Pro-resolving fatty acids and oxylipids in osteoarthritis and rheumatoid arthritis

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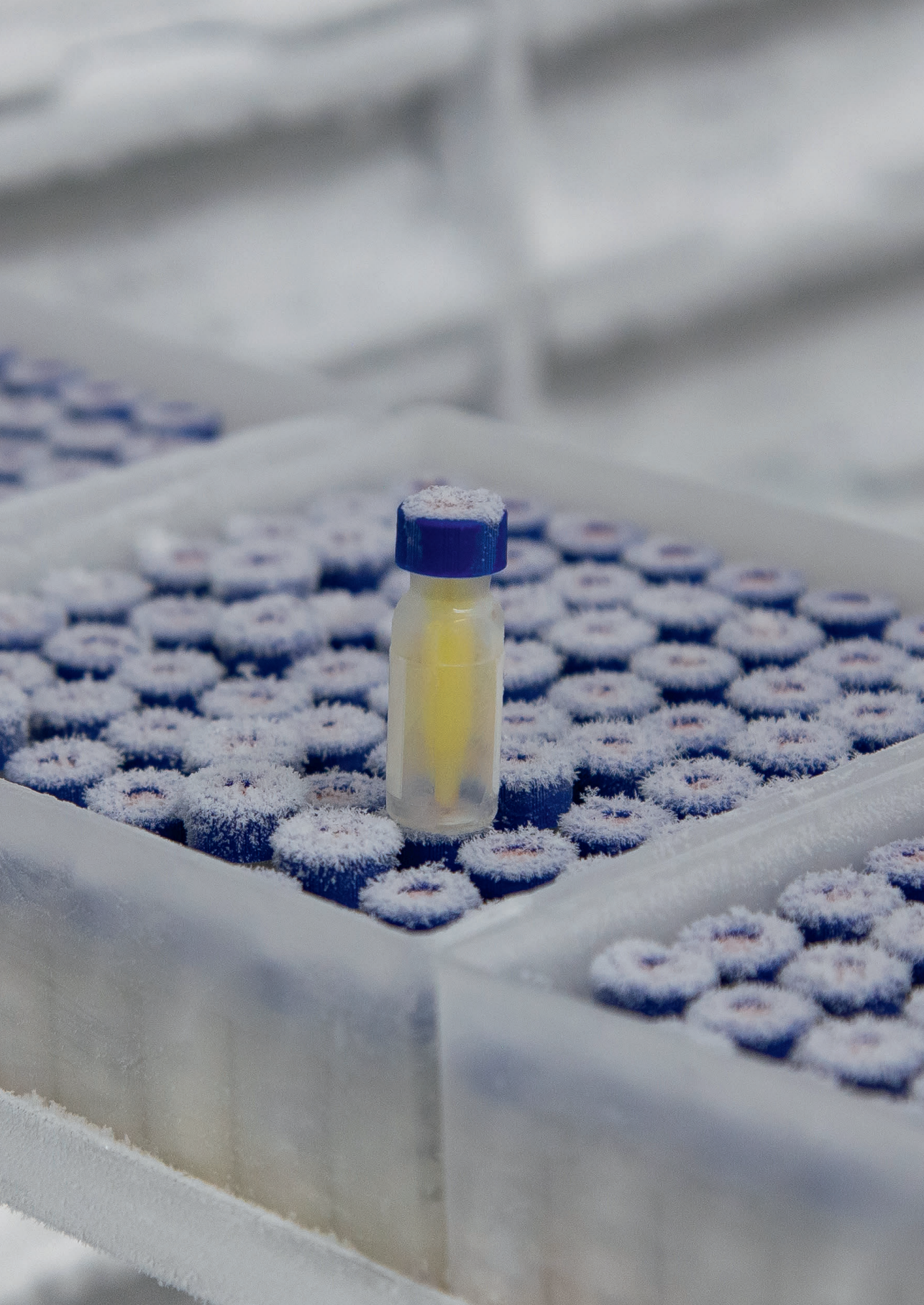

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General introduction

Rheumatoid arthritis and osteoarthritis

Arthritis is a collective name for a wide range of diseases involving inflammation in the joint. The most common type of arthritis is osteoarthritis (OA), affecting 8,7% of the Dutch population(1). The most well-known type of arthritis is rheumatoid arthritis (RA), affecting approximately 1% of the Dutch population.

