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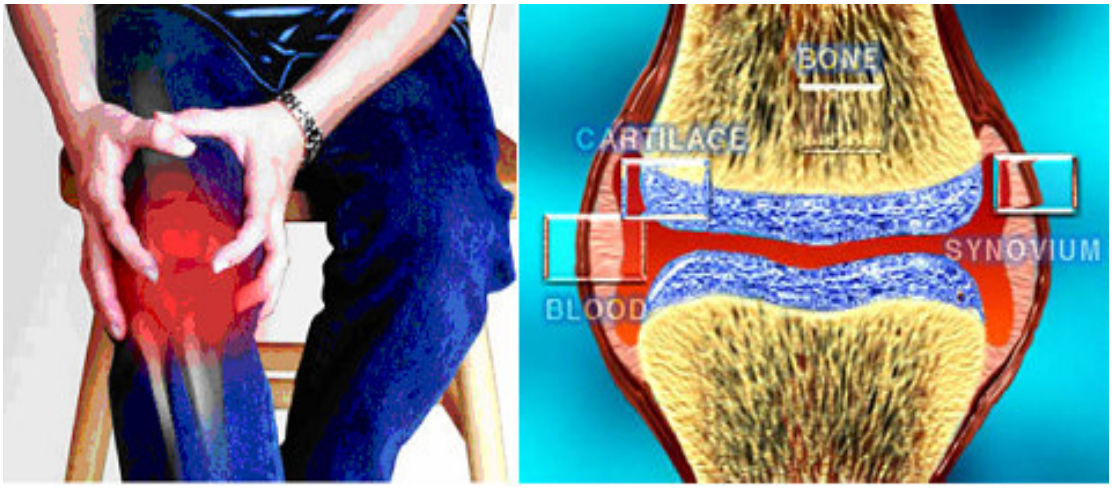

BY

RUBINA OLSEN

MASTER THESIS IN HUMAN NUTRITION

2008

INSTITUTE OF MEDICINE, UNIVERSITY OF BERGEN (UIB)

NATIONAL INSTITUTE OF NUTRITION AND SEAFOOD RESEARCH (NIFES)

HAUKELAND UNIVERSITY HOSPITAL (HUH)

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Cover figures: On the left a there is a figure of a person painfully holding its knee. The swelling knee joint is shown in red (http://www.allaboutarthritis.com/AllAboutArthritis/layoutTemplates/html/en/contentdisplay/document/condition/arthritis/clinicalArticle/knee_RA_symptoms.htm). On the right there is a figure of the synovial joint. Squares mark different parts of the synovial joint, including the synovium, cartilage, blood vessels and bone (http://www.icr.ac.uk/ieu/e-learning/ra/joint.htm).

Forord

Arbeid med denne masteroppgaven ble gjennomført ved Nasjonalt institutt for ernærings- og sjømatforskning (NIFES) i perioden august 2007 til september 2008. Jeg vil takke direktør Øyvind Lie for kontorplass og tilgang til laboratoriefasiliteter i et inspirerende arbeidsmiljø. Videre vil jeg takke Haukeland universitet sykehus og pasientene for materiale som gjorde denne studien mulig.

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Bergen, September 2008

Rubina Olsen

Table of contents

Abstract

Little attention has been brought on the lipid content and fatty acid composition directly in synovial fluid (SF) from joints of patients with inflammatory joint diseases. Studies indicate that excessive SF lipids and fatty acids found in patients with rheumatoid arthritis are mainly derived from blood. Several studies have demonstrated a relationship between n-6 and n-3 polyunsaturated fatty acids (PUFA) in blood and inflammation. Generally, a high content of n-6 PUFA, and an increased ratio between the n-6 to n-3 PUFA increases tissue formation of more potent pro-inflammatory eicosanoids and cytokines, whereas an increased content of n-3 long chain PUFA (LCPUFA), may have anti-inflammatory effects. Little is known about these fatty acids with respect to effects in the actual site of inflammation and joint destruction.

We have performed a cross-sectional study of synovial fluid (SF) from 86 patients with various inflammatory joint diseases, including rheumatoid arthritis (RA), psoriatic arthritis (PsA), reactive arthritis (ReA), and ankylosing spondylitis (AS). The three latter diseases are all included in the main group of diseases, the spondyloarthropaties (SpA). The SF n-6 and n-3 PUFA levels, and the ratios between them, were correlated to the specific inflammatory markers, i.e C-reactive protein (CRP) in serum, and interleukin (IL)-6 and IL-12 in SF. There were found significant correlations of n-6 and n-3 PUFA levels, and ratios between them, to levels of inflammatory markers in SF, which included the patient groups with chronic inflammatory joint diseases, i.e the RA, PsA and AS groups. The correlations included most frequently positive correlation of n-6 arachadonic acid (AA) precursors, including linoleic acid (LA) and dihomo-γ-linolenic acid (DGLA), to levels of IL-6.

The levels of inflammatory markers (serum CRP, SF IL-6 and IL-12) and the correlation between them, and the genereal SF fatty acid composition were also studied in the same patients. There were significantly higher levels of serum CRP in RA patients and AS compared with the PsA group. No differences were found concerning the levels of IL-6 in SF between groups, which represented the most abundant cytokine in SF. As for levels of IL-12, there were found significantly higher levels of the cytokine in SF from RA patients as compared with PsA group and the SpA main group. From the correlation of inflammatory markers, a significant weakly negative correlation between SF IL-12 and IL-6 in RA patients was found. Further, significant weakly positive correlations between serum CRP and SF IL-6 in all patients and the SpA group were found. Approximately half of the fatty acid

composition in SF of all patients constitutes of PUFA, mostly n-6, whereas the other half is divided nearly equally between saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA). Significantly differences were only found between the RA and AS subgroup or more frequently SpA main group. All statistically significant differences were seen between the n-6 and n-3 PUFA levels. The RA group had generally higher content of n-3 fatty acids contributing to a lower n-6 to n-3 ratio.

List of abbreviations

AA - Arachidonic acid
ALA - Alpha-linolenic acid
APP - Acute phase proteins
AS - Ankylosing Spondylitis
CH2 - Methylene groups
CH3 – Methyl group
COOH –Carboxyl group
COX – Cyclooxygenase
CRP – C-reactive proteins
DGLA - Dihomo-γ-linolenic acid
DHA – Docosahexaenoic acid
DMARDs – Disease modifying anti-rheumatic drugs
DPA – Docosapentaenoic acid
ELISA – Enzyme linked Immunosorbent Assay
EPA – Eicosapentaenoic acid
FID - Flame ionization detector
GLC - Gas Liquid Chromatography
HLA – Human leucocyte antigene
HUH – Haukeland University Hospital
IL – Interleukin
INF – Interferon
IR - Interquartile Range
r-hu –recombinant human
IUPAC - International Union of Pure and Applied Chemistry
KM – Control material
LA – Linoleic acid
LCPUFA – Long chain polyunsaturated fatty acid
LDL - Low density lipoprotein
LOD – Limit of detection
LOQ – Limit of quantification
LOX – Lipoxygenase
LT - Leukotrienes
MHC- Major histocompatibility complex
MMP – Matrix metalloproteinases
MUFA – Monounsaturated fatty acids
MWBc Mann-Whitney U test with Bonferroni's correction
(n-) - n minus
NFκB – Nuclear Factor kappa B
NK – Natural killers
NO – Nitric oxide
NSAIDs – Non-steroidal anti-inflammatory drugs
OA – Osteoarthritis
PAF – Platelet Aggregator factors
PG – Prostaglandin
PL - Phospholipids
PsA – Psoriatic Arthritis
PUFA - Polyunsaturated fatty acid
RA – Rheumatoid arthritis
RBC – Red blood cells
ReA – Reactive arthritis
RF – Rhematoid Factor
Rho - Spearman's rank order coefficients
ROS – Reactive oxygen species
SF – Synovial fluid
SFA - Saturated fatty acid
SpA - Spondyloarthropaties
TAG – Triacylglycerol
Th – T helper cell
TMB – Tetramethyl Benzidine
TNF – Tumor necrosis factor
TX - Tromboxane (ω-) omega minus
(ω-) omega minus

I. Introduction

BACKGROUND

The human diet has been represented by a hunter and gatherer lifestyle since the human emergence 1.7 million years ago. However, after the Agricultural evolution over the past 10 000 years, major changes of the Western diet has appeared, and particularly during the Industrial period the last 100 years or so. Among others, the ratio of n-6 to n-3 polyunsaturated fatty acids (PUFA) has increased notably, particular due to increased use of cheap bulkavailable n-6 PUFA rich plant oils (Simopoulos, 2002a). Various lipids are a significant substance of the body tissues, e.g adipose tissue and cell membranes, and a high dietary intake of n-6 to n-3 PUFA is reflected in blood and various tissues (Simopoulos, 2002b). Studies indicate that n-6 and n-3 PUFA generally have different immunomodulatory activities. A high content of n-6 PUFA, and an increased ratio between the n-6 to n-3 PUFA increases tissue formation of more potent pro-inflammatory eicosanoids and cytokines, whereas an increased content of long chain n-3 PUFA, may have anti-inflammatory effects (James *et al.*, 2003; Grimble & Tappia, 1998). Further, pathogenesis of many diseases, including inflammatory joint diseases, have been associated with these proinflammatory mediators and a physiological influence of an increased dietary and thus tissue ratio of n-6 to n-3 PUFA (Simopoulos, 2002a; Adam, 2003; James *et al.*, 2003). On the contrary, n-3 PUFA supplementation may improve clinical disease outcome (Goldberg & Katz, 2007). Studies show that lipids and fatty acids in synovial fluid (SF) are mainly blood-derived (Prete *et al.*, 1995; Navarro *et al.*, 2000). However, little attention has been given towards the lipid content and fatty acid composition directly in SF from joints of patients with inflammatory joint diseases (Wise *et al.*, 1987), despite local joint inflammation resulting in pain and swelling (Cleland *et al.*, 2003). Generally, the literature in this field concerns Rheumatoid arthritis (RA), whereas there is little knowledge about the spondyloarthropaties (SpA) (Cleland *et al.*, 2003; Prete *et al.*, 1995; Navarro *et al.*, 2000). Analyses of SF from patients with RA showed an altered lipid composition and lipoprotein pattern, as well as a general increased content of lipids (Prete *et al.*, 1995; Bole, 1962). A decrease of n-3 PUFA in both plasma and SF of inflamed joints from RA patients is also reported (Navarro *et al.*, 2000). Further, elevated levels of specific pro-inflammatory cytokines in both serum and SF in RA patients have been found, which are involved in tissue destruction in inflammatory diseases (Ribbens *et al.*, 2000; Punzi *et al.*, 2002; Cronstein, 2007).

1.1 AIMS OF THE STUDY

Certain n-6 and n-3 PUFA are found to be immunomodulating in blood and tissue. However, little attention has been brought on the levels of these n-6 and n-3 PUFA directly in synovial tissues from patients with inflammatory joint diseases, and their relation to inflammation. Further, there is a lack of studies of the general fatty acid composition of SF from these patients. The general aim of this cross-sectional study was to investigate:

- n-6 and n-3 PUFA in relation to inflammation: Do levels of specific n-6 and n-3 PUFA, or the n-6 to n-3 ratio in SF correlate with inflammatory disease markers, i.e cytokines interleukin (IL)-6 and IL-12 in SF and C-reactive protein (CRP) in serum, from patients with various inflammatory joint diseases?

- To evaluate the inflammatory activity, and to document the fatty acid profile in SF from patients with inflammatory joint diseases, the following parameters were measured:
    - Inflammatory disease markers: The level of the cytokines IL-6 and IL-12 in SF, and CRP in serum from these patients, and correlations between them.
    - Fatty acid composition: The general fatty acid profile in SF from these patients, with specific interest in the content of n-6 and n-3 PUFA.

- Additionally, since we had access not only to RA patient samples, which is most commonly studied, it was essential to study if there were differences between various groups within inflammatory joint diseases. The patients were grouped at two different levels; as subgroups and main groups:
    - Subgroups; Rheumatoid Arthritis (RA), PsoriaticArthritis (PsA), Reactive Arthritis (ReA) and Ankylosing Spondylitis (AS),
    - Main groups: Rheumatoid Arthritis (RA) and the Spondylarhropathies (SpA) (which includes the three latter subgroups above)

THEORETICAL OVERVIEW

The following introduction reviews the general theory about lipids and fatty acids (Section 1.2), followed by a description of inflammation (Section 1.3) and chronic outcomes with emphasis on inflammatory n-6 and n-3 PUFA and cytokines (Section 1.4). The inflammatory joint diseases are further described (Section 1.5), before a final review of the synovial joint (section 1.6).

1.2 LIPIDS AND FATTY ACIDS

1.2.1 Lipids

Lipids are a class of organic compounds which are generally soluble in organic solvents and insoluble in water. They provide the body with energy, but they are also important for isolation, mechanical support and communication in cell membranes, and as precursors for important signal molecules and tissue hormones. Lipids are a large and diverse group of fatty substances, which vary in polarity. They include the neutral lipid classes, including triacylglycerols (TAG), cholesterol esters, cholesterol, but also the more polar phospholipids (PL), and others. The lipids often consist of fatty acids. For instance, the body storage fat, TAG, is the main lipid found in diet, and is composed of one glycerol molecule bound to three fatty acids. Further, the phospholipids are composed of glycerol bound to two fatty acids, with a phosphate group attached, and are the major component of the cell membrane. The chemical composition of lipids will influence their physiological properties, which also relates to the composition of fatty acids incorporated in the compound (Jones & Kubow, 2006).

1.2.2 Fatty acid structure

Fatty acids are hydrocarbon chains bound to a carboxyl functional group (–COOH) at one end and a methyl group (-CH_3) at the other end (Figure 1.2.1). Saturated fatty acids (SFA) consist of hydrocarbons attached together exclusively by single bonds, whereas monounsaturated fatty acids (MUFA) have one double bond, and PUFA contain more than one double bond in the chain (Jones & Kubow, 2006). Fatty acids are also characterized by size/chain length. Generally, short and medium chain fatty acids have less than eight and 16 carbons in their

backbone, respectively, and comprise of mainly SFA. Long chain fatty acids have more than 16 fatty acids in their backbone, which generally include the bulk of the MUFA and all PUFA (Leonard *et al.*, 2004; Beermann *et al.*, 2003; Jones & Kubow, 2006). The term long chain PUFA (LCPUFA) commonly includes PUFA with 20 or more carbon atoms in their backbone (Sellmayer & Koletzko, 1999)

1.2.3 Fatty acid nomenclature

Fatty acids can be named in many ways. They are often referred to by their trivial names, but are more correctly identified by their systematic nomenclature according to the International Union of Pure and Applied Chemistry (IUPAC) rules for the nomenclature of organic chemistry. According to IUPAC, fatty acids are named by numbering the first, and further the next double bonds, counted from the carboxyl end (IUPAC, 1978). However, in nutritional and biological context the double bonds may be more commonly designated from the methyl end. It may be an easier way of numbering unsaturated fatty acids by only defining the location of the first double bond, since they generally are separated by methylene groups (CH_2). The first double bond is given by the term n minus (n-), or omega minus (ω-). Both numeric systems initiate the numeric names by numbering total carbon atoms next to the number of double bonds. Thus, linoleic acid is referred to as 18:2n-6 (18:2ω-6) by n-(ω-) system (Figure 1.2.1), and all fatty acids are referred to by the n- system or by abbreviations throughout this thesis.

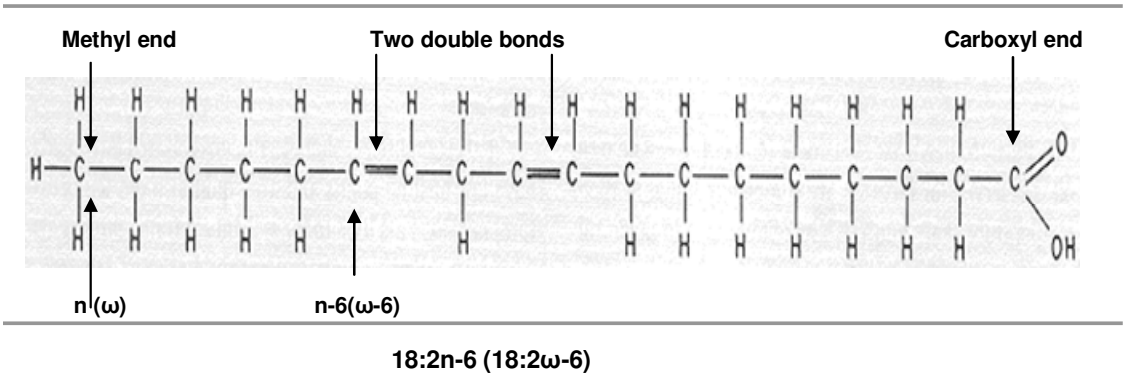

Figure 1.2.1: The structure and the n-(ω-) system nomenclature of linoleic acid. The first double bond is designated from the methyl end. Adapted and modified from the International research Livestock Institute Website (http://www.ilri.org/InfoServ/Webpub/Fulldocs/ILCA_Manual4/Milkchemistry.htm, 22/6-08).

Some common fatty acids are listed in Table 1.2.1. SFA, such as palmitic acid (16:0), are written only with total carbons relative to zero, since they do not contain any double bonds. Typically, the MUFA have a double bond located at the n-7 or n-9 position, whereas PUFA containing 18 or more carbons in their backbone are restricted to having their first double bond only at n-3, n-6 or n-9 position (Jones & Kubow, 2006; Smith, 2001).

Table 1.2.1: Fatty acid nomenclature commonly used in nutritional and biological context (Jones & Kubow, 2006).

Trivial name	Abbreviation	Numeric name n- system
Palmitic acid		16: 0
Stearic acid		18: 0
Oleic acid	OA	18:1n-9
Linoleic acid	LA	18:2n-6
α-Linolenic acid	ALA	18:3n-3
Dihomo γ-linolenic acid	DGLA	20:3n-6
Arachidonic acid	AA	20:4n-6
Eicosapentaenoic acid	EPA	20:5n-3
Docosahexaenoic acid	DHA	22:6n-3

1.2.4 The function of n-6 and n-3 PUFA

The n-6 and n-3 PUFA are both important components of the cell membrane, e.g. they influence the physical and functional properties of the membrane-bound enzymes and receptors, and they are important in signal transduction and activation of transcription factors(Calder, 2003; Haag, 2003). Further, LC PUFA have shown an increased effect on fluidity and functioning of the components in the membrane (Haag, 2003). Noteworthy fatty acids from the two families are; dihomo-γ-linolenic acid (DGLA, 20:3n-6), arachidonic acid (AA, 20:4n-6), eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), which also possess different immunomodulatory activities, mainly by serving as precursors for bioactive lipid mediators, such as eicosanoids, lipoxins and resolvins in blood and tissue. Some of them are discussed later in the chapter (Calder, 2007; Calder, 2003; Yaqoob & Calder, 2007)

1.2.5 The metabolism of n-6 and n-3 PUFA

The PUFA linoleic acid (LA, 18:2n-6) and alpha-linolenic acid (ALA, 18:3n-3) are essential fatty acids, and are the precursors of the family of n-6 and n-3 PUFA, respectively. In contrast

to plants, the human body lack the desaturase enzymes to introduce double bonds in the n-3 and n-6 position, and are dependent on supplement of these two essential fatty acids through the diet (De Lorgeril, 2007). However, the body can convert LA and ALA to the more important physiological active members of n-6 and n-3 families, through elongation and desaturation. The families are not interconvertible and may have opposing physiological effects (Simopoulos, 2002a). Also, the two fatty acid families are competitive, since both employ the same set of desaturase enzymes to form double bonds, and elongase enzymes to elongate the carbon chain, as well as they compete for incorporation into and realise from the cell membranes (Cleland *et al.*, 2003). Figure 1.2.2 describes the desaturation and elongation of n-6 and n-3 PUFA from parent essential fatty acids.

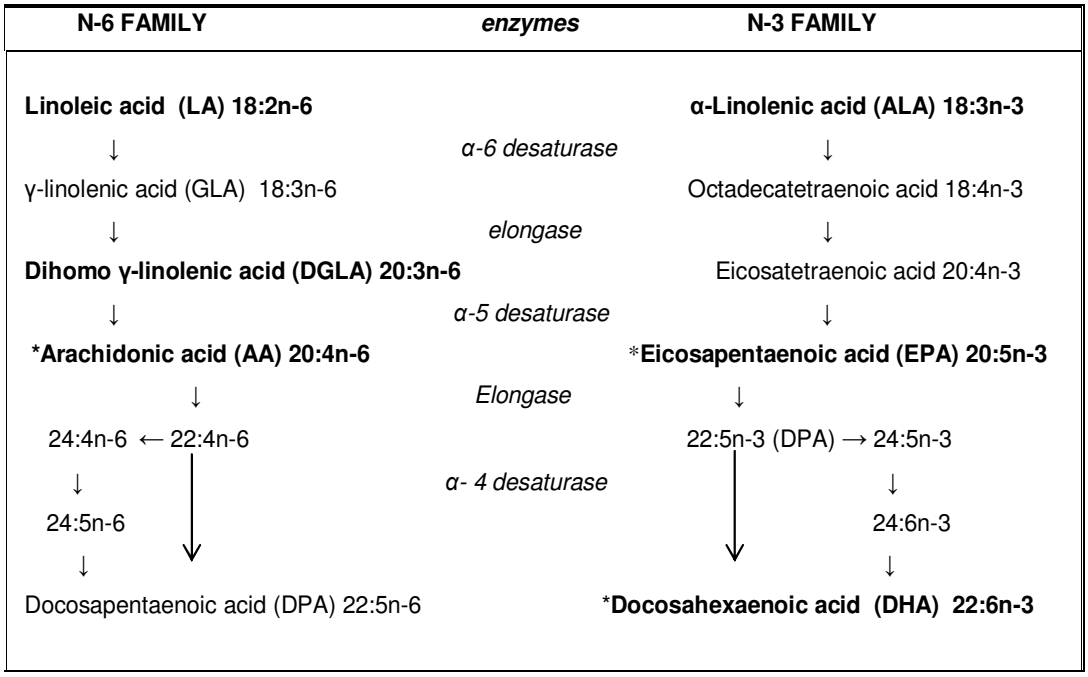

Figure 1.2.2: The elongation and desaturation pathway of n-6 and n-3 PUFA from linoleic acid and alpha-linolenic acid, through competition of the same set of enzymes. Fatty acids in bold text are either the essential fatty acids, or their fatty acid products with immunomodulatory activity. Asterisk show the most common products from each family. Adapted and modified from Haag et al (2003). Abbr: DPA; Docosapentaenoic acid.

The enzymes have greater affinity for more unsaturated fatty acids like n- 3 PUFA than n-6 PUFA, and theoretically one would expect higher contents of n-3 fatty acids in tissues. However, there is generally a much higher content of n-6 PUFA in tissues due to high availability in current Western diets (Simopoulos, 2002a; Jones & Kubow, 2006). With the high intake of LA, particularly AA is a major n-6 metabolite, which is a precursor for more

potent pro-inflammatory mediators. ALA on the other hand, is less consumed in the Western diet and the conversion to EPA is relatively low, and even lower to DHA. Thus, dietary intake of preformed LCPUFA like EPA and DHA, which both possess anti- inflammatory effects and may increase membrane function, is essential (Davis & Kris-Etherton, 2003; Simopoulos, 2002a; Simopoulos, 2002b).

1.2.6 Dietary intake of n-6 and n-3 PUFA

Dietary sources of the essential fatty acids LA and ALA are different plants that synthesise them. Vegetable seed oils per se, particularly sunflower, soybean, maize and safflower, and margarine made with sunflower or soybean oil are rich in LA. Oils from flax seed in particular, and also canola oil, nuts and green vegetables are the main sources of ALA. Further, LCPUFA may also be obtained directly from diet, which is a more efficient pathway for incorporation into the cell membrane, rather than metabolism from the essential fatty acids (De Lorgeril, 2007; James *et al.*, 2003). Dietary EPA and DHA are found in marine fatty fish, but also in lean fish in less content, which is obtained from phytoplankton (algae), and may be consumed as marine oil (fish oil most commonly) products (Sargent, 1997). Generally, there is a high content of AA in traditional Western diets, and common sources are meat and dietary products besides what is metabolised from high intake of LA (Simopoulos, 2002a).

1.3 INFLAMMATION

Inflammation is the immediate reaction of the body to an environmental infectious agent or injury. The infectious agent is recognised by the components of the immune system, and series of events is initiated to eliminate the foreign substance and to repair damaged tissue. The major cellular components of the immune system are monocytes/macrofages, polymorphonuclear cells, mast cells and the lymphocytes (Harbige, 2003).

Generally, the innate immune system recognizes the pathogen through macrophages and polymorphonuclear cells in the tissue. This activates the macrophages to produce various inflammatory mediators, including the main cytokines interleukin (IL)-1, Tumor necrosis Factor (TNF)-α and interleukin (IL) -6, eicosanoids, platelet aggregator factors (PAF), enzymes degrading extracellular matrix (MMPs), and adhesion molecules (Calder, 2003). These mediators trigger vasodilation, increase vascular permeability, perform chemotaxic effect directly or by up-regulating adhesion molecules, and further recruit monocytes and polymorphonuclear cells from blood to the inflammation site. The leucocytes at the site

eliminate the infectious components or damaged tissue commonly by phagocytosis. Further, there is an accumulation of other cells, proteins and plasma fluid at the site to aid in the signalling and elimination. Various inflammatory mediators act on each other in a complex manner and produce a cascade of inflammatory signals, eventually causing local oedema. If increased levels of macrophage-derived cytokines in plasma are obtained, a systemic inflammatory response will be triggered. Thus, cytokines migrate in blood and affect other organs, particularly stimulation of liver and brain to produce acute-phase proteins and "body rest" responses causing fever, anorexia and pain (Wood, 2006; Calder, 2003). The adaptive immune system also interacts with the innate immune system by making the infectious agents recognizable, after initial infection. It consists of lymphocytes, characterized by specificity of immunoglobulin antibodies, which recognize and coat antigens from infectious agents, making them recognizable for degradation by specific leucocytes. Further, they produce clones of the lymphocytes with the specific immunoglobulins matching the antigens. Lymphocytes are primary B-cells and T-cells. The main difference between the two, is that the T-cells are dependent upon the major histocompatibility complex (MHC) molecules from other cells to introduce the foreign antigen to their receptors before degradation (Harbige, 2003). The lymphocytes enter the tissue through the lymphatic vessels and are also a producer of several cytokines (Wood, 2006)

1.4 CHRONIC INFLAMMATION AND INFLAMMATORY MEDIATORS

1.4.1 Chronic inflammation

As a properly functioning immune system is essential for good health, malfunction of the system may result in long-term uncontrolled inflammation and cause chronic disease (Wood, 2006; Mori & Beilin, 2004). Chronic diseases include the inflammatory joint (rheumatic) diseases, which often possess autoimmunity, meaning that the body attacks its own tissue (Thorsby & Lie, 2005; Marker-Hermann & Schwab, 2000) and are characterized by specific inflammation of synovial tissues (Walsh *et al.*, 2005). However, all inflammatory joint diseases are not chronic, such as ReA which is represented by acute inflammation (Kumar & Clark, 2005). During chronic inflammation, there is overproduction of inflammatory mediators from inflammatory cells (Calder, 2003). Among others, high levels of specific eicosanoids and cytokines are found in synovial tissues (Hishinuma *et al.*, 1999; Punzi *et al.*, 2002), but also elevated levels of the metabolic by-products reactive oxygen species (ROS) and nitric oxide (NO). These mediators are involved in tissue damage and inflammatory

responses, including causing pain, tenderness and swelling of joints. The latter mediators may be modulated by n-6 and n-3 PUFA (Afonso *et al.*, 2007; Adam, 2003).

