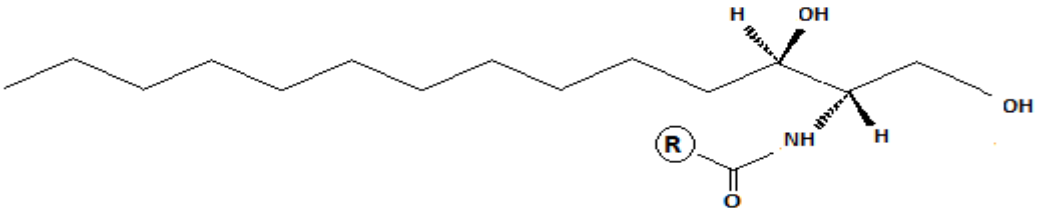


Figure 1.12. General chemical structure of ceramide. R-fatty acid.

Cer are found in high concentrations within cell membranes and regulate many different cellular processes including, cellular stress response, cell proliferation and differentiation, apoptosis and senescence. Interestingly, *in vitro* studies using cultured embryonic rat brain neurons show, that Cer at low concentrations promote survival of cells, whereas higher concentrations of Cer induce apoptosis. Some molecular species of Cer might act as pro-inflammatory mediators in several human disorder for instance, in cystic fibrosis, coronary artery disease, arteriosclerosis, obesity, diabetes and cardiovascular diseases [160]. However, glucosylceramides (GluCer) may inhibit inflammation. Dinitrofluorobenzene-induced mice with induced allergic contact dermatitis in the ears were treated orally with GluCer isolated from maize. Signs of inflammation in the ears were decreased by GluCer, which therefore were thought to possess anti-inflammatory and anti-allergic properties [161]. Another *in vitro* study using human FLS presented, that Cer has influence on proliferation, morphology and apoptosis of FLS. Cells were treated with C2-ceramide, a membrane-permeable Cer analogue (N-acidosphingosine) at concentrations ranging from 6.25 to 50 µM. The results suggest that Cer inhibit cell proliferation in a concentration-dependent manner. Moreover, Cer affect cell morphology resulting in a rounded body cell and induce apoptosis of FLS [162].

In summary, three major components of SF contribute either independently or additively to boundary lubrication: lubricin, HA and SAPLs. SAPLs cover the surface of articular cartilage thereby leading to a microscopically thick bio-film which prevents the cartilage from degradation. Little is known about PL classes and species composition in SF, thus their function in synovial joint has still to be elucidated. Certain PL classes may act as boundary lubricants, the others can posses different functions. For instance, PC has been shown to exhibit anti-inflammatory properties whereas, LPC acts as a pro-inflammatory mediator. PLPE are considered to have notable antioxidant properties. Cer act as pro-inflammatory mediators and, in addition, together with SPM regulate several cellular processes such as cell proliferation and differentiation, cellular response for stress and apoptosis. Taken together it can be concluded, that lipids are very important targets to be studied in order to better understand human biology in health and disease. Thus, “lipidomics” is gaining increasing scientific attention.

2. Aim of the study

Boundary lubrication by SF refers to its ability to reduce the friction between opposite surfaces of articular cartilage. Lubricin, HA and SAPLs contribute either independently or additively to boundary lubrication provided by the SF. Any alterations in the concentration or composition of SF constituents may lead to increased friction, hence, an increased cartilage damage can occur within articular joints. Previous studies already reported about decreased concentrations of HA and lubricin within OA SF. In line of these observations, we tested the hypothesis that OA SF also contains altered amounts of individual PL species, which might contribute to cartilage destruction during OA, and that these alterations are dependent on the stage of the disease. Perspectively, gaining insight into the PLs composition and their alterations during development of OA may identify novel therapeutic targets. Furthermore, getting access to new diagnostic tools may help to diagnose OA in the early stage of the disease and, thus, open a new window to treat OA even during its early onset.

The overall goal of our study was to determine both qualitatively and quantitatively the PL composition of human SF as altered by various joint diseases, and to further elucidate whether these alterations are also dependent on the stage of OA.

The specific aims of the present study were 1. to determine whether differences in the concentrations of all individual PL species present in human SF are dependent on the health status of the joints, 2. to obtain detailed informations about PLs composition of SF collected from different stages of human OA, 3. to analyse qualitatively and quantitatively all PL species of MPs present in human SF, 4. to evaluate whether a canine model of OA reflect the composition of PL species as found in human SF, 5. to determine species dependency with respect to the PL composition in SF obtained from humans, dogs and horses, and 6. to study mechanical impact on PL composition.

It has been hypothesised, that FLS might be the source of SAPLs found in SF since one electromicroscopical study identified LBs within FLS. In order to proof that FLS can indeed synthesise SAPLs, a method for quantitative assessment of PLs present in FLS cell culture media should be established.

Already four studies reported that surfactant proteins might be present in human and equine SF. In order to test the hypothesis, that these proteins are indeed present in human SF and are also locally synthesised, an additional aim of this study was to obtain the protein and mRNA expression pattern of SP-A and -D in different joint tissues and SF.

3. Material and Methods

3.1 Materials

3.1.1 Technical equipment

Autoclave, model 3850 EL	Tuttnauer Europe B.V., Breda, The Netherlands
Automatic pipetting system, Robot Genesis, RSP 150	Tecan, Maennedorf, Switzerland Pipetting
Balance, model 770-12	Kern & Sohn GmbH, Balingen-Frommern, Germany
Balance, model EG2200-2NM	Kern & Sohn GmbH, Balingen-Frommern, Germany
Biological Safety Cabinet, Microflow®	Thermo Scientific Inc., Rockford, IL, USA
Cell culture incubator, model 3039	Forma Scientific Inc., Marietta, OH, USA
Cell culture incubator, Cellstar®	Nunc GmbH, Wiesbaden, Germany
Centrifuge, model 5403	Eppendorf AG, Hamburg, Germany
Drying oven, model 700	Memmert GmbH&Co. KG, Schwabach, Germany
Electrophoresis chamber vertical, Mini PROTEAN® Tetracell	Bio-Rad Laboratories GmbH, Munich, Germany
Electrophoresis chamber horizontal, Wide Min-SUB® Cell GT	Bio-Rad Laboratories GmbH, Munich, Germany
ELISA-Reader, Multiscan Plus MK11	Titertek Instruments, Huntsville, AL, USA
ELISA-Reader, Sunrise™	Tecan, Crailsheim, Germany
Freezer -20°C, model KGE 34422	Bosch GmbH, Gerlingen-Schillerhoehe, Germany
Freezer -86°C, model HFU 486 Top	Thermo Electron GmbH, Langenselbold, Germany
Gel iX Imager INTAS®	Intas Science GmbH, Goettingen, Germany
Horizontal shaker, Polymax 1040	Heidolph Instruments GmbH&Co.KG, Schwabach, Germany
Light microscope, Axiovert® 40 CFL	Carl Zeiss, Goettingen, Germany
Liquid handling roboter system MicroStarlet®	HamiltonRobotics, Bonaduz, Switzerland

Magnetic stirrer, model MR 3002	Scientific Industries Inc., Bohemia, NY, USA
Mass Spectrometer, Ultima Triple™ Quadrupole	Micromass, Manchester, United Kingdom Quattro
Mass Spectrometer, Ultraflex I MALDI TOF/TOF	Bruker Daltonics, Bremen, Germany
Microcentrifuge, model 5415D	Eppendorf AG, Hamburg, Germany
Microplate shaker, model LD-45	Kisker Biotech GmbH&Co.KG, Steinfurt, Germany
Multichannel pipette 8-, 12-channels	Eppendorf AG, Hamburg, Germany
Multipette® plus	Eppendorf AG, Hamburg, Germany
PCR-Mastercycler®, Personal	Eppendorf AG, Hamburg, Germany
pH-meter digital, handylab 1	SI Analytics GmbH, Mainz, Germany
Pipetboy, Easypet®	Eppendorf AG, Hamburg, Germany
Pipette, single channel: 0.5-10 µl, 10-100 µl, 100-10000 µl	Eppendorf AG, Hamburg, Germany
Pipette, single channel	Eppendorf AG, Hamburg, Germany
Power Supply, PowerPac™ HC	Bio-Rad Laboratories GmbH, Munich, Germany
Pump, model 16612	Sartorius, Goettingen, Germany
Refrigerator +4 °C, model KGU66920	Bosch GmbH, Gerlingen-Schillerhoehe, Germany
Thermomixer, Comfort	Eppendorf AG, Hamburg, Germany
Semi-Dry Transfer cell, Trans-blot®SD	Bio-Rad Laboratories GmbH, Munich, Germany
Spot Cutter, ExQuest®	Bio-Rad Laboratories GmbH, Munich, Germany
Universal centrifuge, model 320R	Hettich GmbH&Co. KG, Tufflingen, Germany
UV-visible spectrophotometer, UV-1601	Shimadzu Corporation, Duisburg, Germany
Vacuum concentrator, Christ RVC	Wolf Laboratories Limited, York, United Kingdom
Vortex mixer, Vortex-Genie®2	Scientific Industries Inc., Bohemia, NY, USA
Water bath, AQUAline AL5	DJB Labcare Ltd, Buckinghamshire, UK

X-ray cassette, Hypercasette™ RPN11642	GE Healthcare Europe GmbH, Freiburg, Germany

3.1.2 Consumables

Cartridges, Bond Elut®-NH_2	Agilent Technologies Inc., Santa Clara, CA, USA
Cell culture flask T-75	Greiner bio-one GmbH, Frickenhausen, Germany
Cell strainer, nylon 70 µm, sterile	Becton Dickinson GmbH, Heidelberg, Germany
Combitips plus®: 5 ml, 10 ml	Eppendorf AG, Hamburg, Germany
Conical tubes: 15 ml, 50 ml	Becton Dickinson GmbH, Heidelberg, Germany
Filter Tip FT: 10 µl, 100 µl, 1000 µl	Nerbe plus GmbH, Winsen, Germany
Gel blotting paper, extra thick, 18.5 x 19 cm, Protein® II xi Size	Bio-Rad Laboratories GmbH, Munich, Germany
Neubauer chamber	LO-Laboroptik GmbH, Bad Homburg, Germany
Needles 18G, 23G	Becton Dickinson GmbH, Heidelberg, Germany
PCR tubes: 0.2 ml	Nerbe plus GmbH, Winsen, Germany
Petri dish 94 x 16 mm	Greiner bio-one GmbH, Frickenhausen, Germany
Pipette tip: 10 µl, 100 µl, 300 µl, 1000 µl	Sarstedt AG & Co., Nuembrecht, Germany
Plastic tubes: 5 ml	Sarstedt AG & Co., Nuembrecht, Germany
8-port manifold, autoclavable (#704526)	Fisher Scientific GmbH, Schwerte, Germany
PVDF Membrane, ImmunBlot™	Bio-Rad Laboratories GmbH, Munich, Germany
Radiographic film, Hyperfilm™ ECL 18x24 cm	GE Healthcare Europe GmbH, Freiburg, Germany
Reaction tubes: 1.5 ml, 2 ml	Sarstedt AG & Co., Nuembrecht, Germany
Serological pipette: 2 ml, 5 ml, 10 ml, 25 ml	Becton Dickinson GmbH, Heidelberg, Germany
Sterile scalpel Nr. 21	Feather Safety Razor Co. Ltd., Osaka, Japan
Sterile syringe: 2 ml, 5 ml, 10ml	B. Braun Melsungen AG, Melsungen, Germany
Syringe-driven filter units: 1.2 µm	Whatman Inc. Clifton, NJ, USA

Syringe-driven filter units: 1.2 µm, 5 µm	Sartorius AG, Goettingen, Germany
Syringe filter 0.22 µm, Millex®GP	Millipore GmbH, Schwalbach/Ts., Germany
Syringe for infusion: 50 ml	Dispomed Witt OHG, Gelnhausen, Germany
Tissue culture plates, rectangular 4-well (#176597)	Nalge Nunc Int., Roskilde, Denmark
Tissue culture plates 6-well, Nunclon™	Nalge Nunc Int., Roskilde, Denmark
96-well ELISA plates, MICROLON®	Greiner bio-one GmbH, Frickenhausen, Germany

3.1.3 Reagents

Acetic acid, puriss p.a.	Sigma-Aldrich GmbH, Taufkirchen, Germany (#A6283)
Acrylamide : bisacrylamide (37.5:1) solution, Rotiphorese® Gel 30	Carl Roth GmbH, Karlsruhe, Germany (#3029.2)
Agarose, analytical grade	Promega Corp., Madison, WI, USA (#3121)
Albumine, bovine serum	Invitrogen GmbH, Karlsruhe, Germany (#55213)
Ammonium acetate, ≥98%	Sigma-Aldrich GmbH, Taufkirchen, Germany (#A7330)
Ammonium peroxodisulfate, (APS) ≥ 98%	Sigma-Aldrich GmbH, Taufkirchen, Germany (#09913)
Aqua B. Braun (sterile water for irrigation)	B. Braun Melsungen AG, Melsungen, Germany (#75/12604052/0503)
β-Mercaptoethanol, >99%	Sigma-Aldrich GmbH, Taufkirchen, Germany (#63689)
BD Matrigel™	BD Biosciences, Bedford, MA, USA (#356230)
Bromophenol blue	Sigma-Aldrich GmbH, Taufkirchen, Germany (#B-8026)
Chloroform, ≥99.8%	Merck KGaA, Darmstadt, Germany (#1.02444.1000)
Complete™ Protease Inhibitor, tablets	Roche Diagnostic GmbH, Mannheim, Germany (#04693116001)
Coomassie Brillant Blue R-250 Destaining solution	Bio-Rad Laboratories GmbH, Munich, Germany (#161-0438)
Coomassie Brillant Blue R-250 Staining solution	Bio-Rad Laboratories GmbH, Munich, Germany (#161-0436)
DEPC water	Carl Roth GmbH, Karlsruhe, Germany (#T143)

Dexamethasone, ≥97%	Sigma-Aldrich GmbH, Taufkirchen, Germany (#D4902)
Diethylether, ≥99.5%	Sigma-Aldrich GmbH, Taufkirchen, Germany (#309966)
Dimethyl sulphoxide (DMSO), sterile	Sigma-Aldrich GmbH, Taufkirchen, Germany (#2650)
Dispase II	PAN Biotech GmbH, Aidenbach, Germany (#P10032100)
D-MEM medium	PAN Biotech GmbH, Aidenbach, Germany (#P04-01550)
D-MEM medium w/o Phenol Red	PAN Biotech GmbH, Aidenbach, Germany (#P04-01515)
DNA Ladder (100 bp)	Promega Corporation, Madison, WI, USA (#G210A)
Dulbecco's phosphate buffered saline (PBS) 1x	PAA Laboratories GmbH, Pasching, Austria (#H15-002)
Dulbecco's phosphate buffered saline (PBS) 10x	Bio-Rad Laboratories GmbH, Munich, Germany (#161-0780)
Ethanol absolut , ACS	J. T. Baker, Deventer, The Netherlands (#UN1170)
Ethylendinitrilo-N, N, N', N', -tetra-acetic-acid (EDTA), >99%	Sigma-Aldrich GmbH, Taufkirchen, Germany (#E5513)
Foetal calf serum (FCS)	Sigma-Aldrich GmbH, Taufkirchen, Germany (#F7524)
Formic acid 0.1% in water, LP-MS grade	Carl Roth GmbH, Karlsruhe, Germany (#CP03.1)
Gentamycin sulfate	Calbiochem, Merck KGaA, Darmstadt, Germany (#345814)
Glycerol, ≥99.5% p.a.	Carl Roth GmbH, Karlsruhe, Germany (#3783.1)
Glycine, ≥99% p.a.	Carl Roth GmbH, Karlsruhe, Germany (#3908.2)
Goat polyclonal anti-human SP-C	Santa Cruz Biotechnology Inc., Heidelberg, Germany (#SC-13979)
Hexane, ≥99% p.a.	Sigma-Aldrich GmbH, Taufkirchen, Germany (#34859)
HRP-conjugated rabbit polyclonal anti-goat IgG	Dako Deutschland GmbH, Hamburg, Germany (#P0160)
HRP-conjugated rabbit polyclonal anti-mouse IgG	Dako Deutschland GmbH, Hamburg, Germany (#P0160)
HRP-conjugated swine polyclonal anti-rabbit IgG	Dako Deutschland GmbH, Hamburg, Germany (#P0160)
Hyaluronidase from bovine testes	Sigma-Aldrich GmbH, Taufkirchen, Germany #(3506)

Hydrochloric acid, ≥37% purum p.a.	Sigma-Aldrich GmbH, Taufkirchen, Germany (#84422)
2-(4-2-hydroxyethyl)-piperazinyl-ethansulfonate (HEPES), 1M solution	PAA Laboratories GmbH, Pasching, Austria (#S11-001) 1-
ITS™+Premix	BD Biosciences, Bedford, MA, USA (#354352)
Methanol, min. 99.9%	Sigma-Aldrich GmbH, Taufkirchen, Germany (#34860)
MMLV Reverse Transcriptase	Promega Corp., Madison, WI, USA (#M3681)
Mouse monoclonal anti-human SP-A	Abcam plc, Cambridge, United Kingdom (#ab51891)
Mouse monoclonal anti-human SP A	Dako Deutschland GmbH, Hamburg, Germany (#PE-10)
Mouse monoclonal anti-human SP-D	Abcam plc, Cambridge, United Kingdom (#ab51891)
N,N,N',N'-tetramethyl-ethane-1,2-diamine (TEMED), 99% p.a.	Carl Roth GmbH, Karlsruhe, Germany (#2367.3)
Neomycin sulfate	Calbiochem, Merck KGaA, Darmstadt, Germany (#4801)
Non-fat Milk, blotting grade	Carl Roth GmbH, Karlsruhe, Germany (#T145.3)
Oligo$(dt)_{15}$ Primer	Promega Corp., Madison, WI, USA (#C1101)
PCR Nucleotide Mix	Promega Corp., Madison, WI, USA (#C1141)
Penicillin-streptomycin (100x), solution, penicillin 1000 U/ml, streptomycin 10 mg/ml	PAA Laboratories GmbH, Pasching, Austria (#P11-010)
PeqGOLD TriFast™	Peqlab Biotechnologie GmbH, Erlangen, Germany (#12-6834-00)
Phenol Red	Sigma-Aldrich GmbH, Taufkirchen, Germany (#P3532)
Phosphoric acid, 85% puriss p.a.	Sigma-Aldrich GmbH, Taufkirchen, Germany (#30417)
Precision Plus Protein™ Standards	Bio-Rad Laboratories GmbH, Munich, Germany (#161-0374)
2-Propanol, UV/IR grade	Carl Roth GmbH, Karlsruhe, Germany (#T910.1)
Quinacrine dihydrochloride, min. 90%	Sigma-Aldrich GmbH, Taufkirchen, Germany (#Q3251)
Rabbit polyclonal anti-human SP-A	Millipore GmbH, Schwalbach/TS, Germany (#ab3420)
Rabbit polyclonal anti-human SP-B	Millipore GmbH, Schwalbach/TS, Germany (#07-614)
RNase Away®, Surface Decontaminant	Molecular BioProduct Inc., San Diego, CA, USA (#7000)

RNase-free water	Promega Corp., Madison, WI, USA (#P119C)
RNase Inhibitor, RNasin®	Promega Corp., Madison, WI, USA (#N251B)
Sacharose, ≥99.5% p.a.	Carl Roth GmbH, Karlsruhe, Germany (#4621.1)
Sodium chloride, ≥99.5% p.a.	Carl Roth GmbH, Karlsruhe, Germany (#3957.1)
Sodium chloride, 0.9% solution	B. Braun Melsungen AG, Melsungen, Germany (#2350748)
Sodium dodecyl sulfate (SDS), ≥99%	Carl Roth GmbH, Karlsruhe, Germany (#2326.1)
Surfactant protein–A forward primer	biomers.net GmbH, Ulm, Germany (#00121699-1)
Surfactant protein-A reverse primer	biomers.net GmbH, Ulm, Germany (#00121699-2)
Surfactant protein-D forward primer	biomers.net GmbH, Ulm, Germany (#00099655-1)
Surfactant protein-D reverse primer	biomers.net GmbH, Ulm, Germany (#00099655-2)
Trifluoroacetic acid, 25%	Life Technologies GmbH, Darmstadt, Germany (#400003)
Tris, Pufferan®, ≥99%	Carl Roth GmbH, Karlsruhe, Germany (#5429.3)
Trisodium citrate, ≥99.5%	Sigma-Aldrich GmbH, Taufkirchen, Germany (#71402)
Trypan blue	Merck KGaA, Darmstadt, Germany (#1.11732.0025)
Trypsin/EDTA 0.5%/0.2% in PBS (10x)	PAA Laboratories GmbH, Pasching, Austria (#L11-003)
Tween®20	Carl Roth GmbH, Karlsruhe, Germany (#9127.1)
X-ray developer concentrate	Adefo-Chemie GmbH, Dietzenbach, Germany (#00036/04036)
X-ray fixer concentrate	Adefo-Chemie GmbH, Dietzenbach, Germany (#00036/07036)

3.1.4 Reagent kits

Albumin Removal Kit SwellGel®Blue	Thermo Scientific Inc., Rockford, IL, USA (#89845)
β2-Microglobulin ELISA kit	MP Biomedicals LLC, Orangeburg, NY, USA (#07BC-1061)
Bio-Rad Protein Assay	Bio-Rad Laboratories GmbH, Munich, Germany (#500-0201)
DNase I Kit	Fermentas GmbH, St-Leon-Rot, Germany (#EN0521)

ECL Plus Western Blotting Detection System	GE Healthcare Europe GmbH, Freiburg, Germany (#RPN2132)
GelRed Prestaining Kit	Biotium Inc., Hayward, CA, USA (#31010)
GoTaq®Flexi DNA Polymerase	Promega Corp., Madison, WI, USA (#M8301)
PCR Mycoplasma Test Kit II	PromoKine, PromoCell GmbH, Heidelberg, Germany (#PK-CA20-700-10)
PeqGOLD Total RNA Kit	Peqlab Biotechnologie GmbH, Erlangen, Germany (#12-6834-00)
Quanti Chrom™ Urea Assay Kit	BioAssay Systems, Hayward, CA, USA (#DIUR-500)
RNAqueous™	Ambion Inc., Austin, TX, USA (#1911)

3.1.5 Analysis Software

Compas 1.1 software package	Bruker Daltonics, Bremen, Germany
ELISA reader software, Magellan™	Tecan, Crailsheim, Germany
Graph Pad Prism 5.2	Graph Pad Inc., San Diego, USA
Excel Macros	Dr. G. Liebisch, Regensburg, Germany
Intas® GDS	Intas Science GmbH, Goettingen, Germany
LabImage 1D L340 4.1	Intas Science GmbH, Goettingen, Germany
Mascot	MatrixScience, London, United Kingdom
MassLynx software	Macromass, Manchester, United Kingdom
R version 2.14.0	www.r-project.org

3.1.6 Buffers and solutions

10% APS solution

1 g of APS was dissolved in 10 ml aqua B. Braun.

Blocking solution pH 7.5

Component	**Total volume 200 ml**	**Final concentration**
non-fat dry milk	10 g	5% (w/v)
1x Tris Buffer Saline/Tween®20	up to 200 ml	

The pH was adjusted to 7.5 with HCl

Culture medium-complete DME-M

Component	**Total volume 200 ml**	**Final concentration**
FCS (heat-inactivated)	20 ml	10% (v/v)
HEPES	2 ml	10 mM
Penicillin-streptomycin	2 ml	10 U/ml penicillin
		0.1 mg/ml streptomycin
D-MEM medium	176 ml	

Culture medium – Starvation medium

Component	**Total volume 200 ml**	**Final concentration**
FCS (heat-inactivated)	4 ml	2% (v/v)
HEPES	2 ml	10 mM
Penicillin-streptomycin	2 ml	10 U/ml penicillin
		0.1 mg/ml streptomycin
D-MEM medium	192 ml	

FCS heat-inactivated

500 ml of frozen FCS was kept overnight at 4ºC. Subsequently, completely thawed FCS was incubated in water bath at 56ºC. When fluid temperature was approximately 56ºC, FCS was heat-inactivated for following 30 min.

Protease- Phospholipase Inhibitor Coktail (PPI)

Component	**Total volume 5 ml**	**Final concentration**
Quinacrine dihydrochloride	24 mg	10 mM
Neomycin sulfate	23 mg	5 mM
Gentamycin sulfate	28 mg	10 mM
Aqua B. Braun	5 ml	
Complete™Protease Inhibitor	1 tablet	

Resolving gel solution (1.125 M Tris-HCl) pH 8.8

Component	**Total volume 200 ml**	**Final concentration**
Tris	27.26 g	1.125 M
Sacharose	60 g	30% (w/v)
Aqua B. Braun	up to 200 ml	

The pH was adjusted to 8.8 with HCl.

2x SDS-loading buffer pH 6.8

Component	Total volume 100 ml	Final concentration
Glycerol	50 ml	50% (v/v)
SDS	10 g	10% (w/v)
β-Mercaptoethanol	1 ml	10% (v/v)
Bromophenol blue	10 mg	0.01% (w/v)
125 mM Tris-HCl, pH 6.8	up to 100 ml	

The pH was adjusted to 6.8 with HCl.

10x SDS-running buffer pH 8.3

Component	Total volume 1 l	Final concentration
Tris	30.3 g	250 mM
Glycine	144.1 g	1.92 M
SDS	10 g	0.1% (w/v)
Aqua B. Braun	up to 1 l	

The pH was adjusted to 8.3 with HCl.

1x SDS-running buffer pH 8.3

10x SDS-running buffer was diluted 1:10 in aqua B. Braun.

The pH was adjusted to 8.3 with HCl.

10% SDS stock solution

10 g SDS were dissolved in 100 ml aqua B. Braun.

Stacking gel solution pH 6.8

Component	Total volume 200 ml	Final concentration
Tris	15.14 g	625 mM
Aqua B. Braun	up to 200 ml	

The pH was adjusted to 6.8 with HCl.

10x Tris Buffer Saline/Tween®20 (TBS/T) pH 7.5

Component	Total volume 2 l	Final concentration
Tris	121.14 g	500 mM
NaCl	58.44 g	500 mM
Tween®20	20 g	0.01% (w/v)
Aqua B. Braun	up to 2 l	

The pH was adjusted to 7.5 with HCl.

1x Tris Buffer Saline/Tween®20 (TBS/T) pH 7.5

10x TBS/T was diluted 1:10 in aqua B.Braun.

The pH was adjusted to 7.5 with HCl.

Transfer buffer pH 8.0

Component	Total volume 1 l	Final concentration
Tris	2.42 g	20 mM
Glycine	11.26 g	150 mM
Methanol	200 ml	20% (v/v)
Aqua B. Braun	up to 1 l	

The pH was adjusted to 8.0 with HCl.

3.1.7 Synovial fluid

3.1.7.1 Human synovial fluid

Synovial fluid (SF) was collected in order to investigate the levels of the boundary lubricants PL and surfactant proteins. SF was obtained from the knee joints of 16 cadavers, 23 RA patients and of 85 OA patients (Table 2.1). OA joints were classified into subgroups of early and late stages of OA according to Kellgren/Lawrence (K/L) grades of radiographic severity (range 0-4) and according to the macroscopical appearance of cartilage surfaces as determined by the Outerbridge score [16, 19]. Only patients fulfilling the inclusion criteria and those without the exclusion criteria were included in the study (Table 2.2 and 2.3). SF was collected by direct aspiration during arthroscopy or total knee replacement surgery. RA SF was obtained by Dr. med. U. Käßer (Rheumatologische Schwerpunktpraxis, Internistisches Praxiszentrum am Balserischen Stift, Giessen, Germany) and prepared as described in section 3.2.1 within 30 min from aspiration. Cadaveric SF was collected by the Institute of Forensic Medicine, Giessen, Germany. Samples were aspirated from knee joints within several hours until 5 days after death. Subsequently, SF was stored at 4ºC for not longer than 24 h and further prepared as described in section 3.2.1. All investigations were approved by the local ethics commission (AZ: 62/06 from 18.05.06 with amendment from 16.02.09).

Diagnosis	n	Gender ♂/♀	Age [years] min-max mean ± SD	BMI [kg/m^2] min-max mean ± SD
Human cadavers with healthy joints	15	14/1	17-34 23.8 ± 4.9	18.6-38.3 24.4 ± 4.8
RA	20	5/15	43-86 61.04 ± 14.03	20.7-43.4 29.4 ± 6.3
OA early stage	29	18/11	18-75 41.8 ± 17.2	16.9-37.3 25.9 ± 4.4
OA late stage	26	11/15	44-85 69.6 ± 9.9	19.2-37.4 28.7 ± 4.4

Table 2.1. Characterization of patients included into the study.

Human cadavers	OA early stage	OA late stage	RA
men, women	men, women	men, women	men, women
age < 35	age < 85	age < 85	age < 90
BMI < 40	BMI < 40	BMI < 40	BMI < 45
sudden death	all CRP- levels	all CRP- levels	all CRP- levels
-	Kellgren-Lawrence score ≤ 2	Kellgren-Lawrence score > 2	ACR criteria
healthy knee joints	OA	OA	RA

Table 2.2. Inclusion criteria.

Human cadavers	OA	RA
joint related	**joint related**	**joint related**
knee joint surgery	knee joint surgery within last 6 months (e.g. arthroscopy)	knee joint surgery within last 6 months (e.g. arthroscopy)
any knee joint damage due to e.g. OA, RA, gout, trauma	other surgeries within last 3 months	other surgeries within last 3 months
-	fracture of joints within last 24 months	fracture of joints within last 24 months
-	inflammatory arthritides in other joints (like rheumatoid arthritis, psoriasis arthritis, spondylitis ankylosans), tendinitis	-
-	collagenosis in other joints (like lupus erythematodes, skleroderma)	-
-	joint infection	joint infection
-	any joint diseases like gout, trauma within last 3 months	any joint diseases like gout, trauma within last 3 months
Other diseases	**Other diseases**	**Other diseases**
osteoporosis	severe liver disease (GPT or GOT > 100 U/L)	severe liver disease (GPT or GOT > 100 U/L)
osteopenia	severe kidney disease (creatinine > 1,5 mg/dl)	severe kidney disease (creatinine > 1,5 mg/dl)
tumor/cancer (near joint)	Diabetes mellitus	Diabetes mellitus
chronic disease resulting in death	tumor/cancer (near joint)	tumor/cancer (near joint)
HIV-infection	thyroid gland diseases (hypothyroidism, hyperthyroidism, euthyroidism)	thyroid gland diseases (hypothyroidism, hyperthyroidism, euthyroidism)
-	HIV-infection	HIV-infection
Drugs	**Drugs**	**Drugs**
intraarticular drugs	drug abuser	drug abuser
-	Hyaluronat (within last 6 months)	Hyaluronat (within last 6 months)
-	immunosuppressive drugs (within last 6 months)	-
-	corticosteroids (within last 6 months)	corticosteroids (within last 6 months)

Table 2.3. Exclusion criteria.

3.1.7.2 Canine synovial fluid

Healthy SF obtained from human cadavers was aspirated from knee joints within several hours until even 5 days after death. During this time degradation processes might occur. Thus, we also obtained normal healthy SF aspirated directly after death, however this time from dogs using SF from the canine groove model of OA. Additionally, canine SF obtained from distraction experiments were analysed in order to test whether mechanical loading has an influence on PL composition in SF. The canine groove model and distraction experiment was accomplished by Prof. Dr. F. Lafeber and S. Mastbergen, PhD (Department of Rheumatology & Clinical Immunology, University Medical Center Utrecht, The Netherlands). Mixed breed canines (n=21 females, mean age 1.8 ± 0.5 years, weighing 17.5 ± 1.3 kg) were obtained from the animal laboratory of Utrecht University, The Netherlands. The study was approved by the Utrecht University Medical Ethical Committee for animal studies.

OA was induced in the right knee joint of all 20 dogs according to the method described elsewhere [72-74]. Under general anaesthesia, surgery was performed through a 2-2.5 cm medial incision close to the *ligamentum patellae*. In utmost flexion, approximately ten longitudinal and diagonal grooves were made on the parts of the femoral condyles using a 1.5 mm diameter Kirschner-wire.

Joint distraction was performed using the right knee joint of 9 dogs. Ten weeks after OA induction, the external fixation frame was placed with three bone pins drilled into the femur and the tibia. Distraction of the joints was carried out using the screw threaded connecting rods. In order to control for the influence of the frame alone on the dogs loading behaviour of joints, additional 5 dogs received only a frame without distraction of the knee joint. After five days of recovery, all dogs were allowed to use the patio. After 8 weeks, distraction of joints was terminated. The severity of OA was evaluated 45 weeks after induction of OA in all animals.

At the end of the experiment all dogs were sacrificed with an intravenous injection of pentobarbital Euthesate®. Both hind limbs were amputated and processed within 2 h. SF was obtained by direct aspiration from both knee joints and prepared as described in section 3.2.1. Subsequently the samples were frozen at -80°C, shipped on dry ice to our laboratory where they were stored frozen at -86°C until further analysis.

3.1.7.3 Equine synovial fluid

In order to obtain healthy SF and to compare PL composition in different species, SF was aspirated from 14 horses by direct aspiration from knee joint after euthanasia (mean age ± SD: 12.7 ± 4.7; mean weight ± SD: 518.6 ± 81.7 kg, 6 female and 8 male). Only SF from horses with healthy knee joints and no arthritic disease history were used. Equine SF sampling was performed by Dr. med. vet. Martina Sperling (Veterinary Clinic for Horses, Justus-Liebig-University of Giessen, Germany). SF was stored at 4ºC and prepared as described in section 3.2.1 within a time period not longer than 12 h after aspiration.

3.1.8 Human cells and tissues

3.1.8.1 Human fibroblast-like synoviocytes

Human fibroblast-like synoviocytes (FLS) were isolated and cultured in order to investigate 1. whether synthesis and/or secretion of PL can be measured also *in vitro* and 2. whether FLS express the *sp-a* and *sp-d* genes. FLS were isolated from synovial membranes obtained from 9 OA patients (3 male, 6 female; age 57-77 years, mean age ± SD: 68.1 ± 7.3) undergoing total knee replacement surgery, as described in section 3.2.7.1. All investigations were approved by the local ethics commission (AZ: 106/03 from 08.01.04 with amendment from 21.07.09) and written informed consent was obtained from all patients.

3.1.8.2 Human tissues

Human synovial membranes and cartilage specimens were obtained from 3 OA patients (4 female, 2 male; age 61-76, mean age ± SD: 69 ± 7.5) undergoing total knee replacement surgery in order to test the hypothesis that *sp-a* and *sp-d* genes are expressed in those tissues. All investigations were approved by the local ethics commission (AZ: 106/03 from 08.01.04 with amendment from 21.07.09) and written informed consent was obtained from all patients.

3.2 Methods

3.2.1 Sampling of synovial fluid

Aspirated SF samples were macroscopically analyzed according to colour, turbidity and contamination. Samples contaminated with blood and characterised by high turbidity were discarded. Samples with a volume lower than 2 ml were diluted by the addition of 2 ml sterile 0.9 % NaCl-solution. Samples were incubated for 30 min at 37°C, followed by filtration through 1.2 µm filter. Light microscopical analysis were performed using a Neubauer chamber to verify that 10 µl SF samples were free from cells and cellular debris. SF samples containing cells were filtrated again as described above. Subsequently, 10% (v/v) of PPI was added, samples were aliquoted, frozen at -86°C and stored for a maximum duration of 2 years for further analysis.

3.2.2 Isolation of microparticles

Microparticles (MP) were isolated in order to determine PL composition of MPs present in SF which was further compared with PL from MP-free SF supernatant. MPs from SF and cell culture media were isolated according to Berckmans *et al.* with slight modifications [67]. Briefly, 250 µl aliquots of SF and cell culture media were centrifuged for 45 min at 16.100 x g and 20°C to pellet the MPs. Supernatant (225 µl) was discarded or stored frozen at -86°C for further analysis. 25 µl of pellet was resuspended in 225 µl PBS (pH 7.4) containing 10.9 mM trisodium citrate and centrifuged again as described above. Supernatant (225 µl) was again removed and MPs were resuspended in 25 µl of PBS/citrate buffer resulting in a final volume of 50 µl. Isolated MPs were stored frozen at -86°C until analyzed.