OA and RA both affect more women than men and the mean age of both patient groups is comparable (Table 1). However, disease symptoms and treatment options are different. RA presents with painful, warm swelling of the joints which can eventually lead to bone erosion and deformities. OA presents with pain and stiffness caused by structural damage to the cartilage and bone.

RA patients can be effectively treated with a wide range of DMARDs and biologicals, aiming at symptom alleviation and disease modification. Examples of DMARDs are methotrexate and leflunomide, which both act as immunosuppressants by interfering with cell functions essential for generating adaptive immune responses. Examples of biologicals are tocilizumab and adalimumab or etanercept which directly interfere with interleukin-6 (IL-6) and tumour necrosis factor (TNF) signalling in patients respectively, lowering inflammation. Next to these DMARDs and biologicals, RA patients are prescribed corticosteroids such as prednisolone for short term pain relief and reduction of inflammation.

In contrast to the long list of treatment options for RA patients, there are only limited options for OA patients aiming at symptom alleviation. Currently no disease modifying drugs exist. The core treatment for OA patients consists of education, exercise and weight management, and prescription of NSAIDs to relieve pain(2). As these options merely alleviate symptoms, there is a desperate need for disease modifying drugs. Effectiveness of biologicals targeting TNF or IL-1 has been studied for years in clinical trials but the majority of studies did not show promising results(3–5). Of interest are the developments in the field of regenerative medicine. Platelet-rich plasma injections and bone marrow concentrate injections both aim to induce tissue regeneration in the erosive joint. Although a fair number of clinical trials were performed, the results are inconsistent, most likely due to the poor standardization of the cellular products(6,7).

Not only treatment options differ considerably between RA and OA, but so does their aetiology. RA is an autoimmune disease, in which the immune system of the patient is targeting the body's own tissues. Consequently, RA is characterized by the presence of autoantibodies, such as rheumatoid factor (RF), targeting the Fc part of an IgG antibody, as well as autoantibodies recognizing post-translationally modified proteins, such as

anti-citrullinated protein antibodies (ACPA). RA is characterized by a strongly activated immune system shown by elevated numbers of immune cells in the joints, and often high levels of CRP, cytokines, chemokines as well as other inflammatory markers in circulation. Identification of specific inflammatory pathways is RA potentially allows for their effective targeting using disease modifying DMARDs and biologicals(8).

There is no evidence for autoimmunity in the aetiology of OA. For instance, the prevalence of autoantibodies in OA is similar to healthy controls and is not associated with structural damage(9). The development of OA however is correlated with increasing age, trauma and obesity(10). All these risk factors can cause abnormal loading of the joint, which was long thought to be the mayor contributor to the development of OA. However by combining a variety of imaging and molecular techniques to study OA etiology, it is now evident that also cell stress, innate immune activation, low grade systemic inflammation and maladaptive repair responses are contributors(11). For instance, obesity is also associated with OA in the non-weight baring joints of the hand(12). As obesity is not only characterized by increased bodyweight but also by low grade inflammation and adiposity leading to altered levels of adipokines, lipids and cytokines, it is proposed that these soluble mediators play a role in the etiology of osteoarthritis(13–15).