1.4.2 Pro-inflammatory cytokines (with specific emphasis on IL-6 and IL-12)

Cytokines are small proteins from mainly leucocytes, which act in both local and systemic inflammation by binding to cell membrane receptors, which regulate gene expression, and thus alter the behaviour of the target cells (Punzi *et al.*, 2002; Wood, 2006). These may possess both anti-inflammatory and pro-inflammatory effect, may act upon each other, and are involved in several complex intercellular relationships. TNF - α and IL-1 are the main triggers of a cascade of cytokines, among others IL-6, being produced. The three cytokines are the predominant cytokine products of the immune system cells (Punzi *et al.*, 2002; Grimble & Tappia, 1998). They share common inflammatory features during joint inflammation, including stimulation of acute phase response, osteoclast activity, increased release of eicosanoids, and differentiation and proliferation of immune cells and their mediators in the tissue (Grimble & Tappia, 1998; Gil, 2002). IL-6 is specifically known as a strong inducer of hepatocytes producing acute-phase proteins, such as C-reactive protein (CRP) in systemic inflammatory response, but is also an important activator of local tissue response, including B-cell differentiation and T-cell activation and proliferation (Cronstein, 2007). IL-12 is another important cytokine, which act later and is important in determining the T-helper (Th) 1 cell development in joints (Ribbens *et al.*, 2000; Wood, 2006). It activates Th1 cells and other lymphocytes for production of Interferon (INF)-γ, which among other inflammatory effects is a strong macrophage activator (Paunovic *et al.*, 2008; Punzi *et al.*, 2002; Wood, 2006). The four cytokines and their effect in inflammatory joints are listed in Table 1.4.1.

Table.1.4.1: Cytokines relevant in inflammatory joint disease.

Cytokine (main cell source)	**Inflammatory property on cells**	**Principal inflammatory effects in joint disease**
IL-1 (M, T,F, other)	Activation of leukocytes, endothelial cells, and synoviocytes	↑APP;↑other cytokines; ↑MMP ↑PGE2; ↑COX-2; ↑endothelial adhesion molecules; ↑osteoclast activation; ↑procoagulant
TNF-α (M,T,F, other)	Activation of leukocytes, endiothelial cells, and synoviocytes	↑ APP;↑other cytokines; ↑MMP ↑PGE2; ↑COX-2; ↑ endothelian adhesion molecules; ↑procoagulant
IL-6* (M, T,F,other)	Activation of leukocytes, endiothelial cells, and synoviocytes	↑ APP (including CRP); ↑MMP; ↑ endothelian adhesion molecules ↑osteoclast activation
IL-12** (M, other)	Activation of Th1 cell pathway and NK cells	↑ IFNγ (attracting and activating macrophages; ↑ other cytokines)

Table adapted from Punzi et al. (2002) and modified from Adam (2003) *Modified from Cronstein (2007).**modified from Paunovic et al. (2008) and Schroder et al. (2004). Abbr: NK: natural killers, APP:Acute phase proteins, MMP: Matrix metalloproteinases, PGE2: Prostaglandin E_2, COX-2: cyclooxygenase-2, M:Monocyte/Macrophage, T: T cell, F: Fibroblast

1.4.3 n-6 and n-3 polyunsaturated fatty acids in inflammation

Overall, the n-6 and n-3 PUFA may modulate the inflammation process and cytokines in two ways mentioned below (Harbige, 2003; Calder, 2003).

1.4.3.1 N-6 and n-3 PUFA as precursors for eicosanoids

Eicosanoids are short-lived local hormones, which play an important role in the immune system for rapid cellular response in damaged tissue (Adam, 2003). Specific leucocytes release fatty acids in membranes upon activation. Generally, AA is the precursor of the pro-inflammatory eicosanoids, including prostaglandins (PG) and tromboxane (TX) 2- series and leukotriene (LT) 4-series. Among others, they are involved in release of the pro-inflammatory cytokines IL-1, TNF, and IL-6 (Table 1.4.1). EPA is the precursor of PG and TX 3-series and LT 5-series eicosanoids, which are involved in similar processes, but they generally possess less inflammatory potent features, including inhibiting effect upon production of cytokines. The fatty acids employ the same set of enzymes, cyclooxygenase (COX) and lipoxygenase (LOX), and they compete for release and formation at the levels of their enzymes (Calder, 2003; Gil, 2002; Simopoulos, 2002b; Mori & Beilin, 2004). Studies performed with Dihomo-γ-linolenic acid (DGLA) and its eicosanoids indicate a similar competitive and anti-inflammatory effect upon AA by production of less inflammatory eicosanoids using the same set COX and LOX enzymes (Zurier, 1998).

1.4.3.2 Influence of n-3 PUFA on inflammatory gene expression

Various studies have demonstrated that n-3 PUFA can influence the inflammatory pattern by effects on gene expression (Calder, 2003). It is indicated that n-3 PUFA are involved in lowering the activation of nuclear factor κB (NFκB) transcriptor factors. These factors induce expression of different pro-inflammatory products including the cytokines such as TNF-α, IL-1 and IL-6 (De Caterina & Massaro, 2005). Overall, DHA has demonstrated the most potent inhibitory effect (Rahman *et al.*, 2008; De Caterina & Massaro, 2005).

1.4.3.3 N-3 supplementation

There are strong indications that use of long chain n-3 PUFA supplementation increase competition between EPA and AA for eicosanoid synthesis and enhance n-3 fatty acid incorporation into cell membranes (Sands *et al.*, 2005; Calder, 2007; Itomura *et al.*, 2008). This may further influence on the physiological properties of membranes and alter the inflammatory activity, including cytokines in blood and tissue (Simopoulos, 2002b; Adam, 2003). Figure 1.4.1 summarizes the suppressive effect of n-3 PUFA on n-6 PUFA and cytokines during inflammation. Increased presence of cellular n-3 PUFA through diet does not reduce AA. They may rather inhibit AA proinflammatory products through their role as competitive substrates in the LOX and COX pathway, which generally produce eicosanoids with anti-inflammatory effect. EPA and especially DHA may also down regulate cellular transcription of inflammatory mediators including proinflammatory cytokines. Thus, reducing the n-6 to n-3 ratio in cells through consumptation of fish may reduce the AA products and other inflammatory mediators during inflammation (James *et al.*, 2003; De Caterina & Massaro, 2005; Calder, 2003).

Several clinical fish oil trials have demonstrated a beneficial effect on pain and swollen joints of EPA and DHA in patients with inflammatory joint diseases. Most of the studies have been carried out on patients with RA, whereas a few studies have been performed on the SpA (Henderson & Panush, 1999; Cleland *et al.*, 2003; Goldberg & Katz, 2007). In some cases of the RA fish trials, reduced requirement of non-steroidal anti-inflammatory drugs (NSAIDs) compared to the placebo groups were reported (Goldberg & Katz, 2007), which may additionally lower the potential toxic drug side effects (Cleland *et al.*, 2003). There have been few intervention studies regarding the influence of n-6 and n-3 PUFA on serum levels of pro-inflammatory cytokines in RA patients, and probably no present studies of the SF n-6 and n-3 fatty acid relation to cytokine levels in serum or SF (Sijben & Calder, 2007; Stamp *et al.*,

2005). A study found significant clinical improvement and decrease of serum IL-β after 24 weeks of supplementation of DHA and EPA in RA patients, whereas IL-2, IL-6 and IL-8 levels did not change (Kremer *et al.*, 1995). Further, a study carried out by Sundrarjun et al (2004) found a significant increase of both serum EPA and DHA in RA patients after 24 weeks. A significant decrease of TNF-α and IL-6 was also seen in serum from these RA patients, but this was also seen in the placebo group. Additionally, the CRP values in the fish oil group were significantly decreased after 18 weeks (Sundrarjun *et al.*, 2004). One recent fish oil trail on Alzheimer patients further support the relation between n-3 supplemetation and cytokine production by demonstrating a reduced mononuclear blood cell production of IL-1β and IL-6 cytokines, in addition to elevated plasma levels of DHA and EPA, after 6 months of n-3 supplementation (Vedin *et al.*, 2008).

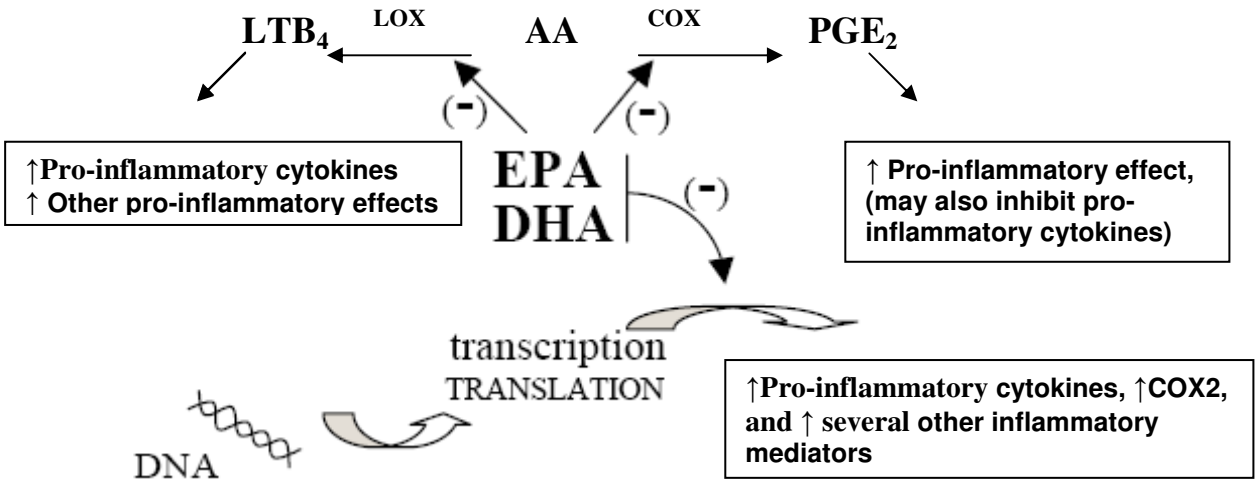

Figure 1.4.1: A simplified summarize of the possible anti-inflammatory effects of n -3 polyunsaturated fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) on inflammatory cellular responses. Adapted from James et al (2003), and modified from Mori & Beilin (2004) and De Caterina & Massaero (2005). Abbr: AA; Arachidonic acid, COX (-2); Cyclooxygenase (-2), LOX; Lipooxygenase, PGE2; Prostaglandin E2, LTB4: Leukotriene B4

1.5 INFLAMMATORY JOINT DISEASES

There are several types of inflammatory joint diseases and there are different ways of classifying them. Generally, one can divide between Rheumatoid Arthritis, the

Spondylarthropaties, and Chrystal arthritis (Hansen, 2003; Haga, 2002). The pathogenic mechanisms of several of the diseases are poorly understood. In various cases the diseases are genetically linked, where specific environmental exposures may be a triggering factor of the abnormal function (Thorsby & Lie, 2005; Gourley & Miller, 2007). Several studies have suggested that the triggering factors include infectious agents, hormonal and dietary factors, lifestyle habits and others (Calvo-Alén & Alarcón, 2006; Gourley & Miller, 2007).

1.5.1 Rheumatoid Arthritis (RA)

RA, the disease typically associated with arthritis, is a chronic disease which mainly attacks the joints, but may also affect the internal organs (James *et al.*, 2003). Symmetric polyarthritis (i.e. inflammation of more than five joints and on both sides of the body), which often include hands and feet, morning stiffness, and nodules beneath the skin, are typical characteristics of the disease. Features of the disease also include erosions of bone and elevated levels of the serum autoantibody rheumatoid factor (RF) (Arnett *et al.*, 1988). Worldwide, approximately 1% of the adult population has RA. The highest prevalence rate is found among women and elderly (Calvo-Alén & Alarcón, 2006). The general theory is that an unknown antigen is presented by the major histocompatability complex (MHC) to CD4 Th cells and cause stimulation of an immune response, eventually causing an auto-inflammatory state (Piet, 1998). Genetic factors may account for as much as 60% of disease susceptibility, which are associated to MHC class II human leukocyte antigen (HLA) DR alleles (Calvo-Alén & Alarcón, 2006).

1.5.2 Spondyloarthropaties (SpA)

The SpA are a heterogenous group of diseases, including ankylosing spondylitis, reactive arthritis, psoriatic arthritis, and arthritis associated with inflammatory bowel disease (IBD). Characteristics within this group are inflammation of the joints and ligaments in the column, inflammation of the sacroiliac joints located between the basis of the spine and pelvis, as well as inflammation of peripheral large joints. Symptoms of the diseases can also be seen in the skin and mucous membranes (Khan & van der Linden, 1990). Unlike RA, SpA are frequently oligoarticular and asymmetrical (i.e. inflammation of two to four joints located differently on each side of the body), affecting both distal and proximal joints (Walsh *et al.*, 2005). In SpA, there is usually no production of serum RF (Kumar & Clark, 2005), and there are higher levels of infiltrating polymorphonuclear leukocytes in the synovial membrane (Ritchlin, 2007). In addition to common clinical and radiographic features of spondyloarthropathies, the

group also shows a similar genetic predisposal to several genes including HLA-B27 alleles, encoded in MHC class I. Similar to RA, the theory is that HLA –B27 antigen presentation to T-cells is abnormal (Breban *et al.*, 2006; Marker-Hermann & Schwab, 2000)

1.5.2.1 Psoriatic Arthritis (PsA)

PsA causes inflammation of the joints in association with the skin disease psoriasis. Clinical characteristics of the disease include asymmetric oligoarthritis or RA-like symmetric polyarthritis, inflammation of sacroiliac joints (i.e joints connecting tail bone and pelvic bones), and swelling in the column or distal joints, the latter often include the upper finger joints. Another clinical pattern within this group is also seen including severe degradation of bones, often involving whole digits referred to as "telescoping fingers" (Moll & Wright, 1973). Other charactheristics are nail lesions, inflammation of the gut and eye (Ritchlin, 2007). The prevalence of PsA has been estimated to involve 0.3 -1% of the population, and concern generally elderly adults of both sexes (Gladman *et al.*, 2005).

1.5.2.2 Reactive Arthritis (ReA)

As opposed to the other inflammatory joint diseases mentioned, ReA is an acute inflammatory joint disease triggered by some infectious bacteria. It is indicated that bacterial antigens is associated with the pathogenesis of the disease after finding their presence in synovial membrane of inflamed joints (Kumar & Clark, 2005). In general, the Gram Negative bacteria Salmonella, Yersina, Shigella and Campylobacter cause infection in the gut, whereas Chlamydia cause infection in the urogenital tract, but there are also other bacteria triggering the disease (Vahamiko *et al.*, 2005; Haga, 2002; Kumar & Clark, 2005). ReA develops in 1-2 % of patients after acute infection of these bacteria, and men are frequently more affected. Approximately 70 % of ReA patients recover from the disease in 6 months, but it is a great chance of relapse (Kumar & Clark, 2005). There are no established criterias for the disease. However, clinical characteristics are asymmetric oligoarthritis or monoarthritis, mainly involving the lower extremities (Vahamiko *et al.*, 2005), and inflammation in tendon attachments. Inflammation of column, sacroiliac joints, eyes, and skin lesions are other symptoms (Kumar & Clark, 2005).

1.5.2.3 Ankylosing Spondylitis (AS)

AS, commonly named Bechterew's disease, is the most classical subgroup of SpA, both in frequency and characteristics (Jacobs & Fehlings, 2008; Breban *et al.*, 2006). It is a chronic

inflammatory joint disease primarily affecting the spine and sacroiliac joints, but also asymmetric peripheral large joints, internal organs and tendon attachments (Kumar & Clark, 2005). The spine transform to be stiff and rigid as the disease progress, and reduced movement in lumbar regions, profound back pain in the daytime, and morning stiffness are common. The prevalence of AS is 0.1-1.4 %, and younger men are more susceptible. Approximately 90-95% of the AS patients are positive for HLA B-27 alleles (Jacobs & Fehlings, 2008).

1.5.3 Social and individual impacts of the disease

Muscoskeletal conditions, including inflammatory joint diseases, are a major burden on individuals, their families and the social health care system, contributing to a large part of chronic conditions (disability, pension etc) in welfare states. Moreover, the physical limitations and pain, such as inflammation and gradual destruction of joints affect social and economic functioning, mental health and quality of life. Economically, treatment and loss of labour force amounts to high costs of illness. Generally, RA is the most disabling inflammatory joint disease with a high mortality rate (Woolf & Pfleger, 2003; NIAMS/NIH, 2004). Patients with PsA have lower tenderness of joints than RA patients and the disease is considered to be less severe (Gladman *et al.*, 2005). In cases of AS, exercise and pain relief increase everyday functioning, and contribute to the fact that 80% of these patients are fully employed (Kumar & Clark, 2005).

1.5.4 Medical treatment

To suppress pain and reduce developmental joint destruction in patients with inflammatory joint diseases, medication may be included in the treatment. There are different medical treatments which include simple analgesics, NSAIDs, disease-modifying anti-rheumatic drugs (DMARDs), among others TNF-α blockage, and glucocorticosteroids. TNF-α blockers inhibit the production of TNF-α, and thus block the cascade of inflammatory mediators triggered by the cytokine. It represents a major therapeutic advance in treatment of RA. Still, the TNF-α blockage drugs are extremely expensive compared to DMARDs. When medical treatment is appropriate, the types of drugs given the patients are based on the disease characteristics. Overall, there are several side-effects of these drugs (Hansen, 2003; Kumar & Clark, 2005). Studies have documented that glucocorticosteroids and different DMARD, but also to some extent NSAIDs, are involved in reducing levels of cytokines, such as IL-6 (Punzi *et al.*, 2002; Cronstein, 2007), and CRP (Menkes, 1993).

1.6 THE SYNOVIAL JOINT

The location of which bones of the skeleton meet is called a joint (Figure 1.6.1 A). The joint provides normal mobility and mechanical support to the body. The synovial joints connect the most mobile parts of the skeleton like shoulders, wrists, fingers, hips and knees. The muscles, ligaments and tendons are the surrounding connective tissue, which support and stabilize the joint. Within the connective tissue there is a capsule, which encircles and supports the joint. Inside the capsule, a membrane, namely the synovium, surrounds the bones, and provides synovial fluid by releasing it into the joint space. The synovial fluid surrounds the articular cartilage surface of the bone endings (Fam *et al.*, 2007; NIAMS/NIH, 2004).

1.6.1 The synovium

The synovium or the synovial membran merges with the joint capsule (Ropes & Bauer, 1953). It has no regulatory covers, which usually are present in the linings of most body cavities (Freemont & Denton, 1991). This provides the tissue membrane with a marked greater permeability than that of other tissues (Blewis *et al.*, 2007). The synovium is composed of different types of cells, mainly macrophage-derived synoviocytes and fibroblast-derived synoviocytes, and lymphatic and blood vessels (Tak, 2006; Blewis *et al.*, 2007). The membrane keeps the fluid free of debris and releases hyaluronan, which is a polysaccharide-like molecule that keeps the SF viscous (Fam *et al.*, 2007; Kumar & Clark, 2005).

1.6.2 Synovial fluid (SF)

SF is a hypocellular transudate of plasma that is filtered through the synovium and accumulates in the synovial cavity (Freemont & Denton, 1991). Normal synovial fluid has a pale yellowish colour, is viscous, and does normally not clot. The fluid allows easy gliding and normal movement of bones, and provides lubrication and nutrition to the articular cartilage (Kumar & Clark, 2005; Fam *et al.*, 2007; Ropes & Bauer, 1953).

1.6.3 Inflammation of the synovial tissues

Figure 1.6.1 present a simple contrast between a normal joint and an inflammatory synovial joint in rheumatoid arthritis (RA). Inflammation of the synovium may increase the cell content from 200 cells/mm^3 of normal synovial fluid to counting over 1000 cells/mm^3 in SF. In some cases of patients with RA and ReA, high counts up to 30 000 cells/mm3 are reported

(Freemont & Denton, 1991). This includes inflammatory activated synoviocytes, leucocytes, and especially polymorphonuclear cells (Fam *et al.*, 2007; Tak, 2006). An overproduction of inflammatory mediators lead to angiogenesis, swelling, and pain in joints (Adam, 2003). As a consequence, the permeability of the synovium and absorption through the lymphatic vessels are increased in inflamed joints versus normal joints. The alterations increase the amount of fluid entering the joint and reduce the capacity of removing colloid particles and its ability of keeping the fluid debris-free (Ropes & Bauer, 1953). With disease progression, the inflammatory mediators, particularly TNF-α, IL-1-β and IL-6 (Table 1.4.1), but also PG, are abundant and are known to gradually destruct the cartilage and bones, which weaken the surrounding muscles and ligaments, eventually leading to joint failure (James *et al.*, 2003; Punzi *et al.*, 2002; NIAMS/NIH, 2004). The activation of osteoclasts by the latter mediators are involved in bone destruction in established RA (Arend & Gabay, 2006), and an increase of their by-products ROS/NO, including peroxidation products of PUFA, cause severe damage of the tissue components (Kinne *et al.*, 2006; Basu *et al.*, 2001; Afonso *et al.*, 2007). Generally, the synovial tissues are characterized by having a gross appearance. The fluid may clot, and are ranged from clear to turbid (Ropes & Bauer, 1953).

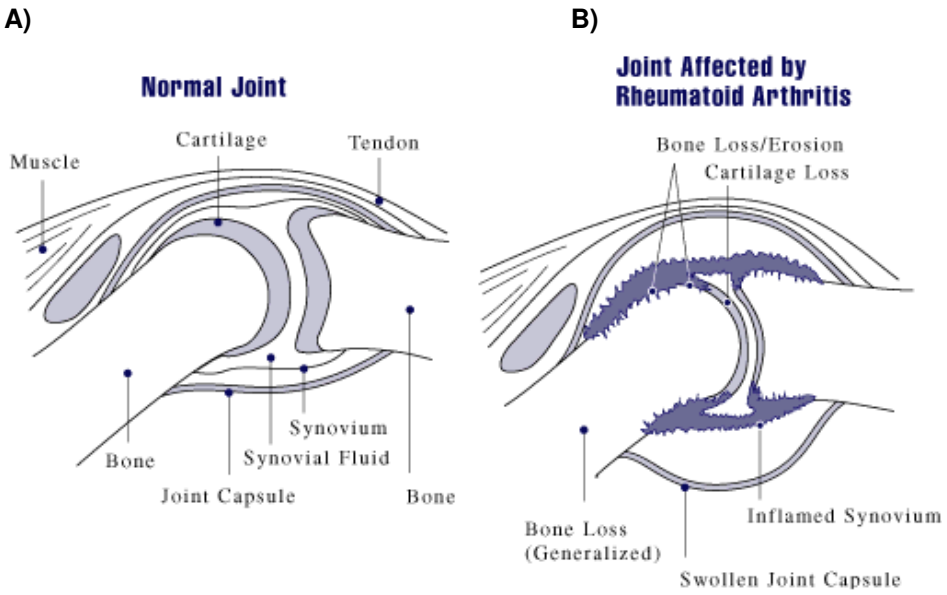

Figure 1.6.1: A) Normal synovial joint. B) Inflammation of synovial joint in Rheumatoid Arthritis (NIAMS/NIH, 2004)

1.6.4 Specific inflammatory markers

Levels of IL-6 and IL-12 (Table 1.3.2) are both elevated in inflamed synovial tissues, and their levels are associated with the degree of disease activity and joint destruction. Whereas IL-12 is involved in the Th1 cell development, which is associated with the autoimmunity of the diseases, IL-6 is more frequently related to disease activity. IL-6 is considered the most abundant cytokine in synovial tissues, and it promotes local osteoclast activity, as well as influence on systemic inflammation by elevating serum CRP. Thus, the SF IL-6 is also found to correlate to serum CRP concentration. Both cytokines have also shown anti-inflammatory features. Whereas IL-6 may protect cartilage during acute disease, but promote excessive bone formation in chronic conditions of RA, IL-12 may rather act pro-inflammatory in early disease state, and anti-inflammatory in late arthritis-like disease state (Punzi *et al.*, 2002; Cronstein, 2007; Matsumoto *et al.*, 2006; Kinne *et al.*, 2006; Ribbens *et al.*, 2000; Paunovic *et al.*, 2008; Petrovic-Rackov & Pejnovic, 2006; Petrovic-Rackov, 2005).

CRP from serum accumulate in the synovial tissue, binds to phosphorylcholin, which is found on the surface of infectious agents and damaged tissue, and presents it to the machrophages that enhances phagocytosis (Wood, 2006). The protein is an inflammatory marker, which directly reflects the levels of cytokines and is used to describe the pathology of the tissue (Dayer *et al.*, 2007; Emery & Luqmani, 1993).Thus, it is an unspecific and sensitive indicator of disease activity in the majority of inflammatory diseases, including, RA, AS, ReA and PsA (van Leeuwen & van Rijswijk, 1994). However, IL-6 may particularly be a better marker of indicating histopathology than CRP (Matsumoto *et al.*, 2006).

1.6.5 Lipids and fatty acids of synovial fluid

Most studies on lipids and fatty acids in SF of patients with inflammatory joint diseases have concerned patients with RA. Compared with normal SF, there was an increase of total lipids to 40-60 % of plasma levels, including cholesterol (15,1%), PL (38, 3%) and more highly neutral lipids in SF of RA patients. Cholesteryl ester (41.5 %) was the major component of the neutral lipids, and TAG (6.4%) was also present, in contrast to nothing found in normal SF. It has also been reported that the composition of lipoproteins, specifically low density lipoproteins (LDL) in RA patients differentiates more from blood when compared to normal synovial fluid (Prete *et al.*, 1995; Bole, 1962). Further, Prete et al (1997) have suggested that the increased content of lipids in SF from RA patients may increase the inflammatory processes through the activation of the AA prostaglandin pathway.

The total fatty acids in SF has been estimated to be one third of the content found in serum of RA patients (Kim & Cohen, 1966). A more detailed study on the fatty acid composition in RA patients carried out by Navarro et al (2000) found approximately 40% PUFA, whereas SFA and MUFA amounted to 31% and 23 %, respectively. The major components found were palmitic acid (16:0), oleic acid (18:1n-9), and LA (18:2n-6), contributing to 60% of the total fatty acid content. Also, AA (20:4n-6) was notably present. Further, it was found a low content of n-3 PUFA, where DHA (22:6n-3) was the major contributor. The n-6 to n-3 ratio can be roughly estimated to 9 and marine n-3 LCPUFA (i.e EPA and DHA) were superior to ALA. Because of lack of a control group with normal SF it is difficult to compare the actual fatty acid changes in inflamed SF. However, Navarro et al (2000) compared the SF fatty acid composition in RA patients by using patients with the less inflammatory disease, osteoarthritis (OA), as a control group. The RA patients show an increase of palmitic acid, a decrease of the n-3 PUFA, ALA and EPA, as well as a decrease of the elongation products of MUFA oleic acid, including 20:1n-9, 22:1n-9 and 24:1n-9. There was no significant change in composition of n-6 PUFA between the two groups.