3.2.3 Extraction of lipids

Lipids were extracted from SF and cell culture media in order to relatively and absolutely quantify PL using electrospray ionization tandem mass spectrometry (ESI-MS/MS). Extraction of PL was performed according to the method of Bligh and Dyer [163] in the presence of non-naturally occurring lipid species which served as internal standards. The following lipid species were added as internal standards: PC 14:0/14:0, PC 22:0/22:0, PE 14:0/14:0, PE 20:0/20:0, PS 14:0/14:0, PS 20:0/20:0, PG 14:0/14:0, PG

20:0/20:0, PI 16:0/16:0, PI 17:0/17:0, LPC 13:0, LPC 16:0, Cer 14:0, Cer 17:0, D_7-FC, CE 17:0 and CE 22:0 [164-166]. 50 µl of cell- and cell debris-free SF, cell-, cell debris- and MPs-free SF or isolated MPs were diluted with 750 µl of water, whereas 800 µl of cell culture media being cell-, cell debris- and MPs-free were used as starting material. PLs were isolated by adding 3 ml of methanol/chloroform (2:1, v/v) followed by 1 h incubation at RT. Separation of phases was performed by adding 1 ml of chloroform and 1 ml of water. Subsequently, samples were centrifuged at 2.500 x g for 10 min at RT. The chloroform phases containing PLs were collected using an automatic pipetting system and were dried in vacuum centrifuge for 1 h. Dried samples were then dissolved in 1 ml methanol/chloroform (3:1, v/v) containing 10 mM ammonium acetate.

3.2.4 Solid phase extraction

Solid phase extraction (SPE) was performed to separate PL fractions from chloroform phase containing the lipids and to eliminate contaminations. 4 ml of cell-, cell debris- and MPs-free cell culture media were taken as starting material. Lipid extraction was performed as described in the previous section. The SPE method was adapted from Kim and Salem [167]. Briefly, extracted lipids were dissolved in 400 µl of chloroform. Subsequently, the total volume of chloroform was loaded onto equilibrated Bond Elut®-NH_2-columns. Thereafter, four elution steps were applied under vacuum using 4 ml of the following elution solutions:

1. Chloroform : 2-propanol (2:1) mixture – elutes neutral lipids
2. Diethylether containing 2% acetic acid - elutes free fatty acids
3. Methanol - elutes neutral PL
4. Hexane : 2-propanol : ethanol : 0.1 M ammonium acetate in water : formic acid (420:350:100:50:0.5) containing 5% phosphoric acid – elutes acidic PL

Additionally, the acidic PL fraction was dried under nitrogen for 10 min to evaporate hexane, dissolved in 1 ml of water and was subsequently extracted three times using 1 ml of chloroform.

3.2.5 Phospholipid mass spectrometry

PL species were quantified by ESI-MS/MS using the mass spectrometer Quattro Ultima™ Triple Quadropole and an analytical setup and strategy described by Liebisch *et al.* [164, 166, 168]. A precursor ion scan of m/z 184 specific for phosphocholine-containing lipids was used for phosphatidylcholine (PC), sphingomyelin (SPM) [166] and lysophosphatidylcholine (LPC) [164]. Neutral loss fragments were used for phosphatidylethanolamine 141 (PE) and phosphatidylserine (PS) 185 [169]. PE-based plasmalogen was analyzed according to method described elsewhere [170]. Ammonium-adduct ions of phosphatidylglycerol (PG) and phosphatidylinositol (PI) were determined by neutral loss scans of *m/z* 189 and 277, respectively [171]. Ceramides (Cer) were measured as a product ion of *m/z* 264 [165]. Quantification was performed by calibration lines generated by addition of non-naturally occurring lipid species served as internal standards. Internal standards from the same lipid class were used to quantity all lipid classes. However, for SPM determination the PC internal standard was used, whereas the PE internal standards were taken as PE-based plasmalogens internal standards. Free cholesterol (FC) and cholesteryl ester (CE) were quantified using a fragment ion of *m/z* 369. Correction of isotopic overlap of lipid species was performed by Excel Macros for all lipid classes according to the method described elsewhere [166]. PL quantification by ESI-MS/MS was performed in Laboratory of Prof. Dr. med. G. Schmitz by Dr. rer. nat. G. Liebisch (Institute for Clinical Chemistry and Laboratory Medicine, University Hospital of Regensburg, Germany).

3.2.6 ESI-MS/MS data correction

The quantitative values of all PL species were corrected for dilution of SF due to possible effusion induced by inflammation. For this purpose the concentrations of β-2-microglobulin and urea were determined in SF samples in order to calculate a dilution factor for SF.

3.2.6.1 Enzyme-linked immunosorbent assay for β-2-microglobulin

β2-microglobulin (β2-MG) levels in SF samples were measured using a commercially available enzyme-linked immunosorbent assay (ELISA) kit according to the instructions provided by the manufacturer. Briefly, 5 µl of standards, controls and samples

were pipetted into the wells of a 96-well microtiter plate coated with anti-β2-MG antibody, diluted with sample diluents and incubated for 30 min at 37°C, which was followed by five washing steps. After 30 min of incubation with enzyme conjugate reagent and repetitive washing, substrate solution was added to each well. After incubation for 20 min in the dark, the reaction was stopped by adding 100 µl of 1 N HCl. The optical density was measured at 450 nm using a microplate ELISA reader. The unknown samples values were calculated from the standard curve. The detection limit for β2-MG was 0.1 µg/ml.

3.2.6.2 Urea determination

Urea concentrations in SF and serum samples were measured using commercially available QuantiChrom™ Urea Assay Kit. Determination was performed according to the manufacturer's instructions. In brief, 5 µl of standards and samples were pipetted into the wells of a 96-well microtiter plate and 200 µl of chromogenic substrate was added. The assay detects urea directly by using o-phthalaldehyde that specifically binds urea and forms a coloured complex with primaquine diphosphate. After 20 min incubation at RT the optical density was measured at 520 nm using a microplate ELISA reader. The unknown urea concentrations of samples were calculated from the standard curve. The detection limit for urea was 0.08 mg/dl (13 µM).

3.2.7 Culture of human fibroblast-like synoviocytes

In order to investigate whether FLS synthesize and/or secrete PL, cells were isolated from synovial membrane and cultured under various cell culture conditions.

3.2.7.1 Isolation of fibroblast-like synoviocytes

Isolation of FLS was performed according to the method previously described [172]. Briefly, synovial membrane was rinsed with sterile phosphate-buffered saline (PBS) and, using sterile technique, tissue containing blood vessels and fat were removed. Excised synovium was chopped with a surgical scalpel in < 1 mm^3 pieces. The minced pieces were digested for 1 h at RT in PBS containing 1% dispase II. The cell suspension was filtered through a 70 µm-mesh cell strainer to obtain single cell suspension. Subsequently cells were collected by centrifugation at *300 x g* for 10 min and plated into cell culture T-75 flasks. FLS were cultured with 15 ml Dulbecco's modified Eagle's medium (DMEM)

supplemented with 10% FCS (heat inactivated), 1% penicillin/streptomycin and 1% 2-(-4-2-hydroxyethyl)-piperazinyl-1-ethansulfonate (HEPES) in a humidified atmosphere of 5% CO_2 at 37°C. To ensure the purity of isolated FLS, cells were morphologically characterised by light microscopy and verified by positive staining for fibroblast-specific antibody SM1214PS using fluorescence-activated cell sorting (FACS). Cells were harvested using EDTA/Trypsin and passaged into new T-75 flasks when attaining 80-90% confluency. All experiments were carried out with FLS from passage 3-6 to ensure stable phenotype and genotype of cultured FLS [172].

3.2.7.2 Effect of various cell culture conditions

In order to optimize cell culture conditions for synthesis and/or release of PL by FLS several different cell culture conditions were applied. Before each experiment cell culture media were tested for mycoplasma contamination using the PCR Mycoplasma Test Kit. Furthermore, cells were trypsinized, counted in a Neubauer chamber and the percentage of viable cells was calculated using the trypan blue method. Subsequently, cells were plated onto 6-well tissue culture dishes at a density of 80.000 cells/well. After cells reached 90% confluency, medium was exchanged and cells were starved in serum-free medium for 16 h. Subsequently medium was exchanged and cells were cultured for 24 h. In order to optimize synthesis and/or release of PL by FLS, the following cell culture conditions were investigated:

1. Serum-free D-MEM medium supplemented with 1% penicillin/streptomycin and 1% HEPES.
2. Serum-free D-MEM medium supplemented with 1% ITS™+Premix, 1% penicillin/streptomycin and 1% HEPES.
3. Serum-free D-MEM medium supplemented with 1% penicillin/streptomycin, 1% HEPES and dexamethasone of different concentrations (10^{-9} mol/ml – 10^{-3} mol/ml).
4. Serum-free D-MEM medium supplemented with 1% penicillin/streptomycin, 1% HEPES. FLS were cultured in 6-well plates coated with 50µl/cm^2 BD Matrigel™ Basement Membrane Matrix Growth Factor Reduced.
5. Serum-free D-MEM medium supplemented with 1% penicillin/streptomycin, 1% HEPES. FLS were cultured in 4-well plates under shear stress conditions on horizontal shaker (5 rpm).

3.2.7.3 Preparation of cell culture media for MS analysis

After a culture period of 24 h, cell culture media from each well were collected and filtrated through a 0.22 µm syringe filters to obtain cell-free samples. After addition of 10% (v/v) of PPI, samples were centrifuged for 45 min at 16.100 x g at RT in order to pellet MPs. Cell-, cell debris- and MPs-free supernatant were aliquoted, and stored frozen at -86°C until further analysis as described in sections 3.2.3 and 3.2.4.

3.2.8 SF preparation for gel electrophoresis

3.2.8.1 Hyaluronidase treatment

SF contains high concentrations of HA which can disturb analysis of surfactant proteins. In order to digest HA and to reduce the viscosity of SF samples, samples were incubated with 320 U/ml of hyaluronidase in a thermomixer for 1 h at 37ºC [109].

3.2.8.2 Albumin depletion

SF contains high concentrations of albumins which can disturb surfactant protein detection. In order to remove albumins from SF samples, SwellGel® Blue Albumin Removal Kit was used according to the instructions provided by the manufacturer. Briefly, 100 µl of SF was loaded onto hydrated SwellGel® Blue Disc. After 2 min incubation at RT, discs were centrifuged at 12.000 x g and then samples were re-applied onto a SwellGel® Blue Disc to repeat described procedure. In order to release unbound proteins other than albumins, 50 µl of washing buffer containing 25 mM Tris and 75 mM NaCl, pH 7.5, was loaded onto the SwellGel® Blue Disc followed by 1 min centrifugation step at 12.000 x g. Samples free from albumins were collected for further analysis.

3.2.8.3 Protein quantification

The concentrations of proteins in SF was first determined in order to calculate the volume of sample and thus the quantity of protein needed to perform a sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) electrophoresis for separating of proteins according to their molecular weight. The concentrations of proteins in each sample are needed in order to load the same amount of proteins per lane as described in section 3.2.9. The concentrations of proteins in SF were spectrophotometrically determined using the Bradford dye reagent [173]. The protein assay is based on the colour change of Coomassie

Brilliant Blue G-250 dye after binding to proteins. The dye binds non-covalently primarily to basic and aromatic amino acids residues. 10 µl of sample was mixed with 200 µl of dye reagent and transferred to a 96-well plate. Bovine serum albumin (BSA) was used as the protein standard in a concentration range of 0.1-1 µg/µl. Standards were mixed with the dye reagent in the same ratio as the samples of unknown concentrations. The reaction mixtures were incubated for 15 min at RT. The absorbance of the samples was measured at 570 nm. The unknown amount of samples protein was calculated from the standard curve.

3.2.9 Separation of proteins with gel electrophoresis

In order to separate proteins according to their size SDS-PAGE electrophoresis was performed. SF, HA-free SF and albumin-free SF samples were reduced by adding 10% β-mercaptoethanol and denatured by heating at 95°C for 10 min in 2x Sodium dodecyl sulfate (SDS)-loading buffer. 50 µg of proteins was loaded onto the gel as described elsewhere [174]. Precision Plus Protein™ Standards was used as a molecular weight marker with range of 10-250 kDa. Separation of proteins was performed in the gel consisting of 5% stacking gel and 10% or 15% resolving gel (Table 2.4). Electrophoresis was carried out in 1x SDS-running buffer with constant 100 V until the bromophenol blue reached the bottom of the resolving gel.

Component	**Stacking gel 5%**		**Resolving gel 10%**		**Resolving gel 15%**	
Aqua B. Braun	6.46 ml	-	3.21 ml	-	6,46 ml	-
acrylamide:bisacrylamide Rotiphorese® Gel 30	1.33 ml	5%	3.3 ml	10%	1.33 ml	15%
10% SDS	100 µl	0.1 %	100 µl	0.1 %	100 µl	0.1 %
Resolving gel solution, pH 8.8	-	-	3.33 ml	-	3.33 ml	-
Stacking gel solution, pH 6.8	2 ml	-	-	-	-	-
10% APS	100 µl	0.1 %	50 µl	0.05%	50 µl	0.05%
TEMED	10 µl	0.01 %	10 µl	0.01 %	10 µl	0.01 %

Table 2.4. 10 ml SDS-gel mixtures [175].

3.2.10 Western blot

Using specific antibodies Western blot were obtained in order to proof whether SP-A and SP-D proteins are present in SF.

3.2.10.1 Protein blotting

Proteins separated on SDS-PAGE were transferred to polyvinylidene fluoride (PVDF) membrane using the semi-dry transfer technique according to the instructions provided by manufacturer. The PVDF membrane was activated by dipping in methanol before used. Electro blotting was performed using a semi-dry system and a transfer buffer pH 8.0 at a constant current of 2 mA/cm^2 for 90 min.

3.2.10.2 Protein detection

The membranes were blocked in blocking solution pH 7.5 at RT for 1 h followed by incubation with the primary antibody at 4°C overnight. Primary antibody concentrations varied depending on the antibody used in the experiment (Table 2.5). Membranes were washed three times for 10 min using 1 x TBS/T pH 7.5. Membranes were incubated with the respective horseradish peroxidase-labeled secondary antibody for 2 h at RT (Table 2.6), and then washed again four times for 15 min with 1 x TBS/T. Proteins were detected using Enhanced Chemiluminescence (ECL) Plus Kit® followed by exposure of the membrane to an ECL film lying in the X-ray cassette. Bands were then visualized by dipping the ECL film into the X-ray developer and fixation solution.

Target	Host	Dilution	Description	Company
Surfactant protein A (SP-A)	Mouse	1:500	monoclonal	Dako
Surfactant protein A (SP-A)	Rabbit	1:20000	polyclonal	Chemicon
Surfactant protein A (SP-A)	Mouse	1:2000	monoclonal	Abcam
Surfactant protein B (SP-B)	Rabbit	1:500	polyclonal	Millipore
Surfactant protein C (SP-C)	Goat	1:2500	polyclonal	Nycomed
Surfactant protein D (SP-D)	Mouse	1:1000	monoclonal	Abcam

Table 2.5. Primary antibodies used in the study.

Antibody	Host	Dilution	Company
HRP-conjugated anti-mouse IgG	Rabbit	1:2000	Dako
HRP-conjugated anti-rabbit IgG	Swine	1:2000	Dako
HRP-conjugated anti-goat IgG	Rabbit	1:2000	Dako

Table 2.6. Secondary antibodies used in the study.

3.2.11 Two-dimensional gel electrophoresis

To investigate whether protein bands obtained by Western blots are indeed the proteins of interest, 2-D gel electrophoresis was performed to separate proteins according to size and isoelectric point (PI). 400 μg protein from SF were applied on immobilized pH gradient (IPG) strips and isoelectric focusing was performed with 32.05 kVh followed by equilibration. For the second dimension IPG strips were placed on top of 12.5% SDS-PAGE gel and electrophoresis was carried out with the following program: 15 min at 15 mA/gel followed by 5 h at 110 mA at 25°C. Gels were stained with Coomassie brilliant blue R-250. 2-D gel electrophoresis was done in the laboratory of Prof. Dr. rer. nat. G. Lochnit (Protein Analytics, Department of Biochemistry, Justus-Liebig-University of Giessen, Germany).

3.2.12 Protein identification (MALDI-TOF)

The two 2-D gel electrophoreses with the same sample were run in parallel. Subsequently, one gel was blotted and Western blot was performed in order to visualise spots detected by specific antibodies. Simultaneously, the second gel was stained with Coomassie blue. The gels were compared and spots detected by the antibodies were localised on the Coomassie blue stained gel and excited with the ExQuest® spot cutter. Further, proteins within the spots were digested with trypsin on a liquid handling automatic system. In order to identify proteins present in excised spots, Matrix-Assisted Laser Desorption Ionization Time-Of-Flight Mass Spectrometry (MALDI-TOF-MS) of the in-gel tryptic digests was applied. MALDI-TOF-MS was performed on an Ultraflex™ I TOF/TOF mass spectrometer with a nitrogen laser. The instrument was operated in the positive-ion (reflectron) mode using 2.5-dihydroxybenzoic acid and methylendiphosphonic acid as a matrix (5 mg/ml each in 0.1% trifluoroacetic acid). Sum spectra consisting of

200-400 single spectra were acquired. For data processing and instrument control the Compass 1.1 software package was used. Proteins were identified by peptide mass fingerprint search using the MASCOT search machine and human IPI database (International Protein Index, version 3.61). All 2-D gels, in-gel digestions and mass spectrometric analyses were performed by Prof. Dr. rer. nat. G. Lochnit (Protein Analytics, Department of Biochemistry, Justus Lieblig University of Giessen, Germany).

3.2.13 RNA isolation

Total RNA was isolated from synovial membranes, cartilage, FLS and cells present in SF to perform reverse transcription and PCR reactions in order to investigate whether *sp-a* and *sp-d* genes are expressed at least on the mRNA level.

3.2.13.1 RNA isolation from synovial tissue and cells

Total RNA was isolated from both synovial membrane and cells according to the manufacturer's instruction provided with peqGOLD TriFast™. For tissue extraction, 100 mg of synovial membrane was used. FLS were extracted by addition of 1 ml of TriFast™ reagent per 10 cm^2 area of culture dish where cells reached 90% confluency. The number of cells used to isolate RNA was approximately 800.000/T75 flask. After 5 min of incubation on ice, samples were collected into 1.5 ml tubes. Subsequently, 0.2 ml chloroform was added to the samples, mixed, kept for 10 min at RT and were then centrifuged with 12.000 x g for 30 min at 4°C. The aqueous phase was transferred to a fresh tube, mixed with 0.5 ml of 2-propanol, incubated for 15 min on ice and again centrifuged with 12.000 x g for 10 min at 4°C. The supernatant was removed, the precipitated pellet containing RNA was washed with 500 µl of 75% ice-cold ethanol and centrifuged again with 7.500 x g for 8 min at 4°C. Washing and centrifugation steps were repeated twice. Obtained RNA pellet was resuspended in RNase-free water. DNA contaminations were removed using DNase I Kit. Concentration and purity of obtained RNA was determined spectrophotometrically at 260 nm and 280 nm wavelengths and the 260/280 ratio was calculated. Samples containing pure RNA with a 260/280 ratio between 1.8 and 2.0 were used for further analysis.

3.2.13.2 RNA isolation from cartilage

Several different RNA isolation methods were used to obtain the highest amount and quality of cartilage RNA. Isolation of total RNA from 100 mg pulverized cartilage was performed according to the manufacturer's instructions provided with peqGOLD TriFast™, peqGOLD Total RNA Kit or RNAqueous™ Ambion Midi Kit. RNA was resuspended or eluted with RNase-free water, respectively. DNA contaminations were removed using DNase I Kit. Concentration and purity of obtained RNA was determined spectrophotometrically at 260 nm and 280 nm wavelength and the 260/280 ratio was calculated. Samples containing pure RNA with a 260/280 ratio between 1.8 and 2.0 were used for further analysis.

3.2.14 Reverse transcription reaction

In order to perform a reverse transcription polymerase chain reaction (RT-PCR) 1 µg isolated RNA was used and RNase-free water was added resulting in a final volume of 16.12 µl [176]. The RNA was heated in a thermomixer at 70°C for 5 min, immediately transferred onto ice, and then pre-mixed RT-PCR components (Table 2.7) were added. The total reaction volume was 25 µl.

To synthesize complementary DNA (cDNA), the reaction mixture was incubated in a PCR thermocycler at 40°C for 10 min, then at 43°C for 50 min and further heated to 94°C for 5 min to inactivate the enzyme. The obtained 25 µl of cDNA was then either used for PCR or stored frozen at -20°C until used.

RT-PCR components	Volume	Final concentration
5 x Reaction buffer	5 µl	1x
10 mM dNTP mix	1.25 µl	0.5 mM
Oligo$(dt)_{15}$ Primer (500 µg/ml)	1 µl	0.02 µg/µl
RNase inhibitor (20 U/µl)	0.63 µl	0.504 U/µl
M-MLV reverse transcriptase (50 U/µl)	1 µl	2 U/µl
RNase-free water	up to 25 µl	

Table 2.7. The RT-PCR mixture.

3.2.15 Semi-quantitative polymerase chain reaction

A semi-quantitative polymerase chain reaction (PCR) was performed according to the manufacturer's instructions provided with the GoTaq® Flexi DNA Polymerase Kit. The PCR reaction was prepared as described in table 2.8.

PCR components	Volume	Final concentration
5 x Reaction buffer (free $MgCl_2$)	10 µl	1x
25 mM $MgCl_2$	3 µl	1.5 mM
10 mM dNTP mix	1 µl	0.2 mM
100 µM forward primer	2 µl	4 µM
100 µM reverse primer	2 µl	4 µM
Taq DNA Polymerase (5 U/µl)	0.25 µl	0.025 U/µl
cDNA template	2 µl	
RNase-free water	up to 50 µl	

Table 2.8. The PCR mixture.

The PCR components were mixed on ice and transferred to a PCR-thermocycler. Using the following program the cDNA was amplified:

PCR step	Time	Temperature
First denaturation	2 min	95°C
Annealing	0.5 min	55-58°C*
Elongation	0.5 min	72°C
Second denaturation	1 min	95°C
Final extension	5 min	72°C

Table 2.9. The PCR cycle program.

* Annealing temperature varied depending on the primers used and was provided by the manufacturer (Table 2.10).

The PCR steps were repeated 30-33 cycles depending on the amplified sequence to obtain appropriate amount of product. The number of cycles was suggested by the manufacturer who provided the primers. Amplicons were stored either at -20°C or immediately separated according to amplicon size by the agarose gel electrophoresis and visualized using GelRed™ Prestaining Kit as described in section 3.2.16.

GeneBank™ Accesion (NCBI)	Forward Primer (5'-3')	Reverse Primer (5'-3')	Annealing temperature [°C]	Amplicon size (bp)
NM_005411 SP-A	ATCTAGATGAGGA GCTCCAAG	CCTCAGTCAGGCCT ACATAGG	56	270
NM_003019 SP-D	CCACAGAACAATG CCCAGTG	TTGCCCTGAGGTCC TATGTTC	55	321
NM_002046 GAPDH	CCTCAAGATCATCA GCAATGCCTCCT	GGTCATGAGTCCTT CCACGATACCAA	58	355

Table 2.10. Primers used to determine the expression of *sp-a* and *sp-d* genes [175].

3.2.16 DNA agarose gel electrophoresis

Agarose gel electrophoresis was performed in order to separate and visualise DNA amplicon obtained by PCR according to size and to visualize possible primer dimers. The experiments were performed in order to detect whether the bp size of bands for *sp-a* and *sp-d* genes are appropriate and thus, whether those genes are expressed in analyzed cells and tissues. 1% agarose gels were used. Agarose was mixed with 1x GelRed™ running buffer. The DNA samples were mixed with 6x GelRed™ loading buffer containing DNA intercalating fluorescent dye GelRed™, that enables visualization of the DNA fragments under ultraviolet light. Electrophoresis was performed in horizontal electrophoresis chamber at 75 V in 1x GelRed™ running buffer for 1 h. The size of amplified DNA was determined by a 100 bp DNA molecular size marker (range 100-1000 bp).

3.2.17 Statistical analysis of data

The non-parametric tests were chosen, since a non-Gaussian distribution of all data presented here was assumed. Statistical significant differences of PL concentrations were determined using the Kruskal-Wallis test. In order to determine statistical significance without possible type I error, adjustment for multiple testing in the series of Kruskal-Wallis tests was done by applaying the false discovery rate (FDR) being 10%. Kruskal-Wallis tests subsequently combined with the FDR were performed using the online available statistical software R version 2.14.0 (www.r-project.org).

The results obtained with the Kruskal-Wallis tests adjusted with FDR were further analysed using the paired Wilcoxon signed-rank test to determine statistical significant differences in median of PL species concentrations between all investigated groups (human: healthy SF vs. OA early SF vs. OA late SF vs. RA SF; dogs: healthy SF vs. OA SF; distraction experiment: OA group vs. frame group vs. distraction group; species dependency: human SF vs. canine SF vs. equine SF). *P*-value of less than 0.05 was considered statistically significant with: * $0.01<p\leq0.05$; ** $0.001<p\leq0.01$; *** $p<0.001$. The paired Wilcoxon signed-rank test was performed using the statistical software R.

Spearman's correlation and linear regression were used to determine the coefficient of determination (R^2). R^2 was calculated in order to correlate (a) the volume of SF with concentrations of β-MG, (b) the concentrations of urea in SF with those in serum and, (c) the concentrations of PL classes with the time period post-mortem. Spearman's correlation and linear regression were performed using the commercially available software Prism 5.2 (GraphPad Inc., San Diego, USA).

Data are presented as a mean ± SD for bars graphs. The notched boxplots show whiskers extending to the minimum and maximum values with circles indicating outliners and 95% confidence intervals for the median. Presented bars graphs were produced using Prism 5.2 software. Moreover, all PL species were analysed individually and graphed as notched boxplots (presented notched boxplots are shown as an example). The notched boxplots were generated with statistical software R.

4. Results

4.1 Estimation of undiluted SF volume

In order to eliminate unknown dilution of SF that might occur during effusion induced by inflammation and to normalise quantitative data obtained from ESI-MS/MS, we were searching for a method to correct our results. For this purpose two independent methods were tested. First using β2-MG concentrations in SF samples and second using urea concentrations in SF and serum samples obtained from the same patient.

4.1.1 ELISA for β2-microglobulins

B2-microglobulins (β2-MG) concentrations were determined in 68 OA SF samples using a commercially available ELISA kit. To test whether there is a correlation between β2-MG level and volume of aspirated SF, β2-MG concentrations were plotted against the corresponding volume of SF. We found no correlation between β2-MG level and total volume of SF (Figure 4.1). Concentrations of β2-MG were in the range of 0.05 and 5.3 µg/ml and those values were independent from the respective total volume of SF. 9 different SF samples had a volume of 12 ml, however, concentrations of β2-MG varied in those samples between 0.4-4.45 µg/ml. This result suggests that the concentration of β2-MG is not a suitable factor to correct for any dilution of SF.

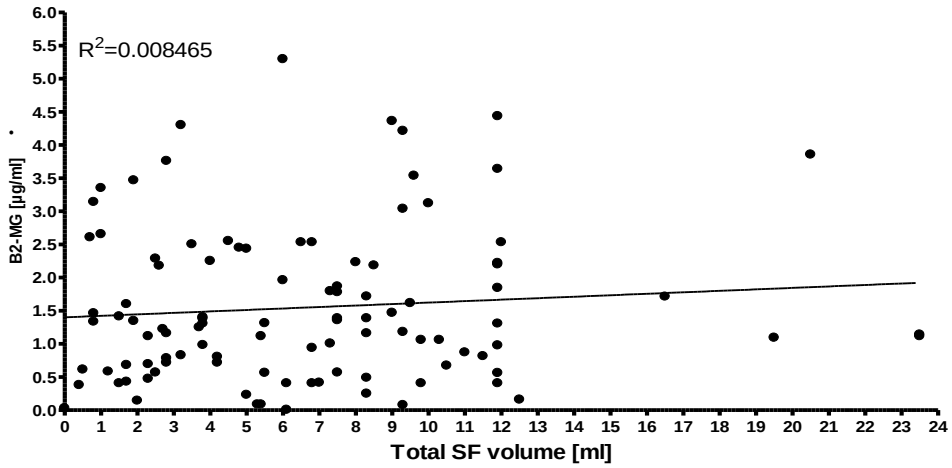


Figure 4.1. Correlation between β2-MG concentrations and corresponding total volume of SF. Variable volumes of SF were obtained from 68 OA patients. B2-MG concentrations were measured using a commercially available ELISA kit. R^2: a linear regression analysis was performed to determine the coefficient of determination. B2-MG: β2-microglobulins.

4.1.2 Urea concentration in serum and SF

Since several studies already [177-179] suggested that the use of urea concentrations is an appropriate method to compensate for variations of SF due to effusion, levels of urea in SF and serum samples were determined using a commercially available QuantiChrom™ Urea Assay Kit. This assay detects urea directly by using o-phthalaldehyde that specifically binds urea and forms a coloured complex with primaquine diphosphate. Optical density was measured at 520 nm using a microplate ELISA reader. Subsequently, urea concentrations in SF were plotted against the corresponding urea concentrations in serum according to the method described by Kraus *et al.* [177]

Correlation was subsequently calculated between the concentrations of urea in SF and those in serum obtained from the same patient (Figure 4.2). In our samples more urea was found in the serum samples compared to SF samples. The dilution factors for all OA and RA SF samples were calculated following previously reported protocols, also applied by Kraus *et al.* to correct dilution of SF [177-178]. The formula to calculate the dilution factor was as follow:

$$\mathrm{DF} = \frac{0.647x[serum\ urea] + 1.752}{SF\ urea}$$

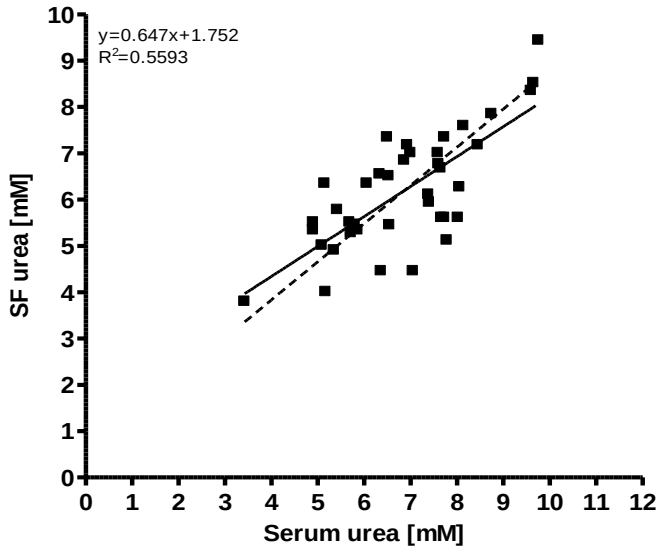


Figure 4.2. Correlation between SF and serum urea concentrations. Urea concentrations were determined in 39 SF samples from early stage of OA with a total volume not being higher than 2.5 ml, which is assumed to be normal, and in 39 serum samples obtained from the same patients. Urea concentrations were determined using commercial available QuantiChrom™ Urea Assay Kit. R^2: a linear regression analysis was performed to determine the coefficient of determination.

Our results are similar to the data reported [177-178], therefore in the present study quantitative values of PL obtained from ESI-MS/MS analysis could be normalised using the urea method.

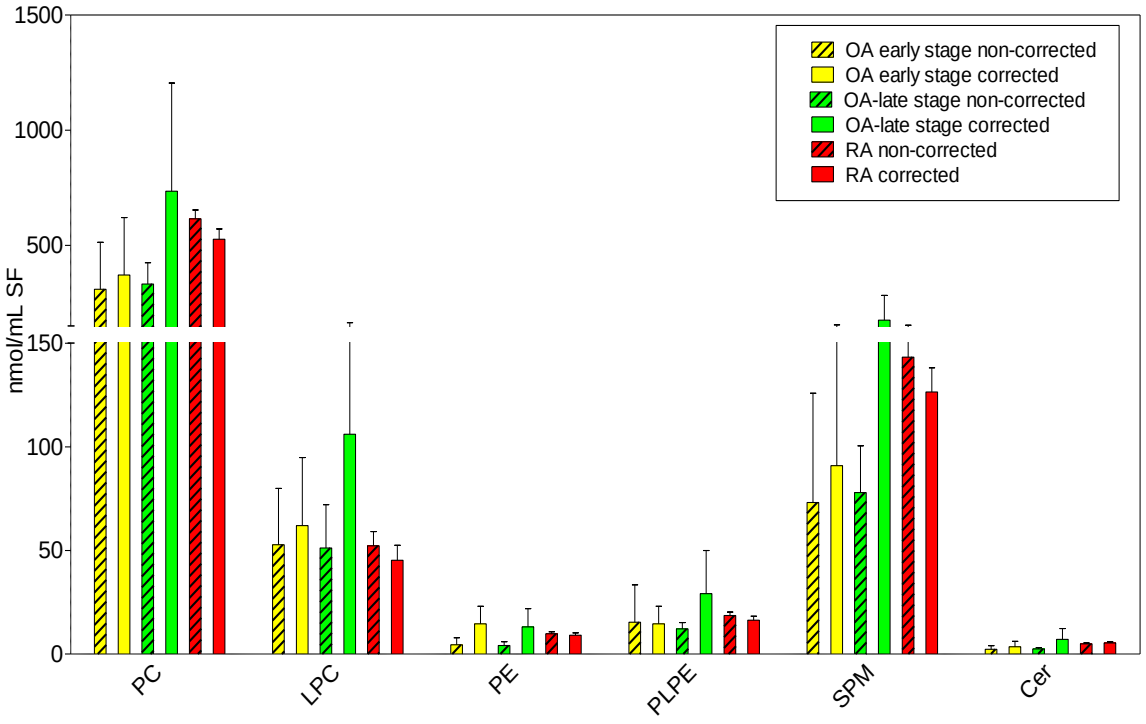


Figure 4.3. Comparison of the concentrations of PL classes between non-corrected and corrected values. The different PL classes were quantified by ESI-MS/MS for 9 healthy SF, 19 OA SF from early stage, 14 OA SF from late stage and 18 RA SF. Presented PLs are phosphatidylcholine (PC), lysophosphatidylcholine (LPC), phosphatidylethanolamine (PE), PE-based plasmalogen (PLPE), sphingomyelin (SPM) and ceramides (Cer). Data are presented as mean ± SD of concentrations of PL classes [nmol/ml]. "Non-corrected"-values before correction. "Corrected"-values after correction for possible dilution using the dilution factor (DF) obtained by the determination of the urea concentrations. OA-osteoarthritis, RA-rheumatoid arthritis.

The quantitative values of all PL classes and individual species obtained from ESI-MS/MS were normalised using the calculated dilution factor derived from concentrations of urea in SF and serum. As Figure 4.3 presents, concentrations of PL classes in SF from late stage of OA are higher after normalisation due to possible dilution using the dilution factor obtained by the determination of the urea concentrations as compared to the non-corrected values. However, analysis of PL species in individual patients revealed that in certain cases the determined concentrations were several fold higher after correction, and in other cases we did not observe any differences between corrected and non-corrected values.

4.2 Analysis of PL composition of human SF

The composition of PL species in human SF and their role in the development of diarthrodial joint diseases are currently largely unknown. There are only several studies which focused on this aspect and not much is known about composition and the physiological and pathophysiological role of PL in the normal and OA joints.