	OA	RA
FEMALE GENDER, %	Female>Male	Female>Male
AGE OF ONSET, YEARS	Middle-age	All ages, mostly between 30-50
RISK FACTORS	Obesity, age, gender, genetics, traumatic injury(16,17)	Gender, genetics, smoking(18)
JOINT RELATED SYMPTOMS	Pain, tenderness, loss of flexibility, bone spurs, swelling(19,20)	Stiffness, tenderness, pain, swelling, redness, warmth
IMAGING FEATURES	Joint space narrowing, sclerosis, osteophytes, erosions, subchondral cysts, bone marrow lesions, synovitis	Joint space narrowing, marginal erosions, periarticular bone loss, bone marrow edema, synovitis
PHARMACOLOGICAL TREATMENT OPTIONS	Symptom alleviation: NSAIDs	Symptom alleviation and disease modification: NSAIDs, DMARDs, biologicals, corticosteroids

Table 1. characteristics RA and OA patients

Structural damage and inflammation in osteoarthritis

In a healthy synovial joint, two strong bone structures are attached to each other to facilitate body motion. The surfaces of the bone are covered with healthy, smooth cartilage and a few cell layers thick synovium forms a cavity surrounding the two surfaces. This synovial cavity is filled with a clear, viscous liquid called synovial fluid. An OA joint is easily identified on radiographic images such as X-ray. At an early phase in the disease process, joint space narrowing indicates loss of cartilage and osteophytes present as bony protrusions at the articulating parts of the bone. In severe stages of OA, there is extensive cartilage loss, the osteophytes are large and osteosclerosis, the abnormal hardening of the bone, is present.

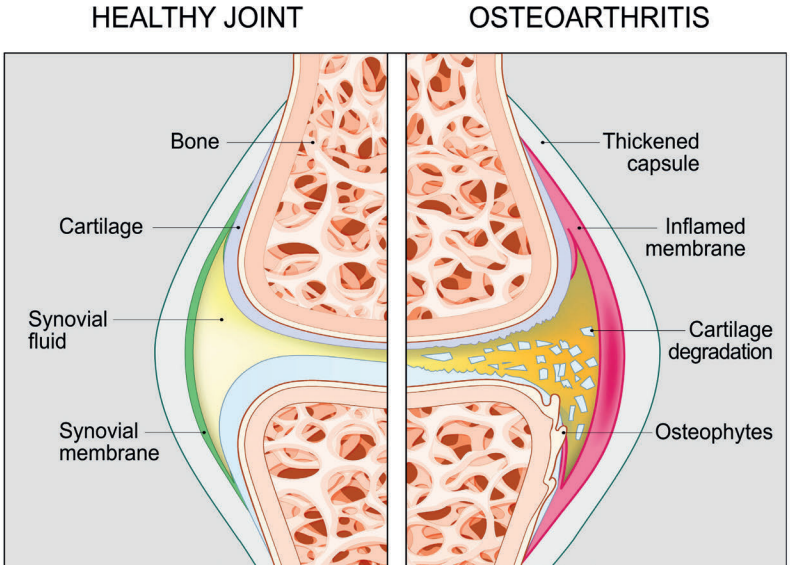

Figure 1. Healthy and OA joint

While X-ray can detect severe structural damage in OA joints, techniques such as MRI and ultrasound can detect synovitis, which is inflammation of the synovial lining, in joints of OA patients(21–24). Using these techniques it became feasible to investigate associations between synovitis and OA symptoms such as pain and structural damage. It was shown that synovitis is associated with pain(21,23). In addition, treating OA patients with the anti-inflammatory glucocorticoid prednisolone during an inflammatory flare reduced pain in hand OA(25). Synovitis is also associated with osteophytes and joint space narrowing in hand osteoarthritis(26). In addition, synovitis, independent of cartilage loss and osteophyte formation, increases the risk of developing structural damage(27,28).