1.4.4 The origin of the lipids and fatty acids in inflamed synovial fluid

Studies have shown that the excessive lipids and fatty acids which are present in the SF of inflamed joints are primary derived from blood. This is indicated from earlier studies, which show an altered permeability of the synovium and its vascular system during inflammation. However, the lipid composition and fatty acids of plasma and SF show some differences in RA patients. Whereas the percentage of PL in total lipids was similar, there was a higher fraction of cholesteryl ester and a lower fraction of TAG in inflamed SF than in serum from patients with RA (Bole, 1962). Further the fatty acid LA was increased and palmitic acid tended to be lowered in SF compared to plasma (Sugiyama & Ono, 1966). This indicates that other factors may also influence the lipid and fatty acid content in inflamed SF in RA patients (Wise *et al.*, 1987; Prete *et al.*, 1995; Navarro *et al.*, 2000; Kim & Cohen, 1966; Sugiyama & Ono, 1966). These factors may include; altered metabolism of the synovium, local synthesis, selective membrane transport of specific lipoproteins into the SF (Wise *et al.*, 1987), reduced capacity of removing excessive lipids, local lymphatic destruction, and release of lipids from degraded components of the inflamed tissue (Prete *et al.*, 1995). Overall, the observations support the theory that fatty acids mainly derive from blood, as well as the composition may to some extent influenced by histological tissue changes.

II. Materials and methods

OUTLINE

SF and blood samples from 86 patients with various inflammatory joint diseases have been collected between June 1992 and March 1994 from the Department of Rheumatology at Haukeland University Hospital (HUH). During this period, patients were consecutively enrolled in the study if synovial fluid aspiration, was clinically indicated. The blood samples were directly forwarded for analysis of CRP at the Laboratory for clinical biochemistry at HUH, while the joint fluid samples were stored in freezer at -70 ˚C at the Department of Rheumatology. In November 2003 and March 2004, the SF samples were forwarded to the Research laboratory at the ear- nose- and throat-department at HUH for measurement of IL-6 and IL-12. In august 2007 the samples were transmitted to National Institute of Nutrition and Seafood Research; NIFES and kept in freezer at -80 ˚C. Table 2 shows an outline of of sample collection, storage and analysis.

Table 2: Time outline of sample collection, handling and storage temperature (ºC), together with different analysis of aliquots of the synovial fluid (SF) samples from all patients.

Temperature (ºC)	Year	Time	History of SF samples
-70	1992	0	Start of collection and storage of aliquots of SF samples
-70	1993	1	
-70	1994	2	All SF samples included on freeze after ended collection
-70	1995	3	
-70	1996	4	
-70	1997	5	
-70	1998	6	
-70	1999	7	
-70	2000	8	
-70	2001	9	
-70	2002	10	
-70	2003	11	IL-6 measurement
-70	2004	12	IL-12 measurement
-70	2005	13	
-70	2006	14	
-70/-80[¶]	2007	15	Fatty acid analysis

[¶]In freezer at -70ºC before transfer to NIFES in August 2007 and kept in freezer at -80ºC before fatty acid analysis

This study took place at NIFES, in the period August 2007 to June 2008. Synovial fluid lipid classes of sampling tests from each of the various diseases were analyzed by laboratory technicians at NIFES by using a high performance thin-layer chromatography (HPTLC)

method. The analysis of the fatty acid composition in synovial fluid I have accomplished by using a gas liquid chromatography (GLC) method after being approved as an analyst at the Laboratory at the Laboratory for Nutrients. The statistical tool SPSS 15.0 for Windows was used as software to compare the data.

2.1 ETHICS AND HUMAN SAMPLES

To protect the rights of privacy and confidentiality of patients, the data material and samples supplied from the hospital did not include access to the personal identity. During the period of collecting personal information and SF the requirement of consent in writing was not yet inducted. Thus, patients gave their verbal consent to use the collected data for research purposes concerning inflammation on rheumatic diseases. Using the data for rheumatologic research was evaluated and approved by the Regional Committee for Medical Research Ethics Western Norway, REK Vest. Further, the extradition of the SF samples to a biobank for research purposes, kept in eppendorf vials in air atmosphere at -70˚C, was approved by the head of the Division of Rheumatology at the time of sample collection.

2.2 PATIENT CHARACTHERISTICS

All patients in the study were enrolled at the time the samples were taken under clinical treatment, either for routine evaluation, or after request due to increased joint symptoms. Joint tap was carried out due to diagnostic or therapeutic reasons, because of high disease activity i.e. one or more symptomatic and swollen joints. The removal of fluid may relieve pain and pressure on joint. Approximately half of the patients were diagnosed with Rheumatoid Arthritis (RA), while the remaining patients were diagnosed with different spondylarthropaties. This includes the diseases mentioned in the introduction: reactive arthritis (ReA), Ankylosing Spondylitis (AS) and psoriatic arthritis (PsA). The classification of RA was based on the committee of the American Rheumatism Association (ARA) revised diagnostic criteria (Arnett *et al.*, 1988). PsA was classified as described by Moll and Wright (1973). AS was diagnosed according to the New York criteria (van der Linden *et al.*, 1984). ReA is arthritis associated with urethral or gastrointestinal infection, and there are no validated classification criteria for the disease. Thus, classification procedures described in textbooks about rheumatology like Kumar and Clark (2005) are followed.

Table 2.2.1 presents an outline of the patient characteristics and number of patients on medical treatments. Nearly all of the patients were using NSAIDs on demand, but the frequency is not accurately registered among each of them. Approximately half of the patients were also using either different disease-modifying anti-rheumatic drugs; DMARDs or the glucocorticosteroid prednisolone. Some of the patients were on both of the latter medical treatments. The DMARD treatments include methotrexate, antimalaria drugs, sulphasalazine, gold salts including myocrisin and ridaura, and cyclosporine and pencillamine. Table 2.2.2 specifies the different medical treatments among the patients.

Table 2.2.1: Patient characteristics, including overall medical treatments other than NSAIDs.

Diagnose group	Patients	Gender		Age (years)		Medical treatment	
		Male	Female	Mean	Range	yes	no
RA	**45**	**15**	**30**	**57**	**20 - 85**	**36**	**8**
SpA	**41**	**22**	**19**	**44**	**23 - 72**	**10**	**30**
PsA	19	8	11	44	23 - 63	8	11
ReA	9	7	2	43	26 - 72	0	8
AS	13	7	6	43	24 - 67	2	11
Total	**86**	**37**	**49**	**51**	**20 - 85**	**46**	**38**
Missing data	0	0		0		2	

Abbr; RA: Rheumatoid arthritis SpA: spondyloarthropaties, PsA: Psoriatic arthritis, ReA: Reactive arthritis, AS: Ankylosing Spondylitis, NSAIDs: non-steroidal anti-inflammatory drugs.

Table 2.2.2: Number of patients on different medical treatments other than NSAIDs.

MEDICAL TREATMENT		Prednisolone		Total data
		Yes	No	
DMARDs:	Mtx	3	14	17
	AntiMal	3	2	5
	Slz	2	2	4
	Other	2	4	6
	Yes	**10**	**22**	**32**
	No	**13**	**39**	**52**
Total data		**23**	**61**	**84**

Abbr: Mtx: Metotrexate AntiMal: Antimalaria drugs Slz: sulphasalazine.

2.3 COLLECTION OF EXPERIMENTAL DATA

SF and blood samples from each patient had been collected before my engagement in the study. To provide information about the level of inflammation, measurements of three inflammatory markers have been performed. We have measurements of IL-6 and IL-12 in SF of all patients, as well as CRP in blood from most of the patients. Table 2.3.1 presents the

frequency of CRP measurements and missing data in all patients and within each patient group.

Table 2.3.1: The frequency of CRP values compared to missing data for the disease groups, together constituting total tests taken, in contrast to missing data.

Diagnosis	CRP	Missing data	Total
RA	**42**	**3**	**45**
SpA	**35**	**6**	**41**
PsA	16	3	19
ReA	8	1	9
AS	11	2	13
Total	**77**	**9**	**86**

Abbr: SpA: Spondylarthropaties, SpA, RA: Rheumatoid Arthritis, PsA: Psoriatic arthritis, ReA: Reactive arthritis, AS: Ankylosing Spondylitis

2.3.1 Blood tests

Blood samples were obtained at the same time as the SF samples. Gel vials were used for separating serum from blood cells. The serum samples were directly forwarded for analysis at the Laboratory for clinical biochemistry at HUH.

2.3.1.1 CRP measurement

CRP values were determined in serum by an immunoturbidimetric method using specific anti-human CRP serum (Orion Diagnostica) and an Axon analyzer. This measures the reduction in light transmission caused by particle formation. The reference area considered for healthy individuals were values below 10 mg/L.

2.3.2 Synovial fluid

The samples of synovial fluid were obtained from knee joints of the examined patients by the following method: First, the skin was disinfected by washing with chlorohexidine. To obtain the synovial fluid a needle was inducted into the joint space and some of the fluid was aspirated into the cylinder containing an anticoagulant, and later centrifuged before aliquots of the SF supernatant from each patient were distributed on several eppendorf tubes. Both the the addition of an anticoagulant and centrifugation procedure will be later discussed (Section 4.1.3.1), as these two procedures are not well-documented. The SF aliquots had been stored in a freezer at -70 ˚C at the Department of Rheumatology. In November 2003 and March 2004, aliquots of the SF samples from each patient were forwarded to the Research

laboratory at the ear- nose- and throat-department at HUH for measurement of the IL-6 and IL-12 levels, respectively.

2.3.2.1 Cytokine measurements

The levels of IL-6 and IL-12 were determined separately by Enzyme-Linked ImmunoSorbent Assay (ELISA). The principle of the assay is based on the principle of antibody-antibody interaction, where a specific monoclonal antibody able to capture the cytokine of interest is coated on a microtiter plate. A second monoclonal antibody, used for detection, binds a different area on the cytokine. When a substrate is added, a colour reaction will develop that is proportional to the bound content of cytokines. The concentration of cytokine (colour intensity) is determined by comparison with standards of known cytokine concentrations (Murphy *et al.*, 2007; Favre *et al.*, 1997).

IL-6 or IL-12 capture and detection antibody pairs were compared to recombinant human (r-hu) IL-6/ IL-12 as standards (R&D Systems Europe Ltd, Abingdon, GB). All procedures were performed according to the pattern of the manufacturer. 96-well microtiter plates (Costar Corning, NY, USA) were coated overnight at room temperature with monoclonal mouse anti-human IL-6 or IL-12 capture antibodies. After blocking, IL-6 and IL-12, respectively diluted to 1:50 and 1:5, and each of their recombinant human standards were added and incubated for two hours at room temperature. This was followed respectively by addition of biotinylated polyclonal goat anti-human IL-6 and IL-12 which allows binding of streptavidin-conjugated horseradish peroxidase. Then, plates were further incubated for 20 minutes at room temperature with the latter enzyme. Tetramethyl-benzidine (TMB) (Sigma) and H_2O_2 were used as substrate. Absorbance values were measured at 450 nm using Softmax Pro version 4.0 on an Emax Precision microtiter plate reader (Molecular Devices, Sunnyvale, CA, USA). The lower detection level was settled at 9.4pg/mL for IL-6 and IL-12.

2.4. FATTY ACID ANALYSIS

The method used for analysis of the fatty acid composition in SF is named NIFES method 354; Fatty acid determination using Gas liquid chromatography (NIFES, 2007). This modified method is based on the conventional accredited method NIFES method 041 (NIFES, 2002), that analyzes polyunsaturated fatty acids through a certain saponification and metylation based on Morrison and Smith (Morrison & Smith, 1964). Modification of this method is based on gathering metylation and extraction in the same working step introduced by Lepage

and Roy (1986), with some simplifications similar of what is described by Kang and Wang (2005) and Masood et al.(2005). The method is time saving and quantitatively less specimens is required, and the risk for sample loss and contamination are reduced (Kang & Wang, 2005). Scientific responsible for the method at NIFES is currently finalising a paper on the method (Araujo *et al*., unpublished).

2.4.1 Principle

By introducing certain samples to the strong base sodium hydroxide under boiling state and in presence of the strong catalyst boron trifluoride in methanol, the conversion of lipids to fatty acid methyl esters will occur. Methylated fatty acids are more volatile and vaporise easier on the column, which is a criteria for quantification by gas liquid chromatography (GLC). The general mechanism involves two steps shown in Figure 2.4.1. First, fatty acids of lipids are esterified by sodium hydroxide, creating glycerol and the salt of the fatty acids. This liberates the fatty acids and is called saponification. Second, transesterification occurs when methanol reacts with the crude soap producing fatty acid methyl esters through methylation (Christie, 1989; NIFES, 2007).

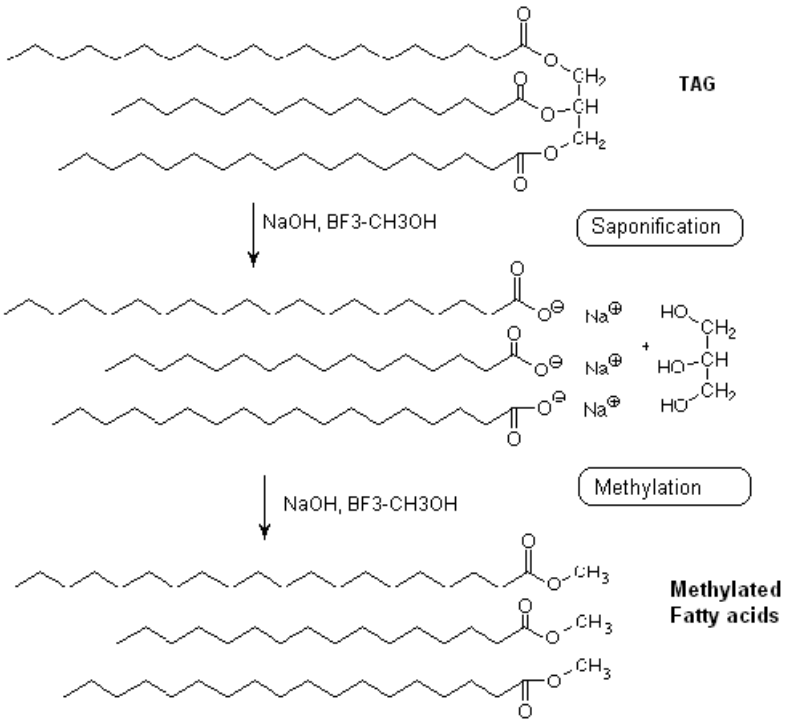

Figure 2.4.1: Methylation and saponification of triacylglycerides under specific conditions. Adapted and modified from the Department of chemistry, Winona State University, USA (http://course1.winona.edu/jfranz/Lab/soaplab.htm, 16/04 2008)

Each fatty acid methyl ester is determined by GLC. The method is based on the chromatography technique which separates compounds between two phases, a mobile phase and a stationary phase. A schematic of a GLC is shown in Figure 2.4.2. GLC is a technique used to separate volatile organic compounds. Due to each specific compounds partitioning behaviour between a gas mobile phase and a liquid stationary phase in a column, a separation is accomplished which can further be quantified.

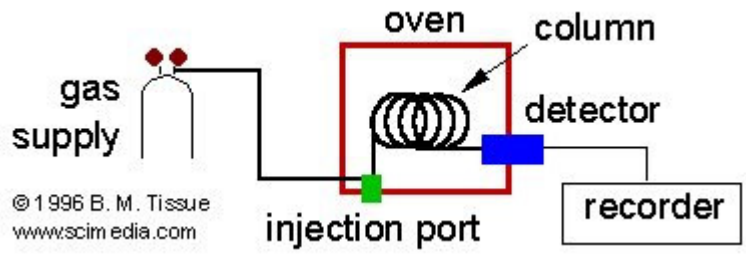

Figure 2.4.2: Rough sketch of Gas Liquid Chromatography and its components. Adapted from the Department of chemistry, The University of Adelaide, Australia (http://www.chemistry.adelaide.edu.au/external/soc-rel/content/gc.htm, 12/03-2007)

The analyte is injected into the mobile phase where it is volatilised. Further it is passed onto the chromatography column. The mobile gas transports the analyte through the stationary phase of liquid, which is distributed as a thin layer over an inert solid matter in the column. The different fatty acids are passed through the gas flow at different rate set by to the adsorption interaction with the stationary phase. Level of interaction depends on the different chemical and physical properties of the various fatty acids, thus each will elute at various retention time. The separation relies also on other factors, among them the properties of the mobile phase and the temperature. The flame ionization detector (FID) is almost universally adopted for registering organic compounds in gas chromatography. Substances emerged from the column are mixed with hydrogen and air. Further they are combusted by a flame, generating ions that conduct electricity. A collector electrode above the flame captures the ion current, and measures the mass signal for each of the eluted fatty acids. The process is monitored by a computer programme (Christie, 1989).

2.4.2 Procedure

All chemicals used in the procedure are purchased at Merck (Darmstadt, Germany) if otherwise is not mentioned.

Parallels of synovial fluid homogenates (≥ 50 µg) were weighed in sovirel vials (10 mL) using an analytical balance (Mettler AT 200, Greifensee, Switzterland) linked to a computer. 1 mL of 0,5 M NaOH dissolved in methanol and 2 mL of BF_3 (20 % in methanol) were added. The vials were capped and shaken 15-20 seconds on Whirlmixer (Heidolph REAX top, Schwabach, Germany), and further heated at 100ºC on block heater (Reacti-Therm III TM, Pierce, Rockford, USA) for 60 minutes. They were shaken again on the whirlmixer after 20 minutes during the boiling state. After boiling, the tubes were cooled down, and 2 mL of n-hexane and 2 mL water distillated from device (RiOs, Millipore SA, Molsheim, France) were added. The mixtures were shaken and then centrifuged with equilibrium for 4 minutes at 3000 rpm (Centrifuge 5804, Eppendorf AG, Hamburg, Germany) for phase separation. The upper phase with n-hexane and methylated fatty acids was collected with Pasteur pipette, and transferred to smaller tubes (4 mL). The n-hexane mixture were further evaporated to dryness, under nitrogen (qual. 5.0), on the block heater. Then, 0,3 mL of n-Hexane was added with a pipette to each tube, concentrating the mixture to the specific capacity of the GLC column (0,20 -0,30 mg/mL). 120 µL of the n-hexane mixture (~ 0,25mg/mL) was transferred with pipette to tubes placed in churns inside the GLC vials (1 mL). The containing fatty acids methyl esters in the vials were separated and analysed by GLC.

2.4.3 The GC instrument

The GLC instrument was the model Autosystem XL from Perkin Elmer, comprising an autosampler (Perkin Elmer, Shelton, USA), where the GLC vials were automatically introduced to an on-column injector (Perkin Elmer, Shelton, USA) attached to a pre-column (Silica 0,53 mm ID, Imperial Eastman Tubing-22-pp-1/8, Santa Clara CA, USA). Within the injector, each sample is being introduced to helium gas flow that sweeps the analyte from the pre-column to the analytical wall coated open-tubular column (CP-sil-88, 50 m WCOT, ID: 0.32, Middelsburg, The Netherlands), which is a narrow bore tubing of fused silica with polymeric material coat on the inner wall as liquid phase. During the analysis the temperature is increased according to a temperature program. The detector connected to the instrument is FID (Perkin Elmer, Shelton, USA), and the chromatography data system managing the instrument is Totalchrom (Perkin Elmer, Shelton, USA). Table 2.4.1 specifies the settings for the instrument.

Table 2.4.1: Information about the GLC Autosystem XL preset parameters.

<table>
<tr><th>GC parameters</th><th>Autosystem XL</th></tr>
<tr><td>Carrier gas, Helium (He)
(quality 6.0, 50L, TESS vest AS, Bergen, Norway)</td><td>Constant flow: 1.5 ml/min
(for samples 3, 6-9, 11-14, 18-20, 23-29, and 38-51)
Constant pressure: 82 kPa
(for the remaining samples)</td></tr>
<tr><td>Injector</td><td>On column</td></tr>
<tr><td>Detector gas, H2
(quality 5.0,50L, TESS vest AS, Bergen, Norway)</td><td>450 mL/min</td></tr>
<tr><td>Detector gas, air
(Atlas Copco Kompressorteknikk AS, Langhus, Norway)</td><td>45 mL/min</td></tr>
<tr><td>Detector temperature</td><td>250 ºC</td></tr>
<tr><td>Injector temperature</td><td>Start: 50 ºC/ 0,1min → 250ºC / 50min</td></tr>
<tr><td>Time analysis</td><td>60 min</td></tr>
<tr><td>Temperature program for the column</td><td>
<table>
<tr><th>Rate (ºC/min)</th><th>Temp. (ºC)</th><th>Isotermic</th></tr>
<tr><td></td><td>60</td><td>1</td></tr>
<tr><td>25</td><td>160</td><td>25</td></tr>
<tr><td>25</td><td>190</td><td>17</td></tr>
<tr><td>5</td><td>220</td><td>5</td></tr>
</table>
</td></tr>
</table>

Abbr: GLC: Gas Liquid Chromatography

2.4.4 Quantification of fatty acids

Cod-liver oil mix prepared from cod-liver oil (Petter Møller, Lysaker, Norway), and a standard solution of methyl esters (Nu-Chek-Prep, Elysian, USA) in hexane (2:10) were used as a standard mixture of fatty acid methyl esters. The cod liver oil mix where included in the GLC analysis to compare the peaks detector signal from the analytical results with the known composition of the standard mixture. Area and percentage of each fatty acid peak were analyzed, and the sum of the graph area, with exception of the internal standard methyl nonadecanoate (19:0), was considered equal 100%. Limit of quantification (LOQ) of each peaks was set to 0,1 %. By addition of internal standard 19:0 (Larodan AB, Malmö, Sweden) in chloroform:methanol (2:1) solution (10 mg/mL), quantification of milligram fatty acids where determined. The internal standard was added each sovirel vials, before addition of the sample material, and evaporated to dryness, under nitrogen. For optimal linearity of the standard curve, 19:0 in chloroform/methanol was diluted and added in a concentration constituting 7- 30 % of the estimated total fatty acid amount of the analysis sample ready for GLC. The linearity of the standard curve was considered between 0,2 -0,3 mg fatty acids per

mL sample. In the method, limit of detection (LOD) was set to 3 mg fatty acids per kg sample, and LOQ was set to 10 mg fatty acids per kg sample. The limit of quantification was determined in the method validation when using a different GLC instrument. The GLC used for analyzing fatty acids in SF in the experiment has a better sensitivity than the latter GLC instrument, and was estimated to the double of mentioned above. Thus, the LOQ is theoretically 5 mg fatty acids per kg sample, which is taken under consideration in the interpretation of the result.

Salmon liver (KM 05-06, NIFES) was added in an additional vial in the procedure used as control material (KM) in the analysis. The quantification of results of area % and mg/g for C16:0, C18:1n-9, C20:5n-3 in KM was compared to their alarm limits on the control chart, to ensure the accuracy of internal reproducibility. The liver was homogenized at NIFES and total amount fat is expected to be around 5-7 %. Because of its increased lipid content, the KM was added 2 mL of 0,5 M NaOH dissolved in methanol. Additionally, it was diluted with n-hexane (1:7) after extraction.

2.5 LIPID CLASS ANALYSIS

To provide an input of the lipids in synovial fluid from patients with inflammatory joint diseases, lipid classes from four specimens available for further examination after fatty acid analysis from each of the various diseases was quantified. The method used for analysis of the lipid classes in the synovial fluid is named NIFES method 230; Lipid class determination in oils, feed, tissue and tissue fluids (NIFES, 2006). The lipid classes were determined by using a high-performance thin-layer chromatography (HPTLC) method applied with a densitometry detector described by Henderson and Tocher (1992). Known amount of lipids standards purchased from Sigma and Avanti were included for comparison, and scanning densitometer (TLC scanner 3, Camag) was used to quantify the charred lipids.

2.6 STATISTICAL ANALYSIS

Microsoft excel 2002 was used to calculate the mean between parallels of each fatty acid sample specimen. A Student's t-test was used to compare total quantified fatty acid composition in percentage and mg/g, as well as calculation of percentage difference between

saturated, monounsaturated and polyunsaturated fatty acids (<10%) was done before accepting the combined parallels from fatty acid analysis.

Software SPSS 15.0 for Windows was used for statistical analysis of all other data. The normality distribution of data was tested by using Saphiro-Wilks for $n < 40$ and Kolmogorov-Smirnov test with Lilliefors correction for $n \geq 40$. Further, Levenes test ($p<0.1$) was used to check for homogenity of variance. Most of the results are presented by median and interquartile range (IR) as measurements of central tendency and dispersion, since data did not sufficiently satisfy one of the above assumptions.

- Mann–Whitney U test was used for comparison between RA and SpA groups. The test requires all the observations to be ranked as if they were from a single sample, and further the average of each sum of the ranks between the two samples are compared. Thus, the test is sensitive for differences in the location between the samples, i.e it is used to test if the median of the groups are equal.

- Kruskal-Wallis H test was used to compare the four subgroups, RA, PsA, ReA and AS. The test is based on the principle of Mann-Whitney U test, but it handles more than two samples.
    - Kruskal-Wallis does not tell how the groups differed, only that they are different in some way. If significant differences between several groups were found, Mann–Whitney U test was used with the Bonferroni's correction ($P' = k \cdot P$) to adjust the p-value for pairwise comparison (in this case: $k=4$). The test is referred to as MWBc.

- Spearman's rank order coefficients (rho), a nonparametric test robust against outliers, were used for analysis of correlation. The rho was compared with published tables for different levels of significance (eg. 0.05, 0.01 etc). Gaussian distribution was relaxed and direct data values are presented in scatter plots with a straight line to provide a figurative association between parameters that had a significant correlation.

If otherwise is not mentioned, the difference was considered significant if the p-value was below 0.05 ($p<0.05$).

III. Results

In general, the results of various analytical parameters measured in blood or SF from all patients with inflammatory joint diseases will be presented as a group, as subgroups RA, PsA, ReA and AS (the three latter subgroups also presented as the main group, SpA). Section 3.1 presents the levels of inflammatory markers CRP, IL-6 and IL-12, respectively in blood or in SF from the patients. Further, the correlation between the three inflammatory markers will be shown. Section 3.2 gives the results from the analysis of the fatty acid composition in SF, in percentage and µg/g. More specific, in part due to large data sets, a focus is set on significantly differences obtained during statistical analysis. Section 3.3 gives the correlation of n-6 and n-3 PUFA and n-6 to n-3 ratios to CRP, IL-6 and IL-12. To provide an input of the lipids in SF from patients with inflammatory joint diseases, lipid class composition from four specimens is briefly mentioned in Section 3.4. Finally, Section 3.5 gives a summary of main results.

3.1 INFLAMMATORY MARKERS

In this section, the concentration of CRP in blood (mg/L) and the cytokines IL-6 and IL-12 in SF (pg/mL) will be presented for all the patients as a group and as comparisons between the sub- and main groups. A presentation of the correlation between the inflammatory markers from all the patients as a group and in sub- and main groups will also be shown, first, between IL-6 and IL-12, secondly, between the latter two and CRP.