4.2.1 Phospholipid content in healthy SF from human cadavers

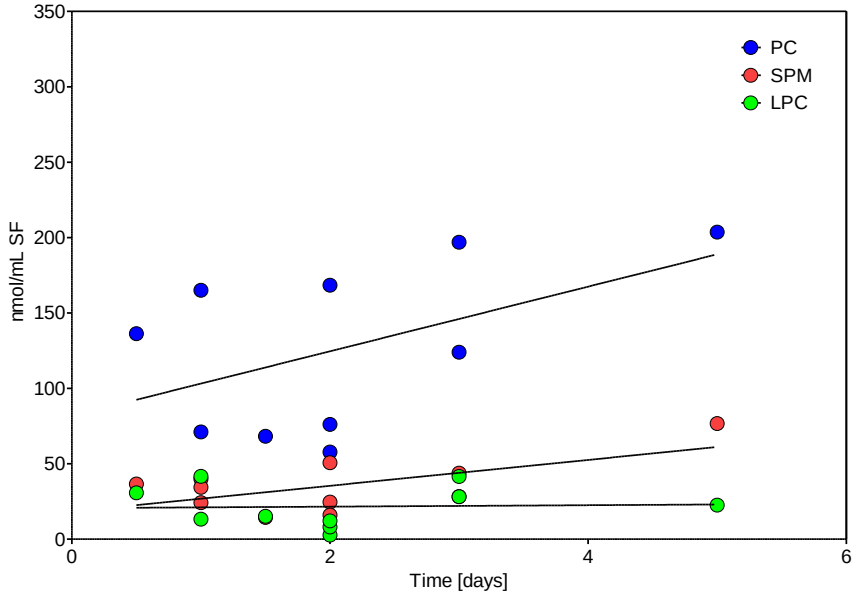
Healthy, normal SF was obtained from 15 human cadavers with healthy joints. Samples were aspirated from knee joints within several hours until 5 days after death. In order to confirm that PL composition in SF is stable until 5 days after death, concentrations of all PL classes were plotted against this time period. Figure 4.4 demonstrates that concentrations of PL classes remained unchanged. The correlation coefficient of all investigated PL classes indicates that PLs are stable until 5 days post-mortem. In addition, similar analysis was performed for the main individual PL species (e.g. PC 34:2, PC 38:4, LPC 16:0, LPC 18:2, PE 36:2, SPM 16:0, SPM 24:1, Cer 24:0). As expected, no changes were identified (data not shown).

4.2.2 Composition of PL in microparticles present in human SF

Extracellular membrane vesicles with diameter of 200-1000 nm, in literature also often called MPs, are present in SF and contain cell membranes composed out of PLs. In order to distinguish between PLs, which are a part of MPs and free, extracellular PLs, MPs were isolated from 10 healthy SF, 19 OA SF from early stage, 14 OA SF from late stage and 18 RA SF. Subsequently, ESI-MS/MS analysis was performed for cell- and cell debris-free SF, cell-, cell debris- and MPs-free SF and isolated MPs. As presented in Table 4.1, no statistical significant difference could be identified between cell- and cell debris-free SF (total SF) and cell-, cell debris- and MPs-free SF (supernatant) for all groups of interest. MPs contain PLs, however, the determined concentrations were low. This is also reflected for all PL classes with the exception of PS where the highest concentration was detected in MPs (Table 4.2). Based on these data, our further analyses were performed using cell-, cell debris- and MPs-free SF (further named “SF”) to determine only the extracellular PLs which may serve as boundary lubricants.

A.

	PC	SPM	LPC
R^2	0.25	0.39	0.0021



B.

	PE	PLPE	PS	PG	Cer
R^2	0.044	0.0049	0.23	0.0064	0.045

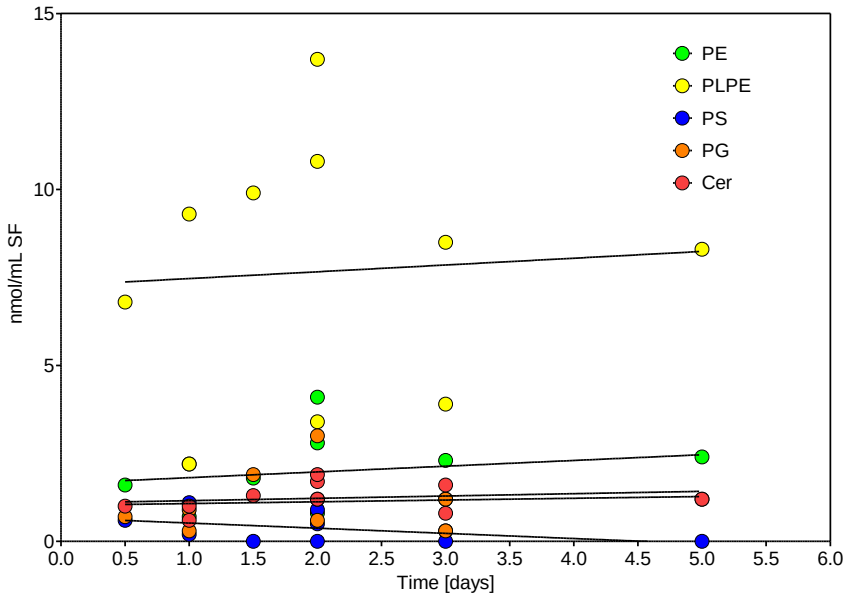


Figure 4.4. Correlation between the concentrations of PL classes and time period post-mortem. The different PL classes were quantified in 10 SF samples obtained from human cadavers by ESI-MS/MS. Panel A shows phosphatidylcholine (PC), sphingomyelin (SPM) and lysophosphatidylcholine (LPC), and panel B shows phosphatidylethanolamine (PE), PE-based plasmalogen (PLPE), phosphatidylserine (PS), phosphatidylglycerol (PG), and ceramide (Cer). R^2: a linear regression analysis was performed to determine the coefficient of determination.

Table 4.1. Total PL content in SF fractions as dependent on the health status of joints.

	Total SF [nmol/ml]	Supernatant [nmol/ml]	MPs [nmol/ml]
Healthy	273.0 (142.6-569.2)	213.4 (102.5-354.7)	126.5 (45.8-226.5)
OA-early stage	597.4 (174.2-1214.8)	556.5 (65.3-1505.3)	294.8 (54.2-730.5)
OA-late stage	1029.9 (375.4-2270.3)	1072.3 (336.8-2612.0)	429.2 (54.5-1147.5)
RA	639.3 (247.8-1006.7)	731.0 (307.7-1178.2)	577.0 (165.2-1397.8)

The total PL content was determined by ESI-MS/MS in 9 healthy SF, 19 OA SF from early stage, 14 OA SF from late stage and 18 RA SF. All values were corrected for possible dilution using the dilution factor (DF) obtained by determination of the urea concentrations. Data are presented as mean concentrations, range of data in parentheses. MPs-microparticles, OA-osteoarthritis, RA-rheumatoid arthritis.

4.2.3 Composition of PL classes in human SF

To investigate potential changes in the concentrations of PL classes in SF, lipids were isolated from 10 healthy SF, 19 OA SF from early stage, 14 OA SF from late stage and 18 RA SF. Subsequently, ESI-MS/MS analysis was performed for cell-, cell debris- and MPs-free SF. Analysed PL classes were as followed: PC, LPC, PE, PLPE, PI, PG, PS, SPM and Cer. All the data presented in this study represent one experiment. However, the reproducibility of the data was tested by a second independent analysis using a different batch of samples, performed at a different time point with newly calibrated instrument settings and other preparation of internal standards. Statistical analysis of the second analysis is summarised in Table 6.2. A lower number of statistical significant differences were found in the second analysis as compared to first one, however, the trends of changes were in both analyses mostly the same indicating good reproducibility.

The composition of PL classes showed low variations between different groups of patients and a characteristic pattern was observed for all SF samples (Figure 4.5 B). As expected, PC was the most abundant PL present, accounting for to 65% of all PLs present. The concentration of PC lied in the range of 135.3 nmol/ml for healthy SF and 735.5 nmol/ml for late stage of OA. However, PG and PS were detected in very low concentrations. Moreover, PC, PE, PLPE, SPM and Cer were increased in SF from late stage of OA as compared to healthy SF (Figure 4.5 A). Determined concentrations in SF from late stage of OA were increased 543.5 ± 346.5% for PC, 289.1 ± 194.3% for PE, 337.3 ± 241.4% for PLPE, 447.3 ± 274.5 for SPM, and 376.2 ± 279.7% for Cer as

compared to healthy SF (=100%). Remarkably, PS is the only PL class with a higher proportion in healthy SF and SF from early stage of OA as compared to SF from late stage OA and RA (Figure 4.5 B).

In addition, the ratios of PL classes were calculated. As illustrated in Figure 4.6 and Table 6.1, PC/LPC ratio as well as SPM/Cer ratio did not differ between the groups with different health status of the joints. Interestingly, PC/PS ratio and PE/PS ratio appear on the highest level in SF from early stage of OA as compared to all others investigated groups.

4.2.4 Composition of PL species in human SF

The decision to determine all individual PL species present in SF, and subsequently to address questions concerning metabolism and possible functions of individual PL species in health and OA, was based on the fact, that there is no detailed study showing PL species composition of human SF until now. It is known, that functions and properties of PL species also depend on the chain length and number of double bounds present in FA. Therefore, a method using ESI-MS/MS was applied, since this technology enables the quantitative determination of all PL species being present in human SF.

PC, PE, PG, PI and PS classes contain two FA. It is important to note, that mass spectrometry analysis used allows only to determinate the sum of carbon atoms and double bonds present in both FA. Thus, for instance a PE 38:4 may represent different combinations of FA such as 18:0/20:4, 16:0/22:4, etc.

All the PL species were analysed individually and graphed as notched boxplots which show 95% confidence intervals for the median as presented in Figure 4.7. However, presentation of all determined PL species would be beyond the allowed volume of this thesis, thus only randomly chosen species of PC are presented individually as notched boxplots in order to illustrate statistical method applied. Statistical significant changes of all individual PL species found between all investigated groups are summarised in Table 4.4.

As shown in Figure 4.8 and Table 4.3, PC in SF was present mostly in highly polyunsaturated form of PC characterised by the presence of three of more double bounds. Unsaturated DPPC, the most abundant compound of the surfactant system in the lung (PC 32:0), was present in very low concentration in OA (3% of all PC). Moreover, healthy SF

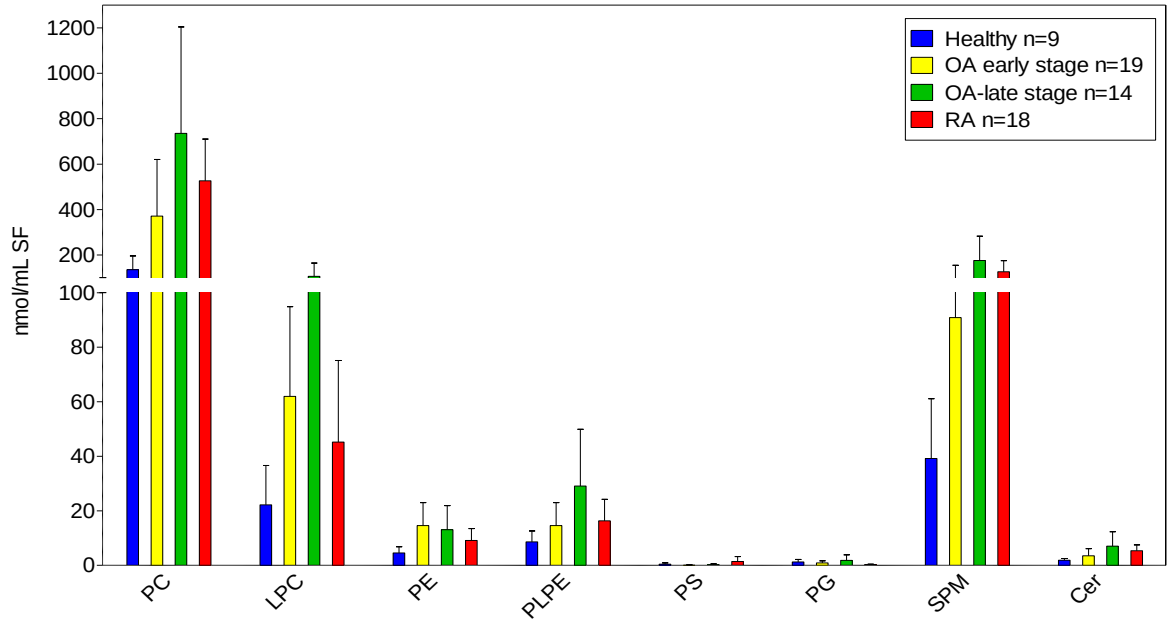
contains higher percentage of saturated PC, however, statistical analysis revealed no significant differences between SF from joints with different health status (Table 4.3).

Table 4.2. PL classes in SF fractions as dependent on the health status of joints.

Healthy (n=9)								
Fraction	**PC [nmol/ml]**	**LPC [nmol/ml]**	**PE [nmol/ml]**	**PLPE [nmol/ml]**	**PS [nmol/ml]**	**PG [nmol/ml]**	**SPM [nmol/ml]**	**Cer [nmol/ml]**
Total SF	168.1	26.0	5.5	11.1	1.9	1.3	56.9	2.1
Supernatant	135.3	22.2	4.5	8.6	0.4	2.4	39.2	1.9
MPs	62.2	9.7	8.8	14.0	7.1	2.4	19.8	2.6
OA early (n=19)								
Fraction	**PC [nmol/ml]**	**LPC [nmol/ml]**	**PE [nmol/ml]**	**PLPE [nmol/ml]**	**PS [nmol/ml]**	**PG [nmol/ml]**	**SPM [nmol/ml]**	**Cer [nmol/ml]**
Total SF	393.5	64.0	8.0	16.3	0.6	1.0	104.9	4.0
Supernatant	371.6	62.0	7.5	14.6	0.07	0.9	90.9	3.5
MPs	186.5	36.2	6.9	12.2	0.5	1.8	46.6	2.9
OA late (n=14)								
Fraction	**PC [nmol/ml]**	**LPC [nmol/ml]**	**PE [nmol/ml]**	**PLPE [nmol/ml]**	**PS [nmol/ml]**	**PG [nmol/ml]**	**SPM [nmol/ml]**	**Cer [nmol/ml]**
Total SF	695.9	97.7	13.6	29.9	1.6	3.8	176.3	7.8
Supernatant	735.5	106.1	13.1	29.1	0.3	1.8	175.4	7.1
MPs	272.6	48.6	11.6	21.6	1.3	3.2	66.8	4.5
RA (n=18)								
Fraction	**PC [nmol/ml]**	**LPC [nmol/ml]**	**PE [nmol/ml]**	**PLPE [nmol/ml]**	**PS [nmol/ml]**	**PG [nmol/ml]**	**SPM [nmol/ml]**	**Cer [nmol/ml]**
Total SF	415.6	34.1	8.3	14.7	3.0	0.3	109.5	13.7
Supernatant	526.8	45.2	9.1	16.3	1.4	0.3	126.4	5.3
MPs	354.1	28.8	14.4	45.7	31.5	1.3	92.8	8.3

The different PL classes were quantified by ESI-MS/MS for 9 healthy SF, 19 OA SF from early stage, 14 OA SF from late stage and 18 RA SF. Presented PLs are phosphatidylcholine (PC), lysophosphatidylcholine (LPC), phosphatidylethanolamine (PE), PE-based plasmalogen (PLPE), phosphatidylserine (PS), phosphatidylglycerol (PG), sphingomyelin (SPM) and ceramides (Cer). Data are presented as mean of concentrations [nmol/ml]. All values were corrected for possible dilution using the dilution factor (DF) obtained by the determination of the urea concentrations. MPs-microparticles, OA-osteoarthritis, RA-rheumatoid arthritis.

A.



B.

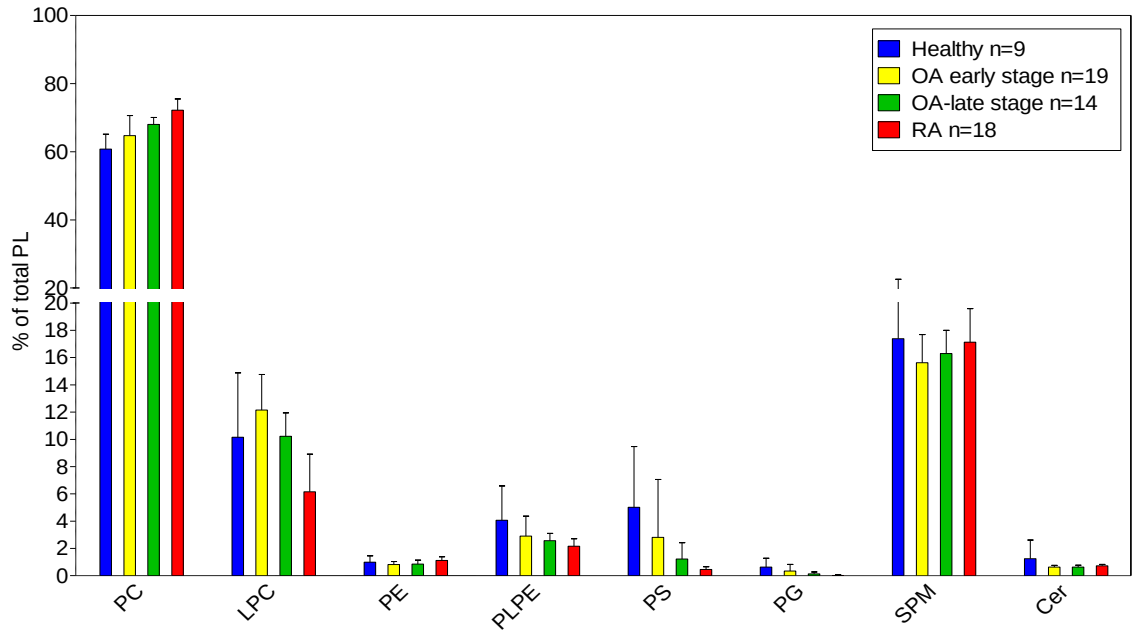


Figure 4.5. Synovial fluid PL classes composition. The different PLs were quantified by ESI-MS/MS. The data shown represent (A) the quantitative values corrected for possible dilution using the dilution factor (DF) obtained by the determination of the urea concentrations and (B) the percentages of the respective PL class of all analysed PLs (=100%). Presented PLs are phosphatidylcholine (PC), lysophosphatidylcholine (LPC), phosphatidylethanolamine (PE), PE-based plasmalogens (PLPE), phosphatidylserine (PS), phosphatidylglycerol (PG), sphingomyelin (SPM), and ceramide (Cer). Values represent mean ± SD of concentrations of PL classes. OA-osteoarthritis, RA-rheumatoid arthritis.

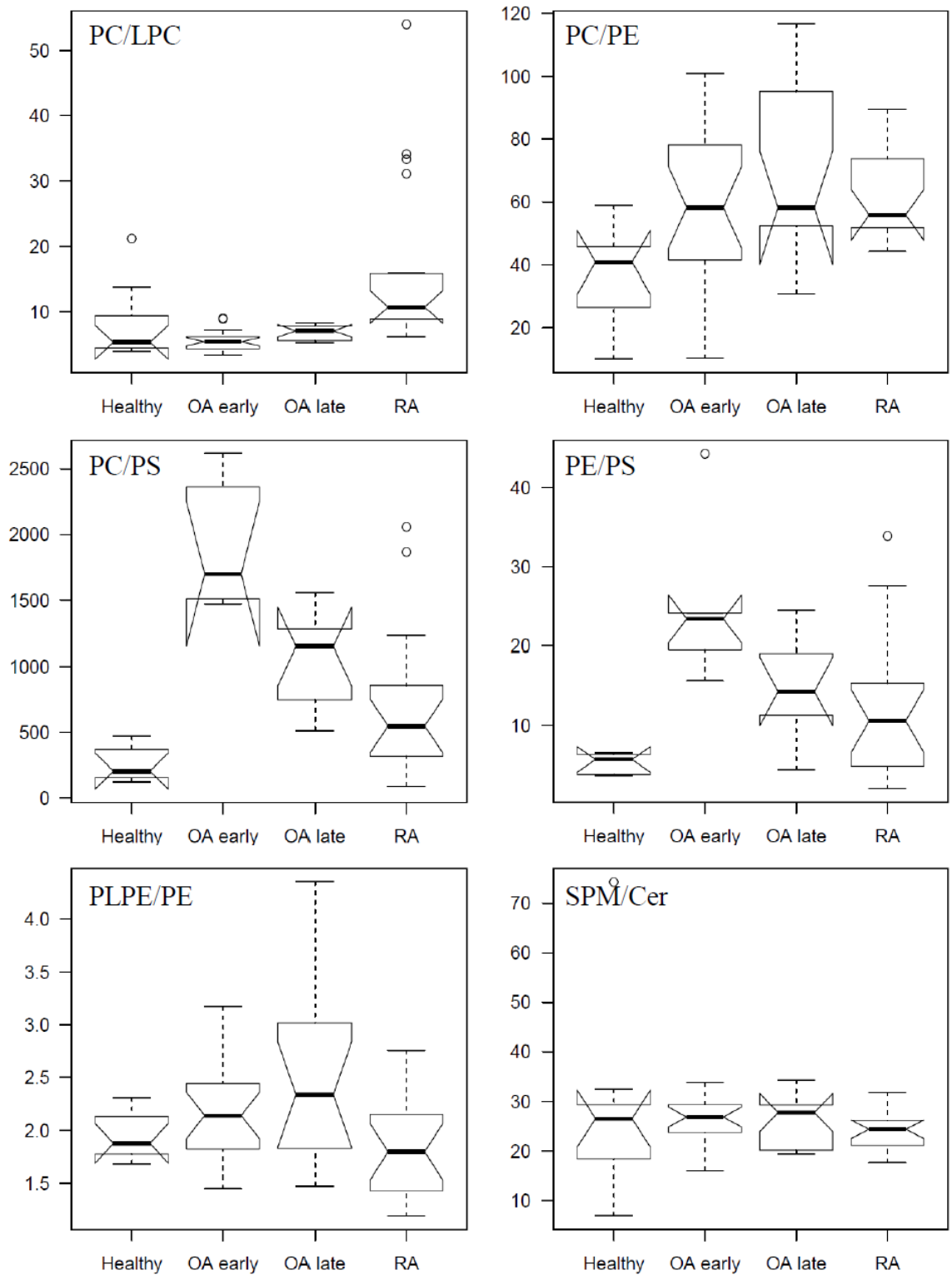


Figure 4.6. Ratios of PL classes in all four groups of patients. The different PL classes were quantified by ESI-MS/MS for 9 healthy SF, 19 OA SF from early stage, 14 OA SF from late stage and 18 RA SF. The quantitative values of PL classes presented in Figure 4.5 B were used to calculate ratios. The notched boxplots show 95% confidence intervals for the median. Circles indicate outliners. Statistical analysis is summarised in Table 6.1. PC-phosphatidylcholine, LPC-lysophosphatidylcholine, PE-phosphatidylethanolamine, PS-phosphatidylserine, PLPE-phosphatidylethanolamine-based plasmalogen, SPM-sphingomyelin, Cer-ceramides, OA-osteoarthritis, RA-rheumatoid arthritis.

The main LPC species present was LPC 16:0 with a concentration range lying between 9.2 nmol/ml and 29.2 nmol/ml. According to the carbon number, the most abundant group present was the FA chain with 18 C-atoms covers 40.4-55.1% of total LPC. Most of the LPC species were detected in higher concentrations in arthritic SF when compared to healthy SF (Figure 4.9 B). For instance, concentrations of the main LPC species increased up to 290.1 ± 153.1% in early OA, 545.1 ± 301.5% in late OA and 297.1 ± 143.4% in RA SF for LPC 16:0, 407.5 ± 277.5% in early OA, 713.4 ± 420.4% in late OA and 419.8 ± 256.9% in RA SF for LPC 18:0, 228.7 ± 113.6% in early OA, 374.5 ± 201.2% in late OA and 217.8 ± 104.5% in RA SF for LPC 18:1, 246.2 ± 132.7% in early OA, 337.6 ± 204.7% in late OA and 204.9 ± 104.3% in RA SF for LPC 18:2, and 204.1 ± 111.2% in early OA, 294.4 ± 146.2 in late OA and 181.7 ± 89.8% in RA SF for LPC 20:4, when compared to healthy SF (=100%).

Similarly to PC, PE species were present mostly in polyunsaturated form (Figure 4.10, Table 4.3). Interestingly, ether-phosphatidylethanolamine (PE O 40:3) was decreased to 60.8 ± 5.1% in RA SF as compared to SF from late stage of OA (=100%).

As Figure 4.11 demonstrates, 39 individual PLPE species were detected. The species were further divided into three groups according to the number of C-atoms and double bounds of the first FA. Similar distribution, dependent on the composition of the second FA was observed in all three groups of PLPE species (Table 4.3). Moreover, detected concentrations of those species were similar in all groups and also dependent from the composition of the second FA.

PI 38:4 was the dominant PI species determined in all analysed samples covers 49.4 ± 5.3 % of total PI. Almost all of the PI species were present in increased concentrations in both OA SF groups as compared to other healthy SF (Figure 4.12 A). Observed increases in the concentrations were as follow, 186.2 ± 65.9 % in early OA and 211.6 ± 117% in late OA SF for PI 36:4, 204 ± 80.5 % in early OA and 229.7 ± 102% in late OA for PI 38:3, 205.4 ± 76.1 % in early OA and 243.7 ± 115% in late OA SF for PI 38:4, and 168.1 ± 61.2 % in early OA and 182.3 ± 116% in late OA SF for PI 38:5, when compared to healthy SF (=100%).

As expected, there was a significant increase of most of Cer species in OA late and RA SF including Cer 16:0, Cer 22:0, Cer 23:0, Cer 24:1 and GluCer 24:1 (Figure 4.12 B). Determined concentrations increased up to 296.7 ± 187.6 % in late OA and 197.8 ± 136.6% in RA SF for Cer 16:0, 395.5 ± 332.8 % in late OA and 246.2 ± 128.4% in RA SF for Cer 22:0, 390.6 ± 341.5 % in late OA and 246.4 ± 133.7% in RA SF for Cer 23:0,

322.2 ± 219.1 % in late OA and 210.2 ± 95% in RA SF for Cer 24:1, and 329.8 ± 224.4 % in late OA and 213.4 ± 130.6% in RA SF for GluCer 24:1,when compared to healthy SF (=100%).

Table 4.3. Distribution of saturated and unsaturated PL species according to FA saturation status in a chosen PL class.

Phosphatidylcholine (PC)	**Healthy**	**OA-early**	**OA-late**	**RA**
Saturated	6.3 ± 5.1%	4.4 ± 0.9%	3.6 ± 1.2%	3.1 ± 1.2%
Unsaturated	86.0 ± 5.6%	88.9 ± 1.8%	90.1 ± 1.8%	90.7 ± 2.2%
Ratio (Saturated/Unsaturated)	0.078 ± 0.074	0.049 ± 0.011	0.040 ± 0.014	0.035 ± 0.014
Lysophosphatidylcholine (LPC)	**Healthy**	**OA-early**	**OA-late**	**RA**
Saturated	55.5 ± 2.7%	60.8 ± 5.3%	67.1 ± 3.2%	72.0 ± 4.0%
Unsaturated	44.5 ± 2.7%	39.2 ± 5.3%	32.9 ± 3.2 %	28.0 ± 4.0 %
Saturated/Unsaturated	1.3 ± 0.1	1.6 ± 0.4	2.1 ± 0.3	2.6 ± 0.5
Phosphatidylethanolamine (PE)	**Healthy**	**OA-early**	**OA-late**	**RA**
Saturated	2.5 ± 0.9%	2.2 ± 1.5%	1.2 ± 0.5%	0.7 ± 0.2%
Unsaturated	97.5 ± 0.9%	97.8 ± 1.5%	98.8 ± 0.5%	99.3 ± 0.2%
Saturated/Unsaturated	0.026 ± 0.010	0.022 ± 0.016	0.012 ± 0.005	0.007 ± 0.002
PE-based plasmalogen (PLPE)	**Healthy**	**OA-early**	**OA-late**	**RA**
16:0	40.6 ± 2.5%	39.7 ± 2.0%	39.7 ± 1.6%	38.9 ± 2.2%
18:1	30.3 ± 2.3%	26.6 ± 3.0%	25.0 ± 2.3%	22.0 ± 2.7%
18:0	29.1 ± 2.5%	33.7 ± 2.7%	35.3 ± 2.4%	39.1 ± 3.2%
Sphingomyelin (SPM)	**Healthy**	**OA-early**	**OA-late**	**RA**
Saturated	61.3 ± 1.9%	62.8 ± 1.5%	63.4 ± 1.5%	63.2 ± 2.5%
Unsaturated	38.7 ± 1.9%	37.2 ± 1.5%	36.6 ± 1.5%	36.8 ± 2.5%
Saturated/Unsaturated	2.0 ± 0.5	1.9 ± 0.4	1.8 ± 0.3	1.9 ± 0.5
Ceramide (Cer)	**Healthy**	**OA-early**	**OA-late**	**RA**
Saturated	70.2 ± 4.2%	74.0 ± 3.0%	74.6 ± 3.0%	74.9 ± 4.0%
Unsaturated	29.8 ± 4.2%	26.0 ± 3.0%	25.4 ± 3.0%	25.1 ± 4.0%
Saturated/Unsaturated	2.4 ± 0.5	2.9 ± 0.5	3.0 ± 0.5	3.1 ± 0.7

PC species were analysed by ESI-MS/MS in 9 healthy SF, 19 OA SF from early stage, 14 OA SF from late stage and 18 RA SF. The values represent the percentage of PL species with the respective FA saturation status of total PL class (=100%) and calculated ratios of total saturated PL and unsaturated PL. Data represent mean ± SD of percentage or ratio. PC-phosphatidylcholine, LPC-lysophosphatidylcholine, PE-phosphatidylethanolamine, PLPE-phosphatidylethanolamine-based plasmalogen, SPM-sphingomyelin, Cer-ceramide, OA-osteoarthritis, RA-rheumatoid arthritis.

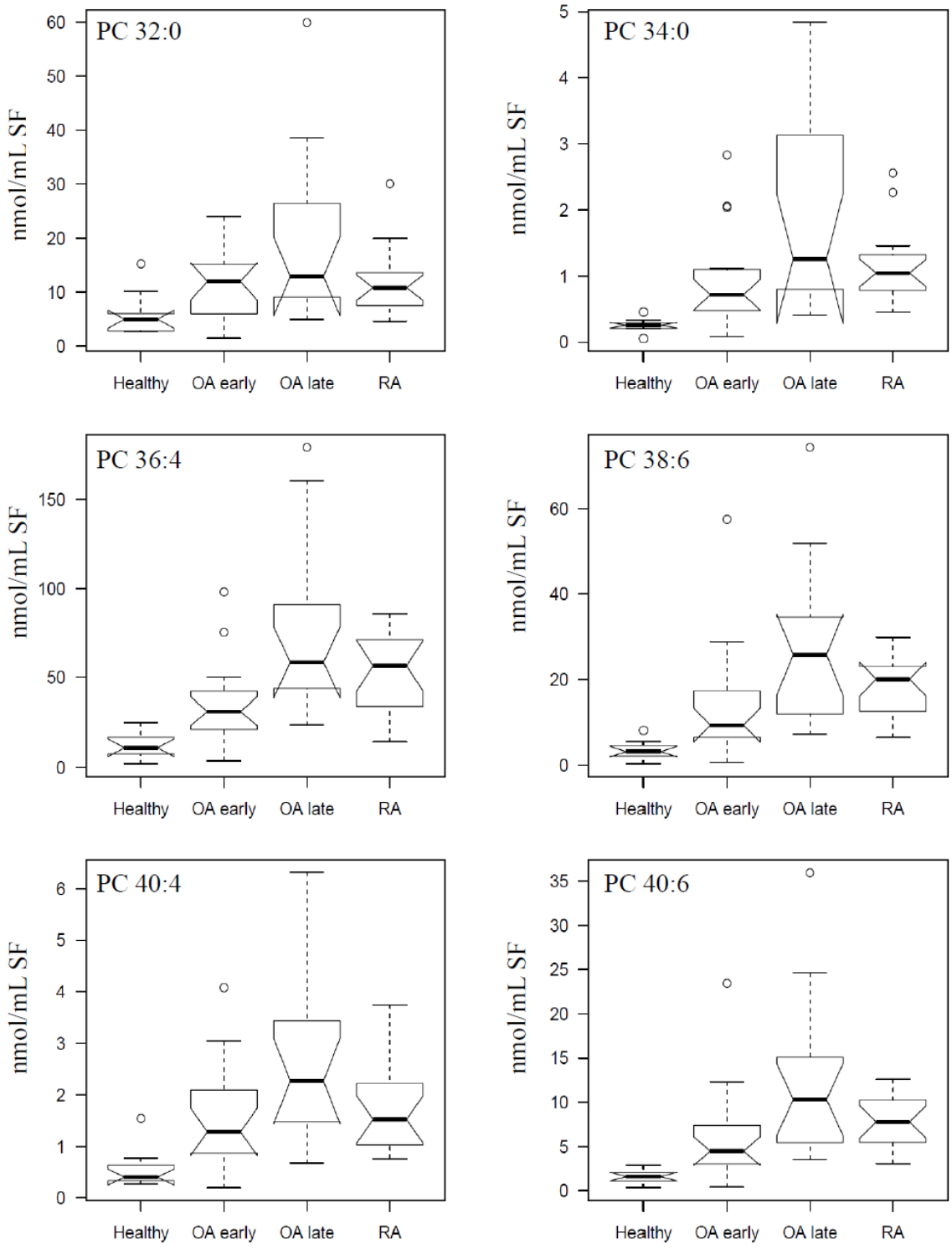


Figure 4.7. Notched boxplots for randomly chosen PC species. PC species were analysed in 9 healthy SF, 19 SF from early stage of OA, 14 SF from late stage of OA and 18 RA SF. The notched boxplots show 95% confidence intervals for the median. Circles indicate outliners. Statistical analysis is summarised in table 4.4. Additional data are also presented in Figure 4.8. OA-osteoarthritis, PC-phosphatidylcholine, RA-rheumatoid arthritis.