One of the most common hypotheses for the development of synovitis, postulates that degradation products of joint tissues can cause a synovial reaction leading to inflammation. Although periods of acute inflammation are critically important, ongoing inflammation can be detrimental to tissue homeostasis and eventually lead to tissue degradation and subsequently disease progression(29). Mechanistically, synovial inflammation is caused by activation and accumulation of immune cells in OA synovium. OA synovium was shown to be infiltrated with several immune cells such as T cells, mast cells and macrophages(24). Synovial macrophages have been most often shown to be associated with disease pathology(30). Depletion of synovial lining macrophages prior to the induction experimental osteoarthritis resulted in a 84% reduction in osteophyte formation(31). In human synovial cultures of OA patients, it was shown that depletion of macrophages drastically decreased the formation of pro-inflammatory cytokines and extracellular matrix (ECM) degrading enzymes such as matrix-metalloproteinases (MMPs) by synovial fibroblasts(32). It was also shown that the presence of synovial macrophages was associated with severity of structural damage(33). Next to reports that show involvement of macrophages in the progression of OA, there are also reports addressing the anti-inflammatory phenotypes of macrophages in OA which could be protective or involved in regeneration(34,35). Thus, although the involvement of macrophages in OA pathology is clear, it has not yet been identified how these macrophages could be targeted to the benefit of the OA patient(30).

Resolution of inflammation

Inflammation can be categorized in three different phases, the induction, progression and resolution phase. The induction phase can be initiated by a large variety of events such as the invasion of a microbe, a damaged cell, toxins or trauma. The induction phase is characterized by the presence of acute inflammatory mediators such as histamine, leukotrienes (LTs), prostaglandins (PGs), and free radicals. These inflammatory mediators cause a rapid influx of innate immune cells such as monocytes and neutrophils leading to quick progression of inflammation. The infiltrated immune cells are programmed to take out the initial trigger that cause the inflammation. In the ideal situation, the period of inflammation is limited to an acute inflammation period, after which the inflammation is resolved and the tissue can return to homeostasis. However, uncontrolled ongoing inflammation can turn into chronic inflammation. Chronic inflammation is characterized by ongoing recruitment of a large variety of immune cells and the excessive production of cytokines and other inflammatory mediators. High burden diseases such as RA, cancer, diabetes, cardiovascular diseases, inflammatory bowel disease (IBD) and chronic obstructive pulmonary disease (COPD) are examples of diseases characterized by ongoing chronic inflammation. Only in the last two decades OA was recognized as

a disease with a chronic inflammatory component as synovitis was added to the list of critical OA symptoms(36).

Chronic inflammation is detrimental to the healthy tissues of the body. The activated immune cells, inflammatory cytokines, chemokines and other mediators damage healthy tissues. By lowering this inflammatory status by for instance effective biologicals in RA, excessive tissue damage can be prevented(37,38). However, when treatment with biologicals is stopped, inflammation can return, showing that inhibiting inflammation alone is not enough to return to homeostasis. In an elegant review it was pointed out that the resolution phase of inflammation is an active process in which the initial trigger of inflammation is eliminated and inflammation resides(39). In this phase, pro-resolving mediators and certain immune cells play a crucial role.

Around the year of 2000, a group of scientists discovered a series of fatty acid derived molecules which were named pro-resolving lipid mediators (SPM)(40). These SPM were described in several mouse models in which the different phases of acute inflammation are clearly defined. An example of such a model is the murine peritonitis model, in which peritonitis is induced by the injection of the glucan zymosan which causes peritonitis. After a few days the mouse recovers without additional intervention. In these mouse models it was shown that the SPM were generated during the resolution phase. It was proposed that these SPM play an active role in the resolution of inflammation as upon administration, these SPM could reduce inflammation and promote return to tissue homeostasis(40).

Specialized pro-resolving mediators in resolution of inflammation

To date, a large number of SPM have been discovered. SPM can be classified in lipoxins, resolvins, protectins and maresins. Lipoxin A (LXA_4) and Lipoxin B (LXB_4) were the first of SPM that were discovered(41,42). Later, E series resolvins (RvE) and D series resolvins (RvD), protectin D1/Neuroprotectin D1 (PD1/NPD1) and Maresin-1 (Mar-1) and Maresin-2 (Mar-2) (43–48). Most of them were shown to inhibit neutrophil migration and promote phagocytosis by macrophages in vitro(44–50). Upon administration, enhanced resolution of inflammation was shown in several mouse models of inflammation, such as murine peritonitis, acute renal failure and arthritis(51,52).