3.1.1 Levels of inflammatory markers in patients

Below, each of the inflammatory markers is presented as box-and-whiskers plots in three separate figures, where total patients and differences between the specific groups are included (Figure 3.1.1-3.1.3). The boxes show the median and interquartile range (IR) of each group, which is the 25^{th} and 75^{th} percentile. The whiskers present values within 1.5 box length from the box edge, which are not considered as outliers, whereas the marked observations outside of these represent outliers. Blue box plots presents the four patient subgroups; RA, PsA, ReA and AS, green box plots present the main diagnostic groups; RA and the SpA, and the beige box plots presents concentration from all the patients. Above each comparison of groups, a textbox with significance level is shown.

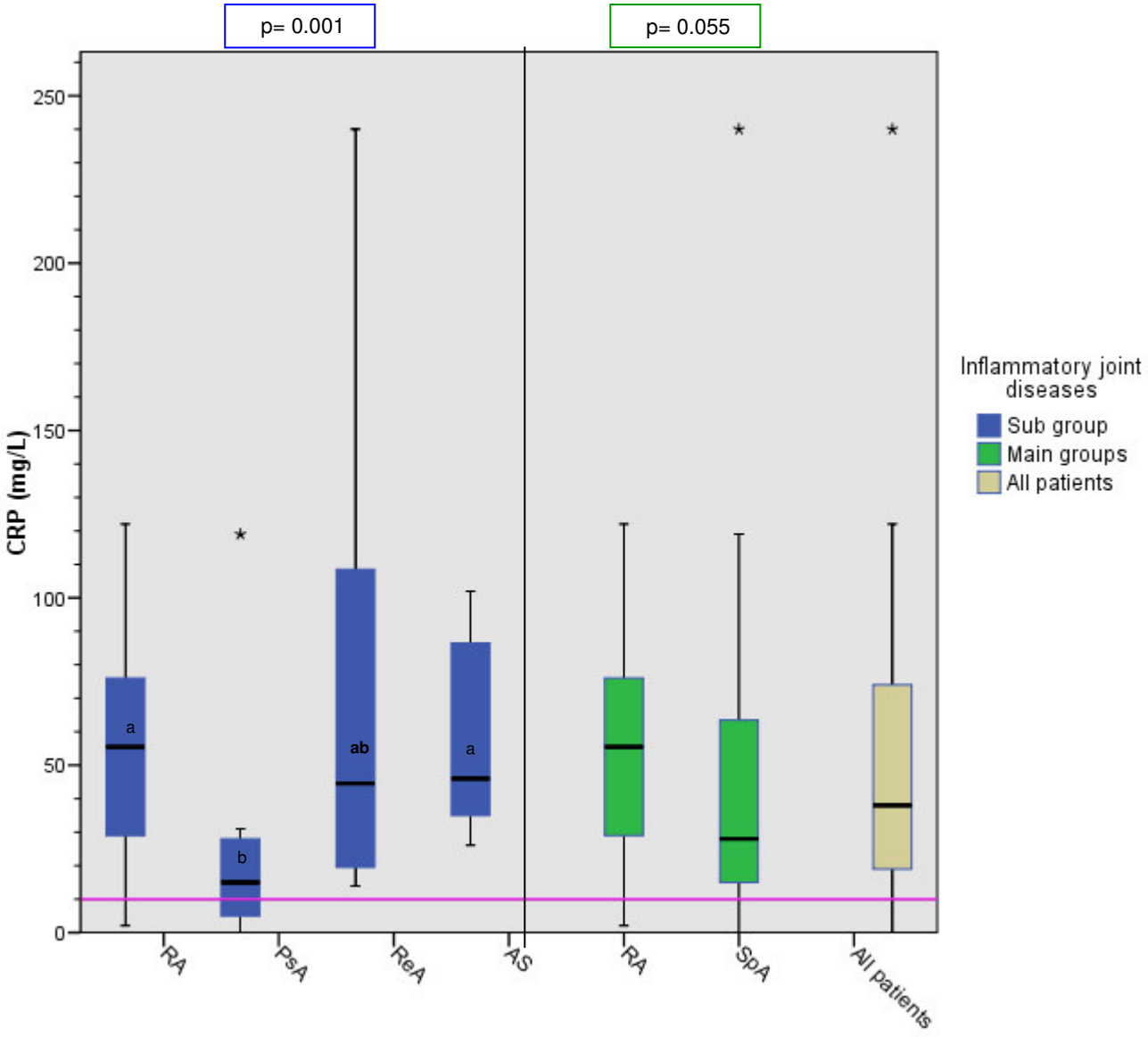

Figure 3.1.1 Concentration of CRP in serum from patients with various inflammatory diseases. The boxes show the median and interquartile range of each group. Asteriks show cases with values more than three times box lengths from the edge of the box, and are considered as extreme outliers. The pink line is the CRP reference value, 10mg /L. The area below is the concentration considered for healthy individuals. Blue and green textboxes above show significance level between groups, respectively Kruskal-Wallis test and Mann-Whitney test, $p < 0.05$. Different small letters (a,b) inside blue boxes indicate significant group differences, Mann-Whitney test with Bonferroni correction, $p < 0.05$. Identical letters indicate not significantly different, and both a and b indicate no significant group differences. Missing data: n=9. Abbr: RA: Rheumatoid arthritis (n=42), SpA: spondyloarthropaties (n=35), PsA: Psoriatic arthritis (n=16), ReA: Reactive arthritis (n=8), AS: Ankylosing Spondylitis (n=11).

- *CRP:* Figure 3.1.1 presents the serum levels of CRP from all the patients. The median was estimated to 38 mg/L (interquartile range 19-75) for all patients. This is generally above the reference concentration area for healthy individuals (below 10 mg/L). Of the total ten patients with CRP concentrations below 10 mg/L, seven of them were from the PsA disease group. Thus, approximately half of the PsA patient group is in the considered healthy reference area.

  CRP was significantly lower in PsA group (median 15 mg/L, IR 5-28) compared with the RA group (median 56 mg/L, IR 28-76) and AS group (median 46 mg/L, IR 32-88), $p<0.000$ (MWBc).

  The ReA group did not differ significantly from neither of the other groups (median 46 mg/L, IR 19-110). Further, there were no statistically significant differences between the main groups RA and SpA (median 28 mg/L, IR 55-76), $p =0.055$.

- *IL-6*: The levels of IL-6 in SF are presented in Figure 3.1.2. The median IL-6 concentration of all the patients was 9372 pg/mL (IR 2746 - 27 916). There were no significant differences between neither the main groups RA (median 9754 pg/mL, IR 4737- 26087) and SpA (median 5851 pg/mL, IR 1834 -32 321) nor the subgroups RA, PsA (median 5044 pg/mL, IR 1012 -16 228), ReA (median 16 166 pg/mL, 3306-50 003) and AS (median 4396 pg/mL, IR 1834-32 321).

- *IL-12:* The levels of SF IL-12 show seven cases with extreme outliers (Figure 3.1.3). The highest extreme value was 21 597 pg/mL, which is approximately hundred times greater than the median from all the patients. As well, this high extreme value is four or eight times above the three other high extreme outliers, respectively 4568 pg/mL, 2664 pg/mL and 2572 pg/mL. Even though the rank methods used are little affected by extreme outliers, the highest extreme outlier was chosen to be excluded from the statistical analysis, and from the boxplot to provide a figurative outline. Thus, after exclusion, the median IL-12 in SF of all patients was 215pg/mL (IR103-369).

  As with CRP levels in blood, there was a significantly higher level of IL-12 in SF of the RA group (median 296 pg/mL, IR 176-416) compared with PsA group (median 105 pg/mL, IR 85-240), $p=0.036$, MWBc. The IL-12 level of RA group was also

significantly higher compared with SpA group (median 149, IR 86-254), p=0.003, MWBc. In between RA and PsA, the levels of IL-12 in AS (median 215 pg/mL, IR 106-324) tended to be higher than that of patients with ReA (median 142 pg/mL, IR 53-206), p=0.080, MWBc.

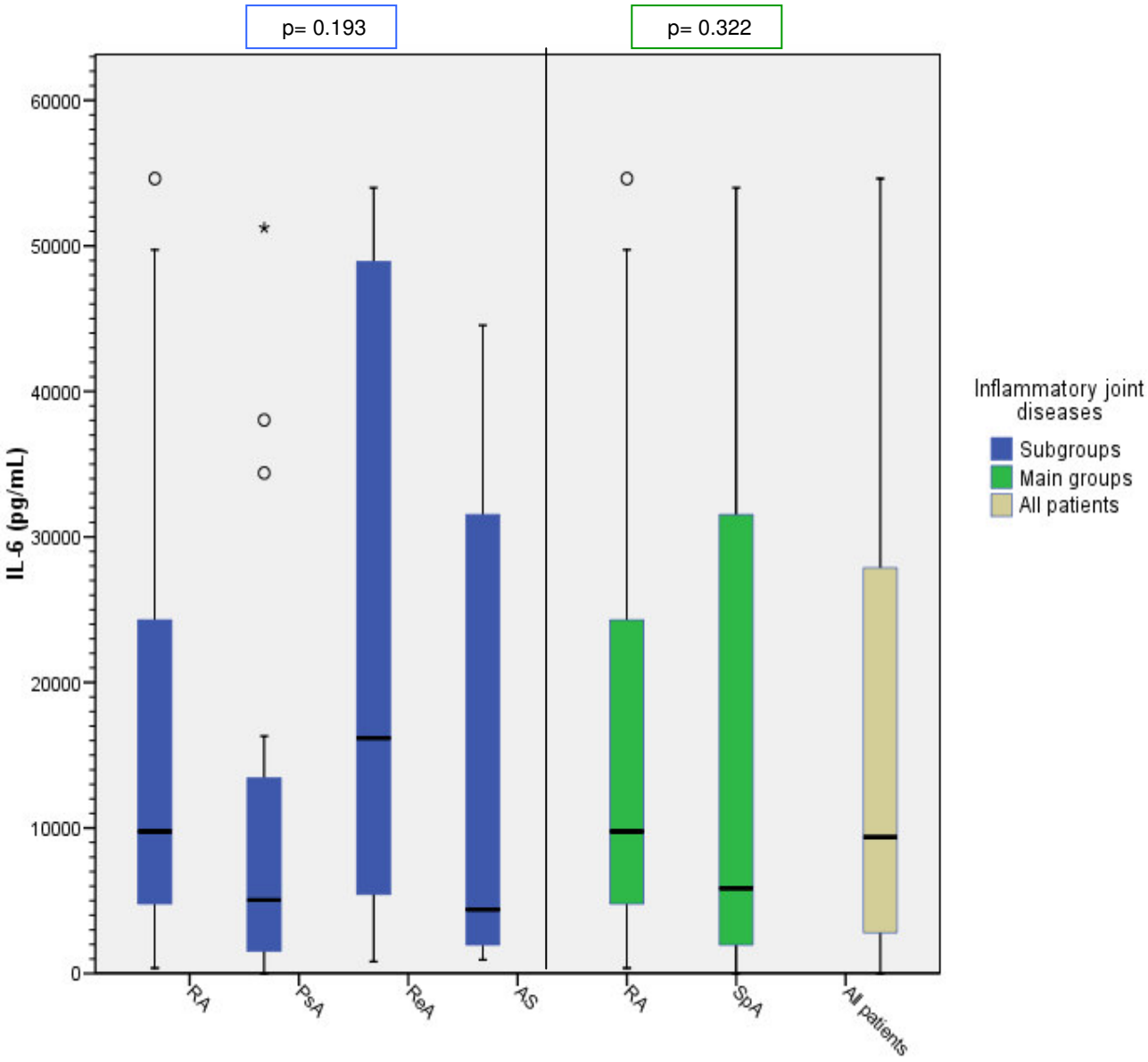

Figure 3.1.2 Concentration of IL-6 in synovial fluid of patients with various inflammatory diseases. The boxes show the median and interquartile range of each group. Circles show outlier cases with values 1.5 - 3 times the box lengths from the edge of the box. Asteriks shows cases with values more than three times the box lengths from the edge of the box, and are considered as extreme outliers. Blue and green textboxes above show significance level between groups, Kruskal-Wallis test and Mann-Whitney test respectively, p< 0.05. Abbr: RA: Rheumatoid arthritis (n=45), SpA: spondyloarthropaties (n=41), PsA: Psoriatic arthritis (n=19), ReA: Reactive arthritis (n=9), AS: Ankylosing Spondylitis (n=13).

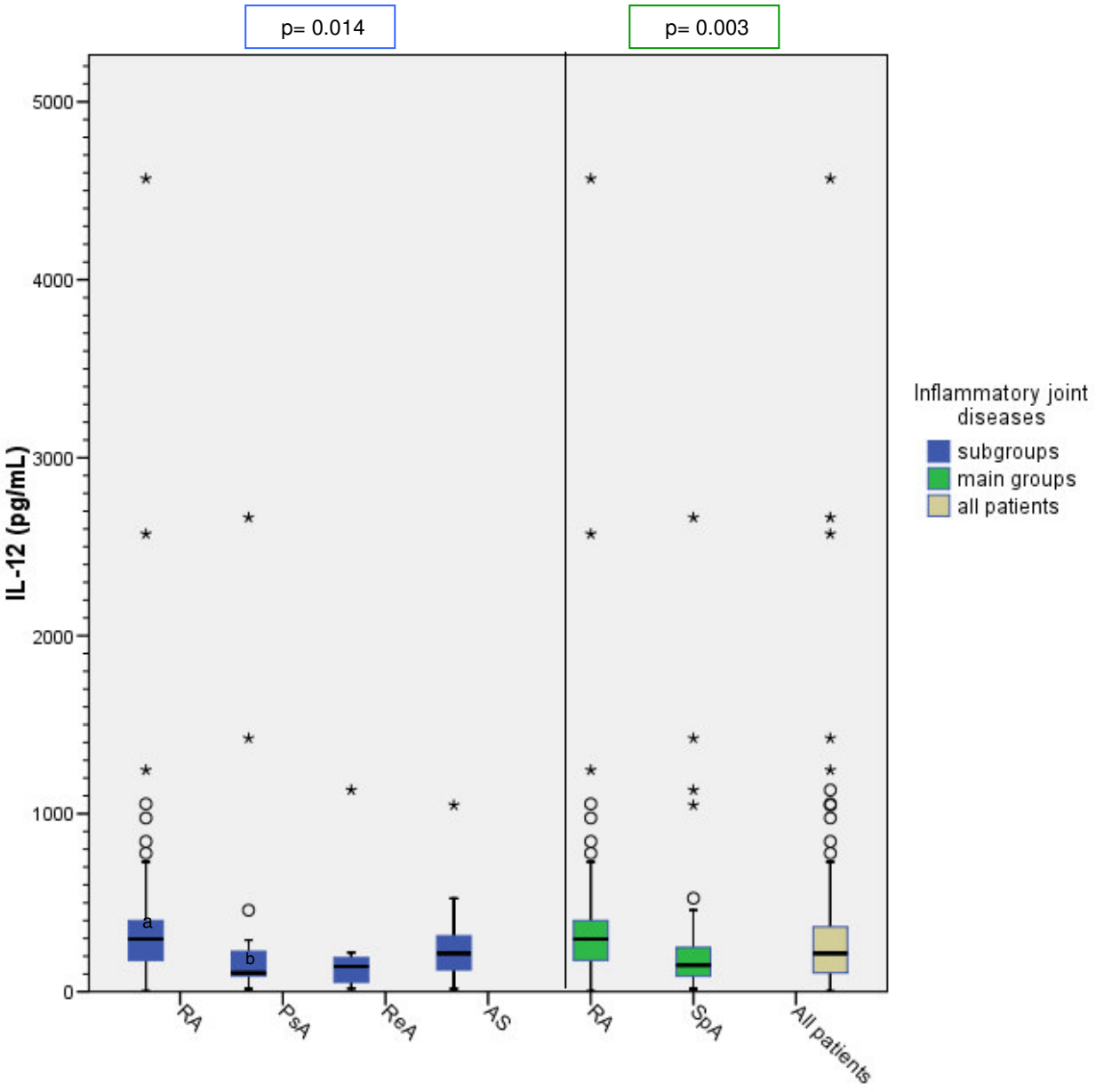

Figure 3.1.3 Concentration of IL-12 in synovial fluid of patients with various inflammatory diseases. The boxes show the median and interquartile range of each group. Circles show outlier cases with values 1.5 - 3 times the box lengths from the edge of the box. Asteriks show cases with values more than three times the box lengths from the edge of the box, and are considered as extreme outliers. One extreme value was excluded from the plot; 21 597 pg/mL (AS patient). The significance between the groups was approximately the same when statistical analysis included this high extreme value. Blue and green textboxes above show significance level between groups, Kruskal-Wallis test and Mann-Whitney test respectively, $p< 0.05$. Different small letters (a,b) inside blue boxes indicate significant group differences, Mann Whitney U test with Bonferroni correction, $p< 0.05$. Identical letters or no letters inside blue boxes indicate no statistically significant differences. Abbr: RA: Rheumatoid arthritis (n=43), SpA: spondylarthropaties (n=39), PsA: Psoriatic arthritis (n=18), ReA: Reactive arthritis (n=9), AS: Ankylosing Spondylitis (n=12)

3.1.2 Correlation between inflammatory markers

Correlations between pg/mL IL-12 and IL-6 in SF, and between mg/L CRP in serum and the two cytokines are presented in Table 3.1. Scatter plots with a straight regression line are presented below to visualize the statistically significant correlations between the parameters (Figure 3.1.4). It is important to have in mind that Spearman's test use the rank order association and does not look at the levels directly as shown in Figure 3.1.4 A and B, as well as the correlation do not have to be linear. To better fit the robustness test to extreme outliers in Spearman's test, the four most extreme IL-12 outliers were not visualised in the figures. Thus, only 82 cases are shown when correlating the parameter IL-12, even though statistics included 85 samples, excluding only the highest extreme value.

Table 3.1: An outline of statistically significant correlations between the inflammatory markers

	Spearman's rank-order correlation coeffisient rho (p-value)					
Correlation	**RA**	**PsA**	**ReA**	**AS**	**SpA**	**All patients**
IL-12 and IL-6	**-0.42 (0.005**)**	-0.04 (0.886)	-0.30 (0.433)	0.11 (0.729)	0.01 (0.927)	-0.13 (0.228)
CRP[¶] and IL-12	-0.20 (0.193)	0.20 (0.465)	0.38 (0.352)	0.24 (0.498)	0.22 (0.219)	0.03 (0.778)
CRP[¶] and IL- 6	0.21 (0.175)	0.40 (0.126)	0.52 (0.183)	0.38 (0.283)	**0.36 (0.033*)**	**0.34 (0.002**)**

Boldface numbers show significant correlations: * P<0.05 (two-tailed); **P<0.01 (two tailed). One extreme value was xcluded from the plot; 21 597 pg/mL (AS patient). [¶] CRP; 9 missing values; n=77 (RA; n=42, SpA; n = 35, PsA; n =16, ReA n=8, Bech; n=11). Abbr: RA: Rheumatoid arthritis (n=45), SpA: spondylarthropaties (n=41), PsA: Psoriatic arthritis (n=19), ReA: Reactive arthritis (n=9), AS: Ankylosing Spondylitis (n=13).

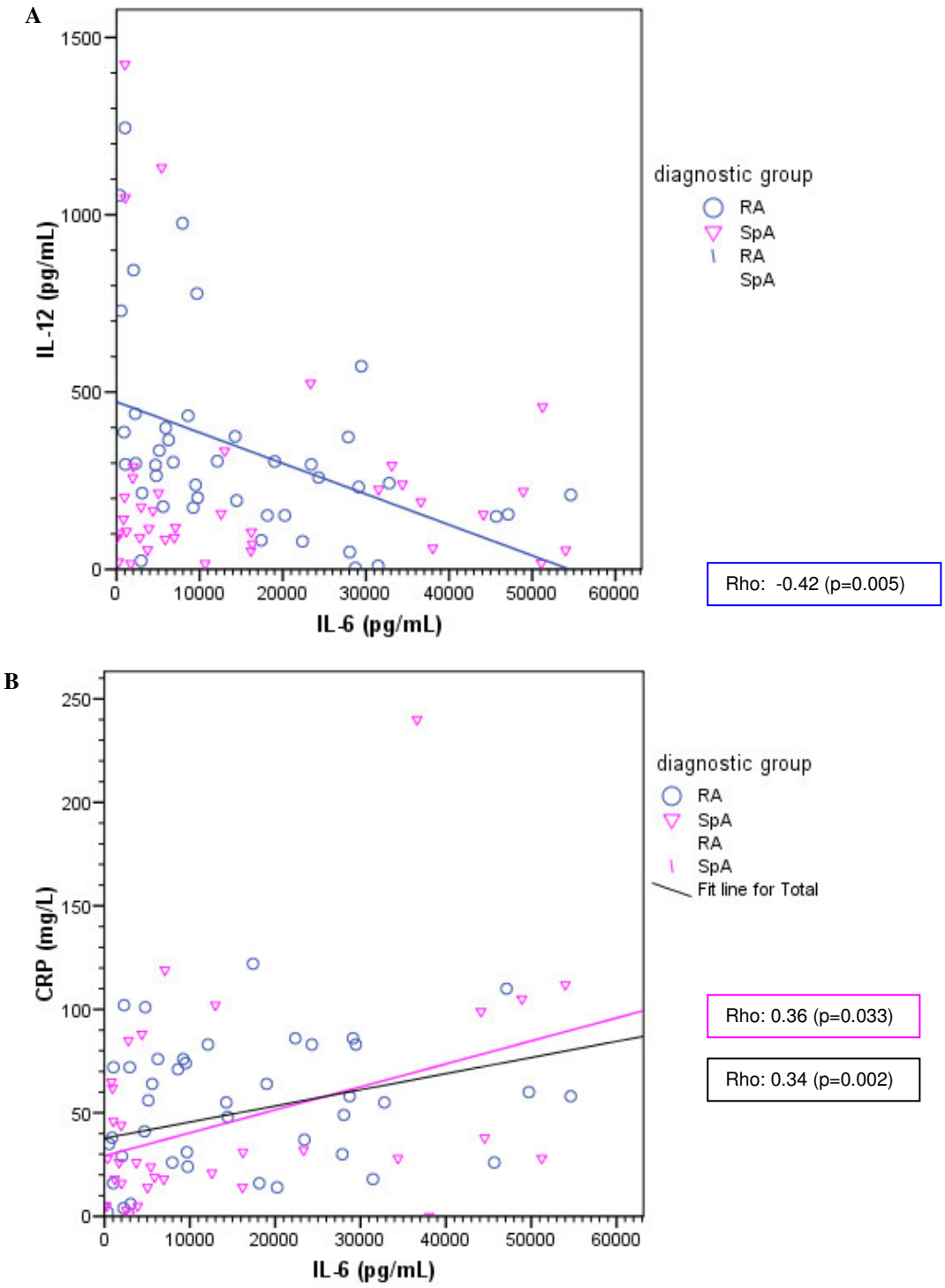

Figure 3.1.4: Significant correlations between inflammatory markers. Straight lines represents the groups with significant correlations, which may include SpA (pink line), RA (blue line) or all patients (the black line, refered to as fit line for total). The depicted symbols represent SpA (pink triangles) and RA (blue dots) together constituting all patients. Text boxes give the the Spearman's rank correlation coefficient (rho), with p-value in parenthesis. **(A)** The statistically negative correlation between IL-12 and IL-6 in RA group. Four high extreme values are not visualized in the plot; 21 597 pg/mL (AS) 4568 pg/mL (RA), 2664 pg/mL (PsA) and 2572 pg/mL (RA). RA n=43, SpA: n=39, PsA: n=18, ReA: n=9, AS: n=11. **(B)** The statistically positive correlation between C-reactive protein (CRP) in serum and IL-6 in synovial fluid in the SpA group and total patients. CRP; 9 missing values; n=77. Abbr: Rheumatoid Arthritis; RA (n=42), Spondylarthropaties; SpA (n=35), Psoriasis Arthritis; PsA (n=16), Reactive Arthritis; ReA (n=8), Ankylosing Spondylitis;AS (n=11)

- *Pattern:* There were no significant correlations between the inflammatory markers within the three subgroups PsA, ReA and AS (Table 3.1).

- *IL-12 and IL-6 in SF:* There was a significant but moderate negative correlation between the markers in the RA group (-0.42), meaning that higher levels of IL-12 may represent lower levels of IL-6 (Figure 3.1.4 A). The correlation coefficients had a tendency to be weakly negative in all patients as a group, as well as in PsA and ReA subgroups (Table 3.1) (not statistically significant).

- *Serum CRP and SF IL-12:* There were no significant correlations between levels of CRP in serum and IL-12 in SF. Notably, all associations between the two, except of the RA group, were positive (Table 3.1).

- *Serum CRP and SF IL-6:* There were two significant positive correlations of low magnitude in both the SpA group, and all the patients as a group (Figure 3.1.4 B) meaning that higher levels of CRP are associated with higher levels of IL-6 in these patient groups. In general, all correlation coefficients within both the groups and total patients as a group tended to have positive correlation coefficients.

3.2 FATTY ACID COMPOSITION OF THE SYNOVIAL FLUID

Only results from combined parallels that had no significantly differences in total quantified fatty acid composition (% and µg/g) as well as combined parallels that had percent difference between SFA, MUFA and PUFA below 10%, were accepted. If differing from these rules, those results will be discussed specifically.

Throughout this section, the fatty acid composition in SF from patients with various inflammatory diseases will be presented. The fatty acid composition in all patients as a group and in various patient groups will be presented in the same manner as the inflammatory markers in Section 3.1.1, but in five different tables (Table 3.2.1- 3.2.3 and 3.2.5- 3.2.6). Both percentage and µg/g values are included. This is to provide an overall picture of the fatty acids. Additionally, the statistically significant differences between the groups, which all concern the n-6 and n-3 fatty acids, will be studied in detail in two additional tables (Table 3.2.4 and 3.2.7).

Data included in various tables: In general, tables in this section show the fatty acid composition by median and interquartile range (IR). Table 3.2.1 includes all data on percentage fatty acids, which had an average and median above 0.2 % of total 100% fatty acids. The same specific fatty acids are presented in µg/g, but values below LOD and limit of quantification LOQ were subsequently marked. The exception was 24:1n-9, 18:3n-3 and 20:5n-3 in µg/g, which were above the theoretical LOQ for the GC instrument explained in Material and method Section 2.4.6. The same specific fatty acids will also be presented in the group comparison tables, except for 24:1n-9 µg/g, which is of no specific interest.

3.2.1 Total patients

The fatty acid composition in SF shows the same tendency between percentage and absolute values (µg/g) from all patients with inflammatory joint diseases (Table 3.2.1). The sum total fatty acids in absolute values have a median of 1153 µg/g (IR 871-1421), which in percentage is added up to 100%. Because of this low amount of total fatty acids, and the LOQ is set to 10 µg/g , it would be expected that percentage fatty acid composition quantified in the lower area of 0.9 %, will be below LOQ for the specific fatty acid composition quantified in µg/g.

PUFA>SFA and MUFA: Approximately half of the fatty acids found in the SF are PUFA (median 546µg/g, IR 424-700). The remaining fatty acids are quantified as SFA (median 287µg/g, IR 226-356) and MUFA (median 271µg/g, IR 193-343) in nearly equal ratio.