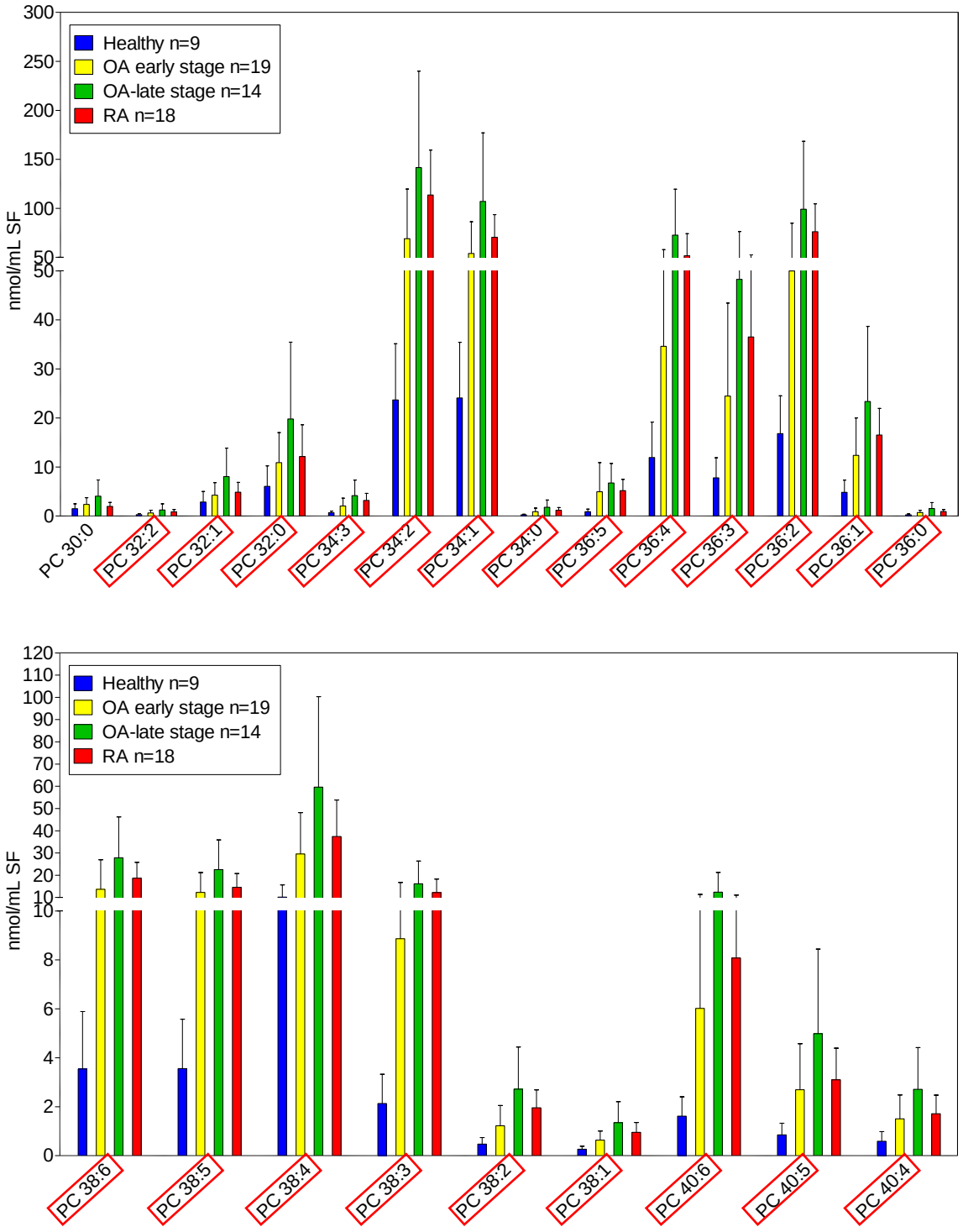
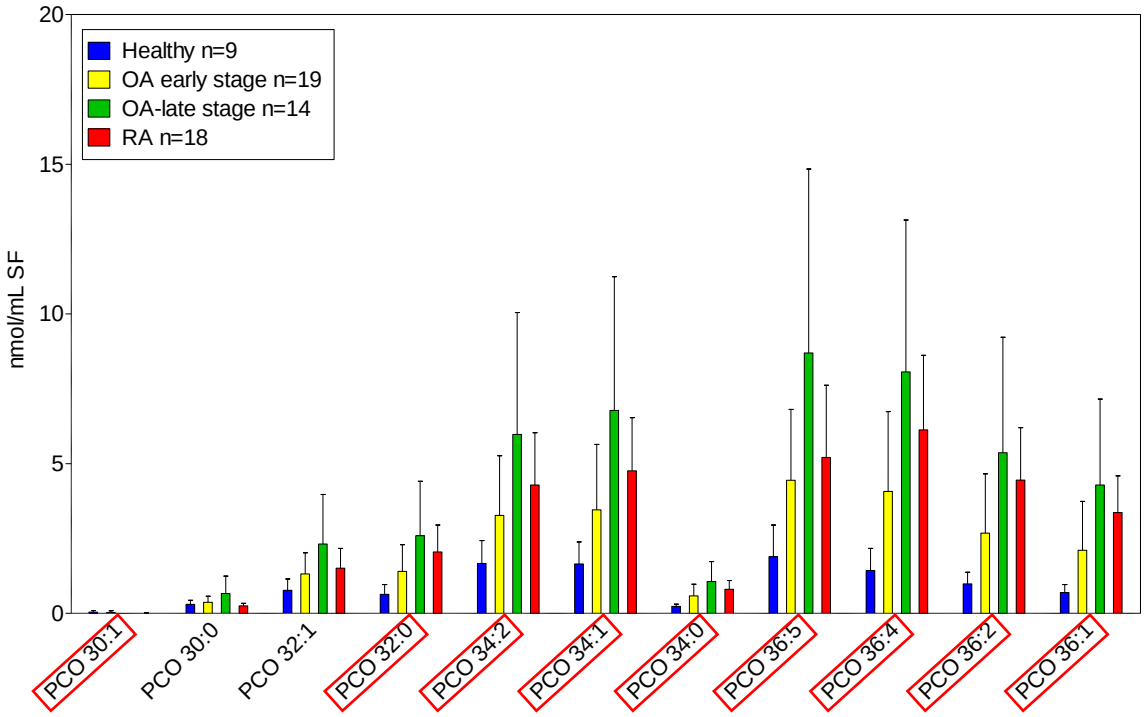


Figure 4.8. Phosphatidylcholine species composition of SF as dependent on the health status of joints. The different PL species were quantified by ESI-MS/MS. All values were corrected for possible dilution using the dilution factor (DF) obtained by the determination of the urea concentrations. Values are expressed as mean ± SD of concentrations of PC species. PL species showing any significant differences between groups are marked with red boxes. Statistical analysis is summarised in table 4.4. OA-osteoarthritis, PC-phosphatidylcholine, RA-rheumatoid arthritis.

A.



B.

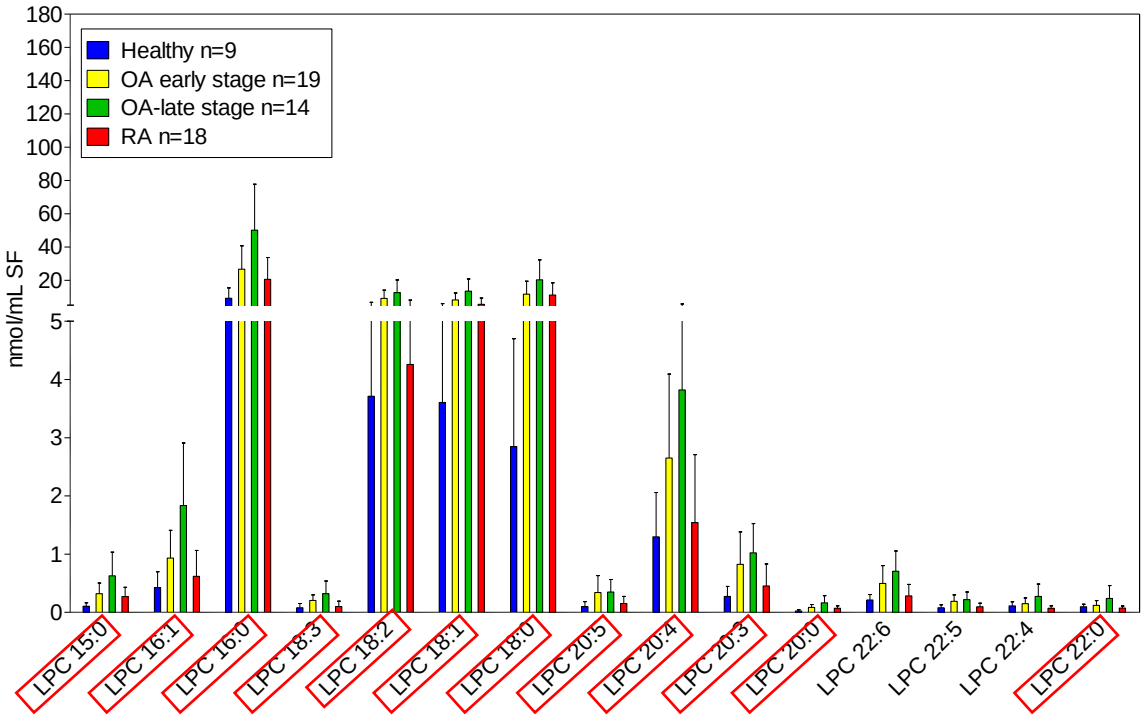


Figure 4.9. A. Ether-phosphatidylcholine and B. Lysophosphatidylcholine species composition of SF as dependent on the health status of joints. The different PL species were quantified by ESI-MS/MS. All values were corrected for possible dilution using the dilution factor obtained by the determination of the urea concentrations. Values represent mean ± SD of concentrations of PL species. PL species showing any significant differences between groups are marked with red boxes. Statistical analysis is summarised in table 4.4. LPC-lysophosphatidylcholine, OA-osteoarthritis, PC O-ether phosphatidylcholine, RA-rheumatoid arthritis.

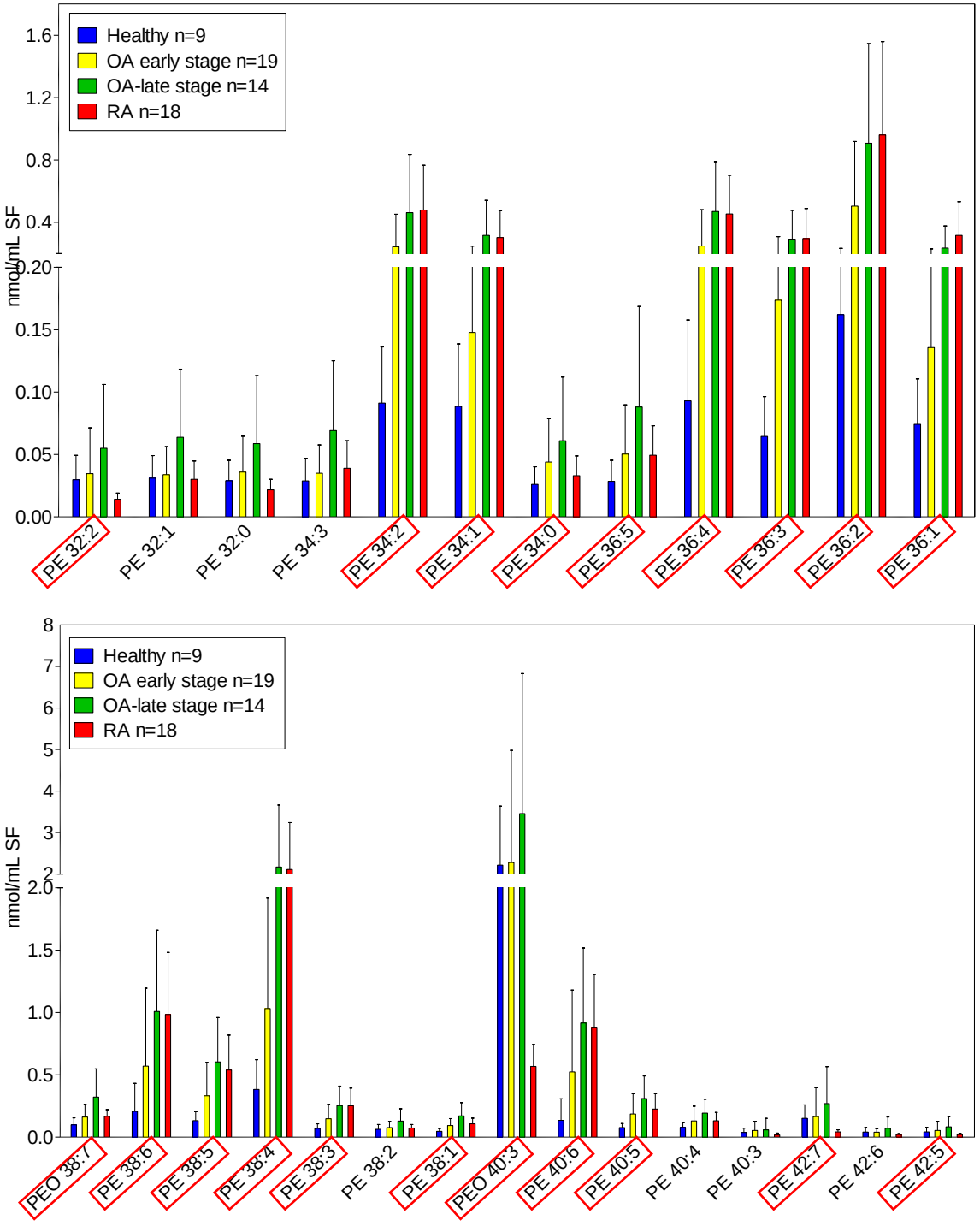


Figure 4.10. Phosphatidylethanolamine species composition of SF as dependent on the health status of joints. The different PL species were quantified by ESI-MS/MS. All values were corrected for possible dilution using the dilution factor (DF) obtained by the determination of the urea concentrations. Values represent mean ± SD of concentrations of PE species. PL species showing any significant differences between groups are marked with red boxes. Statistical analysis is summarised in table 4.4. OA-osteoarthritis, PE-phosphatidylethanolamine, RA-rheumatoid arthritis.

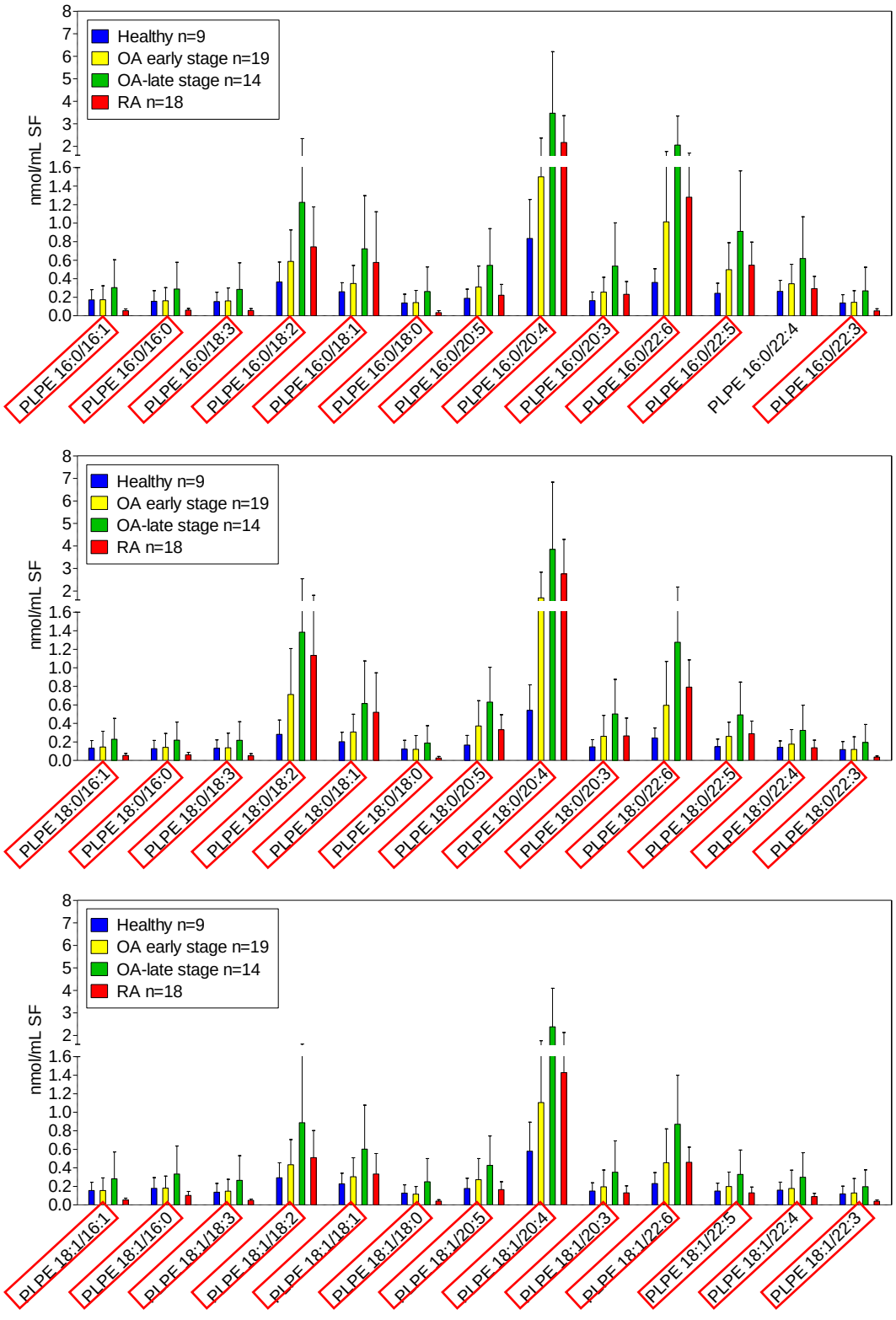


Figure 4.11. Phosphatidylethanolamine-based plasmalogens species composition of SF as dependent on the health status of joints. The different PL species were quantified by ESI-MS/MS. All values were corrected for possible dilution using the dilution factor (DF) obtained by the determination of the urea concentrations. Values represent mean ± SD of concentrations of PLPE species. PL species showing any significant differences between groups are marked with red boxes. Statistical analysis is summarised in table 4.4. OA-osteoarthritis, PLPE-phosphatidylethanolamine-based plasmalogen, RA-rheumatoid arthritis.

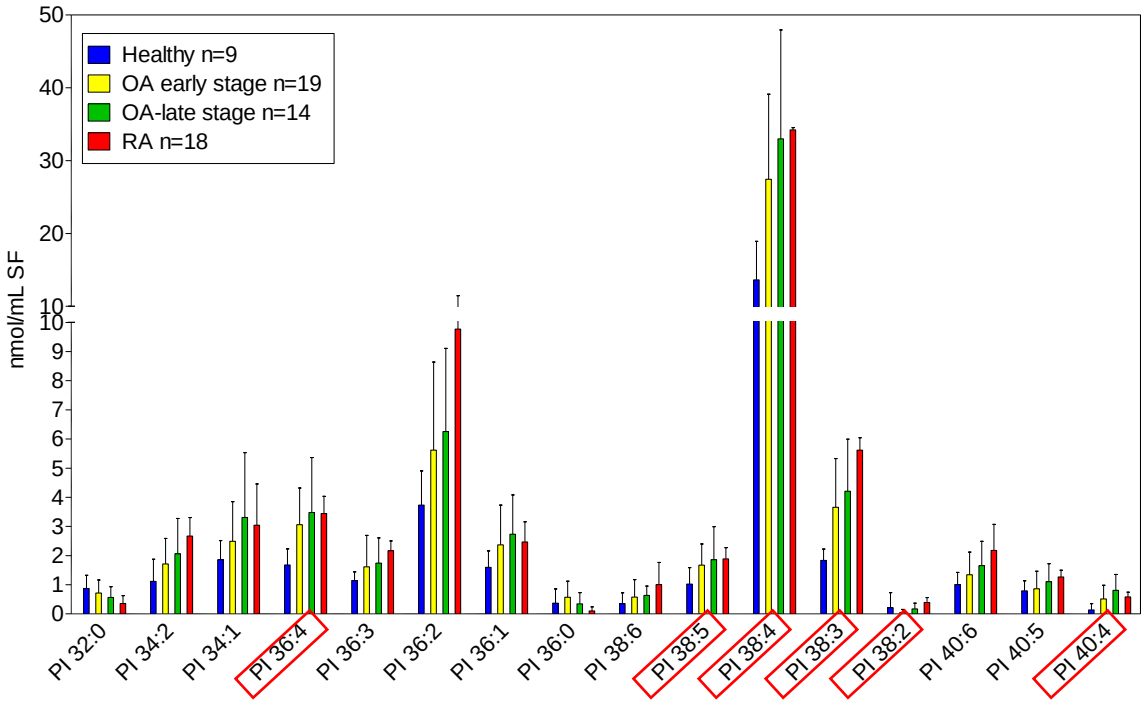
Compared to other PL classes, only three PG and two PS species could be detected. The values of the PG and PS species were close to the detection limit, thus, no individual species composition are presented on any graphs.

Similarly to the LPC class, saturated species characterised by 16 C-atoms in FA were the dominant species found in the SPM class accounting for 50.2 ± 3.4 % of total SPM (Figure 4.13). In addition, the SPM species had increased concentrations in SF from early and late stage of OA and in RA SF as compared to healthy SF. Observed increases in the concentrations of the main SPM species were as follow, 216 ± 144.4% in early OA, 405 ± 241.3% in late OA and 256.6 ± 102% in RA SF for SPM 16:0, 250 ± 189.3% in early OA, 527.5 ± 385.3% in late OA and 336.5 ± 183.5% in RA SF for SPM 18:0, 275.7 ± 208.1% in early OA, 555.6 ± 356.3% in late OA and 344.8 ± 164.2% in RA SF for SPM 22:1, 329.5 ± 287.2% in early OA, 600.9 ± 406% in late OA and 395.4 ± 230.7% in RA SF for SPM 24:0, 227.1 ± 162.4% in early OA, 430.6 ± 262.1% in late OA and 278.9 ± 139% in RA SF for SPM 24:1, and 199.2 ± 125.2% in early OA, 378.5 ± 232.1 in late OA and 237.9 ± 97.9% in RA SF for SPM 24:2, when compared to healthy SF (=100%). Opposite to PC species, a higher percentage of saturated SPM species was found in SF from all investigated groups (Table 4.3).

Additionally, the relative contribution of PL species with various length of the FA chains to the total amount of respective PL class was calculated. As evident from Table 4.5 and Table 6.3, due to the development of OA and RA the FA chains are getting elongated in PC, PE, PLPE and PI. In healthy SF, a higher percentage of FA with shorter chains was observed (≤34) whereas, in OA ad RA SF a higher percentage of FA with longer chains was observed (>34).

To summarise, our result clearly show that there are significant differences between healthy and arthritic SF. Interestingly, SF from late stage of OA exhibited significantly higher concentrations of most of the depicted lipid species when compared with healthy SF and SF from early stage of OA. More importantly, significant differences in PL composition were also observed between early and late stage of OA (Table 4.4).

A.



B.

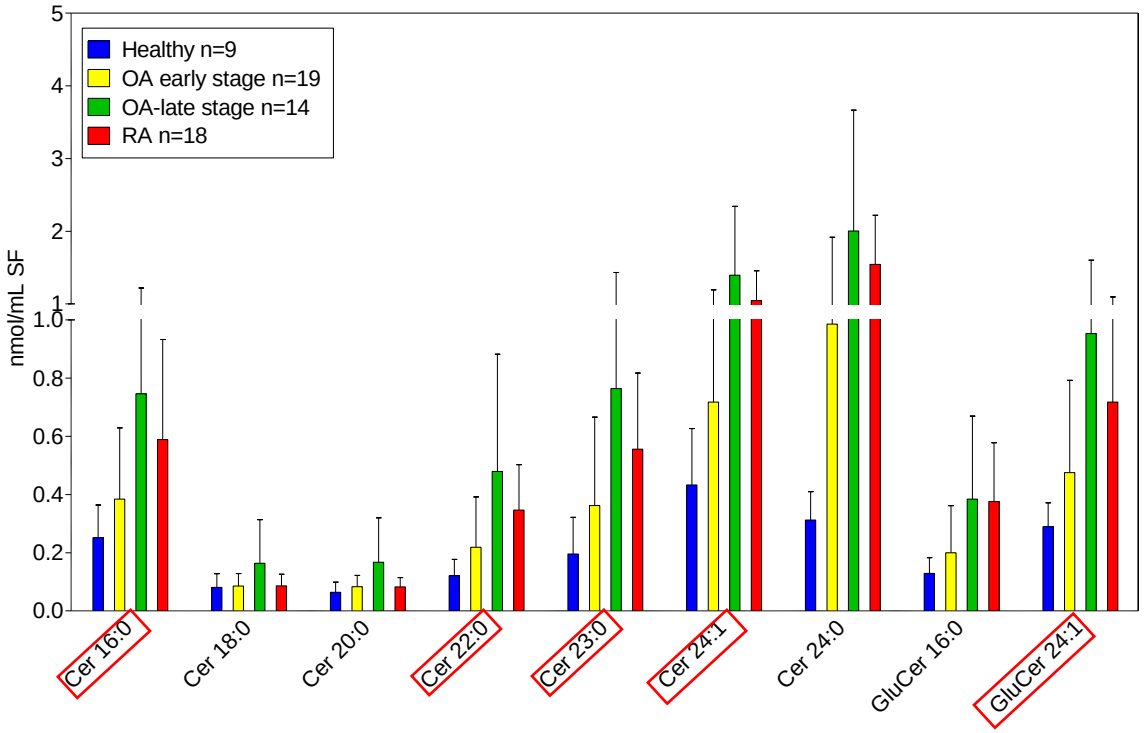


Figure 4.12. A. Phosphatidylinositol and B. Ceramide species composition of SF as dependent on the health status of joints. The different PL species were quantified by ESI-MS/MS. All values were corrected for possible dilution using the dilution factor (DF) obtained by the determination of the urea concentrations. Values represent mean ± SD of concentrations of PL specie. PL species showing any significant differences between groups are marked with red boxes. Statistical analysis is summarised in table 4.4. Cer-ceramide, GluCer-glucosylceramide, OA-osteoarthritis, PI-phosphatidylinositol, RA-rheumatoid arthritis.

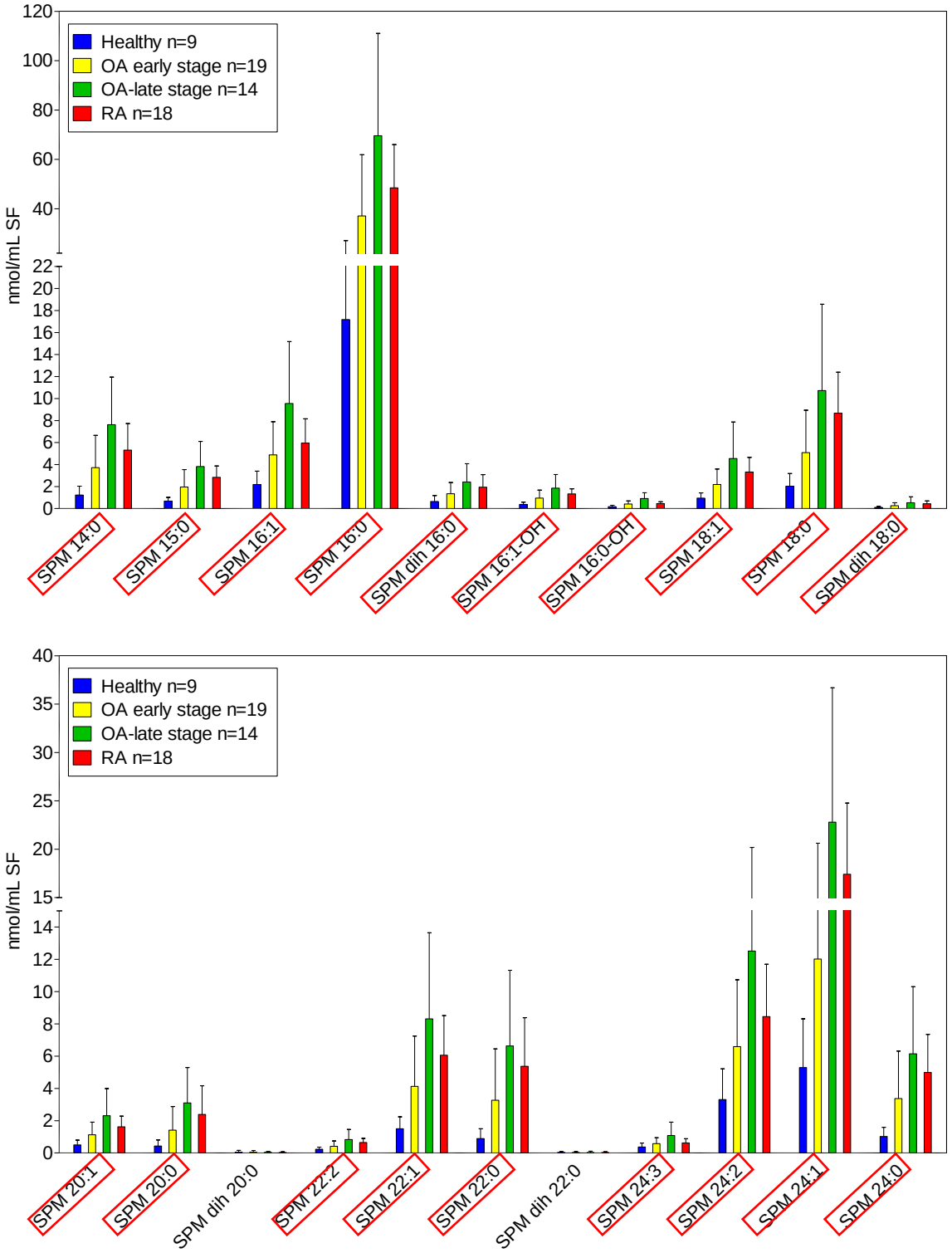


Figure 4.13. Sphingomyelin species composition of SF as dependent on the health status of joints. The different PL species were quantified by ESI-MS/MS. All values were corrected for possible dilution using the correction factor obtained by the determination of urea concentrations. Values represent mean ± SD of concentrations of SPM species. PL species showing any significant differences between groups are marked with red boxes. Statistical analysis is summarised in table 4.4. OA-osteoarthritis, RA-rheumatoid arthritis, SPM-sphingomyelin.

Table 4.4. Summary of statistically significant changes of the concentrations of individual PL species as dependent on the health status of joints.

PL - specie	OA early vs. Healthy	OA late vs. Healthy	RA vs. Healthy	OA early vs. OA late	OA early vs. RA	OA late vs. RA
Phosphatidylcholine (PC)						
PCO 30:0	NS	***↓	** ↓	** ↑	* ↑	NS
PC 32:0	NS	**↑	** ↑	NS	NS	NS
PCO 32:0	* ↑	***↑	***↑	NS	* ↓	NS
PC 32:1	NS	** ↑	** ↑	NS	NS	NS
PCO 32:1	NS	***↑	** ↑	NS	NS	NS
PC 32:2	NS	***↑	***↑	NS	NS	NS
PC 34:0	**↑	***↑	***↑	*↓	* ↓	NS
PCO 34:0	* ↑	***↑	***↑	NS	* ↓	NS
PC 34:1	* ↑	***↑	***↑	*↓	*↓	NS
PCO 34:1	*↑	***↑	***↑	*↓	*↓	NS
PC 34:2	**↑	***↑	***↑	**↓	**↓	NS
PCO 34:2	*↑	***↑	***↑	*↓	NS	NS
PC 34:3	*↑	***↑	***↑	*↓	*↓	NS
PC 36:0	**↑	***↑	***↑	**↓	NS	NS
PC 36:1	**↑	***↑	***↑	*↓	NS	NS
PCO 36:1	**↑	***↑	***↑	**↓	**↓	NS
PC 36:2	**↑	***↑	***↑	**↓	**↓	NS
PCO 36:2	**↑	***↑	***↑	**↓	**↓	NS
PC 36:3	**↑	***↑	***↑	**↓	*↓	NS
PC 36:4	**↑	***↑	***↑	**↓	*↓	NS
PCO 36:4	**↑	***↑	***↑	**↓	*↓	NS
PC 36:5	**↑	***↑	***↑	*↓	NS	NS
PCO 36:5	**↑	***↑	***↑	*↓	NS	NS
PC 38:1	**↑	***↑	***↑	**↓	*↓	NS
PC 38:2	**↑	***↑	***↑	**↓	**↓	NS
PC 38:3	**↑	***↑	***↑	**↓	NS	NS
PC 38:4	**↑	***↑	***↑	**↓	NS	NS
PC 38:5	**↑	***↑	***↑	*↓	NS	NS
PC 38:6	**↑	***↑	***↑	**↓	*↓	NS
PC 40:4	**↑	***↑	***↑	*↓	NS	NS
PC 40:5	**↑	***↑	***↑	**↓	NS	*↑
PC 40:6	**↑	***↑	***↑	**↓	*↓	NS
Lysophosphatidylcholine (LPC)						
LPC 15:0	***↑	***↑	**↑	*↓	NS	**↑
LPC 16:0	***↑	***↑	*↑	**↓	NS	***↑
LPC 16:1	**↑	***↑	NS	*↓	*↑	***↑
LPC 18:0	***↑	***↑	***↑	**↓	NS	**↑
LPC 18:1	**↑	***↑	NS	*↓	NS	***↑
LPC 18:2	**↑	***↑	NS	NS	**↑	***↑
LPC 18:3	**↑	**↑	NS	NS	***↑	***↑
LPC 20:0	***↑	***↑	**↑	NS	NS	**↑

Table 4.4. Continuation.

PL - specie	OA early vs. Healthy	OA late vs. Healthy	RA vs. Healthy	OA early vs. OA late	OA early vs. RA	OA late vs. RA
Lysophosphatidylcholine (LPC)						
LPC 20:3	**↑	***↑	NS	NS	*↑	**↑
LPC 20:4	*↑	***↑	NS	NS	*↑	***↑
LPC 20:5	**↑	**↑	NS	NS	*↑	**↑
LPC 22:0	NS	NS	NS	NS	*↑	**↑
LPC 22:4	NS	NS	NS	NS	**↑	***↑
LPC 22:5	**↑	**↑	NS	NS	**↑	**↑
LPC 22:6	**↑	***↑	NS	NS	*↑	***↑
Phosphatidylethanolamine (PE)						
PE 32:2	NS	NS	**↓	NS	**↑	*↑
PE 34:0	NS	NS	NS	NS	NS	NS
PE 34:1	NS	**↑	***↑	*↓	**↓	NS
PE 34:2	*↑	***↑	***↑	*↓	*↓	NS
PE 36:1	NS	***↑	***↑	*↓	***↓	NS
PE 36:2	**↑	***↑	***↑	*↓	**↓	NS
PE 36:3	**↑	***↑	***↑	NS	NS	NS
PE 36:4	*↑	***↑	***↑	**↓	*↓	NS
PE 36:5	NS	*↑	*↑	NS	NS	NS
PE 38:1	*↑	***↑	**↑	*↓	NS	NS
PE 38:3	*↑	***↑	***↑	*↓	*↓	NS
PE 38:4	*↑	***↑	***↑	**↓	**↓	NS
PE 38:5	*↑	***↑	***↑	*↓	*↓	NS
PE 38:6	*↑	***↑	***↑	*↓	**↓	NS
PE O 38:7	NS	**↑	**↑	*↓	NS	NS
PE O 40:3	NS	NS	***↓	NS	***↑	**↑
PE 40:5	**↑	***↑	***↑	*↓	NS	NS
PE 40:6	*↑	***↑	***↑	*↓	**↓	NS
PE 42:5	NS	NS	NS	NS	*↑	NS
PE 42:7	NS	NS	***↓	NS	***↑	*↑
PE-based plasmalogen (PLPE)						
PLPE 16:0/16:0	NS	NS	**↓	NS	***↑	**↑
PLPE 16:0/16:1	NS	NS	***↓	NS	***↑	***↑
PLPE 16:0/18:0	NS	NS	***↓	NS	***↑	**↑
PLPE 16:0/18:1	NS	**↑	*↑	NS	NS	NS
PLPE 16:0/18:2	NS	**↑	*↑	*↓	NS	NS
PLPE 16:0/18:3	NS	NS	**↓	NS	***↑	**↑
PLPE 16:0/20:3	NS	**↑	NS	NS	NS	*↑
PLPE 16:0/20:4	*↑	***↑	***↑	**↓	NS	NS
PLPE 16:0/20:5	NS	**↑	NS	*↓	NS	**
PLPE 16:0/22:3	NS	NS	**↓	NS	***↑	***↑
PLPE 16:0/22:5	*↑	***↑	**↑	*↓	NS	NS
PLPE 16:0/22:6	***↑	***↑	***↑	**↓	NS	*↑
PLPE 18:0/16:0	NS	NS	*↓	NS	*↑	***↑

Table 4.4. Continuation.