In addition to enhancing resolution, SPM Mar-1 was shown to induce tissue regeneration in flatworms (Planaria) and human tooth extraction sockets(53,54). In recent years, it was also shown that SPM could affect adaptive immunity by reducing T cell cytokine production, modulate T cell differentiation and decrease antibody production by B cells(55–58).

At this point in time, there is one report showing therapeutic effects of SPM in humans. In eczema patients is was shown that topical application of LXA_4 was effective in limiting severity and induction of recovery(59). An approach which is explored far more often, is the administration of fatty acids, which are the precursors of SPM.

Fatty acids and SPM synthesis

SPM are oxidized fatty acids, also named oxylipids(60). They are synthesized from free fatty acids by lipoxygenases (LOX), cyclooxygenases (COX) and cytochrome P450 (CYP) enzymes. Figure 2 shows an overview of a selection of known SPM and their fatty acid precursors. Fatty acids are precursors of pro-resolving, as well as pro-inflammatory oxylipids.

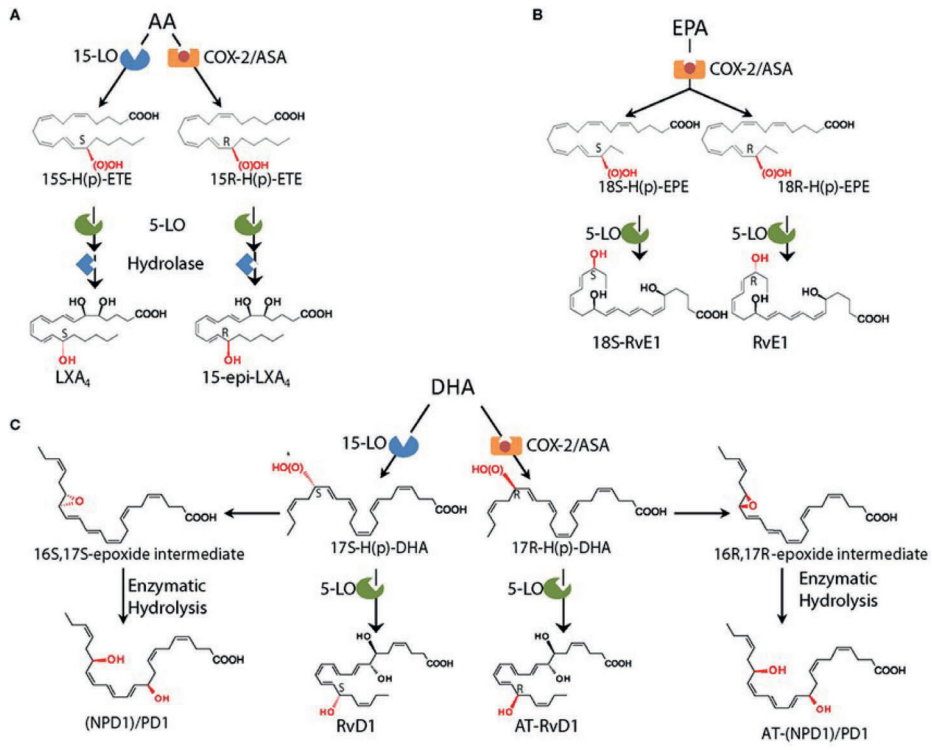

Figure 2. Biosynthesis of SPM. Reprinted from(61). AT: Aspirin-triggered; COX-2/ASA: Aspirin acetylated COX-2; H(p)-ETE: hydro(per)oxyeicosatetraenoic acid; H(p)-EPE: hydro(per) oxyeicosapentaenoic acid; H(p)-DHA: hydro(per)oxydocosahexaenoic acid; LO: lipoxygenase

Fatty acids are a class of fatty acyls and are present in free and bound form in vivo. In essence, a fatty acid is a carbon chain with hydrogen atoms and one carboxyl group (-COOH). Fatty acids can be saturated or unsaturated, the latter meaning that one (monounsaturated) or more (polyunsaturated) double bonds exist in the carbon chain.