Linoleic acid >> oleic acid> palmitic acid: Of the single fatty acids; the n-6 PUFA, LA (18:2n-6), was the major contributor of fatty acids present in SF and constitutes approximately a median 31.9 % (IR, 28.4-34.8), which amount to in µg/g a median at 360 µg/g (IR 269-471) of total fatty acids. The MUFA oleic acid (18:1n-9), and the SFA palmitic acid (16:0), were two other large contributors to the fatty acids in SF from total patients, with respectively a median at 220 µg/g (IR 152-285) and 179 µg/g (IR 77-116) . Together, these three fatty acids constituted approximately 66% of total fatty acids.

Table 3.2.1: Fatty acid composition in synovial fluid from all patients

	SYNOVIAL FLUID (n=86)	
Fatty acid	**All patients (%)**	**All patients (µg/g)**
∑ SFA	25.4 (23.9-26.7)	287 (226-356)
14:0	0.16 (0.06-0.24)	<LOD
16:0	15.3 (13.4-16.6)	179 (77-116)
17:0	0.30 (0.26-0.34)	<LOQ
18:0	8.2 (7.5-9.1)	95 (77-116)
20:0	0.26 (0.21-0.33)	<LOQ
22:0	0.56 (0.40-0.73)	<LOQ
24:0	0.34 (0.26-0.53)	<LOQ
∑ MUFA	23.2 (21.1-25.5)	271 (193-343)
16:1n-7	1.0 (0.8-1.4)	12 (7-19)
16:1n-9	0.28 (0.21-0.38)	<LOQ
18:1n-9	18.7 (16.7-20.8)	220 (152-285)
18:1n-7	1.6 (1.4-1.7)	18 (13-22)
20:1n-9	0.25 (0.19-0.37)	<LOQ
22:1n-9	0.25 (0.06-0.54)	<LOQ
24:1n-9	0.62 (0.41-0.93)	7¶ (5-8)
∑ PUFA	48.4 (45.6-51.8)	546 (424-700)
18:2n-6	31.9 (28.4-34.8)	360 (269-471)
20:2n-6	0.27 (0.25-0.35)	<LOQ
20:3n-6	1.7 (1.4-2.0)	20 (14-27)
20:4n-6	7.0 (6.0-7.9)	76 (60-104)
18:3n-3	0.53 (0.43-0.62)	6¶ (4-8)
20:5n-3	1.7 (1.0-2.6)	17 (11-27)
22:5n-3	0.76 (0.61-0.95)	8¶ (6-11)
22:6n-3	3.9 (3.0-4.8)	41 (30-55)
		0
∑ n-6	41.0 (37.4-44.2)	459 (344-608)
∑ n-3	7.0 (5.3-9.0)	74 (56-102)
∑ vegetable n-3	0.53 (0.43-0.62)	6 (4-8)
∑ marine n-3	6.6 (4.5-8.2)	67 (52-93)
n-6/n-3	5.8 (4.2-7.7)	6.0 (4.3-8.2)
AA/EPA	4.1 (2.6-6.7)	4.1 (2.6-6.7)
n-6/vegetable n-3	76.6 (66.6-101.1)	66.8 (52-85)
n-6/marine n-3	6.5 (4.6-8.9)	6.5 (4.6-9.1)
∑ various	3.0 (2.3-3.6)	32 (21-41)
∑ identified	97.0 (96.4-97.7)	1118 (839-1395)
∑ total	100	1153 (871-1421)

¶Value above the theoretical LOQ for the GC instrument used in this experiment (5 µg/g)
LOD; Limit of detection (3 µg/g). LOQ; Limit of quantification (10 µg/g). Vegetable n-3: 16:4n-3, 18:3n-3 and 18:4n-3. Marine n-3; 20:5n-3, 22:5n-3 and 22:6n-3. Various: unidentified fatty acids. Abbr: SFA; Saturated Fatty acids; MUFA; Monosaturated fatty acids, PUFA; Polysaturated fatty acids. AA; Arachidonic acid (20:4n-6), EPA: Eicosapentaenoic acid (20:5n-3). Values are given as median and interquartile range.

n-6 >> n-3 PUFA: Most of the PUFA come from n-6 PUFA (459µg/g, IR 344-608), with a ratio of n-6/n-3 at approximately 6. Second to LA(18:2n-6), AA (20:4n-6) is the other n-6 PUFA noticeably present, but in clearly lower amount (median 76 µg/g, IR 60-104). Of the low content of n-3 PUFA present (median 74µg/g, IR 56-102), marine PUFA (i.e sum of EPA, DPA and DHA), and especially DHA, (22:6n-3, median 41µg/g, IR 30-55), were the major contributors found. The PUFA ALA (18:3n-3) was present in extremely low amount (median 6µg/g, IR 4-8) and was the only n-3 PUFA of vegetable origin quantified above theoretical limit of quantification.

3.2.2 A comparison between the four different subgroups

Table 3.2.2 and 3.2.3 show the fatty acid composition in synovial fluid in the disease subgroups, RA, PsA, ReA and AS respectively, in percentage and µg/g. RA and AS were the only groups that had significantly different fatty acid composition.

3.2.2.1 Patients with Ankylosing Spondylitis and Rheumatoid Arthritis

Differences between the AS and RA-group were just seen among the amount of n-3 PUFA. The differences between the two groups and the specific significance level are summarized in Table 3.2.4.

- *Percentage and µg/g:* All significant differences between the groups in µg/g included also differences in percentage. As seen in Table 3.2.2 and 3.2.3, it only involved the ratios including ratio of n-6 to n-3, n-6 to marine n-3, and n-6 to vegetable n-3 PUFA (percentage and µg/g*)* are all significantly lower in synovial fluid of patients with RA compared to the patients with AS disease.
- *Percentage:* Additionally, in percentage there was a significantly higher level of DHA (22:6n-3) in the RA group (median 4.2%, IR 3.4-5.5) compared to AS group (median 3.4%, IR 2.4-3.9). There were no other significant differences between singular fatty acids. Nevertheless, there were significantly higher levels of both total percentage n-3 PUFA and total percentage marine n-3 PUFA in the RA group (respectively, median 7.8%, IR 5.9 -10.3 and median 7.0%, IR 5.5-9.5) compared to AS (respectively, median 5.0%, IR 4.2-7.3 and median 5.0%, IR 3.7-6.7). The latter nearly amounts to all of the n-3 PUFA, and high amount in the RA group contributing to lower n-6 to n-3 ratios of the RA group.

Table 3.2.2: Fatty acid composition (%) in synovial fluid from patients with Rheumatiod arthritis (RA), Psoriasis Arthritis (PsA), Reactive Arthritis (ReA) and Ankylosing Spondylitis (AS)

	SYNOVIAL FLUID				
Fatty acid (%)	**RA, n=45**	**PsA, n=19**	**ReA, n=9**	**AS, n=13**	**p-value**
∑ SFA	25.5 (24.0-27.1)	25.1 (23.8-26.3)	25.4 (23.7-27.1)	25.5 (24.5-26.0)	0.74
14:0	0.14 (0.06-0.30)	0.17 (0.00-0.26)	0.16 (0.13-0.20)	0.14 (0.08-0.21)	0.9
16:0	15.1 (13.2-16.7)	14.8 (13.5-16.1)	16.2 (13.4-17.4)	15.6 (14.3-16.4)	0.54
17:0	0.31 (0.26-0.37)	0.28 (0.25-0.31)	0.30 (0.27-0.33)	0.30 (0.28-0.34)	0.12
18:0	8.4 (7.7-9.4)	7.7 (7.4-8.5)	8.6 (6.4-9.1)	8.0 (7.5-8.9)	0.33
20:0	0.26 (0.21-0.32)	0.26 (0.21-0.33)	0.27 (0.20-0.34)	0.25 (0.21-0.38)	0.98
22:0	0.55 (0.42-0.69)	0.68 (0.39-0.86)	0.44 (0.34-0.57)	0.60 (0.48-0.95)	0.25
24:0	0.31 (0.19-0.54)	0.35 (0.26-0.69)	0.33 (0.29-0.46)	0.42 (0.29-0.50)	0.72
∑ MUFA	23.2 (20.3-25.4)	23.2 (21.3-26.7)	24.9 (22.8-25.8)	23.0 (21.9-25.8)	0.54
16:1n-7	1.0 (0.8-1.5)	1.3 (1.0-1.4)	1.0 (0.7-1.5)	0.8 (0.7-1.0)	0.13
16:1n-9	0.28 (0.20-0.37)	0.26 (0.20-0.40)	0.31 (0.26-0.37)	0.27 (0.21-0.37)	0.9
18:1n-9	18.4 (16.4-20.1)	18.8 (16.6-21.3)	20.8 (18.7-21.4)	19.1 (17.5-21.1)	0.25
18:1n-7	1.6 (1.4-1.7)	1.6 (1.3-1.8)	1.5 (1.4-1.6)	1.6 (1.4-1.7)	0.75
20:1n-9	0.22 (0.18-0.43)	0.25 (0.21-0.36)	0.23 (0.14-0.46)	0.26 (0.21-0.40)	0.83
22:1n-9	0.24 (0.00-0.53)	0.21 (0.14-0.47)	0.28 (0.00-0.66)	0.29 (0.10-0.60)	0.82
24:1n-9	0.61 (0.42-0.98)	0.63 (0.39-0.96)	0.61 (0.38-0.80)	0.63 (0.43-0.95)	0.9
∑ PUFA	48.1 (44.7-52.2)	49.6 (46.5-52.1)	47.1 (44.0-49.1)	49.4 (45.7-51.4)	0.77
18:2n-6	30.7 (27.0-34.5)	31.8 (30.0-36.7)	31.5 (27.7-34.5)	33.7 (32.3-35.2)	0.053
20:2n-6	0.27 (0.25-0.35)	0.28 (0.25-0.35)	0.25 (0.21-0.38)	0.27 (0.24-0.33)	0.79
20:3n-6	1.7 (1.4-2.0)	1.6 (1.3-2.2)	1.7 (1.5-2.0)	1.7 (1.4-1.9)	0.89
20:4n-6	7.3 (5.9-8.2)	7.1 (6.7-7.7)	6.1 (5.6-7.4)	6.3 (5.5-8.1)	0.47
18:3n-3	0.55 (0.45-0.63)	0.53 (0.46-0.64)	0.53 (0.38-0.62)	0.45 (0.36-0.58)	0.22
20:5n-3	2.0 (1.2-3.0)	1.5 (0.9-2.5)	1.8 (0.9-2.7)	1.6 (0.8-1.9)	0.1
22:5n-3	0.89 (0.63-1.02)	0.69 (0.61-0.95)	0.67 (0.51-0.80)	0.64 (0.54-0.88)	0.07
22:6n-3	**4.2 (3.4-5.5)** [a]	4.0 (3.1-4.6)	3.9 (2.6-4.4)	**3.0 (2.4-3.9)** [b]	**0.023***
∑ n-6	40.7 (35.3-44.1)	41.0 (38.7-44.4)	40.0 (35.0-43.9)	42.0 (41.1-45.2)	0.24
∑ n-3	**7.8 (5.9 -10.3)** [a]	7.0 (5.6-8.8)	7.0 (4.7-8.4)	**5.0 (4.2-7.3)** [b]	**0.027***
∑ vegetable n-3	0.55 (0.45-0.63)	0.53 (0.46-0.64)	0.53 (0.38-0.62)	0.45 (0.36-0.58)	0.22
∑ marine n-3	**7.0 (5.5 -9.5)** [a]	6.0 (4.5-8.2)	6.6 (4.1-8.0)	**5.0 (3.7-6.7)** [b]	**0.046***
n-6/n-3	**4.9 (3.6-6.9)** [a]	6.0 (4.9-7.3)	7.0 (4.5-9.0)	**8.0 (6.1-9.9)** [b]	**0.006***
AA/EPA	3.8 (2.1-5.7)	4.0 (3.3-7.8)	5.0 (2.4-8.1)	5.0 (3.6-8.7)	0.16
n-6/vegetable n-3	**73.9 (66.4-85.8)** [a]	84.9 (57.3-106.3)	77.3 (66.9-100.9)	**98.7 (78.0-114.6)** [b]	**0.060***
n-6/marine n-3	**5.6 (3.9 - 7.6)** [a]	7.1 (5.4-8.9)	7.8 (4.8-10.4)	**8.2 (6.5-11.5)** [b]	**0.016***

Boldface numbers show significant correlations. * Significance level ($p < 0.05$), Kruskal-Wallis test. Mann-Whitney U test with Bonferroni correction ($p < 0.05$). Identical letters or no letters indicate no significant differences. Abbr: SFA; Saturated Fatty acid; MUFA;Monosaturated fatty acid, PUFA; Polysaturated acid (20:4n-6), AA; Arachidonic acid, EPA: Eicosapentaenoic acid (20:5n-3), Vegetable n-3: 18:3n-3. Marine n-3; 20:5n-3, 22:5n-3 and 22:6n-3.

Table 3.2.3: Fatty acid composition (µg/g) in synovial fluid from patients with Rheumatiod arthritis (RA), Psoriasis Arthritis (PsA), Reactive Arthritis (ReA) and Ankylosing Spondylitis (AS)

	SYNOVIAL FLUID				
Fatty acid (µg/g)	**RA, n=45**	**PsA, n=19**	**ReA, n=9**	**AS, n=13**	**p-value**
∑ SFA	281 (206-357)	287 (248-452)	328 (298-402)	269 (199-324)	0.269
16:0	159 (102-227)	185 (135-284)	198 (174-272)	164 (118-201)	0.338
18:0	99 (71-118)	96 (79-131)	111 (93-130)	84 (67-102)	0.167
∑ MUFA	249 (178-334)	328 (190-380)	299 (284-407)	242 (175-309)	0.089
16:1n-7	12 (7-19)	16 (10-23)	11 (8-23)	9 (7-14)	0.236
18:1n-9	17 (12-21)	19 (14-24)	21 (17-25)	18 (11-21)	0.072
18:1n-7	200 (140-227)	271 (149-317)	249 (235-343)	206 (137-264)	0.241
24:1n-9	6 (5-8)	6 (4-10)	8 (6-10)	7 (5-9)	0.484
∑ PUFA	532 (372-674)	574 (495-826)	608 (497-857)	495 (354-684)	0.329
18:2n-6	329 (213-459)	382 (338-514)	366 (332-601)	366 (253-473)	0.225
20:3n-6	19 (13-26)	21 (13-28)	21 (17-39)	14 (12-28)	0.267
20:4n-6	73 (57-98)	79 (69-104)	71 (64-126)	70 (49-93)	0.484
18:3n-3	6 (4-8)	6 (4-10)	7 (5-10)	4 (3-7)	0.368
20:5n-3	19 (13-30)	14 (11-23)	23 (11-41)	12 (11-20)	0.213
22:5n-3	8 (6-11)	8 (6-11)	8 (7-11)	8 (6-9)	0.474
22:6n-3	45 (32-56)	41 (31-55)	47 (34-73)	29 (22-47)	0.088
∑ n-6	435 (289-577)	482 (438-675)	454 (418-768)	447 (317-606)	0.213
∑ n-3	78 (61-112)	67 (56-97)	87 (60-131)	56 (46-87)	0.092
∑ vegetable n-3	6 (4-8)	6 (4-10)	7 (5-10)	4 (3-7)	0.368
∑ marine n-3	71 (55-106)	62 (51-90)	81 (52-123)	53 (41-78)	0.098
n-6/n-3	**5.1 (3.8-6.9)** [a ¶]	6.5 (4.9-8.3)[¶]	7.2 (4.5-9.1)	**7.6 (6.1-9.86)** [b]	**0.004***
AA/EPA	3.9 (2.2-5.7)	4.4 (3.3-7.8)	4.9 (1.7-8.1)	4.9 (3.6-8.7)	0.195
n-6/vegetable n-3	**64.4 (48.4-74.5)** [a]	71.5 (45.1-86.9)	65.9 (61.1-91.9)	**85.2 (71.2-108.6)** [b]	**0.023***
n-6/marine n-3	**5.6 (4.1 -7.6)** [a]	7.1 (5.4-8.9)	7.7 (4.8-10.3	**8.2 (6.5-11.5)** [b]	**0.021***

Boldface numbers show significant correlations.* Significance level ($p < 0.05$), Kruskal-Wallis test. Comparison between subgroups: Mann-Whitney U test with Bonferroni correction ($p < 0.05$).Different letters significant no differences. Identical letters or no letters indicate no significant differences.¶ Not significant between groups after Bonferroni correction. Abbr: SFA; Saturated Fatty acid; MUFA; Monosaturated fatty acid, PUFA; Polyunsaturated fatty acid, AA; Arachidonic acid,(20:4n-6), EPA: Eicosapentaenoic acid (20:5n-3), Vegetable n-3: 18:3n-3, Marine n-3; 20:5n-3, 22:5n-3 and 22:6n-3.

Table 3.2.4: A simplified table of the statistically significant differences in fatty acid composition between patients with Rheumatoid Arthritis (RA) and Ankylosing Spondylitis (AS)

Fatty acid	%			µg/g		
	RA	AS	p-value	RA	AS	p-value
DHA (22:6 n-3)	↑	↓	0.012			
∑ n-3	↑	↓	0.016			
∑ marine n-3	↑	↓	0.040			
n-6/n-3	↓	↑	0.003	↓	↑	0.004
n-6/ vegetable n-3	↓	↑	0.021	↓	↑	0.006
n-6/marine FA	↓	↑	0.009	↓	↑	0.011

Arrows pointing upwards or downwards indicate statistically significant differences, respectively higher or lower than the other group. Significance level ($p < 0.05$), Mann-Whitney U test with Bonferroni's correction.

3.2.3 A comparison between Rheumatoid Arthritis and Spondyloarthropathies

Comparison of the SF fatty acid composition between the RA patients and the different SpA as one group is presented in Table 3.2.5 and 3.2.6, in percentage and µg/g. There were somewhat similar significant differences between RA and SpA as seen between RA and the specific subgroup AS. The significant differences between the RA and SpA groups are summarized in Table 3.2.7.

- *Percentage and µg/g:* All significantly differences between the groups in µg/g, also included differences in percentage. Both SpA and the subgroup AS had higher SF ratios of n-6 to n-3 and the n-6 to marine n-33, and n-6 to vegetable n-3 PUFA than the RA group. Further, the SpA group had a significantly elevated ratio of n-6 to vegetable n-3 as compared with the RA group.
- *Percentage:* Similar to the differences between RA and AS, the percentage level of DHA, the total n-3 PUFA and the total marine n-3 PUFA were also found to be significantly higher in the RA group compared with the SpA. Additionally, there was a significantly elevated percentage level of the singular n-3 PUFA EPA and DPA in the synovial fluid of the RA group, which may contribute to a significantly decreased ratio of AA to EPA compared to the SpA group. LA (18:2n-6) was also significantly different between the two groups, but the levels were decreased in RA group compared with SpA. A trend of decrease of AA to EPA and LA in the RA group, both in µg/g was also noted.

Table 3.2.5: Fatty acid composition (%) in synovial fluid of patients with Rheumatoid arthritis (RA) and Spondyloarthropathies (SpA).

SYNOVIAL FLUID					
Fatty acid (%)	**RA, n=45**		**SpA, n=41**		**p-value**
∑ SFA	25.5	(24.0-27.1)	25.4	(23.9-26.1)	0.240
14:0	0.14	(0.06-0.30)	0.16	(0.08-0.21)	0.990
16:0	15.1	(13.2-16.7)	15.4	(13.8-16.5)	0.688
17:0	0.31	(0.26-0.37)	0.30	(0.26-0.32)	0.081
18:0	8.4	(7.7-9.4)	8.0	(7.3-8.9)	0.069
20:0	0.26	(0.21-0.32)	0.26	(0.21-0.34)	0.739
22:0	0.55	(0.42-0.69)	0.56	(0.40-0.85)	0.439
24:0	0.31	(0.19-0.54)	0.35	(0.30-0.54)	0.274
∑ MUFA	23.2	(20.3-25.4)	23.2	(21.9-26.2)	0.236
16:1n-7	1.0	(0.8-1.5)	1.0	(0.8-1.4)	0.799
16:1n-9	0.28	(0.20-0.37)	0.27	(0.21-0.38)	0.990
18:1n-9	18.4	(16.4-20.1)	19.1	(17.4-21.2)	0.152
18:1n-7	1.6	(1.4-1.7)	1.5	(1.3-1.7)	0.514
20:1n-9	0.22	(0.18-0.43)	0.25	(0.21-0.36)	0.548
22:1n-9	0.24	(0.00-0.53)	0.26	(0.10-0.55)	0.572
24:1n-9	0.61	(0.42-0.98)	0.63	(0.40-0.88)	0.733
∑ PUFA	48.1	(44.7-52.2)	49.1	(45.8-51.4)	0.993
18:2n-6	**30.7**	**(27.0-34.5)**	**33.4**	**(30.4-35.3)**	**0.017***
20:2n-6	0.27	(0.25-0.35)	0.27	(0.24-0.34)	0.990
20:3n-6	1.7	(1.5-2.0)	1.7	(1.42-1.93)	0.928
20:4n-6	7.3	(5.9-8.2)	6.8	(6.0-7.6)	0.212
18:3n-3	0.55	(0.45-0.63)	0.52	(0.37-0.59)	0.282
20:5n-3	**2.0**	**(1.2-3.0)**	**1.6**	**(0.9-2.3)**	**0.020***
22:5n-3	**0.89**	**(0.63-1.02)**	**0.68**	**(0.56-0.89)**	**0.019***
22:6n-3	**4.2**	**(3.36-5.50)**	**3.8**	**(2.59-4.32)**	**0.009***
∑ n-6	40.7	(35.3-44.1)	41.6	(39.5-44.4)	0.151
∑ n-3	**7.8**	**(5.9-10.3)**	**6.8**	**(4.8-8.0)**	**0.008***
∑ vegetable n-3	0.55	(0.45-0.63)	0.52	(0.38-0.60)	0.282
∑ marine n-3	**7.0**	**(5.48-9.50)**	**6.2**	**(4.3-7.5)**	**0.014***
n-6/n-3	**4.9**	**(3.6-6.9)**	**6.5**	**(5.1-8.6)**	**0.002***
AA/EPA	**3.8**	**(2.1-5.7)**	**4.9**	**(3.2-8.2)**	**0.038***
n-6/vegetable n-3	**73.9**	**(66.4-85.8)**	**84.9**	**(68.7-108.3)**	**0.046***
n-6/marine n-3	**5.6**	**(3.9-7.6)**	**7.4**	**(5.5-10.3)**	**0.004***

Boldface numbers show significant correlations. *Significance level ($p < 0.05$), Mann-Whitney U test. Vegetable n-3: 16:4n-3, 18:3n-3 and 18:4n-3. Marine n-3; 20:5n-3, 22:5n-3 and 22:6n-3. Abbr: SFA; Saturated Fatty acids; MUFA; Monosaturated fatty acids, PUFA; Polysaturated fatty acids. AA; Arachidonic acid (20:4n-6), EPA: Eicosapentaenoic acid (20:5n-3) RA: Rheumatoid arthritis (n=45), SpA: spondyloarthropaties, including patients with Psoriatic arthritis, Reactive arthritis and Ankylosing Spondylitis (n=41)

Table 3.2.6: Fatty acid composition (µg/g) in synovial fluid of patients with Rheumatoid arthritis and Spondyloarthropathies

SYNOVIAL FLUID					
Fatty acid (µg/g)	**RA , n=45**		**SpA, n=41**		**p-value**
∑ SFA	281	(206-357)	288	(248-365)	0.301
16:0	159	(102-227)	182	(144-227)	0.259
18:0	99	(71-118)	94	(83-117)	0.619
∑ MUFA	249	(178-334)	293	(215-364)	0.105
16:1n-7	12	(7-19)	12	(8-20)	0.662
18:1n-9	17	(12-21)	19	(14-23)	0.168
18:1n-7	200	(140-227)	244	(175-309)	0.080
24:1n-9	6	(5-8)	7	(5-9)	0.318
∑ PUFA	532	(372-674)	574	(466-755)	0.208
18:2n-6	329	(213-459)	371	(308-519)	0.058
20:3n-6	19	(13-26)	21	(13-28)	0.348
20:4n-6	73	(57-98)	77	(64-107)	0.508
18:3n-3	6	(4-8)	6	(4-9)	0.921
20:5n-3	19	(13-30)	14	(11-25)	0.779
22:5n-3	8	(6-11)	8	(6-10)	0.143
22:6n-3	45	(32-56)	40	(29-52)	0.675
∑ n-6	435	(289-577)	478	(401-664)	0.071
∑ n-3	78	(61-112)	67	(51-94)	0.508
∑ vegetable n-3	6	(4-8)	6	(4-9)	0.921
∑ marine n-3	71	(55-106)	62	(46-86)	0.205
n-6/n-3	**5.1**	**(3.8-6.9)**	**6.9**	**(5.1-9.1)**	**0.002***
AA/EPA	3.9	(2.2-5.7)	4.9	(3.2-8.2)	0.060
n-6/vegetable n-3	**64.4**	**(48.4-74.5)**	**76.8**	**(60.7-95.2)**	**0.033***
n-6/marine n-3	**5.6**	**(4.1-7.6)**	**7.4**	**(5.5-10.3)**	**0.006***

Boldface numbers show significant correlations. *Significance level ($p < 0.05$), Mann-Whitney U test. Vegetable n-3: 16:4n-3, 18:3n-3 and 18:4n-3. Marine n-3; 20:4n-3, 20:5n-3, 22:5n-3 and 22:6n-3. Abbr: SFA; Saturated Fatty acid; MUFA; Monosaturated fatty acid, PUFA; Polysaturated fatty acid. AA; Arachidonic acid (20:4n-6), EPA: Eicosapentaenoic acid (20:5n-3) RA: Rheumatoid arthritis (n=45), SpA: spondyloarthropaties, including patients with Psoriatic arthritis, Reactive arthritis and Ankylosing Spondylitis (n=41).

Table 3.2.7: A simplified summarize of the significant differences in fatty acid composition between patients with Rheumatoid Arthritis (RA) and Spondyloarthropaties (SpA)

	%			µg/g		
Fatty acid	**RA**	**SpA**	**p-value**	**RA**	**SpA**	**p-value**
AA (18:2n-6)	↓	↑	0.017			
EPA (20:5 n-3)	↑	↓	0.02			
DPA (22:5n-3)	↑	↓	0.019			
DHA (22:6n-3)	↑	↓	0.009			
∑ n-3	↑	↓	0.008			
∑ marine n-3	↑	↓	0.014			
n-6/ n-3	↓	↑	0.002	↓	↑	0.002
AA/ EPA	↓	↑	0.038			
n-6/ vegetable n-3	↓	↑	0.046	↓	↑	0.033
n-6/ marine FA	↓	↑	0.004	↓	↑	0.006

Arrows pointing upwards or downwards indicate significant differences, respectively higher or lower levels of the specific fatty acid compared to the other group. Significance level ($p < 0.05$), Mann-Whitney U test.