PL - specie	OA early vs. Healthy	OA late vs. Healthy	RA vs. Healthy	OA early vs. OA late	OA early vs. RA	OA late vs. RA
PE-based plasmalogen (PLPE)						
PLPE 18:0/16:1	NS	NS	**↓	NS	**↑	**↑
PLPE 18:0/18:0	NS	NS	***↓	NS	***↑	*↑
PLPE 18:0/18:1	NS	**↑	**↑	*↓	*↓	NS
PLPE 18:0/18:2	*↑	***↑	***↑	*↓	NS	NS
PLPE 18:0/18:3	NS	NS	**↓	NS	**↑	*↑
PLPE 18:0/20:3	NS	**↑	NS	*↓	NS	*↑
PLPE 18:0/20:4	**↑	***↑	***↑	**↓	*↓	NS
PLPE 18:0/20:5	*↑	***↑	**↑	*↓	NS	*↑
PLPE 18:0/22:3	NS	NS	***↓	NS	**↑	*↑
PLPE 18:0/22:5	**↑	***↑	**↑	*↓	NS	NS
PLPE 18:0/22:6	***↑	***↑	***↑	***↓	*↓	*↑
PLPE 18:1/16:0	NS	NS	NS	NS	**↑	**↑
PLPE 18:1/16:1	NS	NS	***↓	NS	***↑	**↑
PLPE 18:1/18:0	NS	NS	***↓	NS	***↑	**↑
PLPE 18:1/18:1	NS	*↑	NS	NS	NS	NS
PLPE 18:1/18:2	NS	**↑	NS	*↓	NS	NS
PLPE 18:1/18:3	NS	NS	***↓	NS	***↑	**↑
PLPE 18:1/20:3	NS	NS	NS	NS	NS	*↑
PLPE 18:1/20:4	*↑	***↑	***↑	**↓	NS	NS
PLPE 18:1/20:5	NS	*↑	NS	NS	NS	*↑
PLPE 18:1/22:3	NS	NS	***↓	NS	***↑	**↑
PLPE 18:1/22:4	NS	NS	*↓	NS	*↑	**↑
PLPE 18:1/22:5	NS	NS	NS	NS	NS	*↑
PLPE 18:1/22:6	*↑	***↑	**↑	*↓	NS	**↑
Phosphatidylglycerol (PG)						
PG 32:0	NS	NS	***↑	NS	***↑	**↑
PG 34:1	NS	NS	**↑	NS	*↓	***↓
PG 36:2	NS	NS	NS	NS	*↓	*↓
Phosphatidylserine (PS)						
PS 36:1	*↑	NS	*↑	*↓	***↓	***↓
PS 36:2	NS	NS	NS	NS	*↓	*↓
Sphingomyelin (SPM)						
SPM 14:0	*↑	***↑	***↑	**↓	*↓	NS
SPM 15:0	**↑	***↑	***↑	**↓	*↓	NS
SPM 16:0	*↑	***↑	***↑	**↓	NS	NS
SPM dih 16:0	*↑	***↑	***↑	**↓	*↓	NS
SPM –OH 16:0	*↑	***↑	***↑	**↓	NS	**↑
SPM 16:1	*↑	***↑	***↑	**↓	NS	NS
SPM –OH 16:1	*↑	***↑	***↑	**↓	*↓	NS
SPM 18:0	*↑	***↑	***↑	**↓	**↓	NS
SPM dih 18:0	NS	**↑	***↑	*↓	*↓	NS

Table 4.4. Continuation.

PL - specie	OA early vs. Healthy	OA late vs. Healthy	RA vs. Healthy	OA early vs. OA late	OA early vs. RA	OA late vs. RA
Sphingomyelin (SPM)						
SPM 18:1	**↑	***↑	***↑	**↓	*↓	NS
SPM 20:0	*↑	***↑	***↑	**↓	NS	NS
SPM 20:1	*↑	***↑	***↑	**↓	*↓	NS
SPM 22:0	**↑	***↑	***↑	**↓	*↓	NS
SPM 22:1	**↑	***↑	***↑	**↓	*↓	NS
SPM 22:2	NS	**↑	***↑	*↓	*↓	NS
SPM 24:0	**↑	***↑	***↑	*↓	*↓	NS
SPM 24:1	*↑	***↑	***↑	*↓	*↓	NS
SPM 24:2	*↑	***↑	***↑	**↓	NS	NS
SPM 24:3	NS	*↑	*↑	NS	NS	NS
Ceramide (Cer)						
Cer 16:0	NS	***↑	***↑	**↓	*↓	NS
GluCer 16:0	NS	***↑	***↑	**↓	**↓	NS
Cer 22:0	NS	***↑	***↑	**↓	**↓	NS
Cer 23:0	NS	***↑	***↑	*↓	*↓	NS
Cer 24:0	**↑	***↑	***↑	**↓	**↓	NS
Cer 24:1	NS	***↑	***↑	**↓	*↓	NS
GluCer 24:1	NS	***↑	***↑	**↓	*↓	NS

Statistical analysis was performed by the non-parametric Kruskal-Wallis test followed by the false discovery rate (FDR) correction. Subsequently, paired Wilcoxon signed-rank test was applied to identify significant changes between each group. *P*-values of less than 0.05 were considered statistically significant with: *$0.01<p\leq0.05$; **$0.001<p\leq0.01$; ***$p<0.001$; NS-not significant. ↑:increase ↓:decrease. Chosen individual PL species are presented in Figures 4.8-4.13. PC-phosphatidylcholine, PC O-ether phosphatidylcholine, LPC-lysophosphatidylcholine, PE-phosphatidylethanolamine, PLPE-phosphatidylethanolamine-based plasmalogen, PG-phosphatidylglycerol, PI-phosphatidylinositol, PS-phosphatidylserine, SPM-sphingomyelin, Cer-ceramide, GluCer-glucosylceramide, OA-osteoarthritis, , RA-rheumatoid arthritis.

Table 4.5. FA chain length according to the number of C-atoms in a chosen PL class.

PC	**30**	**32**	**34**	**36**	**38**	**40**	**≤ 34**	**> 34**	**≤ 36**	**> 36**
Healthy	1.4%	8.8%	39.4%	33.3%	15.0%	2.2%	49.6%	50.5%	82.9%	17.2%
OA-early	0.7%	5.1%	36.4%	36.6%	18.5%	2.7%	42.2%	57.8%	78.8%	21.2%
OA-late	0.6%	4.2%	36.1%	37.0%	19.2%	2.9%	40.9%	59.1%	77.9%	22.1%
RA	0.4%	3.9%	38.3%	37.0%	17.7%	2.7%	42.6%	57.4%	79.6%	20.4%
PC-O	**30**	**32**	**34**	**36**	**38**	**40**	**≤ 34**	**> 34**	**≤ 36**	**> 36**
Healthy	3.1%	14.2%	34.6%	48.1%	/	/	51.9%	48.1%	100%	/
OA-early	1.9%	11.4%	30.6%	56.1%	/	/	43.5%	56.1%	99.6%	/
OA-late	1.4%	10.4%	29.7%	58.4%	/	/	41.5%	58.4%	99.9%	/
RA	0.9%	11.5%	30.6%	57.1%	/	/	43.0%	57.1%	100.1 %	/
PC+PC-O	**30**	**32**	**34**	**36**	**38**	**40**	**≤ 34**	**> 34**	**≤ 36**	**> 36**
Healthy	1.5%	9.2%	39.0%	34.5%	13.8%	2.0%	45.2%	50.3%	84.2%	15.8%
OA-early	0.8%	5.5%	36.1%	37.8%	17.4%	2.5%	42.4%	57.7%	80.2%	19.9%
OA-late	0.6%	4.6%	35.7%	38.3%	18.0%	2.8%	40.9%	59.1%	79.2%	20.8%
RA	0.5%	4.5%	37.8%	38.3%	16.5%	2.5%	42.8%	57.3%	81.2%	19.0%
PE	**30**	**32**	**34**	**36**	**38**	**40**	**≤ 34**	**> 34**	**≤ 36**	**> 36**
Healthy	/	2.2%	6.3%	12.7%	28.7%	50.1%	8.5%	91.5%	21.2%	78.8%
OA-early	/	1.4%	6.7%	16.0%	35.6%	40.2%	8.1%	91.8%	24.1%	75.8%
OA-late	/	2.7%	7.9%	16.0%	38.6%	34.8%	10.6%	89.4%	26.6%	73.4%
RA	/	0.8%	8.8%	24.2%	43.6%	22.7%	9.6%	90.5%	3.8%	66.3%
PLPE	**30**	**32**	**34**	**36**	**38**	**40**	**≤ 34**	**> 34**	**≤ 36**	**> 36**
Healthy	/	3.5%	15.7%	33.6%	33.9%	13.5%	19.2%	81.0%	21.2%	47.4%
OA-early	/	2.2%	12.5%	30.1%	41.9%	13.3%	14.7%	85.2%	24.1%	55.1%
OA-late	/	1.9%	11.3%	31.4%	42.6%	12.8%	13.2%	86.8%	26.6%	55.4%
RA	/	0.8%	11.1%	32.4%	43.5%	12.3%	11.9%	88.2%	33.8%	55.8%
PI	**30**	**32**	**34**	**36**	**38**	**40**	**≤ 34**	**> 34**	**≤ 36**	**> 36**
Healthy	/	5.4%	11.3%	31.0%	52.3%	5.4%	11.3%	88.7%	42.3%	57.7%
OA-early	/	5.8%	7.8%	25.0%	61.4%	5.8%	7.8%	92.2%	32.8%	67.2%
OA-late	/	5.3%	8.2%	23.0%	63.5%	5.3%	8.2%	91.8%	31.2%	68.6%
RA	/	4.7%	9.0%	25.2%	61.1%	4.7%	9.0%	91.0%	34.2%	65.8%

PC species were analysed by ESI-MS/MS in 9 healthy SF, 19 OA SF from early stage, 14 OA SF from late stage and 18 RA SF. The values represent the percentage of PL species with the respective FA chain length of total PL class (=100%). Statistical analysis is presented in table 6.3. OA-osteoarthritis, PC-phosphatidylcholine, PC O-ether phosphatidylcholine, PE-phosphatidylethanolamine, PLPE-phosphatidylethanolamine-based plasmalogen, PI-phosphatidylinositol, RA-rheumatoid arthritis.

4.3 Phospholipid composition of healthy and OA canine SF

In order to confirm whether a canine OA-model represent our finding from human SF, lipids were isolated from canine SF obtained from 7 healthy and 7 contralateral OA-induced knee joints.

Similarly to human SF, PC is the most abundant PL class present in canine SF, accounting for to 77% of all PLs present (Figure 4.14). In contrast to the data obtained from human samples, ESI-MS/MS analysis of canine SF revealed no significant differences between surgically induced OA-like knee joints and contralateral healthy control joints. However, the observed slight differences in the canine model of OA are similar with respect to their trends to those obtained from humans SF, where most of PL classes were present in higher concentrations in OA SF when compared to healthy SF (Figure 4.14). Data from individual PL species are not shown.

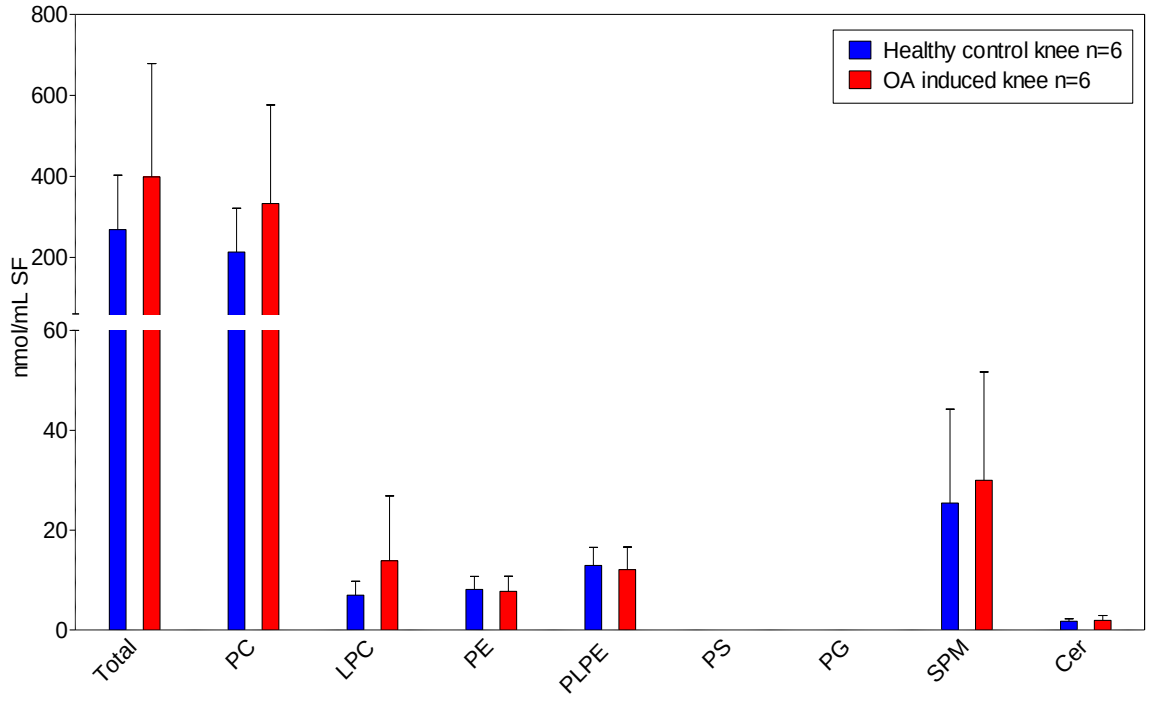


Figure 4.14. PL classes in healthy and contralateral canine OA SF. The different PL classes were quantified by ESI-MS/MS. The displayed values were corrected for possible dilution using the dilution factor (DF) obtained by the determination of the urea concentrations. SF was obtained from healthy, control knee (blue bars) and contralateral knee with induced OA (red bars). Values represent mean ± SD from 6 dogs. Shown PLs are phosphatidylcholine (PC), lysophosphatidylcholine (LPC), phosphatidylethanolamine (PE), phosphatidylethanolamine-based plasmalogen (PLPE), phosphatidylserine (PS), phosphatidylglycerol (PG), sphingomyelin (SPM), and ceramide (Cer). OA-osteoarthritis.

4.4 Effect of joint distraction on composition of PL in SF

According to the data published elsewhere, joint distraction used to treat human OA may be of long-term clinical benefits with respect to both, symptoms and structure modification [180]. Therefore, induction of OA was performed in the group of 9 dogs followed by joint distraction. The effect of joint distraction on PL composition was evaluated by determining potential changes between OA induced knee joint and OA induced knee followed by joint distraction.

As shown in Figure 4.15, the concentrations of PE and PLPE were significantly elevated (161.8 ± 87.5 % for PE and 168.6 ± 90.5 % for PLPE) in distracted joints compared to untreated OA joints (=100%). Other PL classes remained within the control level since the delta between treated and control knee joints was near 0.

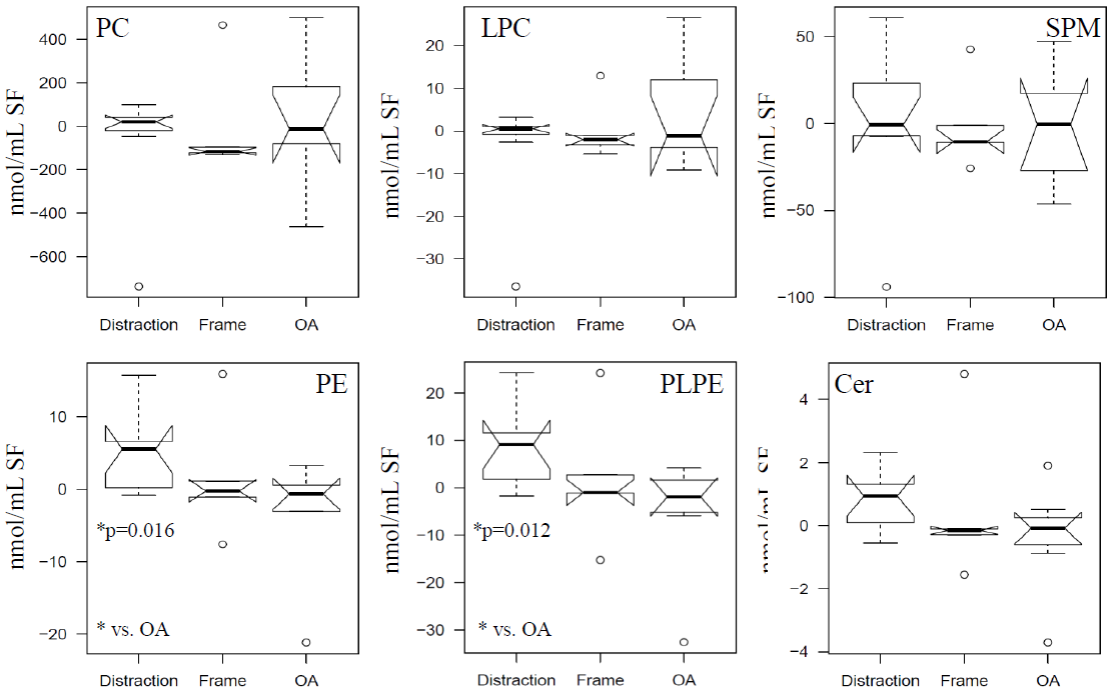


Figure 4.15. Effect of joint distraction on PL composition of OA SF. The different PL classes were quantified by ESI-MS/MS in SF obtained from healthy, control knee (n=21), OA knee without treatment (OA group; n=7), OA framed knee (frame group; n=5) and OA distracted knee (distraction group; n=9). Quantitative PL values were corrected for possible dilution using the dilution factor (DF) obtained by the determination of the urea concentrations. The displayed values are shown as delta between treated and healthy, control knee joints of the same dog. The notched boxplots show 95% confidence intervals for the median. Circles indicate outliners. Statistical analysis was performed by the non-parametric Kruskal-Wallis test followed by the false discovery rate (FDR) correction. Subsequently, paired Wilcoxon signed-rank test was applied to identify significant changes between each groups. *P*-values of less than 0.05 were considered statistically significant. PC-phosphatidylcholine, LPC-lysophosphatidylcholine, SPM-sphingomyelin, PE-phosphatidylethanolamine, PLPE-phosphatidylethanolamine-based plasmalogen, Cer-ceramide, OA-osteoarthritis.

4.5 Composition of PL in various mammalian species

In order to elucidate potential differences between various mammalian species and to answer the question whether PL composition of healthy SF is species-dependent, lipids were extracted from 9 human SF, 21 canine SF and from 14 equine SF and ESI-MS/MS analysis was performed. All subjects were characterised by the lack of arthritic disease history in the knee of interest. Almost all of PL species present in human SF were also detected in canine and equine SF (data not shown). The relative distribution show approximately equal levels of PL classes in all investigated groups (Figure 4.16). However, quantitative data revealed significant changes between several PL classes (Figure 4.17 and Table 6.4). Interestingly, the PC levels did not differ between humans, dogs and horses.

Additionally, human, dogs and horses present similar PL species with the exception of LPC where only 4 species were determined in equine SF. In contrast, human and canine SF possess 15 different LPC species (data not shown). Moreover, decreased concentrations of LPC, PE, PLPE and Cer were found in equine SF as compared to human and canine SF (Figure 4.17).

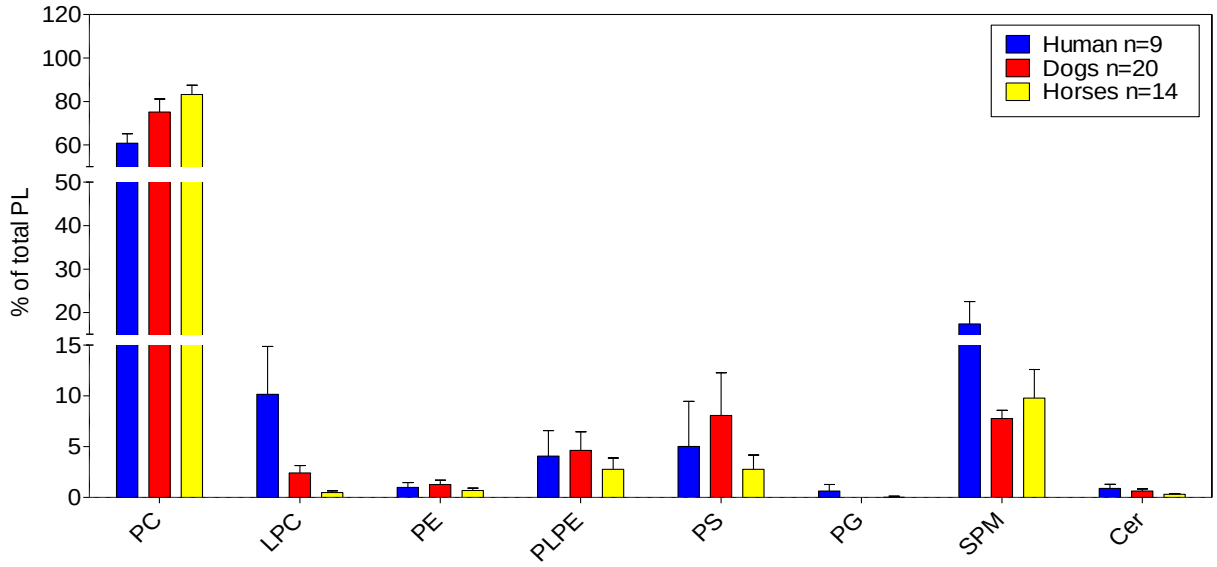


Figure 4.16. Relative distribution of PL classes in SF of various mammalian species. The different PL classes were quantified by ESI-MS/MS in healthy human SF (n=9; blue bars), healthy canine SF (n=20; red bars) and healthy equine SF (n=14; yellow bars). The displayed values are shown as a percentage of the respective PL class of all analysed PL (=100%) and are expressed as mean ± SD. Cer-ceramide, LPC-lysophosphatidylcholine, PC-phosphatidylcholine, PE-phosphatidylethanolamine, PG-phosphatidylglycerol, PL-phospholipids, PLPE-phosphatidylethanolamine-based plasmalogen, PS-phosphatidylserine, SPM-sphingomyelin.

The concentrations determined for equine SF were decreased down to 3 ± 1.7% for LPC, 26.6 ± 16.5% for PE, 43.5 ± 18.9 % for PLPE and 22.1 ± 7.7% for Cer when compared to human SF (=100%), and 8 ± 4.4% for LPC, 20.4 ± 9.2% for PE, 29 ± 12.6 % for PLPE and 27 ± 9.4% for Cer when compared to canine SF (=100%). It is also worth to note, that PL composition is comparable in human and canine SF.

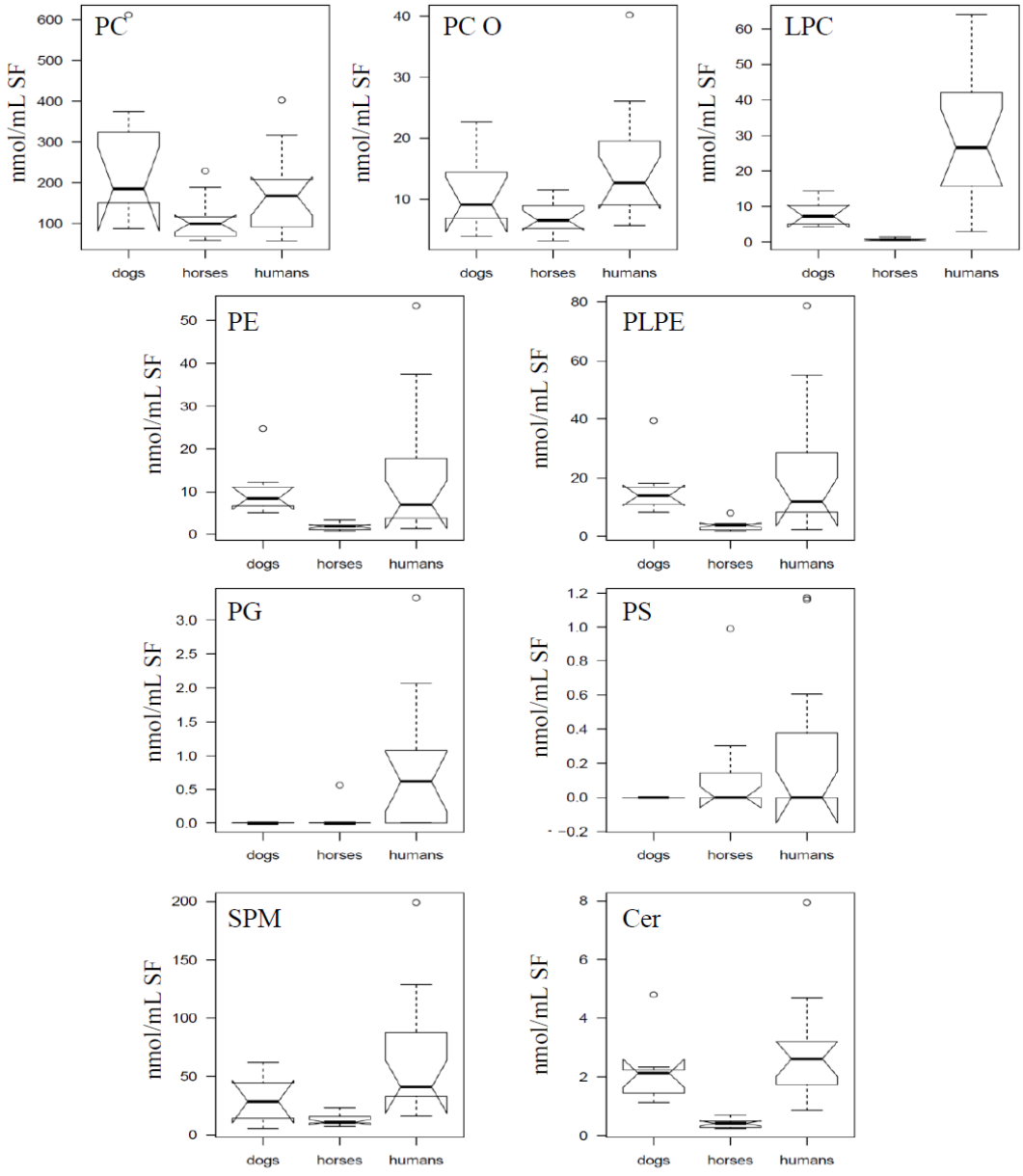


Figure 4.17. PL classes in SF of various mammalian species. The different PL classes were quantified by ESI-MS/MS in healthy human SF (n=9), healthy canine SF (n=20) and healthy equine SF (n=14). The notched boxplots show 95% confidence interval for the median. Circles indicate outliners. Statistical analysis is presented in table 6.4. PC-phosphatidylcholine, PC O-ether phosphatidylcholine LPC-lysophosphatidylcholine, PE-phosphatidylethanolamine, PLPE-phosphatidylethanolamine-based plasmalogen, PG-phosphatidylglycerol, PS-phosphatidylserine, SPM-sphingomyelin, Cer-ceramide.

4.6 Fibroblast-like synoviocytes cell culture

4.6.1 Optimisation of FLS culture conditions

To optimise FLS culture conditions in order to measure synthesis and/or release of PLs, the different cell culture conditions were studied. FLS were cultured with 15 ml D-MEM medium supplemented with 10% FCS, 1% penicillin/streptomycin and 1% HEPES. Subsequently, cells were plated onto 4- or 6-well plates at a density 80.000 cells/well. After cells reached 90% confluency, medium was exchanged and cells were starved in serum-free medium for 16 h. Further, medium was exchanged and cells were cultured for 24 h in serum-free medium at conditions as follow:

1. Supplemented with 1% of ITS™.
2. Supplemented with 10-5 mol/ml dexamethasone
3. FLS were cultured in 6-well plates coated with 50µl/cm^2 BD Matrigel™ Basement Membrane Matrix Growth Factor Reduced.
4. FLS were cultured in 4-well plates under shear stress conditions on horizontal shaker (5 rpm).

Dexamethasone has been previously described as a drug which may be able to induce PLs synthesis in equine knee joint [123]. Therefore, the effect of dexamethasone on the PLs synthesis and/or release by FLS was also investigated. However, only culture on Matrigel™ Basement Membrane which enabled a 3-dimension culture of FLS (Figure 4.18) slightly increased the total PL concentration as compared to blank medium from 0.68 ± 0.062 to 1.5 ± 0.12 nmol/ml/2x10^{5}cells (Figure 4.19). These preliminary data indicate that PLs were synthesised and/or released by FLS. However, all obtained values from all analysed sampled were near the limit of quantification.

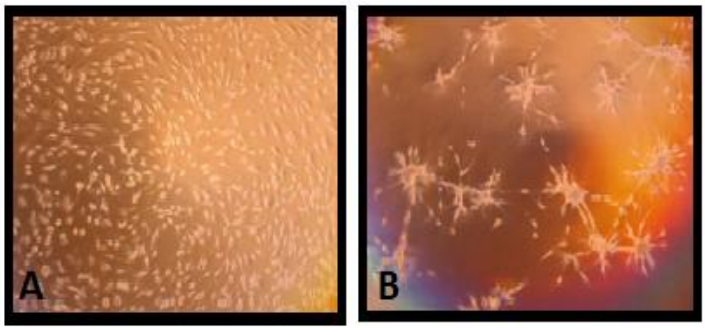


Figure 4.18 Effect of three-dimensional FLS culture on cell morphology. A–FLS growing in monolayer, **B**-FLS growing in 3-D culture. FLS cells were cultured in 6-well plates coated with 50 µl/cm^2 BD Matrigel™ Basement Membrane Matrix Growth Factor Reduced for 24 h. Cell morphology was examined using light microscopy (magnifications 50x). FLS-fibroblast-like synoviocytes.

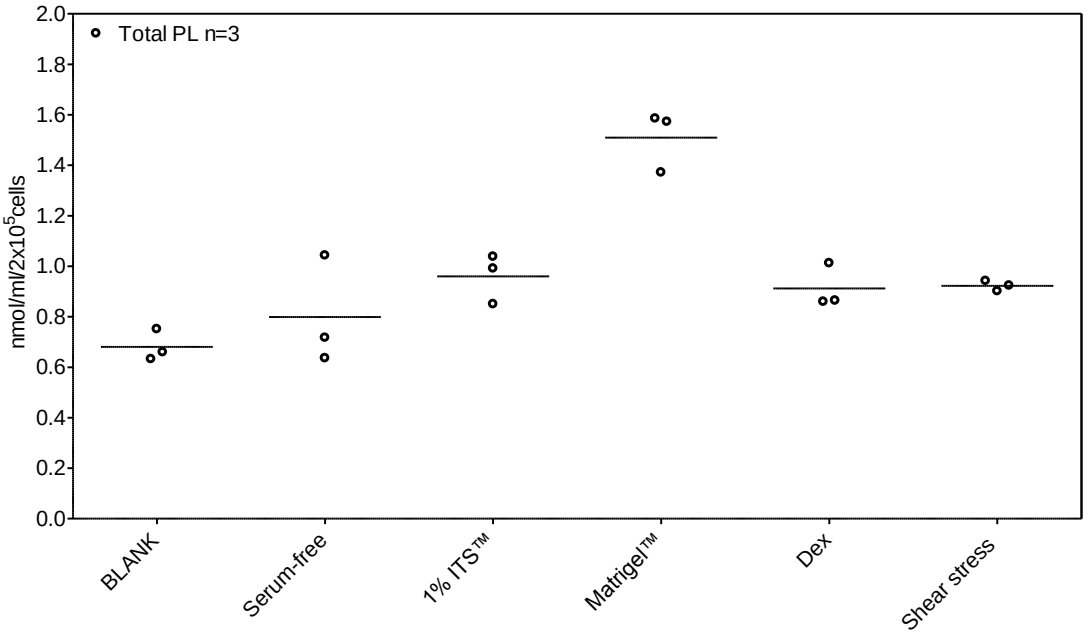


Figure 4.19. Concentrations of total PL in nutrient media from FLS culture. The presented results were obtained after 24 h using cells from different cell cultures. PL were quantified by ESI-MS/MS and normalised to number of cells investigated (200.000). Each dot represents individual wells with different cell preparations. PL-phospholipids, ITS™+Premix-Insulin-Transferrin-Selenium, Dex.-Dexamethasone.

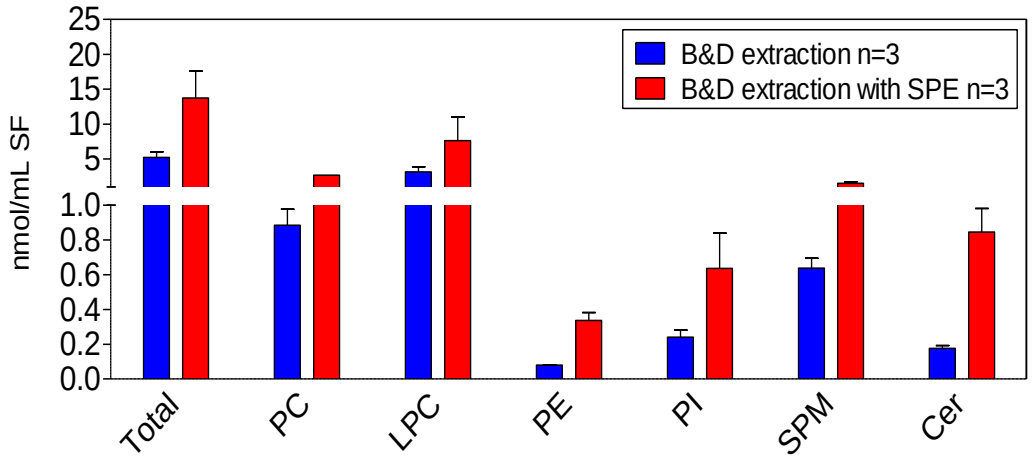
4.6.2 Development of a method for PL detection in cell culture media

The results of our preliminary experiments indicate that FLS synthesise and/or release PL. Nonetheless, the concentrations of all PL classes were very low. Our next aim was to prove, that the ESI/MS-MS method was suitable to determine PL quantitatively in FLS culture media. In order to concentrate and purify PLs present in media sample, SPE was performed as outlined in Material & Methods section.

The data obtained of SPE purification, indicated a 2-4-folds increase in each PL class analysed in lipid extracts acquired after SPE, compared to lipid extracts from standard Bligh & Dyer method (Figure 4.20 A).

To further analyse PL composition of each obtained SPE fraction and to address the question, whether SPE is a suitable method to purify PLs present in media samples, all four fractions were analysed separately by ESI/MS-MS. Accordingly to a previously published protocol [167], PLs should be present in fraction 3 containing neutral PLs and in fraction 4 containing acidic PLs. As expected, fraction 3 and 4 contained the highest amount of detected PL (Figure 4.20 B). However, in fraction 1, which according to literature contains neutral lipids [167], a high amount of Cer was detected. In fraction 2, which contains free FA, no detectable amounts of PLs were found.

A.



B.

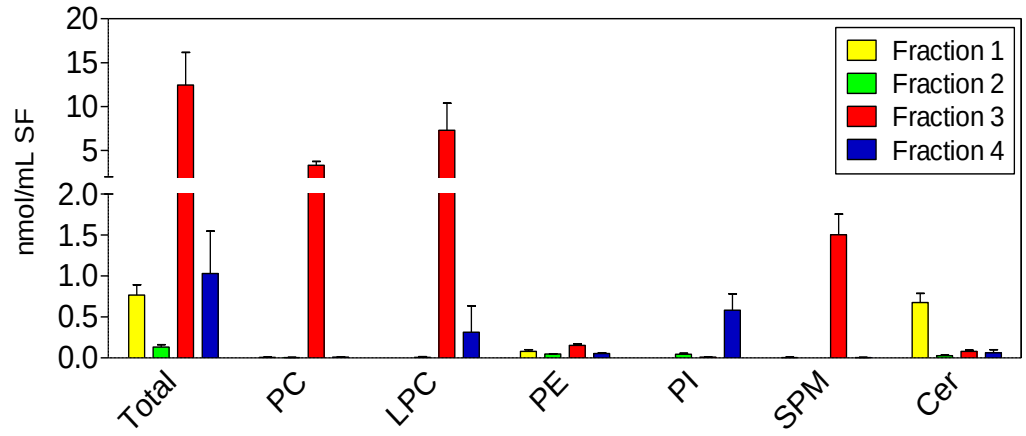


Figure 4.20. Purification of PL in FLS cell culture media using SPE. Panel A compares PL classes present in nutrient media after B&D lipid extraction and B&D lipid extraction followed by SPE. Panel B shows PL composition in each extracted fraction after SPE. Values are presented as mean ± SD from three independent FLS cell preparations. PC-phosphatidylcholine, LPC-lysophosphatidylcholine, PE-phosphatidylethanolamine, PI-phosphatidylinositol, SPM-sphingomyelin, Cer-ceramide, B&D-lipid extraction according to Bligh and Dyer, SPE-solid phase extraction.

4.7 Detection of surfactant proteins in human knee joints

4.7.1 Western blot analysis of surfactant proteins in human SF

In order to assess whether human SF contains surfactant proteins, Western blot analysis was performed. SP-A, -B, -C and -D protein expression was investigated by immunoblotting of SF, albumin-free SF, HA-free SF and lung lavage using specific antibodies (Table 2.5).