The name of the fatty acid reveals the number of carbons and the number of double bonds, together with the carbon position where the first double bond occurs. For instance, arachidonic acid (AA) ($C20:4_{\omega-6}$) is a 20 carbon fatty acid with 4 double bonds, the first starting at carbon atom 6 counted from the omega (carbon) end.

Fatty acids can be e.g. bound to a glycerol backbone to form glycerolipids or phospholipids, the latter also contain a polar head group next to the glycerol backbone. Glycerolipids and phospholipids are both main structures of cell membranes. Acyl hydrolases hydrolyse phospholipid structures resulting in the cleavage of fatty acids and production of free fatty acids(62). The best known acyl hydrolase activated during inflammation is phospholipase A_2 (PLA_2) as this enzyme initiates production of pro-inflammatory leukotrienes and prostaglandins and also lipoxins by cleavage of AA(63).

Multiple enzymes and multiple hydrolyzation steps are involved in the formation of an SPM and other oxylipids from a free fatty acid. For example, to form LXA_4, arachidonic acid (AA) is hydrolysed by 15-LOX at carbon 15 to form 15-HETE. Subsequently, 15-HETE is hydrolysed by 5-LOX or 12-LOX to form LXA_4. Next to this route, LXA_4 could also be formed by 5-LOX hydrolyzation, followed by 15-LOX hydrolysation and subsequently 5-LOX or 12-LOX hydrolysation. The intermediate products such as 15-HETE are called monohydroxylated products. The generation of SPM can be a result of transcellular processes and combined triggering of cells which express the enzymes involved. For instance, it was shown that LXA_4 could be formed by AA oxidation by 5-LOX in activated neutrophils leading to leukotriene B_4 (LTA_4) followed by oxidation by 12-LOX in activated platelets resulting in LXA_4(64). In addition, macrophages alone can produce LXA_4 by stimulation with LPS and ATP(65).

The production of SPM is dependent on the availability of the precursor fatty acid and the expression of several enzymes. The expression of the enzymes seems to be regulated by the presence of different stimuli acting on the different immune cells as was shown for both neutrophils and macrophages(65–68). As the pro-inflammatory and pro-resolving mediators share some of the same precursor fatty acids and the same enzymatic pathways, these oxylipid production pathways should be tightly regulated and can easily interact. Indeed, investigating the kinetics of LXA_4 production during a self-resolving inflammatory model in mice laid the basis for the so called lipid mediator switch hypothesis(69). It was shown that the acute phase inflammatory mediator PGE_2 could initiate LXA_4 production in neutrophils. Lipid mediator class-switch is now a well-established term to describe the process whereby pro-inflammatory mediators prompt the production of pro-resolving mediators to initiate the resolution phase of inflammation(40).

So far it has been proven difficult to induce resolution during the course of chronic inflammation by administration of fatty acids. A possible explanation could be the versatile nature of the enzymatic pathways leading to oxylipid synthesis, which are highly dependent on inflammatory cells and stimuli.

Technical aspects of bodily fluid collection and storage for lipid analysis

Lipidomics is the term which is used to describe the research focussed on the characterization of the role of fatty acids and other lipids in health and disease. Lipidomics has evolved very rapidly due to the development of mass spectrometry techniques. Using these techniques, it became possible to measure a whole range of metabolites such as fatty acids and oxydized lipids but also complex glycan structures derived from antibodies and methylation patterns of DNA. With the implementation of these new techniques in medical biology research, it became of essence to investigate the adequate collection and storage conditions.

It is well known and accepted that human-derived biomaterial should be collected and stored in a reproducible and controlled manner. For example, bacterial load in stool samples collected and stored in rural areas without access to refrigerators cannot be compared to collection in a western hospital setting where samples are immediately refrigerated after collection due to variable outgrowth of bacteria at high temperatures. Similarly, fasted blood samples show different glucose levels compared to blood samples just taken after a meal . The type of collection tube used is also important. For instance, serum and heparin plasma cause chemical noise in the mass spectra of liquid chromatography tandem mass spectrometry (LC-MS) in contrast to EDTA plasma(70,71).