3.3 CORRELATION OF N-6 AND N-3 PUFA, AND THE RATIOS BETWEEN THEM, TO INFLAMMATORY MARKERS

In this section, the cross-sectional correlations between the ratios of the n-6 and n-3 PUFA and the three inflammatory markers, CRP, IL-6, IL -12, will be presented. The correlation will be shown for all patients, the four subgroups RA, PsA, ReA and AS, and between RA and the three other subgroups represented as the SpA. The fatty acid parameters will involve all the single n-6 and n-3 PUFA, in addition to the sum of n-6 or n-3 (Table 3.3.1), and the ratios of total n-6 to n-3, AA to EPA and n-6 to marine FA (Table 3.3.2) in correlation to the inflammatory markers CRP, IL-6 and IL-12. The fatty acid correlation will only include absolute values (µg/g) from the quantification to avoid compensatory percent changes between n-6 and n-3 PUFA.

- *ReA:* There were no significant correlations between the parameters in the ReA group
- All significant correlations were of generally low or moderate magnitude.

- ***Single n-6 and n-3 PUFA***:

There were mainly positive significant correlations between n-6 and n-3 PUFA to IL-6 in SF.

- No correlation between these PUFA and CRP of blood in SF.
- **n-6 PUFA and IL-6**: All significant correlation of n-6 PUFA were positive to IL-6 (meaning that high levels of n-6 PUFA may also represent high levels of IL-6) and included mainly the n-6 AA precursors, i.e LA (18:2n-6), DGLA (20:3n-6), or sums of n-6 PUFA in all patients, SpA, AS or PsA. However, only the n-6 PUFA DGLA in SF from the RA group, correlated significantly to IL-6. A positive correlation was also found between AA and IL-6 in PsA.
- **n-3 PUFA and IL-6:** Two fatty acid correlations were found significant to inflammatory parameters, and both were positive to levels of IL-6. First, a significant correlation between ALA and IL-6 in SF found in all patients, SpA, AS and the RA group. Second, a significant positive correlation between the marine PUFA, DHA (22:6n-3) and IL-6 in all patients.
- **n-6 PUFA and IL-12:** The significant correlations between PUFA and IL-12 were less than to IL-6, they all involved the n-6 PUFA in RA patients (including sum n-6 PUFA, DGLA and AA) and AS patients (including sum of total n-6 PUFA and LA). The significant correlations were found weakly negative in the RA group and positive in the AS group.

Table 3.3.1: An outline of statistically significant correlations between the n-6 to n-3 ratios and the inflammatory markers

Correlation		Spearman's rank-order correlation coeffisient rho (p-value)					
FA	**Marker**	**RA**	**PsA**	**ReA**	**AS**	**SpA**	**All patients**
∑ **n-6**	CRP¶	0.01 (0.962)	0.23 (0.388)	-0.21 (0.610)	0.44 (0.174)	0.05 (0.792)	0.01 (0.962)
	IL-6	0.27 (0.074)	**0.61 (0.005******)**	-0.28 (0.460)	**0.61 (0.027*)**	**0.36 (0.020*)**	**0.27 (0.011*)**
	IL-12	**-0.30 (0.047*)**	-0.02 (0.932)	-0.23 (0.546)	**0.63 (0.022*)**	0.15 (0.365)	-0.15 (0.182)
18:2n-6	CRP¶	0.02 (0.893)	0.20 (0.450)	-0.12 (0.779)	0.38 (0.252)	0.09 (0.609)	0.03 0.806)
	IL-6	0.27 (0.069)	**0.54 (0.018*)**	-0.23 (0.546)	**0.59 (0.033*)**	**0.35 (0.027*)**	**0.27 (0.012*)**
	IL-12	-0.27 (0.075)	-0.04 (0.858)	-0.27 (0.488)	**0.66 (0.013*)**	0.15 (0.357)	-0.13 (0.218)
20:3n-6	CRP¶	0.01 (0.961)	0.40 (0.124)	-0.45 (0.260)	0.54 (0.085)	0.15 (0.377)	0.07 (0.558)
	IL-6	**0.34 (0.024*)**	**0.59 (0.008******)**	-0.40 (0.286)	**0.76 (0.002******)**	**0.48 (0.002******)**	**0.39 (0.000******)**
	IL-12	**-0.30 (0.049*)**	0.02 (0.943)	-0.20 (0.606)	0.41 (0.162)	0.10 (0.516)	-0.13 (0.242)
20:4n-6	CRP¶	-0.18 (0.251)	0.00 (1.000)	-0.50 (0.207)	0.53 (0.091)	-0.03 (0.859)	-0.09 (0.457)
	IL-6	0.06 (0.675)	**0.50 (0.029*)**	-0.53 (0.139)	0.51 (0.074)	0.27 (0.084)	0.15 (0.171)
	IL-12	**-0.30 (0.049*)**	0.14 (0.571)	-0.25 (0.516)	0.30 (0.325)	0.09 (0.592)	-0.13 (0.251)
∑ **n-3**	CRP¶	-0.18 (0.249)	-0.10 (0.706)	-0.55 (0.160)	0.00 (0.989)	-0.21 (0.219)	-0.16 (0.167)
	IL-6	0.10 (0.513)	0.28 (0.250)	-0.12 (0.765)	0.22 (0.471)	0.22 (0.174)	0.18 (0.093)
	IL-12	-0.17 (0.278)	0.04 (0.881)	-0.55 (0.125)	0.44 (0.133)	-0.05 (0.766)	-0.07 (0.507)
18:3n-3	CRP¶	0.18 (0.258)	0.00 (0.991)	0.17 (0.693)	0.19 (0.573)	-0.01 (0.943)	0.09 (0.464)
	IL-6	**0.35 (0.017*)**	0.37 (0.114)	0.12 (0.765)	**0.69 (0.009******)**	**0.39 (0.012*)**	**0.35 (0.001******)**
	IL-12	-0.25 (0.103)	-0.16 (0.518)	-0.22 (0.576)	0.62 (0.025)	0.05 (0.781)	-0.10 (0.376)
∑marine	CRP¶	-0.21 (0.181)	-0.10 (0.706)	-0.50 (0.207)	-0.07 (0.831)	-0.22 (0.195)	-0.20 (0.090)
	IL-6	0.08 (0.602)	0.32 (0.180)	-0.05 (0.898)	0.16 (0.603)	0.21 (0.194)	0.16 (0.138)
	IL-12	-0.16 (0.287)	0.10 (0.673)	-0.57 (0.112)	0.32 (0.280)	-0.04 (0.789)	-0.07 (0.517)
20:5n-3	CRP¶	-0.20 (0.203)	-0.15 (0.575)	-0.52 (0.183)	-0.03 (0.936)	-0.21 (0.237)	-0.17 (0.134)
	IL-6	-0.02 (0.885)	0.24 (0.312)	0.00 (1.000)	-0.23 (0.448)	0.07 (0.646)	0.06 (0.574)
	IL-12	-0.37 (0.810)	0.14 (0.571)	-0.60 (0.088)	0.13 (0.681)	-0.08 (0.637)	-0.04 (0.744)
22:5n-3	CRP¶	-0.19 (0.233)	-0.22 (0.413)	-0.14 (0.736)	0.30 (0.369)	-0.17 (0.319)	-0.15 (0.186)
	IL-6	0.01 (0.971)	0.29 (0.235)	-0.20 (0.606)	0.32 (0.280)	0.16 (0.308)	0.09 (0.434)
	IL-12	-0.04 (0.810)	-0.04 (0.858)	-0.38 (0.308)	-0.01 (0.972)	-0.11 (0.486)	-0.13 (0.227)
22:6n-3	CRP¶	-0.18 (0.245)	-0.10 (0.722)	-0.50 (0.207)	-0.08 (0.811)	-0.27 (0.111)	-0.20 (0.083)
	IL-6	0.15 (0.313)	0.28 (0.249)	0.02 (0.966)	0.23 (0.459)	0.24 (0.125)	**0.22 (0.039*)**
	IL-12	-0.23 (0.126)	0.05 (0.836)	-0.58 (0.099)	0.38 (0.194)	-0.04 (0.816)	-0.09 (0.404)

Correlations: Boldface numbers show significant correlations. * P<0.05 (two-tailed); **P<0.01 (two tailed). ¶ nine missing values; n=77 (RA; n=42, SpA; n=35, PsA; n =16, ReA; n=8, AS; n=11). Abbr: AA; Arachidonic acid (20:4n-6), EPA: Eicosapentaenoic acid (20:5n-3),18:3n-3; sum vegetale fatty acids, Marine; Marine n-3 fatty acids (20:5n-3, 22:5n-3 and 22:6n-3). RA: Rheumatoid arthritis (n=45), SpA: spondylarthropaties (n=41), PsA: Psoriatic arthritis (n=19), ReA: Reactive arthritis (n=9), AS: Ankylosing Spondylitis(n=13)

Table 3.3.2: An outline of statistically significant correlations between the n-6 to n-3 ratios and the inflammatory markers

Correlation		Spearman's rank-order correlation coeffisient rho (p-value)					
FA	**Markers**	**RA**	**PsA**	**ReA**	**AS**	**SpA**	**All patients**
n-6/n-3	CRP¶	0.00 (0.983)	0.42 (0.106)	0.31 (0.456)	0.40 (0.228)	**0.44 (0.008**)**	0.15 (0.208)
	IL-6	0.14 (0.369)	0.24 (0.329)	-0.27 (0.488)	0.46 (0.117)	0.15 (0.355)	0.08 (0.474)
	IL-12	-0.07 (0.659)	0.32 (0.182)	0.32 (0.406)	0.26 (0.394)	0.29 (0.065)	0.00 (0.991)
AA/EPA	CRP¶	0.01 (0.948)	0.21 (0.429)	0.21 (0.610)	0.28 (0.408)	0.25 (0.154)	0.08 (0.511)
	IL-6	0.06 (0.715)	0.11 (0.642)	-0.13 (0.732)	**0.60 (0.029*)**	0.11 (0.491)	0.01 (0.900)
	IL-12	-0.14 (0.358)	0.16 (0.518)	0.27 (0.488)	0.02 (0.943)	0.20 (0.215)	-0.02 (0.882)
n-6/vegetable	CRP¶	-0.24 (0.121)	0.29 (0.274)	-0.02 (0.955)	0.21 (0.527)	0.25 (0.142)	-0.03 (0.783)
	IL-6	-0.16 (0.308)	0.27 (0.265)	-0.35 (0.356)	-0.51 (0.078)	-0.10 (0.552)	-0.13 (0.222)
	IL-12	-0.07 (0.653)	0.41 (0.080)	0.25 (0.516)	**-0.62 (0.025*)**	0.18 (0.258)	-0.02 (0.829)
n-6/marine	CRP¶	0.11 (0.509)	0.40 (0.125)	0.29 (0.493)	0.40 (0.228)	**0.39 (0.021*)**	0.18 (0.119)
	IL-6	0.18 (0.227)	0.20 (0.405)	-0.23 (0.546)	0.42 (0.150)	0.13 (0.432)	0.10 (0.353)
	IL-12	-0.15 (0.325)	0.30 (0.217)	0.38 (0.308)	0.26 (0.384)	0.30 (0.055)	-0.02 (0.862)

Correlations: Boldface numbers show significant correlations. * P<0.05 (two-tailed); **P<0.01 (two tailed). ¶ nine missing values; n=77 (RA; n=42, SpA; n=35, PsA; n =16, ReA; n=8, AS; n=11). Abbr: AA; Arachidonic acid (20:4n-6), EPA: Eicosapentaenoic acid (20:5n-3), Vegetable; vegetable n-3 (18:3n-3), Marine; Marine n-3 (20:5n-3, 22:5n-3 and 22:6n-3). RA:Rheumatoid arthritis (n=45), SpA: spondylarthropaties (n=41), PsA:Psoriatic arthritis (n=19), ReA: Reactive arthritis (n=9), AS: Ankylosing Spondylitis (n=13)

- ***Ratios of n-6 to n-3 PUFA***:
  - Four correlations were found between ratios of n-6 to n-3 PUFA and inflammatory parameters. The correlation only involved the SpA (including n-6 to n-3 ratio and the n-6 to marine n-3 ratio in positive correlation to CRP) and AS (AA to EPA ratio in positive correlation to IL-6, and n-6 to vegetable n-3 PUFA (18:3n-3) in negative correlation to IL-12)

3.4 LIPID COMPOSITION OF SYNOVIAL FLUID

Because of the low amount of fatty acids in µg/g compared to total lipids expected to find in synovial fluid and a general interest of the lipid composition of the synovial fluid, an analysis of the lipid class composition in synovial fluid from four patients with inflammatory joint diseases was completed.

- Overall, approximately three quarter of the lipids in SF from four random sample specimens were mainly composed of neutral lipids. Among these, there was a high content of cholesteryl ester, which amounts to 39 % of total lipids (Appendix I).

IV. Discussion

This cross-sectional study of the fatty acid composition of SF from 86 patients with various inflammatory joint diseases was aimed to correlate specifically the n-6 and n-3 PUFA in SF to certain inflammatory markers in SF and serum (i.e IL-6 and IL-12 in SF, and CRP in serum), as well as to document the general fatty acid composition of SF in these patients. Additionally, the level of and correlation between the stated inflammatory markers were studied to evaluate inflammatory activity. The focus of the study was on all patients as a group, but also on significant differences between the four subgroups Rheumatoid Arthritis (RA), Psoriatic Arthritis (PsA), Reactive Arthritis (ReA) and Ankylosing Spondylitis (AS), as well as differences between the main diagnostic group spondyloarthropathies (SpA), (which include the latter three subgroups), and the RA group. There is a lack of studies regarding the fatty acid profile of SF from these patients, and probably no studies have elucidated the SF n-6 and n-3 PUFA, in relation to local (SF) and systemic (serum) inflammatory markers. Certain n-6 and n-3 PUFA are found to be immunomodulating in blood and tissue, but little attention has been brought on their direct inflammatory correlation in SF.

The discussion chapter is divided in four sections. It begins with a discussion of the materials (Section 4.1) and methods (Section 4.2), before the discussion of results (Section 4.3), and ends by a discussion of future remarks (4.4).

4.1 DISCUSSION OF THE MATERIALS

4.1.1 Patients

The patients were recruited 13-15 years before this study took place. In retrospect we have limited information about the clinical examination of these patients, besides the fact that patients had considerable symptoms related to the knee joint, and need of SF aspiration due to diagnostic or therapeutic reasons. No additional information about the disease history, including other illnesses and intake of supplements or lifestyle habits, such as diet, physical activity and smoking are registered.

The patient subgroups varied in size from number of patients being 45 in the RA group, 19 in the PsA group, 13 in the AS group to nine patients in the ReA group. The results from the SpA group are thus most influenced by the PsA group, which constitutes nearly half of the

patients. Ideally, the groups should have been equally distributed, but the study included all available material within each defined diagnose. Originally there where 46 RA SF samples, but one was excluded due to quantitative shortage of sample material. From patients with inflammatory joint diseases, there were also SF from two patients with crystal arthritis and one patients with inflammatory bowel disease-associated arthritis, but they were excluded due to their sample sizes.

There was no control group included in the study, i.e SF from healthy individuals or other patients, to compare with the disease groups. It is not ethically acceptable to drain SF from healthy individuals. The procedure implies a risk for introducing bacteria into the joint and thus possibly inducing infection. Earlier studies carried out on both lipid composition and fatty acid composition in SF from rheumatic patients generally lack a control group. However, osteoarthritis (OA) is sometimes used in comparison with inflammatory RA (Prete *et al.*, 1995; Navarro *et al.*, 2000; Kim & Cohen, 1966). Even though both OA and inflammatory joint diseases share in common the feature of producing damage and even destruction of the joint, the principal mechanism of OA is different due to being related to disruption of the cartilage itself that further may cause limited signs of inflammation, and thus lack systemic manifestions of inflammation (Mary B. Goldring, 2007). Yet, there were not sufficient sample materials available from this group that only included four patients.

4.1.2 The effect of medical treatment

The RA disease group is considered as a more disabling joint disease (Haga, 2002; Woolf & Pfleger, 2003). Patients with high disease activity, which also may reflect elevated levels of inflammatory markers, are often medically treated (Hansen, 2003; Punzi *et al.*, 2002). Medication, mainly DMARDs and glucocorticosteroids, but also NSAIDs, may reduce inflammatory markers, such as CRP and IL-6 (Punzi *et al.*, 2002). Thus, the medically treated patients, primarily those on DMARDs and glucocorticosteroids, may reflect patients with increased levels of inflammatory markers as a starting point, and in this study include mostly the RA patients. Even though inflammatory markers generally reflect inflammatory/disease activity, the initial high levels (baseline levels before medication) of inflammatory markers may be reduced in medicated patients. As a consequence, the correlations between inflammatory markers, or between fatty acids in SF and inflammatory markers, may also be impaired/ disturbed in medically treated patients. The treatment bias is unavoidable in studies of inflammation, because of ethical reasons that one may not leave patients involved in the

study “untreated”. However, in the early nineties it was probably less ongoing medical treatments of patients than today (Table 2.2.2), which is advantageous in analysis of levels of inflammatory markers.

Since there are different frequency of use of DMARD and glucocorticosteroids between groups of patients (Table 2.2.1), i.e high frequency in RA and low frequency in SpA group, including no frequency in the ReA group, little frequency in the AS group and below half on medical use in the PsA group, medication may influence the statistical results. This includes levels of inflammatory markers and correlations primarily of the highly medicated RA group. However, the impact of medical treatment, both in all patients and comparisons between groups, in relation to analytical values and correlations will not be further discussed throughout this thesis as it is beyond the scope of the work.

4.1.3 SF samples

4.1.3.1 Anticoagulants and centrifugation

SF from patients with inflammatory joint diseases has a tendency to clot, and transfer of samples to tubes containing an anticoagulant, more specifically lithium heparin, and centrifugation is recommended. An advantage of centrifugation before freezing may be that it makes the material easier to handle during analysis by avoiding clotting factors (Freemont & Denton, 1991). Notes from the specific project and common procedures during this time indicate strongly that an anticoagulant was added to our SF samples, and that there is a high possibility that the samples were centrifuged before stored in freeze. However, in retrospect there exist some uncertainties if the anticoagulant added was EDTA or heparine.

Since there is a small possibility that specifically the centrifugation was not completed, the effect this may have upon analyses will be discussed. In several other studies, the SF samples are centrifuged before freezing. In order to test the effect of centrifugation, Aman et al (1999) compared the content of specific collagen propeptides in five samples of SF in patients with inflammatory joint diseases before and after centrifugation, and found a slight decrease of propeptides. Four of five centrifuged SF samples had between 0.2 -1.4 % less propeptide content than native SF samples. Further, Bole et al (1962) analysed the lipid content of both the cell poor supernatant and the solid cell pellet of SF after centrifugation, and found that the

cell pellet accounted for 10 % of the value of total lipids and lipid classes in the cell-poor fraction of SF.

4.1.3.3 Effect of storage conditions and stability of cytokines and fatty acids

Some aliquots of the SF samples have been stored in the freezer at -70 ºC between nine to twelve years before cytokine analyses, and other aliquots additionally three years at -70 ºC before transferred to NIFES and kept in freezer at -80 ºC in maximum five months before fatty acid analysis. No antioxidants have been added to the SF samples before storage. SF is a transudate of plasma and there is some literature regarding blood samples and stability and effects of both cytokines and fatty acids after freezing and storage time.

Cytokines: Studies on freeze and thaw cycles and different storage temperature of both plasma and serum find cytokines relatively stable, including TNF-α, IL-6, IL-α, IFN- α and IL-β,. However, TNF-α is the least stable of the cytokines, and in one case IL-6 is also suggested as a less stable cytokine (Flower *et al.*, 2000; Thavasu *et al.*, 1992; Aziz *et al.*, 1999). Plasma or serum levels of TNF-α are both lowered at room temperature in comparison to 4 ºC and -70 ºC frozen material after 20 days (Aziz *et al.*, 1999). On the contrary, Flower et al (2000) found an increase of TNF-α levels after three freeze and thaw cycles with samples stored at -70 ºC (left one hour in room temperature between cycles). Generally, to avoid breakdown of certain cytokines, prompt processing of serum or specific EDTA-plasma stored at -70 ºC has been recommended (Flower *et al.*, 2000; Aziz *et al.*, 1999; Thavasu *et al.*, 1992). Kenis et al (2002) carried out an accelerated stability testing protocol, which included time-delay, sample processing, clotting temperature and freeze and thaw cycles on serum IL-6 and IL-10 from post-surgical patients before enzyme immunoassay, and estimated that IL-6 and IL-10 can be stored at -20 ºC and -70 ºC respectively and be stable for decades.
The different studies carried out on plasma and serum indicate that storage of SF at -70 ºC without freeze and thaw cycles before ELISA analysis generally should not influence the relative IL-6 and IL-12 cytokine stability. However, the SF cytokine stability is dependent on type of cytokine, and may be improved if the materials have been centrifuged before freezing (Flower *et al.*, 2000; Freemont & Denton, 1991).

Fatty acids: Moilanen and Nikkari (1981) showed that the fatty acid composition of plasma measured shortly after collection and after one year storage without antioxidant at -60 ºC is approximately the same. The small changes that occurred were mostly believed to be minor

modification of the GLC methodology during the year, and few were believed to be due to lipid peroxidation (Moilanen & Nikkari, 1981). Further, Hodson et al (2002) found similar fatty acids of TAG in plasma to be stable at - 80 ºC, first analysed 1-1.5 years after collection, then the same samples were frozen and thawed for a repeated analysis 2-2.5 years later. Given these observations, it seems that fatty acids of plasma may be stable for at least four years at such low temperatures, even with freeze and thaw cycles. Stability studies on SF fatty acid profile are lacking, and even though our samples have only been exposed to two freeze thaw procedures, it should be emphasised that our SF samples have been stored for 12-15 years. Thus, we can not exclude if SF samples have been degraded or not, but other sample materials are often stored for decades in biobanks.

Further, studies found total amount of fatty acids (mg/L) to decrease significantly in red blood cells (RBC) at higher temperatures (-20 ºC) after nine weeks. After 17 weeks, the percentage of all fatty acids were significantly changed, including a decreasion of all PUFA and a increasion of all SFA at baseline. Both hemolysis and lipid peroxidation may cause the lowered content of fatty acids. On the other hand, an addition of an antioxidant prevented the significantly changes of total fatty acid amount (mg/L) and fatty acid percentage after 17 weeks at -20ºC (Magnusardottir & Skuladottir, 2006).

Concerning the the inflamed SF, lipid peroxidation is found to be increased (Basu *et al.*, 2001). Overall these studies indicate that the fatty acid composition may be relatively stable in samples after storage at -70 ºC, but addition of antioxidant should be considered even for such low levels as -70 ºC, particularly due to increased lipid peroxidation in SF (Moilanen & Nikkari, 1981; Hodson *et al.*, 2002; Magnusardottir & Skuladottir, 2006; Basu *et al.*, 2001). Still, our SF samples have been stored under the same conditions and if possible changes have occurred it would concern all samples.

4.2.3 Blood samples

CRP in serum is measured instantly, and may be a more reliable marker than the long-term stored cytokines. However, it is an indirect measurement of the inflammation of the SF as it is a systemic inflammatory marker of joint inflammation/disease, which appears in the acute phase response. Though, from earlier studies it appears to exist a good correlation between

disease activity and levels of CRP in the inflammatory joint diseases, and the turbidometric assays are widely used in clinical practice (van Leeuwen & van Rijswijk, 1994).

4.2 DISCUSSION OF THE METHODS

4.2.1 ELISA

Several studies have shown that different methodologies, as well as biological and pre-analytical factors, can contribute to differences in determination of cytokines (Aziz *et al.*, 1999; Kenis *et al.*, 2002). However, ELISA has since the 1980s been an exceedingly used method for analytical and clinical investigations of innumerable analytes in patient samples, which include cytokines and SF (Lequin, 2005; Kenis *et al.*, 2002). Three to four years before this study was initiated, the cytokine analyses were performed according to guidelines of the kit manufacturer by recognised analysts at HUH. Since the notes does not inform about any experimental errors we assume the two specific analyses were successfully completed. Generally, during ex vivo ELISA processing, including sample collection and storage, the cytokines are exposed to degradation and induction of cytokines may also occur (Kenis *et al.*, 2002). A disadvantage of the indirect measurement is that cross-reactivity may occur with the secondary antibody, resulting in nonspecific signal.

4.2.2 Fatty acid analysis of synovial fluid

Due to problems during the experimental procedure, including instrumental errors and addition of internal standard in proper concentration to the fatty content in different samples, all the SF samples went through two freeze and thaw cycles before analysis. To solve the instrumental problems, modification on the GLC instrument was also done between analyses, which resulted in some samples being analysed under constant flow and some samples being analysed under constant pressure (Table 2.4.1)

It has arisen some difficulties during one-step analysis when compared to two-step analysis, more specifically low values of 16:0, and high values of 20:5n-3 fatty acids for KM on the control chart. The risk for sample loss, lipid peroxidation, and contamination are reduced during procedure, thus increased recovery of LCPUFA resulting from higher levels of 20:5n-3 and lower levels of 16:0 fatty acids are expected on control chart. On the other hand, the measurement uncertainty of NIFES one-step method is somewhat higher for KM at control chart than traditional method.

The one-step method was used for analysis since it may quantify fatty acids in limited amount of sample of low fatty content, such as in the case of SF samples. Synovial fluid is considered to have extremely low amount of lipids, which makes it even more difficult to quantify. On the contrary, the NIFES one-step method has also been suggested as less efficient in dissolving human matrices, as there were observed increased variation between parallels of RBC. Thus, homogenization during the boiling step is specially important for these matrices. One challenge was proper homogenization of SF samples before weight, as the matrice viscosity may differ between patient samples and may be difficult to handle. Eventhough, the homogenization process was highly emphasised during the procedure, the separation phase of SF samples was less clear in comparison to in KM samples.

Parallels were used to monitor homogenization of the material and quality of the method etc. During the time of analysis there were not specifically set accepted differences between parallels. At present the same accepted differences between parallels as the traditional method are used for the one-step method. This is only temporarily, since also the parallels of different material are commonly seen to have increased difference in the one-step method. I did not follow the same acceptance rules for difference between parallels, as the one-step method is not accredited. On the other hand, a Student's t-test was used to compare total quantified fatty acid composition in percentage and mg/g, as well as calculation of percentage difference between saturated, monounsaturated and polyunsaturated fatty acids (<10%) was done before accepting the combined parallels from fatty acid analysis. I might have controlled the accordance between parallels more strictly according to the traditional method, which including percentage difference between each fatty acid of parallels from all 86 SF samples. However, both differences between microgram per gram and percentage for each fatty acid were also overviewed by myself and other analysts at the laboratory to avoid inclusion of parallels that differentiated within the SFA, MUFA and PUFA. Overall, there were no significant differences between total quantified fatty acids in % and µg/g according to Student t-test. In a few cases, parallels may differ more than 10% between sum of SFA, MUFA and PUFA, but due to earlier analyses with similar results (first freeze and thaw cycle), the results were kept.