Mouse monoclonal anti-human SP A (Dako Deutschland GmbH) revealed two bands. In the lung samples, one of the bands had an apparent molecular weight of 37 kDa (Figure 4.21). Antibody to SP-B stained only one band in the lung lavage samples with molecular weight of 15 kDa, with a clear lack of SP-B in SF samples. We could detect SP-C neither in lung lavage nor in SF samples. SP-D antibody revealed one band in lavage lung samples with a molecular weight of 47 kDa. Bands detected by SP-A and SP-D antibodies in SF samples did not have the expected molecular weight. Hence, expression of SP-A and SP-D was further analysed using a 2D-gels system. Detected proteins were identified using MALDI-TOF/MS after in gel trypsynization. Expression of SP-B and SP-C was not further investigated.

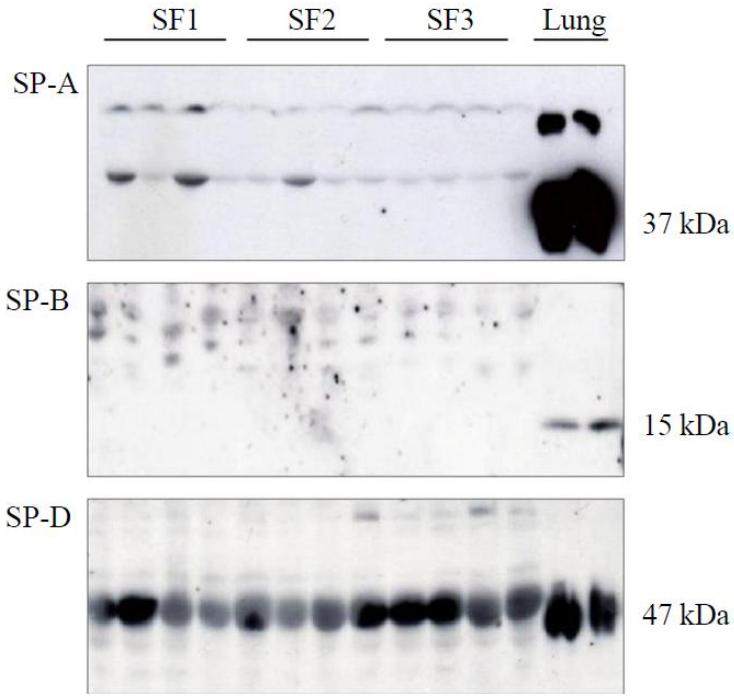


Figure 4.21. Detection of SP-A, SP-B and SP-D on immunoblots. SF and lung lavage was prepared and separated on SDS-PAGE. Immunoblotting with specific antibodies was performed. For each antibody used, one representative blot out of 3-5 is shown. SF-synovial fluid, SF 1-albumin-free synovial fluid, SF 2-hyualuronic acid-free synovial fluid, SP-A-surfactant protein A, SP-B-surfactant protein B, SP-D-surfactant protein D.

4.7.2 MALDI-TOF/MS protein identification

Analysis of Western blots indicated, that SP-A and SP-D might be present in human SF. In order to confirm, whether the proteins detected by the antibodies on immunoblots are indeed surfactant proteins, additional two-dimensional gel electrophoresis was performed using the same antibodies (Figure 4.22). A second gel electrophoresis was performed using the same samples and conditions, and the gel was stained with Coomassie blue. Both gels were compared and the corresponding spots to those showing immunoreactivity with specific antibodies were excited. Subsequently, proteins present in selected spots were identified by MALDI-TOF/MS.

As presented in Table 4.6 none of investigated spots contained surfactant proteins. Most of the proteins detected by immunoblots were albumins. These results confirmed previous findings, that human SF does not contain surfactant proteins.

The finding obtained from Western blot analysis as well as from protein identification by MALDI-TOF/MS was further validated by RT-PCR analysis as described further in section 4.7.3.2.

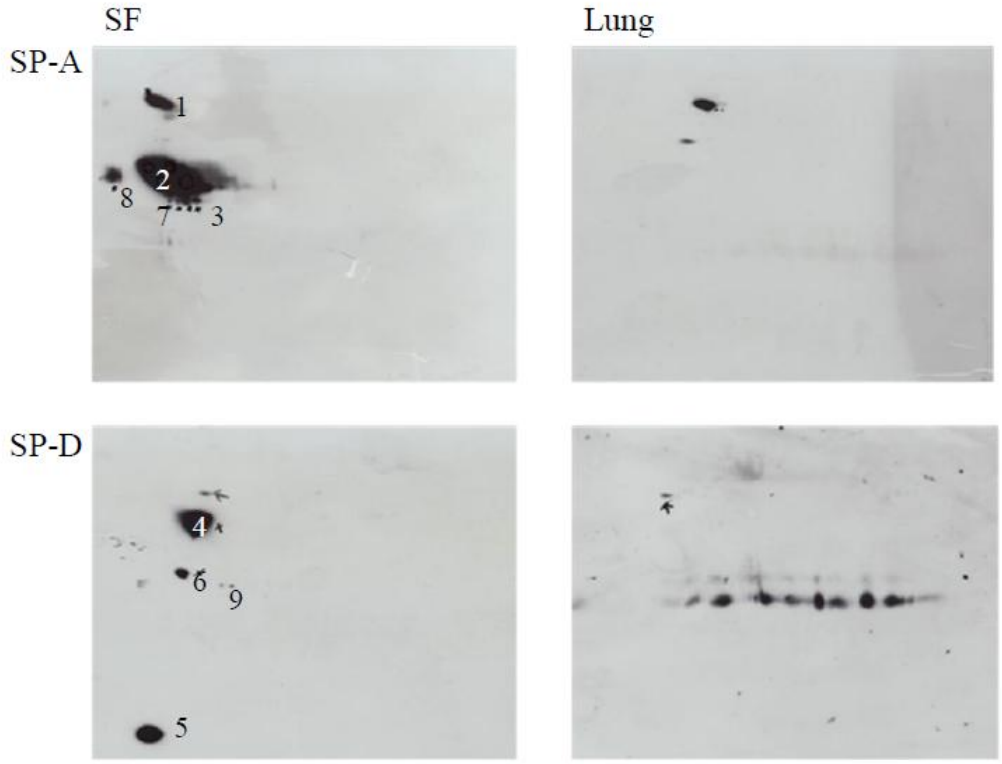


Figure 4.22. Detection of SP-A and SP-D on immunoblots after 2D-PAGE. SF and lung lavage were prepared and separated on 2D-PAGE. Immunoblotting with specific antibodies was performed. Picture is representative of two independent experiments. SF-synovial fluid, SP-A-surfactant protein A, SP-D-surfactant protein D. Number #1-#9-excised spots.

Spot	Identified protein
1	Serum albumin, chain A-Human
2	Serum albumin-Human
3	Serum albumin-Human
4	Serum albumin-Human
5	Hypothetical protein DKFZp686M04163-Human
6	Fibronectin 1.-Human
7	Fibronectin 1.-Human
8	Alpha-2-macroglobulin precursor-Human
9	Interferon regulatory factor 5 variant 11-Human

Table 4.6. Proteins identified with MALDI-TOF/MS from the spots excised from 2D gels which correspond to those spots identified by their immunoreactivity.

4.7.3 Expression of surfactant proteins

To support our previous observation and further investigate whether human SF does not contain surfactant proteins, semi-quantitative PCR was performed. For this purpose total RNA was isolated from all the tissues of synovial joint which might be a source for production of SF components such as cells dispersed in SF, synovial membrane, cartilage and from cultured FLS.

4.7.3.1 Total RNA isolation from human cartilage

Healthy human as well as OA cartilage has a low cell content, which is 1-2% of the total cartilage mass [181]. The remaining 98-99% is composed of water and extracellular matrix with a high concentration of aggrecan, hence, isolation of total cartilage RNA is challenging. To obtain appropriate amount of good quality total RNA and to perform reverse transcription, three independent methods were applied. Using commercial available kits RNAquenous™ (Ambion Inc.) and PeqGOLD Total RNA (Peqlab Biotechnologie GmbH), or a modified phenol-chloroform method with use of PeqGOLD TriFast™ (Peqlab Biotechnologie GmbH). Concentration of isolated RNA was determined spectrophotometrically at 260 nm (Figure 4.23). Appropriate amount of RNA was obtained using the modified phenol-chloroform method of the PeqGOLD TriFast™. This RNA was used for further analysis.

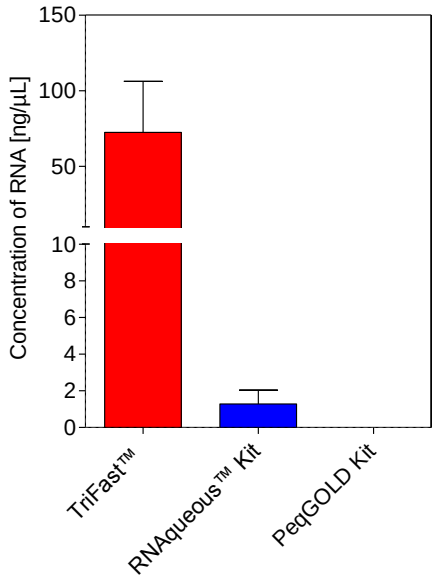


Figure 4.23. Total RNA isolated from human cartilage. Total RNA was isolated from human OA cartilage obtained from three different patients using commercially available kits: RNAqueous™ and PeqGold Total RNA or a modified phenol-chloroform method using PeqGOLD TriFast™. The concentrations of isolated RNA were determined spectrophotometrically at 260 nm.

4.7.3.2 Expression of surfactant protein analysed by RT-PCR

RNA from cells dispersed in SF, cultured FLS, synovial membrane and the cartilage was isolated with a modified phenol-chloroform method using peqGOLD TriFast™. Subsequently, obtained RNA were used to perform transcription to cDNA. Gene expression profiles of SP-A and SP-D was analysed by RT-PCR using 2 µl of cDNA obtained from cells and tissue mentioned above as well as healthy lung. GAPDH serves as a reference gene for all samples. GAPDH mRNA was detected in all analysed samples (Figure 4.24).

SP-A and SP-D mRNA was only detected in healthy lung samples which served as positive control. Electrophoresis revealed a single band with the expected molecular weight (270 bp for SP-A and 321 bp for SP-D). Analysed cells derived from the synovial joint tissues did not express detectable mRNA levels of SP-A or SP-D (Figure 4.24). Samples without cDNA template were used as a negative control. In those samples no product was detected. In the line with Western blot analysis, it was concluded that SP-A and SP-D are absent in human SF.

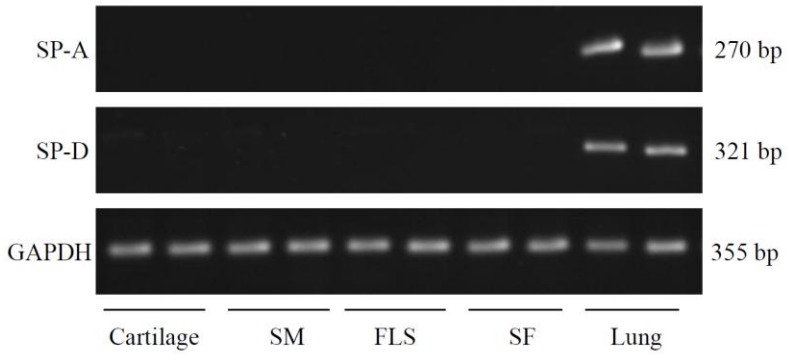


Figure 4.24. Agarose gel of SP-A and SP-D in human joint tissues, FLS and healthy lung. RT-PCR was performed for human OA cartilage (n=3), SM (n=3), cultured FLS (n=3), cells dispersed in SF (n=3), and healthy lung (n=2). In negative control, where no cDNA template was added, no product was observed. GAPDH served as reference gene. Displayed pictures are representative of three independent experiments. bp-base pairs, FLS-fibroblast-like synoviocytes, GAPDH-glyceraldehyde 3-phosphate dehydrogenase, SF-cells dispersed in synovial fluid, SM-synovial membrane, SP-A-surfactant protein A, SP-D-surfactant protein D.

5. Discussion

Our research focus on SF components which can contribute to the boundary lubrication, their changes due to the development of OA and RA and possible physiological and pathophysiological role in normal and OA joints. Of particular interest for this study was the analysis of the composition of SF PL and changes in quantities of individual PL species present in SF from different stages of OA.

5.1 Problems and limitations

Healthy knee joints contain only about 0.5-4 ml of SF. Healthy SF is also very viscous, thus, it is difficult to aspirate SF completely from the joint. Due to ethical restrictions to obtain SF from healthy individuals, we used post-mortem SF from human cadavers for our study. Previous biochemical analysis of post-mortem SF revealed a stable content of calcium, chloride, urea and creatinine up to 5 days after death [182]. We performed similar correlations this time focusing on the concentrations of PL classes and main PL species. Our results show that the concentrations of PL classes and even PL species remained unchanged up to 5 days after death. Our data indicate that SF obtained during this interval can be included in our study.

Extracellular microvesicles, in literature often called MPs represent a heterogeneous population of membrane-bound small vesicles released from the cells by exocytotic blebbing during cell activation or early apoptosis. MPs are thought to have a function in the development of many human diseases including RA, where they trigger FLS to produce pro-inflammatory mediators (for review see [69, 183]). It is known that MPs are present in SF and contain cell membranes composed out of PLs. However, the aim of our study was to determine only extracellular PLs which may contribute to the boundary lubrication in synovial joints. Therefore, MPs were separated from SF samples with a method described elsewhere [67]. PS is known to be the main PL present on the surface of MPs [69, 183-184]. Since we determined PS as the only one PL class present at higher concentration in the MPs fraction as compared to other fractions of SF, and as cell-, cell-debris and MPs-free fraction of SF contained very negligible amount of PS, it seemed that separation of MPs from SF was effective.

One of the clinical signs of OA can be the influx of water into the joint cavity due to effusion, resulting in increased total volume of SF of up to 20-fold, therefore, measurements per unit volume, are often disturbed by variable dilution of SF. In order to eliminate unknown dilution of SF, we were searching for a method to correct our results. Our results about the concentrations of β2-MG indicated that there are independent from the total volume of SF, thus, β2-MG is not a suitable factor to correct for any dilution of SF. A recently published method used urea to compensate for various dilution of SF [177-178]. Urea is produced in the liver and is neither synthesised nor metabolised by joint tissues. This small molecule is transported across synovium through unrestricted diffusion, thus, it is a good marker of unknown dilution [185]. Since our correlation analysis of SF and serum concentrations of urea is in accordance with data published previously, we used the method described above to correct our quantitative values of PL obtained from ESI-MS/MS analysis for any possible dilution due to effusion.

Synovial membrane is a permeable, living "dialysis" membrane which excludes large molecules from SF while allowing smaller components to cross the synovium in both directions and freely equilibrate between SF and serum. It has been shown that concentrations of small molecules such as electrolytes, glucose, uric acid and antibiotics are equal in SF and serum [186]. Unfortunately, sampling of SF often occurs several days after effusion. It is plausible that during longer time of effusion, the concentration of urea equilibrate by diffusion with the concentration of the urea present in serum. Supposing that described equilibrium had occurred in some knee joints of patients included in the present study, concentrations of the urea determined in those SF samples would be overestimated. Subsequently, this would lead to under-calculated dilution factor, and by that fact quantitative values of PL classes and species should be higher in OA and RA SF then reported here. Analysis of PL species in individual patients revealed that in certain cases the determined concentrations were several fold higher after correction, and in other cases we did not observe any differences between corrected and non-corrected values. However, according to our knowledge the use of urea to compensate possible dilution of SF is up to date the best available method. Nevertheless, possible calculation error would not severely change results presented in this thesis.

The reproducibility of our data was confirmed by a second independent analysis where a different batch of samples, different time point, changed instrument settings and other preparation of internal standards were applied. Statistical analysis of the second analysis revealed decreased number of statistical significant differences as compared to the first one. An explanation for it can lie in a lower number of patients included in the second analysis. However, the trends of changes found in both analyses were mostly the same indicating good reproducibility.

5.2 Boundary lubrication provided by PL

Only few components were identified in SF as possible factors contributing to boundary lubrication namely lubricin, HA and SAPLs [63, 84, 187]. Various theories about which of them is mainly responsible for boundary lubrication provided by SF have been advanced. Originally, the main boundary lubricant was thought to be HA, however, further investigations indicated that HA has several properties inconsistent with serving as an active boundary lubricant [63, 81]. It has been shown, that the major function of HA is not lubrication *per se* but rather providing a high viscosity to SF. Indeed, high concentrations of HA makes SF very slippery, but does not provide boundary lubricating ability to SF [81-82].

The question whether lubricin or SAPLs provide main boundary lubrication of SF is a controversial subject under intensive discussion since at least 43 years [188]. Several investigations demonstrated that enzymatic digestion with trypsin destroyed the ability of SF to lower friction on the surfaces of articular cartilage. Therefore, it was concluded that boundary lubrication is related to the protein present in SF, most probably, lubricin [189-190]. Jay *et al* [90, 95, 191] in many of his reports support the concept that synovial boundary lubrication is a function of lubricin. According to the theory of boundary lubrication from 1936 [192], the lubricant must be reversibly bound to the surface. Since the surface of articular cartilage is highly hydrophobic, it was proposed that the major role in lubrication is assigned to SAPLs which possess hydrophilic head groups. Enzymatic digestion of SF with phospholipase C (PLC) resulted in an elimination of the lubricating ability of SF [191, 193]. However, PLC might possess trypsin-like activity, hence, may digest proteins as well. To further confirm that lipid component of SF is responsible for boundary lubrication, similar experiments were performed using highly purified PLA_2. Enzymatic digestion of SF with PLA_2 resulted also in increased friction coefficient in a

concentration-dependent manner [81]. It is known, that PLC and PLA_2 affect mostly PC, however, it is not yet specified whether PC provides most of the boundary lubricating properties of SF. The lipid solvent, which has been shown to efficiently remove all PL present on the surfaces of tendon further increased the effect in a wear test [193]. This suggests that PL other than PC may also provide lubricating abilities. If we take all of the above mentioned findings into consideration, it seems that SAPLs strongly contribute to the boundary lubrication in synovial joints. Lubricin as a water-soluble component of SF, acts as the carrier for insoluble SAPLs [194], hence, lubricin digestion also results in an increased friction [191].

Although a growing number of studies have investigated the role, function and chemical structure of HA and lubricin [63, 80, 96, 120, 195-196], little is known about the composition, molecular structure and source of SAPLs present in SF. Besides free cholesterol, which is known to be the main lipid in most human tissues and fluids, 9 different lipid classes were identified in SF. The major group of PL present in SF are PL containing choline namely PC, LPC and SPM. Several studies which focused on PL composition in synovial joints revealed similar results [51, 128, 187]. PC was the main PL class found, covering approximately half of all present PL. The second main PL class was SPM. PE was shown to cover about 30% of total PL [128]. However, our results differ from this previous finding. We determined only a small amount of PE which was approximately 1.5% of total PL. An explanation for the observed differences can lie in the source of material used, since the previous investigation [128] as well as the others [51, 187] were performed using PLs harvested from the superficial surface of articular cartilage. A more recent study using MALDI-TOF/MS analysis show, that the main classes present in human SF are PC, LPC and SPM which corroborates with our findings [197]. Recently, Hills *et al*. [138] demonstrated that the articular surface in joint afflicted with OA is deficient in SAPLs. However, we determined that during development of OA, total PL content as well as concentrations of main PL classes are increased in SF. As an explanation for this discrepancy, one might put forward that again it is this different composition of PL within SF and PL harvested from the surfaces of articular cartilage [198].

In the publication from 1953 [186] Ropes and Bauer reported that an increased accumulation of synovial lipids appears in those joints, where excessive tissue destruction occurs. There are several evidences [189, 199-200] to support this observation. It has been shown that SF of RA contains severely increased content of PL, whereas normal SF

contains small amounts of lipids. Therefore, it was concluded, that increased levels of PL for instance SPM, LPC and PI contribute to and may stimulate the inflammatory processes during development of RA [201-203]. Our results are in accordance with the previous reports, since we determined higher concentrations of major PL classes including PC, LPC, SPM and Cer in OA SF and RA SF compared to healthy SF.

Results of our study show, that the percentage composition of PL in normal, OA and RA SF is comparable. In addition, the ratios of PL classes were calculated to investigate the relationship between PL classes. Previously published results indicated that the PC/LPC ratio in SF might be a good diagnostic marker for RA [142]. The ratio of PC/LPC was decreased in patients with RA as compared to healthy individuals [142]. In contrast to this previous observation [142], we found a significant increase of PC/LPC ratio in RA SF compared to healthy SF and OA SF. An explanation for this discrepancy can lie in the applied mass spectrometry technique, since in the previous study MALDI-TOF/MS, which is at best a semi-quantitative method, was used. The SMP/Cer ratio remained unchanged in all investigated group due to increased concentrations of both those classes, the SMP and Cer, in OA SF and RA SF as compared to healthy SF.

In summary, total PL content and concentrations of major PL classes are increased in SF from late stage of OA compared to healthy SF and SF from early stage of the disease. Currently, the mechanisms lying behind these changes are not known. We can only assume the reasons for these results:

1. SAPLs are considered to build a bio-film on the surfaces of articular cartilage [131-132, 204]. During the development of OA, the superficial zone of articular cartilage is getting destructed. It might be possible, that during this process SAPLs bound to the surfaces of articular cartilage are released and therefore can be detected in SF in higher concentrations.
2. During the development of OA an increased accumulation of fluid in synovial joints might occur contributing to the joint effusion. Therefore, components of SF might be highly diluted. The diluted amount of PLs present in OA SF might be a signal for FLS to produce and secrete higher amounts of PLs.
3. It has been previously reported, that the articular cartilage at the early stage of degradation can increase the synthesis of extracellular matrix components, which is interpreted as a repair response [31]. It is tempting to speculate, that with SAPLs a similar process may occur. Cells responsible for the synthesis and release of SAPLs

increase their production to prevent cartilage from further degradation by increasing boundary lubrication.

4. MPs might serve as the transport vehicles for extracellular PLs. It can be speculated that MPs are adsorbed to the surfaces of articular cartilage. Since OA is characterised by narrowing of the joint space, one might put forward that MPs which contain PLs, can be crushed between surfaces of articular cartilage thus, released PLs can be determined in SF.

5.3 Composition of PL species

Some studies focusing on the PL composition of cells were also addressing the questions about the composition and possible functions of individual PL species [197, 205-207]. It is known, that the biophysical properties of PL species depend on the chain length and number of double bonds present in FA, hence, it is important to gain insight about molecular structure of PL species to fully understand their properties and possible functions. Recent developments of high sensitivity and high throughput ESI-MS/MS method have opened new analytical possibilities to determine molecular structure and thus, single individual PL species in different cell types and body fluids [164-166, 168, 170].

Our present knowledge concerning extracellular SAPLs stems mostly from the surfactant system present in the lung. Not much is known about SAPLs present in synovial joints, thus, questions addressing SF PL composition and functions of individual PL species remain unanswered.

Our study is the first showing the detailed composition of individual PL species present in SF. From 339 individual PL species being analysed, 182 could be quantitatively determined in SF. Although the functions of most molecular PL species are not known and need to be still explored, it can be postulated that all PL species, composed out of different head groups, FA chain length and number of double bounds, are synthesised for specific biological reasons. This issue would be interesting to be further investigated, since two studies already suggested that the friction between surfaces is decreased when a chain length of FA in PC is increased and that higher unsaturation of PL species further reduce the friction [208-209].

The diagnosis of early stage of OA is challenging. Moreover, it is difficult to distinguish between different stages of the disease. This knowledge is important, since it is assumed that most therapies might be effective only in early stage of OA. Our study is the

first demonstrating significant changes in PL composition between healthy, OA and RA SF. Furthermore, present results indicate, that the concentrations of individual PL species present in SF are impaired at different stages of OA. It is tempting to speculate that the PL pattern can reflect a certain stage of OA progression. Taken together, the determined differences could serve as a useful tool for diagnosis and prognosis of OA, even in early stages. With use of chemometrick techniques, a model including all PL species specifically changed in SF from different stages of OA can be build up. Since we found that the concentrations of 86 individual PL species are significantly increased in SF from late stage of OA as compared to SF from early OA, it can be assumed that this chemometrick model would prevent false diagnosis.

As already mentioned, PC is the prominent PL class found in SF. Studies using an animal model of RA [140], as well as a clinical study with OA patients [210], indicated that PC posses anti-inflammatory properties. Important processes which occur during activation and progression of OA are upregulation of inflammatory cytokines followed by increased enzymatic collagen degradation by MMPs. Our results demonstrate that most of PC molecular species were present in higher concentrations in OA and RA SF as compared to normal SF. It can be speculated that increased production of PC is the response of joint tissue to elevated amounts of pro-inflammatory mediators.

Most studies investigating PC as possible boundary lubricant were investigating the effect of saturated form of PC, known as DPPC. DPPC provides most of the surfactant activities in the lung [211], thus, it was originally thought to be the SF constituent mainly responsible for boundary lubrication. Previously, it has been shown that PC is present in SF mostly in the saturated form [212]. However, the effect of DPPC on decreasing friction was negligible [213]. Only very high concentrations of DPPC resulted in effective boundary lubrication [214-216]. In our present study a higher percentage of polyunsaturated PC species was determined. These results taken together with the evidence from recent publications, where only 8%-11% of DPPC was found on the surface of articular cartilage [129, 217], point on polyunsaturated PC species as those responsible for boundary lubrication. In addition, unsaturated PC species possess better lubrication properties than those provided by DPPC [129].

Plasmalogens probably do not provide lubricating abilities to SF, nevertheless, this is an interesting group to be studied, since numerous PLPE species were found in SF from all investigated patients groups. PLPE possess extraordinary antioxidant properties (for review see [218]) and cells deficient in plasmalogens are more susceptible to free radicals

[219]. In our study all individual species of PLPE were found to be increased in RA and OA SF compared to normal SF. Increased presence of reactive oxidative species (ROS) may contribute to cartilage degeneration during OA [135, 220-221]. An explanation for the observed increased PLPE concentrations in OA SF can be, that PLPE prevent cartilage from further damages induced by ROS. Thus, an increased production of PLPE might be a protective response to extracellular ROS.

Sphingolipids regulate many different cellular processes, including cellular stress response, cell proliferation and differentiation, apoptosis and senescence [157]. Already one study indicated that SPM might play a role in the development of RA, since sphingomyelinase pathway was described to induce apoptosis of cultured human FLS. However, the role of sphingolipids in synovial joints remains to be investigated. Although, sphingolipids do not possess boundary lubricating abilities, it is of interest to investigate whether their composition is impaired in OA SF from different stages of the disease and to assess their possible function in synovial joints.

Collagen type II cleavage is primarily mediated by collagenases like MMP-1 and MMP-13, whereas aggrecan is initially degraded by several aggreacanases and MMP-3 [33, 222]. It has been shown that Cer stimulate chondrocyte expression of MMP-1, -3 and -13 [223] as well as trigger aggrecanase-mediated degradation of aggrecan in cartilage [224]. Cer are also mediators which induce production of pro-inflammatory cytokines. We found higher concentrations of Cer 16:0, 22:0, 23:0 and 24:0 in RA SF and SF from late stage of OA. Our results together with evidences from the literature as described above indicate that Cer could be important factors which contribute to the development of OA. Our findings also support the previous speculation that OA, next to RA, can be a unique inflammatory disease [24].

Several studies already suggested that the friction is decreased when a chain length of FA in PC is increased. The authors also concluded that unsaturation of PL species further reduce the friction [208-209]. Our presented results indicate that during development of OA the chain is elongated in PC, PE, PLPE and PI. In normal SF we found a higher percentage of PL species with shorter chains of FA (≤34 C atoms). Since more unsaturated PL species with longer FA chains were found in the late stage of OA as compared to other investigated PL classes, it could be speculated, that observed remodelling of PL species occurs contributing to decreased friction in late stage of OA.

A recently established canine model of human OA [72-75] corroborates the results obtained from human. In the dog model of OA higher concentrations of most PL species

were also found in OA SF as compared to healthy SF. Additional comparison of PL composition in human, canine and equine SF, revealed that the relative distribution of PL molecular species as well as their quantitative values are comparable in human and canine SF but not in human and equine SF. Equine SF was characterised by a very low content of LPC, which is in agreement with data reported previously [197]. The reason why equine SF contains a decreased concentration of LPC remains to be investigated.

5.4 Joint distraction to study mechanical impact on PL

Currently no therapies are available to stop or slow down the progression of OA, therefore, the final therapeutic approach for OA is total joint replacement surgery. Recently, unloading of joints by joint distraction was proposed as a possible therapeutic strategy for OA, aimed to postpone joint replacement surgery [180, 225-227]. Distraction is a procedure in which ends of bone in the joint are separated to a certain extend for a certain period of time. It has been reported that joint distraction can stop or slow down the progression of OA in human ankle and knee joints [225, 227]. It has been shown that human cartilage thickened 0.9 mm/year and that the ratio of collagen type II synthesis to breakdown was also increased, indicating presence of newly formatted cartilage [227]. Taken together these results suggest that joint distraction seems to be a promising new structure modifying therapy for OA.

Preliminary results from joint distraction study using the canine ‘groove’ model of OA indicated that, distraction treatment resulted in a significant decrease of proteoglycan release from cartilage and a decreased damage of collagen. Macroscopical and histological analyses have shown improvement of the histologically discernible integrity of articular cartilage [228]. Concerning previous reports as presented recently [229], it was interesting for us to test, whether a distraction treatment changes the PL composition in canine SF. Especially we wanted to know, whether mechanical factors might have any impact on PL synthesis and/or composition.

The findings of our analysis indicate, that joint distraction can effect PL composition of OA SF. Significant increase in the concentrations of PE and PLPE was found in the distraction group as compared to OA group. The other PL classes remained unchanged. It would be tempting to speculate, that similar to the results obtained from human, increased production of PLPE might be a protective response to extracellular ROS, since increased amount of ROS may contribute to cartilage degeneration during OA.

However, it remains to be established whether other mechanical parameters are also able to modulate PL biosynthesis and/or release.

5.5 Phospholipid synthesis and release by FLS

The functional role of FLS, also called type B synoviocytes, is still not fully understood. Several studies suggested that the main function of FLS in joint is production and secretion of e.g. inflammatory mediators, fibronectin [230], HA [43, 47], and lubricin [49-50] into the joint cavity. Dobbie *et al.* [44-45] have shown that FLS possess LBs. This unique feature, which differ FLS from others types of fibroblasts, was later verified and confirmed using electron microscopy [51]. Based on these results, it has been hypothesised, that these cells are also responsible for the production and secretion of SAPLs. Beside published results showing the presence of LBs in FLS and assumption that FLS may synthesise and release SAPLs, up to day there is no experimental proof to substantiate the hypothesis, that FLS are indeed the source of SAPLs present in SF.

We investigated the effect of different cell culture conditions on PL synthesis and/or release by FLS. Treatment with dexamethasone, which has been shown [123] to influence the PL secretion revealed no changes compared to blank medium. Three-dimentional culture of FLS on BD Matrigel™ Basement Membrane Matrix Growth Factor Reduced, resulted in a slight increase in total PL concentration. Reliable measurement of extracellular PL in nutrient media is challenging. Obtained values from the analysed samples were close to the limit of quantification, hence, these findings were not sufficient to be a proof for PL synthesis and/or release by FLS. The potential weakness of our experiments is signal suppression, so that ESI-MS/MS is not sensitive enough to detect PL present in cell culture media at the low, depressed concentrations. Therefore, this issue of signal suppression has still to be solved.

Although, our preliminary results are unsatisfactory, this is a good basis to elucidate this problem in the future. From the discovery point of view it would be of interest to investigate how the biosynthesis and release of SAPLs from FLS are regulated. Monitoring of *de novo* synthesised individual PL species and their possible remodelling could enhance our knowledge and understanding of PL metabolic pathways. Additional treatment with cytokines, growth factors and drugs could answer the questions how PL biosynthesis might be regulated *in vivo*.

5.6 Surfactant proteins in human SF

Surfactant proteins next to SAPLs play intrinsic function in the surfactant system of the lung. Differences in content of surfactant proteins trigger severely lung disorders for instance, idiopathic pulmonary fibrosis [231], pneumonitis or [232] pulmonary sarcoidosis [233]. Moreover, serum surfactant proteins levels can be a good biomarker for those diseases. Since the surfactant system in the joint might be similar to the one found in the lung, it is tempting to infer that surfactant proteins may be also present in SF where they might play similar functions like in the lung.

Previously, surfactant proteins were thought to be present only in the lung. However, Kankavi *et al.* [104, 106-108] over the past 15 years provided some evidence that SP-A and SP-D are present also in several non-pulmonary sites of the body. Kankavi *et al.* determined these proteins in human skin, human organ of Corti, human Eustachian tube, human spermatozoa, human kidney and also in vagina, cervix, uterus, oviduct and ovaries of mares and prepuce, smegma, testis, prostate and vesicular glands of stallions [104, 106-108].

There are only few publications which provide valuable evidence for the presence of SP-A and SP-D in the synovial joints [109-111, 234]. Only Kankavi successfully detected SP-A and SP-D in human and equine SF using immunoblotting method [109-110]. To test the previously published results, three different antibodies for SP-A, including the one used by Kankavi (PE-10, Dako Deutschland GmbH), the same method of SF preparation and the same Western blot conditions as applied by Kankavi, were used in this study. Used antibodies revealed the bands, however, none of which had the expected molecular weight. Furthermore, two-dimensional separations of proteins followed by MALDI-TOF/MS protein identification revealed that spots stained by used antibodies do not contain surfactant proteins. These results are in contradiction to the data published by Kankavi. The explanation for these observed differences in results may be that (a) the used antibodies are not only specific for SP-A and SP-D, (b) Western blot alone is an insufficient method to confirm presence of proteins in SF, and (c) SF does not contain surfactant proteins. Other published studies using ELISA method reported, that SP-A [111] and SP-D [234] are present in human SF. Moreover, authors suggested that the level of surfactant proteins is decreased in SF obtained from rheumatoid arthritic patients. Nevertheless, specific and unspecific binding of antibody measured by ELISA results in absorbance. Since no additional experiment testing specificity of used antibodies is

provided, measured absorbance is not reliable proof for presence of surfactant proteins in SF.

The joint cavity is a closed structure and SF is an ultrafiltrate of blood with additives produced by the surrounding tissues. Since it is known that FLS possess LBs, Dobbie *et al.* [45, 101] and Schwarz and Hills [51] implicated, that those cells are responsible for the synthesis of surfactant proteins. To further confirm or contradict our first observations that SF does not contain surfactant protein, mRNA expression of *sp-a* and *sp-d* was determined. Our findings from Western blot analysis were confirmed on the gene level using RT-PCR method. Expression of *sp-a* and *sp-d* was not detectable in cells derived from synovial joint tissues including cultured FLS, not further identified cells suspended in SF and chondrocytes from human cartilage.

To summarise, one- and two-dimensional separations of proteins in SF followed by Western blot detection with antibodies and MALDI-TOF/MS protein identification and additionally, SP-A and SP-D mRNA expression analysis by RT-PCR, indicate that surfactant proteins are not present in human SF. Altogether our findings suggest, that SP-A and SP-D do not participate in boundary lubrication system in synovial joints as it was previously suggested [109-110]. According to our knowledge, this is the first study determines the absence of SP-A and SP-D in SF by using immunoblotting techniques followed by confirmation with mass spectrometry. Moreover, it is also the first study investigating mRNA expression of *sp-a* and *sp-d* in cells derived from synovial tissues.

5.7 Summary, conclusions and future perspectives

Summary and conclusions

1. The current study identified 9 different lipid classes present in SF with PC being the main PL class present. Moreover, PC was, in contrast to the lung, present mostly in the polyunsaturated form.

2. Total PL content was determined in higher concentrations in SF from late stage of OA compared to healthy SF and SF from early stage of OA. Moreover, most of the main PL classes were present in higher concentrations in SF from late stage of OA compared to other investigated groups. These findings indicate that increased PLs content is involved in the pathogenesis of OA.

3. One hundred eighty two molecular PL species were determined to be present in human SF. Based on our knowledge, this study is the first report showing the detailed composition of PL species in SF. Although functions of most molecular PL species are not known and still need to be explored, it can be postulated that all PL species, even though they differ with respect to the head group, FA chain length and number of double bounds, are synthesised for specific biological reasons.