Moreover, the storage conditions are important. It was shown that the recovery of cytokines is quickly altered after freeze-thaw cycles(72). Likewise, while it is safe to store isolated DNA at minus 20°C, viable cells need to be stored below -130°C to keep cellular structures intact. It was shown that after long term storage at minus 80°C (>16 years), altered levels of EDTA plasma metabolites such as amino acids and fatty acids were found compared to baseline(73). In addition, storing blood samples at room temperature altered metabolite levels, including lipids, carbohydrates, amino acids and signalling molecules such as dopamine within hours(70,74,75).

Depending on the desired future analyses and the type of bodily fluid used, the processing of the material also varies. While DNA is best obtained from whole blood or tissue as this includes the cells containing DNA, plasma or serum are more suited for soluble mediator analyses. Importantly, contamination of the serum or plasma with cells should be avoided as dying or broken cells can alter the levels of soluble mediators.

Outline of the thesis

The aim of the studies described in this thesis is to investigate the potential therapeutic effects of SPM in RA and OA. The secondary aim of the studies in this thesis is to investigate optimal processing and storage conditions of bodily fluids to obtain reliable results from the techniques used in part 1 of this thesis.

Part 1 Fatty acids in arthritis

The literature review in **chapter 2** set the basis for the studies described in this thesis by reviewing existing literature on the detection and therapeutic potential of fatty acids and their derivatives in RA and OA patients(76). This review reports on the detection of fatty acids and their derivatives in serum and synovial fluid, their effect on human RA and OA tissues and their association with clinical characteristics. In addition, the results from intervention studies using fatty acids performed in humans and mice are summarized. As evidence for the activation of resolution pathways was scarce, the activation of resolution pathways in RA and OA patients was investigated in **chapter 3**. In this study, fatty acids and their derivatives oxylipids, including SPM were analysed in the synovial fluid of RA and OA patients using an in-house developed LC-MS/MS technique(77,78). In addition, the study investigated the activity of enzymatic pathways and which cell types could be involved in the production of SPM in OA patients. As some results from the study described in chapter 3 were not in agreement with previous literature, the effect of sample storage conditions on lipidomic analysis were analysed in the studies described in part 2 of this thesis.

In the study described in **chapter 4** , the therapeutic potential of SPM in a murine OA model was evaluated. Mice were fed a high fat diet which was previously reported to induce OA(79,80). The level of inflammation in these mice was evaluated as well as whether this inflammation correlated with structural damage. Next, the effect of SPM on human synovial cells and the effect on in vivo disease progression was evaluated.

The study in **chapter 5** describes a detailed molecular mapping of a self-resolving peritonitis mouse model in search of potential novel pro-resolving mediators. This mapping revealed a potential pro-resolving role for fatty acid adrenic acid (AdA). The pro-resolving capacity of AdA was extensively investigated by performing various in vitro experiments to study the effect of AdA on neutrophil chemoattractant production by neutrophils, neutrophil migration and macrophage phagocytosis. Thereafter we investigate possible mechanisms by which AdA acts as a pro-resolving mediator using a quantitative differential mobility spectrometry (DMS) lipidomics platform(Lipidyzer™) (81). Finally, we investigate the potential pro-resolving function of AdA in the K/BxN serum transfer arthritis model in vivo.

Part 2 Technical aspects of bodily fluid collection

Previously, a set of SPM was detected in synovial fluid of RA patients, which could not be not confirmed by the study described in chapter 3(78). To test whether this discrepancy could be due to differences in storage conditions, the effect of storage temperatures, storage time and pre-treatment options on lipidomic analyses of plasma in **chapter 6(**82**).**

The only SPM that could be detected in study described in chapter 3, was detected in the insoluble fraction of the synovial fluid. These results triggered the study described in **chapter 7**. In this study, the importance of homogenization of synovial fluid before analysis by flowcytometry, ELISA, Luminex and lipidomic analysis was investigated(83).

Chapter 8 summarizes and discusses the results obtained from the work presented in this thesis.

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