4.3 DISCUSSION OF RESULTS

The discussion of results is presented in same order as the aims of the study and starts off with the discussion of the main aim. The results of the correlation of n-3 and n-6 PUFA to inflammatory markers only includes absolute values, i.e PUFA in µg/g, and thus only PUFA in µg/g will be discussed (Section 4.3.1).. This is to avoid the inclusion of compensatory changes as a percentage of one n-6 fatty acid may be higher even though the amount of this fatty acid do not differ from SF of another patient, given amount of another fatty acid are lower. Further, the discussion of the results on inflammatory markers will be presented (Section 4.3.2), before the final discussion of the fatty acid composition (Section 4.3.3). For the same reason as mentioned above (to avoid inclusion of compensatory changes) the discussion of the fatty acid composition results will emphasise on absolute values. However, percentage fatty acid composition in SF in patients, mainly in the RA group, will also be discussed to relate our results to existing literature. Furthermore, multiple statistical comparisons of the results have been completed, which is unavoidable in relation to the fatty acid profile. Thus, the results should be cautiously interpreted to avoid highlighting significant differences that might have arisen by chance due to multiple statistical analyses.

4.3.1 Correlation between n-6 and n-3 PUFA in SF and inflammatory markers

ReA was the only patient group that did not have any correlation between their n-6 and n-3 PUFA and inflammatory parameters. Also, ReA was the only acute inflammatory joint disease group included, and patients normally recover from the disease in 6 months. Concerning the three other patient groups, RA, PsA and AS, all are recognized as chronic inflammatory joint diseases involving long-term overproduction of inflammatory markers. In these chronic conditions there were found significant correlations of levels of several n-6 and n-3 PUFA, but also in cases between levels of n-6 to n-3 ratios, to inflammatory markers. The significant correlations of n-6 and n-3 PUFA were mostly positively associated to IL-6 in SF, involving all chronic disease groups, but were observed less frequently in SF from the RA group.

The bulk of the significant correlations did not concern the immunomodulating PUFA; AA, EPA and DHA, and in cases involved opposite significant correlations than expected. Rather, the significant correlations concerned LA and DGLA positively associated to IL-6, further contributing to positive correlations between total sum of n-6 and IL-6 in the groups.

Like EPA, DGLA have been associated with anti-inflammatory effects through its eicosanoid products (Navarro *et al.*, 2000; Zurier, 1998). Yet, in this cross-sectional study, DGLA was the only fatty acid which correlated positively to pro-inflammatory IL-6 in SF for all patient groups (except for ReA) meaning that high levels of DGLA are associated with high levels of the pro-inflammatory IL-6 suggesting otherwise for this particular fatty acid in local inflamed synovial tissue. However, high levels of DGLA are found in plasma of RA compared to plasma of healthy individuals (Navarro *et al.*, 2000), and elevated levels have also been seen in colorectal mucosa tissues of diseased cancer patients compared with paired normal mucosa (Fernandez-Banares *et al.*, 1996). Navarro et al (2000) and other studies have suggested that high levels of DGLA in RA plasma will attenuate inflammation, through their less inflammatory eicosanoids, and that the conversion step from DGLA to AA might be more more slowly in these patient. Thus, preservation of DGLA may serve as a protective mechanism (Zurier, 1998; Chilton *et al.*, 2008). On the other hand Fernandez-Banares et al (Fernandez-Banares *et al.*, 1996) speculated about the DGLA present in colorectal mucosa tissue and its participation in carcinogenesis.

As with high levels of LA, also high levels of DGLA might be expected in association to inflammatory markers, since there generally are expected a conversion of these fatty acids to AA (Lin & Salem, 2005; Chilton *et al.*, 2008) However, levels of AA were commonly not correlated to inflammatory markers (except to IL-6 in the PsA group). From literature concerning blood and immune cells, high levels of AA may be linked to increased inflammation, and thus increased disease activity (Harbige, 2003). It has also been speculated that in tissues of active inflammation there might be a consumptation of AA due to increased synthesis of prostaglandins and thus levels might be reduced (Esteve-Comas *et al.*, 1992; Arslan *et al.*, 2007). In this way it might be difficult to determine the relation between the AA to IL-6 in tissues without any further data on prostaglandin levels.

Concerning correlation og n-3 PUFA to inflammatory markers there were found positive correlations both between the essential PUFA ALA (18:3n-3) and IL-6 in several groups, and one positive correlation of DHA (22:6n-3) to IL-6 in all patients, meaning high levels of these fatty acids in SF also represent higher levels of pro-inflammatory IL-6. The correlation of DHA to IL-6 in all patients was unexpected, but it is difficult to make any assumptions as it is only seen in one incidence of the lowest magnitude, and thus might be statistically significant by chance. However, it should not be ignored, as other expected correlations are

trusted. As for ALA, levels are reported to be decreased in inflamed SF of RA patients (Navarro *et al.*, 2000). In contrast to our findings related to IL-6, higher levels of ALA are normally associated to anti-inflammatory activity in blood through conversion to EPA and DHA, though this pathway appears to be limited (Williams & Burdge, 2006).

All n-6 fatty acids which were correlated to IL-12, were also found to correlate significantly to the cytokine, either in the RA group or the AS group, suggesting a relationship between the n-6 PUFA and IL-12. Unexpectedly, over half of these correlations concern a negative correlation to the RA group. In the RA group DGLA correlated negatively to IL-12, whereas it correlated positive to the more studied and accepted inflammatory marker IL-6. In Section 3.2.1 the possible antagonistic effect between IL-12 and IL-6 will be discussed, suggesting IL-12 to possess late anti-inflammatory effect. Thus, high levels of n-6 PUFA and low IL-12 levels do not necessarily represent less inflammation in the RA group. On the contrary, these two cytokines show similar positive properties in SF from the AS group. This two findings is supported by other studies which suggest IL-12 have both early pro-inflammatory and late anti-inflammatory effects in arthritis-like disease (Paunovic *et al.*, 2008). Overall, even though n-6 PUFA is related to the IL-12, which may further influence the Th1 pattern, the cytokine is somewhat controversial in the inflammatory process, and little can be concluded on disease activity from these associations.

There were few statistically significant correlations between n-6 to n-3 ratios and the inflammatory markers. Of 72 correlations, only four significant correlations were seen in either the AS group or the SpA main group. This might indicate statistical significance by chance however they were generally as expected according to theory. The correlations involved that increased n-6 to n-3 ratios represented either higher levels of the inflammatory markers CRP in serum or IL-6 in SF, or low levels of IL-12 in SF. These correlations to the two cytokines were probably influenced by levels of n-6 PUFA, LA or DGLA.

The correlation between absolute values of fatty acids and inflammatory markers may be significantly positive in cases of IL-6, because inflammation is accompanied by excessive levels of lipids (Bole, 1962), and further elevated levels of fatty acids in SF. Hence, there may be a higher level of lipid-derived fatty acids, no matter type of fatty acids (more commonly seen in ALA, DGLA and LA), which may rather represent excessive lipids not their potential immunomodulating effect, or the possible preservativion of n-3 PUFA and DGLA in SF to protect the tissue. If this should be the case, further speculations to why there are no

correlations between LCPUFA to inflammatory markers might be that excessive entering of these fatty acids are further consumed due to oxidative stress and prostaglandin synthesis in inflamed SF tissue, or that SF n-3 and n-6 PUFA simply does not reflect inflammatory activity. Correlation of percentage fatty acids to inflammation may be a better way to illustrate the relation between the two fatty acid families, and not the possible excessive fatty acids entering the synovial fluid. Then again, the result may be based on compensatory changes of percent values. Both these factors make it difficult to draw conclusions about the single fatty acids. However, the ratios of n-6 to n-3 fatty acids in micrograms per gram may represent better their relationship to inflammation in SF. This is because they may represent excessive fatty acids in both families as a response to inflammation, and avoids compensatory changes. Thus, if presumed that both n-3 and n-6 PUFA increases due to excessive lipids levels of inflamed SF, these results should be emphasised in the evaluation of inflammation in SF.

4.3.2 Inflammatory markers

4.3.2.1 Levels of inflammatory markers

Levels of inflammatory markers did not differ significantly between RA and AS in the same manner as the n-6 and n-3 fatty acids. Further, our results, together with what is described in the literature, indicate that IL-6 might be a more relevant marker for describing inflammatory activity in SF for all patients and groups in comparison to CRP and IL-12. Below, the results will be discussed together with findings from other studies.

CRP: Significantly higher levels of CRP in the RA (median 56 mg/L, IR 28-76) and AS groups (46 mg/L, IR 32-88) were found in comparison to PsA group (median 15 mg/L, IR5-28). These findings are in accordance with the literature where CRP levels are described as high in RA and AS, respectively with mean levels of CRP of 69.2±36.4 mg/L and 50 mm/L previously reported in some patient populations (van Leeuwen & van Rijswijk, 1994; Kumar & Clark, 2005). Further, Mease et al (Mease *et al.*, 2005) reported that CRP is not consistently elevated in PsA patients even with active inflammation, and thus may be a less reliable inflammatory marker in serum of these patients. From our CRP measurements, as much as seven of 16 patients with PsA, were within the reference area of healthy individuals (below 10mg/L). Also, studies find a generally lower disease activity of the PsA group in comparison to the RA group (Gladman *et al.*, 2005)

IL-6: IL-6 has been suggested as a better marker of indicating histopathology than CRP, including infiltration of inflammatory cells in synovial tissues (Matsumoto *et al.*, 2006). Additionally, studies have compared IL-6 levels in RA and PsA to less inflammatory OA. Significantly higher levels are found in both serum and SF in RA and SF of PsA patients (Hermann *et al.*, 1989; Matsumoto *et al.*, 2006), and it may be the most abundant cytokine in inflamed synovial tissues (Punzi *et al.*, 2002). We found similarly high IL-6 levels in SF from the subgroups and the main groups which indicate that IL-6 indicate inflammation for all groups, and median levels in all patients were at 9372 pg/mL (IR, 2746-27 916). Yet, Steiner et al (1999) found IL-6 levels in SF from RA and ReA patients at a much lower median measured to 4500 pg/mL, with a range of 400-80 000 pg/mL for almost all patients. IL-6 is considered to be relatively stable (Kenis *et al.*, 2002; Thavasu *et al.*, 1992; Flower *et al.*, 2000). The IL-6 levels seems to be widely distributed in both patient groups, and the range of our patient group was more narrow (0 – 54 626pg/mL) in comparison to patients studied by Steiner et al (1999). Still our results support high IL-6 levels with a broad range are present in inflamed SF of these inflammatory joint disease patients.

IL-12: Elevated levels of IL-12 have been seen in both serum and SF of RA patients (Petrovic-Rackov & Pejnovic, 2006; Ribbens *et al.*, 2000; Petrovic-Rackov, 2005). In a study carried out by Ribbens et al.(2000), levels of IL-12 in SF had a median of 269 pg/mL (interquartile range IR, 143-355) in RA patients, and 516 pg/mL (IR, 197-984) in ReA patients. IL-12 levels in our RA patients were measured at similar levels (median 296 pg/mL, IR 176-416), whereas ReA patients had a tendency of lower levels. We found one statistically significant difference between subgroups, namely lower levels in the PsA group (median 105 pg/mL, IR85-240), which together with ReA group contributed to significantly lower levels of IL-12 in the SpA group (median 149 pg/mL, IR 86-254), in comparison to the RA group. Yet, not much is known about the influence of high IL-12 levels and disease activity in SF of SpA, whereas SF IL-12 from RA patients has both shown good and poor correlation with disease activity. Further it may possess both proinflammatory and anti-inflammatory effects. (Petrovic-Rackov & Pejnovic, 2006; Petrovic-Rackov, 2005; Ribbens *et al.*, 2000). As a result it is difficult to make any associations about disease activity in these patients.

4.3.2.2 Correlation of inflammatory markers

There was found no correlation between the inflammatory markers within the subgroups of SpA.

SF IL-12 and IL-6: IL-12 and IL-6 are both produced by macrophages and reflect different pathological tissue events, IL-12 being of higher influence on the Th1-cell pattern and IL-6 being in direct relationship between systemic and local inflammation (Cronstein, 2007; Paunovic *et al.*, 2008). Even though high levels in SF of all patients, the levels do not necessarily reflect severity of the disease. In our result, there was observed a negative correlation between IL-12 and IL-6 in the RA group. There are high levels of both of these cytokines and they are generally recognised to possess pro-inflammatory effects. Still they have shown anti-inflammatory features. Whereas IL-6 may protect cartilage during acute disease, but promote excessive bone formation in chronic conditions of RA, IL-12 may rather act early pro-inflammatory, and late anti-inflammatory in arthritis-like diseases (Kinne et al., 2006; Paunovic et al., 2008). This might explain the negative correlation between these to cytokines in the SF from RA patients, where the more studied IL-6 probably reflects late pro-inflammatory effects, whereas IL-12 may possess anti-inflammatory features. Still, no positive correlation between SF IL-12 and IL-6 levels in AS group was found, although both cytokines were significantly elevated in correlation to AA precursors in these patients. Furthermore, IL-12 is probably more dependent upon other cytokines (Paunovic *et al.*, 2008).

SF IL-12 and serum CRP: There were found no correlations between CRP in serum and IL-12 in SF. This might be expected as they represent different pathways in inflammation, where CRP are primarily related to systemic inflammation and IL-12 may reflect local synovitis and development of Th1 pattern.

SF IL-6 and serum CRP: These two inflammatory markers are directly related to each other during acute phase response. High levels of IL-6 in SF are expected to leak out into serum and increase levels of CRP (Cronstein, 2007). In our study we found significantly positive correlations between CRP and IL-6 in SF in SpA group, also contributing to a significant correlation between these two inflammatory in all patients. This was not observed in the RA patient group, and might be due to a high frequency of medical treatment in RA patients in comparison to the SpA group.

4.3.3 Fatty acid composition in synovial fluid

4.3.3.1 Lipids and total fatty acids in all patients

Lipid class analysis of four random SF sample specimens in this study show that neutral lipid content varied between 74-79%, whereas phospholipids varied between 21-26 percent. The TAG contributed to 12% of total lipids in two patients, whereas for two other patients the percentage was as high as 18% and 21% (Appendix I). Similar percentage of lipid classes, including high total cholesterol levels, were also found in a study carried out by Bole et al (Bole, 1962) on SF from RA patients.

There were observed similar trends in the fatty acid composition both in percentage and microgram per gram in all patients. Generally, it was found an extremely low content of fatty acids in SF of all patients (1153 μg/g, IR 871-1421). This low amount of fatty acids may be seen in relation to the also extremely low content of lipids, as well as the high constitution of total cholesterol in SF. Bole et al (Bole, 1962) found extremely low content of SF lipids constituting highly of cholesterol and cholesterol ester (56.6%), and only 0.73 % of total cholesterol contributed to the ester fraction in SF. Still, there are expected to be a considerably lower amount of the different lipid classes (including the absence of TAG) in SF of healthy individuals (Bole, 1962), which also indicate a lower amount of fatty acids in healthy human SF fluid.

4.3.3.2 Rheumatoid Arthritis

To the best of our knowledge no other studies have quantified fatty acids as absolute values in SF. Still, there are a few studies which have quantified the percentage fatty acid profile in SF from RA pasients (Navarro *et al.*, 2000; Kim & Cohen, 1966; Sugiyama & Ono, 1966). In this study, percentage fatty acid composition of SF from RA patients will be compared to the percentage values from the study carried out by Navarro et al (2000), and the results of differences between groups will be discussed. It is easier to make a realistic comparison with the study carried out by Navarro et al (Navarro *et al.*, 2000), as the study is explained in detail, it quantifies single and not sums of fatty acids using a similar one-step method and median values.

The percentage fatty acid composition in RA patients determined in our study was somewhat similar to the analysis done by Navarro et al (2000). Still, there were several differences. In our study, there was a lower fraction of SFA, and specifically a lower percentage of 16:0, and a higher fraction of PUFA, including noticeably increased percentage of LA. Moreover, ALA, marine fatty acids and sum of n-3 PUFA had all duplicated percentage levels. Even though low percentage, DPA (22:5n-3) was also identified in our analysis. On the contrary there were less n-6 fatty acids included in our fatty acid analysis, including γ-linolenic acid (18:3n-6) and docosatetraenoic acid (22:4n-6) in comparison to the analysis carried out by Navarro et al (2000). The different fatty acid inclusion may also enhance differences in fatty acid composition between the two studies due to compensatory percentage changes. This may also give some explanation of the difference in ratio between the studies. In our study the median of n-6 to n-3 ratio was at 4.9 (IR 3.6-6.9), which is much lower than Navarro et al (2000), estimated roughly to be 9. Generally, the lower ratio in our patients may be influated by having less n-6 PUFA and more n-3 fatty acid standards than Navarro et al (2000).

Another perspective of the differences between the groups may be biological, such as disease activity and dietary factors, and pre-analytical methodologies. Both fatty acid analyses were performed by similar one-step procedure, but storage conditions was dissimilar. Whereas our fatty acids were long-term stored at principally -70ºC, Navarro et al (2000) stored fatty acids at -20ºC for unknown period of time, after synovial fluid aspiration to heparinized tubes and centrifugation. Whereas the two latter factors was probably completed in our study, and may increase the homogenisation of the material, a storage without antioxidant at -20 ºC has shown a decrease of PUFA in red blood cell samples from baseline after 17 weeks, and the fatty acids have been shown to be unstable in plasma samples for periods of 12 months and 3 years (Magnusardottir & Skuladottir, 2006; Hodson *et al.*, 2002). Thus, this may indicate a possible ongoing oxidation of (in the case of the RBC study, hemolysis may also be an important influence on) PUFA and n-3 PUFA in SF contributing to lower levels in the fatty acid profile, which was found in RA SF by Navarro et al (2000)

4.3.3.3 Group differences

There was only found statistically significant differences between the subgroups RA and the AS. Also similar tendency of ReA and PsA groups towards AS group contribute to a significant difference between SpA and RA groups. AS and SpA groups are significantly different from RA in fatty acid composition, which all involve the n-6 and n-3 fatty acids. There are significantly higher ratios of n-6 to n-3 fatty acids in µg/g in AS and SpA groups, including total n-6 to n-3 ratio, n-6 to vegetable n-3, and n-6 to marine n-3 PUFA. There are also more differences found in percentage fatty acids between the groups. Increased levels of percentage DHA in SF from the RA group may influence the significantly higher marine n-3 PUFA and sums of n-3 PUFA, which may additionally influence the significantly lowered ratios between n-6 to n-3 PUFA in this group compared with AS and SpA groups. The RA group also had significantly higher percentage values of other marine n-3 PUFA (i.e EPA and DPA), as well as lower levels of both LA and AA, which may contribute to a significantly lower n-6 to n-3 PUFA ratios in both percentage and µg/g. These results indicate that RA patients generally have a higher content of n-3 PUFA in their fatty acid composition and lower n-6 to n-3 ratios compared to AS and more frequently the SpA.

Disease activity and histopathology in synovial tissue from RA patients have been shown to be aggressive, and level of inflammatory activity has been shown to influence the local changes, including permeability and angiogenesis of the synovium. This further increases the lipid and fatty acid levels entering the synovial fluid, as well as increases lipid peroxidation (Basu *et al.*, 2001; Bole, 1962; Navarro *et al.*, 2000; Ropes & Bauer, 1953).

There are uncertainties if fatty acids in SF may reflect diet. Still, an excessive increase of lipids and fatty acids are mainly believed to arise from plasma (Prete *et al.*, 1995; Bole, 1962). There is also an increase of immune cells in SF from RA patients. The levels are observed to increase fivefold, but the increased cell content may be even more extreme (Freemont & Denton, 1991). Further, the fatty acids in the membrane of immune cells have also been shown to reflect dietary changes (Calder, 2007). Even though the synovial fluid in these patients was obtained in the early nineties and n-3 supplementation was more controversial/less establish, the RA patients might have been more familiar to such supplementations, and excessive lipids and fatty acids derived from blood may introduce higher levels of n-3 PUFA. A possible preservation of n-3 PUFA in SF to protect tissue with high inflammatory activity, might also explain higher n-3 PUFA in SF from RA patients in comparison to less inflammatory active(painful) AS.

Further, even less is known about the lipid and fatty acid pattern of AS and SpA patients. One may speculate that these patients have different local inflammation patterns in SF, which cause differences in n-3 and n-6 PUFA composition. SpA patients, and specifically AS patients, had low frequency of medical treatment in comparison to RA patients, which may suggest that these patients have less local inflammation.

4.3.4 Dietary fatty acids and synovial fluid

There have been few intervention studies regarding the influence of n-6 and n-3 PUFA on serum levels of pro-inflammatory cytokines in RA patients, and probably no present studies of the SF n-6 and n-3 fatty acid relation to cytokine levels in serum or SF (Sijben & Calder, 2007; Stamp *et al.*, 2005). Fish oil studies have demonstrated increased composition of EPA and DHA in blood, and enhanced clinical outcomes in patients with rheumatoid arthritis (Henderson & Panush, 1999; Cleland *et al.*, 2003; Goldberg & Katz, 2007). Little is known about these fatty acids with respect to effects in the actual site of inflammation and joint destruction. In our cross-sectional study, there were found significant correlations of n-6 and n-3 PUFA levels, and ratios between them, to levels of inflammatory markers in SF in chronic inflammatory joint diseases (i.e RA, PsA and AS). Disregarded the possible correlation due to excessive lipid entering during inflammation, this may indicate that these n-6 and n-3 PUFA interfere with the chronic inflammatory activity in SF in these patients. Thus, an alteration of dietary fatty acids introduced to blood might influence fatty acid composition and inflammation of SF.

4.4 FUTURE REMARKS

The cross-sectional study described in this thesis indicates that there is an association between an increased content of specifically n-6 AA precursor fatty acids and inflammation through the IL-6 levels in SF, but little is associated between the SF fatty acids and serum CRP. As for the role of IL-12, these n-6 PUFA also show associations to this cytokine. Still, IL-12 might not indicate overall inflammation as studies have shown alternate effect in inflamed SF. Thus, further studies should be performed on this cytokine in SF before using it as an inflammatory marker.

In future studies concerning fatty acids in relation to inflammation in SF, it should be considered to use percentage values for singular fatty acids (even though risk for correlation of compensatory changes) due to excessive lipids and fatty acids entering the fluid. Thus, in this study it was difficult to make any assumptation for each of the specific n-6 and n-3 PUFA.

Little is known about fatty acids with respect to effects in the actual site of inflammation and joint destruction. Later studies should focus on recruiting SF from patients in need of joint tap, and use animal models to study the actual effect of the n-6 and n-3 fatty acids on local synovial tissue. Basically, the synovium have marked greater permeability than other tissues, and an inflammation of the synovial tissue additionally increases the permeability of the synovium, and probably makes the tissue even more exposed to destruction. Even though studies implicate that the excessive lipids and fatty acids originates from the blood, more data is needed in this area about the specific effect of these substances upon the tissue.

Intervention studies to study the effect of n-3 supplementation on SF are expensive and recruiting control groups is ethically difficult as synovial fluid aspirations should be generally avoided if it is not necessary, since it might result in an infection. However, such studies would provide more certain data on n-6 and n-3 PUFA changes in SF in relation to inflammation. Furthermore, a more controlled study is needed, that provide clinical and dietary history, and blood and synovial fluid samples in the same manner as Navarro et al (2000). Inclusion of cell counting, and analysis of disease marker like cytokines, prostaglandins and oxidative stress should also be accomplished. This is important to provide a possible association between n-6 and n-3 PUFA and inflammation. More established and analytically stable pro-inflammatory markers, such as IL-1β in synovial fluid should also be chosen to provide inflammatory data. However, even though medication may disturb the results, stopping medication is ethically unavoidable.

Additionally, it would be interesting to study fatty acids in inflamed synovial tissue of all groups within the inflammatory joint diseases, specifically the chronic conditions, since little is known about these disease groups and there were found correlations of n-6 and n-3 PUFA on inflammation. The apparent difference in n-6 and n-3 PUFA composition of RA patients and AS patients seen in this study is of particular interest.

Today, it is a great focus on n-3 supplementation and its possible anti-inflammatory effect in various diseases. It is important to have in mind that rheumatic diseases, such as RA, PsA, ReA and AS, are mostely resulted in genetically susceptible individuals. However, the possibility that several enviromental factors, including diet, namely prolonged n-3 supplementation might hold a preventive effect, and reduce inflammatory mediators in synovial tissue and improve clinical outcome must not be ignored. Specially, since the medical treatments may give side effects, and is highly expensive.

Conclusion

N-6 and n-3 PUFA in relation to inflammation: We found significant correlations of n-6 and n-3 fatty acids to inflammatory markers in the chronic inflammatory diseases; RA, PsA and AS, whereas no correlations were found in the acute inflammatory joint disease, ReA, suggesting that these fatty acids may influence on chronic inflammation.

The significant correlations of single and sum of PUFA was of low or moderate magnitude. However, the correlation generally did not include the immunomodulatory AA, EPA and DHA. Overall, there was a higher frequency of positive correlations of n-6 AA precursor PUFA and IL-6 (13 correlations), which indicate that there are a stronger association between high levels of n-6 fatty acids and the inflammatory marker IL-6. There was also found unexpected positive significant correlations of ALA to IL-6 in several groups, which is a n-3 PUFA related to less inflammatory features.

Significant correlations between the n-6 PUFA and SF IL-12 were found less frequently in comparison to SF IL-6. However, the n-6 PUFA correlated negatively to IL-12 in the RA group, including DGLA that also correlated positive to IL-6. On the other hand, there was found positive correlations of n-6 PUFA and IL-12 in the same manner as the correlations to IL-6 in the AS group. In evaluation of n-6 PUFA and inflammation in SF, these findings support that IL-12 might possess antagonistic effects, being both anti-inflammatory and proinflammatory, whereas n-6 PUFA in correlation to IL-6 give more reliable indications about inflammation in SF.

Between ratios of n-6 to n-3 PUFA and inflammatory markers, only four of totally 72 correlations were found to be significant. The significant correlations only involved the SpA and AS group, three were positive correlations to either CRP or IL-6, and one was negative correlation to IL-12. Even though few correlations, they all points to high levels of n-6 to n-3 ratios represent high levels of inflammation.

Inflammatory disease markers: There are much higher levels of IL-6 than IL-12 in SF in all groups, which support its abundance in inflamed SF and its importance in inflammatory activity of SF. It may be a better marker to describe inflammatory activity in local synovial tissue inflammation than serum CRP and SF IL-12 for all groups. There were no significant

differences, concerning the levels of IL-6 in SF between groups, and the high levels comes with a broad range. As for serum CRP, there were found significantly higher levels of serum CRP in RA patients and AS versus the PsA group. This is coexistent with literature, which find levels of CRP elevated in both RA and AS, whereas PsA patients have lower levels of serum CRP due to both lower disease activity and CRP being a less reliable marker of active inflammation in this group. There was also a significantly higher level of IL-12 in SF of the RA group compared with PsA group and the main group SpA.

No correlation between IL-12 and CRP were found, which might be more expected as they represents different inflammatory pathways. Regarding correlations of IL-6 and CRP, a significant weakly positive correlation between these inflammatory markers was found, in all patients and the SpA, which support the upregulation of serum CRP as a result of high levels of SF IL-6. Between the two SF cytokines, levels of IL-12 correlated negatively to levels of IL-6 in SF from RA patient groups, which may enhance the theory about its possession of chronic late-anti-inflammatory effects. Thus, significantly higher levels of IL-12 found in RA patients as compared with PsA group and the SpA, might represent higher anti-inflammatory activity, but little can be concluded on levels of disease activity from these associations.