4. Significant changes in concentrations of 86 individual PL species were found between healthy, OA and RA SF. More interestingly, significant differences in PL composition were observed at different stages of OA. Therefore, it can be concluded that specific PL pattern reflect a stage of the disease progression. These results may help to develop the chemometrick model which can serve as a useful tool for diagnosis and prognosis of OA.

5. Analysis of canine healthy and OA SF revealed slight differences between those two groups but also demonstrated similar trends to the results obtained from humans, where most of PL classes were present in higher concentrations in OA SF when compared to healthy SF. Moreover, we found that relative PL distribution is similar in human and dogs with comparable concentrations of PL classes and PL species. These results obtained with a dog model of OA confirm our observation that an altered PLs content, remodeling and/or metabolism seems to be involved in the pathogenesis of OA.

6. The findings of our analysis indicate that joint distraction can effect PL composition of OA SF. Significant increase in the concentrations of PE and PLPE, which can serve as protective agent against extracellular ROS contributing to cartilage degeneration during OA, was found in the distraction group as compared to OA group. The other PL classes remained unchanged. Therefore, it can be conclude that mechanical factors might have an impact on PL synthesis and/or composition.

7. Our preliminary results from cultured FLS indicate that these cells might be a source of PL being present in SF. However, detected concentrations of PL present in cell culture media were very low. The potential weakness of our experiments is signal suppression, so that ESI-MS/MS is not sensitive enough to detect PL present in cell culture media at the low, depressed concentrations. Therefore, this has to be further elucidated by the additional experiments to solve the problem with signal suppression.

8. We used several different methods to confirm the hypothesis that surfactant proteins are present in human SF. Nevertheless, we could not provide any evidence to substantiate this hypothesis. Hence, we can only conclude that surfactant proteins are absent in human SF and do not play any role in boundary lubrication of joints.

Future perspective

Diagnosis of early stage of OA is challenging. Moreover, it is difficult to distinguish between stages of the disease. This knowledge is important, since it is assumed that therapies might be effective only during the early stage of OA. Our results may help to develop the chemometrick model which can further serve as a useful tool for diagnosis and prognosis of OA.

Preliminary results from cultured FLS indicate that FLS can be a source of PL present in SF. It would be very interesting to investigate whether and how biosynthesis and release of SAPL from FLS are regulated. Monitoring of *de novo* synthesised individual PL species and their possible remodelling could enhance our knowledge and understanding of PL metabolic pathways thus, providing novel targets to treat OA.

6. Appendix

Table 6.1. *P*-values for ratios of PL class in SF as compared between joints with different health status.

Ratio	OA early vs. Healthy	OA late vs. Healthy	RA vs. Healthy	OA early vs. OA late	OA early vs. RA	OA late vs. RA
PC/LPC	NS	NS	*↑	*↓	***↓	***↓
PC/PE	*↑	**↑	**↑	NS	NS	NS
PC/PS	**↑	***↑	*↑	**↑	**↑	*↑
PE/PS	**↑	**↑	NS	*↑	**↑	NS
PLPE/PE	NS	NS	NS	NS	*↑	*↑
SPM/Cer	NS	NS	NS	NS	NS	NS

Statistical analysis was performed by the non-parametric Kruskal-Wallis test followed by the false discovery rate (FDR) correction. Subsequently, paired Wilcoxon signed-rank test was applied to identify significant changes between each groups. *P*-values of less than 0.05 were considered statistically significant with: *$0.01<p\leq0.05$; **$0.001<p\leq0.01$; ***$p<0.001$; NS-not significant. ↑:increase ↓:decrease. Data are presented as the graphs in Figure 4.6. Cer-ceramides, LPC-lysophosphatidylcholine, OA-osteoarthritis, PC-phosphatidylcholine, PE-phosphatidylethanolamine, PLPE-PE-based plasmalogen, PS-phosphatidylserine, RA-rheumatoid arthritis, SPM-sphingomyelin.

Table 6.2. Summary of statistically significant changes of the concentrations of individual PL species as dependent on the health status of joints – second independent ESI/MS-MS analysis.

PL-specie	OA early vs. Healthy	OA late vs. Healthy	OA early vs. OA late
Phosphatidylcholine (PC)			
PCO 30:0	NS↓	NS↓	NS↑
PC 32:0	* ↑	** ↑	NS
PCO 32:0	** ↑	** ↑	NS
PC 32:1	NS	* ↑	NS
PCO 32:1	NS	NS↑	NS
PC 32:2	* ↑	** ↑	NS
PC 34:0	*** ↑	** ↑	NS↓
PCO 34:0	** ↑	*** ↑	NS
PC 34:1	NS↑	** ↑	NS↓
PCO 34:1	** ↑	** ↑	NS↓
PC 34:2	* ↑	*** ↑	NS↓
PCO 34:2	NS↑	* ↑	NS↓
PC 34:3	** ↑	*** ↑	NS↓
PC 36:0	* ↑	** ↑	NS↓
PC 36:1	* ↑	** ↑	NS↓
PCO 36:1	** ↑	*** ↑	NS↓
PC 36:2	* ↑	*** ↑	NS↓
PCO 36:2	** ↑	*** ↑	NS↓

Table 6.2. Continuation.

PL-specie	OA early vs. Healthy	OA late vs. Healthy	OA early vs. OA late
Phosphatidylcholine (PC)			
PC 36:3	** ↑	*** ↑	NS↓
PC 36:4	** ↑	*** ↑	NS↓
PCO 36:4	* ↑	*** ↑	NS↓
PC 36:5	** ↑	*** ↑	NS↓
PCO 36:5	** ↑	*** ↑	NS↑
PC 38:1	NS↑	* ↑	NS↑
PC 38:2	NS↑	** ↑	NS↓
PC 38:3	* ↑	*** ↑	NS↓
PC 38:4	* ↑	*** ↑	NS↓
PC 38:5	** ↑	*** ↑	NS↓
PC 38:6	** ↑	*** ↑	NS↓
PC 40:4	* ↑	** ↑	NS↓
PC 40:5	* ↑	*** ↑	NS↓
PC 40:6	* ↑	*** ↑	NS↓
Lysophosphatidylcholine (LPC)			
LPC 15:0	** ↑	** ↑	NS↓
LPC 16:0	** ↑	*** ↑	NS↑
LPC 16:1	NS↑	NS↑	NS↑
LPC 18:0	*** ↑	*** ↑	NS↑
LPC 18:1	* ↑	* ↑	NS↑
LPC 18:2	** ↑	NS	** ↑
LPC 18:3	** ↑	NS	** ↑
LPC 20:0	* ↑	NS↑	NS
LPC 20:3	*** ↑	** ↑	NS
LPC 20:4	* ↑	* ↑	NS
LPC 20:5	* ↑	** ↑	NS
LPC 22:0	NS	NS	NS
LPC 22:4	NS	NS	NS
LPC 22:5	* ↑	** ↑	NS
LPC 22:6	** ↑	** ↑	NS
Phosphatidylethanolamine (PE)			
PE 32:2	NS	* ↓	NS
PE 34:0	NS	NS	NS
PE 34:1	NS	NS↑	NS↓
PE 34:2	NS↑	NS↑	NS↓
PE 36:1	NS	NS↑	NS↓
PE 36:2	NS↑	* ↑	NS↓
PE 36:3	NS↑	NS↑	NS
PE 36:4	NS↑	* ↑	NS↓
PE 36:5	NS	NS↑	NS
PE 38:1	NS↑	NS↑	NS↑
PE 38:3	NS↑	NS↑	NS↓

Table 6.2. Continuation.

PL-specie	OA early vs. Healthy	OA late vs. Healthy	OA early vs. OA late
Phosphatidylethanolamine (PE			
PE 38:4	NS↑	* ↑	NS↓
PE 38:5	NS↑	NS↑	NS↓
PE 38:6	NS↑	** ↑	NS↓
PE O 38:7	NS	NS↓	NS↑
PE O 40:3	NS	NS	NS
PE 40:5	NS↓	NS↑	NS↓
PE 40:6	NS↑	** ↑	NS↓
PE 42:5	NS	NS	NS
PE 42:7	NS	NS	NS
PE-based plasmalogen (PLPE)			
PLPE 16:0/16:0	NS	NS	NS
PLPE 16:0/16:1	NS	NS	NS
PLPE 16:0/18:0	NS	NS	NS
PLPE 16:0/18:1	NS	NS↑	NS
PLPE 16:0/18:2	NS	NS↑	NS↓
PLPE 16:0/18:3	NS	NS	NS
PLPE 16:0/20:3	NS	NS↓	NS
PLPE 16:0/20:4	NS↑	NS↑	NS↓
PLPE 16:0/20:5	NS	NS↑	NS↑
PLPE 16:0/22:3	NS	NS	NS
PLPE 16:0/22:5	NS↑	NS↑	NS↑
PLPE 16:0/22:6	NS↑	** ↑	NS↓
PLPE 18:0/16:0	NS	NS	NS
PLPE 18:0/16:1	NS	NS	NS
PLPE 18:0/18:0	NS	NS	NS
PLPE 18:0/18:1	NS	NS↑	NS↑
PLPE 18:0/18:2	NS↑	NS↑	NS↓
PLPE 18:0/18:3	NS	NS	NS
PLPE 18:0/20:3	NS	NS↓	NS↓
PLPE 18:0/20:4	* ↑	* ↑	NS↓
PLPE 18:0/20:5	NS↑	NS↑	NS↑
PLPE 18:0/22:3	NS	NS	NS
PLPE 18:0/22:5	NS↑	NS↑	NS↑
PLPE 18:0/22:6	NS↑	NS↑	NS↑
PLPE 18:1/16:0	NS	NS	NS
PLPE 18:1/16:1	NS	NS	NS
PLPE 18:1/18:0	NS	NS	NS
PLPE 18:1/18:1	NS	NS↓	NS

Table 6.2. Continuation.

PL-specie	OA early vs. Healthy	OA late vs. Healthy	OA early vs. OA late
PE-based plasmalogen (PLPE)			
PLPE 18:1/18:2	NS	NS↑	NS↑
PLPE 18:1/18:3	NS	NS	NS
PLPE 18:1/20:3	NS	NS	NS
PLPE 18:1/20:4	NS↑	NS↑	NS↓
PLPE 18:1/20:5	NS	NS↓	NS
PLPE 18:1/22:3	NS	NS	NS
PLPE 18:1/22:4	NS	NS	NS
PLPE 18:1/22:5	NS	NS	NS
PLPE 18:1/22:6	NS↑	NS↑	NS↑
Phosphatidylglycerol (PG)			
PG 32:0	NS	NS	NS
PG 34:1	NS	NS	NS
PG 36:2	NS	NS	NS
Phosphatidylinositol (PI)			
PI 36:1	NS	* ↑	NS
PI 36:4	* ↑	* ↑	NS
PI 38:3	* ↑	** ↑	NS
PI 38:4	* ↑	** ↑	NS
PI 38:5	* ↑	NS	NS
PI 40:4	NS	** ↑	NS
Phosphatidylserine (PS)			
PS 36:1	NS	NS	NS
PS 36:2	NS	NS	NS
Sphingomyelin (SPM)			
SPM 14:0	* ↑	*** ↑	NS↓
SPM 15:0	* ↑	*** ↑	NS↓
SPM 16:0	NS↑	** ↑	NS↓
SPM dih 16:0	NS↑	* ↑	NS↓
SPM –OH 16:0	* ↑	** ↑	NS
SPM 16:1	* ↑	** ↑	NS
SPM –OH 16:1	* ↑	** ↑	NS
SPM 18:0	* ↑	** ↑	NS
SPM dih 18:0	-	* ↑	NS
SPM 18:1	* ↑	** ↑	NS
SPM 20:0	NS↑	NS↑	NS↓
SPM 20:1	* ↑	** ↑	NS↓
SPM 22:0	* ↑	*** ↑	NS↑
SPM 22:1	* ↑	*** ↑	NS↓
SPM 22:2	NS	* ↑	NS↓
SPM 24:0	* ↑	** ↑	NS↑

Table 6.2. Continuation.

PL-specie	OA early vs. Healthy	OA late vs. Healthy	OA early vs. OA late
Sphingomyelin (SPM)			
SPM 24:1	NS↑	** ↑	NS↓
SPM 24:2	NS↑	* ↑	NS↓
SPM 24:3	NS	NS↑	NS
Ceramide (Cer)			
Cer 16:0	NS	* ↑	NS↓
GluCer 16:0	NS	NS↑	NS↓
Cer 22:0	* ↑	** ↑	NS↓
Cer 23:0	** ↑	*** ↑	NS↓
Cer 24:0	** ↑	*** ↑	NS↓
Cer 24:1	NS	** ↑	NS↓
GluCer 24:1	NS	NS↑	NS↓

PC species were analysed by ESI-MS/MS in 6 healthy SF, 10 OA SF from early stage, 11 OA SF from late stage and 2 RA SF. Statistical analysis was performed by the non-parametric Kruskal-Wallis test followed by the false discovery rate (FDR) correction. Subsequently, paired Wilcoxon signed-rank test was applied to identify significant changes between each groups. *P*-values of less than 0.05 were considered statistically significant with: *$0.01<p\leq0.05$; **$0.001<p\leq0.01$; ***$p<0.001$; NS-not significant. ↑:increase ↓:decrease. When statistical significance was found in the first analysis, and not in the second, box is marked with grey background and arrows indicate observed trend. Boxes marked with bold line indicate that observed trend is opposite to those found in the first analysis. Data from RA SF are not shown due to only three patients available for second analysis. PC-phosphatidylcholine, PC O-ether phosphatidylcholine, LPC-lysophosphatidylcholine, PE- phosphatidylethanolamine, PLPE-phosphatidylethanolamine-based plasmalogen, PG-phosphatidylglycerol, PI-phosphatidylinositol, PS-phosphatidylserine, SPM-sphingomyelin, Cer-ceramide, GluCer-glucosylceramide, OA-osteoarthritis, , RA-rheumatoid arthritis.

Table 6.3. *P*-values for FA chain length according to the number of C-atoms in a chosen PL class.

Number of C-atoms	OA early vs. Healthy	OA late vs. Healthy	RA vs. Healthy	OA early vs. OA late	OA early vs. RA	OA late vs. RA
Phosphatidylcholine (PC)						
PC 30	NS	**↓	***↓	*↑	***↑	NS
PC 32	NS	**↓	***↓	*↑	***↑	NS
PC 34	NS	NS	NS	NS	NS	NS
PC 36	**↑	**↑	***↑	NS	*↓	NS
PC 38	*↑	*↑	NS	NS	*↑	NS
PC 40	NS	NS	NS	NS	NS	NS
Phosphatidylcholine Ether (PCO)						
PC O 30	*↓	***↓	***↓	NS	***↑	**↑
PC O 32	*↓	*↓	*↓	NS	NS	NS
PC O 34	**↓	**↓	***↓	NS	NS	NS
PC O 36	***↑	***↑	***↑	*↓	*↓	NS

Table 6.3. Continuation.

Number of C-atoms	OA early vs. Healthy	OA late vs. Healthy	RA vs. Healthy	OA early vs. OA late	OA early vs. RA	OA late vs. RA
PC + PCO						
PC + PCO 30	*↓	**↓	***↓	*↑	***↑	NS
PC + PCO 32	NS	**↓	***↓	*↑	***↑	NS
PC + PCO 34	**↓	NS	NS	NS	NS	NS
PC + PCO 36	**↑	**↑	***↑	NS	*↓	NS
PC + PCO 38	*↑	**↑	NS	NS	NS	NS
PC + PCO 40	NS	NS	NS	NS	NS	NS
Phosphatidylethanolamine (PE)						
PE 32	*↓	**↑	***↓	NS	***↑	***↑
PE 34	NS	**↑	***↑	NS	***↓	***↓
PE 36	*↑	***↑	***↑	NS	**↓	***↓
PE 38	NS	***↑	***↑	NS	***↓	*↓
PE 40	*↓	***↓	***↓	NS	***↑	***↑
PE-based plasmalogen (PLPE)						
PLPE 32	***↓	NS	**↓	***↑	NS	NS
PLPE 34	***↓	NS	*↓	NS	**↑	NS
PLPE 36	*↓	***↓	*↓	NS	NS	NS
PLPE 38	***↑	*↑	**↑	NS	**↓	NS
PLPE 40	NS	NS	NS	NS	NS	NS
Phosphatidylinositol (PI)						
PI 32	NS	***↓	NS	NS	NS	NS
PI 34	NS	NS	NS	NS	NS	NS
PI 36	*↓	***↓	NS	NS	NS	NS
PI 38	**↑	***↑	NS	NS	NS	NS
PI 40	NS	NS	NS	NS	NS	NS

Statistical analysis was performed by the non-parametric Kruskal-Wallis test followed by the false discovery rate (FDR) correction. Subsequently, paired Wilcoxon signed-rank test was applied to identify significant changes between each groups. *P*-values of less than 0.05 were considered statistically significant with: *$0.01<p\leq0.05$; **$0.001<p\leq0.01$; ***$p<0.001$; NS-not significant. ↑:increase ↓:decrease. OA-osteoarthritis, PC-phosphatidylcholine, PC O-ether phosphatidylcholine, PE-phosphatidylethanolamine, PLPE-phosphatidylethanolamine-based plasmalogen, RA-rheumatoid arthritis.

Table 6.4. *P*-values for PL classes in SF of various mammalian species.

	Human vs. Dogs	Human vs. Horses	Horses vs. Dogs
Phosphatidylcholine Ether (PCO)	NS	**↑	NS
Phosphatidylcholine (PC)	NS	NS	*↓
Lysophosphatidylcholine (LPC)	**↑	***↑	***↓
Phosphatidylethanolamine (PE)	NS	***↑	***↓
PE-based plasmalogen (PLPE)	NS	***↑	***↓
Phosphatidylglycerol (PG)	*↑	**↑	NS
Phosphatidylinositol (PI)	NA	NA	NA
Phosphatidylserine (PS)	NS	NS	*↑
Sphingomyelin (SPM)	NS	***↑	NS
Ceramide (Cer)	NS	***↑	***↓

The different PL classes were quantified by ESI-MS/MS in healthy human SF (n=9), healthy canine SF (n=20) and healthy equine SF (n=14). Statistical analysis was performed by the non-parametric Kruskal-Wallis test followed by the false discovery rate (FDR) correction. Subsequently, paired Wilcoxon signed-rank test was applied to identify significant changes between each groups. *P*-values of less than 0.05 were considered statistically significant with: *$0.01<p\leq0.05$; **$0.001<p\leq0.01$; ***$p<0.001$. Data are presented as the graphs in Figure 4.17. NA-not analysed.

7. References

1. Excellence, N.I.f.C., *National clinical guideline for care and managment in adults.* Cited 4 Sept 2009. Available from: http://www.nice.org.uk/nicemedia/pdf/CG059FullGuideline.pdf, 2009.
2. **Wieland, H.A.**, et al., *Osteoarthritis - an untreatable disease?* Nat Rev Drug Discov, 2005. **4**(4): p. 331-44.
3. *NIH Consensus Statement on total knee replacement December 8-10, 2003.* J Bone Joint Surg Am, 2004. **86-A**(6): p. 1328-35.
4. **Garstang, S.V.** and **T.P. Stitik**, *Osteoarthritis: epidemiology, risk factors, and pathophysiology.* Am J Phys Med Rehabil, 2006. **85**(11 Suppl): p. S2-11; quiz S12-4.
5. **Buckwalter, J.A.** and **J.A. Martin**, *Osteoarthritis.* Adv Drug Deliv Rev, 2006. **58**(2): p. 150-67.
6. **Kean, W.F.**, **R. Kean**, and **W.W. Buchanan**, *Osteoarthritis: symptoms, signs and source of pain.* Inflammopharmacology, 2004. **12**(1): p. 3-31.
7. **Hannan, M.T.**, **D.T. Felson**, and **T. Pincus**, *Analysis of the discordance between radiographic changes and knee pain in osteoarthritis of the knee.* J Rheumatol, 2000. **27**(6): p. 1513-7.
8. **Wluka, A.E.**, **F.M. Cicuttini**, and **T.D. Spector**, *Menopause, oestrogens and arthritis.* Maturitas, 2000. **35**(3): p. 183-99.
9. **Felson, D.T.**, *Risk factors for osteoarthritis: understanding joint vulnerability.* Clin Orthop Relat Res, 2004(427 Suppl): p. S16-21.
10. **Felson, D.T.**, *Obesity and vocational and avocational overload of the joint as risk factors for osteoarthritis.* J Rheumatol Suppl, 2004. **70**: p. 2-5.
11. **Felson, D.T.**, et al., *The effect of body weight on progression of knee osteoarthritis is dependent on alignment.* Arthritis Rheum, 2004. **50**(12): p. 3904-9.
12. **Felson, D.T.** and **M.C. Nevitt**, *Epidemiologic studies for osteoarthritis: new versus conventional study design approaches.* Rheum Dis Clin North Am, 2004. **30**(4): p. 783-97, vii.
13. **Sharma, L.**, et al., *Does laxity alter the relationship between strength and physical function in knee osteoarthritis?* Arthritis Rheum, 1999. **42**(1): p. 25-32.
14. **Anderson, J.J.** and **D.T. Felson**, *Factors associated with osteoarthritis of the knee in the first national Health and Nutrition Examination Survey (HANES I). Evidence for an association with overweight, race, and physical demands of work.* Am J Epidemiol, 1988. **128**(1): p. 179-89.
15. **Zhang, Y.**, et al., *Prevalence of symptomatic hand osteoarthritis and its impact on functional status among the elderly: The Framingham Study.* Am J Epidemiol, 2002. **156**(11): p. 1021-7.
16. **Kellgren, J.H.** and **J.S. Lawrence**, *Radiological assessment of osteoarthrosis.* Ann Rheum Dis, 1957. **16**(4): p. 494-502.
17. **Schiphof, D.**, et al., *Impact of different descriptions of the Kellgren and Lawrence classification criteria on the diagnosis of knee osteoarthritis.* Ann Rheum Dis, 2011. **70**(8): p. 1422-7.
18. **Schiphof, D.**, et al., *Good reliability, questionable validity of 25 different classification criteria of knee osteoarthritis: a systematic appraisal.* J Clin Epidemiol, 2008. **61**(12): p. 1205-15.
19. **Outerbridge, R.E.**, *The etiology of chondromalacia patellae.* J Bone Joint Surg Br, 1961. **43B**: p. 752-57.
20. **Berenbaum, F.**, *Osteoarthritis year 2010 in review: pharmacological therapies.* Osteoarthritis Cartilage, 2011. **19**(4): p. 361-5.
21. **Jordan, K.M.**, et al., *EULAR Recommendations 2003: an evidence based approach to the management of knee osteoarthritis: Report of a Task Force of the Standing Committee for*

International Clinical Studies Including Therapeutic Trials (ESCISIT). Ann Rheum Dis, 2003. **62**(12): p. 1145-55.
22. **Hunter, D.J.** and **F. Eckstein**, *Exercise and osteoarthritis.* J Anat, 2009. **214**(2): p. 197-207.
23. **Hawker, G.A.**, et al., *Osteoarthritis year 2010 in review: non-pharmacologic therapy.* Osteoarthritis Cartilage, 2011. **19**(4): p. 366-74.
24. **Attur, M.G.**, et al., *Osteoarthritis or osteoarthrosis: the definition of inflammation becomes a semantic issue in the genomic era of molecular medicine.* Osteoarthritis Cartilage, 2002. **10**(1): p. 1-4.
25. **Hunter, D.J.** and **D.T. Felson**, *Osteoarthritis.* BMJ, 2006. **332**(7542): p. 639-42.
26. **Jadin, K.D.**, et al., *Depth-varying density and organization of chondrocytes in immature and mature bovine articular cartilage assessed by 3d imaging and analysis.* J Histochem Cytochem, 2005. **53**(9): p. 1109-19.
27. **Hunziker, E.B.**, **T.M. Quinn**, and **H.J. Hauselmann**, *Quantitative structural organization of normal adult human articular cartilage.* Osteoarthritis Cartilage, 2002. **10**(7): p. 564-72.
28. **Blewis, M.E.**, et al., *Microenvironment regulation of PRG4 phenotype of chondrocytes.* J Orthop Res, 2007. **25**(5): p. 685-95.
29. **Laver-Rudich, Z.** and **M. Silbermann**, *Cartilage surface charge. A possible determinant in aging and osteoarthritic processes.* Arthritis Rheum, 1985. **28**(6): p. 660-70.
30. **Mow, V.C.**, **C.C. Wang**, and **C.T. Hung**, *The extracellular matrix, interstitial fluid and ions as a mechanical signal transducer in articular cartilage.* Osteoarthritis Cartilage, 1999. **7**(1): p. 41-58.
31. **Hardingham, T.**, *Chondroitin sulfate and joint disease.* Osteoarthritis Cartilage, 1998. **6 Suppl A**: p. 3-5.
32. **Sauerland, K.** and **J. Steinmeyer**, *Intermittent mechanical loading of articular cartilage explants modulates chondroitin sulfate fine structure.* Osteoarthritis Cartilage, 2007. **15**(12): p. 1403-9.
33. **Tetlow, L.C.**, **D.J. Adlam**, and **D.E. Woolley**, *Matrix metalloproteinase and proinflammatory cytokine production by chondrocytes of human osteoarthritic cartilage: associations with degenerative changes.* Arthritis Rheum, 2001. **44**(3): p. 585-94.
34. **Nagase, H.** and **M. Kashiwagi**, *Aggrecanases and cartilage matrix degradation.* Arthritis Res Ther, 2003. **5**(2): p. 94-103.
35. **Stanton, H.**, et al., *ADAMTS5 is the major aggrecanase in mouse cartilage in vivo and in vitro.* Nature, 2005. **434**(7033): p. 648-52.
36. **Naito, S.**, et al., *Expression of ADAMTS4 (aggrecanase-1) in human osteoarthritic cartilage.* Pathol Int, 2007. **57**(11): p. 703-11.
37. **Yatabe, T.**, et al., *Hyaluronan inhibits expression of ADAMTS4 (aggrecanase-1) in human osteoarthritic chondrocytes.* Ann Rheum Dis, 2009. **68**(6): p. 1051-8.
38. **Steinmeyer, J.**, **J. Kordelle**, and **H. Stürz**, *In vitro inhibition of aggrecanase activity by tetracyclines and proteoglycan loss from osteoarthritic human articular cartilage.* J Orthop Res, 2010. **28**(6): p. 828-33.
39. **Belcher, C.**, et al., *Synovial fluid chondroitin and keratan sulphate epitopes, glycosaminoglycans, and hyaluronan in arthritic and normal knees.* Ann Rheum Dis, 1997. **56**(5): p. 299-307.
40. **Campion, G.V.**, et al., *Levels of keratan sulfate in the serum and synovial fluid of patients with osteoarthritis of the knee.* Arthritis Rheum, 1991. **34**(10): p. 1254-9.
41. **Fawthrop, F.**, et al., *Chondroitin and keratan sulphate epitopes, glycosaminoglycans, and hyaluronan in progressive versus non-progressive osteoarthritis.* Ann Rheum Dis, 1997. **56**(2): p. 119-22.
42. **Hardingham, T.** and **M. Bayliss**, *Proteoglycans of articular cartilage: changes in aging and in joint disease.* Semin Arthritis Rheum, 1990. **20**(3 Suppl 1): p. 12-33.
43. **Iwanaga, T.**, et al., *Morphology and functional roles of synoviocytes in the joint.* Arch Histol Cytol, 2000. **63**(1): p. 17-31.

44. **Dobbie, J.W.**, et al., *Lamellar bodies in synoviocytes, mesothelium and specific epithelia as possible site of auto-antigen in rheumatoid disease.* Br J Rheumatol, 1994. **33**(6): p. 508-19.
45. **Dobbie, J.W.**, et al., *Lamellar body secretion: ultrastructural analysis of an unexplored function of synoviocytes.* Br J Rheumatol, 1995. **34**(1): p. 13-23.
46. **Shikichi, M.**, et al., *Three-dimensional ultrastructure of synoviocytes in the horse joint as revealed by the scanning electron microscope.* Arch Histol Cytol, 1999. **62**(3): p. 219-29.
47. **Pitsillides, A.A.**, et al., *Uridine diphosphoglucose dehydrogenase activity in normal and rheumatoid synovium: the description of a specialized synovial lining cell.* Int J Exp Pathol, 1993. **74**(1): p. 27-34.
48. **Flannery, C.R.**, et al., *Articular cartilage superficial zone protein (SZP) is homologous to megakaryocyte stimulating factor precursor and is a multifunctional proteoglycan with potential growth-promoting, cytoprotective, and lubricating properties in cartilage metabolism.* Biochem Biophys Res Commun, 1999. **254**(3): p. 535-41.
49. **Schumacher, B.L.**, et al., *A novel proteoglycan synthesized and secreted by chondrocytes of the superficial zone of articular cartilage.* Arch Biochem Biophys, 1994. **311**(1): p. 144-52.
50. **Hills, B.A.** and **R.W. Crawford**, *Normal and prosthetic synovial joints are lubricated by surface-active phospholipid: a hypothesis.* J Arthroplasty, 2003. **18**(4): p. 499-505.
51. **Schwarz, I.M.** and **B.A. Hills**, *Synovial surfactant: lamellar bodies in type B synoviocytes and proteolipid in synovial fluid and the articular lining.* Br J Rheumatol, 1996. **35**(9): p. 821-7.
52. **Ayral, X.**, et al., *Synovitis: a potential predictive factor of structural progression of medial tibiofemoral knee osteoarthritis -results of a 1 year longitudinal arthroscopic study in 422 patients.* Osteoarthritis Cartilage, 2005. **13**(5): p. 361-7.
53. **Benito, M.J., et al.**, *Synovial tissue inflammation in early and late osteoarthritis.* Ann Rheum Dis, 2005. **64**(9): p. 1263-7.
54. **Konttinen, Y.T.**, et al., *The microenvironment around total hip replacement prostheses.* Clin Orthop Relat Res, 2005(430): p. 28-38.
55. **Henrotin, Y.**, **L. Pesesse**, and **C. Sanchez**, *Subchondral bone in osteoarthritis physiopathology: state-of-the art and perspectives.* Biomed Mater Eng, 2009. **19**(4-5): p. 311-6.
56. **Gilbertson, E.M.**, *Development of periarticular osteophytes in experimentally induced osteoarthritis in the dog. A study using microradiographic, microangiographic, and fluorescent bone-labelling techniques.* Ann Rheum Dis, 1975. **34**(1): p. 12-25.
57. **Liu, X.H.**, et al., *Interactive effect of interleukin-6 and prostaglandin E2 on osteoclastogenesis via the OPG/RANKL/RANK system.* Ann N Y Acad Sci, 2006. **1068**: p. 225-33.
58. **Sanchez, C., et al.**, *Phenotypic characterization of osteoblasts from the sclerotic zones of osteoarthritic subchondral bone.* Arthritis Rheum, 2008. **58**(2): p. 442-55.
59. **Heilmann, H.H.**, **K. Lindenhayn**, and **H.U. Walther**, *[Synovial volume of healthy and arthrotic human knee joints].* Z Orthop Ihre Grenzgeb, 1996. **134**(2): p. 144-8.
60. **Swann, D.A.**, et al., *The molecular structure and lubricating activity of lubricin isolated from bovine and human synovial fluids.* Biochem J, 1985. **225**(1): p. 195-201.
61. **Jay, G.D.**, et al., *Homology of lubricin and superficial zone protein (SZP): products of megakaryocyte stimulating factor (MSF) gene expression by human synovial fibroblasts and articular chondrocytes localized to chromosome 1q25.* J Orthop Res, 2001. **19**(4): p. 677-87.
62. **Su, J.L.**, et al., *Detection of superficial zone protein in human and animal body fluids by cross-species monoclonal antibodies specific to superficial zone protein.* Hybridoma, 2001. **20**(3): p. 149-57.
63. **Ogston, A.G.** and **J.E. Stanier**, *The physiological function of hyaluronic acid in synovial fluid; viscous, elastic and lubricant properties.* J Physiol, 1953. **119**(2-3): p. 244-52.

64. **Mazzucco, D.**, **R. Scott**, and **M. Spector**, *Composition of joint fluid in patients undergoing total knee replacement and revision arthroplasty: correlation with flow properties.* Biomaterials, 2004. **25**(18): p. 4433-45.
65. **Balazs, E.A.**, et al., *Hyaluronic acid in synovial fluid. I. Molecular parameters of hyaluronic acid in normal and arthritis human fluids.* Arthritis Rheum, 1967. **10**(4): p. 357-76.
66. **Berckmans, R.J.**, et al., *Cell-derived microparticles in synovial fluid from inflamed arthritic joints support coagulation exclusively via a factor VII-dependent mechanism.* Arthritis Rheum, 2002. **46**(11): p. 2857-66.
67. **Berckmans, R.J.**, et al., *Synovial microparticles from arthritic patients modulate chemokine and cytokine release by synoviocytes.* Arthritis Res Ther, 2005. **7**(3): p. R536-44.
68. **Messer, L.**, et al., *Microparticle-induced release of B-lymphocyte regulators by rheumatoid synoviocytes.* Arthritis Res Ther, 2009. **11**(2): p. R40.
69. **Beyer, C.** and **D.S. Pisetsky**, *The role of microparticles in the pathogenesis of rheumatic diseases.* Nat Rev Rheumatol, 2010. **6**(1): p. 21-9.
70. **Pond, M.J.** and **G. Nuki**, *Experimentally-induced osteoarthritis in the dog.* Ann Rheum Dis, 1973. **32**(4): p. 387-8.
71. **Brandt, K.D.**, et al., *Osteoarthritic changes in canine articular cartilage, subchondral bone, and synovium fifty-four months after transection of the anterior cruciate ligament.* Arthritis Rheum, 1991. **34**(12): p. 1560-70.
72. **Marijnissen, A.C.**, et al., *The canine 'groove' model, compared with the ACLT model of osteoarthritis.* Osteoarthritis Cartilage, 2002. **10**(2): p. 145-55.
73. **Marijnissen, A.C.**, et al., *Steady progression of osteoarthritic features in the canine groove model.* Osteoarthritis Cartilage, 2002. **10**(4): p. 282-9.
74. **Mastbergen, S.C.**, et al., *The canine 'groove' model of osteoarthritis is more than simply the expression of surgically applied damage.* Osteoarthritis Cartilage, 2006. **14**(1): p. 39-46.
75. **Intema, F.**, et al., *The canine bilateral groove model of osteoarthritis.* J Orthop Res, 2008. **26**(11): p. 1471-7.
76. **Da Silva, J.A.** and **G.M. Hall**, *The effects of gender and sex hormones on outcome in rheumatoid arthritis.* Baillieres Clin Rheumatol, 1992. **6**(1): p. 196-219.
77. **Goldring, S.R.**, *Pathogenesis of bone and cartilage destruction in rheumatoid arthritis.* Rheumatology (Oxford), 2003. **42 Suppl 2**: p. ii11-6.
78. **Lefevre, S.**, et al., *Synovial fibroblasts spread rheumatoid arthritis to unaffected joints.* Nat Med, 2009. **15**(12): p. 1414-20.
79. **Arnett, F.C.**, et al., *The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis.* Arthritis Rheum, 1988. **31**(3): p. 315-24.
80. **Schmidt, T.A.**, et al., *Boundary lubrication of articular cartilage - Role of synovial fluid constituents.* Arthritis Rheum, 2007. **56**(3): p. 882-91.
81. **Hills, B.A.** and **M.K. Monds**, *Enzymatic identification of the load-bearing boundary lubricant in the joint.* Br J Rheumatol, 1998. **37**(2): p. 137-42.
82. **Wilkins, J.**, *Proteolytic destruction of synovial boundary lubrication.* Nature, 1968. **219**(5158): p. 1050-1.
83. **Nitzan, D.W.**, et al., *The role of hyaluronic acid in protecting surface-active phospholipids from lysis by exogenous phospholipase A(2).* Rheumatology (Oxford), 2001. **40**(3): p. 336-340.
84. **Jay, G.D.**, **D.E. Britt**, and **C.J. Cha**, *Lubricin is a product of megakaryocyte stimulating factor gene expression by human synovial fibroblasts.* J Rheumatol, 2000. **27**(3): p. 594-600.
85. **Schumacher, B.L.**, et al., *Immunodetection and partial cDNA sequence of the proteoglycan, superficial zone protein, synthesized by cells lining synovial joints.* J Orthop Res, 1999. **17**(1): p. 110-20.

86. **Warman, M.L.**, et al., *CACP, encoding a secreted proteoglycan, is mutated in camptodactyly-arthropathy-coxa vara-pericarditis syndrome.* Nat Genet, 1999. **23**(3): p. 319-22.
87. **Ikegawa, S.**, et al., *Isolation, characterization and mapping of the mouse and human PRG4 (proteoglycan 4) genes.* Cytogenet Cell Genet, 2000. **90**(3-4): p. 291-7.
88. **Swann, D.A.**, et al., *The lubricating activity of synovial fluid glycoproteins.* Arthritis Rheum, 1981. **24**(1): p. 22-30.
89. **Jay, G.D.**, **D.A. Harris**, and **C.J. Cha**, *Boundary lubrication by lubricin is mediated by O-linked beta(1-3)Gal-GalNAc oligosaccharides.* Glycoconj J, 2001. **18**(10): p. 807-15.
90. **Jay, G.D.**, *Characterization of a bovine synovial fluid lubricating factor 1. Chemical, surface-activity and lubricating properties.* Connect Tissue Res, 1992. **28**(1-2): p. 71-88.
91. **Jay, G.D.**, et al., *Association between friction and wear in diarthrodial joints lacking lubricin.* Arthritis Rheum, 2007. **56**(11): p. 3662-9.
92. **Teeple, E.**, et al., *Coefficients of friction, lubricin, and cartilage damage in the anterior cruciate ligament-deficient guinea pig knee.* J Orthop Res, 2008. **26**(2): p. 231-7.
93. **Fleming, B.C.**, et al., *Comparison of differential biomarkers of osteoarthritis with and without psttraumatic injury in the Hartley guinea pig model.* J Orthop Res, 2010. **28**(7): p. 900-6.
94. **Teeple, E.**, et al., *Effects of supplemental intra-articular lubricin and hyaluronic acid on the progression of posttraumatic arthritis in the anterior cruciate ligament-deficient rat knee.* Am J Sports Med, 2011. **39**(1): p. 164-72.
95. **Jay, G.D.**, et al., *Prevention of cartilage degeneration and restoration of chondroprotection by lubricin tribosupplementation in the rat following anterior cruciate ligament transection.* Arthritis Rheum, 2010. **62**(8): p. 2382-91.
96. **Flannery, C.R.**, et al., *Prevention of cartilage degeneration in a rat model of osteoarthritis by intraarticular treatment with recombinant lubricin.* Arthritis Rheum, 2009. **60**(3): p. 840-7.
97. **Kuroki, Y.** and **T. Akino**, *Pulmonary surfactant protein A (SP-A) specifically binds dipalmitoylphosphatidylcholine.* J Biol Chem, 1991. **266**(5): p. 3068-73.
98. **Holmskov, U.**, et al., *Collectins: collagenous C-type lectins of the innate immune defense system.* Immunol Today, 1994. **15**(2): p. 67-74.
99. **Mason, R.J.**, **K. Greene**, and **D.R. Voelker**, *Surfactant protein A and surfactant protein D in health and disease.* Am J Physiol, 1998. **275**(1 Pt 1): p. L1-13.
100. **Takahashi, H.**, et al., *Pulmonary surfactant proteins A and D: innate immune functions and biomarkers for lung diseases.* Curr Pharm Des, 2006. **12**(5): p. 589-98.
101. **Dobbie, J.W.**, *Surfactant protein A and lamellar bodies: a homologous secretory function of peritoneum, synovium, and lung.* Perit Dial Int, 1996. **16**(6): p. 574-81.
102. **Hills, B.A.**, *A common physical basis for the gastric mucosal barrier and the action of sucralfate.* Am J Med, 1991. **91**(2A): p. 43S-51S.
103. **Schmitz, G.** and **G. Muller**, *Structure and function of lamellar bodies, lipid-protein complexes involved in storage and secretion of cellular lipids.* J Lipid Res, 1991. **32**(10): p. 1539-70.
104. **Kankavi, O.**, et al., *Presence and subcellular localizations of surfactant proteins A and D in human spermatozoa.* Fertil Steril, 2008. **90**(5): p. 1904-9.
105. **Mo, Y.K.**, et al., *Surfactant protein expression in human skin: evidence and implications.* J Invest Dermatol, 2007. **127**(2): p. 381-6.
106. **Kankavi, O.**, **A. Ata**, and **O. Gungor**, *Surfactant proteins A and D in the genital tract of mares.* Anim Reprod Sci, 2007. **98**(3-4): p. 259-70.
107. **Kankavi, O.**, *Immunodetection of surfactant proteins in human organ of Corti, Eustachian tube and kidney.* Acta Biochim Pol, 2003. **50**(4): p. 1057-64.
108. **Kankavi, O.**, **A. Ata**, and **M. Akif Ciftcioglu**, *Surfactant protein A and D in the reproductive tract of stallion.* Theriogenology, 2006. **66**(5): p. 1057-64.
109. **Kankavi, O.**, *Increased expression of surfactant protein A and D in rheumatoid arthritic synovial fluid.* Croat Med J, 2006. **47**(1): p. 155-61.

110. **Kankavi, O.** and **M.S. Roberts**, *Detection of surfactant protein A (SP-A) and surfactant protein D (SP-D) in equine synovial fluid with immunoblotting.* Can J Vet Res, 2004. **68**(2): p. 146-9.
111. **Trinder, P.K.**, et al., *Humoral autoreactivity directed against surfactant protein-A (SP-A) in rheumatoid arthritis synovial fluids.* Clin Exp Immunol, 2000. **120**(1): p. 183-7.
112. **Meyer, K.** and **J.W. Palmar**, *The polysaccharide of the vitreous humour.* J Biol Chem, 1934. **107**: p. 629-34.
113. **Weissmann, B.**, et al., *Isolation of oligosaccharides enzymatically produced from hyaluronic acid.* J Biol Chem, 1954. **208**(1): p. 417-29.
114. **Dahl, L.B.**, et al., *Concentration and molecular weight of sodium hyaluronate in synovial fluid from patients with rheumatoid arthritis and other arthropathies.* Ann Rheum Dis, 1985. **44**(12): p. 817-22.
115. **Fraser, J.R.**, **T.C. Laurent**, and **U.B. Laurent**, *Hyaluronan: its nature, distribution, functions and turnover.* J Intern Med, 1997. **242**(1): p. 27-33.
116. **Weigel, P.H.**, **V.C. Hascall**, and **M. Tammi**, *Hyaluronan synthases.* J Biol Chem, 1997. **272**(22): p. 13997-14000.
117. **Levick, J.R.**, et al., *Role of hyaluronan chain length in buffering interstitial now across synovium in rabbits.* J Physiol, 2000. **526**(2): p. 425-34.
118. **Bellamy, N.**, et al., *Viscosupplementation for the treatment of osteoarthritis of the knee.* Cochrane Database Syst Rev, 2006(2): p. CD005321.
119. **Zhang, W.**, et al., *OARSI recommendations for the management of hip and knee osteoarthritis: part III: Changes in evidence following systematic cumulative update of research published through January 2009.* Osteoarthritis Cartilage, 2010. **18**(4): p. 476-99.
120. **Wang, C.T.**, et al., *High molecular weight hyaluronic acid down-regulates the gene expression of osteoarthritis-associated cytokines and enzymes in fibroblast-like synoviocytes from patients with early osteoarthritis.* Osteoarthritis Cartilage, 2006. **14**(12): p. 1237-47.
121. **Bleijerveld, O.B.**, et al., *Metabolipidomics: profiling metabolism of glycerophospholipid species by stable isotopic precursors and tandem mass spectrometry.* Anal Biochem, 2006. **352**(1): p. 1-14.
122. **Postle, A.D.**, *Phospholipid lipidomics in health and disease.* Eur J Lipid Sci Technol 2009. **111**: p. 2-13.
123. **Hills, B.A.**, **M.T. Ethell**, and **D.R. Hodgson**, *Release of lubricating synovial surfactant by intra-articular steroid.* Br J Rheumatol, 1998. **37**(6): p. 649-52.
124. **Birken, E.A.** and **K.H. Brookler**, *Surface tension lowering substance of the canine Eustachian tube.* Ann Otol Rhinol Laryngol, 1972. **81**(2): p. 268-71.
125. **Bernhard, W.**, et al., *Composition of phospholipid classes and phosphatidylcholine molecular species of gastric mucosa and mucus.* Biochim Biophys Acta, 1995. **1255**(2): p. 99-104.
126. **Paananen, R.**, et al., *Eustachian tube surfactant is different from alveolar surfactant: determination of phospholipid composition of porcine eustachian tube lavage fluid.* J Lipid Res, 2002. **43**(1): p. 99-106.
127. **Chen, Y.**, **B.A. Hills**, and **Y.C. Hills**, *Unsaturated phosphatidylcholine and its application in surgical adhesion.* ANZ J Surg, 2005. **75**(12): p. 1111-4.
128. **Sarma, A.V.**, **G.L. Powell**, and **M. LaBerge**, *Phospholipid composition of articular cartilage boundary lubricant.* J Orthop Res, 2001. **19**(4): p. 671-6.
129. **Chen, Y.**, **R.W. Crawford**, and **A. Oloyede**, *Unsaturated phosphatidylcholines lining on the surface of cartilage and its possible physiological roles.* J Orthop Surg Res, 2007. **2**: p. 14.
130. **Chen, Y.**, **R.W. Crawford**, and **A. Oloyede**, *Preliminary evaluation of the capacity of surface-active phospholipids to provide semipermeability in a saline filtration environment.* Med Sci Monit, 2007. **13**(4): p. BR101-5.
131. **Hills, B.A.**, *Oligolamellar nature of the articular surface.* J Rheumatol, 1990. **17**(3): p. 349-56.

132. **Crockett, R.**, *Boundary Lubrication in Natural Articular Joints.* Tribol Lett, 2009. **35**: p. 77-84.
133. **Hills, B.A.**, *Remarkable anti-wear properties of joint surfactant.* Ann Biomed Eng, 1995. **23**(2): p. 112-5.
134. **Ghio, A.J.**, et al., *Synthetic surfactant scavenges oxidants and protects against hyperoxic lung injury.* J Appl Physiol, 1994. **77**(3): p. 1217-23.
135. **Burkhardt, H.**, et al., *Oxygen radicals as effectors of cartilage destruction. Direct degradative effect on matrix components and indirect action via activation of latent collagenase from polymorphonuclear leukocytes.* Arthritis Rheum, 1986. **29**(3): p. 379-87.
136. **Dieppe, P.A.**, et al., *Intra-articular steroids in osteoarthritis.* Rheumatol Rehabil, 1980. **19**(4): p. 212-7.
137. **Torday, J.S.**, **B.T. Smith**, and **C.J. Giroud**, *The rabbit fetal lung as a glucocorticoid target tissue.* Endocrinology, 1975. **96**(6): p. 1462-7.
138. **Hills, B.A.** and **M.K. Monds**, *Deficiency of lubricating surfactant lining the articular surfaces of replaced hips and knees.* Br J Rheumatol, 1998. **37**: p. 143-7.
139. **Li, Z.** and **D.E. Vance**, *Phosphatidylcholine and choline homeostasis.* J Lipid Res, 2008. **49**(6): p. 1187-94.
140. **Eros, G.**, et al., *Oral phosphatidylcholine pretreatment alleviates the signs of experimental rheumatoid arthritis.* Arthritis Res Ther, 2009. **11**(2): p. R43.
141. **Schmitz, G.** and **K. Ruebsaamen**, *Metabolism and atherogenic disease association of lysophosphatidylcholine.* Atherosclerosis, 2010. **208**: p. 10-18.
142. **Fuchs, B.**, et al., *The phosphatidylcholine/lysophosphatidylcholine ratio in human plasma is an indicator of the severity of rheumatoid arthritis: investigations by 31P NMR and MALDI-TOF MS.* Clin Biochem, 2005. **38**(10): p. 925-33.
143. **Vance, J.E.**, *Phosphatidylserine and phosphatidylethanolamine in mammalian cells: two metabolically related aminophospholipids.* J Lipid Res, 2008. **49**: p. 1377-87.
144. **Nagan, N.** and **R.A. Zoeller**, *Plasmalogens: biosynthesis and functions.* Prog Lipid Res, 2001. **40**(3): p. 199-229.
145. **Mandel, H.**, et al., *Plasmalogen phospholipids are involved in HDL-mediated cholesterol efflux: insights from investigations with plasmalogen-deficient cells.* Biochem Biophys Res Commun, 1998. **250**(2): p. 369-73.
146. **Allan, D.**, *Mapping the lipid distribution in the membranes of BHK cells (mini-review).* Mol Membr Biol, 1996. **13**(2): p. 81-4.
147. **Kuge, O.** and **M. Nishijima**, *Phosphatidylserine synthase I and II of mammalian cells.* Biochim Biophys Acta, 1997. **1348**(1-2): p. 151-6.
148. **Delwaide, P.J.**, et al., *Double-blind randomized controlled study of phosphatidylserine in senile demented patients.* Acta Neurol Scand, 1986. **73**(2): p. 136-40.
149. **Suzuki, S.**, et al., *Oral administration of soybean lecithin transphosphatidylated phosphatidylserine improves memory impairment in aged rats.* J Nutr, 2001. **131**(11): p. 2951-6.
150. **Mozzi, R.**, **S. Buratta**, and **G. Goracci**, *Metabolism and functions of phosphatidylserine in mammalian brain.* Neurochem Res, 2003. **28**(2): p. 195-214.
151. **Downes, C.P.**, **A. Gray**, and **J.M. Lucocq**, *Probing phosphoinositide functions in signaling and membrane trafficking.* Trends Cell Biol, 2005. **15**: p. 259-68.
152. **De Matteis, M.A.** and **A. Godi**, *PI-loting membrane traffic.* Nat Cell Biol, 2004. **6**(6): p. 487-92.
153. **Rothenberg, R.J.** and **H. Cheung**, *Rabbit synoviocyte inositol phospholipid metabolism is stimulated by hydroxyapatite crystals.* Am J Physiol, 1988. **254**(4 Pt 1): p. C554-9.
154. **Lewis, J.F.** and **A.H. Jobe**, *Surfactant and the adult respiratory distress syndrome.* Am Rev Respir Dis, 1993. **147**(1): p. 218-33.
155. **Vance, J.E.** and **D.E. Vance**, *Phospholipid biosynthesis in mammalian cells.* Biochem Cell Biol, 2004. **82**(1): p. 113-28.
156. **Nag, K.**, et al., *SP-B refining of pulmonary surfactant phospholipid films.* Am J Physiol 1999. **277**: p. L1179-L1189.

157. **Lahiri, S.** and **A.H. Futerman**, *The metabolism and function of sphingolipids and glycoshingolipids.* Cell Mol Life Sci, 2007. **64**: p. 2270-84.
158. **Cutler, R.G.** and **M.P. Mattson**, *Sphingomyelin and ceramide as regulators of development and lifespan.* Mech Ageing Dev, 2001. **122**(9): p. 895-908.
159. **Gerritsen, M.E.**, **C.P. Schen**, and **C.A. Perry**, *Ceramide, apoptosis, and TNF-α signaling in synovial fibroblasts.* AJP, 1998. **152**(2): p. 505-12.
160. **Dechecchi, M.C.**, et al., *Modulators of sphingolipid metabolism reduce lung inflammation.* Am J Respir Cell Mol Biol, 2011. **45**(4): p. 825-33.
161. **Duan, J.**, et al., *Oral glucosylceramide reduces 2,4-dinitrofluorobenzene induced inflammatory response in mice by reducing TNF-alpha levels and leukocyte infiltration.* Lipids, 2011. **46**(6): p. 505-12.
162. **Wen, F.Q.**, et al., *c-myc proto-oncogene expression in hemophilic synovitis: in vitro studies of the effects of iron and ceramide.* Blood, 2002. **100**(3): p. 912-6.
163. **Bligh, E.G.** and **W.J. Dyer**, *A rapid method of total lipid extraction and purification.* Can J Biochem Physiol, 1959. **37**(8): p. 911-7.
164. **Liebisch, G.**, et al., *High-throughput quantification of lysophosphatidylcholine by electrospray ionization tandem mass spectrometry.* Clin Chem, 2002. **48**(12): p. 2217-24.
165. **Liebisch, G.**, et al., *Quantitative measurement of different ceramide species from crude cellular extracts by electrospray ionization tandem mass spectrometry (ESI-MS/MS).* J Lipid Res, 1999. **40**(8): p. 1539-46.
166. **Liebisch, G.**, et al., *High-throughput quantification of phosphatidylcholine and sphingomyelin by electrospray ionization tandem mass spectrometry coupled with isotope correction algorithm.* Biochim Biophys Acta, 2004. **1686**(1-2): p. 108-17.
167. **Hee-Young, K.** and **S. Norman**, *Separation of lipid classes by solid phase extraction.* J Lipid Res, 1990. **31**: p. 2285-9.
168. **Liebisch, G.**, et al., *High throughput quantification of cholesterol and cholesteryl ester by electrospray ionization tandem mass spectrometry (ESI-MS/MS).* Biochim Biophys Acta, 2006. **1761**(1): p. 121-8.
169. **Brugger, B.**, et al., *Quantitative analysis of biological membrane lipids at the low picomole level by nano-electrospray ionization tandem mass spectrometry.* Proc Natl Acad Sci U S A, 1997. **94**(6): p. 2339-44.
170. **Zemski Berry, K.A.** and **R.C. Murphy**, *Electrospray ionization tandem mass spectrometry of glycerophosphoethanolamine plasmalogen phospholipids.* J Am Soc Mass Spectrom, 2004. **15**(10): p. 1499-508.
171. **Matyash, V.**, et al., *Lipid extraction by methyl-tert-butyl ether for high-throughput lipidomics.* J Lipid Res, 2008. **49**(5): p. 1137-46.
172. **Neumann, E.**, et al., *Cell culture and passaging alters gene expression pattern and proliferation rate in rheumatoid arthritis synovial fibroblasts.* Arthritis Res Ther, 2010. **12**(3): p. R83.
173. **Bradford, M.M.**, *A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding.* Anal Biochem, 1976. **72**: p. 248-54.
174. **Laemmli, U.K.**, *Cleavage of structural proteins during the assembly of the head of bacteriophage T4.* Nature, 1970. **227**(5259): p. 680-5.
175. **Korfei, M.**, et al., *Epithelial endoplasmic reticulum stress and apoptosis in sporadic idiopathic pulmonary fibrosis.* Am J Respir Crit Care Med, 2008. **178**(8): p. 838-46.
176. **Innis, M.A.**, *A guide to methods and applications.* Academic Press PCR protocols, 1990.
177. **Kraus, V.B.**, et al., *Urea as a passive transport marker for arthritis biomarker studies.* Arthritis Rheum, 2002. **46**(2): p. 420-7.
178. **Kraus, V.B.**, et al., *Measurement of synovial fluid volume using urea.* Osteoarthritis Cartilage, 2007. **15**(10): p. 1217-20.
179. **Gough, M.R.**, G.A. Munroe, and I.G. Mayhew, *Urea as a measure of dillution of equine synovial fluid.* Equine Vet J, 2002. **34**(1): p. 76-9.
180. **Lafeber, F.P.**, et al., *Unloading joints to treat osteoarthritis, including joint distraction.* Curr Opin Rheumatol, 2006. **18**(5): p. 519-25.

181. **Duff, S.R.**, *Dyschondroplasia/osteochondrosis of the femoral trochanter in the fowl.* J Comp Pathol, 1985. **95**(3): p. 363-71.
182. **Madea, B.**, **C. Kreuser**, and **S. Banaschak**, *Postmortem biochemical examination of synovial fluid--a preliminary study.* Forensic Sci Int, 2001. **118**(1): p. 29-35.
183. **Distler, J.H.**, et al., *Microparticles as regulators of inflammation: novel players of cellular crosstalk in the rheumatic diseases.* Arthritis Rheum, 2005. **52**(11): p. 3337-48.
184. **Wuthier, R.E.**, *Lipid composition of isolated epiphyseal cartilage cells, membranes and matrix vesicles.* Biochim Biophys Acta, 1975. **409**(1): p. 128-43.
185. **Simkin, P.A.** and **J.E. Pizzorno**, *Transynovial exchange of small molecules in normal human subjects.* J Appl Physiol, 1974. **36**(5): p. 581-7.
186. **Ropes, M.W.** and **W. Bauer**, *Synovial Fluid Changes in Joint Disease.* J Bone Joint Surg, 1954. **36**(1): p. 208-b-208
187. **Hills, B.A.** and **B.D. Butler**, *Surfactants identified in synovial fluid and their ability to act as boundary lubricants.* Ann Rheum Dis, 1984. **43**(4): p. 641-8.
188. **Little, T.**, **M.A.R. Freeman**, and **S.A.V. Swanson**, *Experiments on friction in the human hip joint.* Lubrication and Wear in Joints, V. Wright (Ed.), 1969: p. 110-116.
189. **Bole, G.G.**, *Synovial fluid lipids in normal individuals and patients with rheumatoid arthritis.* Arthritis Rheum, 1962. **5**: p. 589-601.
190. **Linn, F.C.** and **E.L. Radin**, *Lubrication of animal joints. 3. The effect of certain chemical alterations of the cartilage and lubricant.* Arthritis Rheum, 1968. **11**(5): p. 674-82.
191. **Jay, G.D.** and **C.J. Cha**, *The effect of phospholipase digestion upon the boundary lubricating ability of synovial fluid.* J Rheumatol, 1999. **26**(11): p. 2454-7.
192. **Hardy, W.B.**, *Collected papers of Sir William Hardy.* Cambridge: Cambridge University Press, 1936.
193. **Sun, Y.**, et al., *The effect of hyaluronidase, phospholipase, lipid solvent and trypsin on the lubrication of canine flexor digitorum profundus tendon.* J Orthop Res, 2008. **26**(9): p. 1225-9.
194. **Schwarz, I.M.** and **B.A. Hills**, *Surface-active phospholipid as the lubricating component of lubricin.* Br J Rheumatol, 1998. **37**(1): p. 21-6.
195. **Yielding, K.L.**, **G.M. Tomkins**, and **J.J. Bunim**, *Synthesis of hyaluronic acid by human synovial tissue slices.* Science, 1957. **125**(3261): p. 1300.
196. **Carpten, J.D.**, et al., *The secreted glycoprotein lubricin protects cartilage surfaces and inhibits synovial cell overgrowth.* J Clin Invest, 2005. **115**(3): p. 622-31.
197. **Fuchs, B.**, et al., *Phospholipid compositions of sera and synovial fluids from dog, human and horse: a comparison by ^{31}P-NMR and MALDI-TOF MS.* J Anim Physiol Anim Nutr (Berl), 2009. **93**(4): p. 410-22.
198. **Gopinath, B.**, et al., *A questionnaire survey about doctor-patient communication, compliance and locus of control among south Indian people with epilepsy.* Epilepsy Res, 2000. **39**(1): p. 73-82.
199. **Prete, P.E.**, A. Gurakar-Osborne, and M.L. Kashyap, *Synovial fluid lipoproteins: review of current concepts and new directions.* Semin Arthritis Rheum, 1993. **23**(2): p. 79-89.
200. **Chung, A.C.**, **J.R. Shanahan**, and **E.M. Brown**, Jr., *Synovial fluid lipids in rheumatoid and osteoarthritis.* Arthritis Rheum, 1962. **5**: p. 176-83.
201. **Punzi, L.**, et al., *Phospholipids in inflammatory synovial effusions.* Rheumatol Int, 1986. **6**(1): p. 7-11.
202. **Prete, P.E.** and **A. Gurakar-Osborne**, *The contribution of synovial fluid lipoproteins to the chronic synovitis of rheumatoid arthritis.* Prostaglandins, 1997. **54**(4): p. 689-98.
203. **Purbach, B.**, **B.A. Hills**, and **B.M. Wroblewski**, *Surface-active phospholipid in total hip arthroplasty.* Clin Orthop Relat Res, 2002(396): p. 115-8.
204. **Hills, B.A.**, *Oligolamellar lubrication of joints by surface active phospholipid.* J Rheumatol, 1989. **16**(1): p. 82-91.
205. **Leidl, K.**, et al., *Mass spectrometric analysis of lipid species of human circulating blood cells.* Biochim Biophys Acta, 2008. **1781**(10): p. 655-64.

206. **Castro-Perez, J.M.**, et al., *Comprehensive LC-MS E lipidomic analysis using a shotgun approach and its application to biomarker detection and identification in osteoarthritis patients.* J Proteome Res, 2010. **9**(5): p. 2377-89.
207. **Postle, A.D.**, et al., *Lipidomics of cellular and secreted phospholipids from differentiated human fetal type II alveolar epithelial cells.* J Lipid Res, 2006. **47**(6): p. 1322-31.
208. **Fuller, D.D.**, *Boundary lubrication.* Theory and practice of lubrication for engineers, 1956. **1**(New York: Chapman & Hall): p. 342-72.
209. **Rogers, J.M.**, et al., *Effect of phospholipidic boundary lubrication in rigid and compliant hemiarthroplasty models* Proc Inst Mech Eng H, 1999. **213**: p. 5-18.
210. **Belcaro, G.**, et al., *Efficacy and safety of Meriva(R), a curcumin-phosphatidylcholine complex, during extended administration in osteoarthritis patients.* Altern Med Rev, 2010. **15**(4): p. 337-44.
211. **Tonks, A.**, et al., *Surfactant phospholipid DPPC downregulates monocyte respiratory burst via modulation of PKC.* Am J Physiol Lung Cell Mol Physiol, 2005. **288**(6): p. L1070-80.
212. **Higaki, H.**, et al., *The lubricating ability of biomembrane models with dipalmitoyl phosphatidylcholine and gamma-globulin.* Proc Inst Mech Eng H, 1998. **212**(5): p. 337-46.
213. **Schmidt, T.A.**, et al., *Boundary lubrication of articular cartilage: role of synovial fluid constituents.* Arthritis Rheum, 2007. **56**(3): p. 882-91.
214. **Forsey, R.W.**, et al., *The effect of hyaluronic acid and phospholipid based lubricants on friction within a human cartilage damage model.* Biomaterials, 2006. **27**(26): p. 4581-90.
215. **Kawano, T.**, et al., *Mechanical effects of the intraarticular administration of high molecular weight hyaluronic acid plus phospholipid on synovial joint lubrication and prevention of articular cartilage degeneration in experimental osteoarthritis.* Arthritis Rheum, 2003. **48**(7): p. 1923-9.
216. **Vecchio, P.**, **R. Thomas**, and **B.A. Hills**, *Surfactant treatment for osteoarthritis.* Rheumatology (Oxford), 1999. **38**(10): p. 1020-1.
217. **Gale, L.R.**, et al., *Boundary lubrication of joints: characterization of surface-active phospholipids found on retrieved implants.* Acta Orthop, 2007. **78**(3): p. 309-14.
218. **Brosche, T.** and **D. Platt**, *The biological significance of plasmalogens in defense against oxidative damage.* Exp Gerontol, 1998. **33**(5): p. 363-9.
219. **Morand, O.H.**, **R.A. Zoeller**, and **C.R. Raetz**, *Disappearance of plasmalogens from membranes of animal cells subjected to photosensitized oxidation.* J Biol Chem, 1988. **263**(23): p. 11597-606.
220. **Yudoh, K., et al.**, *Potential involvement of oxidative stress in cartilage senescence and development of osteoarthritis: oxidative stress induces chondrocyte telomere instability and downregulation of chondrocyte function.* Arthritis Res Ther, 2005. **7**(2): p. R380-91.
221. **Parkes, H.G.**, et al., *Oxidative damage to synovial fluid from the inflamed rheumatoid joint detected by 1H NMR spectroscopy.* J Pharm Biomed Anal, 1991. **9**(1): p. 75-82.
222. **Mort, J.S.** and **C.J. Billington**, *Articular cartilage and changes in arthritis: matrix degradation.* Arthritis Res, 2001. **3**(6): p. 337-41.
223. **Sabatini, M.**, et al., *Effects of ceramide on apoptosis, proteoglycan degradation, and matrix metalloproteinase expression in rabbit articular cartilage.* Biochem Biophys Res Commun, 2000. **267**(1): p. 438-44.
224. **Sabatini, M.**, et al., *Effects of ceramide on aggrecanase activity in rabbit articular cartilage.* Biochem Biophys Res Commun, 2001. **283**(5): p. 1105-10.
225. **Marijnissen, A.C.**, et al., *Clinical benefit of joint distraction in the treatment of severe osteoarthritis of the ankle: proof of concept in an open prospective study and in a randomized controlled study.* Arthritis Rheum, 2002. **46**(11): p. 2893-902.
226. **Ploegmakers, J.J.**, et al., *Prolonged clinical benefit from joint distraction in the treatment of ankle osteoarthritis.* Osteoarthritis Cartilage, 2005. **13**(7): p. 582-8.
227. **Intema, F.**, et al., *Tissue structure modification in knee osteoarthritis by use of joint distraction: an open 1-year pilot study.* Ann Rheum Dis, 2011. **70**(8): p. 1441-6.
228. **Intema, F.**, *Loading and unloading in the development and treatment of osteoarthritis.* Thesis, Utrecht University, the Netherlands, 2010.

229. **Wiegant, K.**, et al., *Joint distraction in treatment of canine experimentally induced osteoarthritis leads to cartilage repair accompanied by sustained relieve in pain.* Orthopaedic Research Society (ORS) Annual Meeting 2012: p. Poster. No. 0792, San Francisco, USA, 04-07 February 2012
230. **Mapp, P.I.** and **P.A. Revell**, *Fibronectin production by synovial intimal cells.* Rheumatol Int, 1985. **5**(5): p. 229-37.
231. **Greene, K.E.**, et al., *Serum surfactant proteins-A and -D as biomarkers in idiopathic pulmonary fibrosis.* Eur Respir J, 2002. **19**(3): p. 439-46.
232. **Takahashi, H.**, et al., *Diagnostic significance of surfactant proteins A and D in sera from patients with radiation pneumonitis.* Eur Respir J, 2001. **17**(3): p. 481-7.
233. **Kunitake, R.**, et al., *KL-6, surfactant protein A and D in bronchoalveolar lavage fluid from patients with pulmonary sarcoidosis.* Respiration, 2001. **68**(5): p. 488-95.
234. **Christensen, A.F.**, et al., *Circulating surfactant protein -D is low and correlates negatively with systemic inflammation in early, untreated rheumatoid arthritis.* Arthritis Res Ther, 2010. **12**(2): p. R39.

8. Declaration

“I declare that I have completed this dissertation single-handedly without the unauthorized help of a second party and only with assistance acknowledge therein. I have appropriately acknowledged and referenced all text passages that are derived literally from or are based on the content of published or unpublished work of others, and all information that relates to verbal communications. I have abided by the principles of good scientific conduct laid down in the charter of the Justus-Liebig-University of Giessen in carrying out the investigations described in the dissertation.”

Giessen, 18.04.2012 Marta Kosinska

Der Lebenslauf wurde aus der elektronischen Version der Arbeit entfernt.

The curriculum vitae was removed from the electronic version of the paper.

10. Acknowledgements

In the last chapter of my thesis, I would like to express my sincere acknowledgements to all people who have encouraged me with their support and suggestions during the long way of my PhD.

First and foremost I would like to thank my supervisor Prof. Dr. rer. nat. Jürgen Steinmeyer for giving me the unique opportunity to begin my research carrier in his laboratory. I am eternally grateful for his guidance, unreserved support, endless patience, constructive criticism and long scientific discussions in nice atmosphere.

Secondly, I would like to gratefully acknowledge Prof. Dr. med. Gerd Schmitz (Department of Clinical Chemistry and Laboratory Medicine, University Hospital Regensburg, Regensburg, Germany) for offering me the cooperation opportunity and possibility to get insight in lipidomic knowledge. I am also grateful to Dr. rer. nat. Gerhard Liebisch (Department of Clinical Chemistry and Laboratory Medicine, University Hospital Regensburg, Regensburg, Germany) for helping me with ESI-MS/MS analysis and giving answers for all my questions.

My sincere acknowledgements go to the surgery team from our clinic for providing me with OA SF, synovial tissues and cartilage used in this study and helping me with medical evaluation of the patients (Prof. Dr. med. Markus Rickert, Prof. Dr. med. Henning Stürz, Dr. med. Heiko Klein, PD Dr. med. Jens Kordelle).

I would like to express my gratitude to Prof. Floris Lafeber, PhD and Simon Mastbergen, PhD (both from Department of Rheumatology and Clinical Immunology, University Medical Center Utrecht, Utrecht, The Netherlands) for providing me with canine SF obtained from distraction experiments.

I am also grateful to Dr. med. Ulrich Käβer (Internistisches Praxiszentrum am Balserischen Stift, Giessen, Germany) for providing me with RA SF and for evaluating medically the patients.

I would like to acknowledge Prof. Dr. rer. nat. Günter Lochnit (Department of Biochemistry, Justus-Liebig-University of Giessen, Giessen, Germany) for 2-D gels electrophoresis, SPE and MALDI-TOF/MS analysis.

My special thanks go to Dr. Jochen Wilhelm (Medical Clinic II/IV, Justus-Liebig-University of Giessen, Giessen, Germany) for guiding me through statistical labyrinth.

I would like to thank people from Institute of Forensic Medicine (Justus-Liebig-University of Giessen, Giessen, Germany) for providing me with cadaveric SF (Prof. Dr. med. Dr. jur. Reinhard Dettmeyer, Prof. Dr. med. Marcel A. Verhoff, Dr. med. Gabriele Lasczkowski).

My thanks go also to Dr. med. vet. Martina Sperling (Veterinary Clinic for Horses, Justus-Liebig-University of Giessen, Germany) for providing me with equine SF.

It was particular pleasure to work together with people from Laboratory for Experimental Orthopaedics. I offer my heartfelt thanks to Manuela Döller, Christiane Hild, Anne Staubitz and Magdalena Singer for all their help and support and for creating nice and warm atmosphere in the lab every day.

Special thanks I address to my best friends, Ewa Jablonska for more than 20 years of friendship, Joanna Wilczynska for all help and time spend together and Izabela Chrobak for support behind the wall and across the ocean. During four years spend in Giessen I have met a lot of great people. Alex, Diya, Djuro, Kate, Lukas, Oleg and Swati -thank you all for making life abroad much easier.

My warmest gratitude goes to my partner Sylwester. Thank you for all your love, support, encourages, patients and for making me laugh.

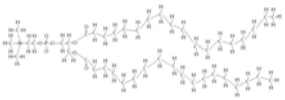
Finally, nothing would have been possible without my Mom and my Father. Thank you for your unlimited love, support and all help I got. Without you I would not be here.

Foremost I would like to thank my good angels, my Grandmother and Grandfather, without whom I would not be this person I am today. I know that you are watching and protecting me every day. All my work I am dedicating to you.

Moje najszczersze podziękowania kieruję do mojego partnera Sylwestra. Dziękuję Ci za całą Twoją miłość, wsparcie, cierpliwość, zachęcanie mnie do pracy i codzienny uśmiech. Za zielone banany i maślanki…

Na koniec, to wszystko nie byłoby możliwe bez mojej Mamy i mojego Taty. Dziękuję Wam za Waszą miłość, wsparcie i pomoc. Bez Was nie byłoby mnie tutaj.

Dziękuję również moim dobrym Aniołom, Babci i Dziadkowi, bez których nie byłabym tą osobą którą jestem dzisiaj. Wiem że na mnie patrzycie i ochraniacie mnie każdego dnia. Całą moją pracę dedykuję Wam.



édition scientifique
VVB LAUFERSWEILER VERLAG

VVB LAUFERSWEILER VERLAG
STAUFENBERGRING 15
D-35396 GIESSEN

Tel: 0641-5599888 Fax: -5599890
redaktion@doktorverlag.de
www.doktorverlag.de

ISBN: 978-3-8359-5952-1

Cover photo: Medical Art Inc. - iStockPhoto