Fatty acid composition: The fatty acid composition was somewhat similar of that reported in the literature (related to RA). However, the SF material and the extreme low amount of lipids, which consist highly of cholesterol makes it difficult to quantify the fatty acids. Significantly differences were only found between the RA and AS subgroup or more frequently SpA main group. All statistically significant differences were seen between the n-6 and n-3 PUFA. The RA group had generally higher content of n-3 fatty acids contributing to a lower n-6 to n-3 ratio. RA patients might have been more familiar to n-3 supplementations, and excessive lipids and fatty acids derived from blood may introduce higher levels of n-3 fatty acids, as well as different local SF inflammation, are suggestions as reasons for these group differences.

References

Adam, O. (2003) Dietary fatty acids and immune reactions in synovial tissue. *Eur J Med Res,* **8,** 381-387.

Afonso, V., Champy, R., Mitrovic, D., Collin, P. & Lomri, A. (2007) Reactive oxygen species and superoxide dismutases: role in joint diseases. *Joint Bone Spine,* **74,** 324-329.

Aman, S., Risteli, J., Luukkainen, R., Risteli, L., Kauppi, M., Nieminen, P. & Hakala, M. (1999) The value of synovial fluid analysis in the assessment of knee joint destruction in arthritis in a three year follow up study. *Ann Rheum Dis,* **58,** 559-562.

Arend, W.P. & Gabay, C. (2006) Cytokine networks. In *Rheumatoid Arthritis* (Firestein, G.S.*, et al.* Oxford University Press, Oxford.

Arnett, F.C., Edworthy, S.W., Bloch, D.A., McShane, D.J. & Fries, J.F. (1988) The American rheumatism association 1987 revised criteria for the classification of rheumattoid arthritis. *Arthritis and Rheumatism,* **31,** 315-324.

Arslan, G., Erichsen, K., Milde, A.M., Helgeland, L., Bjørkkjær, T., Frøyland, L. & Berstad, A. (2007) No protecction against DSS-induced Colitis by short-term pretreatment with seal or fish oils in rats. *Integrative Medisine Insighths,* **2,** 25-34.

Aziz, N., Nishanian, P., Mitsuyasu, R., Detels, R. & Fahey, J.L. (1999) Variables that affect assays for plasma cytokines and soluble activation markers. *Clin Diagn Lab Immunol,* **6,** 89-95.

Basu, S., Whiteman, M., Mattey, D.L. & Halliwell, B. (2001) Raised levels of F(2)-isoprostanes and prostaglandin F(2alpha) in different rheumatic diseases. *Ann Rheum Dis,* **60,** 627-631.

Blewis, M.E., Nugent-Derfus, G.E., Schmidt, T.A., Schumacher, B.L. & Sah, R.L. (2007) A model of synovial fluid lubricant composition in normal and injured joints. *Eur Cell Mater,* **13,** 26-39.

Bole, G.G. (1962) Synovial fluid lipids in normal individuals and patients with rheumatoid arthritis. *Arthritis Rheum,* **5,** 589-601.

Breban, M., Miceli-Richard, C., Zinovieva, E., Monnet, D. & Said-Nahal, R. (2006) The genetics of spondyloarthropathies. *Joint Bone Spine,* **73,** 355-362.

Calder, P.C. (2003) N-3 polyunsaturated fatty acids and inflammation: from molecular biology to the clinic. *Lipids,* **38,** 343-352.

Calder, P.C. (2007) Immunomodulation by omega-3 fatty acids. *Prostaglandins Leukot Essent Fatty Acids,* **77,** 327-335.

Calvo-Alén, J. & Alarcón, G.S. (2006) Epidemiology and determinants of susceptibility. In *Rheumatoid Arthritis* (Firestein, G.S.*, et al.* Oxford University Press, New York.

Chilton, F.H., Rudel, L.L., Parks, J.S., Arm, J.P. & Seeds, M.C. (2008) Mechanisms by which botanical lipids affect inflammatory disorders. *Am J Clin Nutr,* **87,** 498S-503.

Christie, W.W. (1989) Gas chromatography: theoretical aspects and instrumentation. Preparation of Methyl ester and other Derivatives. In *Gas chromatography and Lipids: A practical guide* (Christie, W.W. eds), s. 24-47. Oil Press Ltd, Bridgewater.

Cleland, L.G., James, M.J. & Proudman, S.M. (2003) Omega-6/omega-3 fatty acids and arthritis. *World Rev Nutr Diet,* **92,** 152-168.

Cronstein, B.N. (2007) Interleukin-6--a key mediator of systemic and local symptoms in rheumatoid arthritis. *Bull NYU Hosp Jt Dis,* **65 Suppl 1,** S11-15.

Davis, B.C. & Kris-Etherton, P.M. (2003) Achieving optimal essential fatty acid status in vegetarians: current knowledge and practical implications. *Am J Clin Nutr,* **78,** 640S-646S.

Dayer, E., Dayer, J.M. & Roux-Lombard, P. (2007) Primer: the practical use of biological markers of rheumatic and systemic inflammatory diseases. *Nat Clin Pract Rheumatol,* **3,** 512-520.

De Caterina, R. & Massaro, M. (2005) Omega-3 fatty acids and the regulation of expression of endothelial pro-atherogenic and pro-inflammatory genes. *J Membr Biol,* **206,** 103-116.

De Lorgeril, M. (2007) Essential polyunsaturated fatty acids, inflammation, atherosclerosis and cardiovascular diseases. *Subcell Biochem,* **42,** 283-297.

Emery, P. & Luqmani, R. (1993) The validity of surrogate markers in rheumatic disease. *Br J Rheumatol,* **32 Suppl 3,** 3-8.

Esteve-Comas, M., Ramirez, M., Fernandez-Banares, F., Abad-Lacruz, A., Gil, A., Cabre, E., Gonzalez-Huix, F., Moreno, J., Humbert, P., Guilera, M. & et al. (1992) Plasma polyunsaturated fatty acid pattern in active inflammatory bowel disease. *Gut,* **33,** 1365-1369.

Fam, H., Bryant, J.T. & Kontopoulou, M. (2007) Rheological properties of synovial fluids. *Biorheology,* **44,** 59-74.

Favre, N., Bordmann, G. & Rudin, W. (1997) Comparison of cytokine measurements using ELISA, ELISPOT and semi-quantitative RT-PCR. *Journal of Immunological Methods,* **204,** 57-66.

Fernandez-Banares, F., Esteve, M., Navarro, E., Cabre, E., Boix, J., Abad-Lacruz, A., Klaassen, J., Planas, R., Humbert, P., Pastor, C. & Gassull, M.A. (1996) Changes of the mucosal n3 and n6 fatty acid status occur early in the colorectal adenoma-carcinoma sequence. *Gut,* **38,** 254-259.

Flower, L., Ahuja, R.H., Humphries, S.E. & Mohamed-Ali, V. (2000) EFFECTS OF SAMPLE HANDLING ON THE STABILITY OF INTERLEUKIN 6, TUMOUR NECROSIS FACTOR-[alpha] AND LEPTIN. *Cytokine,* **12,** 1712-1716.

Freemont, A.J. & Denton, J. (1991) Section 2: Evaluation: signs and symptoms. Chapter 23: Synovial fluid analysis. In *Atlas of synovial fluid cytopathology. Current histophatology vol 18.* Kluwer Academic Publishers Group, Dordrecht.

Gil, A. (2002) Polyunsaturated fatty acids and inflammatory diseases. *Biomed Pharmacother,* **56,** 388-396.

Gladman, D.D., Antoni, C., Mease, P., Clegg, D.O. & Nash, P. (2005) Psoriatic arthritis: epidemiology, clinical features, course, and outcome. *Ann Rheum Dis,* **64,** ii14-17.

Goldberg, R.J. & Katz, J. (2007) A meta-analysis of the analgesic effects of omega-3 polyunsaturated fatty acid supplementation for inflammatory joint pain. *Pain,* **129,** 210-223.

Goldring, Mary B.,S.R.G. (2007) Osteoarthritis, Vol. 213, s. 626-634.

Gourley, M. & Miller, F.W. (2007) Mechanisms of disease: Environmental factors in the pathogenesis of rheumatic disease. *Nat Clin Pract Rheumatol,* **3,** 172-180.

Grimble, R.F. & Tappia, P.S. (1998) Modulation of pro-inflammatory cytokine biology by unsaturated fatty acids. *Z Ernahrungswiss,* **37 Suppl 1,** 57-65.

Haga, H.-J. (2002) Kompendium i Reumatologi. Rikshospitalet, Bergen.

Hansen, M.H. (2003) Reumatologi. Munksgaard Danmark, Copenhagen.

Harbige, L.S. (2003) Fatty acids, the immune response, and autoimmunity: a question of n-6 essentiality and the balance between n-6 and n-3. *Lipids,* **38,** 323-341.

Henderson, C.J. & Panush, R.S. (1999) Diets, dietary supplements, and nutritional therapies in rheumatic diseases. *Rheum Dis Clin North Am,* **25,** 937-968, ix.

Henderson, R.J. & Tocher, D.R. (1992) Thin-layer chromatography. In *Lipid Analysis. A Practical Approach* (Hamilton, R.J.H.S. eds), s. 65-111. IRL Press, Oxford.

Hermann, E., Fleischer, B., Mayet, W.J., Poralla, T. & Meyer zum Buschenfelde, K.H. (1989) Correlation of synovial fluid interleukin 6 (IL-6) activities with IgG concentrations in patients with inflammatory joint disease and osteoarthritis. *Clin Exp Rheumatol,* **7,** 411-414.

Hishinuma, T., Nakamura, H., Sawai, T., Uzuki, M., Itabash, Y. & Mizugaki, M. (1999) Microdetermination of prostaglandin E2 in joint fluid in rheumatoid arthritis patients using gas chromatography/selected ion monitoring. *Prostaglandins Other Lipid Mediat,* **58,** 179-186.

Hodson, L., Skeaff, C.M., Wallace, A.J. & Arribas, G.L.B. (2002) Stability of plasma and erythrocyte fatty acid composition during cold storage. *Clinica Chimica Acta,* **321,** 63-67.

Haag, M. (2003) Essential fatty acids and the brain. *Can J Psychiatry,* **48,** 195-203.

Itomura, M., Fujioka, S., Hamazaki, K., Kobayashi, K., Nagasawa, T., Sawazaki, S., Kirihara, Y. & Hamazaki, T. (2008) Factors influencing EPA+DHA levels in red blood cells in Japan. *In Vivo,* **22,** 131-135.

IUPAC (1978) The nomenclature of lipids (Recommendations 1976) IUPAC-IUB Commission on Biochemical Nomenclature. *Biochem J,* **171,** 21-35.

Jacobs, W.B. & Fehlings, M.G. (2008) Ankylosing spondylitis and spinal cord injury: origin, incidence, management, and avoidance. *Neurosurg Focus,* **24,** E12.

James, M.J., Proudman, S.M. & Cleland, L.G. (2003) Dietary n-3 fats as adjunctive therapy in a prototypic inflammatory disease: issues and obstacles for use in rheumatoid arthritis. *Prostaglandins Leukot Essent Fatty Acids,* **68,** 399-405.

Jones, P.J.H. & Kubow, S. (2006) Lipids, sterols, and their metabolites. In *Modern nutrition in health and disease* (Shils, M.D.e.a. eds), s. 92-122. Wiliams & Wilkinson, Baltimore.

Kang, J.X. & Wang, J. (2005) A simplified method for analysis of polyunsaturated fatty acids. *BMC Biochem,* **6,** 5.

Kenis, G., Teunissen, C., De Jongh, R., Bosmans, E., Steinbusch, H. & Maes, M. (2002) STABILITY OF INTERLEUKIN 6, SOLUBLE INTERLEUKIN 6 RECEPTOR, INTERLEUKIN 10 AND CC16 IN HUMAN SERUM. *Cytokine,* **19,** 228-235.

Khan, M.A. & van der Linden, S.M. (1990) A wider spectrum of spondyloarthropathies. *Semin Arthritis Rheum,* **20,** 107-113.

Kim, I.C. & Cohen, A.S. (1966) Synovial fluid fatty acid composition in patients with rheumatoid arthritis, gout and degenerative joint disease. *Proc Soc Exp Biol Med,* **123,** 77-80.

Kinne, R.W., Stuhlmüller, B., Palombo-Kinne, E. & Burmester, G.-R. (2006) The role of macrophages in rheumatoid arthritis. In *Rheumatoid arthritis* (Firestein, G.S.*, et al.* Oxford University Press, Oxford.

Kremer, J.M., Lawrence, D.A., Petrillo, G.F., Litts, L.L., Mullaly, P.M., Rynes, R.I., Stocker, R.P., Parhami, N., Greenstein, N.S., Fuchs, B.R. & et al. (1995) Effects of high-dose fish oil on rheumatoid arthritis after stopping nonsteroidal antiinflammatory drugs. Clinical and immune correlates. *Arthritis Rheum,* **38,** 1107-1114.

Kumar, P. & Clark, M. (2005) Rheumatology and bone disease. In *Clinical medicine* (Kumar, P. & Clark, M. eds), s. 529-602. Elsevier Saunders, London.

Lepage, G. & Roy, C.C. (1986) Direct transesterification of all classes of lipids in a one-step reaction. *J Lipid Res,* **27,** 114-120.

Lequin, R.M. (2005) Enzyme Immunoassay (EIA)/Enzyme-Linked Immunosorbent Assay (ELISA). *Clin Chem,* **51,** 2415-2418.

Lin, Y.H. & Salem, N., Jr. (2005) In vivo conversion of 18- and 20-C essential fatty acids in rats using the multiple simultaneous stable isotope method. *J. Lipid Res.,* **46,** 1962-1973.

Magnusardottir, A.R. & Skuladottir, G.V. (2006) Effects of storage time and added antioxidant on fatty acid composition of red blood cells at -20 degrees C. *Lipids,* **41,** 401-404.

Marker-Hermann, E. & Schwab, P. (2000) T-cell studies in the spondyloarthropathies. *Curr Rheumatol Rep,* **2,** 297-305.

Masood, A., Stark, K.D. & Salem, N., Jr. (2005) A simplified and efficient method for the analysis of fatty acid methyl esters suitable for large clinical studies. *J Lipid Res,* **46,** 2299-2305.

Matsumoto, T., Tsurumoto, T. & Shindo, H. (2006) Interleukin-6 levels in synovial fluids of patients with rheumatoid arthritis correlated with the infiltration of inflammatory cells in synovial membrane. *Rheumatol Int,* **26,** 1096-1100.

Mease, P.J., Antoni, C.E., Gladman, D.D. & Taylor, W.J. (2005) Psoriatic arthritis assessment tools in clinical trials. *Ann Rheum Dis,* **64 Suppl 2,** ii49-54.

Menkes, C.J. (1993) Effects of disease-modifying anti-rheumatic drugs, steroids and non-steroidal anti-inflammatory drugs on acute-phase proteins in rheumatoid arthritis. *Br J Rheumatol,* **32 Suppl 3,** 14-18.

Moilanen, T. & Nikkari, T. (1981) The effect of storage on the fatty acid composition of human serum. *Clin Chim Acta,* **114,** 111-116.

Moll, J.M. & Wright, V. (1973) Psoriatic arthritis. *Semin Arthritis Rheum,* **3,** 55-78.

Mori, T.A. & Beilin, L.J. (2004) Omega-3 fatty acids and inflammation. *Curr Atheroscler Rep,* **6,** 461-467.

Morrison, W.R. & Smith, L.M. (1964) Preparation of Fatty Acid Methyl Esters and Dimethylacetals from Lipids with Boron Fluoride--Methanol. *J Lipid Res,* **5,** 600-608.

Murphy, K.M., Travers, P. & M., W. (2007) The detection, measurement, and characterization of
antibodies and their use as research and diagnostic tools. In *Janeway's Immunobiology*
(Murphy, K.M*., et al.* eds), s. 740-756. Garland Science Publishing, New York.

Navarro, E., Esteve, M., Olive, A., Klaassen, J., Cabre, E., Tena, X., Fernandez-Banares, F., Pastor, C. & Gassull, M.A. (2000) Abnormal fatty acid pattern in rheumatoid arthritis. A rationale for treatment with marine and botanical lipids. *J Rheumatol,* **27,** 298-303.

NIAMS/NIH (2004) Handout on Health: Rheumatoid Arthritis. . National Institute of Arthritis and Musculoskeletal and Skin Diseases. National Institutes of Health, USA.

NIFES (2002) Metode 041 - Fettsyrebestemmelse av totalfettsyrer v.h.a GLC. In *Håndbøker*. Nasjonalt institutt for ernærings- og sjømatforskning, Bergen.

NIFES (2006) Lipid class determination in oils, feed, tissue and tissue fluids. In *Hand books* (sjømatforskning, N.i.f.e.-o. Nasjonalt institutt for ernærings- og sjømatforskning, Bergen.

NIFES (2007) Metode 354 - Fettsyrebestemmelse v.h.a GLC. In *Håndbøker*. Nasjonalt institutt for ernærings og sjømatforskning, Bergen.

Paunovic, V., Carroll, H.P., Vandenbroeck, K. & Gadina, M. (2008) Signalling, inflammation and arthritis: crossed signals: the role of interleukin (IL)-12, -17, -23 and -27 in autoimmunity. *Rheumatology (Oxford),* **47,** 771-776.

Petrovic-Rackov, L. (2005) Cytokines in rheumatoid arthritis and osteoarthrosis. *Med Pregl,* **58,** 245-251.

Petrovic-Rackov, L. & Pejnovic, N. (2006) Clinical significance of IL-18, IL-15, IL-12 and TNF-alpha measurement in rheumatoid arthritis. *Clin Rheumatol,* **25,** 448-452.

Piet, P.G. (1998) n-3 Fatty acids in the treatment of arthritis. In *Medicinal fatty acids in inflammation* (Kremer, J.M. Birkhäuser Verlag, Berlin.

Prete, P.E., Gurakar-Osborne, A. & Kashyap, M.L. (1995) Synovial fluid lipids and apolipoproteins: a contemporary perspective. *Biorheology,* **32,** 1-16.

Punzi, L., Calo, L. & Plebani, M. (2002) Clinical significance of cytokine determination in synovial fluid. *Crit Rev Clin Lab Sci,* **39,** 63-88.

Rahman, M.M., Bhattacharya, A. & Fernandes, G. (2008) Docosahexaenoic acid is more potent inhibitor of osteoclast differentiation in RAW 264.7 cells than eicosapentaenoic acid. *J Cell Physiol,* **214,** 201-209.

Ribbens, C., Andre, B., Kaye, O., Kaiser, M.J., Bonnet, V., de Groote, D., Franchimont, N. & Malaise, M.G. (2000) Increased synovial fluid levels of interleukin-12, sCD25 and sTNF-RII/sTNF-RI ratio delineate a cytokine pattern characteristic of immune arthropathies. *Eur Cytokine Netw,* **11,** 669-676.

Ritchlin, C. (2007) Psoriatic disease--from skin to bone. *Nat Clin Pract Rheumatol,* **3,** 698-706.

Ropes, M.W. & Bauer, W. (1953) Synovial fluid changes in joint diseases. oxford university press, London.

Sands, S.A., Reid, K.J., Windsor, S.L. & Harris, W.S. (2005) The impact of age, body mass index, and fish intake on the EPA and DHA content of human erythrocytes. *Lipids,* **40,** 343-347.

Sargent, J.R. (1997) Fish oils and human diet. *Br J Nutr,* **78 Suppl 1,** S5-13.

Schroder, K., Hertzog, P.J., Ravasi, T. & Hume, D.A. (2004) Interferon-{gamma}: an overview of signals, mechanisms and functions. *J Leukoc Biol,* **75,** 163-189.

Sellmayer, A. & Koletzko, B. (1999) Long-chain polyunsaturated fatty acids and eicosanoids in infants--physiological and pathophysiological aspects and open questions. *Lipids,* **34,** 199-205.

Sijben, J.W. & Calder, P.C. (2007) Differential immunomodulation with long-chain n-3 PUFA in health and chronic disease. *Proc Nutr Soc,* **66,** 237-259.

Simopoulos, A.P. (2002a) The importance of the ratio of omega-6/omega-3 essential fatty acids. *Biomed Pharmacother,* **56,** 365-379.

Simopoulos, A.P. (2002b) Omega-3 fatty acids in inflammation and autoimmune diseases. *J Am Coll Nutr,* **21,** 495-505.

Smith, A.D.e.a. (2001) Oxford dictionary of biochemistry and molecular biology. Oxford University Press Inc, New York.

Stamp, L.K., James, M.J. & Cleland, L.G. (2005) Diet and rheumatoid arthritis: a review of the literature. *Semin Arthritis Rheum,* **35,** 77-94.

Steiner, G., Tohidast-Akrad, M., Witzmann, G., Vesely, M., Studnicka-Benke, A., Gal, A., Kunaver, M., Zenz, P. & Smolen, J.S. (1999) Cytokine production by synovial T cells in rheumatoid arthritis. *Rheumatology (Oxford),* **38,** 202-213.

Sugiyama, Y. & Ono, S. (1966) Fatty acid metabolism in the synovial fluid in the patients with rheumatoid arthritis and osteoarthritis. *Nippon Geka Hokan,* **35,** 1020-1025.

Sundrarjun, T., Komindr, S., Archararit, N., Dahlan, W., Puchaiwatananon, O., Angthararak, S., Udomsuppayakul, U. & Chuncharunee, S. (2004) Effects of n-3 fatty acids on serum interleukin-6, tumour necrosis factor-alpha and soluble tumour necrosis factor receptor p55 in active rheumatoid arthritis. *J Int Med Res,* **32,** 443-454.

Tak, P. (2006) Examination of the synovium and synovial fluid. In *Rheumatoid arthritis* (Firestein, G.S.*, et al.* eds), s. xvii, 575 s. Oxford University Press, Oxford.

Thavasu, P.W., Longhurst, S., Joel, S.P., Slevin, M.L. & Balkwill, F.R. (1992) Measuring cytokine levels in blood. Importance of anticoagulants, processing, and storage conditions. *J Immunol Methods,* **153,** 115-124.

Thorsby, E. & Lie, B.A. (2005) HLA associated genetic predisposition to autoimmune diseases: Genes involved and possible mechanisms. *Transpl Immunol,* **14,** 175-182.

Vahamiko, S., Penttinen, M.A. & Granfors, K. (2005) Aetiology and pathogenesis of reactive arthritis: role of non-antigen-presenting effects of HLA-B27. *Arthritis Res Ther,* **7,** 136-141.

van der Linden, S., Valkenburg, H.A. & Cats, A. (1984) Evaluation of diagnostic criteria for ankylosing spondylitis. A proposal for modification of the New York criteria. *Arthritis Rheum,* **27,** 361-368.

van Leeuwen, M.A. & van Rijswijk, M.H. (1994) Acute phase proteins in the monitoring of inflammatory disorders. *Baillieres Clin Rheumatol,* **8,** 531-552.

Vedin, I., Cederholm, T., Freund Levi, Y., Basun, H., Garlind, A., Faxen Irving, G., Jonhagen, M.E., Vessby, B., Wahlund, L.O. & Palmblad, J. (2008) Effects of docosahexaenoic acid-rich n-3 fatty acid supplementation on cytokine release from blood mononuclear leukocytes: the OmegAD study. *Am J Clin Nutr,* **87,** 1616-1622.

Walsh, N.C., Crotti, T.N., Goldring, S.R. & Gravallese, E.M. (2005) Rheumatic diseases: the effects of inflammation on bone. *Immunol Rev,* **208,** 228-251.

Williams, C.M. & Burdge, G. (2006) Long-chain n-3 PUFA: plant v. marine sources. *Proc Nutr Soc,* **65,** 42-50.

Wise, C.M., White, R.E. & Agudelo, C.A. (1987) Synovial fluid lipid abnormalities in various disease states: review and classification. *Semin Arthritis Rheum,* **16,** 222-230.

Wood, P. (2006) Understanding immunology. Pearson Education Limited, Harlow.

Woolf, A.D. & Pfleger, B. (2003) Burden of major musculoskeletal conditions. *Bull World Health Organ,* **81,** 646-656.

Yaqoob, P. & Calder, P.C. (2007) Fatty acids and immune function: new insights into mechanisms. *Br J Nutr,* **98 Suppl 1,** S41-45.

Zurier, R.B. (1998) Gammalinolenic acid treatment of rheumatoid arthritis. In *Medicinal fatty acids in inflammation* (Kremer, J.M. eds), s. 29-43. Birkhäuser Verlag, Berlin.

Appendix

I. LIPID CLASS COMPOSITION OF SYNOVIAL FLUID

Lipid class composition of four random SF samples one from each of the different diagnoses, RA, PsA, ReA and AS.

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Figure A1 presents the average of the percentage lipid composition of SF in patients with inflammatory joint diseases. The SF from all four patients as a group contained a high fraction of neutral lipids constituting 77 % of total lipids. Based upon this determination, disregarded the extremely small values quantified for other phospholipids between the subgroups, there was an agreement between lipid classes of the four patients with different diagnosis. Studying the main groups, neutral lipid content varied between 74-79%, whereas phospholipids varied between 21-26 percent. The TAG contributed to 12% of total lipids in two patients, whereas for two other patients the percentage was as high as 18% and 21%.

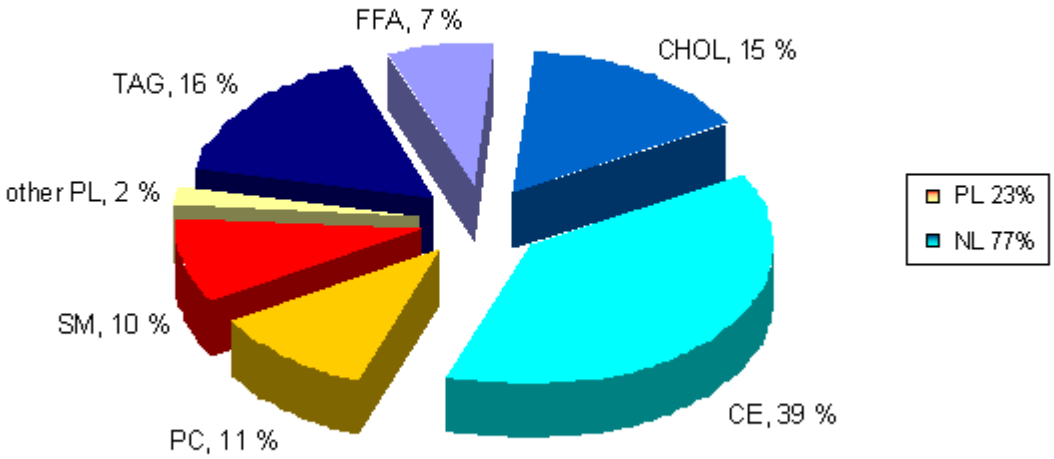

Figure A.1: Average percentage lipid class composition in synovial fluid of patients, n=4.
The bluish pies are different neutral lipid classes, whereas the red and yellowish pies are different phospholipids. Other phospholipids include phosphatidylinositol (PI), phosphatidylethanolamine (PE) and phosphatidylserine (PS). Abbr; PL: Phospholipids, PC: Phosphatidylcholine, SM: Sphingomyelin, NL: Neutral lipids, TAG: Triacylglycerol, FFA: Free fatty acids, CHOL: cholesterol, CE: cholesteryl ester.